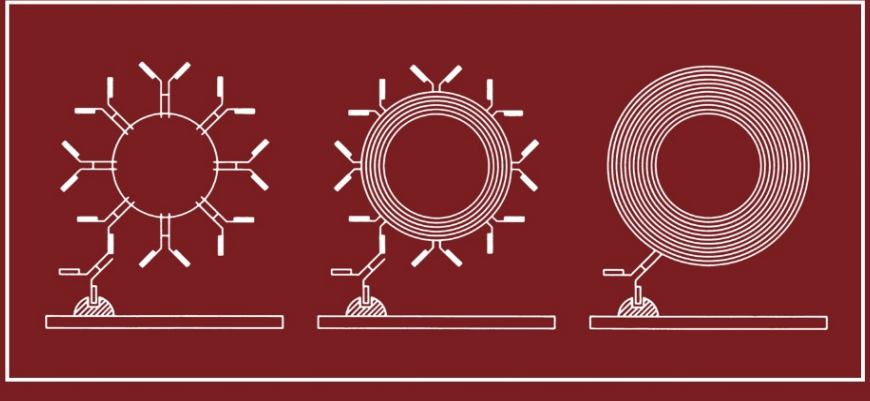
Methods in Molecular Biology

Volume 32

BASIC PROTEIN AND PEPTIDE PROTOCOLS

Edited by

John M. Walker



Basic Protein and Peptide Protocols

Methods in Molecular Biology

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Methods in Molecular Biology • 32

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Humana Press Totowa, New Jersey

999 Riverview Drive, Suite 208
Totowa, New Jersey 07512

Printed in the United States of America. 10 9 8 7 6 5 4 3 2 1

Library of Congress Cataloging in Publication Data

Main entry under title:

Methods in molecular biology.

Basic protein and peptide protocols / edited by John M. Walker.
p. cm. — (Methods in molecular biology ; 32)
Includes index.
ISBN 0-89603-268-X. —ISBN 0-89603-269-8 (comb)
1. Proteins—Analysis—Methodology. 2. Peptides—Analysis—Methodology. I. Walker, John M., 1948– . II. Series: Methods in molecular biology (Totowa, NJ) ; 32.
QP551.B268 1994
574.19'245—dc20 93-48929
CIP

Preface

The first volume in this *Methods in Molecular Biology* series, *Proteins* (1984), concentrated on basic techniques for the analysis and purification of peptides and proteins. As the series developed, more specialized volumes on proteins were introduced, such as those on *Immunochemical Protocols* (vol. 10), *Practical Protein Chromatography* (vol. 11), *Glycoprotein Analysis in Biomedicine* (vol. 14), *Protein–DNA Interactions* (vol. 30), *Biomembrane Protocols* (vols. 19 and 27), *Spectroscopic Methods and Analyses* (vol. 17), and *Optical Spectroscopy, Microscopy, and Macroscopic Techniques* (vol. 22). Further specialist volumes on peptides, monoclonal antibodies, immunoassays, ELISA, protein engineering, protein stability, mass spectrometry of proteins, automated sequence analysis, and protein NMR are currently in preparation.

Since it is now a decade since the initial volume was published, it seems an especially appropriate moment to extensively reorganize, update, and revise the earlier volume. In an attempt to be more comprehensive in our coverage, this current volume, *Basic Protein and Peptide Protocols,* is totally committed to basic analytical methods; a planned companion volume will later concentrate on preparative techniques. Those analytical techniques requiring expensive specialized instrumentation, such as NMR, mass spectrometry, X-ray crystallography, spectroscopy, and automated sequence analysis, are not described here, but in the appropriate specialized volumes listed above. Not surprisingly, a number of new analytical techniques have been developed since the predecessor work was published. This is reflected, for example, in the inclusion here of such newer techniques as capillary electrophoresis, the use of immobilized pH gradients in isoelectric focusing, the use of immunogold reagents, and the BCA assay for proteins. Additionally, over the years the range of many techniques has been expanded by the application of new technologies,

such as the development of chemiluminescent systems for detecting protein blots or the use of lectins to detect glycoproteins on blots. Most other techniques have gained from modifications that improve their sensitivities, resolution, speed, or ease of use. A few techniques have become redundant: For example, the analysis of peptides by paper chromatography or paper electrophoresis has been effectively totally superseded by HPLC technology. As a consequence of these changes and improvements over the years, little that appeared in the earlier volume remains unchanged here. Finally, one or two obvious omissions from the earlier volume—the analysis of glycoproteins and of disulfide bridges—have been redressed.

In keeping with the established format of the *Methods in Molecular Biology* series, each chapter starts with a description of the basic theory behind the method being described. The Materials section lists all the chemicals, reagents, buffers, and other materials necessary for carrying out the protocol. Since the main aim of the book is to provide active experimentalists with a full account of the practical steps necessary for carrying out each protocol successfully, the Methods section contains detailed step-by-step descriptions of every protocol that should result in successful execution of the method. The Notes section complements the Methods material by indicating how best to deal with any problem or difficulties that may arise when using the technique, and how to go about making the widest variety of modifications or alterations to the protocol.

Basic Protein and Peptide Protocols is intended as a benchtop manual and guide for both those who are new to the protein chemistry laboratory and those more established workers who wish to try a new technique for the first time.

John M. Walker

Contents

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CHAPTER 1

The Lowry Method for Protein Quantitation

Jaap H. Waterborg and Harry R. Matthews

1. Introduction

The most accurate method of determining protein concentration is probably acid hydrolysis followed by amino acid analysis. Most other methods are sensitive to the amino acid composition of the protein, and absolute concentrations cannot be obtained. The procedure of Lowry et al. *(1)* is no exception, but its sensitivity is moderately constant from protein to protein, and it has been so widely used that Lowry protein estimations are a completely acceptable alternative to a rigorous absolute determination in almost all circumstances where protein mixtures or crude extracts are involved.

The method is based on both the Biuret reaction, where the peptide bonds of proteins react with copper under alkaline conditions producing Cu^{+}, which reacts with the Folin reagent, and the Folin–Ciocalteau reaction, which is poorly understood but in essence phosphomolybdotungstate is reduced to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids. The reactions result in a strong blue color, which depends partly on the tyrosine and tryptophan content. The method is sensitive down to about 0.01 mg of protein/mL, and is best used on solutions with concentrations in the range 0.01–1.0 mg/mL of protein.

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2. Materials

1. Complex-forming reagent: Prepare immediately before use by mixing the following three stock solutions A, B, and C in the proportion 100:1:1 (v:v:v), respectively.

   Solution A: 2% (w/v) Na_2CO_3 in distilled water.
   Solution B: 1% (w/v) $CuSO_4 \cdot 5H_2O$ in distilled water.
   Solution C: 2% (w/v) sodium potassium tartrate in distilled water.

2. 2*N* NaOH.
3. Folin reagent (commercially available): Use at 1*N* concentration.
4. Standards: Use a stock solution of standard protein (e.g., bovine serum albumin fraction V) containing 4 mg/mL protein in distilled water stored frozen at –20°C. Prepare standards by diluting the stock solution with distilled water as follows:

Stock solution, µL	0	1.25	2.50	6.25	12.5	25.0	62.5	125	250
Water, µL	500	499	498	494	488	475	438	375	250
Protein conc., µg/mL	0	10	20	50	100	200	500	1000	2000

3. Method

1. To 0.1 mL of sample or standard (*see* Notes 1–3), add 0.1 mL of 2*N* NaOH. Hydrolyze at 100°C for 10 min in a heating block or boiling water bath.
2. Cool the hydrolyzate to room temperature and add 1 mL of freshly mixed complex-forming reagent. Let the solution stand at room temperature for 10 min (*see* Notes 4 and 5).
3. Add 0.1 mL of Folin reagent, using a vortex mixer, and let the mixture stand at room temperature for 30–60 min (do not exceed 60 min) (*see* Note 6).
4. Read the absorbance at 750 nm if the protein concentration was below 500 µg/mL or at 550 nm if the protein concentration was between 100 and 2000 µg/mL.
5. Plot a standard curve of absorbance as a function of initial protein concentration and use it to determine the unknown protein concentrations (*see* Notes 7–10).

4. Notes

1. If the sample is available as a precipitate, then dissolve the precipitate in 2*N* NaOH and hydrolyze as in step 1. Carry 0.2-mL aliquots of the hydrolyzate forward to step 2.

2. Whole cells or other complex samples may need pretreatment, as described for the Burton assay for DNA *(2)*. For example, the PCA/ethanol precipitate from extraction I may be used directly for the Lowry assay, or the pellets remaining after the PCA hydrolysis step (step 3 of the Burton assay) may be used for Lowry. In this latter case, both DNA and protein concentration may be obtained from the same sample.
3. Peterson *(3)* has described a precipitation step that allows the separation of the protein sample from interfering substances and also consequently concentrates the protein sample, allowing the determination of proteins in dilute solution. Peterson's precipitation step is as follows:
   a. Add 0.1 mL of 0.15% deoxycholate to 1.0 mL of protein sample.
   b. Vortex, and stand at room temperature for 10 min.
   c. Add 0.1 mL of 72% TCA, vortex, and centrifuge at 1000–3000*g* for 30 min.
   d. Decant the supernatant and treat the pellet as described in Note 1.
4. The reaction is very pH-dependent, and it is therefore important to maintain the pH between 10 and 10.5. Take care, therefore, when analyzing samples that are in strong buffer outside this range.
5. The incubation period is not critical and can vary from 10 min to several hours without affecting the final absorbance.
6. The vortex step is critical for obtaining reproducible results. The Folin reagent is only reactive for a short time under these alkaline conditions, being unstable in alkali, and great care should therefore be taken to ensure thorough mixing.
7. The assay is not linear at higher concentrations. Ensure, therefore, that you are analyzing your sample on the linear portion of the calibration curve.
8. A set of standards is needed with each group of assays, preferably in duplicate. Duplicate or triplicate unknowns are recommended.
9. One disadvantage of the Lowry method is the fact that a range of substances interferes with this assay, including buffers, drugs, nucleic acids, and sugars. The effect of some of these agents is shown in Table 1 in Chapter 2. In many cases, the effects of these agents can be minimized by diluting them out, assuming that the protein concentration is sufficiently high to still be detected after dilution. When interfering compounds are involved, it is, of course, important to run an appropriate blank. Interference caused by detergents, sucrose, and EDTA can be eliminated by the addition of SDS *(4)*.
10. Modifications to this basic assay have been reported that increase the sensitivity of the reaction. If the Folin reagent is added in two portions, vortexing between each addition, a 20% increase in sensitivity is

achieved *(5)*. The addition of dithiothreitol 3 min after the addition of the Folin reagent increases the sensitivity by 50% *(6)*.

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CHAPTER 2

The Bicinchoninic Acid (BCA) Assay for Protein Quantitation

John M. Walker

1. Introduction

The bicinchoninic acid (BCA) assay, first described by Smith et al. *(1)* is similar to the Lowry assay, since it also depends on the conversion of Cu^{2+} to Cu^{+} under alkaline conditions (*see* Chapter 1). The Cu^{+} is then detected by reaction with BCA. The two assays are of similar sensitivity, but since BCA is stable under alkali conditions, this assay has the advantage that it can be carried out as a one-step process compared to the two steps needed in the Lowry assay. The reaction results in the development of an intense purple color with an absorbance maximum at 562 nm. Since the production of Cu^{+} in this assay is a function of protein concentration and incubation time, the protein content of unknown samples may be determined spectrophotometrically by comparison with known protein standards. A further advantage of the BCA assay is that it is generally more tolerant to the presence of compounds that interfere with the Lowry assay. In particular it is not affected by a range of detergents and denaturing agents such as urea and guanidinium chloride, although it is more sensitive to the presence of reducing sugars. Both a standard assay (0.1–1.0 mg protein/mL) and a microassay (0.5–10 µg protein/mL) are described.

2. Materials

2.1. Standard Assay

1. Reagent A: sodium bicinchoninate (0.1 g), $Na_2CO_3 \cdot H_2O$ (2.0 g), sodium tartrate (dihydrate) (0.16 g), NaOH (0.4 g), $NaHCO_3$ (0.95 g), made up

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Edited by: J. M. Walker

to 100 mL. If necessary, adjust the pH to 11.25 with $NaHCO_3$ or NaOH (*see* Note 1).
2. Reagent B: $CuSO_4 \cdot 5H_2O$ (0.4 g) in 10 mL of water (*see* Note 1).
3. Standard working reagent (SWR): Mix 100 vol of regent A with 2 vol of reagent B. The solution is apple green in color and is stable at room temperature for 1 wk.

2.2. Microassay

1. Reagent A: $Na_2CO_3 \cdot H_2O$ (0.8 g), NaOH (1.6 g), sodium tartrate (dihydrate) (1.6 g), made up to 100 mL with water, and adjusted to pH 11.25 with 10*M* NaOH.
2. Reagent B: BCA (4.0 g) in 100 mL of water.
3. Reagent C: $CuSO_4 \cdot 5H_2O$ (0.4 g) in 10 mL of water.
4. Standard working reagent (SWR): Mix 1 vol of reagent C with 25 vol of reagent B, then add 26 vol of reagent A.

3. Methods

3.1. Standard Assay

1. To a 100-µL aqueous sample containing 10–100 µg protein, add 2 mL of SWR. Incubate at 60°C for 30 min (*see* Note 2).
2. Cool the sample to room temperature, then measure the absorbance at 562 nm (*see* Note 3).
3. A calibration curve can be constructed using dilutions of a stock 1 mg/mL solution of bovine serum albumin (BSA) (*see* Note 4).

3.2. Microassay

1. To 1.0 mL of aqueous protein solution containing 0.5–1.0 µg of protein/mL, add 1 mL of SWR.
2. Incubate at 60°C for 1 h.
3. Cool, and read the absorbance at 562 nm.

4. Notes

1. Reagents A and B are stable indefinitely at room temperature. They may be purchased ready prepared from Pierce, Rockford, IL.
2. The sensitivity of the assay can be increased by incubating the samples longer. Alternatively, if the color is becoming too dark, heating can be stopped earlier. Take care to treat standard samples similarly.
3. Following the heating step, the color developed is stable for at least 1 h.
4. Note, that like the Lowry assay, response to the BCA assay is dependent on the amino acid composition of the protein, and therefore an absolute concentration of protein cannot be determined. The BSA stan-

Table 1
Effect of Selected Potential Interfering Compounds[a]

Sample (50 µg BSA) in the following	BCA assay (µg BSA found)		Lowry assay (µg BSA found)	
	Water blank corrected	Interference blank corrected	Water blank corrected	Interference blank corrected
50 µg BSA in water (reference)	50.00	—	50 00	—
0.1*N* HCl	50.70	50.80	44.20	43.80
0.1*N* NaOH	49.00	49.40	50.60	50.60
0.2% Sodium azide	51.10	50 90	49 20	49.00
0.02% Sodium azıde	51.10	51 00	49 50	49 60
1.0*M* Sodium chloride	51.30	51.10	50.20	50 10
100 m*M* EDTA (4 Na)	No color		138.50	5.10
50 m*M* EDTA (4 Na)	28.00	29.40	96.70	6.80
10 m*M* EDTA (4 Na)	48.80	49.10	33.60	12.70
50 m*M* EDTA (4 Na), pH 11 25	31 50	32.80	72.30	5.00
4.0*M* Guanidine HCl	48.30	46.90	Precipitated	
3.0*M* Urea	51.30	50.10	53.20	45.00
1 0%Triton X-100	50.20	49.80	Precipitated	
1.0% SDS (lauryl)	49.20	48.90	Precipitated	
1 0% Brij 35	51.00	50 90	Precipıtated	
1.0% Lubrol	50.70	50.70	Precipitated	
1.0% Chaps	49 90	49.50	Precipıtated	
1.0% Chapso	51.80	51.00	Precipitated	
1.0% Octyl glucoside	50.90	50.80	Precipitated	
40.0% Sucrose	55.40	48.70	4.90	28.90
10.0% Sucrose	52 50	50.50	42 90	41 10
1.0% Sucrose	51 30	51.20	48 40	48 10
100 m*M* Glucose	245 00	57.10	68.10	61.70
50 mM Glucose	144.00	47.70	62.70	58.40
10 m*M* Glucose	70.00	49.10	52.60	51.20
0 2*M* Sorbitol	42.90	37.80	63.70	31.00
0.2*M* Sorbitol, pH 11 25	40.70	36.20	68.60	26.60
1 0*M* Glycine	No color		7.30	7.70
1.0*M* Glycıne, pH 11	50.70	48.90	32.50	27.90
0.5*M* Tris	36.20	32.90	10.20	8.80
0.25*M* Tris	46.60	44.00	27.90	28.10
0.1*M* Tris	50.80	49 60	38.90	38.90
0.25*M* Tris, pH 11 25	52.00	50.30	40 80	40.80
20.0% Ammonium sulfate	5 60	1.20	Precipitated	
10 0% Ammonium sulfate	16.00	12.00	Precipitated	
3.0% Ammonium sulfate	44.90	42 00	21.20	21.40
10.0% Ammonıum sulfate, pH 11	48.10	45.20	32 60	32 80
2.0*M* Sodıum acetate, pH 5 5	35.50	34.50	5.40	3 30
0 2*M* Sodium acetate, pH 5.5	50.80	50 40	47.50	47.60
1.0*M* Sodıum phosphate	37.10	36.20	7.30	5.30
0.1*M* Sodium phosphate	50 80	50 40	46.60	46.60
0.1*M* Cesium bicarbonate	49.50	49.70	Precipitated	

[a]Reproduced from ref. *1* with permission from Academic Press Inc.

dard curve can only therefore be used to compare the relative protein concentration of similar protein solutions.

5. Some reagents interfere with the BCA assay, but nothing like as many as with the Lowry assay (*see* Table 1). The presence of lipids gives excessively high absorbances with this assay *(2)*. Variations produced by buffers with sulfhydryl agents and detergents have been described *(3)*.
6. Since the method relies on the use of Cu^{2+}, the presence of chelating agents such as EDTA will of course severely interfere with the method. However, it may be possible to overcome such problems by diluting the sample as long as the protein concentration remains sufficiently high to be measurable. Similarly, dilution may be a way of coping with *any* agent that interferes with the assay (*see* Table 1). In each case it is of course necesary to run an appropriate control sample to allow for any residual color development. A modification of the assay has been described that overcomes lipid interference when measuring lipoprotein protein content *(4)*.
7. A modification of the BCA assay, utilizing a microwave oven, has been described that allows protein determination in a matter of seconds *(5)*.

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CHAPTER 3

The Bradford Method for Protein Quantitation

Nicholas J. Kruger

1. Introduction

A rapid and accurate method for the estimation of protein concentration is essential in many fields of protein study. An assay originally described by Bradford *(1)* has become the preferred method for quantifying protein in many laboratories. This technique is simpler, faster, and more sensitive than the Lowry method. Moreover, when compared with the Lowry method, it is subject to less interference by common reagents and nonprotein components of biological samples (*see* Note 1).

The Bradford assay relies on the binding of the dye Coomassie blue G250 to protein. The cationic form of the dye, which predominates in the acidic assay reagent solution, has a λ max of 470 nm. In contrast, the anionic form of the dye, which binds to protein, has a λ max of 595 nm *(2)*. Thus, the amount of dye bound to the protein can be quantified by measuring the absorbance of the solution at 595 nm.

The dye appears to bind most readily to arginyl residues of proteins (but does not bind to the free amino acid) *(2)*. This specificity can lead to variation in the response of the assay to different proteins, which is the main drawback of the method. The original Bradford assay shows large variation in response between different proteins *(3–5)*. Several modifications to the method have been developed to overcome this problem (*see* Note 2). However, these changes generally result in a less robust assay that is often more susceptible to

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Edited by J M Walker

interference by other chemicals. Consequently, the original method devised by Bradford remains the most convenient and widely used formulation. Two types of assay are described here: the standard assay, which is suitable for measuring between 10–100 µg protein, and the microassay for detecting between 1–10 µg protein.

2. Materials

1. Reagent: The assay reagent is made by dissolving 100 mg of Coomassie blue G250 in 50 mL of 95% ethanol. The solution is then mixed with 100 mL of 85% phosphoric acid and made up to 1 L with distilled water (*see* Note 3).

   The reagent should be filtered through Whatman No. 1 filter paper and then stored in an amber bottle at room temperature. It is stable for several weeks. However, during this time dye may precipitate from the solution and so the stored reagent should be filtered before use.
2. Protein standard (*see* Note 4). Bovine γ-globulin at a concentration of 1 mg/mL (100 µg/mL for the microassay) in distilled water is used as a stock solution. This should be stored frozen at –20°C. Since moisture content of solid protein may vary during storage, the precise concentration of protein in the standard solution should be determined from its absorbance at 280 nm. The absorbance of a 1 mg/mL solution of γ-globulin, in a 1-cm light path, is 1.35. The corresponding values for two alternative protein standards, bovine serum albumin and ovalbumin, are 0.66 and 0.75, respectively.
3. Plastic and glassware used in the assay should be absolutely clean and detergent-free. Quartz (silica) spectrophotometer cuvets should not be used, since the dye binds to this material. Traces of dye bound to glassware or plastic can be removed by rinsing with methanol or detergent solution.

3. Methods

3.1. Standard Assay Method

1. Pipet between 10 and 100 µg of protein in 100 µL total volume into a test tube. If the approximate sample concentration is unknown, assay a range of dilutions (1, 1/10, 1/100, 1/1000). Prepare duplicates of each sample.
2. For the calibration curve, pipet duplicate volumes of 10, 20, 40, 60, 80, and 100 µL of 1 mg/mL γ-globulin standard solution into test tubes, and make each up to 100 µL with distilled water. Pipet 100 µL of distilled water into a further tube to provide the reagent blank.

3. Add 5 mL of protein reagent to each tube and mix well by inversion or gentle vortexing. Avoid foaming, which will lead to poor reproducibility.
4. Measure the A_{595} of the samples and standards against the reagent blank between 2 min and 1 h after mixing (*see* Note 5). The 100 µg standard should give an A_{595} value of about 0.4. The standard curve is not linear and the precise absorbance varies depending on the age of the assay reagent. Consequently, it is essential to construct a calibration curve for each set of assays (*see* Note 6).

3.2. Microassay Method

This form of the assay is more sensitive to protein. Consequently, it is useful when the amount of the unknown protein is limited (*see* Note 7).

1. Pipet duplicate samples containing between 1–10 µg in a total volume of 100 µL into 1.5-mL polyethylene microfuge tubes. If the approximate sample concentration is unknown, assay a range of dilutions (1, 1/10, 1/100, 1/1000).
2. For the calibration curve, pipet duplicate volumes of 10, 20, 40, 60, 80, and 100 µL of 100 µg/mL γ-globulin standard solution into microfuge tubes, and adjust the volume to 100 µL with water. Pipet 100 µL of distilled water into a tube for the reagent blank.
3. Add 1 mL of protein reagent to each tube and mix gently, but thoroughly. Measure the absorbance of each sample between 2–60 min after addition of the protein reagent. The A_{595} value of a sample containing 10 µg γ-globulin is 0.45. Figure 1 shows the response of three common protein standards using the microassay method.

4. Notes

1. The Bradford assay is relatively free from interference by most commonly used biochemical reagents. However, a few chemicals may significantly alter the absorbance of the reagent blank or modify the response of proteins to the dye (Table 1). The materials that are most likely to cause problems in biological extracts are detergents and ampholytes *(2,6)*. These should be removed from the sample solution, for example, by gel filtration or dialysis. Alternatively, they should be included in the reagent blank and calibration standards at the same concentration as in the sample. The presence of base in the assay increases absorbance by shifting the equilibrium of the free dye toward the anionic form. This may present problems when measuring protein

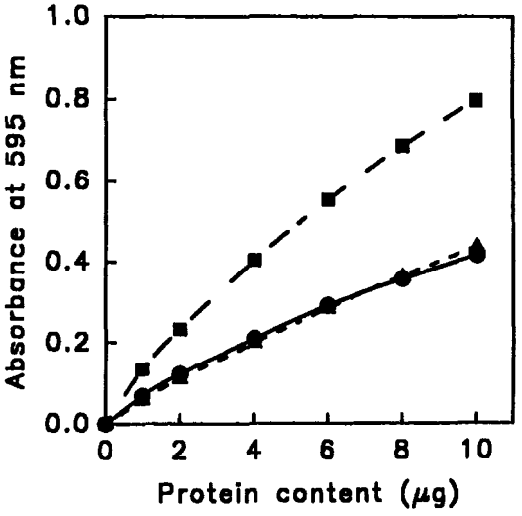

Fig. 1. Variation in the response of proteins in the Bradford assay. The extent of protein–dye complex formation was determined for bovine serum albumin (■), γ-globulin (●), and ovalbumin (▲) using the microassay. Each value is the mean of four determinations. These data allow comparisons to be made between estimates of protein content obtained using these protein standards.

content in concentrated basic buffers *(2)*. Guanidine hydrochloride and sodium ascorbate compete with dye for protein, leading to underestimation of the protein content *(2)*.

2. The assay technique described here is subject to variation in sensitivity between individual proteins (*see* Table 2). Several modifications have been suggested that reduce this variability *(3–5,7)*. Generally, these rely on increasing either the dye content or the pH of the solution. In one variation, adjusting the pH by adding NaOH to the reagent improves the sensitivity of the assay and greatly reduces the variation observed with different proteins *(5)*. However, the optimum pH is critically dependent on the source and concentration of the dye (*see* Note 3). Moreover, the modified assay is far more sensitive to interference from detergents in the sample.
3. The amount of soluble dye in Coomassie blue G250 varies considerably between sources, and suppliers' figures for dye purity are not a reliable estimate of the Coomassie blue G250 content *(8)*. Generally, Serva blue G is regarded to have the greatest dye content and should be used in the modified assays discussed in Note 2. However, the quality of the dye is not critical for routine protein determination using the

Table 1
Effects of Common Reagents on the Bradford Assay[a]

Compound	Absorbance at 600 nm	
	Blank	5 μg Immunoglobulin
Control	0.005	0.264
0.02% SDS	0.003	0.250
0.1% SDS	0.042*	0.059*
0.1% Triton	0.000	0.278
0.5% Triton	0.051*	0.311*
1*M* β-Mercaptoethanol	0.006	0.273
1*M* Sucrose	0.008	0.261
4*M* Urea	0.008	0.261
4*M* NaCl	–0.015	0.207*
Glycerol	0 014	0.238*
0.1*M* HEPES (pH 7.0)	0.003	0.268
0.1*M* Tris (pH 7.5)	–0.008	0.261
0.1*M* Citrate (pH 5.0)	0.015	0.249
10 m*M* EDTA	0.007	0.235*
1*M* $(NH_4)_2SO_4$	0.002	0 269

[a]Data were obtained by mixing 5 μL of sample with 5 μL of the specified compound before adding 200 μL of dye-reagent. Data taken from ref. *5*.

*The asterisks indicate measurements that differ from the control by more than 0.02 absorbance unit for blank values or more than 10% for the samples containing protein.

method described in this chapter. The data presented in Fig. 1 were obtained using Coomassie brilliant blue G (C.I. 42655; Product code B-0770, Sigma Chemical Co., St. Louis, MO).

4. Whenever possible the protein used to construct the calibration curve should be the same as that being determined. Often this is impractical and the dye-response of a sample is quantified relative to that of a "generic" protein. Bovine serum albumin is commonly used as the protein standard because it is inexpensive and readily available in a pure form. The major argument for using this protein is that it allows the results to be compared directly with those of the many previous studies that have used bovine serum albumin as a standard. However, it suffers from the disadvantage of exhibiting an unusually large dye-response in the Bradford assay and, thus, may underestimate the protein content of a sample. Increasingly, bovine γ-globulin is being advanced as a more suitable general standard since the dye binding

Table 2
Comparison of the Response
of Different Proteins in the Bradford Assay

Protein[a]	Relative absorbance	
	Assay 1	Assay 2
Myelin basic protein	139	—
Histone	130	175
Cytochrome c	128	142
Bovine serum albumin	100	100
Insulin	89	—
Transferrin	82	—
Lysozyme	73	—
α-Chymotrypsinogen	55	—
Soybean trypsin inhibitor	52	23
Ovalbumin	49	23
γ-Globulin	48	55
β-Lactoglobulin A	20	—
Trypsin	18	15
Aprotinin	13	—
Gelatin	—	5
Gramicidin S	5	—

[a]For each protein, the response is expressed relative to that of the same concentration of bovine serum albumin. The data for Assays 1 and 2 are recalculated from refs. *3* and *5*, respectively.

capacity of this protein is closer to the mean of those proteins that have been compared (Table 2). Because of this variation, it is essential to specify the protein standard used when reporting measurements of protein amounts using the Bradford assay.

5. Generally, it is preferable to use a single new disposable polystyrene semimicro cuvet that is discarded after a series of absorbance measurements. Rinse the cuvet with reagent before use, zero the spectrophotometer on the reagent blank and then do not remove the cuvet from the machine. Replace the sample in the cuvet gently using a disposable polyethylene pipet.
6. The standard curve is nonlinear at high protein levels because the amount of free dye becomes depleted. If this presents problems, the linearity of the assay can be improved by plotting the ratio of absorbances at 595 and 465 nm, which corrects for depletion of the free dye *(9)*.

7. For routine measurement of the protein content of many samples the microassay may be adapted for use with a microplate reader *(5,10)*. The total volume of the modified assay is limited to 210 µL by reducing the volume of each component. Ensure effective mixing of the assay components by pipeting up to 10 µL of the protein sample into each well before adding 200 µL of the dye-reagent.

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CHAPTER 4

Nondenaturing Polyacrylamide Gel Electrophoresis of Proteins

John M. Walker

1. Introduction

SDS-PAGE (Chapter 5) is probably the most commonly used gel electrophoretic system for analyzing proteins. However, it should be stressed that this method separates denatured protein. Sometimes one needs to analyze native, nondenatured proteins, particularly if wanting to identify a protein in the gel by its biological activity (for example, enzyme activity, receptor binding, antibody binding, and so on). On such occasions it is necessary to use a nondenaturing system such as described in this chapter. For example, when purifying an enzyme, a single major band on a gel would suggest a pure enzyme. However this band could still be a contaminant; the enzyme could be present as a weaker (even nonstaining) band on the same gel. Only by showing that the major band had enzyme activity would you be convinced that this band corresponded to your enzyme. The method described here is based on the gel system first described by Davis *(1)*. To enhance resolution a stacking gel can be included (*see* Chapter 5 for the theory behind the stacking gel system).

2. Materials

1. Stock acrylamide solution: 30 g acrylamide, 0.8 g *bis*-acrylamide. Make up to 100 mL in distilled water and filter. Stable at 4°C for months (*see* Note 1). **Care: Acrylamide Monomer Is a Neurotoxin.** Take care in handling acrylamide (wear gloves) and avoid breathing in acrylamide dust when weighing out.

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Edited by: J M Walker

2. Separating gel buffer: 1.5*M* Tris-HCl, pH 8.8.
3. Stacking gel buffer: 0.5*M* Tris-HCl, pH 6.8.
4. 10% Ammonium persulfate in water.
5. *N,N,N',N'*-tetramethylethylenediamine (TEMED).
6. Sample buffer (5X). Mix the following:
   a. 15.5 mL of 1*M* Tris-HCl pH 6.8;
   b. 2.5 mL of a 1% solution of bromophenol blue;
   c. 7 mL of water; and
   d. 25 mL of glycerol.

   Solid samples can be dissolved directly in 1X sample buffer. Samples already in solution should be diluted accordingly with 5X sample buffer to give a solution that is 1X sample buffer. Do not use protein solutions that are in a strong buffer that is not near to pH 6.8 as it is important that the sample is at the correct pH. For these samples it will be necessary to dialyze against 1X sample buffer.
7. Electrophoresis buffer: Dissolve 3.0 g of Tris base and 14.4 g of glycine in water and adjust the volume to 1 L. The final pH should be 8.3.
8. Protein stain: 0.25 g Coomassie brilliant blue R250 (or PAGE blue 83), 125 mL methanol, 25 mL glacial acetic acid, and 100 mL water.

   Dissolve the dye in the methanol component first, then add the acid and water. Dye solubility is a problem if a different order is used. Filter the solution if you are concerned about dye solubility. For best results do not reuse the stain.
9. Destaining solution: 100 mL methanol, 100 mL glacial acetic acid, and 800 mL water.
10. A microsyringe for loading samples.

3. Method

1. Set up the gel cassette.
2. To prepare the separating gel (*see* Note 2) mix the following in a Buchner flask: 7.5 mL stock acrylamide solution, 7.5 mL separating gel buffer, 14.85 mL water, and 150 µL 10% ammonium persulfate.

   "Degas" this solution under vacuum for about 30 s. This degassing step is necessary to remove dissolved air from the solution, since oxygen can inhibit the polymerization step. Also, if the solution has not been degassed to some extent, bubbles can form in the gel during polymerization, which will ruin the gel. Bubble formation is more of a problem in the higher percentage gels where more heat is liberated during polymerization.
3. Add 15 µL of TEMED and gently swirl the flask to ensure even mixing. The addition of TEMED will initiate the polymerization reaction,

and although it will take about 20 min for the gel to set, this time can vary depending on room temperature, so it is advisable to work fairly quickly at this stage.

4. Using a Pasteur (or larger) pipet, transfer the separating gel mixture to the gel cassette by running the solution carefully down one edge between the glass plates. Continue adding this solution until it reaches a position 1 cm from the bottom of the sample loading comb.
5. To ensure that the gel sets with a smooth surface, *very carefully* run distilled water down one edge into the cassette using a Pasteur pipet. Because of the great difference in density between the water and the gel solution, the water will spread across the surface of the gel without serious mixing. Continue adding water until a layer about 2 mm exists on top of the gel solution.
6. The gel can now be left to set. When set, a very clear refractive index change can be seen between the polymerized gel and overlaying water.
7. While the separating gel is setting, prepare the following stacking gel solution. Mix the following quantities in a Buchner flask: 1.5 mL stock acrylamide solution, 3.0 mL stacking gel buffer, 7.4 mL water, and 100 µL 10% ammonium persulfate. Degas this solution as before.
8. When the separating gel has set, pour off the overlaying water. Add 15 µL of TEMED to the stacking gel solution and use some (~2 mL) of this solution to wash the surface of the polymerized gel. Discard this wash, then add the stacking gel solution to the gel cassette until the solution reaches the cutaway edge of the gel plate. Place the well-forming comb into this solution and leave to set. This will take about 30 min. Refractive index changes around the comb indicate that the gel has set. It is useful at this stage to mark the positions of the bottoms of the wells on the glass plates with a marker pen.
9. Carefully remove the comb from the stacking gel, remove any spacer from the bottom of the gel cassette, and assemble the cassette in the electrophoresis tank. Fill the top reservoir with electrophoresis buffer ensuring that the buffer fully fills the sample loading wells, and look for any leaks from the top tank. If there are no leaks, fill the bottom tank with electrophoresis buffer, then tilt the apparatus to dispel any bubbles caught under the gel.
10. Samples can now be loaded onto the gel. Place the syringe needle through the buffer and locate it just above the bottom of the well. Slowly deliver the sample (~5–20 µL) into the well. The dense sample solvent ensures that the sample settles to the bottom of the loading well. Continue in this way to fill all the wells with unknowns or standards, and record the samples loaded.

11. The power pack is now connected to the apparatus and a current of 20–25 mA passed through the gel (constant current) (*see* Note 3). Ensure that the electrodes are arranged so that the proteins are running to the anode (*see* Note 4). In the first few minutes the samples will be seen to concentrate as a sharp band as it moves through the stacking gel. (It is actually the bromophenol blue that one is observing, not the protein but, of course, the protein is stacking in the same way.) Continue electrophoresis until the bromophenol blue reaches the bottom of the gel. This will usually take about 3 h. Electrophoresis can now be stopped and the gel removed from the cassette. Remove the stacking gel and immerse the separating gel in stain solution, or proceed to step 13 if you wish to detect enzyme activity (*see* Notes 5 and 6).
12. Staining should be carried out, with shaking, for a minimum of 2 h and preferably overnight. When the stain is replaced with destain, stronger bands will be immediately apparent and weaker bands will appear as the gel destains. Destaining can be speeded up by using a foam bung, such as those used in microbiological flasks. Place the bung in the destain and squeeze it a few times to expel air bubbles and ensure the bung is fully wetted. The bung rapidly absorbs dye, thus speeding up the destaining process.
13. If proteins are to be detected by their biological activity, duplicate samples should be run. One set of samples should be stained for protein and the other set for activity. Most commonly one would be looking for enzyme activity in the gel. This is achieved by washing the gel in an appropriate enzyme substrate solution that results in a colored product appearing in the gel at the site of the enzyme activity (*see* Note 7).

4. Notes

1. The stock acrylamide used here is the same as used for SDS gels (*see* Chapter 5) and may already be availabe in your laboratory.
2. The system described here is for a 7.5% acrylamide gel, which was originally described for the separation of serum proteins *(1)*. Since separation in this system depends on both the native charge on the protein *and* separation according to size owing to frictional drag as the proteins move through the gel, it is not possible to predict the electrophoretic behavior of a given protein the way that one can on an SDS gel, where separation is based on size alone. A 7.5% gel is a good starting point for unknown proteins. Proteins of mol wt >100,000 should be separated in 3–5% gels. Gels in the range 5–10% will separate proteins in the range 20,000–150,000, and 10–15% gels will separate proteins in the range 10,000–80,000. The separation of smaller polypeptides is

described in Chapter 8. To alter the acrylamide concentration, adjust the volume of stock acrylamide solution in Section 3., step 2 accordingly, and increase/decrease the water component to allow for the change in volume. For example, to make a 5% gel change the stock acrylamide to 5 mL and increase the water to 17.35 mL. The final volume is still 30 mL, so 5 mL of the 30% stock acrylamide solution has been diluted in 30 mL to give a 5% acrylamide solution.

3. Because we are separating native proteins, it is important that the gel does not heat up too much, since this could denature the protein in the gel. It is advisable therefore to run the gel in the cold room, or to circulate the buffer through a cooling coil in ice. (Many gel apparatus are designed such that the electrode buffer cools the gel plates.) If heating is thought to be a problem it is also worthwhile to try running the gel at a lower current for a longer time.
4. This separating gel system is run at pH 8.8. At this pH most proteins will have a negative charge and will run to the anode. However, it must be noted that any basic proteins will migrate in the opposite direction and will be lost from the gel. Basic proteins are best analyzed under acid conditions, as described in Chapter 7.
5. It is important to note that concentration in the stacking gel *may* cause aggregation and precipitation of proteins. Also, the pH of the stacking gel (pH 6.8) may affect the activity of the protein of interest. If this is thought to be a problem (e.g., the protein cannot be detected on the gel), prepare the gel without a stacking gel. Resolution of proteins will not be quite so good, but will be sufficient for most uses.
6. If the buffer system described here is unsuitable (e.g., the protein of interest does not electrophorese into the gel because it has the incorrect charge, or precipitates in the buffer, or the buffer is incompatible with your detection system) then one can try different buffer systems (without a stacking gel). A comprehensive list of alternative buffer systems has been published *(2)*.
7. The most convenient substrates for detecting enzymes in gels are small molecules that freely diffuse into the gel and are converted by the enzyme to a colored or fluorescent product within the gel. However, for many enzymes such convenient substrates do not exist, and it is necessary to design a linked assay where one includes an enzyme together with the substrate such that the products of the enzymatic reaction of interest is converted to a detectable product by the enzyme included with the substrate. Such linked assays may require the use of up to two or three enzymes and substrates to produce a detectable product. In these cases the product is usually formed on the surface of the

gel because the coupling enzymes cannot easily diffuse into the gel. In this case the zymogram technique is used where the substrate mix is added to a cooled (but not solidified) solution of agarose (1%) in the appropriate buffer. This is quickly poured over the solid gel where it quickly sets on the gel. The product of the enzyme assay is therefore formed at the gel–gel interface and does not get washed away. A number of review articles have been published which described methods for detecting enymes in gels *(3–7)*. A very useful list also appears as an appendix in ref. *8*.

8. In addition to the specific problems identified above, the technique is susceptible to the normal problems associated with any polyacrylamide gel electrophoresis system. These problems and the identification of their causes are described in Table 1, Chapter 5.

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CHAPTER 5

SDS Polyacrylamide Gel Electrophoresis of Proteins

Bryan John Smith

1. Introduction

Probably the most widely used technique for analyzing mixtures of proteins is SDS polyacrylamide gel electrophoresis. In this technique, proteins are reacted with the anionic detergent, sodium dodecylsulfate (SDS, or sodium lauryl sulfate) to form negatively charged complexes. The amount of SDS bound by a protein, and so the charge on the complex, is roughly proportional to its size. Commonly, about 1.4 g SDS is bound per 1 g protein, although there are exceptions to this rule. The proteins are generally denatured and solubilized by their binding of SDS, and the complex forms a prolate elipsoid or rod of length roughly proportionate to the protein's mol wt. Thus, proteins of either acidic or basic p*I* form negatively charged complexes that can be separated on the bases of differences in charges and sizes by electrophoresis through a sieve-like matrix of polyacrylamide gel.

This is the basis of the SDS gel system, but it owes its popularity to its excellent powers of resolution that derive from the use of a "stacking gel." This system employs the principles of isotachophoresis, which effectively concentrates samples from large volumes (within reason) into very small zones, that then leads to better separation of the different species. The system is set up by making a stacking gel on top of the "separating gel," which is of a different pH. The sample is introduced to the system at the stacking gel. With an electric field

From: *Methods in Molecular Biology, Vol 32: Basic Protein and Peptide Protocols*
Edited by J M Walker

applied, ions move towards the electrodes, but at the pH prevailing in the stacking gel, the protein–SDS complexes have mobilities intermediate between the Cl^- ions (present throughout the system) and glycinate ions (present in the reservoir buffer). The Cl^- ions have the greatest mobility. The following larger ions concentrate into narrow zones in the stacking gel, but are not effectively separated there. When the moving zones reach the separating gel, their respective mobilities change in the pH prevailing there and the glycinate ion front overtakes the protein–SDS complex zones to leave them in a uniformly buffered electric field to separate from each other according to size and charge. More detailed treatments of the theory of isotachophoresis and electrophoresis generally are available in the literature (e.g., *1*).

The system of buffers used in the gel system desrcibed below is that of Laemmli *(2)*, and is used in a polyacrylamide gel of slab shape. This form allows simultaneous electrophoresis of more than one sample, and thus is ideal for comparative purposes.

2. Materials

1. The apparatus required may be made in the workshop, say, to Studier's design *(3)*, or is available from commercial sources. For safety reasons, the design should deny access to the gel or buffers while the circuit is complete. The gel is prepared and run in a narrow chamber formed by two glass plates separated by spacers of narrow strips of perspex or other suitable material, arranged on the side and bottom edges of the plates as indicated in Fig. 1. The thickness of the spacers clearly dictates the thickness of the gel. The sample wells into which the samples are loaded are formed by a template "comb" that extends across the top of the gel and is of the same thickness as the spacers. Typically, the "teeth" on this comb will be 1 cm long, 2–10 mm wide, and separated by 3 mm. The chamber may be sealed along its edges with white petroleum jelly (Vaseline), sticky tape (electrical insulation tape), or silicone rubber tubing between the glass plates. A dc power supply is required.
2. Stock solutions. Chemicals should be analytical reagent (Analar) grade and water should be distilled. Stock solutions should all be filtered. Cold solutions should be warmed to room temperature before use.
   a. Stock acrylamide solution (total acrylamide content, %T = 30% w/v, ratio of crosslinking agent to acrylamide monomer, %C = 2.7% w/w): 73 g acrylamide and 2 g *bis*-acrylamide. Dissolve and make

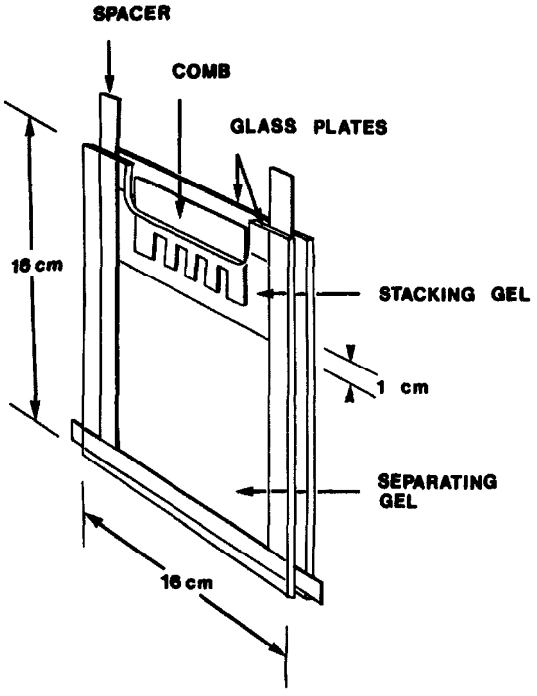

Fig. 1. The construction of a slab gel, showing the positions of the glass plates, the spacers, and the comb.

up to 250 mL in water. This stock solution is stable for weeks in brown glass, at 4°C.

b. Stock separating gel buffer: 1.0 g SDS and 45.5 g Tris buffer [2-amino-2-(hydroxymethyl)- propane-1,3-diol]. Dissolve in <250 mL of water, adjust the pH to 8.8 with HCl, and make the volume to 250 mL. This stock solution is stable for months at 4°C.
c. Stock ammonium persulfate: 1.0 g ammonium persulfate. Dissolve in 10 mL of water. This stock solution is stable for weeks in brown glass, at 4°C.
d. Stock stacking gel buffer: 1.0 g SDS and 15.1 g Tris base. Dissolve in <250 mL of water, adjust the pH to 6.8 with HCl, and make up to 250 mL. Check the pH before use. This stock solution is stable for months at 4°C.
e. Reservoir buffer (0.192*M* glycine, 0.025*M* Tris, 0.1% [w/v] SDS): 28.8 g glycine, 6.0 g Tris base, and 2.0 g SDS. Dissolve and make to

2 L in water. The solution should be at about pH 8.3 without adjustment. This solution is readily made fresh each time.

f. Stock (double strength) sample solvent: 0.92 g SDS, 2 mL β-mercaptoethanol, 4.0 g glycerol, 0.3 g Tris base, and 2 mL bromophenol blue (0.1% [w/v] solution in water). Dissolve in <20 mL of water, adjust the pH to 6.8 with HCl, and make to 20 mL. Check the pH before use. Exposed to oxygen in the air, the reducing power of the β-mercaptoethanol wanes with time. Periodically (after a few weeks) add extra agent or renew the solution. This stock solution is stable for weeks at 4°C.

g. Protein stain: 0.25 g PAGE blue 83, 125 mL methanol, 25 mL glacial acetic acid, and 100 mL water. Dissolve the dye in the methanol component first, then add the acid and water. If dissolved in a different order, the dye's staining behavior may differ. The stain is best used when freshly made. For best results do not reuse the stain—its efficacy declines with use.

h. Destaining solution: 100 mL methanol, 100 mL glacial acetic acid, and 800 mL water. Mix thoroughly. Use when freshly made.

3. Method

1. Thoroughly clean and dry the glass plates and three spacers, then assemble them as shown in Fig. 1, with the spacers set 1–2 mm in from the edes of the glass plates. Hold the construction together with bulldog clips. White petroleum jelly (melted in a boiling water bath) is then applied around the edges of the spacers to hold them in place and seal the chamber. Clamp the chamber in an upright, level position.
2. A sufficient volume of separating gel mixture (say 30 mL for a chamber of about 14 × 14 × 0.1 cm) is prepared as follows. Mix the following: 15 mL stock acrylamide solution and 7.5 mL distilled water. Degas on a water pump, and then add: 7.5 mL stock separating gel buffer, 45 µL stock ammonium persulfate solution, and 15 µL *N,N,N',N'*-tetramethylethylenediamine (TEMED). Mix gently and use immediately (because polymerization starts when the TEMED is added). The degassing stage removes oxygen, which inhibits polymerization by virtue of mopping up free radicals, and also discourages bubble formation caused by warming when the gel polymerizes.
3. Carefully pipet or pour the freshly mixed solution into the chamber without generating air bubbles. Pour to a level about 1 cm below where the bottom of the well-forming comb will come when it is in position. Carefully overlayer the acrylamide solution with butan-2-ol without

mixing (to eliminate oxygen and generate a flat top to the gel). Leave the mixture until it is set (0.5–1.5 h).

4. Prepare stacking gel (5 mL) as follows. Mix the following: 0.75 mL stock acrylamide solution and 3 mL distilled water. Degas on a water pump, then add: 1.25 mL stock stacking gel buffer, 15 µL stock ammonium persulfate solution, and 5 µL TEMED. Mix gently and use immediately.

   Pour off the butan-2-ol from the polymerized separating gel, wash the gel top first with water and then with a little stacking gel mixture, and fill the gap remaining in the chamber with the stacking gel mixture. Insert the comb and allow the gel to stand until set (about 0.5–1 h).
5. When the stacking gel has polymerized, remove the comb without distorting the shapes of the wells. Remove the clips holding the plates together, and install the gel in the apparatus. Fill apparatus with reservoir buffer. The reservoir buffer can be circulated between anode and cathode reservoirs, to equalize their pH values if required, but usually this is not necessary. The buffer can also be cooled (by circulating it through a cooling coil in ice), so that heat evolved during electrophoresis is dissipated and does not affect the size or shape of protein zones (or bands) in the gel. Push out the bottom spacer from the gel and remove bubbles from both the top and underneath of the gel, for they could partially insulate the gel and distort electrophoresis. Check the electrical circuit by turning on the power (dc) briefly, with the cathode at the stacking gel end of the gel (i.e., the top). Use the gel immediately.
6. While the gel is polymerizing (or before making the gel), prepare samples for electrophoresis. A dry sample may be dissolved directly in single-strength sample solvent (i.e., the stock solution diluted twofold with water) or dissolved in water and diluted with 1 vol of stock double-strength sample solvent. The concentration of sample in the solution should be such as to give a sufficient amount of protein in a volume not greater than the size of the sample well. Some proteins may react adequately with SDS within a few minutes at room temperature, but as a general practice, heat sample solutions in boiling water for 2 min. Cool the sample solution before loading it. The bromophenol blue dye indicates when the sample solution is acidic by turning yellow. If this happens, add a little NaOH, enough to just turn the color blue.
7. Load the gel. Take up the required volume of sample solution in a microsyringe or pipet and carefully inject it into a sample well through the reservoir buffer. The amount of sample loaded depends on the method of its detection *(see below)*. Having loaded all samples with-

out delay, start electrophoresis by turning on power (dc). On a gel of about 0.5 –1 mm thickness and about 14 cm length, an applied voltage of about 150 V gives a current of about 20 mA or so (falling during electrophoresis if constant voltage is employed). The bromophenol blue dye front takes about 3 h to reach the bottom of the gel. Greater voltage speeds up electrophoresis, but generates more heat in the gel.

8. At the end of electrophoresis (say, when the dye front reaches the bottom of the gel), protein bands in the gel may be visualized by staining. Remove the gel from between the glass plates and immerse it in the protein stain immediately (although delay of 1 h or so is not noticeably detrimental in a gel of 15%T). The gel is left there with gentle agitation until the dye has penetrated the gel (about 1.5 h for 15%T gels of 0.5–1 mm thickness). Dye that is not bound to protein is removed by transferring the gel to destaining solution. After about 24 h, with gentle agitation and several changes of destaining agent, the gel background becomes colorless and leaves protein bands colored blue, purple, or red. PAGE blue 83 visibly stains as little as 0.1–1 μg of protein in a band of about 1 cm width.

4. Notes

1. The reducing agent in the sample solvent reduces intermolecular disulfide bridges and so destroys quarternary structure and separates subunits, and also reduces intramolecular disulfide bonds to ensure maximal reaction with SDS. The glycerol is present to increase the density of the sample, to aid the loading of it onto the gel. The bromophenol blue dye also aids loading of the sample, by making it visible, and indicates the position of the front of electrophoresis in the gel. The dye also indicates when the sample solution is acidic by turning yellow.
2. The polymerization of acrylamide and *bis*-acrylamide is initiated by the addition of TEMED and persulfate. The persulfate activates the TEMED and leaves it with an unpaired electron. This radical reacts with an acrylamide monomer to produce a new radical that reacts with another monomer, and so on, to build up a polymer. The *bis*-acrylamide is incorporated into polymer chains this way and so forms cross-links between them.
3. The gel system described is suitable for electrophoresis of proteins in the mol wt range of 10,000–100,000. Smaller proteins move at the front or form diffuse, fast-moving bands, whereas larger proteins hardly enter the gel, if at all. Electrophoresis of larger proteins requires gels of larger

pore size, which are made by dilution of the stock acrylamide solution (reduction of %T) or by adjustment of %C (the smallest pore size is at 5%C, whatever the %T). The minimum %T is about 3%, useful for separation of proteins of mol wts of several millions. Such low %T gels are extremely weak and may require strengthening by the inclusion of agarose to 0.5% (w/v). Smaller pore gels, for electrophoresis of small proteins, are prepared by increasing %T and adjustment of %C. Such adjustment of %T and %C may be found empirically to improve resolution of closely migrating species.

A combination of large and small pore gels, suitable for electrophoresis of mixtures of proteins of wide-ranging sizes, can be made in a gradient gel, prepared with use of a gradient-making apparatus when pouring the separating gel (*see* Chapter 6).

4. Since proteins (or rather, their complexes with SDS) are resolved largely on the basis of differences in their sizes, electrophoretic mobility in SDS gels may be used to estimate the mol wt of a protein by comparison with proteins of known size (as described in ref. *1*). However, it should be remembered that some proteins have anomalous SDS-binding properties, and hence anomalous mobilities in SDS gels.
5. If necessary, the gel may be stored for 24 h (preferably in the cold) either as the separating gel only, under a buffer of stock-separating gel buffer diluted fourfold in water, or together with the stacking gel with the comb left in place to prevent drying out.
6. Proteins dissolved in sample solvent are stable for many weeks if kept frozen (at –10°C or below), although repeated freezing and thawing causes protein degradation.
7. The result of electrophoresis in SDS gels ideally has protein(s) as thin, straight band(s) that are well-resolved from other bands. This may not always be so, however. Some faults and their remedies are given in Table 1. Some examples are shown in Fig. 2.
8. Be wary of the dangers of electric shock and of fire, and of the neutrotoxic acrylamide monomer. Ready-made solutions of acrylamide and *bis*-acrylamide are commercially available and reduce the hazards from handling of these agents. Safer still are the ready-made gels that are available commercially. They may come either as the separating gel, to which the user adds the stacking gel (and sample wells), or as a gel without any stacking gel but with wells formed in the separating gel itself. The latter type is sufficient for many purposes but critical separations may require the added benefit that comes from using a stacking gel.

Table 1
Some Problems that May Arise During the Preparation and Use of SDS Gels

Fault	Cause	Remedy
Failure or decreased rate of gel polymerization	Oxygen is present	Degas the solutions
	Stock solutions (especially acrylamide and persulfate) are aged	Renew the stock solutions
Formation of a sticky top to the gel	Penetration of the gel by butan-2-ol	Overlayer of the gel solution with butan-2-ol without mixing them. Do not leave butan-2-ol to stand on a polymerized gel
Poor sample wells	The stacking gel resists the removal of the comb	Remove the comb carefully or use a gel of lower %T
The wells are distorted or broken		
The wells contain a loose webbing of polyacrylamide	The comb fits loosely	Replace the comb with a tighter-fitting one
Unsatisfactory staining		
The staining is weak	The dye is bound inefficiently	Use a more concentrated dye solution, a longer staining time, or a more sensitive stain
		The stain solution should contain organic solvent (e.g., methanol), which strips the SDS from the protein to which the dye may then bind
The staining is uneven	The dye penetration or destaining is uneven	Agitate the gel during staining and destaining. Increase the staining/destaining time
Stained bands become decolorized	The dye has been removed from the protein	Restain the gel. Reduce the destaining time or use a dye that stains proteins indelibly, e.g., Procion navy MXRB (*see* Chapter 14)

(continued)

Table 1 *(continued)*

Fault	Cause	Remedy
The gel is marked nonspecifically by the dye	Solid dye is present in the staining solution	Ensure full dissolution of the dye, or filter the solution before using it
Contaminants are apparent	The apparatus and/or stock solutions are contaminated	Clean or renew them as required
	Nonproteinaceous material in the sample (e.g., nucleic acid) has been stained	Try another stain that will not stain the contaminants
	Samples have crosscontaminated each other because of their overloading or their sideways seepage between the gel layers	Do not overfill sample wells. Ensure good adherence of the gel layers to each other by thorough washing of the polymerized gel before application of subsequent layers
Protein bands are not sufficiently resolved	Insufficient electrophoresis	Prolong the run
	The separating gel's pore size is incorrect	Alter the %T and/or %C of the separating gel
There are small changes in standard proteins' electrophoretic mobilities from time to time	The amounts loaded differ greatly	Keep the loadings roughly similar in size each time
	The constituents of the gel vary in quality from batch to batch or with age	Use one batch of a chemical for as long as possible. Replace aged stock solutions and reagents
Distortion of bands: Bands have become smeared or streaked	Proteins in the sample are insoluble or remain aggregated in the sample solvent	Use fresh sample solvent and/or extra SDS and reducing agent in it (especially for concentrated sample solutions)
	There is insoluable matter or a bubble in the gel that has interfered with protein band migration	Filter the stock solutions before use and remove any bubbles from the gel mixtures
	The pore size of the gel is inconsistent	Ensure that the gel solutions are well

(continued)

Table 1 *(continued)*

Fault	Cause	Remedy
		mixed and that polymerization is not very rapid (to slow it down, reduce the amount of persulfate added)
Protein migration has been uneven (bands are bent)	Part of the gel has been insulated	Remove any bubbles adhering to the gel before electrophoresis
	Electrical leakage	Ensure that the side spacers are in place
	Cooling of the gel is uneven (allowing one part of the gel to run more quickly than another)	Improve the cooling of the gel, or reduce the heating by reducing the voltage or ionic strength of buffers
	The band and/or its neighbors are overloaded	Repeat the electrophoresis, but with smaller loadings. Leave gaps (i.e., unloaded sample wells) between neighboring heavily loaded samples. If necessary, alter the system (e.g., *see* Protein Bands Are Not Sufficiently Resolved) and so the relative mobilities of bands, so that they do not interfere with each other
	The sample well used was at the very end of the row of wells (the "end well effect")	Avoid using the end wells
Bands are not of uniform thickness	The sample was loaded unevenly	Check that the sample well bottoms are straight and horizontal (*see* Poor Sample Wells)

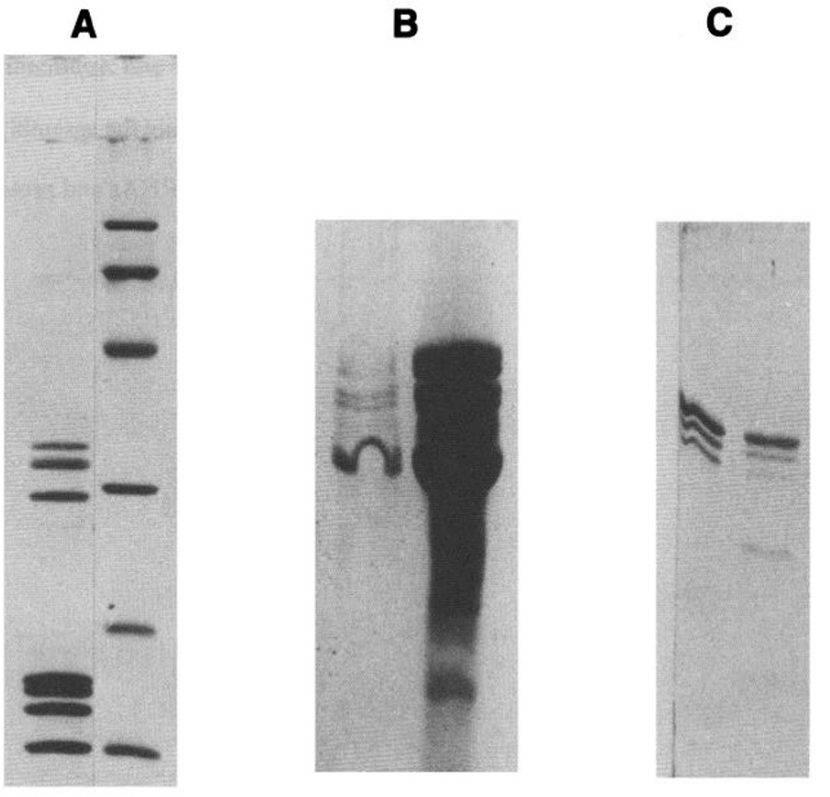

Fig. 2. Examples of proteins electrophoresed on SDS polyacrylamide (15%T) gels and stained with Coomassie brilliant blue R 250 as described in the text. Electrophoresis was from top to bottom. **(A)** Good electrophoresis. Sample, left, 15-μg loading of histone proteins from chicken erythrocyte nuclei. Sample right, a 5-μg loading (total) of mol-wt marker proteins (obtained from Pharmacia, Uppsala, Sweden). The mol wts are, from top to bottom: phosphorylase b, 94,000; albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; trypsin inhibitor, 20,100; α-lactalbumin, 14,400. **(B)** Examples of artifacts. Sample, left, the fastest (bottom) band has distorted as it encountered a region of high polyacrylamide density (which arose during very rapid gel polymerization). Sample, right, the effect on protein overloading of increasing a band's size (the sample proteins are as in sample, left). Extreme overloading may also cause narrowing of faster-migrating bands, as has happened here to some extent (cf fast bands' widths with widths of bands in sample, left). **(C)** Example of an artifact. Sample, left, the "end well effect" of distortion of the sample loaded into the very end well, not seen in samples in other wells (e.g., sample, right).

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Chapter 6

Gradient SDS Polyacrylamide Gel Electrophoresis of Proteins

John M. Walker

1. Introduction

The preparation of fixed-concentration polyacrylamide gels has been described in Chapter 5. However, the use of polyacrylamide gels that have a gradient of increasing acrylamide concentration (and hence decreasing pore size) can sometimes have advantages over fixed-concentration acrylamide gels. During electrophoresis in gradient gels, proteins migrate until the decreasing pore size impedes further progress. Once the "pore limit" is reached, the protein banding pattern does not change appreciably with time, although migration does not cease completely. There are three main advantages of gradient gels over linear gels:

1. The advancing edge of the migrating protein zone is retarded more than the trailing edge, thus resulting in a sharpening of the protein bands.
2. The gradient in pore size increases the range of mol wts that can be fractionated in a single gel run.
3. Proteins with close mol wt values are more likely to separate in a gradient gel than a linear gel.

The usual limits of gradient gels are 3–30% acrylamide in linear or concave gradients. The choice of range will of course depend on the size of proteins being fractionated. The system described here is for a 5–20% linear gradient using SDS polyacrylamide gel electrophoresis. The theory of SDS polyacrylamide gel electrophoresis has been decribed in Chapter 5.

From: *Methods in Molecular Biology, Vol 32: Basic Protein and Peptide Protocols*
Edited by: J. M. Walker

2. Materials

1. Stock acrylamide solution: 30% acrylamide, 0.8% *bis*-acrylamide. Dissolve 75 g of acrylamide and 2.0 g of *N,N'*-methylene *bis*-acrylamide in about 150 mL of water. Filter and make the volume to 250 mL. Store at 4°C. The solution is stable for months.
2. Buffers:
   a. 1.875*M* Tris-HCl, pH 8.8.
   b. 0.6*M* Tris-HCl, pH 6.8.
   Store at 4°C.
3. Ammonium persulfate solution (10% [w/v]). Make fresh as required.
4. SDS solution (10% [w/v]). Stable at room temperature. In cold conditions, the SDS can come out of solution, but may be redissolved by warming.
5. *N,N,N',N'*-Tetramethylene diamine (TEMED).
6. Gradient forming apparatus (*see* Fig. 1). Reservoirs with dimensions of 2.5 cm id and 5.0 cm height are suitable. The two reservoirs of the gradient former should be linked by flexible tubing to allow them to be moved independently. This is necessary since although equal volumes are placed in each reservoir, the solutions differ in their densities and the relative positions of A and B have to be adjusted to balance the two solutions when the connecting clamp is opened.

3. Method

1. Prepare the following solutions:

	Solution A, mL	Solution B, mL
1.875*M* Tris-HCl, pH 8.8	3.0	3.0
Water	9.3	0.6
Stock acrylamide, 30%	2.5	10.0
10% SDS	0.15	0.15
Ammonium persulfate (5%)	0.05	0.05
Sucrose	—	2.2 g (equivalent to 1.2 mL volume)

2. Degas each solution under vacuum for about 30 s and then, when you are ready to form the gradient, add TEMED (12 µL) to each solution.
3. Once the TEMED is added and mixed in, pour solutions A and B into the appropriate reservoirs (*see* Fig. 1.)
4. *With the stirrer stirring,* fractionally open the connection between A and B and adjust the relative heights of A and B such that there is no

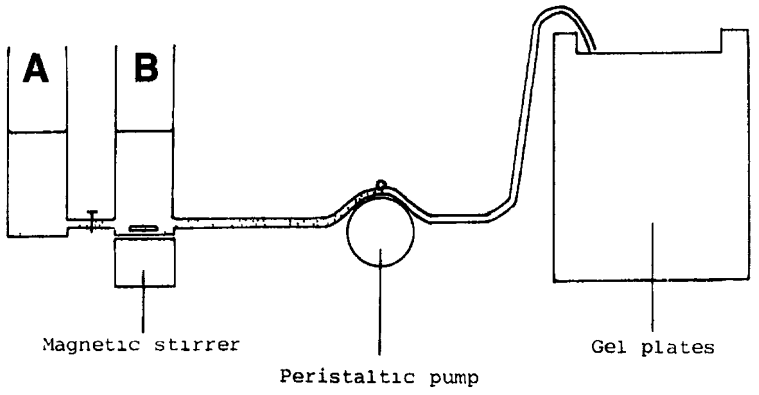

Fig. 1 Diagram of an apparatus for forming gradient gels.

flow of liquid between the two reservoirs (easily seen because of the difference in densities). Do not worry if there is some mixing between reservoirs—this is inevitable.

5. When the levels are balanced, completely open the connection between A and B, turn the pump on, and fill the gel apparatus by running the gel solution down one edge of the gel slab. Surprisingly, very little mixing within the gradient occurs using this method. A pump speed of about 5 mL/min is suitable. If a pump is not available, the gradient may be run into the gel under gravity.
6. When the level of the gel reaches about 3 cm from the top of the gel slab, connect the pump to distilled water, reduce pump speed, and overlay the gel with 3–4 mm of water.
7. The gradient gel is now left to set for 30 min. Remember to rinse out the gradient former before the gel sets in it.
8. Prepare a stacking gel by mixing the following:
   a. 1.0 mL 0.6*M* Tris-HCl, pH 6.8;
   b. 1.35 mL Stock acrylamide;
   c. 7.5 mL Water;
   d. 0.1 mL 10% SDS;
   e. 0.05 mL Ammonium persulfate (10%).
9. Degas this mixture under vacuum for 30 s and then add TEMED (12 µL).
10. Pour off the water overlayering the gel and wash the gel surface with about 2 mL of stacking gel solution and then discard this solution.
11. The gel slab is now filled to the top of the plates with stacking gel solution and the well-forming comb placed in position (*see* Chapter 5).

12. When the stacking gel has set (~15 min), carefully remove the comb. The gel is now ready for running. The conditions of running and sample preparation are exactly as described for SDS gel electrophoresis in Chapter 5.

4. Notes

1. The total volume of liquid in reservoirs A and B should be chosen such that it approximates to the volume available between the gel plates. However, allowance must be made for some liquid remaining in the reservoirs and tubing.
2. As well as a gradient in acrylamide concentration, a density gradient of sucrose (glycerol could also be used) is included to minimize mixing by convectional disturbances caused by heat evolved during polymerization. Some workers avoid this problem by also including a gradient of ammonium persulfate to ensure that polymerization occurs first at the top of the gel, progressing to the bottom. However, we have not found this to be necessary in our laboratory.

Chapter 7

Acetic Acid–Urea Polyacrylamide Gel Electrophoresis of Proteins

Bryan John Smith

1. Introduction

In SDS polyacrylamide gel electrophoresis, proteins are separated essentially on the basis of their sizes, by the sieving effect of the polyacrylamide gel matrix (*see* Chapter 5). In the absence of SDS, the proteins would still be subject to the sieving effect of the gel matrix, but their charges would vary according to their amino acid content. This is because the charge on a protein at any particular pH is the sum of the charges prevailing on the side chain groups of its constituent amino acid residues, and the free amino and carboxyl groups at its termini (although these are relatively trivial in anything other than a very small peptide). Thus, in an ionic detergent-free gel electrophoretic system, both the molecular size and charge act as bases for effective protein separation. The pH prevailing in such a system might be anything, but is commonly about pH 3. Since the pK_a values of the side chain carboxyl groups of aspartic and glutamic acids are about 3.8 and 4.2, respectively, even these amino acids will contribute little to the negative charge on a protein at this pH. Thus at pH 3, all proteins are likely to be positively charged and to travel toward the cathode in an electric field.

In such an acid–polyacrylamide gel electrophoresis system, two proteins of similar size but different charge may be separated from each other. Since SDS gels may be unable to achieve this end, these two electrophoresis systems usefully complement each other for analy-

From. *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
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sis of small amounts of proteins. Proteins that might be usefully studied in the acid–gel system are minor primary structure variants (of slightly different charge), or modified forms of the same protein. Thus, a protein that has had some threonine or serine side chains phosphorylated, or lysine side chains acetylated, will be more acidic (or less basic) than the unmodified form of the same protein, and so will have a different electrophoretic mobility in the appropriate acid–gel system (e.g., *see* the acetylated derivatives of H4 in Fig. 1).

Commonly, the hydrogen bond-breaking agent urea is added to the simple acid–gel electrophoresis system in amounts traversing its entire range of solubility. This denaturant increases the frictional coefficient of proteins and so alters their electrophoretic mobilities. This has often proved useful in obtaining optimal resolution of proteins of interest and so urea is included in the system described below, which uses 2.5*M* urea. The system is buffered to about pH 3 with acetic acid, and is similar to the system described by Panyim and Chalkley *(1)*.

2. Materials

1. The apparatus required for running slab gels is available commercially or may be made in the workshop, but is usually of the type described by Studier *(2)*. The gel is cast and used in a chamber formed between two glass plates, as are SDS gels (for further details, *see* Chapter 5). A dc power supply is required.
2. Stock solutions. Use analytical grade (Analar) reagents and distilled water. Filter stock solutions and warm to room temperature before use.
   a. Stock acrylamide solution (total acrylamide content, %T = 30% [w/v], ratio of crosslinking agent to acrylamide monomer, %C = 1.5% [w/w]): 73.8 g acrylamide and 1.1 g *bis*-acrylamide. Dissolve and make to 250 mL in water. Filter the solution before use. This stock solution is stable for weeks in brown glass at 4°C.
   b. Stock ammonium persulfate: 1 g ammonium persulfate. Dissolve in 10 mL of water. This stock solution is stable for weeks in brown glass at 4°C.
   c. Reservoir buffer, pH 3 (0.9*M* acetic acid): 51.5 mL acetic acid (glacial). Make up to 1 L with water. Can be stored at room temperature.
   d. Sample solvent: 1 mL HCl (1*M*), 0.5 mL β-mercaptoethanol, 5.4 g urea, and 0.5 mL pyronin Y (0.4% [w/v] solution in water). Add 4.5 mL distilled water and fully dissolve the urea. The final volume is

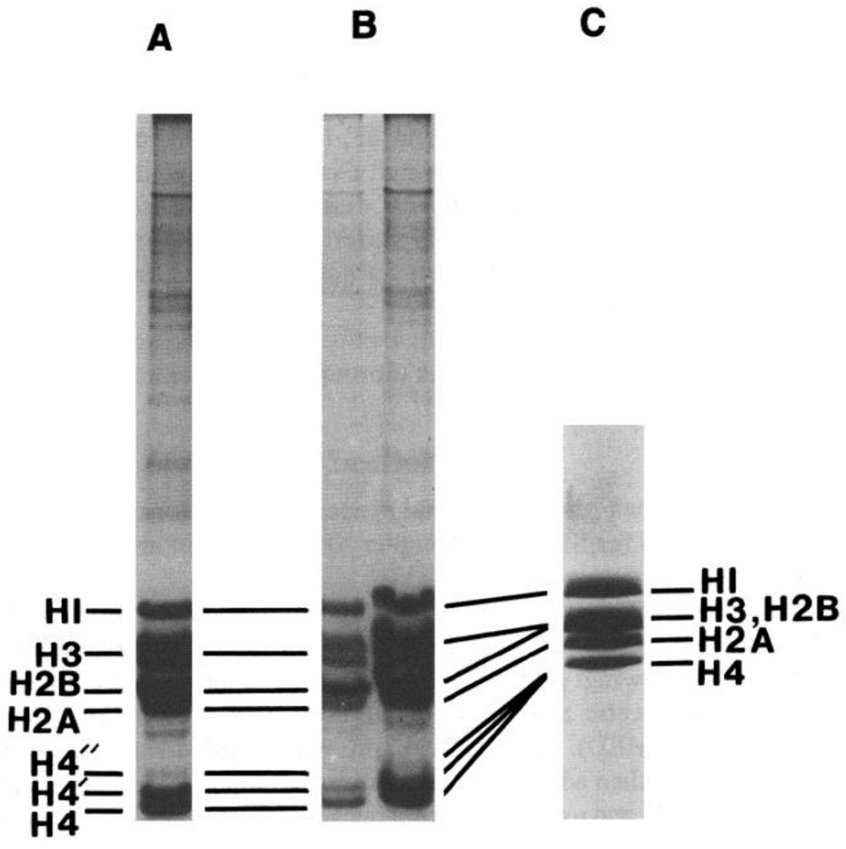

Fig. 1. Examples of electrophoresis on acetic acid (0.9*M*, pH 3)–polyacrylamide (20%T, 1.5%C) gels. **(A)** Slab gel containing 2.5*M* urea, stained with Coomassie brilliant blue R250, as described in the text. Sample: 8 μg of a mouse liver nuclei extract. The histones are identified. The H3 band probably also contains another protein, H1°. Note the mono- and the faint band of diacetylated forms of H4 (H4' and H4", respectively) migrating behind the nonacetylated H4. **(B)** Slab gel containing 2.5*M* urea, stained with Procion navy MXRB as described in Chapter 14. Sample: mouse liver nuclei extract. Left, loading 8 μg; right, loading 24 μg. Note the different sensitivity of the stain (cf Fig. 1A) and the distortion of bands that occurs with heavier loading. **(C)** Rod gel (5-mm diameter) containing *no* urea, stained with Procion navy MXRB as in ref. *10*. Sample: 50 μg of pig thymus histones. Note alteration of H2B mobility relative to other histones because of the omission of urea.

10 mL. Although it is probably best to use this solution when it is fresh, it may be stored frozen at –20°C for weeks without apparent adverse effect (*see* Note 1).

e. Protein stain: 0.25 g PAGE blue 83, 125 mL methanol, 25 mL acetic acid (glacial), and 100 mL water. Dissolve the dye in the methanol, then add the acid and water. If dissolved in a different order the dye's staining behavior may differ. The stain is best used when freshly made. For best result do not reuse the stain—its efficacy declines with use.

f. Destaining solution: 100 mL methanol, 100 mL acetic acid (glacial), and 800 mL water. Mix thoroughly. Make when required and use fresh.

3. Method

1. Assemble clean glass plates and spacers into the form of a chamber (as described for SDS gels, Chapter 5). Clamp it in an upright, level position.
2. Prepare a sufficient volume of separating gel mixture (30 mL for a chamber of about 14 × 14 × 0.1 cm allows for some wastage), as follows. Mix the following: 20 mL stock acrylamide solution, 4.5 g urea, 1.57 mL glacial acetic acid, and 100 µL *N,N,N',N'*-tetramethylethylenediamine (TEMED). Make to 29.45 mL with distilled water, degas on a water pump, then add stock ammonium persulfate solution (0.55 mL).

   Mix gently and use immediately because polymerization starts when the persulfate is added (*see* Note 2). The degassing removes oxygen, which can inhibit polymerization (by virtue of mopping up free radicals), and also discourages bubble formation when pouring the gel.
3. Carefully pipet or pour freshly made solution into the chamber and remove any bubbles present. Pour to a level about 0.5 cm below where the bottom of the well-forming template ("comb") will come when it is in position. Carefully overlayer the acrylamide solution with butan-2-ol, without mixing. This insulates the solution from oxygen and generates a flat top to the gel. The acrylamide should polymerize in an hour or so at room temperature (*see* Note 3).
4. Prepare the upper gel layer (5 mL) as follows (*see* Note 4). Mix the following: 1.25 mL stock acrylamide solution, 0.75 g urea, 78.2 µL glacial acetic acid, and 25 µL TEMED. Make to 4.75 mL with water, degas on water pump, then add stock ammonium persulfate solution (0.24 mL). Mix gently and use immediately. Pour off the butan-2-ol from the polymerized separating gel, wash the gel top with water and then a little of the upper gel mixture, and then fill the gap remaining in

the chamber with upper gel mixture. Insert the well-forming comb and allow to stand until set (about 0.5–1 h) (*see* Note 5).

5. When the upper gel has set, remove the comb without breaking or distorting the sample wells. Install the gel in the apparatus and fill the reservoirs with buffer (0.9*M* acetic acid). The gel may be run at room temperature without buffer circulation or cooling. Cooling tends to slow up the rate of electrophoresis and so the advantage of the decreased rate of protein diffusion (which causes band widening) is counteracted by increased diffusion occurring during the longer time required to complete the run. Push out the bottom spacer from the chamber and, with the cathode at the bottom end of the gel, turn on the dc power supply, to give about 180 V. Continue this electrophoresis without added samples ("pre-electrophoresis") at a constant voltage until the current falls to a steady level (say, from 25 to 5 mA for a 0.5–1-mm-thick slab of this sort), or alternatively, at constant current until the voltage increases to a steady level. This process may take about 5 h, but may be done conveniently overnight. The pre-electrophoresed gel may be stored under fresh 0.9*M* acetic acid for at least several days at room temperature (*see* Note 6).
6. While the gel is polymerizing, prepare samples for electrophoresis. Dry samples will be dissolved directly in sample solvent or aqueous solutions may be diluted with not less than 1 vol of sample solvent. The concentration of protein in the solution should be as great as possible so that the volume of solution loaded onto the gel is as small as possible.
7. Prepare to load the gel. Use fresh reservoir buffer in the apparatus. Take up the required volume of sample solution in a microsyringe or pipet and carefully inject it into a sample well, loading it through the reservoir buffer without mixing. The amount of sample loaded depends partly on the sensitivity of the method of detection *(see below),* but in any case it is often found that good, even, and straight bands are obtained most frequently when samples are lightly loaded, say about 1 μg/1 cm wide band in a 0.5–1-mm-thick gel. Having loaded the samples without delay, start electrophoresis at 180 V. The pyronin Y will take 4–5 h to migrate to the bottom of a thin, 14-cm-long gel of the type described here. Decreasing the voltage will prolong the run, whereas an increased voltage will generate more heat, which may not benefit the appearance of the protein bands (*see* Note 7).
8. At the end of electrophoresis, remove the gel and immerse it in protein stain for at least several hours with gentle agitation. The dye that is then not bound to the protein bands may be removed by washing the

gel in several changes of destaining solution. After destaining, protein bands are seen to be colored blue to purplish-red. Excessive destaining will decolorize these protein bands, but they may be restained. PAGE blue 83 visibly stains as little as 0.1–1 μg of protein in a 1-cm-wide band.

9. Problems that can arise from using this gel system and possible modifications to the system are discussed in Notes 8–14.

4. Notes

1. The 9*M* urea in the sample solvent has two functions. First, to disrupt aggregates, and second, to increase the density of the solution (which aids in the loading of the sample beneath the less dense reservoir buffer). The β-mercaptoethanol that is also present reduces inter- and intramolecular disulfide bonds and so, together with the urea, destroys higher-order protein structures. The pyronin Y dye is present to aid sample application by making it visible, and also to mark the approximate position of the front of electrophoresis, near which it runs.
2. Addition of TEMED and persulfate to the gel mixture initiates its polymerization. This occurs by their interaction and formation of a TEMED radical that reacts with an acrylamide monomer. This in turn produces a radical that reacts with another acrylamide monomer, or occasionally one half of a *bis*-acrylamide molecule. Incorporation of *bis*-acrylamide molecules into different chains forms crosslinks between them.
3. The 20%T gel described is suitable for electrophoresis of smaller proteins (say, of M_r below 50,000). For larger proteins a gel of larger pore size is more convenient by virtue of allowing greater mobility. The pore size may be increased by reduction of %T by simple dilution of the stock acrylamide solution, or by adjustment of %C (which gives the smallest pores when at 5%). It may be found that adjustment of the gel's pore size in this way may also improve the resolution of proteins of interest.
4. The purpose of the upper, weak polyacrylamide, gel is to provide a medium in which sample wells can be formed and from which the well-forming comb can be readily removed (20%T gel tends to break when the comb is removed). Since the upper gel contains weaker acid than does the separating gel, its pH is slightly higher. However, the purpose of this design is that the upper gel has lower conductivity than the rest of the system and when the electric field is applied this has a small band-sharpening effect similar to that produced by the "stacking gel" used in SDS gels (*see* Chapter 5).
5. A more typical stacking gel for 0.9*M* acetic acid/urea gels has been described by Spiker *(3)*. It has been found in this laboratory that

although bands may have an improved appearance, they may instead become smeared.

6. The pre-electrophoresis treatment of the gel before addition of the samples removes persulfate and other ions that would otherwise slow up the rate of sample electrophoresis and also spoil the resolution of protein bands.
7. As mentioned earlier, protein bands in this system are often slightly misshapen, and this problem is exacerbated by heavy loading (*see* Fig. 1). Thus, slab gels may give unsatisfactory resolution for quantification purposes (*see* Chapter 14). As an alternative, gels may be made in the forms of rods by casting them in tubes of glass. The result has the proteins as discs running through the rod (e.g., *see* Fig. 1). These discs suffer less from distortion than do bands on slabs. The separating gel for rod gels is prepared as for slab gels, and polymerized in glass tubes that have been siliconized beforehand. No upper gel is added, but sample is applied directly onto the separating gel. The gel may be extruded from the glass tube, as described in *(4)*, or removed after cracking the glass in a vice or with a hammer. The apparatus required for running rod gels is available commercially or may be made to a simple design (*see* ref. *4*).
8. If the resolution of the proteins of interest is not good enough, then alteration of pH of the system may be beneficial, by virtue of altering proteins' respective charges by titration of the side chain groups (e.g., *see* ref. *1*). The literature describes polyacrylamide gel systems of various pH values, e.g., pH 4.5 *(5,6)*; pH 7.1 *(7)*; pH 8.9 *(8)*.
9. The concentration of urea in the gel may be altered, to alter the relative mobilities of proteins in the system. The effects of altering the urea concentration have to be determined empirically. An example is shown in Fig. 1, in which it may be seen that in the absence of urea (as in 1*M* urea) histone proteins H2B and H3 comigrate, whereas in 2.5*M* urea they are resolved from each other (*see also* ref. *1*).
10. In addition to urea, the nonionic detergent Triton X-100 (or Triton DF-16) may be added to the gel *(9)*. This agent binds to proteins in proportion to their hydrophobicity, and alters their electrophoretic mobilities accordingly. This technique has proved useful in the study of proteins that differ slightly in their hydrophobic character and that are not separated by ordinary acid/urea or SDS gel electrophoresis.
11. The acid–urea system described may be adapted for separation of nondenatured proteins, which may be specifically detected in the gel (or blots there from) by their biological activity (e.g., enzymatic activity as reviewed in ref. *10*). For this purpose, denaturants (urea and Tri-

ton) are omitted and instead of the sample solvent given earlier, with its urea and reducing agent, the 0.9*M* acetic acid, 30% (w/v) sucrose sample solvent of Panyim and Chalkley *(1)* may be used.

12. PAGE blue 83 may not penetrate sufficiently to stain the centers of bands of concentrated protein, especially in thicker slabs and rod gels. Procion navy MXRB does not suffer this defect, although it is several-fold less sensitive (*see* Fig. 1). The method of staining with Procion navy described by Goodwin et al. *(10)* is suitable for rod gels, but the heated destaining process is inconvenient for slabs. However, the method described in Chapter 14 gives an equivalent result and is suitable for all forms of gel.
13. Various problems may arise to spoil the otherwise perfect gel. Failure of the gel to polymerize, distortion of sample wells, and other problems that are general to the process of polyacrylamide gel electrophoresis are dealt with in Chapter 5. The main failing with the present system in particular is the common, uneven shape of the protein bands especially in conditions of heavy loading (e.g., *see* Fig. 1). As mentioned earlier, use of a stacking gel may alleviate this problem, but generally speaking light loading (about 1 µg/band or less) and small sample volumes go a long way to promote good results. Avoid using the sample wells at the very ends of the row of wells, for samples in these often suffer distortion during the electrophoresis.
14. Be wary of the dangers of electric shock and of fire, and of the neurotoxic acrylamide monomer. Ready-made solutions of acrylamide and *bis*-acrylamide are commercially available, which reduce the hazards associated with handling of these agents.

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CHAPTER 8

Electrophoresis of Peptides

Ralph C. Judd

1. Introduction

The separation of proteins and peptides by sodium dodecylsulfate—polyacrylamide gel electrophoresis (SDS-PAGE) has proven to be one of the most valuable tools yet developed in the area of molecular biology (*see* Chapter 5). The advent of the "slab" gel, using a discontinuous buffer system, has made it possible to readily separate complex mixtures of molecules (e.g., tissues, cells) in order to visualize and compare their component parts. The separation of proteins offers the researcher the ability to quantify the amount of a particular protein in a sample, obtain fairly reliable mol-mass information, and, by combining SDS-PAGE with immunoelectroblotting, evaluate protein antigenicity. Since SDS-PAGE is a powerful separation system, it can also be used as a preparative purification technique (*see* ref. *1* and Chapter 22).

Parameters that influence the resolution or proteins in SDS-PAGE separations include the ratio or acrylamide to crosslinker (*bis*-acrylamide), the percentage of acrylamide/crosslinker used to form the stacking and separation gels, the pH of, and the components in, the stacking and separation buffers, and the method of sample preparation. The systems that employ glycine in the running buffers (e.g., Laemmli *[2]*, Dreyfuss et al. *[3]*) can reliably separate proteins ranging in molecular mass from over 200,000 Daltons (200 kDa) down to about 3 kDa. Separation of proteins and peptides below 3 kDa requires slightly different procedures to prevent band broadening and to obtain reliable mol wts. In addition, the increased use of SDS-PAGE to purify

From. *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
Edited by J M Walker Copyright ©1994 Humana Press Inc , Totowa, NJ

peptides for N-terminal sequence analysis demands that glycine, which drastically interferes with automated sequence technology, be replaced with noninterfering buffer components.

This chapter describes a modification of the tricine gel system of Schagger and von Jagow *(4)*. Proper application allows for the separation of peptides as small as 500 Daltons. This allows SDS-PAGE to be used for peptide mapping (*see* Chapter 23), epitope mapping *(5)*, and protein and peptide separation for N-terminal sequence analyses. It is important to remember that all forms of SDS-PAGE are denaturing, and are therefore unsuitable for separation of proteins or peptides to be used in functional analyses (e.g., enzymes, receptors).

2. Materials

2.1. Equipment

1. SDS-PAGE gel apparatus.
2. Power pack.
3. Blotting apparatus.

2.2. Reagents

1. Separating/spacer gel acrylamide (1X crosslinker): 48 g acrylamide, 1.5 g *N,N'*-methylene-*bis*-acrylamide, bring to 100 mL, then filter through qualitative paper to remove cloudiness (*see* Note 1).
2. Separating gel acrylamide (2X crosslinker): 48 g acrylamide, 3 g *N,N'*-methylene-*bis*-acrylamide, bring to 100 mL, then filter through qualitative paper to remove cloudiness (*see* Note 2).
3. Stacking gel acrylamide: 30 g acrylamide, 0.8 g *N,N'*-methylene-*bis*-acrylamide, bring to 100 mL, then filter through qualitative paper to remove cloudiness.
4. Separating/spacer gel buffer: 3*M* Trizma base, 0.3% sodium dodecylsulfate (*see* Note 3); bring to pH 8.9 with HCl.
5. Stacking gel buffer: 1*M* Trizma base; bring to pH 6.8 with HCl.
6. Cathode (top) running buffer (10X stock): 1*M* Trizma base, 1*M* Tricine, 1% SDS (*see* Note 3). Dilute 1:10 immediately before use. Do not adjust pH, it will be about 8.25.
7. Anode (bottom) buffer (10X stock): 2*M* Trizma base, bring to pH 8.9 with HCl. Dilute 1:10 immediately before use.
8. 0.2*M* tetrasodium EDTA.
9. 10% ammonium persulfate.

10. TEMED.
11. Glycerol.
12. 25% Isopropanol, 7% glacial acetic acid (fixer/destainer).
13. 1% Coomassie brilliant blue in fixer/destainer.
14. Sample solubilization buffer: 2 mL 10% SDS (w/v) in dH_2O, 1.0 mL glycerol, 0.625 mL 1*M* Tris-HCl, pH 6.8, 6 mL H_2O, bromophenol blue to color.
15. Dithiothreitol (DTT).
16. 2% Agarose.
17. Molecular-mass markers, e.g., low-mol wt kit (Bio-Rad, Inc., Richmond, CA, or equivalent) and peptide mol-mass markers (Pharmacia Inc., Piscataway, NJ, or equivalent).
18. PVDF (nylon) membranes.
19. Methanol.
20. Blotting transfer buffer: 20 m*M* phosphate buffer, pH 8.0: 94.7 mL 0.2*M* Na_2HPO_4 stock, 5.3 mL 0.2*M* NaH_2PO_4 stock in 900 mL H_2O.
21. Filter paper for blotting (Whatman #1, or equivalent).
22. Distilled water (dH_2O).

2.3. Gel Recipes

2.3.1. Separating Gel Recipe

Add reagents in order given (*see* Note 4):

Water	6.7 mL
Separating/spacer gel buffer	10 mL
Separating/spacer gel acrylamide (1X or 2X crosslinker)	10 mL
Glycerol	3.2 mL
TEMED	10 µL
10% Ammonium persulfate	100 µL

2.3.2. Spacer Gel Recipe

Add reagents in order given (*see* Note 4):

Water	6 9 mL
Separating/spacer gel buffer	5.0 mL
Separating/spacer gel acrylamide (1X crosslinker only)	3.0 mL
TEMED	5 µL
10% Ammonium persulfate	50 µL

2.3.3. Stacking Gel Recipe

Add reagents in order given (*see* Note 4):

Water	10.3 mL
Stacking gel buffer	1.9 mL
Stacking gel acrylamide	2.5 mL
EDTA	150 µL
TEMED	7.5 µL
10% Ammonium persulfate	150 µL

3. Methods

3.1. Sample Solubilization

Boil samples in sample solubilization buffer for 10–30 min. Solubilize sample at 1 mg/mL and run 1–2 µL/lane (1–2 µg/lane) (*see* Note 5). For sequence analysis, as much sample as is practical should be separated.

3.2. Gel Preparation/Electrophoresis

1. Assemble the gel apparatus (*see* Note 6). Make two marks on the front plate to identify top of separating gel and top of spacer gel (*see* Note 7). Assuming a well depth of 12 mm, the top of the separating gel should be 3.5 cm down from the top of the *back* plate and the spacer gel should be 2 cm down from the top of the *back* plate, leaving a stacking gel of 8 mm (*see* Note 8).
2. Combine the reagents to make the separating gel, mix gently, and pipet the solution between the plates to lowest mark on the plate. Overlay the gel solution with 2 mL of dH_2O by gently running the dH_2O down the center of the inside of the front plate. Allow the gel to polymerize for about 20 min. When polymerized, the water–gel interface will be obvious.
3. Pour off the water and dry between the plates with filter paper. Do not touch the surface of the separating gel with the paper. Combine the reagents to make the spacer gel, mix gently, and pipet the solution between the plates to second mark on the plate. Overlay the solution with 2 mL of dH_2O by gently running the dH_2O down the center of the inside of the front plate. Allow the gel to polymerize for about 20 min. When polymerized, the water–gel interface will be obvious.
4. Pour off the water and dry between the plates with filter paper. Do not touch the surface of spacer gel with the paper. Combine the reagents to make the stacking gel and mix gently. Place the well-forming comb between the plates leaving one end slightly higher than the other. Slowly

add the stacking gel solution at the raised end (this allows air bubble to be pushed up and out from under the comb teeth). When the solution reaches the top of the back plate, gently push the comb all the way down. Check to be sure that no air pockets are trapped beneath the comb. Allow the gel to polymerize for about 20 min.

5. When the stacking gel has polymerized, carefully remove the comb. Straighten any wells that might be crooked with a straightened metal paperclip. Remove the acrylamide at each edge to the depth of the wells. This helps prevent "smiling" of the samples at the edge of the gel. Seal the edges of the gel with 2% agarose.
6. Add freshly diluted cathode running buffer to the top chamber of the gel apparatus until it is 5–10 mm above the top of the gel. Squirt running buffer into each well with a Pasteur pipet to flush out any unpolymerized acrylamide. Check the lower chamber to insure that no cathode running buffer is leaking from the top chamber, then fill the bottom chamber with anode buffer. Remove any air bubbles from the under edge of the gel with a bent-tip Pasteur pipet. The gel is now ready for sample loading.
7. After loading the samples and the mol-mass markers, connect leads from the power pack to the gel apparatus (the negative lead goes on the top, the positive lead goes on the bottom). Gels can be run on constant current, constant voltage, or constant power settings. When using the constant current setting, run the gel at 50 mA. The voltage will be between 50–100 V at the beginning and will slowly increase during the run. For a constant voltage setting, begin the electrophoresis at 50 mA. As the run progresses, the amperage will decrease, so adjust the amperage to 50 mA several times during the run or the electrophoresis will be very slow. The electrophoresis should be run at 5–7 W on the constant power setting. Each system varies, so empirical information should be used to modify the electrophoresis conditions so that electrophoresis is completed in about 4 h (*see* Note 9).
8. When the dye-front reaches the bottom of the gel, turn off the power, disassemble the gel apparatus, and place the gel in 200–300 mL of fixer/destainer. Gently shake for 16 h (*see* Note 10). Pour off spent fixer/destainer and add Coomassie brilliant blue. Gently shake for 30 min. Destain the gel in several changes of fixer/destainer until the background is almost clear, then place the gel in dH_2O and gently mix until the background is completely clear. The peptide bands will become a deep purple-blue. The gel can now be photographed or dried. To store the gel wet, soak the gel in 7% glacial acetic acid for 1 h and seal in a plastic bag.

Figure 1 demonstrates the mol-mass range of separation of a 1X crosslinker tricine gel. Whole cell (WC) lysates and 1X and 2X purified (*see* Chapter 23, Sections 3.1.1. and 3.1.2.) 44 kDa proteins of *Neisseria gonorrhoeae*, Bio-Rad low-mol-wt markers (mw), and Pharmacia peptide markers (pep mw), were separated and stained with Coomassie brilliant blue (CBB). The top of the gel in this figure is at the spacer gel-separating gel interface. Proteins larger than about 100 kDa remained trapped at the spacer/separating gel interface, resulting in the bulging of the outside lanes. Smaller proteins all migrated into the gel but many remained tightly bunched at the top of the separating gel. The effective separation range is below 40 kDa. Comparison of this figure with Fig. 1 in Chapter 23, which shows gonococcal whole cells and the two mol-wt marker preparations separated in a standard 15% Laemmli gel *(2)*, demonstrates the tremendous resolving power for low-mol-wt components by this tricine gel system.

3.3. Blotting of Peptides

Separated peptides can be electroblotted to PVDF membranes for sequencing or immunological analyses (*see* Note 11).

1. Before the electrophoresis is complete, prepare enough of the 20 m*M* sodium phosphate transfer buffer, pH 8.0 (*see* Note 12) to fill the blotting chamber (usually 2–4 L). Degas about 1 L of transfer buffer for at least 15 min before use. Cut two sheets of filter blotting paper to fit blotting apparatus and cut a piece of PVDF membrane a little larger than the gel. Place the PVDF membrane in 10 mL of methanol until it is wet (this takes only a few seconds), then place the membrane in 100 mL of degassed transfer buffer.
2. Following the gel electrophoresis, remove the gel from the gel apparatus and place it on blotting filter paper that is submersed in the degassed transfer buffer. Immediately overlay the other side of the gel with the wetted PVDF membrane, being sure to remove all air pockets between the gel and the membrane. Overlay the PVDF membrane with another piece of blotting filter paper and place the gel "sandwich" into the blotting chamber using the appropriate spacers and holders.
3. Connect the power pack electrodes to the blotting chamber (the positive electrode goes on the side of the gel having the PVDF membrane). Electrophorese for 16 h at 25 V, 0.8 A. Each system varies, so settings may be somewhat different than those described here.

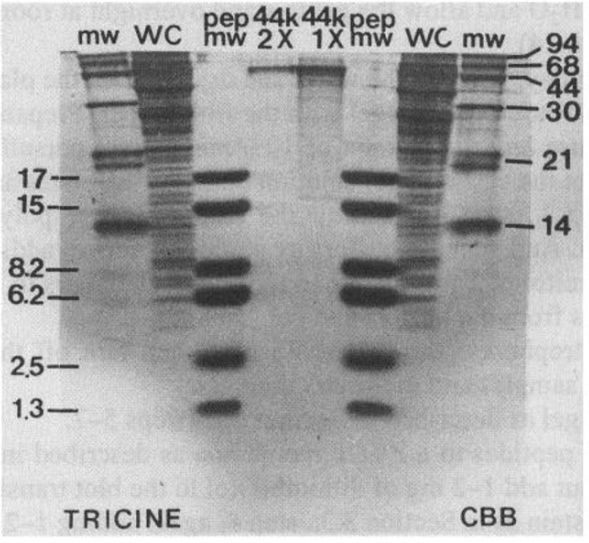

Fig. 1. Separation of whole cell (WC) lysates and 1X and 2X pure (*see* Chapter 23, Sections 3.1.1. and 3.1.2.) 44 kDa protein of *Neisseria gonorrhoeae*, Bio-Rad low-mol-wt markers (mw) (1 μg of each protein), and Pharmacia peptide markers (pep mw), to which the 1.3 kDa protein kinase C substrate peptide (Sigma) was added (3 μg of each peptide), were separated in 1X crosslinker tricine gel, fixed, and stained with Coomassie brilliant blue (CBB). Molecular masses are given in thousands of daltons.

4. Following blotting electrophoresis, disconnect the power, disassemble the blotting chamber, and remove the PVDF membrane from the gel (*see* Note 13). The PVDF membrane can be processed for immunological analyses (*see* Chapters 25–28) or placed in Coomassie brilliant blue in fixer/destainer to stain the transferred peptides. Remove excess stain by shaking the membrane in several changes of fixer/destainer until background is white. Peptide bands can be excised, rinsed in dH_2O, dried, and subjected to N-terminal sequencing.

3.4. Modifications for Peptide Sequencing

Peptides to be used in N-terminal sequence analyses must be protected from oxidation which can block the N-terminus. Several simple precautions can help prevent this common problem.

1. Prepare the separation and spacer gel the day before electrophoresing the peptides. After pouring, overlay the spacer gel with several millili-

ters of dH_2O and allow the gel to stand overnight at room temperature (*see* Note 14).

2. The next day, pour off the water and dry between the plates with filter paper. Do not touch the gel with the filter paper. Prepare the stacking gel but use *half* the amount of 10% ammonium persulfate (*see* Note 15). Pipet the stacking gel solution between the plates as described in Section 3.2., step 4 and allow the stacking gel to polymerize for at least 1 h. Add running buffers as described above, adding 1–2 mg of dithiothreitol to both the upper and lower chambers to scavenge any oxidizers from the buffers and gel.
3. Pre-electrophorese the gel for 15 min, then turn off the power, and load the samples and mol-mass markers.
4. Run the gel as described in Section 3.2., steps 5–7.
5. Blot the peptides to a PVDF membrane as described in Section 3.3., step 2, but add 1–2 mg of dithiothreitol to the blot transfer buffer.
6. Fix and stain as in Section 3.3., step 4, again adding 1–2 mg of dithiothreitol to the fixer/destainer, Coomassie brilliant blue, and dH_2O used to rinse the peptide-containing PVDF membrane.

4. Notes

1. The working range of separation is about 40 kDa down to about 1 kDa.
2. The working range of separation is about 20 kDa down to less than 500 daltons.
3. Use electrophoresis grade SDS. If peptide bands remain diffuse, try British Drug House SDS.
4. Degassing of gel reagents is *not* necessary.
5. Coomassie staining can generally visualize a band of 0.5 µg. This may vary considerably based on the properties of the particular peptide (some peptides do not stain at all with Coomassie). Some peptides do not bind SDS well and may never migrate exactly right when compared to mol-wt markers. Fortunately, this is rare.
6. Protocols are designed for a standard 13 cm × 11 cm × 1.5 mm slab gel. Dimensions and reagent volumes can be proportionally adjusted to accommodate other gel dimensions.
7. Permanent marks with a diamond pencil can be made on the back of the back plate if the plate is dedicated to this gel system.
8. The depth of the spacer gel can be varied from 1 to 2 cm. Trial and error is the only way to determine the appropriate dimension for each system.
9. It is wise to feel the front plate several times during the electrophoresis to check for overheating. The plate will become pleasantly warm as

the run progresses. If it becomes too warm the plates might break, so turn down the power!

10. Standard sized gels can be fixed in as little as 4 h with shaking.
11. It is best to blot peptides to PVDF membranes rather than nitrocellulose membranes since small peptides tend to pass through nitrocellulose without binding. Moreover, peptides immobilized on PVDF membranes can be directly sequenced in automated instrumentation equipped with a "blot cartridge" *(6)*.
12. The pH of the transfer buffer can be varied from 5.7 to 8.0 if transfer is inefficient at pH 8.0 *(7)*.
13. Wear disposable gloves when handling membranes.
14. Do not refrigerate the gel. It will contract and pull away from the plates resulting in leaks and poor resolution.
15. Do not pour the stacking gel the day before electrophoresis, it will shrink allowing the samples to leak from the wells.

Acknowledgments

I thank Joan Strange for her assistance in developing this system. I also thank the Public Health Service, NIH, NIAID (grants RO1 AI21236 and KO4 AI00834) and UM Research Grant Program for their continued support.

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CHAPTER 9

Isoelectric Focusing of Proteins in Ultra-Thin Polyacrylamide Gels

John M. Walker

1. Introduction

Isoelectric focusing (IEF) is an electrophoretic method for the separation of proteins, according to their isoelectric points (pI), in a stabilized pH gradient. The method involves casting a layer of support media (usually a polyacrylamide gel but agarose can also be used) containing a mixture of carrier ampholytes (low-mol-wt synthetic polyamino-polycarboxylic acids). When an electric field is applied across such a gel, the carrier ampholytes arrange themselves in order of increasing pI from the anode to the cathode. Each carrier ampholyte maintains a local pH corresponding to its pI and thus a uniform pH gradient is created across the gel. If a protein sample is applied to the surface of the gel, it will also migrate under the influence of the electric field until it reaches the region of the gradient where the pH corresponds to its isoelectric point. At this pH, the protein will have no net charge and will therefore become stationary at this point. Should the protein diffuse slightly toward the anode from this point, it will gain a weak positive charge and migrate back to its position of zero charge. Similarly diffusion toward the cathode results in a weak negative charge that will direct the protein back to the same position. The protein is therefore trapped or "focused" at the pH value where it has zero charge. Proteins are therefore separated according to their charge, and not size as with SDS gel electrophoresis. In practice the protein samples are loaded onto the gel before the pH gradient is formed.

From *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
Edited by J. M. Walker

When a voltage difference is applied, protein migration and pH gradient formation occur simultaneously.

Traditionally, 1–2 mm thick isoelectric focusing gels have been used by research workers, but the relatively high cost of ampholytes makes this a fairly expensive procedure if a number of gels are to be run. However, the introduction of thin-layer isoelectric focusing (where gels of only 0.15 mm thickness are prepared, using a layer of electrical insulation tape as the "spacer" between the gel plate) has considerably reduced the cost of preparing IEF gels, and such gels are therefore described in this chapter. The tremendous resolution obtained with IEF can be further enhanced by combinations with SDS gel electrophoresis in the form of 2-D gel electrophoresis. Various 2-D gel systems are described in Chapters 11–13.

2. Materials

1. Stock acrylamide solution: acrylamide (3.88 g), *bis*-acrylamide (0.12 g), sucrose (10.0 g). Dissolve these components in 80 mL of water. This solution may be prepared some days before being required and stored at 4°C.
2. Riboflavin solution: This should be made fresh, as required. Stir 10 mg riboflavin in 100 mL water for 20 min. Stand to allow undissolved material to settle out (or briefly centrifuge).
3. Ampholytes: pH range 3.5–9.5 (*see* Note 1).
4. Electrode wicks: 22 × 0.6 cm strips of Whatman No. 17 filter paper.
5. Sample loading strips: 0.5 cm square pieces of Whatman No. 1 filter paper, or similar.
6. Anolyte: 1.0*M* H_3PO_4.
7. Catholyte: 1.0*M* NaOH.
8. Fixing solution: Mix 150 mL of methanol and 350 mL of distilled water. Add 17.5 g sulfosalicylic acid and 57.5 g trichloroacetic acid.
9. Protein stain: 0.1% Coomassie brilliant blue R250 in 50% methanol, 10% glacial acetic acid. N.B. Dissolve the stain in the methanol component first.
10. Glass plates: 22 × 12 cm. These should preferably be of 1 mm glass (to facilitate cooling), but 2 mm glass will suffice.
11. PVC electric insulation tape: The thickness of this tape should be about 0.15 mm and can be checked with a micrometer. The tape we use is actually 0.135 mm.

3. Methods

1. Thoroughly clean the surfaces of two glass plates, first with detergent and then methylated spirit. It is *essential* for this method that the glass plates are clean.
2. To prepare the gel mold, stick strips of insulation tape, 0.5 cm wide, along the four edges of one glass plate. Do not overlap the tape at any stage. Small gaps at the join are acceptable, but do *not* overlap the tape at corners as this will effectively double the thickness of the spacer at this point.
3. To prepare the gel solution, mix the following: 9.0 mL acrylamide, 0.4 mL ampholyte solution, and 60.0 µL riboflavin solution.

   N.B. Since acrylamide monomer is believed to be neurotoxic, steps 4–6 must be carried out wearing protective gloves.
4. Place the glass mold in a spillage tray and transfer ALL the gel solution with a Pasteur pipet along one of the short edges of the glass mold. The gel solution will be seen to spread slowly toward the middle of the plate.
5. Take the second glass plate and place one of its short edges on the taped edge of the mold, adjacent to the gel solution. Gradually lower the top plate and allow the solution to spread across the mold. Take care not to trap any air bubbles. If this happens, carefully raise the top plate to remove the bubble and lower it again.
6. When the two plates are together, press the edges firmly together (NOT the middle) and discard the excess acrylamide solution spilled in the tray. Place clips around the edges of the plate and thoroughly clean the plate to remove excess acrylamide solution using a wet tissue.
7 Place the gel mold on a light box (*see* Note 2) and leave for at least 3 h to allow polymerization (*see* Note 3). Gel molds may be stacked at least three deep on the light box during polymerization. Polymerized gels may then be stored at 4°C for at least 2 mo, or used immediately. If plates are to be used immediately they should be placed at 4°C for ~15 min, since this makes the separation of the plates easier.
8. Place the gel mold on the bench and remove the top glass plate by inserting a scalpel blade between the two plates and *carefully* twisting to remove the top plate. (N.B. Protect eyes at this stage.) The gel will normally be stuck to the side that contains the insulation tape. Do *not* remove the tape. Adhesion of the gel to the tape helps fix the gel to the plate and prevents the gel coming off in the staining/destaining steps (*see* Note 4).
9. Carefully clean the underneath of the gel plate and place it on the cooling plate of the electrophoresis tank. Cooling water at 10°C should be passing through the cooling plate.

10. Down the full length of one of the longer sides of the gel lay electrode wicks, uniformly saturated with either 1.0*M* phosphoric acid (anode) or 1.0*M* NaOH (cathode) (*see* Note 5).
11. Samples are loaded by laying filter paper squares (Whatman No. 1, 0.5 × 0.5 cm), wetted with the protein sample, onto the gel surface. Leave 0.5 cm gaps between each sample. The filter papers are prewetted with 5–7 µL of sample and applied across the width of the gel (*see* Notes 6 and 7).
12. When all the samples are loaded, place a platinum electrode on each wick. Some commercial apparatus employ a small perspex plate along which the platinum electrodes are stretched and held taut. Good contact between the electrode and wick is maintained by applying a weight to the perspex plate.
13. Apply a potential difference of 500 V across the plate. This should give current of about 4–6 mA. After 10 min increase the current to 1000 V and then to 1500 V after a further 10 min. A current of ~4–6 mA should be flowing in the gel at this stage, but this will slowly decrease with time as the gel focuses.
14. When the gel has been running for about 1 h, turn off the power and carefully remove the sample papers with a pair of tweezers. Most of the protein samples will have electrophoresed off the papers and be in the gel by now (*see* Note 8). Continue with a voltage of 1500 V for a further 1 h. During the period of electrophoresis, colored samples (myoglobin, cytochrome c, hemoglobin in any blood samples, and so on) will be seen to move through the gel and focus as sharp bands (*see* Note 9).
15. At the end of 2 h of electrophoresis, a current of about 0.5 mA will be detected (*see* Note 10). Remove the gel plate from the apparatus and *gently* wash it in fixing solution (200 mL) for 20 min (overvigorous washing can cause the gel to come away from the glass plate).

    Some precipitated protein bands should be observable in the gel at this stage (*see* Note 11). Pour off the fixing solution and wash the gel in destaining solution (100 mL) for 2 min and discard this solution (*see* Note 12). Add protein stain and gently agitate the gel for about 10 min. Pour off and discard the protein stain and wash the gel in destaining solution. Stained protein bands in the gel may be visualized on a light box and a permanent record may be obtained by simply leaving the gel to dry out on the glass plate overnight (*see* Note 13).

16. Should you wish to stain your gel for enzyme activity rather than staining for total protein, immediately following electrophoresis gently agitate the gel in an appropriate substrate solution (*see* Note 7, Chapter 4).

4. Notes

1. This broad pH range is generally used because it allows one to look at the totality of proteins in a sample (but note that very basic proteins will run off the gel). However, ampholytes are available in a number of different pH ranges (e.g., 4–6, 5–7, 4–8, and so on) and can be used to expand the separation of proteins in a particular pH range. This is necessary when trying to resolve proteins with very similar pI values. Using the narrower ranges it is possible to separate proteins that differ in their pI values by as little as 0.01 of a pH unit.
2. Ensure that your light box does not generate much heat as this can dry out the gel quite easily by evaporation through any small gaps at the joints of the electrical insulation tape. If your light box is a warm one, stand it on its side and stand the gels adjacent to the box.
3. It is not at all obvious when a gel has set. However, if there are any small bubbles on the gel (these can occur particularly around the edges of the tape) polymerization can be observed by holding the gel up to the light and observing a "halo" around the bubble. This is caused by a region of unpolymerized acrylamide around the bubble that has been prevented from polymerizing by oxygen in the bubble. It is often convenient to introduce a small bubble at the end of the gel to help observe polymerization.
4. If the gel stays on the sheet of glass that does not contain the tape, discard the gel. Although usable for electrofocusing you will invariably find that the gel comes off the glass and rolls up into an unmanageable "scroll" during staining/destaining.
5. The strips must be fully wetted but must not leave a puddle of liquid when laid on the gel. (Note that in some apparatus designs application of the electrode applies pressure to the wicks, which can expel liquid.)
6. The filter paper must be fully wetted but should have no surplus liquid on the surface. When loaded on the gel this can lead to puddles of liquid on the gel surface which distorts electrophoresis in this region. The most appropriate volume depends on the absorbancy of the filter paper being used but about 5 µL is normally appropriate. For pure pro-

teins load approx 0.5–1.0 µg of protein. The loading for complex mixtures will have to be done by trial and error.

7. Theoretically, samples can be loaded anywhere between the anode or cathode. However, if one knows approximately where the bands will focus it is best not to load the samples at this point since this can cause some distortion of the band. Similarly, protein stability is a consideration. For example, if a particular protein is easily denatured at acid pH values, then cathodal application would be appropriate.
8. Although not absolutely essential, removal of sample strips at this stage is encouraged since bands that focus in the region of these strips can be distorted if strips are not removed. Take care not to make a hole in the gel when removing the strips. Use blunt tweezers (forceps) rather than pointed ones When originally loading the samples it can be advantageous to leave one corner of the filter strip slightly raised from the surface to facilitate later removal with tweezers.
9. It is indeed good idea to include two or three blood samples in any run to act as markers and to confirm that electrophoresis is proceeding satisfactorily. Samples should be prepared by diluting a drop of human blood approx 1:100 with distilled water to effect lysis of the erythrocytes. This solution should be pale cherry in color. During electrophoresis, the red hemoglobin will be seen to electrophorese off the filter paper into the gel and ultimately focus in the central region of the gel (pH 3.5–10 range). If samples are loaded from each end of the gel, when they have both focused in the sample place in the middle of the gel one can be fairly certain that isoelectrofocusing is occurring and indeed that the run is probably complete.
10. Theoretically, when the gel is fully focused, there should be no charged species to carry a current in the gel. In practice there is always a slow drift of buffer in the gel resulting in a small (~0.5 mA) current even when gels are fully focused. Blood samples (*see* Note 9) loaded as markers can provide additional confirmation that focusing is completed.
11. It is not possible to stain the IEF gel with protein stain immediately following electrophoresis since the ampholytes will stain giving a uniformly blue gel. The fixing step allows the separated proteins to be precipitated in the gel, while washing out the still soluble ampholytes.
12. This brief wash is important. If stain is added to the gel still wet with fixing solution, a certain amount of protein stain will precipitate out. This brief washing step prevents this.

13. If you wish to determine the isoelectric point of a protein in a sample, then the easiest way is to run a mixture of proteins of known pI in an adjacent track (such mixtures are commercially available). Some commercially available kits comprise totally colored compounds that also allows one to monitor the focusing as it occurs. However, it is just as easy to prepare ones own mixture from individual purified proteins. When stained, plot a graph of protein pI vs distance from an electrode to give a calibration graph. The distance moved by the unknown protein is also measured and its pI read from the graph. Alternatively, a blank track can be left adjacent to the sample. This is cut out prior to staining the gel and cut into 1 mm slices. Each slice is then homogenized in 1 mL of water and the pH of the resultant solution measured with a micro electrode. In this way a pH vs distance calibration graph is again produced.

CHAPTER 10

The In Vivo Isotopic Labeling of Proteins for Polyacrylamide Gel Electrophoresis

Jeffrey W. Pollard

1. Introduction

Detection of radiolabeled proteins separated by gel electrophorersis can be rapidly achieved using either autoradiography that offers a convenient, quick and cheap means of quantifying proteins, or, if access to a machine is available, by using a phosphorimager. In both cases, for metabolic experiments, proteins must be labeled with a radioactive isotope in vivo prior to isolation and subsequent electrophoretic analysis. The isotope chosen, of course, must correspond to the question to be investigated, but those in general use are the beta-emitters ^{3}H, ^{14}C, ^{32}P, and ^{35}S. The maximum energies and half lives are: 0.019 MeV (12.28 yr), 0.156 MeV (5730 yr), 1.706 MeV (14.3 d), 0.167 MeV (87.4 d), respectively. Tritium is a popular isotope for biological use, potentially available at high specific activity, but its low energy precludes direct detection by autoradiography on X-ray film and it needs to be either treated for fluorography or quantified by gel slicing and scintillation counting. Thus, the higher energy [^{14}C]- and [^{35}S]-labeled amino acids are commonly used as protein labels since they can be directly detected by autoradiography or on the phosphorimager. The low specific activity of [^{14}C]-labeled amino acids often prevents their use, with the result that [^{35}S]-methionine or [^{35}S]-cysteine labeling, either alone or in combination, are more commonly used. Nevertheless, it is worth noting that Bravo and Celis *(1)*

From: *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
Edited by: J. M. Walker

were able to detect 28% more proteins by labeling with a mixture of 16 [^{14}C]-amino acids than could be detected with [^{35}S]-methionine and that such labeling with [^{14}C]-amino acids allows the long-term storage of data on gels. Unfortunately, the cost in isotopes demanded by [^{14}C]-labeling is often prohibitive.

In this chapter, methods for labeling both cells in culture and tissues in vitro with [^{35}S]-methionine, [^{3}H]-, or [^{14}C]-amino acids, and ^{32}P for phosphoproteins are described, with particular emphasis on sample preparation for two-dimensional polyacrylamide gel electrophoresis (ref. *2*, *see also* Chapter 11). But these techniques may easily be modified to encompass other uses, such as labeling with the isotopes ^{59}Fe, ^{67}Cu, ^{125}I, or ^{131}I for specific studies of particular proteins.

2. Materials

1. Tissue culture medium: I use a medium rich in amino acids, α-minimal essential medium, but lacking in methionine. Store at 4°C. Any defined tissue culture medium lacking any single, or combination, of amino acids may be used.
2. Dialyzed fetal calf serum (DFCS): Serum is serially dialyzed against two changes of phosphate-buffered saline to remove amino acids and stored in aliquots at –20°C. Do not store this dialyzed serum at 4°C because endogenous proteolytic activity will result in a relatively high concentration of amino acids.
3. Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS): 0.14*M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4.
4. 0.1% (w/v) trypsin in PBS citrate (PBS containing 20 m*M* sodium citrate).
5. Lysis buffer: 9.5*M* urea, 2% (v/v) Nonidet, 2% (v/v) ampholines (1.6%, pH range 5–7; 0.4%, pH range 3.5–10), 5% (v/v) β-mercaptoethanol. Stored frozen at –20°C in 0.5-mL aliquots (*see* Chapter 11).
6. Isotopes: [^{35}S]-Methionine, [^{35}P]-orthophosphate, and [^{3}H]-amino acids are used as supplied. [^{14}C]-Amino acids are lyophilized and resuspended in a medium lacking amino acids at 500 μCi/mL.

3. Methods

3.1. Cells in Monolayers

1. Plate the cells directly into microwells at about 2000 cells/well in 0.25 mL of growth medium. Leave them to attach for at least 5 h, but preferably overnight (*see* Note 1).

2. Remove the media, wash the cells with 0.5 mL of medium lacking methionine and add 0.1 mL medium supplemented with 10% (v/v) dialyzed fetal calf serum, lacking methionine, but containing 100 µCi [^{35}S]-methionine and 1 mg/L unlabeled methionine to each well.
3. Label the cells for up to 20 h at 37°C in a humidified incubator (wrap the microtiter plates in cling film to reduce evaporation).
4. Following incubation, remove the medium, wash the cells twice with ice-cold PBS (*see* Note 2) to remove serum proteins and, if a total cell extract for two-dimensional gel electrophoresis is required, lyse the cells with 20 µL lysis buffer (*see* Note 3). The samples may be stored in small vials or in the microtiter wells at –70°C, or loaded directly onto isoelectric focusing gels (*see* Chapters 11–13).
5. Alternatively, cells may be trypsinized and processed according to the analytical technique required. To retrieve the cells, add 200 µL of ice-cold 0.1% trypsin and incubate on ice until cells begin to detach (approx 15–20 min for most fibroblasts). Neutralize the trypsin with 20 µL of calf serum and pipet gently to completely detach the cells. Retrieve the cells by centrifugation at 350*g* for 3.5 min and wash them twice with ice-cold PBS before dissolving the cell pellet in lysis buffer.
6. Cells may also be adequately labeled for up to about 4 h in a medium completely lacking unlabeled methionine. But under these circumstances, equilibrium labeling may not be achieved.
7. It is difficult to maintain tight physiological control (*see* Notes 4–6) of cells growing in microwells and the cell number obtained is often too small for adequate fractionation. Thus in some experiments, where better control of cell physiology or an increased yield of cells is required, cells growing in larger flasks (25–125 cm^2) may be labeled in medium containing 10% DFCS and methionine at 1 mg/L containing 100–200 µCi of ^{35}S-methionine (2 mL of medium to a 25 cm^2 flask or pro rata according to flask size). Pre-equilibrate flasks with a 95% air/5% CO_2 mix and incubate with occasional rocking to prevent desiccation of the cells. After labeling, wash the cells twice with PBS and collect cells either by scraping with a rubber policeman, or by trypsinization using 2 mL/25 cm^2 flask at 4°C and process as before.

3.2. Cells Growing in Suspension

1. Collect cells growing in suspension by centrifugation (300*g* for 3.5 min), wash them in methionine-free medium, and regain the cells by centrifugation.

2. Resuspend the cells at about 25% of their saturation density in 5 mL medium containing 10% DFCS lacking methionine, but supplemented with 400 µCi mL [^{35}S]-methionine in a 15-mL Falcon round-bottom plastic snap-cap tube previously gassed with a 5% CO_2/95% air mixture. Agitate the cell suspension with a small magnetic flea. This agitation can either be achieved by using a magnetically stirred water bath or a magnetic stirrer set up in an incubator. Alternatively, cells can be agitated by rolling in a temperature regulated roller apparatus. But remember that temperature equilibration is achieved most rapidly in a water bath.
3. Label cells for 1–4 h at 37°C, collect the cells by centrifugation, and wash twice with PBS before processing as before (*see* Note 5).

3.3. Tissues

1. Excise 100–200 µg of the tissue to be labeled, blot it briefly onto filter paper, and chop it into small pieces (approx 2-mm diameter).
2. Place these tissue pieces into 1 mL of tissue culture medium containing 10% dialyzed fetal calf serum, lacking methionine but supplemented with 200 µCi/mL ^{35}S-methionine in glass scintillation vials gassed with a 5% CO_2-95% air mixture.
3. Place the vials in a shaking water bath at 37°C and label for 1 h; thereafter, process the sample as appropriate.

4. Notes

1. For complete details of tissue culture procedures, *see* vol. 5 in this series.
2. Because protein synthesis involves many enzymatic reactions flooding with ice-cold PBS immediately inhibits protein synthesis to very low levels (> 2%) and allows very precise control over timing of incubations.
3. To determine the number of counts incorporated, remove a small aliquot (1–5 µL) from the final sample, add it to 0.5 mL of water containing 10 µg of bovine serum albumin, and precipitate it with 0.5 mL of ice-cold 20% (w/v) trichloroacetic acid (TCA). Allow the precipitate to develop for 10 min and then collect it onto Whatman glass fiber discs under vacuum followed by four washings of 5 mL of 5% (w/v) TCA. Wash the discs finally with ethanol, dry them and count them in a compatible scintillation fluid. Process ^{3}H-labeled samples by overnight digestion with a commercial tissue solubilizer (or 1*M* KOH) before counting. Determine ^{32}P by Cerenkov counting.
4. Accurate protein synthetic rates over short periods may be determined concurrently in parallel flasks by measuring the rate of incorporation of a mixture of three [^{3}H]- or [^{14}C]-labeled essential amino acids (used at 1 µCi or 0.2 µCi/mL, respectively) into acid insoluble counts per

cell. In this case, following labeling aspirate the radioactive medium, wash the cells with ice-cold PBS and trypsinize the cells with 2 mL of 0.1% trypsin (for a 25 cm^2 flask) on ice. After cells have begun to detach (10–15 min) neutralize the trypsin with 200 µL of calf serum, pipet the cells up and down five times and transfer them to a centrifuge tube. Wash the flasks with a further 2 mL of trypsin, combine the supernatants and regain the cells by centrifugation at 300*g* for 3.5 min. Remove the supernatant, dry the tube walls with a cotton bud and resuspend the cells in 2 mL of PBS containing 0.2% FCS. Pipet up and down to disperse the cells, remove 0.2 mL for determination of cell number on a Coulter counter (0.2–7.8 mL PBS +0.2% calf serum to give a 1/40 dilution in a 15 mL snap-cap round bottom Falcon tube). Precipitate the remaining cell suspension with an equal vol of 20% (w/v) TCA and process for scintillation counting as described in Note 3.

5. There are considerable pitfalls in measuring the true rate of protein synthesis using isotopic methods. Accurate protein synthesis measurements may be achieved only when very small amounts of radioactive precursors are added to reduced volumes of the same conditioned growth medium removed from the growing culture and which contains large amounts of unlabeled precursors (*see* ref. *3* for a full discussion). It must also be remembered that mammalian cells show large changes in the rate of synthesis of proteins when growth conditions are altered. For example, amino acid deprivation results in an inhibition of the rate of protein synthesis because of a reduction in the initiation rate of ribosomes onto mRNA *(4)*. This effect also results in the preferential synthesis of proteins whose mRNAs have high intrinsic rates of initiation. The conditions for maintaining protein synthetic rates at steady state levels are clearly not met when fresh medium, often lacking methionine, is used to label proteins for gel electrophoresis! Considerable care should therefore be exercised in standardizing growth and labeling conditions for any experiments involving comparison of different samples and in relating the interpretation of the data to normal, physiological conditions. Often a compromise has to be effected between high levels of incorporation and the physiological constraints of maintaining precursor pool sizes.
6. For determination of protein synthetic rates parallel tubes can be incubated with a mixture of [^{3}H]- or [^{14}C]-labeled amino acids at 1 µCi or 0.2 µCi/mL, respectively in 5 mL of media. At appropriate times remove 1 mL and add it to 9 mL of ice-cold growth medium lacking serum. Regain the cells by centrifugation. Thereafter process the cells as described in Note 4.

7. The handling of cells to be labeled with [^{3}H]- or [^{14}C]-amino acids or [^{32}P]-orthophosphate, is identical. The [^{3}H]- or [^{14}C]-amino acids are exposed to cells in 0.1 mL of medium at 500 μCi/mL in medium lacking the appropriate amino acids or to [^{32}P]-orthophosphate at 2 μCi/mL in medium lacking phosphate.
8. The only other problems that may be encountered, providing care is taken over sterility, pH, and temperature regulation, is the toxicity of isotopes. This is rarely a problem for a day's labeling, but could potentially be so if proteins are labeled for longer. Fibroblasts will survive in 0.5 mCi ^{32}P/mL in medium containing 0.2 m*M* phosphate and in mutant isolation, 1.5 dpm of ^{3}H-amino acid incorporation per cell is considered lethal, but only after a period in the cold for accumulation of radioactive damage. Thus, toxicity will not be a problem if the above labeling procedures are followed.
9. The procedures given above will result in [^{35}S]-methionine-labeled proteins with specific activities of around 10^5 cpm/μg of protein. But obviously the optimal conditions and resultant specific activity of proteins will depend on the cell type, growth conditions and specific activity of the isotope. It is also worth noting that, following two-dimensional gel electrophoresis, a 1 mm^2 spot containing 3 dpm of [^{35}S]-methionine may be readily detected (>0.01 OD above background) after 1 wk.

Acknowledgments

This chapter was prepared while the author's research was supported by grants from NIH HD27372 and the Albert Einstein Cancer Center grant P30-CA1330.

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CHAPTER 11

Two-Dimensional Polyacrylamide Gel Electrophoresis of Proteins

Jeffrey W. Pollard

1. Introduction

Since O'Farrell *(1)* introduced the improved technique for high resolution two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), it has become one of the most powerful tools for the separation and quantification of proteins from complex mixtures. The major reason for this success is that 2-D PAGE employs separation of denatured proteins according to two different parameters, mol wt and isoelectric point. Consequently, it has sufficient resolution to separate individual proteins as discrete spots on the gel. Each parameter may also be varied and therefore, with the modification of nonequilibrium pH-gradient electrophoresis (NEPHGE) to analyze basic proteins *(2)*, almost any polypeptide may be investigated. Thus to date, the O'Farrell 2-D gel system has no serious rivals, with the possible exception of the Kaltschmidt and Wittmann *(3)* gel system for analyzing ribosomal proteins. Ribosomal proteins, however, may be adequately separated with NEPHGE.

It has been estimated from mRNA complexity studies that individual mammalian cells may each contain 10,000 different polypeptides out of the possible 50–100,000 proteins expressed in the organism as a whole. These peptides may range in abundance from 10^9 copies to a few hundred per cell. However, by 2-D PAGE analysis only about 1800 individual proteins can be detected *(4)*, even after long exposures. This is usually interpreted as the inability to

From: *Methods in Molecular Biology, Vol. 32: Basic Proteins and Peptide Protocols*
Edited by: J. M Walker

detect minor proteins, but Duncan and McConkey *(5)* have argued that 2000 is close to the number of proteins in a cell and that the remaining rare mRNAs are rarely, if ever, translated. If this is the case, then, 2-D PAGE represents an even more powerful technique than previously expected for investigating changes in cellular physiology.

Because of its resolution, the 2-D PAGE technique has been applied to a great number of biological problems ranging from the analysis of proteins in different tissues under various hormonal states and at different stages of development, to cells in culture under different growth conditions, to polypeptides within a single cell (*see* reviews in ref. *4*). Another powerful application of the 2-D PAGE system is the detection of proteins that contain single amino acid substitutions, which confer a change in isoelectric point on the protein. This has resulted in definitive identification of mis-sense mutations within proteins *(1)* and the visualization of mistranslated proteins *(6,7)*. The methodology has also allowed for the determination of the states of phosphorylation or glycosylation of proteins *(4)*.

The sophistication of 2-D PAGE has increased dramatically since its introduction. Of particular consequence in the development of data bases of 2D-PAGE patterns and their use in the identification of proteins and for the analysis of changing patterns of gene expression under varying physiological stimuli and in pathological conditions *(8–10)*. These improvements require access to sophisticated equipment and graphic analysis computer packages. However, considerable information can still be obtained using basic commercial, or even homemade apparatuses. In this chapter a straightforward, extremely reliable protocol is described that can be used in almost any laboratory in the world. If the more sophisticated techniques prove necessary, the reader is referred to recent books or journal issues wholly devoted to the 2-D PAGE method *(4,11–13)*.

2. Materials

2.1. First Dimension, Isoelectric Focusing

1. Lysis buffer: 9.5*M* urea, 2% (v/v) Nonidet P-40 (NP-40), 2% (v/v) ampholytes (1.6%, pH range 5–7; 0.4%, pH range 3–10), and 5% (v/v) β-mercaptoethanol (or 100 m*M* DTT).

This buffer may be stored frozen at –80°C for several months in 0.5-mL aliquots. But do not continually freeze and thaw the buffer. Use an aliquot once and discard the remainder.

2. 30% Acrylamide stock solution: 28.38% (w/v) acrylamide and 1.62% (w/v) *N,N'*-methylene-*bis*-acrylamide.

   All acrylamide solutions are light-sensitive and should be filtered and stored in the dark at 4°C. It is usable for at least 1 mo. Acrylamide is a potent neurotoxin, so precautions should be taken to protect against contact with the dust. It should *never* be mouth-pipeted.
3. 10% (w/v) Nonidet P-40 in water.
4. Anode electrode solution: 0.01*M* phosphoric acid. This should be made fresh from a 1*M* stock (63.64 mL phosphoric acid to 1 L water).
5. Cathode electrode solution: 0.02*M* sodium hydroxide. This should be made fresh from a 1*M* stock and degassed.
6. Sample overlay buffer: 8*M* urea and 1% ampholytes (0.8% [v/v], pH range 5–7; 0.2% [v/v], pH range 3–10). This solution may be stored in aliquots at –80°C.
7. Sodium dodecylsulfate (SDS) equilibration buffer: 0.06*M* Tris-HCl, pH 6.8, at 20°C, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol (or 100 m*M* DTT), and 10% (v/v) glycerol.
8. Ampholytes are used as supplied in 40% solutions. They should be kept sterile and stored at 4°C.
9. Ammonium persulfate: 10% (w/v) solution made up fresh every week and stored at 4°C.
10. Pancreatic ribonuclease (50 μg/mL) and deoxyribonuclease (1 mg/mL) in sonication buffer. Store frozen in aliquots.
11. Sonication buffer: 0.01*M* Tris-HCl, pH 7.4, at 20°C, and 0.005*M* $MgCl_2$.

2.2. Second-Dimension, SDS-Slab Electrophoresis

12 30% Acrylamide solution: 29.2% (w/v) acrylamide and 0.8% (w/v) *N,N'*-methylene-*bis*-acrylamide.
13. Running buffer: 0.025*M* Tris base, 0.192*M* glycine, and 0.1% (w/v) SDS. For 3 L: 43.2 g glycine, 9.0 g Trisma base, and 3.0 g SDS gives the correct pH; do not titrate this or add any salt.
14. Separating gel buffer: 1.5*M* Tris-HCl, pH 8.8, at 23°C, and 0.4% (w/v) SDS.
15. Stacking gel buffer: 0.5*M* Tris-HCl, pH 6.8, at 23°C, and 0.4% (w/v) SDS.

16. Bromophenol blue: 0.1% (w/v) bromophenol blue in water. Solutions 2, 3, 7, 8, 9, 11, 12, 14, and 15 are stored at 4°C, and 4, 5, 13, and 16 at room temperature. All other solutions are stored as indicated.

3. Method

3.1. Sample Preparation

The principle to follow here is to avoid causing any chemical modification of proteins, since any charge change will be detected on the gel and will result in an aberrant pattern (*see* Note 1). The following method should give satisfactory results.

1. Resuspend pellets of cells in 100 µL or less of sonication buffer at 4°C. Sonicate the suspended cells with the microtip of an 150W MSE sonicator for six 5-s bursts at 8 µm peak to peak. Be very careful not to overheat the sample.
2. Add 2 µL of RNase and DNase to 100 µL and incubate for 5 min at 4°C.
3. Add solid urea to bring the sample to ~9*M* (100 mg urea/100 µL of sample), which approx doubles the volume. Add to this mixture 1 vol of lysis buffer, take it off the ice, and solubilize the urea with the palm of the hand. Do not heat above 37°C, since this can result in modifications (mainly carbamylation) of the protein. Samples can now be used directly or stored at –70°C until needed (*see* Notes 2 and 3).
4. Alternatively, lyse cells grown and radioactively labeled in microtiter plates directly with 20–40 µL lysis buffer and store the samples at –70°C until use (*see* Chapter 10). In this case, because of the small amount of DNA and RNA in the sample it is not necessary to treat it with nucleases.

3.2. First-Dimension Gels; Isoelectric Focusing

1. Thoroughly clean standard glass tubes, 1–1.5 mm internal diameter and 10–15 cm long, in chromic acid (5% Decon may be used, but beware of precipitation). The tubes should all be of the same length and diameters to ensure run reproducibility.
2. If using chromic acid, rinse the tubes thoroughly in water, place them in fresh KOH in ethanol (0.4 g KOH to 20 mL ethanol) for 20 min, rinse thoroughly first with distilled water, then ethanol, and finally allow the tubes to air dry.
3. When dry, seal the tube bottoms with three layers of parafilm and line them up vertically around a 250-mL beaker with elastic bands (or in a tight-fitting rack). Mark the tubes to the same point, approx 1 cm from

the top, with a felt-tipped pen to ensure that gel lengths are uniform (this is important to facilitate reproducibility between runs).

4. To make 10 mL of isoelectric focusing gel solution, add to a 125-mL flask: 5.5 g urea, 1.33 mL acrylamide stock (solution 2), 2.0 mL NP-40 (solution 3), 1.7 mL water, 0.6 mL ampholytes (pH 5–7), and 0.115 ampholytes (pH 3–10). Gels may be made easier to remove by increasing the nonidet concentration by 10%.
5. To make nonequilibrium polyacrylamide gels (NEPHGE), weigh out the same quantity of urea, add the same volume of acrylamide stock and NP-40, but add 2.0 mL of water, 0.25 mL ampholytes (pH 7–9), and 0.25 mL ampholytes (pH 8–9.5).
6. Dissolve the urea by swirling in a water bath whose temperature is set no higher than 37°C and then briefly degas under vacuum.
7. Add 7 µL (IEF) or 14 µL (NEPHGE) of TEMED and 10 µL (IEF) or 20 µL (NEPHGE) of 10% ammonium persulfate solution. You now have about 15 min to pour the gels at normal room temperature (20°C), but if room temperatures are substantially higher, reduce the TEMED concentration to about two-thirds.
8. Using a long narrow-gage hypodermic needle (or drawn-out Pasteur pipet) fill the tubes to the mark, being careful to avoid trapping air bubbles (if present these may be removed by carefully tapping the tubes).
9. Overlay the gel mix with 10 µL of water, allow it to polymerize for 2 h, and then carefully remove the parafilm by cutting in order to avoid damage to the bottom of the gel. The parafilm may be replaced by dialysis tubing clipped on with a rubber O-ring to prevent the gel slipping out, but generally this precaution is unnecessary.
10. Place the gels in the electrophoresis tank. (We use an [-shaped tank rather than the conventional round one, since these are easy to manufacture and operate, and can be fitted with locking devices to prevent electric shocks to the operator. However, many commercial devices are now available.)
11. Fill the lower chamber with 10 m*M* phosphoric acid (IEF) or 20 m*M* NaOH (NEPHGE), and remove any trapped air bubbles from the end of the gels by a gentle stream of fluid using a bent syringe.
12. For IEF, remove the water from the top of the gel with a Pasteur pipet and replace it with 10 µL lysis buffer, 10 µL overlay buffer, and enough 20 m*M* NaOH to fill the tubes.
13. Add the cathode solution carefully to the upper chamber, connect it to the cathode and the lower chamber to the anode, and prerun the gels at

200 V for 15 min, 300 V for 30 min, and 400 V for 1 h or more. At the end of this procedure, there should not be more than 1.5 mA/5 tubes or 2 mA/12 tubes. Do not cool the gels during this procedure since the urea will crystallize out.

14. After the prerun remove the NaOH from the upper chamber carefully so as to avoid contact with the gel surface and discard. Remove the liquid from above the gels and wash the tops with three washes of 20 µL of water.
15. Load the samples in a vol of 5–50 µL with a syringe and overlay the sample with 10 µL sample overlay buffer and 20 m*M* NaOH to fill the tubes.
16. Fill the upper electrophoresis chamber with 20 m*M* NaOH, reconnect it to the anode and cathode, and electrophorese it for 18 h at 400 V. One hour before the termination of the run, turn the voltage up to 1000 V to increase the band sharpness, but do not exceed 10,000 V/h since bands will become distorted.
17. Turn off the power pack, wait a few seconds, then remove the tubes and force the gels out onto parafilm troughs with a syringe full of water connected to the tubes via a flexible plastic tubing.
18. Put the gels (using the parafilm to handle them) into capped tubes containing 5 mL of sample buffer and 0.002% bromophenol blue. Leave for at least 20 min without agitation and at this point the gels may be stored at –70°C indefinitely. After defrosting, exchange the sample buffer and leave for a further 30 min before loading onto the second dimension, by which time the second-dimension slab gel will have been prepared.
19. For NEPHGE there is no prerunning since a stable pH gradient is not formed; therefore load the samples directly onto the gels, overlay them with 10 µL of sample overlay buffer and 10 m*M* phosphoric acid to fill the tubes. Fill the upper reservoir with 10 m*M* phosphoric acid and connect it to the anode and run the gels at 400 V for 4.5 h. However, since this is a nonequilibrium procedure, very basic proteins (e.g., some ribosomal proteins or histones) may migrate off the end of the gel. Thus shorter total voltage hours may need to be used.
20. Investigators may wish to test their procedures at this point and gels may be stained as described in Chapter 9 (*see* Note 4). The bands should be razor sharp. pH gradients may also be tested by slicing the gel into 0.5-cm pieces, then equilibrated for 1 h in degassed water, and the pH read using a microelectrode and a pH meter. Similarly, a pH contact

electrode may be used or alternatively visible pH markers may be bought from a range of suppliers. pH ranges should be from about 3.5 to 7.5 for the IEF gels.

3.3. Second-Dimension Gels; SDS-Slab Gel Electrophoresis

1. Assemble the slab-gel apparatus (we use the Bio-Rad/Hoeffler design, which gives consistently good results, but any homemade or commercial slab-gel system is suitable) ensuring that there is a good seal. Make it level and vertical. The plates should have been thoroughly washed in 5% Decon, rinsed with water and ethanol, and air dried. Do not wipe with tissues that leave lint since this will interfere with gel polymerization.
2. Make the SDS separating gel as follows for 100 mL of a 10% gel (for other percentages adjust the ratio of acrylamide to water accordingly, *see* Note 5): 33.3 mL acrylamide stock (solution 12), 41.7 mL water, and 25.0 mL separating gel buffer. Mix the solutions and degas under vacuum.
3. Add 50 µL of TEMED and 333 µL of 10% ammonium persulfate and pour the gel by pipeting the solution down the side of the gel plates to about 2.5 cm from the top. Overlay it with deionized water applied at one end and leave it to polymerize for 1 h until the gel interface can be seen as a sharp straight boundary (*see* Note 6). The gel may be left to stand overnight.
4. Remove the water overlay, wash the gel surface with water, and pour the stacking gel. This is prepared fresh and to make 25 mL of solution add: 4.0 mL acrylamide stock (solution12), 14.75 mL water, and 6.25 mL stacking gel buffer.
5. Degas briefly, add 25 µL TEMED and 87.5 µL ammonium persulfate, and pour (taking care to avoid trapping any air bubbles) up to a level 1 mm below the gel plate edge before inserting a Teflon straightedge. Overlay with water and allow this solution to polymerize for 30 min.
6. Remove the Teflon strip, rinse the surface of the gel with water, and blot dry.
7. Take the defrosted, re-equilibrated isoelectric focusing gel and straighten it in a trough of parafilm. Remove the liquid and apply it directly to the top of the stacking gel (best performed with the gel flat or at a slight angle). Press down gently with a curved spatula tip from one end to ensure the removal of air bubbles, but be careful not to stretch the gel since this is one stage where variability can be introduced into the procedure.

8. I find the gel adheres; however, the more conventional but slower method is to overlay the gel with 2 mL 1% agarose in SDS sample buffer (not too hot) and then to allow it to set for 5 min. If bromophenol blue had not been included in the sample buffer, add a few drops of 0.1% bromophenol blue over the total length of the gel.
9. Assemble the slab-gel system, fill the reservoirs with running buffer, and remove any air bubbles trapped under the gel with a bent syringe needle or Pasteur pipet. Connect the lower gel reservoir to the anode and the upper reservoir to the cathode, and subject the gels to electrophoresis at 9 mA/gel overnight or at 20 mA/gel for 4–5 h until the bromophenol blue reaches the bottom of the gel. It is essential to avoid overheating, and thus if the gels are to be run fast, the whole apparatus can be run in the cold room, or, alternatively, an efficient cooling system may be incorporated into the gel apparatus. After 1–2 h, change the upper reservoir buffer (or alternatively, continuously mix the upper and lower reservoirs with a pump-siphon system) to prevent vertical tailing of the protein spots (*see* Note 7).
10. At the end of the run, turn off the power, remove the plates, separate them with gentle leverage, and process the gel for staining (*see* Chapter 5) or autoradiography (*see* Chapter 20).
11. Briefly for staining, fix gels in 45% (v/v) methanol and 7.5% (v/v) glacial acetic acid (or 15% TCA) and stain with 0.2% (w/v) Coomassie blue (prepared by dissolving it in a small volume of methanol) in 45% (v/v) methanol and 10% (v/v) glacial acetic acid for 30 min to 1 h (*see* Note 8). Destain the gels using several changes in the fixative; the latter may be regenerated by absorbing the Coomassie blue by passing it through an activated charcoal filter.
12. Dry gels down using a gel drier (*see* Chapter 19) and subject them to autoradiography, using Kodak-XO-mat-AR film for several different lengths of time. Owing to the saturation of the film, this allows visualization and qualification of a range of proteins with different abundances.
13. Autoradiographs may be quantified using scanning gel densitometry, by the slower method of quantitative roster scanning or for greater accuracy gels can be directly quantitated with a phosphorimager. However, a better, rapid, and accurate method for handling a large number of proteins is to count the resolved spots in a scintillation counter. To do this prepare transparent plastic sheets (the sort used for overhead projector transparencies) as templates. Mark the position of the protein spots on the autoradiogram on the sheet and use this as a template

to identify the spots from the original dried gel. Punch out appropriate areas of the gel and count these areas in a scintillation counter. Label the plastic template with a suitable code and this then becomes a permanent record of the protein spot position and may be filed with the resultant counts and autoradiograms. This enables long-term handling of data from a larger number of gels for metabolic experiments. A typical gel pattern is shown in Fig. 1 (*see* Note 9).

4. Notes

1. The major problems associated with two-dimensional gels are spot streaking and artifactual charge heterogeneity. The principle charge artifacts are produced by posttranslational modifications of proteins; for example, the spontaneous deamination of asparagine and glutamine or the oxidation of cysteine to cysteic acid. Care should be taken when preparing extracts and they should always be maintained in the cold and stored in lysis buffer. Proteins may also be carbamylated by isocyanate impurities in the urea solution, and therefore it is worth investing in the highest quality urea, preparing it fresh, and storing it at –20°C. It is also advisable to have ampholytes present wherever proteins are in contact with urea and to prerun the isoelectric focusing gels to remove isocyanate contamination. Basic ampholytes may precipitate nucleic acids, which, with the resultant binding of proteins, will produce streaking, but this may be easily solved by treating the sample with ribonuclease and deoxyribonuclease as described. Despite these problems sample preparation by the methods described should result in trouble-free gels.
2. Spot size increases nonlinearly with high protein concentrations and overloading may cause precipitation at the top of the isoelectric focusing gel, which can streak across the pH gradient. Overloading may also cause pH inversions in the isoelectric focusing dimension. The best separation and resolution is therefore obtained when the lowest amounts of protein are applied to the gel, but concentrations up to 100 µg/gel are tolerated. Obviously, the best results are obtained with small amounts of protein having high specific activities. The evolution of highly sensitive silver stains has diminished the requirements for high protein concentrations somewhat. If samples are too dilute or at too low a specific activity, they may be precipitated with ammonium sulfate or better, lyophilized, followed by solubilization of the pellet directly in lysis buffer. But remember that any salt will also be concentrated by these techniques and the salt may cause artifacts on

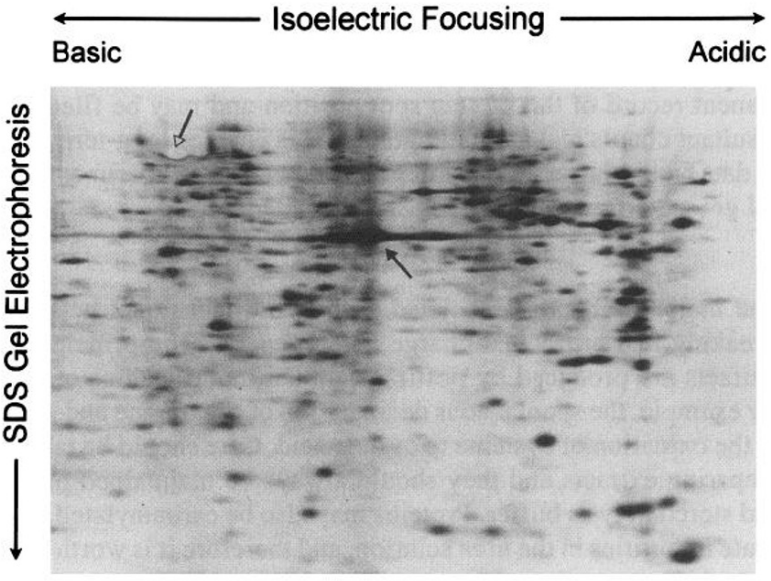

Fig. 1. A typical two-dimensional polyacrylamide gel. An autoradiograph of a 10% two-dimensional polyacrylamide gel of [^{35}S]-methionine labeled chick embryo fibroblast proteins. Cells were radioactively labeled in microtiter plates followed by direct lysis in the well with lysis buffer before loading onto the isoelectric focusing gel. The autoradiogram represents about 2×10^6 cpm of [^{35}S]-methionine labeled proteins exposed for 5 d. The filled arrow shows the position of actin and its isoforms; note the sideways streaking caused by the large concentration of this protein. The open arrow shows the position of unlabeled serum proteins (albumin) that remains from the tissue culture medium despite washing the cell layer with PBS. This albumin because of its high concentration has excluded the labeled proteins in this region.

the gel. Cells, if they are grown in a small area, for example in a microwell (*see* Fig. 1 and Chapter 10), may also be taken up directly in lysis buffer and applied without processing to the gel, consequently avoiding dilution during sample preparation. Overloading can be a problem, particularly with cells grown in serum, because of the enormous concentration of albumin (*see* Fig. 1). Methods such as immunoprecipitation of the major proteins might improve resolution of minor components. Interestingly, it is also possible to polymerize proteins within the isoelectric focusing gel, and this increases capacity, ameliorates the problem of precipitation, and gives acceptable gels.

3. We have found that, when cells in culture are trypsinized, if they are not thoroughly washed, the trypsin may carry over and cause streaking and artifactual gel patterns. Degradation by cellular proteins, particularly of nuclear proteins, has also proved a problem and this has been remedied by adding protease inhibitors such as sodium bisulfite and diisopropylfluorophosphate. These should be omitted from the 2-D sample gel preparation since they may cause modification of proteins.
4. Reproducibility can also be a problem and this is usually caused by batch variation in ampholytes (*see* ref. *4* for extensive discussions). If it can be afforded, batch testing is advisable. Manufacturers have also promised to improve the reproducibility of their ampholytes. To date, LKB manufactures the most consistent product. It is also important to keep gel lengths and run times the same from day-to-day and particularly to exercise care in handling the isoelectric focusing gel to avoid stretching it. During equilibration between 5 and 25% of protein may elute from the gel, and variable amounts of protein may also precipitate at the top of the gel during the run. This can be a problem when comparing, for example, time courses of protein synthesis when it is desirable to add constant amounts of protein to the gels. Low concentrations of protein in the sample reduce the precipitation problem and O'Farrell *(1)* has described a technique for rapid equilibration involving a high SDS buffer added directly to the isoelectric focusing gel already *in situ* on the slab gel.
5. Other ratios of acrylamide to *bis*-acrylamide can also be used to alter the crosslinking and therefore, the gels' resolution (*see* ref. *4* for details).
6. Workers when they first start running gels often obtain poor polymerization. This is usually caused by dirty plates or poor quality *bis*-acrylamide or acrylamide. Commercial electrophoresis-grade chemicals are usually of high enough quality, but acrylamide may be purified by heating 1 L of chloroform in a water bath at 50°C and adding to it 70 g acrylamide. Filter this through Whatman No. 1 grade filter paper while it is hot, and leave the resultant liquor on ice for several hours to recrystallize. Collect crystals in a Buchner funnel and wash with cold chloroform. Leave the crystals to dry. *Bis*-acrylamide may be crystallized by bringing 1 L of acetone to a boil in a water bath, adding 10 g *bis*-acrylamide and reboiling it. Thereafter, continue as described for acrylamide, except that crystallization is performed at –20°C. But remember these chemicals are highly toxic. Finally, to improve polymerization, we make up all solutions in double glass-distilled or deionized water (but be careful of flaking of ion-exchange resin from the column) and we filter all gel solutions through 0.45-μm filters before use.

7. Verticle streaking can also occur, and this is caused either by not changing or recycling the running buffer or, more commonly, by poor equilibration of the first-dimension gels. Occasionally, horizontal streaking can occur owing to poor solubility of proteins, but high concentrations of NP-40 and urea usually increase solubility sufficiently.
8. Ampholines also run as small proteins, are acid precipitable, and will stain. They can be eluted from the gel using the fixative described above and thus masking of small proteins migrating near the gel front is avoided.
9. SDS slab-gel electrophoresis separates according to mol wt since SDS binds to most proteins on a molar basis (1.4 to 1) giving a uniform charge-to-mass ratio. Thus, dependent on the size range of the proteins to be analyzed, a suitable percentage acrylamide gel may be selected. Gradient gels may also be run and these give greater accuracy to mol-wt determinations of glycoproteins. Anomalous migration of unusual proteins can occur, for example those containing a large percentage of basic residues, or those with large amounts of carbohydrate.

Acknowledgments

These techniques were derived from the original O'Farrell papers, simplified with the help of J. Parker, J. Friesen, R. Bravo, and J. Celis. This chapter was prepared and my work was funded by HD27372 and the Albert Einstein Cancer Center grant P30-CA1330.

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CHAPTER 12

Two-Dimensional Polyacrylamide Gel Electrophoresis Using Immobilized pH Gradients in the First Dimension

Michael J. Dunn and Joseph M. Corbett

1. Introduction

The high resolution capacity of two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) based on the method of O'Farrell *(1)* makes it the method of choice for the analysis of complex protein mixtures *(2–4)*. In the standard procedure, cylindrical tube isoelectric focusing (IEF) gels cast in glass capillary tubes are used for the first-dimension separation (*see* Chapter 11). Unfortunately, this system suffers from cathodic drift during IEF, resulting from the very high electroendosmotic flow caused by charged groups on the glass walls of the gel tubes. As a consequence, pH gradients are unstable and rarely extend above pH 8.0, leading to loss of basic proteins from 2-D maps. Gradients can be extended to pH 10 by special treatment of the glass IEF tubes *(5)* or by using horizontal flat-bed IEF (*see* Chapter 13).

In spite of the improvement in resolution of basic proteins that can be achieved with these procedures, difficulties are still associated with the first-dimension IEF gels as a consequence of the characteristic properties of the synthetic carrier ampholytes used to generate the pH gradients. These include problems of batch reproducibility of ampholytes, lack of reproducibility of separations, difficulty in control

From *Methods in Molecular Biology, Vol 32: Basic Protein and Peptide Protocols*
Edited by: J. M Walker

of pH gradient stability and shape (i.e., pH gradient engineering), and the possibility of artifacts caused by protein–ampholyte interactions.

The development of immobilized pH gradients (IPG) *(6)* has provided a solution to these problems, since this technique produces reproducible, stable pH gradients of any desired pH range. The Immobilines (Pharmacia, Milton Keynes, UK) are a series of seven substituted acrylamide derivatives with different pH values. A detailed review of IPG technology can be found in ref. *7*. IPG IEF gels are made by generating a gradient of the appropriate Immobiline solutions, so that during polymerization the buffering groups forming the pH gradient are covalently attached and immobilized via vinyl bonds to the polyacrylamide backbone. This immobilization results in the elimination of pH gradient drift (but *not* electroendosmosis) making pH gradients reproducible and infinitely stable—ideal properties for 2-D PAGE.

Considerable problems were encountered in early attempts to apply IPG technology to the IEF dimension of 2-D PAGE. These problems were caused not by problems with the IPG IEF dimension *per se*, but by problems in elution and transfer of proteins from the first-dimension IPG gels to the second-dimension SDS-PAGE gels, resulting in streaking of spots on 2-D maps. This phenomenon was found to be a result of the presence of fixed charges on the Immobiline matrix, leading to increased electroendosmosis in the region of contact between the IPG IEF gel and the SDS-PAGE gel and resulting in disturbance of migration of proteins from the first- to the second-dimension gel.

The problems associated with the use of IPGs for 2-D PAGE have now been overcome, largely because of the work of Angelika Görg and her colleagues in Munich, and a standardized protocol of 2-D PAGE has been described *(8)*. In this method, IPG gels (0.5 mm thick) are cast on GelBond PAG support films and subsequently dried. The dried gels are cut into strips that are rehydrated in a solution containing urea and nonionic or zwitterionic detergent. These IPG gel strips are used for the IEF dimension of 2-D PAGE using a horizontal flatbed IEF apparatus. The IPG gel strips are, therefore, analogous to the cylindrical IEF gels used in the traditional O'Farrell technique (*see* Chapter 11). After IEF is complete, the IPG strips are equilibrated and applied to second-dimension SDS-PAGE gels using either a vertical or horizontal electrophoresis apparatus.

IPGs were originally optimized for the generation of narrow and ultra-narrow pH gradients not generally suitable for 2-D applications. Recipes are now available for producing wide pH gradients, so that extended pH gradients spanning the range pH 2.5–11 have been applied to 2-D separations *(9)*. Very wide pH gradients are ideal for maximizing the number of proteins resolved on a single 2-D gel and for initially examining the complexity of a particular protein mixture. However, the resulting 2-D maps are generally very complex and regions of the 2-D patterns where large numbers of proteins are distributed tend to be crowded making analysis (both visual and computer-aided) very difficult. We, therefore, suggest that it is better for most applications to use pH gradients spanning three or four pH units. In 2-D PAGE we routinely use pH 4–8 IPG IEF gels for the separation of acidic and neutral polypeptides and pH 6–10 IPG IEF gels for neutral and basic polypeptides.

2. Materials

1. GelBond PAG sheets (20 × 26 cm) (*see* Note 1).
2. Glass plates (20 × 26 cm) with a 0.5 mm thick U-frame for gel casting (Pharmacia).
3. Glass plates (20 × 26 cm) without U-frame (Pharmacia).
4. Reswelling cassette (12.5 × 26 cm) (Pharmacia).
5. Rubber roller.
6. Gradient mixer (2 × 15 mL).
7. Horizontal electrophoresis apparatus (*see* Note 2).
8. Power supply able to provide an output of 3500 V.
9. Thermostatic circulator.
10. IEF sample applicator strip, 53 well/20 µL (Pharmacia).
11. Roll of Parafilm (50 cm × 15 m).
12. Electrode wicks (25 × 0.7 cm) cut from sheets of Whatman Chr17 chromatography paper.
13. Urea (BRL Ultrapure, Life Technologies, Renfrew, Paisley, UK) should be stored dry at 4°C to reduce the rate of breakdown of urea with the formation of cyanate ions, which can react with protein amino groups to form stable carbamylated derivatives of altered charge.
14. Repel-Silane (Pharmacia).
15. Solution A; 9*M* urea (100 mL): Dissolve 54.0 g of urea in 59.5 mL deionized water. Deionize the solution by adding 1 g Amberlite MB-1 monobed resin (Merck, Poole Dorset, UK) and stirring for 1 h. Filter the solution using a sintered glass filter.

16. Solution B; sample lysis buffer: 9*M* urea, 2% (w/v) CHAPS, 1% (w/v) dithiothreitol (DTT), 0.8% (w/v) Pharmalyte (Pharmacia) (*see* Note 3) Add 2.0 g CHAPS, 1.0 g DTT, and 2.0 mL of Pharmalyte 3-10 to 96.0 mL of solution A.
17. Solution C; Acrylamide/Bis solution (30%T, 4%C): Dissolve 28.8 g acrylamide and 1.2 g *N,N'*-methylene *bis*-acrylamide (Bis) in distilled water and make up to 100 mL. Deionize the solution by adding 1 g Amberlite MB-1 resin and stir for 1 h. Filter and store at 4°C for a maximum of 1 wk.
18. Recipe for preparation of pH 4–8 IPG gel (*see* Table 1).
19. Recipe for preparation of pH 6–10 IPG gel (*see* Table 2).
20. Solution D; 8*M* urea solution (40 mL): Dissolve 19.2 g of urea in 25.6 mL deionized water. Deionize the solution by adding 1 g Amberlite MB-1 monobed resin (Merck) and stirring for 1 h. Filter the solution using a sintered glass filter.
21. Solution E (reswelling solution): 8*M* urea, 0.5% (w/v) CHAPS (*see* Note 9), 0.2% (w/v) DTT, 0.2% (w/v) Pharmalyte 3-10. Add 60 mg DTT, 150 mg Triton X-100 and 150 µL Pharmalyte 3-10 to 29.7 mL of solution D.
22. Silicon fluid, Dow Corning 200/10 cs (Merck).
23. Solution F (Catholyte): 10 m*M* lysine.
24. Solution G (Anolyte): 10 m*M* glutamic acid.
25. Solution H (Ultrodex suspension): Swell 30 mg Ultrodex (Pharmacia) in 1 mL sample lysis buffer (solution B) overnight.
26. Solution I (equilibration buffer [100 mL]): 6*M* urea, 30% (w/v) glycerol, 2% (w/v) SDS, 50 m*M* Tris-HCl buffer, pH 6.8. Add 36 g urea, 30 g glycerol, and 2 g SDS to 3.3 mL 1.5*M* Tris-HCl buffer, pH 6.8.
27. Solution J (DTT stock solution): Add 200 mg DTT to 1 mL deionized water. Prepare immediately before use.
28. Solution K; Bromophenol blue solution: Add 30 mg bromophenol blue to 10 mL 1.5*M* Tris-HCl buffer, pH 6.8.

3. Method

1. Sample preparation: Samples of isolated cells can be prepared by suspension in a small volume of lysis buffer, followed by disruption by sonification in an ice bath. Samples of solid tissues should be homogenized while still frozen in a mortar cooled with liquid nitrogen. The resulting powder is then suspended in a small volume of lysis buffer. Plant cells and tissues often require additional treatments *(8)*. The final protein concentration of the samples should be about 10 mg/mL. Protein samples should be used immediately or stored frozen at –80°C.

Table 1
Recipe for Preparation of pH 4–8 IPG IEF Gel

Immobiline pK_a	Acidic dense solution, pH 4.0 (10 mL)	Basic light solution, pH 8.0 (10 mL)
3.6	392 µL	—
4 6	169 µL	369 µL
6.2	157 µL	240 µL
7.0	78 µL	95 µL
8.5	113 µL	223 µL
9.3	—	192 µL
Deionized water	5.8 mL	7.6 mL
For effective polymerization both solutions are adjusted to pH 7.0 before addition of acrylamide and glycerol (the acidic solution with 1N NaOH and the basic solution with 1.5N acetic acid).		
Acrylamide (solution C)	1.3 mL	1.3 mL
Glycerol (100%)	2.5 g	—
	add just before use	
TEMED	6 µL	6 µL
Ammonium persulfate (40%)	10 µL	10 µL

Table 2
Recipe for Preparation of pH 6–10 IPG IEF Gel

Immobiline pK_a	Acidic dense solution, pH 6.0 (10 mL)	Basic light solution, pH 10.0 (10 mL)
3.6	627 µL	67 µL
6.2	182 µL	222 µL
7.0	162 µL	241 µL
8.5	173 µL	159 µL
9.3	188 µL	217 µL
Deionized water	5.4 mL	7 8 mL
For effective polymerization both solutions are adjusted to pH 7.0 before addition of acrylamide and glycerol (the acidic solution with 1N NaOH and the basic solution with 1.5N acetic acid).		
Acrylamide (solution C)	1.3 mL	1.3 mL
Glycerol (100%)	2.5 g	—
	add just before use	
TEMED	6 µL	6 µL
Ammonium persulfate (40%)	10 µL	10 µL

2. Preparation of gel casting cassette: To prevent the gel from sticking to the glass plate with the 0.5 mm U-frame, pour 2 mL of Repel-Silane on the plate and distribute it evenly over the surface with a tissue, and leave it to dry for a few minutes. Then, rinse the plate with distilled water, drain, and leave to air dry. Cut a piece of GelBond PAG film measuring 20 × 26 cm (*see* Note 4). Pour a few milliliters of water on the plain glass plate and lay the GelBond PAG film over it with the hydrophilic side up (*see* Note 5). Use the roller to eliminate air bubbles and seal the film to the plate. Clamp the glass plates together with two clips on each side and along the bottom of the cassette.
3. Preparation of IPG gel: Prepare the two starter solutions appropriate for the pH gradient to be constructed (i.e., pH 4–8, *see* Table 1 or pH 6–10, *see* Table 2). Place the gel casting cassette, which should be precooled in a refrigerator, on a level table. Set up the gradient mixer on a magnetic stirrer. Insert the silicon delivery tube attached to the outlet of the gradient mixer between the glass plates of the cassette. Place a magnetic stirring bar in the mixing chamber of the gradient mixer. Make sure that the valve between the two chambers of the gradient mixer and the stopcock on the delivery tube are closed. Pipet the dense gel solution into the mixing chamber of the gradient mixer and the light gel solution into the reservoir chamber. Turn on the magnetic stirrer and open the valve between the two chambers of the gradient mixer. Open the stopcock on the delivery tube and allow the gel solution to flow into the gel cassette. Leave the cassette at room temperature for 15 min to allow adequate levelling of the density gradient prior to polymerization in an oven for 1 h at 50°C.
4. When polymerization is complete, remove the clamps and disassemble the cassette, leaving the IPG gel on its GelBond PAG support.
5. Wash the IPG gel six times, at 10 min each, with deionized water.
6. Wash the IPG gel with 20% (w/v) glycerol for 10 min.
7. Dry the gel at room temperature in a dust-free cabinet with a ventilator.
8. Cover the dried gel with a sheet of plastic food wrapping film and store at –20°C (*see* Note 6).
9. Preparation of reswelling cassette: The reswelling cassette comes fitted with a 0.5 mm U-frame gasket. However, the thickness of the gasket must be increased to 0.7 mm to allow the IPG gel to rehydrate to its original thickness (i.e., 0.5 mm) and to allow for the thickness of the GelBond support film (0.2 mm). This is achieved by cutting two U-shaped gaskets of the appropriate size from a sheet of Parafilm (0.1

mm thick). Clamp the glass plates and gaskets together to make the reswelling cassette and place it upright on a level table. Fill the cassette with reswelling solution (solution E).

10. Preparation of IPG gel strips: For the IEF dimension of 2-D PAGE, cut the dried slab gel into 3 mm strips (*see* Note 7) using a paper cutter (*see* Note 8). Remove the protective plastic film from the required number of IPG gel strips and insert them into the reswelling cassette. Rehydrate at room temperature for a minimum of 6 h or overnight.
11. IPG IEF dimension: Disassemble the reswelling cassette and carefully remove the rehydrated IPG gel strips. Blot the strips lightly between two sheets of water saturated filter paper to remove excess rehydration solution. Place the IPG gel strips side by side, 2 mm apart, on the flatbed cooling plate (20°C), precoated with a thin film of silicon fluid, of the horizontal IEF apparatus. The film of silicon fluid, which has excellent thermal conductivity properties and a low viscosity, allows for good contact between the gel strips and the cooling plate. The basic end of the IPG strips must be at the cathodic side of the apparatus. Wet the electrode wicks with the electrode solutions (solutions F and G) and apply them across the appropriate ends of the IPG gel strips (i.e., cathodic wick at the basic end and anodic wick at the acidic end of the strips). Apply silicon rubber frames (*see* Note 10), containing 8 × 2 mm sample slots, to the gel surface at a distance of 5 mm from the anode or cathode (*see* Note 11). Apply 20 µL of sample solution, diluted 1:1 with Ultrodex™ suspension (solution H), into the wells in the silicon frames. A 30% (w/v) solution of NaOH should be present within the apparatus to absorb CO_2.
12. IEF running conditions: Run the IPG IEF gels at 0.05 mA/strip, and 5 W limiting. For improved sample entry, limit the initial voltage to 150 V for 30 min (75 Vh) and then 300 V for 60 min (300 Vh). Continue IEF with maximum settings of 3500 V, 2 mA, and 5 W until constant focusing patterns are obtained. The precise running conditions required depend on the pH gradient, the separation distances used, and the type of sample being analyzed (*see* Note 12).
13. After completion of IEF, remove the gel strips from the apparatus. Freeze the strips and store them at –80°C if they are not to be used immediately for the second dimension separation.
14. Equilibration of IPG gel strips: Equilibrate IPG gel strips with gentle shaking for 2 × 15 min in 10 mL equilibration buffer (solution I). Add 500 µL/10 mL DTT stock (solution J) and 30 µL/10 mL bromophenol

blue stock (solution K) to the first equilibration solution. Add 500 mg iodoacetamide/10 mL of the second equilibration solution (final concentration iodoacetamide 5% [w/v]).

15. SDS-PAGE dimension: The second dimension SDS-PAGE separation is carried out using a standard vertical SDS-PAGE system, of the normal Laemmli type *(10)*, as used in the standard 2-D PAGE technique (*see* Chapters 5 and 11) (*see* Note 13). The gels can be either of a suitable constant percentage concentration of polyacrylamide or of a linear or nonlinear polyacrylamide concentration gradient (*see* Chapter 6). We routinely use 1.5 mm thick 12% T SDS-PAGE gels (20 × 25 cm). No stacking gel is used.
16. Rinse the equilibrated IPG gel strips with deionized water and blot them on filter paper to remove excess liquid.
17. Apply the IPG gel strips to the SDS-PAGE gels by filling the space in the cassette above the separation gel with upper reservoir buffer and gently slide the strips into place. Good contact between the tops of the SDS gels and the strips must be achieved and air bubbles must be avoided. Cement the strips in place with 1% (w/v) agarose in equilibration buffer.
18. The gels are run in a suitable vertical electrophoresis apparatus. We use the SDS-PAGE tank of the Investigator 2-D PAGE system (Millipore, Watford, UK) that allows up to 5 large-format (20 × 25 cm) second-dimension SDS-PAGE gels to be electrophoresed simultaneously. The gels are run at 15 mA/gel overnight at 15°C until the bromophenol blue tracking dye reaches the bottom of the gels.
19. The gels can be subjected to any suitable procedure to detect the separated proteins (e.g., electroblotting, autoradiography, staining with silver or Coomassie brilliant blue, and so on). A typical separation of human myocardial proteins using this technique is shown in Fig. 1.

4. Notes

1. We routinely use IPG gels 25 cm wide with an 18 cm pH gradient separation distance, but it is possible to use gels of other sizes (e.g., 11 cm or 4 cm pH gradient separation distance) *(8)*.
2. We use the Multiphor II (Pharmacia) horizontal electrophoresis apparatus, but any horizontal flat-bed IEF apparatus can be used.
3. Lysis buffer should be prepared freshly. Small portions of lysis buffer can be stored at –80°C, but once thawed they should not be frozen again.
4. GelBond PAG film should be washed six times, at 10 min each, with deionized water prior to use to minimize spot streaking.

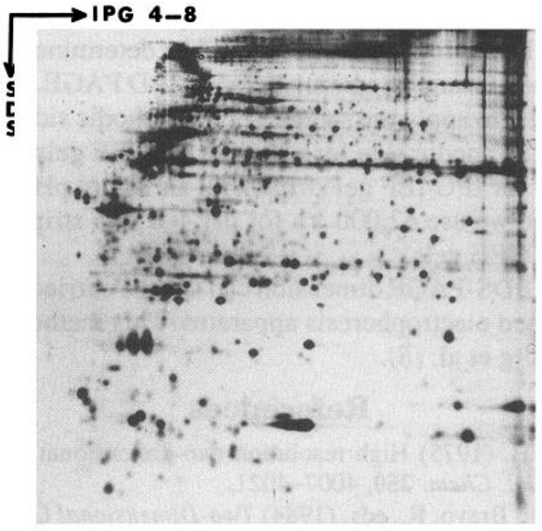

Fig. 1. Silver stained 2-D pattern of human myocardial proteins. A loading of 15 µg protein was used. The first dimension was pH 4–8 IPG IEF and the second dimension was 12% T SDS-PAGE.

5. The gel will adhere only to the hydrophilic surface of GelBond PAG film. Avoid touching this surface as it will reduce gel adherence.
6. Dried IPG gels can be stored frozen for at least one year.
7. It is possible to use IPG gel strips up to 5 mm wide for the IEF dimension of 2-D PAGE, but the best results are obtained with 3 mm strips.
8. Dried IPG gels are commercially available as DryPlates™ (covering the range pH 4–7) or DryStrips™ (pH 4–7 or pH 3–10.5) from Pharmacia). However, at present the pH gradient separation distance is limited to 10 cm. DryPlates must be cut into 3 mm strips before rehydration, whereas DryStrips are already in strip form. After rehydration, these commercial strips are used and handled in the same way as described for homemade IPG gels.
9. We use the zwitterionic detergent CHAPS in the IPG IEF gel as this can give improved solubilization and resolution of certain proteins, but good results can also generally be obtained using nonionic detergents such as Triton X-100 and Nonidet NP-40.
10. The silicon rubber frames are cut from the IEF application strip (*see* Materials, item 10).

11. Samples can be applied at either the cathodic or anodic side of the IPG gels strips. The optimal position should be determined experimentally for each type of sample to be analyzed by 2-D PAGE. For our samples, we find it best to apply the samples at the cathodic side for pH 4–8 IPG IEF and at the anodic side for pH 6–10 IPG IEF gels.
12. As a guide, for IPG IEF gel strips with an 18 cm pH gradient separation distance we use 42,000 Vh for pH 4–8 IPG strips and 35,000 Vh for pH 6–10 IPG gels.
13. The second SDS-PAGE dimension can also be carried out using a horizontal flat-bed electrophoresis apparatus. This method is described in detail by Görg et al. *(8)*.

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CHAPTER 13

Two-Dimensional Polyacrylamide Gel Electrophoresis Using Flat-Bed Isoelectric Focusing in the First Dimension

Michael J. Dunn

1. Introduction

Electrophoretic techniques are, perhaps, the most important group of procedures for the separation and analysis of complex protein mixtures because of their high resolution capacity. This resolving power is maximized in high-resolution, two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) based on the method developed by O'Farrell *(1)*. This methodology involves a combination of a first-dimension separation in cylindrical isoelectric focusing (IEF) gels containing 9*M* urea and 2% (w/v) of the detergent NP-40 with discontinuous polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS-PAGE) in the second dimension. This technique is described in Chapter 11 and is reviewed in refs. *2–4*.

The standard method of 2-D PAGE based on the original O'Farrell *(1)* method using cylindrical IEF gels prepared in glass capillary tubes in the first dimension produces high resolution protein separations, but certain problems are associated with its use. The most important of these problems is severe cathodic drift during IEF resulting from the very high electroendosmotic flow caused by charged groups on the glass walls of the capillary tubes. As a result, pH gradients are unstable and rarely extend above pH 8.0, leading to loss of basic proteins from 2-D maps.

From. *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
Edited by: J. M Walker

To overcome the problem of protein resolution at basic pH, a special system again attributable to O'Farrell *(5)*, and termed nonequilibrium pH gradient electrophoresis (NEPHGE), is commonly used. In this procedure, the sample proteins are applied at the anodic end of the gel and electrophoresed for a relatively short time. The proteins are thus separated electrophoretically according to their mobilities in the presence of a rapidly forming pH gradient. Their separation is dependent on the time of electrophoresis, and their final positions on the 2-D gel do not correspond to their isoelectric points (pI). The nonsteady state nature of this method compared with conventional IEF makes it inherently rather irreproducible and very sensitive to small changes in experimental conditions.

In contrast to cylindrical IEF gels usually used in 2-D PAGE systems, horizontal flat-bed gels are almost universally used for one-dimensional IEF separations (*see* Chapter 9). A significant advantage is that flat-bed apparatus is specifically designed for IEF applications, in contrast to the modified conventional electrophoresis apparatus normally used for tube-gel IEF. Thus, small electrolyte volumes can be used, and the electrodes can be positioned close to the ends of the gels in order to minimize pH gradient drift and loss of proteins from the gels. Perhaps most importantly, flat-bed IEF gels are capable of resolving basic proteins into discretely separated components rather than poorly resolved streaks.

Initial attempts to adapt flat-bed technology to 2-D separations had limited success, probably because of the thick gels that were used *(6–8)*. More successful procedures have been developed, however, using thin *(9)* and ultrathin *(10,11)* IEF gels and this approach has become the method of choice using immobilized pH gradients for the IEF dimension of 2-D PAGE *(12)*.

2. Materials

1. GelBond PAG sheets (17.5 × 23 cm).
2. Glass plates, 17.5 × 23 cm, for casting cassettes.
3. Silicon rubber gasket, 0.5 mm thick × 7 mm wide, to fit around the edge of the glass plates.
4. PVC sheets (17.5 × 23 cm).
5. Rubber roller.

6. Solution A: Acrylamide stock solution (30%T, 4%C): 14.4 g of acrylamide; 0.6 g of *N,N'*-methylene *bis*-acrylamide (Bis) made up to 50 mL with distilled water.

   The solution is deionized with a suitable ion exchange resin (Amberlite MB-1 mono-bed resin, Merck, Poole, Dorset, UK), 1 g/100 mL solution, with stirring for 1 h. After degassing using a vacuum pump, the solution is filtered using a sintered glass filter under vacuum. The solution can be stored at 4°C for a maximum of 1 wk. The deionization step reduces ionic contaminants, particularly acrylic acid, which can increase cathodic drift and pH gradient instability.
7. Urea (BRL Ultrapure, Life Technologies, Renfrew, Paisley, UK) should be stored dry at –20°C to reduce the rate of breakdown of urea with the formation of cyanate ions, which can react with protein amino groups to form stable carbamylated derivatives of altered charge.
8. Solution B (Acrylamide-urea solution): 5.32 mL acrylamide stock (solution A), 19.40 g urea, 14.48 mL distilled water.

   After the urea is completely dissolved, MB-1 ion-exchange resin (1 g/100 mL) is added, and the solution is deionized with stirring for 1 h. This removes charged contaminants from the urea, most importantly any cyanate ions that are present. The solution is filtered under vacuum.
9. Any synthetic carrier ampholyte preparation of a suitable pH range can be used, but we use a mixture (*see* Section 3., step 3) of the following: Pharmalyte 3-10 (Pharmacia, Uppsala, Sweden), Pharmalyte 5-8 (Pharmacia), Servalyt 2-11 (Serva, Heidelburg, Germany), and Ampholine 3.5-10 (Pharmacia).

   The appropriate ampholyte mixture for a particular type of sample must be established (*see* Note 4).
10. Solution C (10% [w/v] 3-[(cholamidopropyl)-dimethylammonio]-1-propane sulfonate [CHAPS]). Commercial preparation of this detergent are not necessarily salt free, so it is recommended to deionize the solution using MB-1 ion-exchange resin (1 g/100 mL) with stirring overnight at 4°C.
11. *N,N,N',N'*-Tetramethylethylenediamine (TEMED).
12. Solution D: Ammonium persulfate, 10% (w/v), made fresh daily.
13. Silicon fluid, Dow Corning 200/10 cs.
14. Electrode wicks (20 × 0.7 cm) cut from sheets of Whatman GF/B glass fiber paper.
15. Solution E: Catholyte, 1*M* NaOH.
16. Solution F: Anolyte, 1*M* oxalic acid.
17. Solution G: Bromophenol blue, 0.1% (w/v) in distilled water.

18. Solution H: Sample lysis buffer: 9*M* urea, 3% (w/v) CHAPS, 20 m*M* dithiothreitol (DTT).
19. Solution I: Equilibration buffer: 11.5 mL 10% (w/v) SDS (final concentration, 2.3%), 6.2 mL 1*M* Tris buffer, pH 6.8 (final concentration, 124 m*M*), 0.154 g DTT (final concentration, 20 m*M*), 0.114 g sodium β-mercaptoacetate (final concentration, 5 m*M*), made up to 50 mL with distilled water.

3. Method

1. Cut a piece of GelBond PAG measuring 17.5 × 23 cm (*see* Note 1). The gel will adhere only to the hydrophilic side of the sheet (*see* Note 2). The reverse side (hydrophobic surface) is marked using a permanent marker pen into individual lanes (13.5 × 1.2 cm). Fifteen lanes can be accommodated, and these are flanked on each side by a further 1-cm wide lane. The sample lanes are numbered (1–15) to aid in identification of the sample strips at the completion of the IEF run.
2. The IEF gel is cast in a vertical cassette fitted with a U-shaped gasket. The GelBond PAG sheet is rolled onto a glass plate (17.5 × 23 cm) with the marked, hydrophobic side in contact with the glass plate. A small film of water is used between the glass and the plastic sheets to ensure good adherence. Small air bubbles must be removed using a roller. On a second glass plate of the same dimensions, a sheet of PVC (17.5 × 23 cm) is rolled. The U-shaped silicon rubber gasket, which provides a leak-proof seal without the use of grease, is placed onto the GelBond PAG/glass plate assembly. The PVC/glass plate is then placed onto the gasket/GelBond PAG/glass plate to form the complete cassette, which is held together with bulldog clips on the two sides and along the bottom edge.
3. Samples for 2-D PAGE are solubilized and denatured using high levels of urea and detergent (*see* solution H) so that the proteins can be separated as individual polypeptide chains. Thus the gels also contain urea and zwitterionic detergent (CHAPS) (*see* Note 3). To 17.5 mL of the acrylamide-urea mixture (solution B) are added the ampholytes (0.46 mL Pharmalyte 3-10, 0.46 mL Servalyt 2-11, 0.46 mL Ampholine 3.5-10, 0.12 mL Pharmalyte 5-8, 0.06 mL Servalyt 4-6) (*see* Note 4). TEMED (15 μL) is then added, and the solution is degassed under vacuum. After degassing, 1 mL of CHAPS (solution C) is added, followed by 60 μL of ammonium persulfate (solution D). The solution is mixed and poured, avoiding the formation of air bubbles, into the cassette using a syringe barrel with a needle fitted with plastic tubing (*see* Note 5). The gel is allowed to polymerize at room temperature for

1 h. The resulting gel contains 8*M* urea, 0.5% (w/v) CHAPS, and 3.12% (w/v) ampholytes. The gel cannot be stored and must be used for IEF immediately.

4. The bulldog clips are removed, and the PVC/glass plate is gently removed. The gel on its GelBond PAG support can now be removed from the cassette.
5. Any flat-bed IEF apparatus can be used. We are currently using a Pharmacia FBE-3000/150 powerpack fitted with a VH-1 volt hour (Vh) integrator. The flat-bed apparatus is cooled to 15°C using a thermostated controlled circulator.
6. A thin film of silicon fluid is applied to the cooling plate. The gel on its plastic support is then placed onto the cooling plate. The film of silicon fluid, which has excellent thermal conductivity properties and a low viscosity, allows for good contact between the gel support and the cooling plate.
7. A cover sheet (20 × 13.5 cm), having the same lane markings as the GelBond PAG sheet and with slits (3 × 10 mm) punched for sample application, is applied to the surface of the gel. The lane markings should be superimposable, and care must be taken to eliminate air bubbles between the surface of the gel and the cover sheet, which is weighted with glass plates (*see* Note 6).
8. A stack of four wicks is used at the anode and cathode. The wicks are moistened (not too wet) with the electrode solutions (solutions E and F). The wicks are then applied at either end of the cover sheet with the cathode arranged to be at the sample application side of the gel. A 1-mm gap should remain between the wicks and the cover sheet to prevent electrical arcing. Bromophenol blue (solution G) (2 µL) is applied at each sample application site, and the electrode assembly is placed on the gel.
9. The gel is prefocused at 600 V, 30 mA, 15 W limiting for 600 Vh. After prefocusing the bromophenol blue dye should have migrated about halfway to the anode, and all the lanes should have migrated with equal velocity.
10. The samples, solubilized in sample lysis buffer (solution H), are then applied in the holes in the cover sheet (*see* Note 7). For radioactively labeled protein samples we load 10^6 dpm of [^{35}S]-methionine-labeled proteins. For detection by silver staining, protein loads of 10–20 µg are required, whereas for Coomassie brilliant blue staining between 150–300 µg are needed. It is best to limit the sample volume to 10 µL. Only the central lanes (3–12) should be used for the samples, with the remainder being loaded with 2 µL of bromophenol blue (solution G).

The gel is focused at 700 V, 30 mA, 15 W limiting for a total of 14,000 Vh. The voltage is then increased to 1000 V (30 mA, 15 W limiting) to sharpen the bands to give a total of 15,000 Vh.

11. After completion of IEF, the gel is removed from the apparatus. The wicks and cover sheet are carefully removed. A lane to which a sample was not applied is cut off for pH gradient determination. The remainder of the gel is covered with food-wrapping film and stored between two glass plates at –70°C. If the sample proteins are radioactively labeled, autoradiography can be used at this stage to identify sample lanes and to monitor the quality of the separation (*see* Section 1.).
12. The pH gradient is determined with a surface electrode (Ingold, Desaga, V. A. Howe, Banbury, Oxon, UK) at 1 cm intervals along the length of the gel strip.
13. If an autoradiograph has been made, sample tracks are excised using the X-ray film as a template. Otherwise, sample lanes must be cut off using the lane markings as a guide.
14. Strips are equilibrated for 7 min in 5 mL of equilibration buffer (solution I).
15. The preparation of second-dimension SDS-PAGE gels will not be described here, since they are of the normal Laemmli *(13)* type, as used in the standard 2-D PAGE technique (*see* Chapter 5). These gels can be either of a suitable constant percentage concentration of polyacrylamide or of a linear or nonlinear polyacrylamide concentration gradient (*see* Chapters 5 and 6). We currently use modified nonlinear (8–20%T) gradient SDS-PAGE gels *(14)*. The gels (0.75 mm thick) are 16 cm wide and 18 cm long. A short (2 cm) stacking gel is used.
16. The IEF strips are applied to the SDS gels by filling the space in the cassettes above the stacking gels with equilibration buffer (solution I) and gently sliding the strips into place. Good contact between the IEF strips and the tops of the stacking gels must be achieved. There is no need to cement the strips in place with agarose.
17. The second-dimension SDS-PAGE gels can be run in any suitable vertical electrophoresis apparatus. The gels are run at 12 mA/gel overnight until the bromophenol blue reaches the bottom of the separating gels.
18. The gels can be subjected to any suitable procedure to detect the separated proteins (e.g., electroblotting, autoradiography, staining with silver or Coomassie brilliant blue, and so on). A typical separation of [^{35}S]-methionine labeled human skin fibroblast proteins using this technique is shown in Fig. 1.

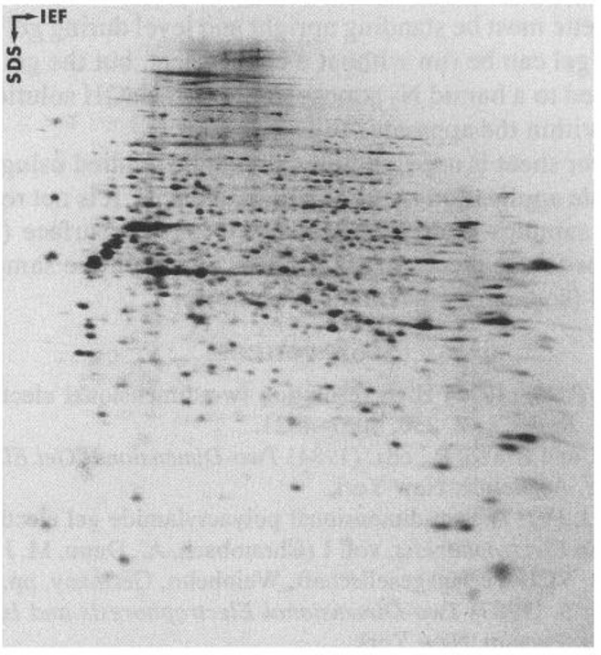

Fig. 1. Autoradiograph of a two-dimensional separation of human skin fibroblast proteins labeled with [^{35}S]-methionine using horizontal flat-bed IEF in the first dimension.

4. Notes

1. Different sizes of IEF gel can be readily used in this technique within the constraints of the size of the cooling plate in the IEF apparatus being used.
2. Avoid touching the hydrophilic surface of GelBond PAG, since this reduces gel adherence.
3. We use the zwitterionic detergent CHAPS in the IEF gel, but it is also possible to use nonionic detergents such as Triton X-100 or NP-40 with equally good results.
4. We use mixtures of different commercial synthetic carrier ampholytes, since the greater diversity of ampholyte species increases resolution. The mixture has been optimized for the separation of human skin fibroblast proteins. Such pH gradient engineering using synthetic carrier ampholytes is a rather empirical process (cf. immobilized pH gradients for IEF, *see* Chapter 12). For any particular type of sample to be separated by 2-D PAGE, it is good practice to establish which ampholyte or mixture of ampholytes produces the best separation for that sample.

5. The cassette must be standing upright and level during gel preparation.
6. The IEF gel can be run without a cover-sheet, but the gel should then be exposed to a humid N_2 atmosphere, or an NaOH solution should be present within the apparatus to absorb CO_2.
7. If no cover sheet is used, samples should be applied using silicon rubber sample application strips or similar devices. It is not recommended to apply samples as droplets directly to the gel surface (they tend to spread) or to use pieces of filter paper on which the sample has been absorbed (some proteins bind irreversibly).

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CHAPTER 14

Quantification of Proteins on Polyacrylamide Gels (Nonradioactive)

Bryan John Smith

1. Introduction

It is frequently necessary in biochemical experiments to quantify proteins. There are various methods for estimation of the concentration of total protein in a sample, such as total amino acid analysis, the Biuret reaction, and the Lowry method (*see* ref. *1*), but these do not allow quantification of one protein in a mixture of several. This may be done by chromatography and estimation of the content of the appropriate peak in the elution profile by virtue of its absorption of light at, say, 220 nm. However, it is usually quicker, easier, and more economical to quantify proteins that have been separated from each other on a polyacrylamide gel. This is done by scanning the gel and by densitometry of the stained bands on it. Microgram quantities of protein may be quantified in this way. The method described herein for quantitative staining uses Procion navy MXRB and is suitable for proteins on acid/urea or SDS polyacrylamide gels (*see* Chapters 5 and 7).

2. Materials

1. A suitable densitometer, e.g., the Gilford 250 spectrophotometer with scanning capability and chart recorder, or the Joyce-Loebl "Chromoscan."
2. Protein stain: 0.4 g Procion navy MXRB. Dissolve in 100 mL methanol, then add 20 mL glacial acetic acid and 80 mL distilled water. Make fresh each time.

From *Methods in Molecular Biology, Vol. 32. Basic Protein and Peptide Protocols*
Edited by: J. M Walker

3. Destaining solution: 100 mL methanol, 100 mL glacial acetic water, and 800 mL distilled water.

3. Method

1. At the end of electrophoresis, immerse the gel in Procion navy stain, and gently agitate until the dye has fully penetrated the gel. This time varies with the gel type (e.g., 1.5 h for a 0.5-mm-thick SDS polyacrylamide [15%T] gel slab), but cannot really be overdone.
2. At the end of the staining period, decolorize the background by immersion in destain, with agitation, and a change of destain whenever the destain becomes deeply colored. This passive destaining may take 24–48 h even for a 0.5-mm-thick gel. Thicker gels will take longer.
3. Measure the degree of dye binding by each band of protein by scanning densitometry of the gel at a wavelength of 580 nm. The total absorption by the dye in each band (proportional to the area of the peak in the scan profile) may be automatically calculated by integration of peaks in the scan profile, but if not then the peaks in the chart recording may be cut out and weighed. For standard curves, the absorption at 580 nm (i.e., peak size) is plotted vs the weight of protein in the various samples.

4. Notes

1. For quantification of proteins on gels, three simple conditions need to be fulfilled:
   a. Protein bands on the gel should be satisfactorily resolved.
   b. The dye used should bind to the protein of interest.
   c. Since one sample will be compared to others, to overcome errors caused by differences in sample sizes, the binding of the dye should be constantly proportional to the amount of protein present over a suitably wide range.
2. Errors that are difficult to eradicate arise when the above condition (a) is not fulfilled. Ideally, another elctrophoresis system that *does* give sufficient resolution should be used, but otherwise the operator has to decide where the division between two overlapping peaks should be and where the baseline is. Use of a narrower beam of light for densitometry will improve resolution, but may worsen the baseline because of the increased effects of bubbles, dust, and so on.
3. Conditions (b) and (c) should be checked by construction of a standard curve for the protein(s) of interest. The range of protein quantities used should cover that to be found in experiments, and for accurate results

the plot of dye-binding (measured by densitometry at a particular wavelength) vs protein quantity should be linear (or approach close to it) over that range.

4. Any stain may be used for quantification provided that it meets the above requirements (b) and (c). However, various factors may influence the choice of dye to be used. This can be illustrated by comparing Coomassie brilliant blue R250 (CBB R-250) (equivalent to PAGE blue 83) with Procion navy MXRB. First, given time, Procion navy can penetrate dense bands of protein and stain them, but CBB R-250 seems unable always to do this, so that the size of band that the protein forms may affect its staining. Thus, since the size of a protein band will generally increase with migration during electrophoresis, the extent of electrophoresis may have a small effect on staining by this dye. Second, when CBB R-250 binds to a protein, the resulting complex may be colored blue or red or anything in between, depending on the chemical structure of the protein. Such production of a variety of colors upon complexing of dye with proteins is called the "metachromatic effect." Maximum sensitivity is achieved in this technique by densitometry at the wavelength at which the dye–protein complex absorbs maximally. The metachromatic effect, therefore, dictates multiple estimations at wavelengths optimal for each complex, or gives suboptimal sensitivity. Procion navy gives the same color with every protein. Third, CBB R-250 binds proteins by electrostatic forces and can be completely removed from protein by extensive destaining. Thus, destaining may introduce an element of variability to the experiment. Results are more consistent with Procion navy, which binds covalently to proteins so that prolonged destaining does not remove it. Fourth, the efficacy of CBB R-250 varies from batch to batch of the solid dye and also with the method of its dissolution in making the stain (*see* Chapter 5). We have not seen this with Procion navy MXRB. It may be seen, then, that Procion navy MXRB (and probably other members of the Procion dye family) is potentially the better choice for quantification purposes. For the proteins studied in our own laboratory, at least, it also gives stoichiometric binding to proteins over a wider range than does CBB R-250, and so it is the dye of choice for quantification, even though it is several-fold less sensitive than CBB R-250.
5. Standard curves of Procion navy MXRB binding to various proteins are straight lines passing through the origin, although they do plateau at higher protein quantities. The upper limit is dependent on the size of

the gel, but in a 0.5-mm-thick gel the standard curve was found to be linear beyond a loading of 6 μg of histone H1, and up to about 30 μg for a mixture of four proteins (histones) together *(2)*. On such gels, a loading of <0.5 μg is detectable and 1 μg is sufficient for quantification.

6. The whole process of quantification should be standardized to reduce variation. Sources of error should be recognized and countered if results are to be accurate. Thus, the densitometer should give a linear response over the range of dye densities measured. Sampling errors are inevitable, but their effect can be reduced by repetition and averaging the results. The process of electrophoresis should be standardized, using samples of the same volume and electrophoresing for the same time at the same voltage in each experiment. Multisample slab gels are ideal for this purpose. The staining time should also be the same each time. Since variation in band widths will still occur, in a slab gel at least, and because irregularities in band shape will occur occasionally in any case, the whole of every stained band should be quantified. If the band width is greater than the width of the densitometer's light beam, and the raster type of scan is not available, the whole gel may be sufficiently reduced in size by equilibration in aqueous ethanol. Too much ethanol causes the whole polyacrylamide gel to become opaque, but if this happens the gel may be rehydrated in a lower-percent ethanol solution. For shrinking 15% polyacrylamide gels, 40% (v/v) ethanol in water for 1 h or so is suitable, reducing their size by about a third. If the sample width is still too great, it can be divided up and each section estimated separately. These estimates are then summed for an estimate of the whole sample.

   Despite efforts to reduce variations between experiments, they may still occur. In this case it may be necessary to construct a standard curve for each experiment. The type of standard curve required differs according to the aim of the experiment, which may be of the following types:

   a. Simple comparison: determination of the relative concentrations of the same protein in two or more samples.
   b. Complex comparison: determination of the relative concentrations of two or more proteins in the same or different samples.
   c. Absolute determination: determination of the amount of a protein in one or more samples (i.e., on a weight or molar basis).

   In experiments of type a the dye-binding capabilities of the (identical) protein in the different samples will be the same and all that is needed is to know that dye binding is stoichiometric over the appropriate range of quantities. Thus, the standard curve can be set up with a

serially diluted (or concentrated) sample, without any accurate protein quantification involved. To compensate for sampling errors, it is often useful to relate the protein of interest to an "internal standard" of another protein that is known to be at the same concentration in all samples. This internal standard will indicate the magnitude of difference in size between samples on the gel, and this factor can then be used to adjust the results of the experimental protein. It must be remembered that the internal standard protein should also have stoichiometric dye-binding capabilities over the appropriate range.

Two different proteins will not necessarily have the same dye-binding capabilities, so if they are to be compared on a weight or molar basis (experiment type b) it is necessary to determine the characteristics of each. Thus, the pure proteins are isolated, and each is accurately quantified (say, by amino acid analysis) and used to construct a standard curve. Experimental samples can be compared with these standard curves to give accurate weight measurements or it may be sufficient to determine the ratio between dye-binding properties of the different proteins for subsequent use with only one of the standard curves.

Experiments of type c are similar to those of type b in that accurately constructed standard curves are required using well-quantified protein standards. Note that simple weighing is usully not accurate enough for such work as this, for it cannot distinguish between protein and nonproteinaceous constituents (such as contaminating dust).

For accurate results with experiment types b and c, standard curves need to be run in each experiment (even if they are relatively crude, with only four or five points).

7. Destaining of Procion navy-stained gels may be speeded up by driving free dye out of the gels by electrophoresis. The protein–dye complex remains in the gel. To do this, immerse the gel in destaining solution. Place a platinum electrode on either side of the gel and apply a dc electric field across the gel. Change the buffer frequently (as it becomes colored) or cycle the buffer through a decolorizing agent (i.e., an agent that binds the dye). Disposable paper tissues have proven satisfactory as a cheap, disposable decolorizing agent.

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CHAPTER 15

Detection of Proteins in Polyacrylamide Gels Using an Ultrasensitive Silver Staining Technique

Michael J. Dunn and Samantha J. Crisp

1. Introduction

Polyacrylamide gel electrophoresis is a versatile and powerful tool for the analysis of biological samples and is capable of good separation and high resolution of complex protein mixtures. Although Coomassie brilliant blue R-250 (CBB R-250) has proved to be ideal as a general protein stain for the more traditional applications of this method, current trends toward thinner gels, decreased sample loading (to improve resolution) and the use of two-dimensional gel electrophoresis have necessitated increasingly sensitive detection methods. The introduction in 1979 by Switzer et al. *(1)* of silver staining of proteins following gel electrophoresis was a major advance providing a detection sensitivity between 20–200 times higher than methods using CBB R-250, being able to detect about 0.1 ng protein/band.

Over 100 publications have appeared since 1979 describing variations on the methodology of silver staining and these are reviewed in *(2,3)*. All silver staining procedures depend on the reduction of ionic silver to its metallic form, but the precise mechanism involved in the staining of proteins has not been fully established. It has been proposed that silver cations complex with protein amino groups, particularly the ε-amino group of lysine *(4)*, and with sulfur residues of cysteine and methionine *(5)*. However, Gersten and colleagues have

From: *Methods in Molecular Biology, Vol 32: Basic Protein and Peptide Protocols*
Edited by: J. M Walker

shown that "stainability" cannot be attributed entirely to specific amino acids, and they have suggested that some element of protein structure, higher than amino acid composition, is responsible for differential silver staining *(6)*.

Silver staining procedures can be grouped into two types of methods depending on the chemical state of the silver ion when used for impregnating the gel. The first group is alkaline methods based on the use of an ammoniacal silver or diamine solution, prepared by adding silver nitrate to a sodium–ammonium hydroxide mixture. The silver ions in the gel complexed to proteins are subsequently developed by reduction to metallic silver by reduction with formaldehyde in an acidified environment, usually using citric acid. In the second group of methods, silver nitrate in a weakly acidic (ca. pH 6) solution is used for gel impregnation. Development is subsequently achieved by the selective reduction of ionic silver to metallic silver by formaldehyde made alkaline with either sodium carbonate or NaOH.

Rabilloud has recently compared several staining methods based on both the silver diamine and silver nitrate types of procedure *(7)*. The most rapid procedures were found to be generally less sensitive than the more time-consuming methods. Methods using glutaraldehyde pretreatment of the gel and silver diamine complex as the silvering agent were found to be the most sensitive. The method we describe here is based on that of Hochstrasser et al. *(8,9)*, together with modifications and technical advice that will enable an experimenter to optimize results. An example of an SDS-PAGE separation of total proteins of the EAHY926 endothelial cell line stained by this procedure is shown in Fig. 1.

2. Materials

1. All solutions should be freshly prepared, and overnight storage is not recommended. Solutions must be prepared using clean glassware and deionized, distilled water.
2. Gel fixation solution: Trichloroacetic acid (TCA) solution, 20% (w/v).
3. Sensitization solution: 10% (w/v) glutaraldehyde solution.
4. Silver diamine solution: 21 mL of 0.36% (w/v) NaOH is added to 1.4 mL of 35% (w/v) ammonia and then 4 mL of 20% (w/v) silver nitrate is added dropwise with stirring. When the mixture fails to clear with the formation of a brown precipitate, further addition of a minimum amount of ammonia results in the dissolution of the precipitate. The

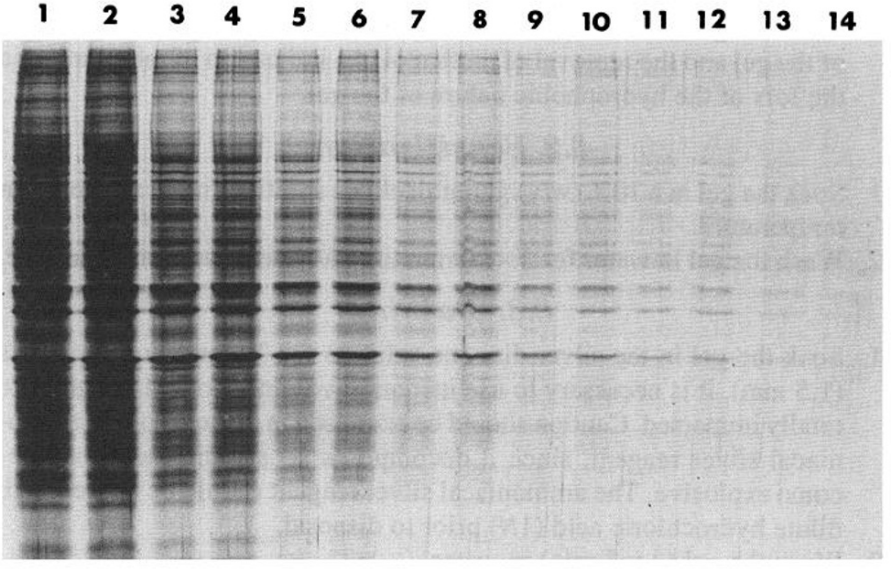

Fig. 1. Separation of total proteins from the EAHY926 endothelial cell line by 10%T SDS-PAGE and visualized by silver staining. The amount of total cellular proteins applied to each lane was: (1, 2) 4 µg, (3, 4) 2 µg, (5, 6) 1 µg, (7, 8) 0.5 µg, (9, 10) 0.25 µg, (11, 12) 0.125 µg, (13, 14) 0.0625 µg.

solution is made up to 100 mL with water. The silver diamine solution is unstable and should be used within 5 min.

5. Developing solution: 2.5 mL of 1% (w/v) citric acid, 0.26 mL of 36% (w/v) formaldehyde made up to 500 mL with water.
6. Stopping solution: 40% (v/v) ethanol, 10% (v/v) acetic acid in water.
7. Farmer's reducer: 0.3% (w/v) potassium ferricyanide, 0.6% (w/v) sodium thiosulfate, 0.1% (w/v) sodium carbonate.

3. Method

Note: All incubations are carried out at room temperature with gentle agitation.

3.1. Fixation

1. After electrophoresis, fix the gel immediately (*see* Note 1) in 200 mL of TCA (*see* Note 2) for a minimum of 1 h at room temperature. High-percentage polyacrylamide and thick gels require an increased period for fixation, and overnight soaking is recommended.
2. Place the gel in 200 mL of 40% (v/v) ethanol, 10% (v/v) acetic acid in water and soak for 2 × 30 min.

3. Wash the gel in excess water for 2 × 20 min, facilitating the rehydration of the gel and the removal of methanol. An indication of rehydration is the loss of the hydrophobic nature of the gel.

3.2. Sensitization

1. Soak the gel in a 10% (w/v) glutaraldehyde solution for 30 min at room temperature.
2. Wash the gel in water for 3 × 20 min to remove excess glutaraldehyde.

3.3. Staining

1. Soak the gel in the silver diamine solution for 30 min. For thick gels (1.5 mm), it is necessary to use increased volumes so that the gels are totally immersed. Caution should be exercised in disposal of the ammoniacal silver reagent, since it decomposes on standing and may become explosive. The ammoniacal silver reagent should be treated with dilute hydrochloric acid (1*N*) prior to disposal.
2. Wash the gel (3 × 5 min) in water.

3.4. Development

3. Place the gel in developing solution. Proteins are visualized as dark brown zones within 10 min (*see* Note 3), after which the background will gradually increase (*see* Note 4). It is important to note that the reaction displays inertia, and that staining will continue for 2–4 min after removal of the gel from the developing solution. Staining times in excess of 20 min usually result in an unacceptable high background (*see* Note 5).
4. Terminate staining by immersing the gel in stopping solution.
5. Wash the stained gel in water prior to storage or drying.

3.5. Destaining

Partial destaining of gels using Farmer's reducing reagent *(5)* is recommended for the controlled removal of background staining that obscures proper interpretation of the protein pattern.

1. Wash the stained gel in water for 5 min to remove the stop solution.
2. Place the gel in Farmer's reducer for a time dependent upon the intensity of the background.
3. Terminate destaining by returning the gel to the stop solution.

4. Notes

1. Gloves should be worn at all stages when handling gels, since silver staining will detect keratin proteins from the skin.

2. Volumes of the solutions used at all stages should be sufficient such that the gels are totally immersed. If the volume of solution is insufficient for total immersion, staining will be uneven and the gel surface can dry out.
3. The silver stain is normally monochromatic producing dark brown spots or bands. However, if image development is allowed to proceed, dense protein zones can become saturated and negative staining can occur, leading to serious problems if quantitative analysis is attempted. In addition, certain proteins stain to give yellow or red zones regardless of protein concentration, and this effect has been linked to the post-translational modification of the proteins. Staining protocols have been developed to exploit these color effects *(11)*, but again these result in problems if quantitative analysis is to be performed.
4. An inherent problem with the staining of gradient SDS-PAGE gels is uneven staining along the concentration gradient. The less concentrated polyacrylamide region develops background staining prior to the more concentrated region. A partial solution to this problem is to increase the time of staining in silver diamine (*see* Section 3.3., step 1).
5. Various chemicals used in one- and two-dimensional electrophoresis procedures can inhibit staining, whereas others impair resolution or produce artifacts. Acetic acid will inhibit staining and should be completely removed prior to the addition of silver diamine solution (Section 3.3, step 1). Glycerol, used to stabilize SDS gradient gels during casting, and urea, used as a denaturing agent in isoelectric focusing (IEF), are removed by water washes. Agarose, often used to embed rod IEF gels onto SDS-PAGE gels in 2-D PAGE procedures, contains peptides that are detected by silver staining as diffuse bands and give a strong background. Tris, glycine, and detergents (especially SDS) present in electrophoresis buffers can complex with silver and must be washed out with water prior to staining. The use of β-mercaptoethanol as a disulfide bond reducing agent should be avoided since it leads to the appearance of two artifactual bands at 50 and 67 kDa on the gel *(10)*.
6. Radioactively labeled proteins can be detected by silver staining prior to autoradiography or fluorography for the majority of the commonly used isotopes (^{14}C, ^{35}S, ^{32}P, ^{125}I). In the case of ^{3}H, however, silver deposition will absorb most of the emitted radiation.

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CHAPTER 16

Identification of Glycoproteins on Nitrocellulose Membranes and Gels

David J. Thornton, Ingemar Carlstedt, and John K. Sheehan

1. Introduction

This chapter will focus on the identification of glycoproteins on gels and, in particular, after their immobilization by slot blotting onto nitrocellulose membranes. The procedures described have been optimized for the analysis of mucus glycoproteins (mucins) but should be applicable to other families of glycoprotein and to molecules that have been transferred to nitrocellulose by dot or Western blotting. Mucins are high-M_r glycoproteins that may contain up to 90% by weight carbohydrate primarily in the form of short chain O-linked oligosaccharides. A number of different mucins can be distinguished, some of which are located at the epithelial cell surface, whereas others are secreted and form mucus. The latter glycoproteins occur typically as macromolecular assemblies, some 5–30 $\times$ 10^6 in mol wt and appear in electron microscopy as long filamentous threads often microns in length *(1)*. Reduction of disulfide bonds yields a major fragment (M_r 2–3 $\times$ 10^6), which we term a subunit. Proteolytic digestion of subunits gives rise to large glycopeptides (M_r 300–500,000), which correspond to the very highly-substituted regions of the protein core.

Because of their dense coat of oligosaccharides, the detection of mucins is targeted principally at these carbohydrate moieties and has relied mainly on colorimetric assays in solution for hexose and/or

From *Methods in Molecular Biology, Vol 32: Basic Protein and Peptide Protocols*
Edited by J. M. Walker

silaic acid *(2–4)*. We have complemented these assays with a membrane-based technique in which we have adapted histological stains used for the identification of mucins in tissue sections to the analysis of the macromolecules on blots *(5–7)*. This approach has the advantage of solvent flexibility and offers greater sensitivity combined with the ability to load the membrane with a relatively large quantity of solution, thus allowing the detection of mucins from very dilute solutions.

When using membrane methods for detection and quantitation a major concern is the selectivity of binding and thus the representativity of the molecules on the membrane. In general, proteins bind strongly to nitrocellulose, whereas highly-glycosylated proteins bind weakly or not at all. Indeed, we have shown that intact mucins bind well to unmodified nitrocellulose membranes, whereas the highly-glycosylated fragments produced by their proteolytic cleavage, which have little exposed protein, bind poorly *(5)*.

We use a number of staining protocols to detect mucins and their relative specificities will be mentioned briefly. The periodic acid/Schiff reagent will stain vicinal diol groups found mainly on peripheral sugars and sialic acids and is, as such, used as a general glycoprotein stain. In contrast, alcian blue reacts with sulfate and carboxylate groups. The interaction between this cationic dye and a polyanionic glycoprotein is influenced by pH. For example, at pH 2.5 alcian blue will bind to both carboxylate and sulfate groups, whereas at pH 1.0 the carboxylate group of the sialic acid residues will be protonated and only sulfate groups will stain. High-iron diamine is used as a "selective" reagent for sulfate groups *(8)*. Lectins and antibodies (mono- and polyclonal) are employed as more specific probes of the carbohydrate moieties on glycoproteins. Lectins are animal and plant proteins/glycoproteins that are carbohydrate-binding molecules. There is a wide range of commercially available lectins, conjugated to a variety of "reporter" groups, whose binding specificities range from simple monosaccharide residues to more complex oligosaccharide structures. We use these, and antibodies directed against carbohydrate epitopes, to detect glycoproteins in what are essentially direct binding enzyme-linked immunosorbent assays (ELISA).

2. Materials

2.1. Periodic Acid/Schiff Staining

2.1.1. Solutions for Staining Gels

1. Solution A: 1.0% (v/v) periodic acid in 3% (v/v) acetic acid (make fresh).
2. Solution B: 0.1% (w/v) sodium metabisulfite in 10 m*M* HCl (make fresh).
3. Schiffs' reagent: We use a commercial reagent purchased from the Sigma Chemical Co. (St. Louis, MO). Alternatively, Schiffs' reagent can be made as follows (taken from ref. *9*).
   a. Dissolve 1 g basic fuchsin in 200 mL of boiling distilled water, stir for 5 min, and cool to 50°C.
   b. Filter and add 20 mL 1*M* HCl to the filtrate.
   c. Cool to 25°C, add 1 g potassium metabisulfite, and leave to stand in the dark for 12–24 h.
   d. Add 2 g activated charcoal, shake for 1 min, and then filter and store at room temperature in the dark.
4. Solution C: 50% (v/v) ethanol.
5. Solution D: 0.5% (w/v) sodium metabisulfite in 10 m*M* HCl (make fresh).
6. Solution E: 7.5% (v/v) acetic acid/5% (v/v) methanol in distilled water.

2.1.2. Solutions for Staining Blots

Solutions A, B, and Schiffs' reagent from above (Section 2.2.1.) and 100 µg/mL Poly-L-lysine (*see* Note 5).

2.2. Alcian Blue Staining (see Note 1)

1. Solution F: 1% (w/v) alcian blue in 3% (v/v) acetic acid, pH 2.5.
2. Solution G: 1% (w/v) alcian blue in 0.1*M* HCl, pH 1.0.

2.3. High-Iron Diamine Staining

1. Dissolve 240 mg *N,N*-dimethyl-meta-phenylenediamine dihydrochloride and 40 mg *N,N*-dimethyl-para-phenylenediamine hydrochoride in 100 mL distilled water, then add 2.8 mL 60% (w/v) ferric chloride. Store in darkened bottle.

2.4. Lectin Staining

A wide range of lectins are available from commercial suppliers, we buy horseradish peroxidase and biotin conjugated-lectins from the Sigma Chemical Co.

2.4.1. Peroxidase-Labeled Lectins

1. 0.01*M* phosphate buffered saline (PBS), pH 6.8.
2. 0.01*M* PBS, pH 6.8 containing 0.1% (v/v) Tween 20.
3. Blocking solution: 0.01*M* PBS, pH 6.8 containing 0.1% (v/v) Tween 20 and 1% (w/v) bovine serum albumin.
4. Substrate: 1 × 10 mg tablet diaminobenzidine hydrochloride (Sigma Chemical Co.) in 40 mL 0.01*M* PBS, pH 6.8. Add 16 µL 30% hydrogen peroxide immediately before use.
5. Stop solution: 1% (w/v) sodium azide.

2.4.2. Biotinylated-Lectins

Solutions as stated in Section 2.4.1.

2.5. Antibody Staining

1. Tris-buffered saline (TBS): 10 m*M* Tris-HCl, 150 m*M* sodium chloride, pH 8.0 containing 0.1% (v/v) Tween 20.
2. Blocking solution: TBS, pH 8.0 containing 0.1% (v/v) Tween 20 and 1% (w/v) skimmed milk powder.
3. Substrate buffer: 100 m*M* Tris-HCl, 150 m*M* sodium chloride, 5 m*M* magnesium chloride, pH 9.5.
4. Substrate: 33 µL Nitroblue tetrazolium (50 mg/mL in 70% (v/v) dimethylformamide) and 16.6 µL 5-bromo-4-chloro-3-indolyl phosphate (50 mg/mL in dimethylformamide)/5 mL substrate buffer.
5. Stop solution: 20 m*M* Tris-HCl, 5 m*M* EDTA, pH 8.0.

3. Methods

3.1. Staining on Gels

3.1.1. Periodic Acid / Schiff Staining (9)

This procedure can be applied to polyacrylamide, agarose, and composite polyacrylamide/agarose gels and should be performed with shaking in a fume cupboard (*see* Notes 2 and 3).

1. Wash the gel in solution C for 30 min.
2. Wash in distilled water for 10 min.
3. Incubate in solution A for 30 min.
4. Wash in distilled water for at least 6 × 5 min. The gel can be left overnight at this stage.
5. Wash in solution B for 2 × 10 min.
6. Incubate in Schiffs' reagent for 1 h in the dark.
7. Immerse in solution B for 1 h in the dark

8. Wash several times in solution D for a total of at least 2 h in the dark.
9. Store the gel in solution E.

3.2. Transfer to Nitrocellulose

We typically transfer aliquots of the fractions obtained from, for example, gel filtration, ion-exchange chromatography or a centrifugal separation method, to nitrocellulose membranes (0.2-μm pore size) using a Minifold II 72-well slot blot apparatus *(5)*. This apparatus allows quantities of 10 μL up to 500 μL to be blotted in a single application although the membrane can be loaded by repeated applications. Alternatively glycoproteins can be separated by an electrophoretic technique and the molecules transferred to nitrocellulose by Western blotting (*see* Chapter 24).

3.3. Staining on Membranes (see *Notes 4–7)*

3.3.1. Periodic Acid / Schiff Staining

All steps in this procedure are performed with shaking and should be carried out in a fume cupboard.

1. Wash the membrane in distilled water for 5 min.
2. Incubate for 30 min in solution A.
3. Wash in distilled water (2 × 5 min).
4. Wash in solution B (2 × 5 min).
5. Incubate for 15 min in Schiffs' reagent.
6. Repeat step 4.
7. Dry in warm air stream (low setting on a hand held hair dryer).

3.3.2. Alcian Blue Staining

All steps should be performed with shaking.

3.3.2.1. Alcian Blue at pH 2.5

1. Wash the membrane in distilled water for 5 min.
2. Incubate in solution F for 15–30 min.
3. Wash in distilled water (3 × 10 min).
4. Dry in warm air stream.

3.3.2.2. Alcian Blue at pH 1.0

1. Wash the membrane in distilled water for 5 min.
2. Incubate in solution G for 15–30 min.
3. Wash in distilled water (3 × 10 min).
4. Dry in warm air stream.

3.3.3. High-Iron Diamine Staining

1. Wash the membrane in distilled water for 5 min.
2. Incubate in the high-iron diamine reagent for 30 min (or longer, can leave overnight).
3. Wash in distilled water (3 × 10 min).
4. Dry in warm air stream.

3.3.4. Lectin Staining

All steps should be performed with shaking.

3.3.4.1. Peroxidase-Labeled Lectins

1. Wash the membrane in distilled water for 5 min.
2. Incubate in blocking solution for 30 min.
3. Incubate for 30 min with a peroxidase-labeled lectin (1–5 μg/mL) in PBS containing 0.1% (v/v) Tween 20.
4. Wash membrane in PBS containing 0.1% (v/v) Tween 20 (3 × 5 min).
5. Wash membrane in PBS (3 × 5 min).
6. Develop color with substrate solution for up to 30 min.
7. Stop the reaction by immersion in stop solution for 15 min.
8. Wash in distilled water (3 × 5 min).
9. Dry in warm air stream.

3.3.4.2. Biotinylated Lectins

1. Wash the membrane in distilled water for 5 min.
2. Incubate in blocking solution for 30 min.
3. Incubate for 30 min with biotinylated-lectin (10 μg/mL) in PBS containing 0.1% (v/v) Tween 20.
4. Wash membrane in PBS containing 0.1% (v/v) Tween 20 (3 × 5 min).
5. Incubate for 30 min with peroxidase-labeled avidin (5 μg/mL) in PBS containing 0.1% (v/v) Tween 20.
6. Repeat step 4.
7. Wash the membrane in PBS (3 × 5 min).
8. Develop color with substrate solution for up to 30 min.
9. Stop the reaction by immersion in stop solution for 15 min.
10. Wash in distilled water (3 × 5 min).
11. Dry in warm air stream.

3.3.5. Antibody Staining

All steps should be performed with shaking.

1. Wash the membrane in distilled water for 5 min.
2. Incubate in blocking solution for 30 min.

3. Incubate for 30 min with first antibody (either raised by us or obtained from commercial suppliers) in TBS containing 0.1% (v/v) Tween 20.
4. Wash the membrane in TBS containing 0.1% (v/v) Tween 20 (3 × 5 min).
5. Incubate for 30 min with alkaline phosphatase-labeled second antibody (obtained from commercial suppliers) in TBS containing 0.1% (v/v) Tween 20.
6. Wash membrane in TBS containing 0.1% (v/v) Tween 20 (3 × 5 min).
7. Develop color with substrate for up to 30 min.
8. Stop the reaction by immersion in stop solution for 15 min.
9. Wash in distilled water (3 × 5 min).
10. Dry in warm air stream.

4. Notes

1. *Alcian blue:* We use alcian blue AB-8GX. This reagent, if certified by the Biological Stain Commission (BSC), gives reliable and reproducible staining.
2. *Washing/incubation:* Washing/staining is performed on a slow rocker that washes a single wave across the membrane. An orbital shaker creates a standing wave pattern and depending on the shape of the container can cause a background staining pattern particularly with the cationic dyes.
3. *Gel staining:* High background staining can occur when staining agarose or composite agarose gels. It is necessary to buy agarose of high quality and test the suitability of each new batch.
4. *Representivity:* The effectiveness of the membrane in binding the molecules may be assessed by examining both sides of the membrane after staining. Staining found on the back of the membrane is clear evidence of penetration by the molecules. Stacking 2–3 membranes may also be used to assess the effectiveness of binding. If radiolabeled material is available then it is possible to do direct autoradiography on the membrane and compare the result with that from scintillation counting (in our experience with direct autoradiography of ^{14}C-labeled mucins we can detect readily 100 dpm/band after an overnight exposure). Alternatively a solution assay can be used to compare with the profile obtained on the membrane. Low binding should always be regarded with suspicion.
5. *Binding to membrane:* Intact mucins and subunits have high affinity for the nitrocellulose, probably because of hydrophobic interaction with the less substituted regions of the molecule. These macromolecules can be blotted in a wide range of solvents, in particular we have no

difficulty with guanidinium chloride concentrations up to 8*M*. In contrast, fragments corresponding to the glycosylated domains have little, if any affinity for nitrocellulose alone but can be immobilized by treating the membrane with poly-L-lysine (5 μg/slot). The binding is now owing to ionic interactions and the ionic strength of the blotting solution must be kept low (<0.1*M*). Binding will also depend on the polyanionic nature of the molecules, and uncharged highly glycosylated proteins will have little affinity for the membrane. Poly-L-lysine on the membrane precludes the use of the lectin and antibody assays as described above because it yields a high nonspecific background color in all slots. Poly-L-lysine will also, to some degree, effect the binding of cationic stains to the molecules. Glycoproteins less glycosylated than mucins will probably stick to untreated membranes. However, with "unknown" glycoproteins it may be necessary to blot with and without polylysine and compare the responses.

When mucin samples contain large amounts of protein, the higher affinity of the nitrocellulose for the protein over the mucin can yield an underrepresentation of the latter. Furthermore, the presence of high quantities of proteins may interfere with the PAS reaction and cause an exclusion of the cationic dye stains. It is also important not to load too much mucin onto the membrane since it is possible, particularly when using lectins and antibodies, to get a gross underrepresentation of the amount of material present.

On Western blots the problem of low affinity for nitrocellulose may be overcome by transfer of highly-glycosylated polyanionic proteins or fragments to positively charged membranes (i.e., Zeta probe or DEAE membranes). We have found with slot blotting that these membranes are not as efficient as poly-L-lysine treated nitrocellulose.

6. *Detergents:* Ionic detergents (e.g., 0.1% [w/v] SDS and 1% [w/v] CHAPS) in the blotting solution do not effect binding of intact mucins to the membrane. In contrast, nonionic detergents (e.g., 0.1% [v/v] Triton X-100 or 0.1% [v/v] Tween 20) can greatly diminish binding. However, once bound the molecules are not removed by washing the membrane with 0.1% (v/v) Tween 20. The effect of detergents on the binding of highly substituted oligosaccharide-rich glycopeptides to poly-L-lysine treated membranes is the reverse of that observed for intact mucins and subunits. Ionic detergents greatly reduce binding, whereas nonionic detergents have little effect.
7. *Quantitation:* It is possible to use transmission densitometry on blots and it can be an advantage to make the nitrocellulose translucent by the use, for example, of household lubricating oil *(10)*. The most reli-

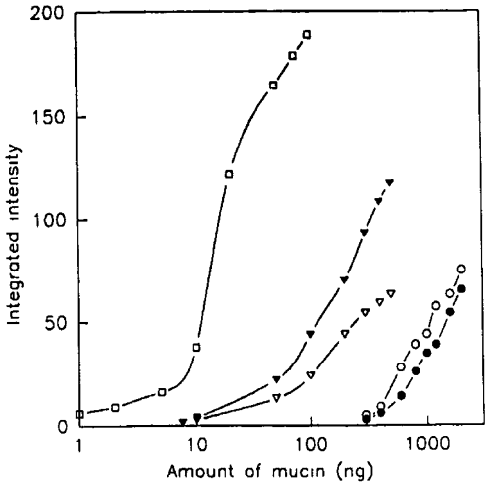

Fig. 1. Standard curves for respiratory mucin preparation. Purified asthmatic respiratory mucins were blotted onto nitrocellulose and probed with PAS (▲), AB pH 1.0 (●), and 2 5 (○), HID (△) and the peroxidase-labeled lectin Ulex Europaeus I (□).

able and precise method we have found for routine quantitation of blots is reflectance densitometry. We use an image analysis system based on an AT specification PC. Our equipment consists of a solid state video camera, image capture card for the computer, basic image analysis software, and monochrome monitor. Using this system for quantitation we show in Fig. 1 standard curves obtained using the different staining protocols described on a purified respiratory mucin preparation.

Acknowledgments

The authors wish to thank the Wellcome Trust, the Cystic Fibrosis Research Trust, UK, the Medical Faculty, University of Lund, Sweden, and the Swedish Medical Research Council (Grant 7902).

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CHAPTER 17

The Release of Oligosaccharides from Glycoproteins

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1. Introduction

The release of oligosaccharides from glycoproteins is performed for two main reasons; first to allow further studies on the core protein, and second to elucidate the structure of the oligosaccharide moeities present. For further studies of the protein to be carried out it is essential that the amino acid peptide bonds remain intact during the release process, whereas this is not essential for further studies on the released oligosaccharides. Here we describe strategies for the release of both *N*-linked and *O*-linked oligosaccharides, which allow further characterization of both protein and oligosaccharide. *N*-linked oligosaccharides can be readily released enzymatically, and rational use of glycosidases, e.g., peptide N glycanase F (PNGase F), endo-β-*N*-acetylglucosaminidase H (Endo H), neuraminidase and endo-α-*N*-acetylgalactosaminidase (*O*-glycosidase) will reveal useful information on the type of oligosaccharide present. *O*-linked chains are more difficult to release as sequential glycosidase digestions (e.g., neuraminidase and *O*-glycosidase) will remove some but not all types of *O*-linked chain. For the release of all *O*-linked chains for further analysis a chemical method is required, which also degrades the protein.

The release methods described here can either be used sequentially, as described (Fig. 1), to give preliminary information on the type of carbohydrate chains present, or individually to prepare oligosaccha-

From: *Methods in Molecular Biology, Vol 32: Basic Protein and Peptide Protocols*
Edited by: J. M. Walker

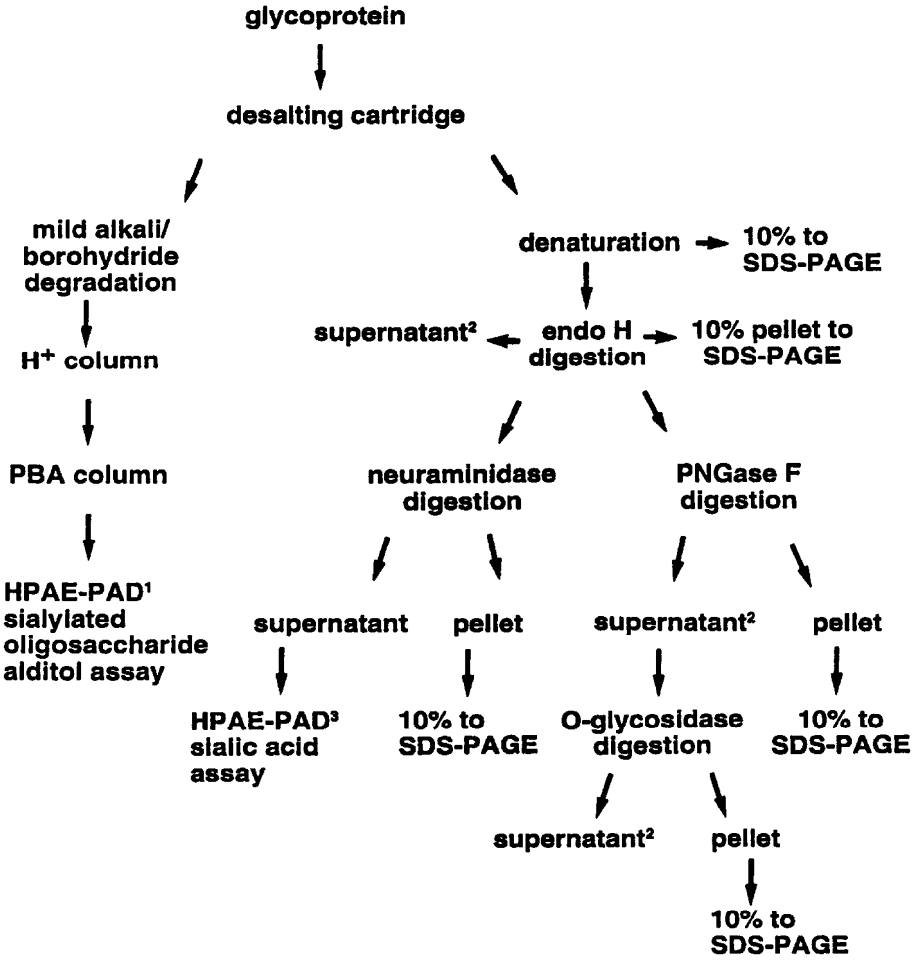

Fig 1. A generalized procedure for the deglycosylation of glycoproteins. [1,2]HPAE-PAD techniques for oligosaccharide profiling as described in the following chapter. [2,3]HPAE-PAD of monosaccharides and sialic acids as described above.

rides for profiling as described in the following chapter in this book (*see also* ref. *1*).

To allow the enzymatic release methods to be fully effective the glycoprotein must be relatively salt free, thus a desalting step is usually carried out before any digestion. In addition enzymatic treatment of glycoproteins often requires the denaturation of the protein first to ensure complete release of oligosaccharides. Once this has been

achieved (by boiling the protein in water) sequential digestion with Endo H (cleaves high-mannose chains), neuraminidase (removes sialic acid), PNGase F (removes all *N*-linked chains), and *O*-glycosidase (removes *O*-linked Gal-GalNAc-Ser/Thr) will sequentially deglycosylate the protein *(2)*. An aliquot of protein is removed after each digestion for SDS-PAGE analysis to monitor the oligosaccharides present by the change in protein mobility on SDS-PAGE *(3)*, whereas the released oligosaccharides can then be analyzed individually (*see* Chapter 18). To determine the degree of deglycosylation, the gel can be blotted and assayed for remaining oligosaccharides as described in Chapter 16.

This method of deglycosylation can also be used to investigate the role of carbohydrates in the function of a glycoprotein. For example, if a glycoprotein is thought to be a receptor for a bacterium during infection then the role of carbohydrates can be studied by overlaying the native and deglycosylated proteins from a gel with the bacterium or bacterial product. The presence or absence of binding to the deglycosylated protein should indicate whether oligosaccharides are involved in the interaction.

As *O*-glycosidase will not release all *O*-linked chains, the alternative chemical method of release is by mild alkali catalyzed β-elimination. This is usually carried out with concommitant reduction in order to recover the oligosaccharides as their stable alditols, however reduction results in limited proteolysis of the core protein *(4)*. The alditols are purified on a H^+ cation exchange column and specifically retained on a phenyl-boronic acid (PBA) column *(5)* for separation from peptides generated by the reduction. The resulting alditols are identified by means of a microhexose assay *(6)* calibrated with 1–80 μg of mannose or galactose.

The release of sialic acids by neuraminidase (or an alternative chemical method, such as mild acid hydrolysis) will result in the release of all forms of sialic acid. This includes both 2–6 and 2–3 linked types as well as *N*-glycolylneuraminic acid and *N*-acetylneuraminic acid. The *N*-acetyl and *N*-glycolyl forms can easily be resolved by use of high pH anion exchange chromatography (HPAE) with pulsed amperometric detection (PAD). The technique utilizes the property of carbohydrates to form anions at high pH giving excellent separation of isomers, with PAD giving pmole sensitivity *(7,8)*.

HPAE-PAD can also be used for the analysis of monosaccharides from oligosaccharide chains. The monosaccharides are generated by acid hydrolysis of either the released oligosaccharides or the intact glycoprotein. This provides more useful data on the nature of the oligosaccharide chains and also on the amount of oligosaccharide present.

2. Materials

2.1. Desalting

1. 1 mL Spectra/Chrom desalting cartridge (Orme, Manchester, UK).
2. HPLC grade H_2O.

2.2. Glycosidases

1. Endoglycosidase H (EC 3.2.1.96) (ex., *E.coli,* Boehringer Mannheim, Lewes, UK). Digestion buffer: 250 m*M* sodium citrate buffer adjusted to pH 5.5 with 1*M* HCl.
2. Test-neuraminidase (EC 3.2.1.18) (ex., *Vibrio cholerae*, Behring Ag, Marburg, Germany). Made up as 1 U/mL enzyme in digestion buffer and stored at 4°C. Digestion buffer: 50 m*M* sodium acetate, 134 m*M* NaCl, 9 m*M* $CaCl_2$.
3. Peptide-*N*-glycosidase F (EC 3.2.2.18) (ex., *Flavobacterium meningosepticum*, Boehringer Mannheim). Digestion buffer: 40 m*M* potassium dihydrogen orthophoshate (KH_2PO_4), 10 m*M* EDTA adjusted to pH 6.2 with 1.0*M* NaOH.
4. *O*-Glycosidase (EC 3.2.1.97) (ex., *Diplococcus pneunomiae,* Boehringer Mannheim). Digestion buffer: 40 m*M* KH_2PO_4/10 m*M* EDTA adjusted to pH 6.0 with 1.0*M* NaOH.
5. Ice cold ethanol.
6. Toluene.

2.3. SDS-PAGE and Silver Staining

1. 8 × 10 cm Mini gel apparatus (Bio-Rad, UK).
2. 1% Agarose.
3. Resolving gel buffer: 1.5*M* Tris-HCl, pH 8.8.
4. Stacking gel buffer: 0.5*M* Tris-HCl, pH 6.8.
5. Running gel buffer: 45 g Tris, 216 g glycine, 7.5 g SDS, 0.15 g sodium azide in 1500 mL ddH_2O.
6. Gel loading buffer: 0.5 µL 0.2% bromophenol blue, 7.5 µL 20% SDS, 1.5 µL 50% sucrose, 0.5 µL β-mercaptoethanol.
7. Resolving gel (12%): 4 mL of 30% acrylamide (Protogel solution, National Diagnostics, Aylesbury, UK), 3.4 mL of ddH_2O, 2.5 mL of

resolving gel buffer, 0.05 mL of 10% SDS, 0.01 mL of TEMED, 0.10 mL of 10% ammonium persulfate, prepared immediately before use.

8. Stacking gel (4%): 1.3 mL of 30% acrylamide, 6.1 mL of ddH_2O, 2.5 mL of stacking gel buffer, 0.05 mL 10% of SDS, 0.01 mL of TEMED, 0.10 mL 10% of ammonium persulfate, prepared immediately before use.
9. Fixers:
   a. Methanol/acetic acid/water (5:1:4 [v:v:v]).
   b. Methanol/acetic acid/water (5:7:88 [v:v:v]).
   c. 10% glutaraldehyde.
10. Reducing agent: 1.62 µL dithiothreitol in 50 mL of ddH_2O.
11. Stain: 0.1% silver nitrate.
12. Developer: 3 g sodium carbonate, 50 mL formaldehyde made up to 100 mL with ddH_2O.
13. Neutralizer: 1.2 g citric acid in 2.5 mL of ddH_2O.
14. Gel loading buffer: 6 µL glycoprotein, 0.5 µL bromophenol blue, 2.25 µL 20% SDS, 1.5 µL 50% sucrose, 0.5 µL β-mercaptoethanol.
15. Gel drying apparatus.

2.4. β-Elimination and Reduction

1. 1*M* $NaBH_4$ (Sigma, Poole, UK) in 50 m*M* NaOH. This is made up fresh each time from 50% (w/v) NaOH and HPLC grade H_2O.
2. Methanol (HPLC grade) containing 1% acetic acid.
3. Acetic acid.
4. 1 mL Dowex H^+ (50W X 12) strong cation ion exchange column (Sigma).
5. Bond elut phenyl boronic acid column (Jones Chromatography, Hengoed, UK).
6. 0.1*M* HCl.
7. 0.2*M* NH_4OH.
8. 0.1*M* Acetic acid.
9. Methanol.
10. H_2O (HPLC grade).
11. 4% Aqueous phenol.
12. Concentrated H_2SO_4.
13. 1 mg/mL Galactose.
14. 1 mg/mL Mannose.

2.5. High pH Anion Exchange Chromatography

1. Biocompatible gradient HPLC system (titanium or PEEK lined), e.g., 2 Gilson 302 pumps with 10-mL titanium pump heads, 802Ti mano-

metric module, 811B titanium dynamic mixer, Rheodyne 7125 titanium injection valve with Tefzel rotor seal, and Gilson 712 Chromatography system control software (all Gilson Medical Electronics, France).
2. IBM PS-2 personal computer (or compatible model) with Microsoft Windows 3.0.
3. Carbopac PA1 separator (4 × 250 mm) and PA1 Guard column (Dionex Camberley, UK).
4. Pulsed amperometric detector with Au working electrode (Dionex), set up with the following parameters:
   a. Time = 0 s E = +0.1 V
   b. Time = 0.5 s E = +0. 1V
   c. Time = 0.51 s E = +0.6 V
   d. Time = 0.61 s E = +0.6 V
   e. Time = 0.62 s E = –0.8 V
   f. Time = 0.72 s E = –0.8 V
5. Reagent reservoir and postcolumn pneumatic controller (Dionex).
6. High purity helium.
7. NaOH 50% w/v.
8. Reagent grade sodium acetate (Camlab, Cambridge,UK).
9. HPLC grade H_2O.
10. 2*M* Trifluoroacetic acid HPLC grade.
11. 0.01*M* HCl.
12. Dowex 50W × 12 H^+ cation exchange resin.
13. 3.5-mL Screw cap septum vials (Pierce, Chester, UK) cleaned with chromic acid (2 L H_2SO_4/350 mL H_2O/100 g Cr_2O_3) (**CARE!, extremely corrosive,** *see* Note 1) and coated with Repelcote (BDH, Poole, UK).
14. Teflon®-backed silicone septa for 3.5-mL vials (Aldrich, Poole, UK).

3. Methods

3.1. Desalting

1. Wash the cartridge with 5 mL of HPLC grade H_2O.
2. Load the sample onto the cartridge in a volume between 50 µL and 200 µL H_2O.
3. Elute the column with 200 µL of H_2O (including sample load).
4. Elute the glycoprotein in 350 µL of H_2O.
5. Elute the salt with a further 1 mL of H_2O.

3.2. Glycosidase Digestions

1. Dissolve 1 nmol of glycoprotein in 100 µL of H_2O and boil for 30 min to denature. Remove a 10% aliquot for SDS-PAGE and dry the remainder by lyophilization (*see* Note 2) *(2)*.

2. Resuspend the glycoprotein in 500 µL of Endo H digestion buffer and 5 mL of toluene. Add 1 mU of Endo H/1 nmol glycoprotein and incubate at 37°C for 72 h (*see* Notes 3 and 4).
3. Precipitate the protein with a twofold excess of ice-cold ethanol and centrifuge at 15,000*g* for 20 min. Wash the pellet a further three times with ice-cold ethanol. Dry the supernatant completely and retain it for oligosaccharide analysis. Dry the protein pellet, and aliquot 10% (relative to original amount) for SDS-PAGE.
4. Dry the remaining pellet and resuspend in neuraminidase/neuraminidase digestion buffer at a concentration of 2 nmol of glycoprotein/10 mL of buffer. Incubate for 18 h at 37°C and then ethanol precipitate and aliquot as in step 3.
5. Resuspend the remaining glycoprotein in 500 µL of PNGase F digestion buffer, 5 µL toluene, and 1 U PNGase F/10 nmol glycoprotein. Incubate at 37°C for 72 h before precipitation and aliquotting as in step 3 (*see* Note 5).
6. Digest the final pellet with *O*-glycosidase under the same conditions as for the PNGase F digestion. Precipitate the pellet from ethanol with washing and dry as described above.
7. Apply all the pellets to SDS-PAGE.

3.3. SDS-PAGE and Silver Staining

1. For a more detailed description of SDS-PAGE *see* Chapter 5 of this book.
2. Pour fresh resolving gel into a gel sandwich (*see* Notes 6 and 7).
3. Overlay with butanol and allow to polymerize.
4. Remove butanol and wash with ddH_2O.
5. Pour fresh stacking gel to the top of the plate and add the required comb.
6. Assemble the gel apparatus and fill the upper and lower reservoirs with running gel buffer (40 mL stock solution diluted with 160 mL ddH_2O).
7. Boil the samples in the gel loading buffer for 3 min.
8. Load the samples into wells, along with one lane of 0.25–0.5 µL molecular markers from 1 µL markers stock + 9 µL gel loading buffer.
9. Place the lid on the gel and connect to a power supply.
10. Run until the gel front is 5–10 mm from base of gel at 20 mA and 200 V.
11. Disconnect leads, remove sandwich from base, and decant off running gel buffer before carefully removing gel and transferring it to a staining tank.
12. Wash with each fixer sequentially for 30 min with stirring.
13. Wash for 2 h in ddH_2O.
14. Wash the gel for 30 min with reducing agent and then stain for 30 min with 50 mL silver nitrate *(3)*.

15. Rinse the gel with 2 × 10 mL of developer, then wash until it is stained to desired intensity in 80 mL of developer.
16. Neutralize the gel with citric acid.
17. Leave for 10 min in neutralizer and then wash with ddH_2O. The gel can now be photographed or dried.
18. To dry the gel place it in 20% methanol/10% acetic acid for 2 h and then in a gel dryer overnight.

3.4. β-Elimination and Reduction

1. Dry the glycoprotein in a screwtopped vial and resuspend in 1*M* $NaBH_4$, 50 m*M* NaOH (*see* Notes 8–10) *(4)*.
2. Incubate at 55°C for 18 h.
3. Quench the reduction by the addition of ice-cold acetic acid until no further effervescence is seen.
4. Dry the reaction mixture down and then wash and dry three times with a 1% acetic acid, 99% methanol solution to remove methyl borate.
5. Resuspend the alditols in H_2O and pass down a 1mL H^+ cation exchange resin. The alditols will not be retained and will elute by washing the column with water.
6. Dry the alditols and resuspend them in 100 µL of 0.2*M* NH_4OH.
7. Equilibrate a phenyl boronic acid (PBA) column prior to sample loading and then regenerate after use as follows *(5)*:
   a. 2 × 1 mL Methanol.
   b. 1 × 1 mL 0.1*M* HCl.
   c. 2 × 1 mL H_2O.
   d. 4 × 1 mL 0.2*M* NH_4OH.
   e. Sample in 100 µL 0.2*M* NH_4OH.
   f. 2 × 100 µL 0.2*M* NH_4OH.
   g. 2 × 100 µL H_2O.
   h. 6 × 100 mL 0.1*M* acetic acid.
   i. 1 × 1 mL 0.1*M* HCl.
   j. 2 × 1 mL H_2O.
7. Collect fractions for steps e–i inclusively, resulting in 12 fractions among which the alditols should be in fractions 5–11.
8. Aliquot 10% of each fraction into a microtiter plate along with a range of concentrations of a hexose standard (galactose or mannose, usually 1–10 µg) (adapted from ref. *6*).
9. Add 25 µL of 4% aqueous phenol to each well, mix thoroughly, and leave for 5 min (*see* Note 11).
10. Add 200 µL of H_2SO_4 to each well (**CARE!** ***see*** **Note 12**) and mix prior to reading on a plate reader at 492 nm (*see* Note 13).

11. Pool the alditols on the basis of hexose present for further analysis.

3.5. HPAE-PAD Chromatography of Sialic Acids

1. Dry the glycoprotein into a clean screw top vial with a Teflon®-backed silicone lid insert (*see* Note 14).
2. Release the sialic acids by hydrolysis with 0.01*M* HCl for 60 min at 70°C in an inert N_2 atmosphere.
3. Dry down the hydrolyzate and wash three times with HPLC grade H_2O. Alternatively, the sialic acids can be released by neuraminidase treatment as previously described.
4. Prepare 500 mL of 100 m*M* NaOH, 1.0*M* sodium acetate (eluant A). (*see* Note 15) *(7)*.
5. Prepare 500 mL of 100 m*M* NaOH (eluant B).
6. Degas eluants by bubbling helium through them in their reservoirs (*see* Note 16)
7. Regenerate the HPLC column in 100% eluant B for 30 min at a flowrate of 1 mL/min.
8. Equilibrate the column in 95% eluant B for 30 min at a flowrate of 1 mL/min (*see* Note 17).
9. Inject approx 0.2 nmol of sialic acid onto the column and elute using the following gradient at a flowrate of 1 mL/min:
   a. Time = 0; 95% eluant B.
   b. Time = 4 mins; 95% eluant B.
   c. Time = 29 mins; 70% eluant B.
   d. Time = 34 min; 70% eluant B.
   e. Time = 35 min; 100% eluant B.
   f. Time = 44 min; 100% eluant B.
   g. Time = 45 min 95% eluant B; 1 mL/min.
10. Quantitate the sialic acids by comparison to known standards run in the same day (Fig. 2).
11. When the baseline has stabilized the system is ready for the next injection.
12. When all the analyses have been completed regenerate the column in eluant B and flush pumps with HPLC grade H_2O (*see* Note 18).

3.6. HPAE-PAD Chromatography of Monosaccharides

1. Dry down glycoprotein or oligosaccharide in a clean screwtop vial with Teflon®-backed silicone lid insert (*see* Note 14).
2. Hydrolyze for 4 h at 100°C with 2*M* TFA in an inert N_2 atmosphere.
3. Dry the hydrolyzate and wash three times with HPLC grade H_2O.

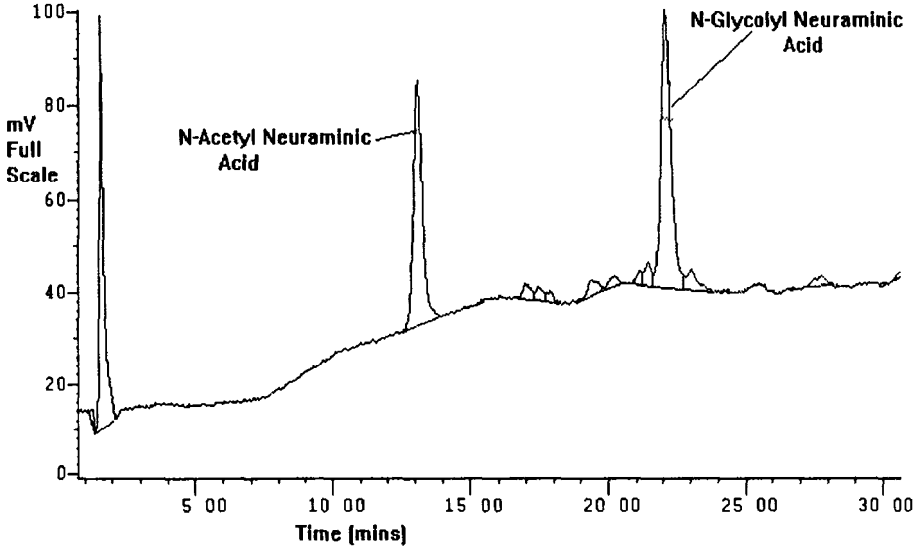

Fig 2. HPAE-PAD chromatography of 100 pmol of *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid; eluted with 100 m*M* NaOH and a gradient of 5–30% 1*M* sodium acetate.

4. Purify on a 1 mL Dowex 50W X 12 H^+ column as described for purification of oligosaccharide alditols (Section 3.4., step 5).
5. Dry down the monosaccharides ready for injection onto the HPLC system.
6. Prepare the following eluants:
   Eluant A = 500 mL HPLC grade H_2O.
   Eluant B = 500 mL of 50 m*M* NaOH, 1.5 m*M* sodium acetate.
   Eluant C = 500 mL of 100 m*M* NaOH.
   Post column reagent = 500 mL of 300 m*M* NaOH.
7. Degas the eluants by bubbling helium through them (*see* Note 16).
8. Place the postcolumn reagent in a pressurized reagent reservoir and use the pneumatic controller to adjust helium pressure to give a flowrate of 1 mL/min (approx 10 psi).
9. Regenerate the column with eluant C at 1 mL/min for 30 min.
10. Equilibrate the column with 98% eluant A, 2% eluant B at a flowrate of 1 mL/min.
11. Add the postcolumn reagent between column and detector cell at a flowrate of 1 mL/min via a mixing tee.
12. Inject approx 200 pmol of monosaccharide and elute isocratically as follows: eluant A = 98%; eluant B = 2%; flowrate = 1 mL/min; for 30 min.

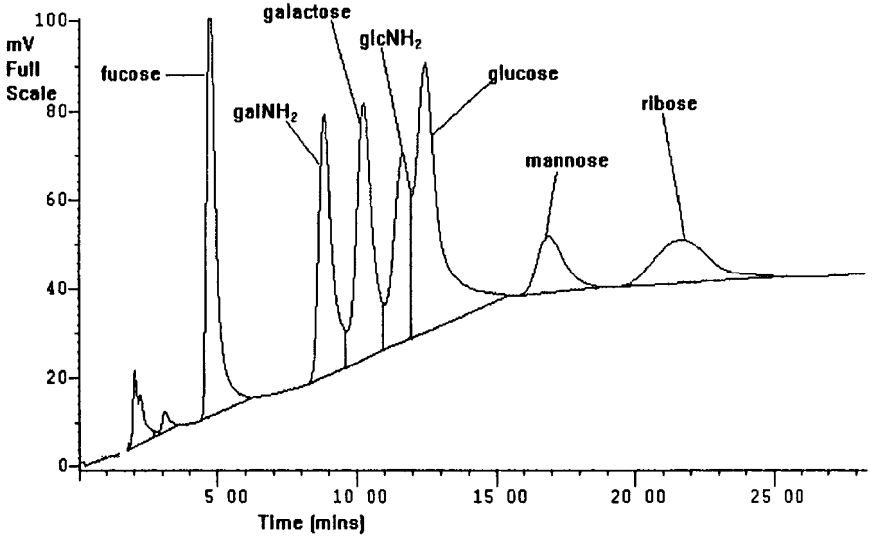

Fig 3. HPAE-PAD chromatography of 100 pmoles of monosaccharide standards eluted isocratically with 2% 50 m*M* NaOH/1 5 m*M* sodium acetate and 98% H_2O

13. Monosaccharide amounts can be calculated by comparision to a range of known monosaccharide standards run on the same day. From this it is possible to infer the type and amount of glycosylation of the glycoprotein (Fig. 3; Note 19).
14. Regenerate the column in eluant C for 30 min at 1 mL/min (*see* Note 20).
15. Re-equilibrate the column with 98% A/2% B before the next injection.
16. At the end of the analysis regenerate the column in eluant C and flush pumps with H_2O (*see* Note 18).

4. Notes

1. If required an equivalent detergent based cleaner may be used.
2. The described procedure assumes approx 10% glycosylation of the glycoprotein. The amount of glycoprotein treated may have to be increased to obtain oligosaccharides for further analysis with less highly glycosylated glycoproteins.
3. PNGase F is stored at –20°C; all other enzymes at 4°C.
4. The toluene is added to prevent bacterial growth.
5. PNGase F digests may also be performed in 0.2*M* sodium phosphate buffer pH 8.4, but this may result in the release of sialic acid residues as monosaccharides.

6. All SDS-PAGE buffers are kept at 4°C, the acrylamide is kept in the dark and treated with caution since it is a neurotoxin.
7. The concentration of the acrylamide may have to be adjusted depending on the MW of the glycoprotein.
   Up to 100 kDa glycoproteins: 12% acrylamide.
   100 kDa to 150 kDa glycoproteins: 8% acrylamide.
   Over 150 kDa glycoproteins: 6% acrylamide.
8. The $NaBH_4$/NaOH solution is made up less than 6 h before it is required.
9. Reduction with NaB^3H_4 allows the incorporation of a radioactive label into the alditol to enable a higher degree of sensitivity to be achieved while profiling.
10. Protein degradation can be minimized by the omission of the $NaBH_4$, although this results in the degradation of sugar chains having a 3-substituted GalNAc-Ser/Thr, i.e., most types. The addition of 6 m*M* cadmium acetate, 6 m*M* Na_2 EDTA to the $NaBH_4$/NaOH solution reduces protein degradation without the loss of oligosaccharide alditols *(9)*.
11. Do not overfill wells during the hexose assay, since the conc. H_2SO_4 will severely damage the microtiter plate reader if it is spilled.
12. Exercise care when adding the conc. H_2SO_4 to the phenol/alditol mixture, since it is likely to "spit," particularly in the presence of salt.
13. Fucosylated oligosaccharides will produce a more orange coloration than nonfucosylated oligosaccharides.
14. If problems with contaminants are encountered it may be necessary to wash the vials with chromic acid overnight, wash them thoroughly with distilled water, and then treat with a hydrophobic coating such as repelcoat.
15. Exercise caution with HPAE-PAD, since NaOH is corrosive.
16. Use polypropylene reagent vessels as far as possible for HPAE-PAD because of the corrosive nature of the NaOH, and to minimize leaching of contaminants from the reservoirs.
17. For maximum efficiency of detection always ensure that the PAD reference electrode is accurately calibrated and the working electrode is clean.
18. Failure to wash out the eluants from the pumps at the end of an analysis may result in crystallization and serious damage to the pump heads.
19. ^3H monosaccharides from metabolically-labeled glycoproteins can be detected by HPAE chromatography. The released ^3H monosaccharides are cochromatographed with ^{14}C-standard monosaccharides and detected with a dual channel (^3H and ^{14}C) radioactivity monitor.
20. Some drift in retention times may be observed during the monosaccharide analysis. This can be minimized by thorough regeneration of the column and use of a column jacket to maintain a stable column temperature.

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CHAPTER 18

Structural Profiling of Oligosaccharides of Glycoproteins

Kevin D. Smith, Michael J. Davies, and Elizabeth F. Hounsell

1. Introduction

The release of oligosaccharides from glycoproteins provides useful information on the type and extent of glycosylation that can be correlated with the effect of glycosylation on the function of the glycoprotein. However, to fully characterize the diversity of oligosaccharide structures within a glycoprotein it is necessary to assign each oligosaccharide structure to a particular glycosylation site (i.e., the site-specific glycosylation pattern). Only when this has been done can accurate models be made of glycoproteins to study the roles of specific oligosaccharides. The complete characterization of the site-specific glycosylation patterns of a glycoprotein requires the fulfillment of three conditions, namely:

1. The isolation of glycopeptides with only one glycosylation site.
2. The identification of the glycosylation site relative to the amino acid sequence of the protein.
3. Characterization of the structures of the oligosaccharide chains and the nature of the carbohydrate–protein linkage at each site.

Here we present a strategy for the complete determination of site-specific glycosylation patterns of glycoproteins *(1)* (Fig. 1). The glycoprotein is first digested with a protease to generate peptides and glycopeptides of suitable size for analysis. Peptides in the mixture

From *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
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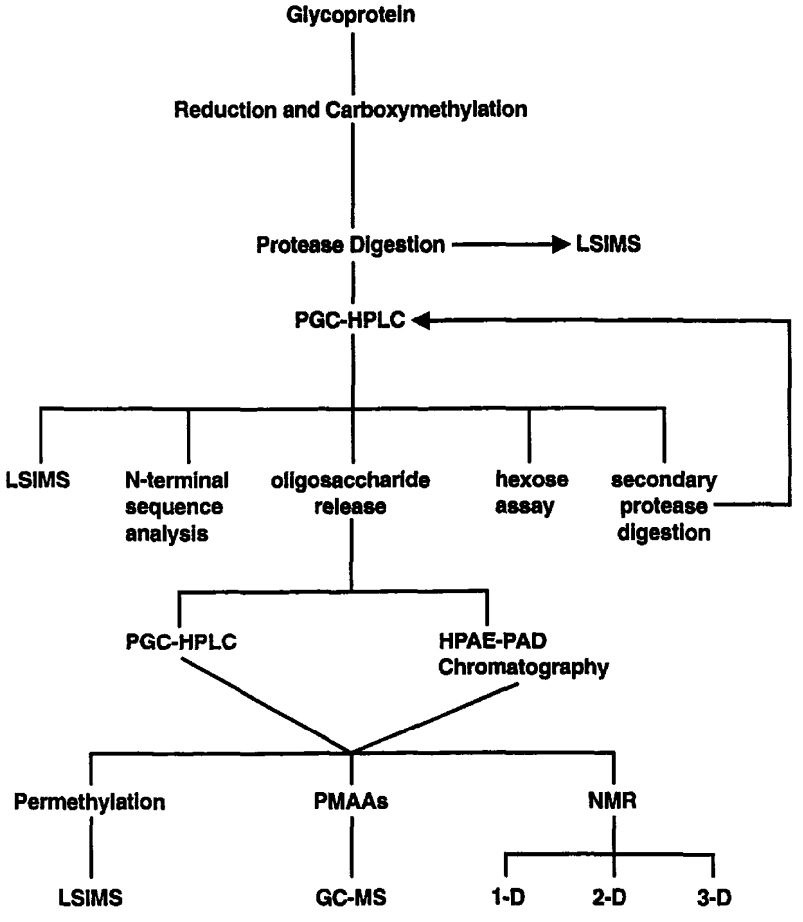

Fig. 1 A protocol for the determination of site-specific glycosylation patterns of glycoproteins.

are identified by liquid secondary ion mass spectrometry (LSIMS). This technique readily identifies nonglycosylated peptides up to 3000 Daltons, but glycopeptides are not detected because of their larger size and hydrophilicity. After fractionation by HPLC on reverse phase (ODS) or porous graphitized carbon (PGC) columns *(2)*, the glycosylated peptides are identified by hexose assay (as described in Chapter 17) and the hexose-positive peaks analyzed by N-terminal peptide sequencing. In the event of a peptide containing two glyco-

sylation sites a secondary protease digest can be performed followed by PGC-HPLC separation.

Once individual glycopeptides have been isolated and their position within the protein established (by comparison of the peptide sequence to that of the intact protein) the oligosaccharides are released according to either a chemical or enzymatic method as described in Chapter 17. The released and purified oligosaccharides can then be analyzed further: *N*-linked oligosaccharides are separated from each other by high pH anion exchange chromatography with pulsed amperometric detection (HPAE-PAD) *(3)*. This provides a structural profile of the oligosaccharides. Additional structural information can be obtained after desalting the oligosaccharides and derivitization either by permethylation for analysis by LSIMS *(4,5)*, or to partially methylated alditol acetates (PMAAs) for gas chromatography-mass spectrometry (GC-MS) *(4)*. LSIMS will provide more information on the type of chain present and the sequences of sugars within the chain, whereas GC-MS provides more detailed information on the chain linkages. Alternatively, the desalted oligosaccharide chains can be analyzed underivatized by nuclear magnetic resonance (NMR) spectroscopy *(6)*. A combination of 1-,2-, and 3-dimensional NMR techniques is often necessary to fully elucidate a complex oligosaccharide structure; this also provides conformational information about the oligosaccharides that can be included in molecular modeling by computer graphics *(7)*.

O-linked oligosaccharide alditols can also be separated by HPAE-PAD, although only sialylated alditols are retained to any useful degree *(8)*. Both sialylated and nonsialylated oligosaccharide alditols can be separated by PGC-HPLC *(2)*. In addition normal and reverse phase HPLC have been used to separate oligosaccharide alditols *(9)*. PGC chromatography is preferable for preparation of sialylated oligosaccharide alditols, since it minimizes the use of complex elution buffers. The resolved oligosaccharides can then be derivitized and analyzed as for those isolated by HPAE-PAD.

2. Materials

2.1. Reduction and Carboxymethylation

1. Buffer: 6*M* guanidine hydrochloride, 0.1*M* Tris-HCl, pH 8.5, 1 m*M* EDTA.
2. 5 mg/mL Dithiothreitol (DTT).

3. 50 m*M* Aqueous iodoacetic acid (stored at 4°C).
4. 3.5 L 50 m*M* Ammonium bicarbonate.
5. Dialysis tubing.

2.2. Protease Digestions

1. TPCK treated, chymotrypsin free trypsin (bovine pancreas) EC 3.4.21.4.
2. Thermolysin (ex., *Bacillus thermoproteolyticus*) EC 3.4.24.4.
3. Trypsin digestion buffer: 100 m*M* ammonium bicarbonate adjusted to pH 8.0 with 1*M* NaOH.
4. Thermolysin digestion buffer: 100 m*M* NH_4HCO_3, 1 m*M* $CaCl_2$ adjusted pH 8.5 with 1*M* NaOH.
5. Toluene.

2.3. PGC-HPLC

1. Gradient HPLC system: e.g., 2 × 302 pumps, 802C manometric module, 811 dynamic mixer, 116 UV detector, 201 fraction collector, 715 chromatography system control software (all Gilson Medical Electronics, France).
2. IBM PS-2 personal computer (or compatible model) with Microsoft Windows 3.0.
3. Hypercarb S HPLC column (100 × 4.6 mm) (Shandon Scientific, Runcorn, Cheshire, England).
4. HPLC grade H_2O.
5. HPLC grade acetonitrile.
6. HPLC grade trifluoroacetic acid (Pierce, Chester, UK).

2.4. HPAE-PAD Chromatography

1. Biocompatible gradient HPLC system (titanium or PEEK lined). E.g., 2 Gilson 302 pumps with 10-mL titanium pump heads, 802Ti manometric module, 811B titanium dynamic mixer, Rheodyne 7125 titanium injection valve with Tefzel rotor seal and Gilson 712 chromatography system control software (all Gilson Medical Electronics, France).
2. IBM PS-2 personal computer (or compatible model) with Microsoft Windows 3.0.
3. Carbopac PA100 separator (4 × 250 mm) and PA100 Guard column (Dionex, Camberley, UK).
4. Pulsed amperometric detector with Au working electrode (Dionex), set up with the following parameters:
   a. Time = 0 s E = +0.1 V
   b. Time = 0.5 s E = +0.1 V
   c. Time = 0.51 s E = +0.6 V

d. Time = 0.61 s E = +0.6 V
e. Time = 0.62 s E = –0.8 V
f. Time = 0.72 s E = –0.8 V

5. Anion Micromembrane Suppressor 2 (AMMS2) (Dionex).
6. Autoregen unit with anion regenerant cartridge (Dionex).
7. High purity helium.
8. NaOH 50% (w/v).
9. Reagent grade sodium acetate (Camlab, Cambridge,UK).
10. HPLC grade H_2O.
11. 500 mL of 50 m*M* H_2SO_4 (reagent grade).

2.5. Permethylation of Oligosaccharides and Preparation of PMAAs

1. Clean (Chromic acid washed; **Care!** ***see*** **Note 1**); 5 mL and 0.5 mL reactivials with Teflon®-backed silicone lid septa and magnetic stirrer (Pierce, Chester, UK).
2. DMSO (Analar grade, BDH).
3. 100 µg powdered NaOH suspended in 1.2 mL DMSO prepared immediately prior to use by crushing NaOH pellets.
4. Methyliodide (Aldrich, Gillingham, UK).
5. H_2O HPLC grade.
6. Chloroform HPLC grade.
7. 2*M* Trifluoracetic acid (TFA) HPLC grade (Pierce) prepared immediately before use.
8. Methanol HPLC grade.
9. 50 m*M* Sodium borodeuteride (Sigma, Poole, UK) in 50 m*M* aqueous NaOH.
10. Glacial acetic acid.
11. Anhydrous pyridine (Aldrich, Gillingham, UK).
12. Acetic anhydride (Aldrich).
13. Hewlett Packard 5890 series 2 gas chromatograph with an Ultra 2 25*M* 0.33 µm 5% phenyl methyl silicone capillary column (Hewlett Packard, UK), HP5971A mass selective detector and He carrier gas.

3. Methods

3.1. Reduction and Carboxymethylation

1. Dissolve the glycoprotein in 500 µL of buffer (*see* Note 2).
2. Add 5 mg/mL DTT such that it is 2 m*M* in excess over the disulfide bonds present.
3. Incubate at 37°C for 2 h under N_2.

4. Neutralize with 50 m*M* iodoacetic acid until a twofold molar excess over the DTT is achieved (*see* Note 3).
5. Incubate at 37°C for a further 2 h.
6. Dialyze against 3.5 L of 50 m*M* ammonium bicarbonate overnight.
7. Lyophilize glycoprotein.

3.2. Primary Protease Digestion

1. Dissolve 1 mg trypsin (*see* Note 4) in 500 µL of trypsin digestion buffer and add 10 µL of toluene (*see* Note 5).
2. Dissolve the glycoprotein in enzyme/buffer solution at 20 µg enzyme/1 mg glycoprotein (*see* Note 6).
3. Incubate at 37°C for 72 h, with a further addition of enzyme (10 µg enzyme/1 mg glycoprotein) after 24 h.
4. Wash the digest with 3 × 100 µL H_2O.
5. Lyophilize the digest.

3.3. PGC-HPLC of Peptides and Glycopeptides (Fig. 2)

1. Prepare eluant A: 500 mL of 0.1% TFA.
2. Prepare eluant B: 250 mL of acetonitrile containing 0.1% TFA.
3. Degas eluants by sparging with helium.
4. Equilibrate the column (*see* Note 7) in 98% eluant A, 2% eluant B at 1 mL/min for 30 min prior to injection of samples.
5. Elute 2 nmol of digested glycoprotein with the following gradient at a flowrate of 1 mL/min and UV detection at 210 nm/0.08 aufs:
   a. Time = 0 min A = 98%; B = 2%
   b. Time = 10 min A = 98%; B = 2%
   c. Time = 90 min A = 18%; B = 82%
   d. Time = 100 min A = 18%; B = 82%
   e. Time = 110 min A = 98%; B = 2%
6. Assay the fractions for hexose as described in Chapter 17 and/or analyze by LSIMS or N-terminal sequence analysis for peptides and glycopeptides.
7. Lyophilize fractions identified as containing single-glycosylation site glycopeptides and release the oligosaccharides as described in Chapter 17.
8. Treat any fractions containing multiple-glycosylation site glycopeptides with a secondary protease digestion.

3.4. Secondary Protease Digestion

1. Dissolve the glycopeptide in 500 µL of thermolysin digestion buffer and 5 µL of toluene.

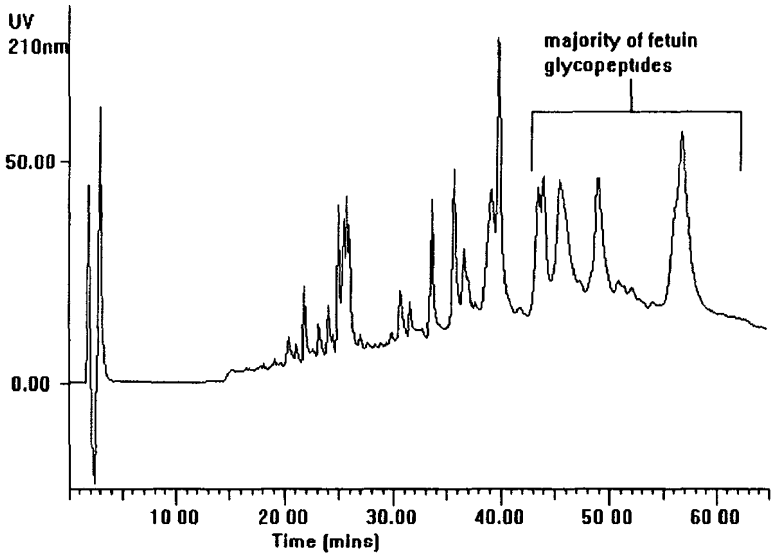

Fig. 2 The separation of 2 nmol of a tryptic digest of bovine fetuin by PGC-HPLC with UV absorbance at 210 nm.

2. Add thermolysin in the same proportions as for the trypsin digestion and incubate at 37°C for 48 h.
3. Lyophilize the glycopeptide before rechromatography by PGC-HPLC and subsequent analysis as before.

3.5. HPAE-PAD Chromatography of Oligosaccharides and Sialylated Oligosaccharide Alditols (Fig. 3)

1. Prepare eluant A: 100 m*M* NaOH, 500 m*M* sodium acetate.
2. Prepare eluant B: 100 m*M* NaOH.
3. Prepare the column by elution with 50% A/50% B for 30 min at a flow of 1 mL/min.
4. Equilibrate column in 5% eluant A/95% eluant B at a flow of 1 mL/min.
5. Connect the AMMS2 to eluant out line and autoregen unit containing 500 mL of 50 m*M* reagent grade H_2SO_4 and pump regenerant at a flow of 10 mL/min (*see* Note 8).
6. Inject 200 pmol of each oligosaccharide or sialylated oligosaccharide alditol (more if required for NMR or LSIMS) and elute with the following gradient at a flow of 1 mL/min:
   a. Time = 0 min A = 5%; B = 95%
   b. Time = 15 min A = 5%; B = 95%

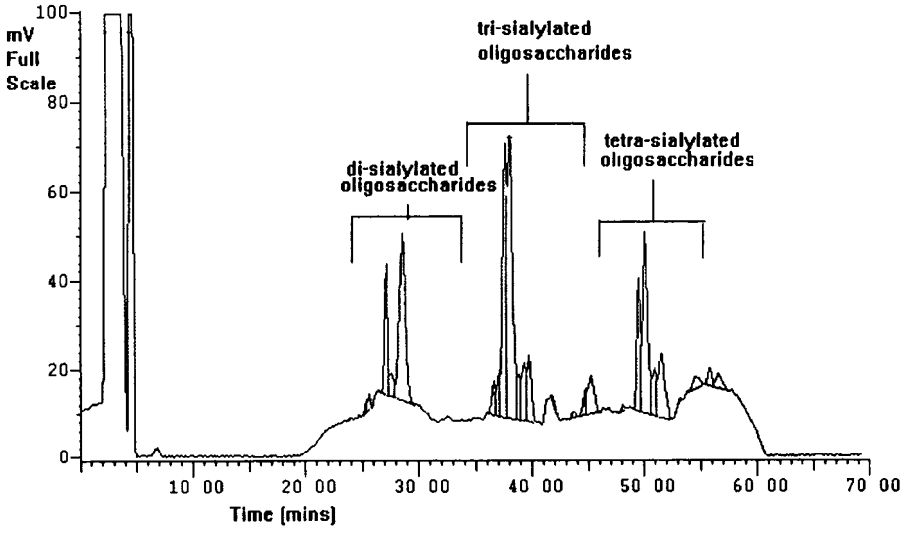

Fig. 3 The separation of *N*-linked oligosaccharides from 1 nmol bovine fetuin (PNGase F treated) by HPAE-PAD chromatography, as described in the text.

c. Time = 50 min A = 40%; B = 60%
d. Time = 55 min A = 40%; B = 60%
e. Time = 58 min A = 0%; B = 100%

7. Equilibrate the column in 5%A/95%B prior to the next injection.
8. At the end of the analyses regenerate the column in 100%B and flush the pumps with H_2O.
9. Desalt oligosaccharide-containing fractions by AMMS and derivatize for LSIMS, and GC-MS *(4)*.

3.6. PGC-HPLC of Oligosaccharide Alditols (Fig. 4)

1. Prepare eluant A: 500 mL of 0.05% TFA.
2. Prepare eluant B: 250 mL of acetonitrile containing 0.05% TFA.
3. Degas eluants by sparging with helium.
4. Equilibrate the column in 100% eluant A, 0% eluant B for 30 min at 0.75 mL/min prior to injection of samples.
5. Elute 10 nmol oligosaccharide alditol (*see* Note 9) with the following gradient at a flowrate of 0.75 mL/min and UV detection at 206 nm/ 0.08 aufs (*see* Note 10):
   a. Time = 0 min A = 100%; B = 0%
   b. Time = 5 min A = 100%; B = 0%

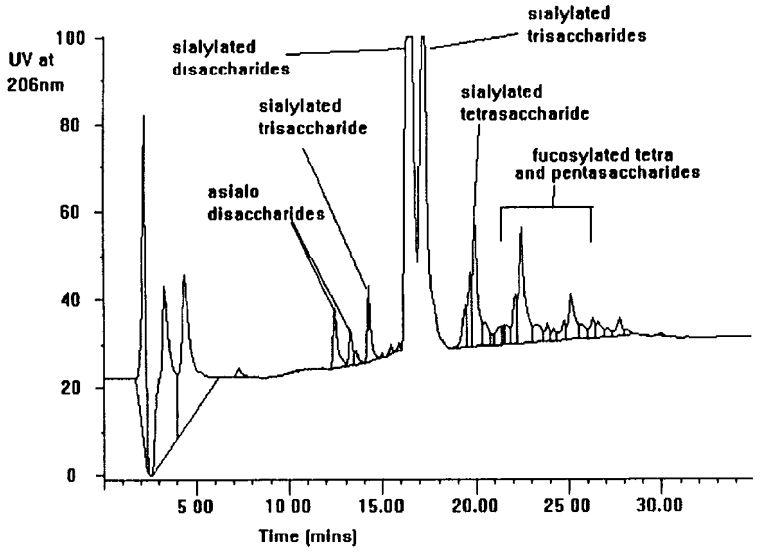

Fig. 4. The separation of oligosaccharide alditols released by mild alkali degradation from 2 mg (approx 5 nmol) bovine submaxillary mucin by PGC-HPLC with UV detection at 206 nm. Printed with permission of *J. Chrom. (9)*.

c. Time = 40 min A = 60%; B = 40%
d. Time = 45 min A = 60%; B = 40%
e. Time = 50 min A = 100%; B = 0%

6. The resulting oligosaccharide containing fractions are then derivatized for LSIMS and GC-MS or analyzed by NMR *(4)*.

3.7. Permethylation of Oligosaccharides and Preparation of PMAAs

1. The methylation procedure described by Hakomori *(10)* has now largely been superseded by that of Ciucanu and Kerek *(11)* as described here.
2. Lyophilize 20 nmol of desalted oligosaccharide (or dry over P_2O_5 *in vacuo*) in a 5 mL reactivial.
3. Dissolve the oligosaccharide in 75 µL DMSO and sonicate for 15 min in an ultrasonic bath in a N_2 atmosphere.
4. Add 75 µL of the NaOH/DMSO suspension (*see* Note 11).
5. Add 50 µL methyl iodide.
6. Incubate sealed vials with a magnetic stirrer for 15 min at 22°C.
7. Add 1 mL HPLC grade chloroform and 3 mL HPLC grade H_2O (*see* Note 12).

8. Mix by vortexing and then centrifuge the reaction mixture momentarily at 2000 rpm to separate the phases.
9. Discard the upper aqueous layer and repeat the washing procedure five further times.
10. Dry the remaining chloroform phase and aliquot one-third for analysis by LSIMS (*see* Note 13).
11. Transfer the remaining permethylated oligosaccharide to a 0.5 mL reactivial and evaporate under N_2.
12. Add 100 µL of 2*M* TFA and hydrolyze the oligosaccharides at 110°C under a N_2 atmosphere for 1 h.
13. Cool the reaction mixture, dry under N_2 and reevaporate three times with 100 µL methanol.
14. Reduce the permethylated monosaccharides with 100 µL of 50 m*M* $NaBD_4$, 50 m*M* NaOH for 4 h at 22°C (*see* Notes 14 and 15).
15. Neutralize the $NaBD_4$ with 25 µL glacial acetic acid at 0°C.
16. Evaporate the reaction mixture and reevaporate three times with 100 µL methanol.
17. Sonicate the residue for 5 min in 75 µL pyridine, 75 µL acetic acid.
18. Incubate at 100°C for 90 min.
19. Cool reaction vial and evaporate the reaction mixture at room temperature under a stream of N_2.
20. Transfer the residue to a 5 mL reactivial and desalt by washing with chloroform/water as described for the permethylated oligosaccharides.
21. Dry the PMAAs (chloroform phase) and resuspend in 10 µL chloroform for GC-MS analysis.
22. Inject 5 nmol of PMAAs into the GC with a splitless injection and a carrier gas pressure of 10 psi.
23. Set the injection port to 250°C and the MS interface at 280°C with the mass selective detector operating with an electron impact source and a scanning range of 50–400 mass units.
24. Separate the PMAAs on a temperature gradient of 50°C for the first minute followed by a gradient of 10°C/min to 260°C, which is then held for a further 8 min. The resulting retention times and molecular ion data are then interpreted to determine the linkages of the monosaccharides (*see* Note 15).

4. Notes

1. Chromic acid (85% H_2SO_4, 15% H_2O, containing 42 g/L Cr_2O_3), is **extremely corrosive as well as being toxic,** and if required an alternative equivalent detergent may be used to wash the reactivials.

2. Reduction and carboxymethylation will aid enzymatic cleavage but prevents elucidation of disulfide-bonding patterns, therefore the rest of the procedure should be attempted before and after reduction and carboxymethylation.
3. Check that the reaction mixture is still alkaline so as to maintain the reactivity of the cysteine thiol groups.
4. The choice of protease used for digestion depends on the glycoprotein being studied. Examination of the amino acid sequence (if known) will reveal the potential proteolytic cleavage sites of the glycoprotein and thus determine the most suitable protease to use *(1)*. The present strategy is for the characterization of bovine fetuin digested with trypsin and thermolysin.
5. Enzyme/buffer solutions are prepared fresh prior to each digest to minimize self-proteolysis.
6. The amount of glycoprotein required is dependent on the degree of glycosylation and the subsequent analyses to be performed. 200 pmol of glycoprotein is sufficient for LSIMS and N-terminal sequence analysis; up to 10 nmol of glycoprotein may be required for complete oligosaccharide analyses; and more than 100 nmol is necessary for NMR analysis.
7. Peptide mapping can also be performed on an ODS-reverse phase column, although a slightly lower resolution is achieved.
8. A better desalting profile may be achieved with an AMMS membrane (rather than AMMS2) if a flowrate of <1 mL/min can be used. In addition it is important that the membranes of the suppressor remain fully hydrated and that the regenerant solution is replaced about once a week.
9. Reversed phase or normal phase (amine bonded silica) HPLC may also be required for the complete separation of some oligosaccharide isomers.
10. Sensitivity of detection can be improved by postcolumn addition of 300 m*M* NaOH and the use of pulsed amperometric detection as described for HPAE-PAD chromatography.
11. The powdered NaOH suspension must be thoroughly mixed immediately prior to addition to the oligosaccharides to minimize the amount of NaOH settling to the bottom of the tube.
12. The permethylated oligosaccharides may also be purified on a Sep-Pak C_{18} column (Waters, UK) by elution of permethylated oligosaccharides with acetonitrile or an acetonitrile–water mixture *(12)*.
13. The reaction with the NaOH/DMSO suspension deprotonates all the free hydroxyl groups and NH of acetamido groups forming an unstable

carbanion. The addition of methyl iodide then rapidly reacts with the carbanions to form O–Me groups and thus permethylate the oligosaccharide. When subjected to LSIMS these permethylated oligosaccharides will fragment about their glycosidic bonds particularly at acetamido residues. This means each oligosaccharide generates a unique fragmentation pattern allowing the determination of the oligosaccharide sequence. For example, the oligosaccharide Hex-HexNAc-HexNAc-Hex will generate the following fragments: Hex-HexNAc and Hex-HexNAc-HexNAc, where Hex denotes a hexose residue and HexNAc an *N*-acetylhexosamine. Sialic acid residues will be degraded by the derivitization, since they do not form carboxy group methyl esters.

14. The reduction may also be carried out overnight (16 h) at 4°C.
15. The hydrolysis step generates monosaccharides with the hydroxyl groups involved in the glycosidic linkages still retaining their protons. Reduction of these monosaccharides with $NaBD_4$ will break the ring structure to form monosaccharide alditols with the anomeric (C_1) carbon being monodeuterated. Acetylation of the free hydroxyls to *O*-acetyl groups completes the derivitization. The retention times of the PMAAs on the GC allow the assignment of the monosaccharide type (galactose, *N*-acetylgalactosamine, and so on). On line mass spectrometric detection identifies fragment ions formed by the cleavage of C–C bonds of the monosaccharide alditols with the preference: methoxy-methoxy>methoxy-acetoxy>acetoxy-acetoxy. The resulting spectra are diagnostic for the substitution pattern and hence the previous position of linkage, e.g., a 2-linked hexose will produce a different set of ions to a 3-linked hexose, and a 2,3-linked hexose being different again. Selected ions from the spectra of all commonly occurring linkages can be used to analyze across the chromatogram (selected ion monitoring).

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CHAPTER 19

Drying Gels

Bryan John Smith

1. Introduction

There are several reasons why it may be desirable to dry a gel after its use for electrophoresis. First, it is a convenient way of storing the end result of the experiment. Second, drying a gel that is fragile may make it easier to handle (say, during optical scanning or display on an overhead projector). Third, and most important, it may be necessary to dry a gel to allow the most efficient detection of radioactive samples on it by autoradiography or fluorography. The method described in this chapter is rapid and generally applicable to all types of slab gel; although the description applies specifically to a thin (0.5–1-mm thick) polyacrylamide (15%) gel being prepared for fluorography (*see* Chapter 20).

2. Materials

1. A high vacuum, oil pump. Protect the pump and its oil from acid and water by inclusion of a cold trap in the vacuum line.
2. A heat source (such as a hot air fan, infrared lamp, electric hot plate, or a steam/hot water bath).
3. A gel dryer, available commercially from various sources or readily made in the laboratory, along the lines of the dryer shown diagrammatically in Fig. 1. Essentially, a gel dryer has the gel placed on top of absorbent paper, which in turn is supported by a firm sheet of porous polyethylene and/or a metal grille. As shown in Fig. 1, the gel is covered with plastic sheet cling film domestic food wrapping, such as Saran Wrap™. This construction is put under vacuum beneath a sheet of silicon rubber and is heated so as to help drive off moisture. Suitable

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Edited by: J M. Walker

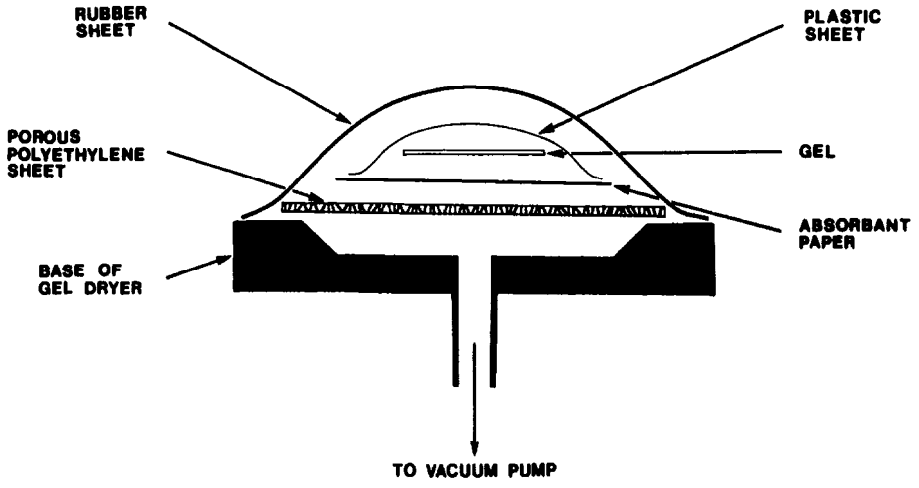

Fig 1 Diagram showing the construction of a simple gel dryer.

polyethylene sheet, absorbent paper, cellophane, and other items may be obtained commercially. Preferably the absorbent paper, to which the dried gel adheres, should be about 1-mm thick. If it is thinner (as is, say, Whatman 3MM paper), it has a tendency to curl up once removed from the gel dryer.

3. Method

1. The gel will already have been run and, if carrying out fluorography, suitably treated and then washed thoroughly with distilled water (*see* Chapter 20).
2. Place the gel on a piece of absorbent paper that is slightly larger than the gel itself. Do not trap air bubbles between them. Place them, gel uppermost, on top of the porous polyethylene sheet and cover the whole with some of the nonporous cling film. Place this construction on the gel dryer base and cover over with the sheet of silicon rubber, as shown in Fig. 1.
3. Apply the vacuum, which should draw all layers tightly together. Check that there is no air leak. After about 10–15 min, apply heat (say, 60°C) evenly over the face of the gel.
4. Continue until the gel is dry, at which point the silicon rubber sheet over the gel should assume a completely flat appearance. The time

taken to dry a gel is dependent on various factors (*see* Notes 2, 5, 6, 8, 9), but a gel of 0.5–1-mm thickness, equilibrated with water, will take 1–2 h to dry.

5. When the gel is completely dry and bound onto the absorbent paper, it may be removed and the cling film over it peeled off and discarded.

4. Notes

1. The plastic cling film layered over the gel is used for two reasons. First, it prevents sticking of the gel to the rubber sheet covering it. Second, and more importantly, it reduces the likelihood of contamination of the dryer by radioactive substances from the gel. After drying, the plastic film may be discarded as radioactive waste.
2. If a suitable high vacuum pump is not available for use with the gel dryer, a good water pump may suffice instead, but in this case the gel will take longer to dry. As another alternative, the gel may be dried in atmospheric conditions, for instance as described in ref. *1*. In that case the gel is equilibrated with 2% (v/v) glycerol in water, covered (with exclusion of air bubbles) with porous cellophane, and kept flat while it dries in the air at room temperature. A gel of about 1-mm thickness with both sides covered with cellophane and exposed to the atmosphere, may take 18–24 h to dry to a thin, tough, and clear sheet. The option of the DMSO solution mentioned in Note 4c, also works satisfactorily.
3. Gels for storage can be dried, as for fluorography, onto paper. However, gels for transmission optical scanning obviously need to be transparent. For this purpose the gel is sandwiched between two sheets of porous cellophane (with no trapped air bubbles), and then taken through the process described above. The dry gel will not adhere to the absorbent paper.
4. The main problem with this method is that gels may crack up and so spoil the end result. This may occur as a chronic process, during the drying. Gels of higher %T acrylamide and of greater thickness are particularly prone to suffer this fate. However, to alleviate this problem the gel may be treated before drying with one of several solutions, which are:
   a. Methanol (70% [v/v]) in water *(2)*. Equilibrate the gel by soaking in this solution (0.5–1 h for thin, 1-mm, gels or longer for thick gels) and then proceed with drying. This treatment will cause the gel to shrink. High %T gels may dehydrate and go opaque. This process may be reversed with water, but if it is too extreme the gel may crack. If this is a danger, use a weaker methanol solution (say, 40%

[v/v]). This treatment speeds up the drying process somewhat since the methanol is driven off fairly quickly.

b. Glycerol (1% [v/v]) and acetic acid (10% [v/v]) in water *(3)*. Equilibrate the gel and proceed with drying.

c. DMSO (2% [v/v]) and acetic acid (10% [v/v]) in water *(3)*. Equilibrate the gel and proceed with drying.

Of these three, the DMSO solution is most likely to prevent cracking of difficult gels and the 70% methanol the least likely, but the former will take the longest time to dry down, and the latter the least time.

5. As a further precaution, and also to speed up drying, a second sheet of porous polyethylene may be used, so that the gel, without the overlaid, nonporous plastic cling film sheet, is sandwiched between the two polyethylene sheets. This arrangement provides a greater surface of gel for drying. However, ensure that the face of the polyethylene sheet that is in contact with the gel is very smooth, for otherwise the gel will dry into it (as well as into the absorbent paper) and they will be difficult to separate. If this remains a problem, employ a sheet of porous cellophane between the gel and polyethylene. The cellophane may be removed after drying.
6. Another precaution is to use a lower temperature during the drying process, so that gradients of temperature and hydration through the system are less extreme.

   Thus, probably the best approach to drying a difficult gel, such as a 3-mm-thick 10% or 15%T acrylamide gel, would be to use the DMSO (2%) soaking of the gel before drying, and two polyethylene sheets and less heat (say, 40°C) during the drying. Under these circumstances, a thick gel may take a whole working day to dry down.
7. Gel cracking may also occur as an acute phenomenon when the vacuum is released from a gel that is not completely dry. Thus, it is important to check for air leaks in the der and not to end the drying (i.e., release the vacuum) too soon. So that this does not happen, it is important to determine (by trial) the time required to dry down a gel in one's own gel-drying equipment. This time increases with increased %T of acrylamide, thickness, and surface area of the gel, and with decreased temperature and vacuum during the drying.
8. A problem may arise if the gel is prepared for fluorography using DMSO as solvent. The gel must be thoroughly washed in water (or other solution, *see* Note 4) to remove the DMSO. This is because DMSO has a boiling point of 189–193°C and is difficult to remove under the conditions of drying. If remaining in the gel in significant amounts, the gel will remain sticky and the photographic film it contacts may become fogged.

9. Agarose gels may be dried in the manner described. Even 3-mm agarose gels dry quickly (about 0.5 h) and without cracking when using only one polyethylene sheet and room temperature for drying. Composite weak acrylamide–agarose gels are likewise readily dried down. When dealing with gels containing agarose, beware the use of DMSO, which dissolves agarose.
10. If using EN^{3}HANCE (New England Nuclear, Boston, MA) in the gel, do not employ temperatures above 70°C for drying, for EN^{3}HANCE is volatile at higher temperatures (boiling point is 117°C).

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CHAPTER 20

Fluorography of Polyacrylamide Gels Containing Tritium

Jaap H. Waterborg and Harry R. Matthews

1. Introduction

Fluorography is the term used for the process of determining radioactivity in gels and other media by a combination of fluorescence and photography. Since most of the radiation of a low energy emitter will largely be absorbed by the gel, in the technique of fluorography a fluor (e.g., PPO) is infiltrated into the gel where it can absorb the radiation and re-emit light that will pass through the gel to the film. The resulting photographic image is analogous to an autoradiograph, but for low energy β-emitting isotope like ^3H, the sensitivity of fluorography is many times the sensitivity of autoradiography. The fluorograph may be used directly, as a qualitative picture of the radioactivity on the gel. It may also be used to locate radioactive bands or spots that can then be cut from the original gel for further analysis, or be scanned to give quantitative information about the distribution of radioactivity. Figure 1 shows an example of a gel that was stained with Coomassie blue and then fluorographed. Notice that there is no loss of resolution in the fluorography of thin gels of normal size.

The procedures described here are based on those described by Laskey and Mills *(1)*, Bonner and Laskey *(2)*, and Randerath *(3)*.

2. Materials

1. –70°C Freezer.
2. Film cassette, preferably with enhancing screen.
3. Small photographic flash unit.

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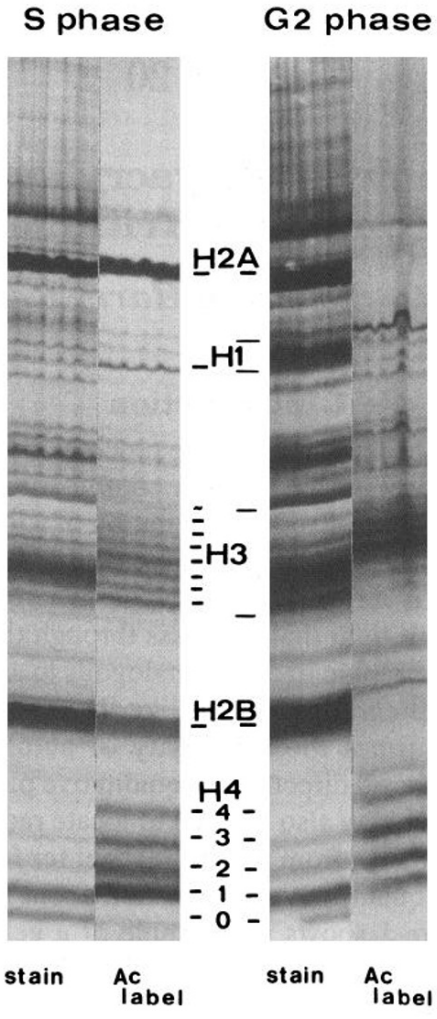

Fig. 1. This shows two examples of fluorography of protein bands labeled with ^3H. Basic nuclear proteins were isolated from the slime mold, *Physarum polycephalum,* pulse-labeled with ^3H-acetate in either S or G_2 phase of the cell cycle. The proteins were analyzed by polyacrylamide gel electrophoresis in acetic acid, urea, and Triton X-100. After electrophoresis, the gel was stained with Coomassie blue, photographed, and then fluorographed. Individual lanes of the gel image were cut from the photograph (negative) and the fluorograph and then printed side-by-side to give the figure shown.

Notice that the stain patterns of the two lanes are practically identical, except for the loading, whereas the radioactivity patterns show major differences, for example, the absence of label in histones H2A and H2B in G_2 phase *(5).*

4. Gel dryer.
5. Film developer.
6. Fixing solution: 7% acetic acid (v/v), 20% methanol (v/v) in distilled water.
7. Acetic acid, 25% (v/v).
8. Acetic acid, 50% (v/v).
9. Acetic acid (glacial), 100%.
10. PPO solution: 20% (w/v) PPO (2,5-diphenyloxazole) in glacial acetic acid.
11. Film: Kodak XAR-5.

3. Method

1. After electrophoresis, remove the gel from the apparatus and fix by soaking in fixing solution for 1 h. If required, the gel may then be stained with Coomassie blue, destained, and photographed.
2. Dehydrate the gel by shaking it for 10 min each in 25% acetic acid, 50% acetic acid, and glacial acetic acid.
3. Completely cover the gel with not less than 4 gel vol of PPO solution and shake the gel in the PPO solution for 2 h.
4. Transfer the gel gently and evenly to a dish of distilled water and shake for 2 h. In water, PPO precipitates so that the gel turns opaque white.
5. Dry the gel completely (*see* Chapter 19), using Saran wrap on one side, on Whatman 3MM paper.
6. Open a film cassette and place the dry gel (after removal of the Saran wrap) in the cassette with the enhancing screen, if used. Leave the top of the cassette beside the part with the gel in it with the white inner lining facing up.
7. In complete darkness, place a sheet of film on the white lining of the open cassette and preflash it (*see* Note 5).
8. Assemble the cassette with the film directly on the gel. Wrap the cassette with aluminum foil and place in the –70°C freezer.
9. Remove the cassette from the freezer after the appropriate exposure times (*see* Note 7) and allow to warm up for about 2 h at room temperature.
10. In complete darkness, open the cassette and develop the film.

4. Notes

1. Other fixing solutions may be used in step 1. For example, formalin can be used to fix peptides covalently in the gel.
2. Coomassie blue staining gives minimal color quenching, but very heavily stained bands may show reduced efficiency for fluorography.

Amido black gives more color quenching and is not recommended. Silver staining has not been tested. It is possible to reswell the gel after fluorography and stain it then, but some loss of resolution occurs.

3. The times given in steps 2 and 3 are for 0.5–1.5-mm thick gels containing 15% acrylamide. Thicker or more concentrated gels will require longer periods in all solutions.
4. Since PPO is expensive, it is normally recycled as follows: Set up four 4 L Erlenmeyer flasks with a large stirring bar and about 2.5 L of distilled water in each. Add about 0.25 L of used PPO solution, slowly, to each Erlenmeyer, stirring continuously. The PPO crystallizes out. Collect the PPO by filtering the solutions. Dry the crystals at 20°C for 2–3 d. Dissolve the crystals in a minimum volume of ethanol and precipitate, filter, and dry again. Finally, dry the PPO in a vacuum oven for about 1 wk, breaking up lumps at intervals. Glassware that was used for PPO should be rinsed in ethanol before washing.
5. *Preflashing* is used to improve the sensitivity and linearity of response of the film *(1)*. Use a small battery-operated, photographic flash unit. Tape a red filter and a diffusing screen of Whatman 3MM paper over the flash window. Experiment with the number of layers of paper required to give an absorbance of about 0.1 when the film is preflashed. To preflash, hold the flash unit directly above the film, about 60 cm away, and press the manual flash button. Use a procedure that you can easily reproduce in the dark. Note that the first flash after the flash unit is switched on may be different from subsequent flashes, so avoid using the first flash. The white inner lining of the film cassette provides a uniform reproducible background for preflashing.
6. Be careful not to place the cassette near penetrating radiation from sources such as a ^{32}P or ^{125}I autoradiograph or radioactive samples. This radiation will fog the fluorograph. Make sure the cassette is light-tight, too.
7. Recommended exposure times are of the order of 24 h for 1000–10,000 dpm ^{3}H and correspondingly longer for lower amounts of radioactivity. Exposure times of several months do not give a significant increase in background. Sensitivity for ^{14}C is reported to be about 10X that for ^{3}H. Note that high acrylamide concentrations such as the 50% gels used for peptide analysis severely quench the fluorescence and drastically reduce the efficiency of fluorography.
8. An automatic developer is most convenient. Manual development is described in ref. *4*.

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CHAPTER 21

The Electrophoretic Elution of Proteins from Polyacrylamide Gels

Jaap H. Waterborg and Harry R. Matthews

1. Introduction

The analytical power of acrylamide gel electrophoresis is one of the keys of modern protein chemistry. It is not surprising, therefore, that many methods have been described for converting that analytical power into a preparative tool. None of the available methods are entirely satisfactory for general use since loss of resolution or low recovery is often involved. The method described here has given both high resolution and good recovery but suffers from the disadvantage of being relatively laborious *(1,2)*. In addition, although the recovered proteins are good for peptide analysis or amino acid composition determination, we have found very low yields on Edman degradation of proteins eluted from gels *(3)*.

The method described below works well for eluting proteins from acid-urea-Triton gels and should work equally well with acid-urea gels (*see* Chapter 6). Wu et al. *(1)* describe an alternative set of buffers that can be used for SDS gels (*see* Note 1).

The method uses the principle of isotachophoresis, as in the stacking gel portion of a discontinuous gel electrophoresis system (*see*, for example, Chapter 5). The gel pieces containing the protein of interest are embedded in agarose above an agarose gel column. A detergent, CTAB, is used to displace the Triton (or SDS) bound to the protein that is electrophoretically eluted into the low-melting-temperature agarose gel column. In the column, the protein is concentrated

From: *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
Edited by J. M. Walker

by stacking between the leading ion (Na^+) and the trailing ion (betaine). The protein dye (Coomassie blue or Amido black) stacks ahead of the protein. The concentrated protein band is cut from the agarose gel and recovered.

2. Materials

1. Lyophilizer.
2. Vacuum pump.
3. Electrophoresis apparatus capable of running tube gels, preferably in glass tubes with small funnels like the Bio-Rad Econocolumn #737-0243 (which is 5-mm inner diameter and 20-cm long) with the lower end cut off.
4. Staining solution (Coomassie): 0.1% (w/v) Coomassie brilliant blue R, 5% (v/v) acetic acid, 40% (v/v) ethanol, 0.1% (w/v) cysteamine in distilled water.
5. Destaining solution (Coomassie): 5% (v/v) acetic acid, 40% (v/v) ethanol, 0.1% (w/v) cysteamine.
6. Staining solution (Amido): 0.1% (w/v) Amido black in destaining solution (Amido) (note: add the cysteamine immediately before use).
7. Destaining solution (Amido): 7% (v/v) acetic acid, 20% (v/v) methanol, 0.1% (w/v) cysteamine.
8. Equilibration buffer: 1*M* acetic acid, 50 m*M* NaOH, 1% (w/v) cysteamine.
9. Siliconizing solution: 1% (v/v) Prosil-28 in distilled water.
10. LMT agarose solution: 0.5% (w/v) low-melting-temperature agarose in 1*M* acetic acid, 50 m*M* NaOH.
11. HMT agarose solution: 1% (w/v) high-melting-temperature agarose, 1*M* acetic acid, 50 m*M* NaOH, 0.0005% (w/v) methyl green in distilled water (*see* Note 2).
12. Upper reservoir buffer: 1*M* acetic acid, 0.1*M* betaine, 0.15% (w/v) cetyl trimethyl ammonium bromide (CTAB) in distilled water. Lower reservoir buffer: 1*M* acetic acid, 50 m*M* NaOH.
13. Acidified acetone: add conc. HCl to acetone to give a concentration equivalent to 0.02*N*.
14. Elution buffer: 0.02*N* HCl with optionally 0.1 (w/v) cysteamine, 0.1*M* *N*-methylmorpholine acetate, pH 8.0.
15. Agarose gels: (can be done 1 d before needed):
    a. Thoroughly clean and dry the tubes and then siliconize them, for example, by immersing in 1% Prosil-28 followed by thorough rinsing with water and drying.

b. Soak small pieces (3-cm square) of dialysis membrane in distilled water and fix one piece over the end of each tube with an elastic band. This is intended to hold the agarose in the tube.
c. Melt LMT agarose solution by heating to 65–100°C. Use 7 mL/tube. Fill each tube carefully, avoiding trapping air bubbles, just to the bottom of the funnel. Let the agarose gel at 4°C.

3. Method

1. Stain the gel just enough to visualize the band(s) of interest. Either Coomassie blue or Amido black staining may be used. If the gel is loaded heavily enough, then 15 min in either staining solution and no destaining is recommended (*see* Note 3).
2. Cut out the bands of interest and soak them for at least 1 h, or overnight, in equilibration buffer (*see* Note 4).
3. Chop the gel bands into small pieces with a razor blade and transfer them to funnels fixed on the tops of the agarose gels in the electrophoresis apparatus.
4. Melt the HMT agarose solution in a 100°C bath and add between 2–5 mL to each funnel. Stir to remove air bubbles and achieve a uniform distribution of gel pieces in the agarose (*see* Notes 2 and 5).
5. Let the agarose solution gel either at room temperature or at 4°C.
6. Place the bottom reservoir of the electrophoresis apparatus in a tray containing melting ice as coolant. Fill the upper and lower reservoir with upper reservoir buffer and lower reservoir buffer, respectively (*see* Notes 6 and 7). Connect the negative terminal of the power supply to the lower electrode and the positive terminal to the upper electrode.
7. Start the electrophoresis, using 2.5–5 mA/tube (about 200 V).
8. During electrophoresis, the staining dye (Coomassie or Amido) migrates fastest, followed by the buffer discontinuity, the protein, and the methyl green marker dye. Continue electrophoresis at least until the staining dye front reaches the middle of the gel. If the buffer discontinuity is clearly separated from the staining dye, then proceed to step 9. Otherwise, continue electrophoresis until the staining dye is clearly separated (*see* Notes 5, 8, and 9).
9. Immediately remove the gel from the tube as follows (*see* Notes 9 and 10):
   a. Use a spatula to remove the acrylamide gel pieces and their supporting HMT agarose gel.
   b. Push the gel column with a plunger from a disposable syringe out of the tube onto a clean glass plate.

10. Immediately cut the gel about 3 mm below the buffer discontinuity (above the staining dye) and about 5 mm above the buffer discontinuity (*see* Note 11). The resulting 8-mm section of gel contains the protein and the methyl green marker dye; the remainder is discarded. Place the gel section in a 1.5-mL polypropylene centrifuge tube.
11. Use the following procedure (steps 12–15) if the presence of agarose, and protein denaturation during drying, do not interfere with subsequent analysis. Otherwise proceed to step 16 (*see* Note 12).
12. Add 1 mL of acidified acetone and leave overnight in the freezer, at −20°C (*see* Note 13).
13. Centrifuge (10,000*g*; 5 min) and slowly decant the acetone, which contains the dye, buffer salts, and detergent.
14. Cap the tube, pierce the cap, and dry the gel under vacuum using a vacuum pump until the vacuum falls below 100 mtorr (*see* Note 14).
15. Rehydrate the agarose with 20–50 µL of distilled water, or appropriate buffer, by incubation at 65°C for a few min. This solution will remain liquid at 37°C, allowing enzymatic digestion, or it may be diluted to give < 0.1% agarose when it will no longer gel. This is the end of this procedure.
16. This step follows step 11 if steps 12–15 were unsuitable. If this procedure is used, then the methyl green marker dye should not be used (*see* Note 2).
17. To the gel slice, add 0.5 mL elution buffer. Mix very gently without breaking up the agarose. Leave at room temperature for at least 1 h and preferably overnight (*see* Note 15).
18. Equilibrate a small (~1 × 10 cm) desalting column of Sephadex G-25 with 0.1*M* *N*-methylmorpholine acetate, pH 8.0.
19. Centrifuge (10,000*g*; 5 min). Carefully decant or pipet off the supernatant and set it aside.
20. Repeat steps 17 and 19, combining the supernatants. The extracted gel may be discarded (*see* Note 16).
21. Load the combined supernatants onto the Sephadex desalting column equilibrated with 0.1*M* *N*-methylmorpholine acetate, pH 8.0 (step 18). Elute with 0.1*M* *N*-methylmorpholine acetate and collect the material eluting in the excluded volume.
22. Lyophilize the protein to dryness. The resulting protein is salt-free since *N*-methylmorpholine acetate is volatile. This ends the alternative procedure.

4. Notes

1. Solutions used to recover proteins from SDS gels *(1)* are as follows:

a. Lower reservoir buffer (pH 8.3): 0.12*M* 2-dimethylaminoethanol, 0.043*M* phosphoric acid, 0.05*M* bicine.
b. Lower reservoir buffer (pH 8.5): 0.4*M* 2-dimethylaminoethanol, 0.15*M* boric acid, 1% SDS.
c. LMT agarose: 0.5% LMT agarose, 0.06*M* 2-dimethylaminoethanol-HCl, pH 8.3.
d. HMT agarose: 0.87% HMT agarose, 0.08*M* 2-dimethylaminoethanol-HCl, pH 8.3, 0.00001% phenol red.
e. Equilibration buffer: 0.08*M* 2-dimethylaminoethanol-HCl, pH 8.3, 1% β-mercaptoethanol.

2. The presence of methyl green in the HMT agarose makes it easy to locate the buffer discontinuity during the subsequent electrophoresis and is mostly removed by acetone precipitation of the protein. Small residual amounts do not interfere with subsequent peptide mapping. However, if the protein is to be isolated and desalted by the Sephadex method (step 16 on) the methyl green should be omitted from the HMT agarose solution. (If methyl green *were* included it would elute partly with the protein and partly immediately after the protein). In this case the buffer discontinuity must be located directly from the refractive index change.
3. Gels that have been normally stained and destained may be used but the time of agarose gel electrophoresis may have to be increased or the capacity of the system will be reduced. (Note that cysteamine should not be included for Coomassie staining of histone H1).
4. For Amido black, but not for Coomassie, the equilibration (step 2) also acts as a destaining step.
5. At step 4, use the minimum volume of agarose required to suspend the gel fragments since the time of electrophoresis depends greatly on the volume of agarose plus gel fragments.
6. Betaine (rather than glycine) is used as the trailing ion because of its higher acetone solubility. It also increases the capacity of the system.
7. If ice cooling is not used during electrophoresis, reduce the current to 2.5 mA/tube.
8. The distance between the lower edge of the staining dye and the buffer discontinuity depends on the amount of stain and how full the funnels are on top of the gel, varying from about 5 to about 40 mm. The distance between the upper edge of the staining dye and the buffer discontinuity is fairly constant at 5–10 mm (reduces to 2–3 mm if the betaine buffer is replaced by glycine).
9. The time of electrophoresis will be 2–4 h at 4 mA/tube for low amounts in the funnels, and up to overnight at 2.5 mA/gel for full funnels. Dif-

ferent tubes may require different running times. In this case, remove the tubes whose electrophoresis is complete as follows:

a. Turn off the power, disconnect the power supply, and empty the upper reservoir buffer into a beaker.
b. Remove the required tube(s), block the resulting hole(s) with rubber bung(s), replace the upper reservoir buffer, and continue the electrophoresis, adjusting the power supply as necessary.

If large volumes of reservoir buffers (e.g., 2 L) are used, then reservoir buffer changes are not needed.

10. It is important to remove the gel immediately after electrophoresis since the staining dye diffuses rapidly once the current is turned off. It may be possible to remove the gel in other ways (e.g., *see* ref. *1*), but we have found the method described in step 9 to be rapid and reliable.
11. Slice the gel immediately after electrophoresis, to prevent loss of resolution by diffusion. In our experience, this 8 mm section of the gel contains at least 98% of the protein.
12. The simple acetone extraction and drying procedure (steps 12–15) is suitable if the protein is to be digested with enzymes and the products analyzed by gel electrophoresis. Note that the procedure described by Cleveland (*see* Chapter 23) provides another peptide mapping approach for some applications. The alternative procedure (step 16 on) is recommended if the protein is to be analyzed for amino acid composition or characterized by nuclear magnetic resonance or other techniques, for example, thin layer analysis of tryptic digests.
13. The protein will precipitate in the gel at step 12, possibly forming a white band, but precipitation may not be quantitative with extremely small amounts of protein.
14. Drying of the extracted gel (step 14) takes several hours. This procedure may result in irreversible denaturation of the protein, but it will probably still be solubilized by enzymic digestion.
15. At step 17, do not vortex or mix vigorously or you will get agarose in the final protein solution. Allow the protein to diffuse out. Do not include urea or other agents that would solubilize the agarose in the elution buffer.
16. The second extraction (step 20) need only be 1 h. In our tests, < 5% of the protein remained in the gel after the two extractions, although the white appearance persisted. The protein concentration in the eluate cannot be determined by the Bradford method (*see* Chapter 3) since CTAB strongly interferes.

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CHAPTER 22

Preparation of Proteins from Gels for Protein Microsequencing

Cathy S. Baker and Michael J. Dunn

1. Introduction

Complex mixtures containing small amounts of protein can be separated and purified in one step using either one- or two-dimensional electrophoresis (1-D or 2-D). Since automated microsequencing has been developed, which requires small amounts of purified proteins, electrophoresis has become the method of choice for protein preparation and purification. Once the proteins are separated, they must be recovered from the gel matrix for further chemical characterization. Two methods available for this include electroelution (*see* Chapter 21) and western blotting (electroblotting) *(1)*.

Electroelution has drawbacks. It is time-consuming and sample recovery can be unreliable. Only a few proteins can be processed at a time and they can become contaminated, degraded, or modified. All of these problems may interfere with further chemical characterization.

Electroblotting transfers essentially all the proteins from the gel in one step to an inert membrane rendering the proteins suitable for chemical characterization. N-terminal sequencing requires a membrane that can withstand the chemicals used in the sequencer. Several commercially available membranes are suitable, but the most robust are the polyvinylidene difluoride (PVDF) based membranes. Nitrocellulose membranes can be used for the preparation of proteins for internal microsequencing, but they cannot be used for direct microsequencing because they dissolve in the sequencer. Protein contamina-

From: *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
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tion, degradation, or modification can be eliminated when pure reagents are used for electrophoresis and electroblotting. For these reasons, electroblotting has become the preferred method for protein preparation and purification from gels.

Proteins prepared by electroblotting are generally sequenced at high efficiencies with very low backgrounds. However, amino acid sequences obtained from electroblotted proteins are of limited value when they are short (10–25 residues). This situation can lead to false matches through homology searches of databases. Also, N-terminal sequences are not obtained from many proteins because they do not have a free α-amino group (i.e., they are N-terminally blocked) *(2)*. To overcome these problems, internal protein microsequencing can be performed. In this technique a chemical or an enzyme is used to cleave the intact polypeptide into peptide fragments, which can be subsequently isolated and sequenced. This method provides additional information for those proteins with short N-terminal sequences to aid in a more extensive homology search and provides sequence information for those proteins possessing a blocked N-terminus.

In the following sections electroblotting methods for recovering proteins separated by gel electrophoresis for microsequencing will be described. In addition a staining procedure for membranes and a procedure for enzymatic digestion for internal microsequencing will be briefly discussed.

2. Material

2.1. Electroblotting

1. Blotting buffers are selected empirically to give the best transfer of the protein(s) under investigation. The following compositions have been used successfully:
   a. For proteins separated by SDS-PAGE with pIs between pH 4 and 7: 6.06 g Tris base, 3.09 g boric acid dissolved in 1 L deionized water and adjusted to pH 8.5 with 10*N* sodium hydroxide *(3)*.
   b. For proteins with pIs between pH 6 and 10, 2.21 g 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) dissolved in 1 L 10% methanol and adjusted to pH 11.0 with 10*N* sodium hydroxide *(4)*.
2. Whatman 3MM filter paper.
3. Blotting membrane (*see* Note 1 and Section 3.3.).

4. Electroblotting equipment. There are numerous types on the market. Methods for use with semidry and wet blotting apparatus are described here.
5. 1-D or 2-D gel previously electrophoresed.

2.2. Protein Staining

1. Destain: 45% (v/v) methanol, 10% (v/v) acetic acid in deionized water.
2. 0.2% (w/v) Coomassie brilliant blue R-250 in destain.
3. 0.15% (w/v) Amido black (Napthalene black 12B) in destain.

2.3. Enzyme Digestion for Internal Microsequencing

1. Polyvinylpyrrolidone (PVP-40).
2. TPCK-treated trypsin (modified trypsin from Promega, Southampton, UK).
3. Glass fiber disks (Applied Biosystems [ABI], Warrington, UK).
4. Polybrene (Applied Biosystems).

3. Methods

3.1. Electroblotting

3.1.1. Semidry Blotting

1. Wet the anode plate with deionized water.
2. Wet three pieces of Whatman 3MM filter paper with blotting buffer and place onto the anode plate. Roll with a glass tube to remove any bubbles.
3. Wet three pieces of Whatman 3MM filter paper in the blotting buffer and place onto the three filter papers on the anode. Roll again to ensure air bubbles are not present.
4. Wet the PVDF-blotting membrane with methanol and then blotting buffer. Place the membrane on top of the filter paper "sandwich" and remove air bubbles with the glass tube.
5. Place the 1-D or 2-D gel (after equilibration in appropriate blotting buffer, 30 min in Tris-borate or 5 min in CAPS [*see* Note 2]) onto the blotting membrane and ensure all air bubbles are removed.
6. Repeat steps 2 and 3 but place the filter stack on top of the gel.
7. Wet the cathode plate with deionized water and place on top of the blotting sandwich.
8. Connect the blotter to a power supply and transfer at 0.8 mA/cm^2 of gel area for 1 h (1.5-mm thick gels) or 4 h (3-mm thick 2-D gels) at room temperature (*see* Note 3).
9. An example of a sample prepared by semidry blotting is shown in Fig. 1.

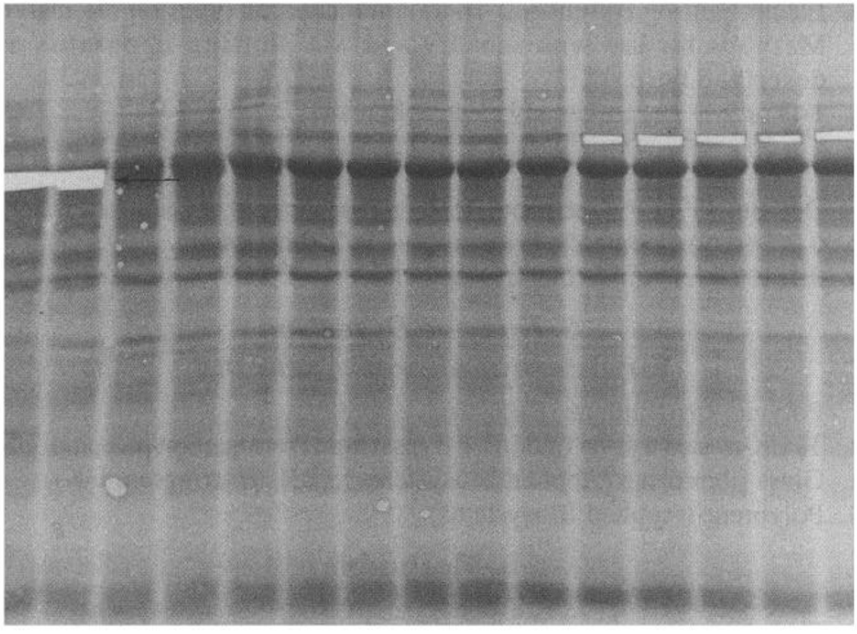

Fig. 1. Shows a 1-D semidry blot of cultured EAHY926 human endothelial cells. The SDS-PAGE was performed on an 8% gel. The arrow shows where one band has been removed and sequenced with the following amino acid (one-letter) sequence result: DAHKSEVAHRFKDLGEENFK. This was searched for homologies and found to correspond to human albumin.

3.1.2. Wet Blotting

1. Place the anode side of the blotting cassette in a dish of blotting buffer.
2. Submerge a sponge pad and place on top of the anodic side of the blotting cassette.
3. Place two pieces of Whatman 3MM filter paper onto the sponge pad. Roll to ensure air bubbles are not present.
4. Wet the PVDF-blotting membrane with methanol and place on top of the blotting sandwich in the blotting buffer.
5. Place the 1-D or 2-D gel on top of the blotting membrane.
6. Place a sponge pad into the blotting buffer and then on top of the gel.
7. Place the cathode side of the blotting cassette on top of the sponge pad and fasten to the anode side.
8. Remove the cassette from the dish of buffer and place into the blotting tank filled with transfer buffer.

9. Connect to the power supply and transfer for 14 h (3-mm thick 2-D gels) or 6 h (1.5-mm thick 1-D gels or 2-D gels) at 500 mA at 10°C (*see* Note 3 for optimization).
10. An example of a sample prepared by wet blotting is shown in Fig. 2.

3.2. Protein Staining for N-Terminal Microsequencing

1. Remove the blotting membrane from sandwich assembly.
2. Place the PVDF membrane into a dish containing the Coomassie blue staining solution for 2 min on a gentle shaker.
3. Place the membrane into destaining solution and onto a shaker for 10–15 min (or until background is pale).
4. Wash with deionized water and place on filter paper and allow to air dry.
5. Place the membrane into a clean plastic bag and seal until required for further analysis (*see* Notes 4 and 5). The membranes can be stored at room temperature indefinitely (over 2 yr in our laboratory) without any apparent adverse effects.

3.3. Enzyme Digestion for Internal Microsequencing

This technique is described by Aebersold *(2)*.

1. Proceed with blotting as in the electroblotting section except use nitrocellulose (*see* Note 6) as the blotting membrane (do not prewet in methanol).
2. Stain the blot with the Amido black staining solution for 2 min and destain until the background is pale (5 min).
3. Rinse with deionized water and do not dry.
4. Excise the protein band or spot of interest and transfer to an Eppendorf tube (1.5 mL) and store at –20°C until further analysis. Use up to five bands (1-D) or 40 spots (2-D) of destained nitrocellulose membrane containing the same protein and place into 1 Eppendorf tube.
5. Place 1.2 mL of 0.5% PVP-40 dissolved in 100 m*M* acetic acid into the tube and incubate at 37°C for 30 min. This step prevents adsorption of the protease to the nitrocellulose. The protease tends to adsorb to the nitrocellulose membrane more in a basic environment.
6. Remove excess PVP-40 by washing with deionized water (5X). PVP-40 absorbs UV light strongly and can interfere with HPLC analysis.
7. Cut the pieces of nitrocellulose into 1 × 1 mm fragments and place back into the same tube.
8. Place 300 µL (or the minimum volume to cover the nitrocellulose pieces)

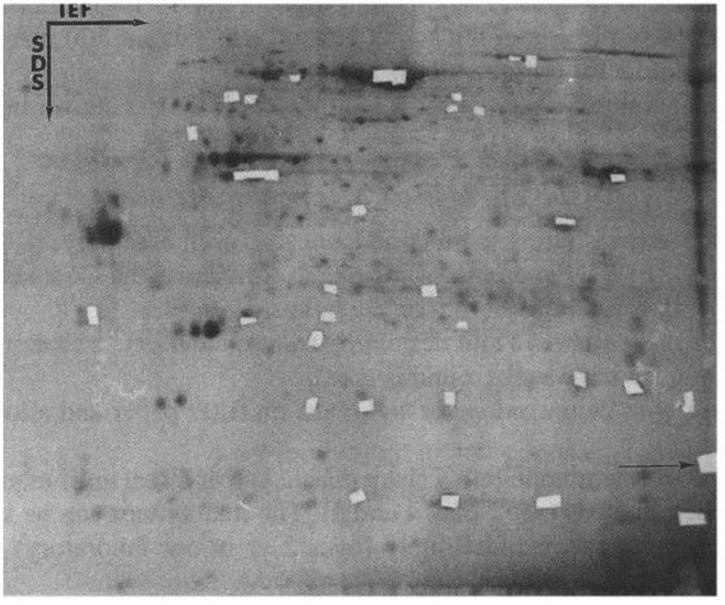

Fig. 2. Shows a 2-D wet blot of a tissue extract of human myocardial protein. The SDS-PAGE was performed on a 12% gel. The arrow points to one spot that has been removed and sequenced with the following amino acid (one-letter) result sequence results: GLSDGEWQLVLNVWGKVEAD. This was searched for homologies and found to correspond to human myoglobin.

of trypsin in 100 m*M* Tris-HCl, pH 8.2:acetonitrile (95:5 [v/v]) into the tube. Keep the enzyme/substrate ratio at 1:20 [w/w] (*see* Note 7).

9. Incubate at 37°C overnight.
10. Freeze the entire reaction mixture at –20°C or prepare according to Notes 8 and 9.

4. Notes

1. There are several PVDF-blotting membranes on the market. We have found optimum protein retention on FluoroTrans (Pall, Portsmouth, UK) or ProBlott (ABI) membranes *(3)*.These are both compatible with the chemistry of the ABI Model 477A Pulse-Liquid Protein Sequencer.
2. Gels are equilibrated in blotting buffer to remove excess glycine and SDS. This step also minimizes swelling effects during protein transfer. Equilibration may result in diffusion of zones and reduced transfer

efficiencies of high-mol-wt proteins. It is important to optimize times for your protein(s) of interest.

3. Blotting times need to be optimized for the particular proteins of interest. Larger proteins usually need a longer transfer time, whereas smaller proteins need less time. The times quoted in this chapter are optimal for our use *(3)*.
4. Microsequencing was performed on an Applied Biosystems Pulse-Liquid Protein Sequencer where the protein from the PVDF blot was excised with a razor blade, rinsed well with deionized water, placed into the Blott Cartridge *(5)* fitted on the protein sequencer. This cartridge, designed specifically for use with blotting membranes, allows for a vertical crossflow of reagents rather than the flow-through design of the traditional ABI sequencing cartridge. This crossflow design overcomes the problem of restricted flow caused by the small pore size of the PVDF membrane allowing for more complete chemistry. Dry the blot with Argon in the cartridge inside the sequencer before proceeding with the Edman degradation. Proceed with the Edman chemistry using the Applied Biosystem's computer program supplied with the Blott cartridge and optimized *(3)* for your blot.
5. To prevent the line between the sequencer and the 120A Analyzer from clogging (possibly with SDS from the blots), rinse the line with methanol (use bottle X2 on the Model 477A Sequencer) after completing each blot.
6. Enzymatic and chemical digests to cleave peptides from proteins can be performed on PVDF membranes *(6)*, however the nitrocellulose technique has been the only successful approach in our laboratory.
7. This ratio of enzyme to substrate was determined to be the optimum in our laboratory. A larger amount of enzyme did not increase the number of peptide fragments, however, less enzyme did produced fewer peptide fragments. This ratio should be optimized for the type of enzyme and substrate being used.
8. Once the digestion has been completed, the reaction mixture should be acidified with 30 µL of 10% trifluoroacetic acid, vortexed, and centrifuged. The peptide fragments can be separated by microbore reverse phase HPLC as described by Aebersold *(2)* (*see also* Chapter 31) and collected manually onto glass fiber disks.
9. When sequencing peptide fragments directly on glass fiber disks, one can use the traditional reaction cartridge supplied with the ABI Model 477A sequencer.

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CHAPTER 23

Comparison of Protein Primary Structures

Peptide Mapping

Ralph C. Judd

1. Introduction

The comparison of the primary structure of proteins is an important facet in the characterization of families of proteins from the same organism, similar proteins from different organisms, and cloned gene products. There are many methods available to establish the similarity/dissimilarity of proteins. A relatively uncomplicated approach is to compare the peptide fragments of proteins generated by enzymatic or chemical cleavage; e.g., peptide mapping (*see* ref. *1* for previous review).

The principle behind peptide mapping is straightforward: If two proteins have the same primary structures, then cleavage of each protein with a specific protease or chemical cleavage reagent will yield identical peptide fragments. However, if the proteins have different primary structures, then the cleavage will generate unrelated peptides. The similarity or dissimilarity of the protein's primary structure is reflected in the similarity or dissimilarity of the peptide fragments.

There are four phases to the peptide mapping process:

1. Identification and purification of the proteins to be compared;
2. Radiolabeling of the proteins, and thus the peptide fragments, to minimize the quantity of protein required;

From: *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
Edited by J M Walker

3. Cleavage of the proteins with specific endopeptidic reagents, either chemical or enzymatic; and
4. Separation and visualization of the peptide fragments for comparison.

Each step can be accomplished in different ways depending on the amount of protein available, the technologies available, and the needs of the researcher. Several basic procedures are presented that have proven reliable. These include procedures that employ sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional thin-layer electrophoresis–thin-layer chromatography (2D-TLE-TLC), and high-performance liquid chromatography (HPLC). The researcher must decide which method best suits his or her abilities and goals. Because peptide mapping is empirical by nature, reaction times, reagent concentrations, and amounts of proteins and peptides may need to be altered to accommodate different research requirements.

2. Materials

2.1. SDS-PAGE

1. SDS-PAGE gel rig and power pack (e.g., EC 500, EC Apparatus, Inc., St. Petersburg, FL) or equivalent.
2. Solubilization buffer: 2 mL 10% SDS (w/v) in H_2O, 1.0 mL glycerol, 0.625 mL 1*M* Tris-HCl, pH 6.8, 6 mL H_2O; bromophenol blue to color.
3. Reagents needed to make separation and stacking gels having the appropriate crosslinker concentrations and acrylamide percentages (*see* Chapter 5).
4. Ethanol: 1*M* Tris-HCl, pH 6.8 (50:50 [v/v]).
5. Laemmli running buffers (*see* Chapter 5).
6. Mol-wt markers, e.g., low-mol-wt kit (Bio-Rad, Inc., Richmond, CA) or equivalent or peptide mol-wt markers (Pharmacia Inc., Piscataway, NJ) or equivalent.

2.2. Electroblotting

1. Blotting chamber with cooling coil (e.g., Transblot chamber, Bio-Rad, Inc.) or equivalent.
2. Power pack (e g , EC 420, EC Apparatus, Inc) or equivalent.
3. Nitrocellulose paper (NCP).
4. PVDF-nylon membrane.
5. Ponceau S: 1–2 mL/100 mL H_2O, 0.1%.
6. Naphthol blue black (NBB) in H_2O.

7. India ink (Pelikan, Hannover, Germany).
8. 0.05% Tween 20 (#P1379, Sigma, St. Louis, MO) in phosphate buffered saline, pH 7.4.
9. 20 m*M* phosphate buffer, pH 8.0: 89 mL 0.2*M* Na_2HPO_4 stock, 11 mL 0.2*M* NaH_2PO_4 stock in 900 mL H_2O.

2.3. Radiolabeling

1. Gamma radiation detector.
2. SpeedVac concentrator (Savant Inst. Inc., Farmingdale, NY) or any other drying system, such as heat lamps, warm air, and so on, will suffice.
3. Carrier free ^{125}I (*see* Note 1).
4. 1,3,4,6-tetrachloro-3α,6α-glycouril (Iodogen). Iodogen-tubes are prepared by placing 10 µL of chloroform containing 1 mg/mL Iodogen in the bottom of 1.5-mL polypropylene microfuge tubes and allowing to air dry. Iodogen tubes can be stored at –20°C for up to 6 mo.
5. Phosphate buffered saline, pH 7.4 (any dilute, neutral buffer should work).
6. Dowex 1-X-8, 20–50 mesh, anion exchange resin (#451421, Bio-Rad).
7. Twenty-four well disposable microtiter plate.
8. Sephadex G-25 or G-50 (Pharmacia).
9. 15% Methanol in H_2O.
10. XAR-5 film (Kodak, Rochester, NY) or equivalent.
11. Lightening Plus intensifying screens (DuPont, Wilmington, DE) or equivalent.

2.4. Protein Cleavage Reagents and Buffers (Table 1)

1. Cleavage reagents (*see* Table 1).
2. 88% or 70% formic acid.
3. 50 m*M* Ammonium bicarbonate adjusted to the appropriate pH with sodium hydroxide.
4. 50% Glacial acetic acid in H_2O.
5. Glacial acetic acid added to H_2O to bring pH to 3.0.

2.5. Peptide Separation

2.5.1. SDS-PAGE

1. *See* Section 2.1. for required SDS-PAGE reagents.
2. Enzyme buffer for Cleveland, et al. *(2)* "in-gel" digestion: 1% SDS, 1 m*M* EDTA, 1% glycerol, 0.1*M* Tris-HCl, pH 6.8.

Table 1
Cleavage Reagents (*see also* Chapters 32 and 33)

Reagent	Site of cleavage	Buffer
Chemical		
Cyanogen bromide[a,d]	carboxy side of Met	70% formate
BNPS-skatole[a] (-bromo-methyl-2-(nitrophenylmercapto)-3H-indole)	carboxy side of Trp	50% H_2O-50% glacial acetic acid
Formic acid	between Asp and Pro	88% in H_2O
Chloramine T[b]	carboxy side of Trp	H_2O
Enzymatic[c]		
α-chymotrypsin	carboxy side of Tyr, Trp, Phe, Leu	50 m*M* ammonium bicarbonate, pH 8.5
Pepsin A	amino side of Phe>Leu	Acetate-H_2O, pH 3.0
Thermolysin	carboxy side of Leu>Phe	50 m*M* ammonium bicarbonate, pH 7.85
Trypsin	carboxy side of Arg, Lys	pH 8 5
V8 protease	carboxy side of Glu, Asp	50 m*M* ammonium bicarbonate, pH 8.5
V8 protease	carboxy side of Glu	pH 6–7 (H_2O)

[a]Cyanogen bromide and BNPS-skatole are used at 1 mg/mL in 70% formate. Room temperature incubation should proceed for 24–48 h under nitrogen in the dark.

[b]Chloramine T is used at 10 mg/mL in H_2O

[c]All enzymes are used at 1 mg/mL in the appropriate buffer.

[d]**CNBr is extremely toxic—handle with care in chemical hood.**

2.5.2. 2D TLE-TLC

1. Forma 2095 refrigerated cooling bath (Forma Scientific, Marietta, OH) or equivalent.
2. Immersion TLE chamber (e.g., Savant TLE 20 electrophoresis chamber, or equivalent).
3. 1200 V power pack.
4. Chromatography chambers.
5. "Varsol" (EC 123, Savant) or equivalent.
6. 0.1-mm Mylar-backed cellulose sheets (E. Merck, MCB Reagents, Gibbstown, NJ) or equivalent.
7. TLE buffer: 2 L H_2O, 100 mL glacial acetic acid, 10 mL pyridine.
8. TLC buffer: 260 mL *n*-butanol, 200 mL pyridine, 160 mL H_2O, 40 mL glacial acetic acid.

9. H_2O containing Tyr, Ile, and Asp (1 mg/mL). These are 2D TLC-TLE amino acid markers.
10. 1% Methyl green.
11. Laboratory sprayer.
12. 0.25% Ninhydrin in acetone.
13. XAR-5 film (Kodak, Rochester, NY) or equivalent.
14. Lightening Plus intensifying screens (DuPont, Wilmington, DE) or equivalent.

2.5.3. High-Performance Liquid Chromatography

1. HPLC capable of generating binary gradients.
2. *Preferred:* In-line UV detector and in-line gamma radiation detector (e.g., Model 170, Beckman, or equivalent). *Alternate:* Manual UV and gamma radiation detectors.
3. Fraction collector.
4. Computing integrator or strip chart recorder.
5. Reverse phase C_{18} column (P/N 27324 S/N, Millipore, or equivalent).
6. 0.005% Trifluoroacetic acid.
7. HPLC grade methanol.
8. H_2O containing Phe, Trp, and Tyr (1 mg/mL). These are HPLC amino acid markers.

3. Methods

3.1. Protein Purification

Any protein purification procedure that results in 95–100% purity is suitable for peptide mapping. For analytical purposes, the discontinuous buffer SDS-PAGE procedure is the best choice *(3,4)* (*see also* Chapter 5). The advantages of SDS-PAGE are: Its exceptional resolving power generally can bring proteins to adequate purity in one separation, whereas a second SDS-PAGE separation almost always provides the required purity for even the most difficult proteins; the simple reliability of the procedure; both soluble and insoluble proteins can be purified, either before or after radiolabeling; apparent mol-mass information, and the ability to probe SDS-PAGE-separated proteins by immunoblotting, help insure that the proper proteins are being studied.

Proteins separated in SDS-PAGE gels can be labeled and cleaved directly in gel slices *(5–7)*. However, labeling and cleavage are *much* more efficient if the proteins are first electroblotted to nitrocellulose

paper (NCP) *(8)*. Proteins can also be intrinsically or extrinsically labeled before SDS-PAGE separation *(9)*. If peptides are to be separated in SDS-PAGE gels (*see* Section 3.1.1. and 3.1.2.) enzymatic cleavage can be performed in the stacking gel with the resultant peptides being directly separated in the gel *(2)*. It is strongly recommended that even highly pure proteins be separated in SDS-PAGE gels and transferred to NCP because of the ease of labeling and cleavage using this system. Blotted proteins can be readily located by staining with Ponceau S in water (preferred), naphthol blue black (NBB) in water, or India ink-0.05% Tween 20-PBS *(10)*. Proteins of interest can then be excised, labeled using ^{125}I and cleaved directly on the NCP *(8)*. The peptides are released into the supernatant and can then be separated by SDS-PAGE (relatively low resolution of peptides but allows for epitope mapping following electroblotting) *(2,3,8)*, by two-dimensional thin-layer electrophoresis allowed by thin-layer chromatography (2D TLE-TLC) *(8)* (very high resolution), or by high-performance liquid chromatography (HPLC) *(8,11–13)* (intermediate resolution but allows for recovery of peptides). Peptides can be visualized by Coomassie brilliant blue (CBB) *(4)* or silver staining *(14)* (for SDS-PAGE, *see* Chapter 15), if sufficient protein is available and the cleavage reagent does not interfere with interpretation, autoradiography *(3,7–9)* (for SDS-PAGE gels and 2D TLE-TLC), gamma radiation emission *(3,7–9)* (for HPLC), or ultraviolet absorption (UV) (for HPLC).

A single SDS-PAGE separation is usually adequate to purify proteins for peptide mapping. Occasionally, a second separation may be required. If ^{125}I-labeling is used, a single protein and from a single lane of a 24-tooth comb is ample material for numerous separations of peptide fragments. Again, labeling and cleavage are greatly facilitated by electroblotting the protein to NCP.

3.1.1. Single SDS-PAGE Separation

1. Samples to be compared can be separated in individual lanes of an SDS-PAGE gel or "preparative" gels, where each sample is loaded over the entire stacking gel *(8)*, may be used.
2. After electrophoresis, fixation, CBB staining, and destaining (*see* Chapter 5), excise the protein bands of interest for use in the "gel slice" methods described below. The preferred method is to electroblot the protein to NCP, at 20 V constant current, 0.6 A for 16 h in degassed 20 m*M* phosphate buffer, pH 8.0 *(15)* (*see also* Chapter 24).

3. To stain the proteins on NCP; shake the NCP in Ponceau S for 15 min, destain with H_2O; or shake in 0.1% NBB in H_2O for 1 h, then destain with H_2O. If the proteins cannot be located by using these stains, place the NCP in 100 mL of 0.05% Tween 20-PBS and mix for 1 h, then add three drops of India ink and mix for another hour. Protein bands will be black, the background white (*see* Note 2).
4. Excise the protein band from the NCP (a 1 × 5 mm band is more than ample) and place excised strip in a 1.5-mL microfuge tube. Wash with H_2O until no stain is released into the supernatant. The protein is now ready for labeling and cleavage (*see* Note 3).

3.1.2. Double SDS-PAGE Separation

1. Separate the samples in individual lanes of an SDS-PAGE gel or in "preparative" SDS-PAGE gels. Fix, stain with CBB, then destain (*see* Chapter 5).
2. Excise the protein bands of interest. Soak the bands in 50% ethanol-50% stacking buffer (1*M* Tris-HCl, pH 6.8) for 0.5 h to shrink the gel strip to facilitate loading onto a second SDS-PAGE gel.
3. Push the excised band into contact with the stacking gel of a second SDS-PAGE gel of a different acrylamide concentration (generally use high concentration in the first gel and lower concentration in the second gel).
4. Separate proteins in second gel (CBB runs just behind the dye front). Stain or electroblot the proteins as above (*see* Section 3.1.1.). The protein is now ready for labeling and cleavage.

3.2. Protein Labeling (see also *Chapters 10 and 45*)

Microgram amounts of peptide fragments can be visualized by in-gel staining (SDS-PAGE), the ninhydrin reaction (2D TLE-TLC), or UV absorbance (HPLC), whereas radiolabeling allows visualization of pico- to nanogram quantities. Proteins can be intrinsically labeled by growing organisms in the presence of a uniform mixture of ^{14}C-amino acids *(9)* (*see* Chapter 10), but this is quite expensive. Intrinsic labeling with individual amino acids, such as ^{35}S-Met or ^{35}S-Cys, will not work since many peptide fragments will not be labeled. Iodination with ^{125}I is inexpensive and reproducible. Iodinated peptides are readily visualized by autoradiography or gamma emission detection *(3,7,8)*. Comparative cleavages of a 40,000 Dalton protein intrinsically labeled with ^{14}C-amino acids extrinsically labeled with ^{125}I showed that 61 of 66 α-chymotryptic peptides were labeled

with ^{125}I, whereas all twenty-two *Staphylococcus aureus* V8 protease (V8 protease)-generated peptides were labeled with ^{125}I, demonstrating the effectiveness of radioiodination.

Iodination mediated by chloramine-T (CT) *(16)* produces extremely high specific activities, but the procedure requires and extra step to remove the CT and can cleave some proteins at tryptophan residues *(17)*. This can be beneficial since it is specific and increases the number of peptides, thus increasing the sensitivity of the procedure (*see* ref. *18* for peptide maps of CT- vs Iodogen-labeled proteins). Unfortunately, small peptides generated by CT cleavage, followed by a second enzymatic or chemical cleavage, can be lost during the removal of the CT and unbound ^{125}I. In addition, CT-labeled peptides yield "noisy" HPLC separations with many small peaks clustering around major peaks *(11)*.

The 1,3,4,6-tetrachloro-3α,6α-glycouril (Iodogen) *(19)* procedure, where the oxidizing agent is bound to the reaction vessel, does not damage the protein and produces high specific activities. Aspiration of the reaction mixture stops the iodination and separates the protein from the oxidant in a single step. For these reasons, iodogen-mediated labeling is the preferred method for radioiodination. (Radioemission of ^{125}I will be expressed as counts per minute [cpm]. This assumes a detector efficiency of 70%. If detector efficiency varies, multiply the cpm presented here by 1.43 to determine decays per minute [dpm], then multiply the dpm by the efficiency of your detector).

3.2.1. NCP Strip (Preferred Method)

1. Put the protein-containing NCP strip in an Iodogen-coated (10 µg) microfuge tube.
2. Add 50–100 µL phosphate buffered saline, pH 7.4 (any dilute, neutral buffer should work) and 50–100 µCi ^{125}I (as NaI, carrier free, 25 µCi/µL) (*see* Notes 1 and 4).
3. Incubate at room temperature for 1 h. Aspirate the supernatant. **Caution: supernatant is radioactive.**
4. Place the NCP strip in a fresh microfuge tube and wash 3–5 times with 1.5 mL H_2O (radioactivity released should stabilize at <10,000 cpm/wash).
5. The protein on the NCP strip is now ready for cleavage (*see* Note 5).

3.2.2. Gel Slice

1. Dry the gel slice containing protein using a SpeedVac concentrator or other drying system, such as heat lamps, warm air, and so on.
2. Put the slice in an Iodogen-coated (10 µg) microfuge tube.
3. Add 100 µL of phosphate buffered saline, pH 7.4 (any dilute, neutral buffer should work) plus 50–100 µCi ^{125}I (as NaI, carrier free, 25 µCi/µL) (*see* Notes 1 and 4).
4. Incubate at room temperature for 1 h. Aspirate the supernatant. **Caution: Supernatant is radioactive.**
5. Remove the gel slice and soak for 0.5–1 h in 1.5 mL of H_2O. Repeat three times.
6. Place 0.5 g Dowex 1-X-8, 20–50 mesh, anion exchange resin and 1.5 mL of 15% methanol in H_2O in the wells of a 24-well microtiter plate.
7. Add the iodinated gel slice to a well with anion exchange resin and incubate at room temperature for 16 h. The resin binds unreacted iodine, becoming *extremely* radioactive.
8. Remove the gel slice from the resin and soak it in 1.5 mL of H_2O. Repeat several times and dry the gel slice. The protein is now ready for cleavage (*see* Note 6).

3.2.3. Lyophilized/Soluble Protein

1. Suspend up to 1 mg/mL of protein in 100–200 µL of phosphate buffered saline, pH 7.4 (any dilute, neutral buffer should work) 1.5-mL microfuge tube containing 10 µg Iodogen (*see* Note 4).
2. Add 100–200 µCi ^{125}I (as NaI, carrier free, 25 µCi/µL) (*see* Note 1).
3. Incubate at room temperature or on ice for 1 h.
4. Remove the protein-containing supernatant and separate the protein from salts and unbound iodine by:
   a. *Preferred method:* Separation on a Sephadex G-25 or G-50 desalting column using H_2O as the elution buffer, and lyophilize.
   b. *Relatively easy method:* Solubilize the sample in 2X sample buffer (10–20 µg/lane) and separate in an SDS-PAGE gel. Stop the electrophoresis before the ion-front reaches the bottom of the gel and cut the gel just above the dye front. Unbound iodine will be in this portion of the gel. Either fix, stain, and destain the gel to locate the protein band or electroblot on to NCP and locate the protein by Ponceau S, NBB, or India ink staining (*see* Section 3.1.1., step 3). Excise the protein band from the gel or NCP.

c. *Excellent if available method:* Separate the protein using reverse phase or molecular exclusion HPLC columns, then dialyze and lyophilize.
d. *Least preferred method:* Dialysis, followed by lyophilization, can be used but it produces excessive radioactive liquid waste.

5. The protein is now ready for cleavage.

3.3. Protein Cleavage

The use of cleavage reagents (e.g., α-chymotrypsin), or combinations of cleavage reagents, which generate many fragments tends to accentuate differences in primary structure, whereas cleavage reagents, which produce small numbers of fragments (e.g., V8 protease, thermolysin, CNBr, BNPS-skatole), emphasize similarities in primary structure.

Enzymatic reagents are often easiest to use, safest, and most reliable but they can interfere with results since they are themselves proteins. Chemical reagents are also easy to use and reliable but can be toxic, requiring careful handling (*see* Note 7). Several practical cleavage represented in Table 1.

Volatile buffers are best for peptide mapping, especially when using 2D TLE-TLC peptide separation, which is negatively affected by salts. For formate and CNBr cleavages, the acid can be diluted in H_2O to 88 or 70%, respectively. BNPS-skatole works well in 50% glacial acetic acid-50% H_2O. 50 m*M* ammonium bicarbonate, adjusted to the appropriate pH with sodium hydroxide, is excellent for enzymes requiring weak base environments (trypsin, α-chymotrypsin, thermolysin, V8 protease). The acid peptidase, Pepsin A, is active in H_2O adjusted to pH 3 with glacial acetic acid. (*See* Section 3.4. and 3.4.1. for cleavage of peptides to be separated in SDS-PAGE gels.)

3.3.1. Protein on NCP Strip

1. Put the NCP strip containing the radiolabeled protein in a 1.5-mL microfuge tube and measure the radioemission using a gamma radiation detector.
2. Add 90 µL of the appropriate buffer and 10 µL of chemical or enzymatic cleavage reagent in buffer (1 mg/mL) to the NCP strip.
3. Incubate with shaking at 37°C for 4 h (for enzymes) or at room temperature for 24–48 h in dark under nitrogen (for chemical reagents).
4. Aspirate the peptide-containing supernatant and count the NCP strip and supernatant. Enzymes should release 60–70% of counts in slice

into the supernatant; CNBr should release >80%, BNPS-skatole rarely releases more than 50% (*see* Notes 8 and 9).
5. Completely dry-down the supernatant in a SpeedVac and wash the sample at least four times by adding 50 µL of H_2O, vortexing, and redrying in a SpeedVac. Alternate drying systems will work.
6. The sample is now ready for peptide separation.

3.3.2. Gel Slice

1. Put the dry gel slice containing the radiolabeled protein in a 1.5-mL microfuge tube and measure the radioemission using a gamma radiation detector.
2. Add 10 µL of cleavage reagent in buffer (1 mg/mL) directly to the dry gel slice. Allow slice to absorb cleavage reagent, then add 90 µL of appropriate buffer.
3. Continue as from step 3, Section 3.3.1. Release of peptides into the supernatant will be less efficient than with the NCP strip.
4. The sample is now ready for peptide separation.

3.3.3. Lyophilized / Soluble Proteins

1. Rehydrate the lyophilized radiolabeled proteins in the appropriate buffer at 1 mg/mL (less concentrated samples can be used successfully).
2. Add up to 25 µL of the appropriate cleavage reagent (1 mg/mL) to 25 µL of suspended protein. If SDS-PAGE is to be used to separate the peptides, use as little enzyme as possible (1:50 enzyme to sample maximum).
3. Continue as from step 3, Section 3.3.1., except there is no strip to count.
4. The sample is now ready for peptide separation. *Be aware that the sample will usually contain uncleaved protein along with the peptide fragments.*

3.4. Peptide Separation

3.4.1. One-Dimensional Peptide Mapping by SDS-PAGE

Moderate separation of peptides can accomplished using this procedure. The technique, first described Cleveland et al. *(2),* is relatively easy, requires no special equipment, and can be combined with Western blotting to locate epitopes (e.g., epitope mapping) *(2,3,8).* Cleavages can be performed prior to loading gel *(preferred)* or they can be accomplished in the stacking gel with the resultant peptides separated directly in the separating gel. Several lanes, with increasing incubation time or increasing concentration of enzyme in each lane, should be run to determine optimal proteolysis conditions. Standard Laemmli SDS-PAGE *(4)* system is able to resolve peptide frag-

ments >3000 daltons *(8)*. Smaller peptides are best separated in the tricine gel system of Schagger and von Jagow *(20)*. Any of the methods described in Section 3.3. can be used to generate peptides.

3.4.1.1. Lyophilized/Soluble Protein

1. Boil the purified protein (~1 mg/mL) for 5 min in SDS-PAGE solubilizing buffer, Section 2.1.2 (*see* Note 10).
2. Place 10–30 µL (10–30 µg) of protein in 1.5-mL microfuge tubes and add the enzyme of choice.
3. Either incubate replicates of each sample at 37°C for increasing times (e.g., 0.25, 0.5, 1.0, and 2 h) or vary the enzyme concentration (from 0.005–0.5 µg/mL) of each sample and incubate for a constant digestion period (e.g., 30 min) (*see* Note 11). Stop the reactions by boiling for 5 min.
4. Add equal volume of 2X solubilizing buffer and boil for 10 min (*see* Note 12).
5. Load 10–30 µL (10–30 µg) in each well of a gel. For peptides greater than 3000 daltons, a 15% (1.33% *N,N*-methylene *bis*-acrylamide crosslinker) Laemmli acrylamide gel can be used, whereas a 22.5% (2.66% crosslinker) acrylamide gel will separate peptides of separate peptides as small as 2000 daltons. Peptides as small as 500 daltons, and peptides to be sequenced, can be separated in tricine gels *(20)* (*see also* Chapter 8). If enzyme cleavage has been used to generate the peptide fragments, be sure to run enzyme controls to distinguish protein fragments from enzyme fragments.
6. Run the gel, fix, stain, destain, or blot to NCP for immunoanalysis (*see* Note 13).

Solubilized proteins can also be loaded directly into wells (10–30 µL), overlaid with enzyme solution, and digested in-gel as described below for proteins in gel slices or on NCP strips.

3.4.1.2. Protein in Gel-Slice or on NCP Strip

For best results, radiolabeled proteins should be used; proteins in gel slices or on NCP will retain adequate SDS to migrate into second gel without further treatment.

1. Soak the gel slices containing labeled protein in 1*M* Tris-HCl, pH 6.8 for 30 min to shrink the gel, making loading on the second gel easier. Place the gel slices into the wells of a second gel. NCP strips can be cut to fit the wells and pushed to the bottom of the wells (*see* Note 14).

2. Overlay the gel slices or NCP strip with enzyme solution (1 mg/mL, add 1, 5, 10, 20 µL to separate wells to establish conditions yielding maximal digestion) in 0.1% SDS, 1 m*M* EDTA, 1% glycerol in 0.1*M* Tris-HCl, pH 6.8. V8 protease (endoproteinase glu-C) works very well in this system. SDS does hinder the activity of trypsin, α-chymotrypsin, and themolysin, so cleavage with these enzymes may be very slow. In-gel cleavage with chemical reagents is not recommended since they are inefficient in neutral, oxygenated environments.
3. Subject the sample to electrophoresis until the dye reaches the bottom of the *stacking* gel. Turn off the power and incubate for 2 h at 37°C. Following incubation, continue electrophoresis until dye reaches the bottom of the gel, fix, stain, and destain, or electroblot onto NCP for immunoanalysis (*see* Note 13).

Once the separation conditions, protein concentrations, and enzyme concentrations have been established, a single digestion lane for each sample can be used for comparative purposes.

Figure 1 is presented to demonstrate the separation of peptides generated by cleavage with BNPS-skatole in an SDS-PAGE gel. These peptide maps indicate that the porin protein (POR) protein is structurally unrelated to the 44 kDa proteins, whereas the 44 kDa proteins from the a sarkosyl insoluble (membrane) extract (44 kDa Mem.) and a periplasmic extract (44 kDa Peri.) appear to have similar primary structures.

3.4.2. Two-Dimensional Thin-Layer Electrophoresis-Thin-Layer Chromatography (2D TLE-TLC)

This is the best method for primary structural comparisons. It is strongly recommended that iodinated samples be used. Peptide maps can be overlaid to facilitate comparisons. Flat-bed electrophoresis can be used, but systems that cool by immersion of the thin-layer sheet in an inert coolant such as "varsol" (such as the Savant TLE 20 electrophoresis chamber, or equivalent) yield superior results. Cooling should be supplied by as large a refrigerated bath as possible, such as the Forma 2095 refrigerated cooling bath. Extra cooling coils, made by bending 1/4" aluminum tubing, are helpful. Peptides migrate based on charge, which is a function of pH, in an electric field. The buffer pH is a function of temperature, therefore, maintenance of the running buffer temperature is crucial. Inconsistent cooling results

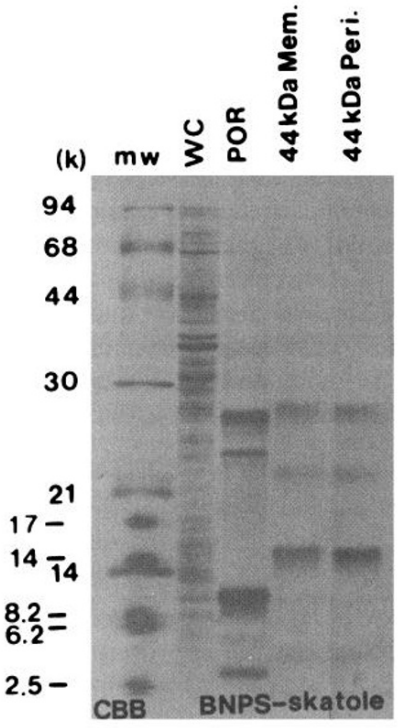

Fig. 1. Example of peptides separated in an SDS-PAGE gel. Whole cells (WC), a sarkosyl insoluble pellet, and a periplasmic extract of *Neisseria gonorrhoeae* were separated in "preparative" 15% SDS-PAGE gels and blotted to NCP as described in Section 3.1.1. The 37,000 Dalton major outer membrane protein (POR) and two 44,000 Dalton (44 kDa) proteins, one isolated from a sarkosyl insoluble (membrane) extract (44 kDa Mem.), the other isolated from a periplasmic extract (44 kDa Peri.) were located on the NCP by Ponceau S staining, excised, radioiodinated using the methods described in Section 3.2.1., and cleaved with BNPS-skatole as described in Section 3.3.1. Approximately 30 μg of peptides of each protein were solubilized and separated in an SDS-PAGE gel along with whole cells (WC), Bio-Rad low-mol-wt markers, and Pharmacia peptide mol-wt markers (mw) (expressed in thousands of daltons [k]). The gel was stained with Coomassie brilliant blue (CBB) to visualize peptides.

in inconsistent peptide migration. For best results, use only 0.1-mm Mylar-backed cellulose sheets of E. Merck. Run two or three peptide maps per 20 × 20 cm sheet. If necessary, increased resolution can be obtained by running one sample/sheet and increasing running times (*see* Note 15).

1. Set the cooling bath at 8.5°C to keep the electrophoresis tank at 10–13.5°C. The temperature of cooling tank should not increase more than 1.5°C during a run.
2. Rehydrate the peptide sample to 10^5 cpm/μL in H_2O containing Tyr, Ile, and Asp (1 mg/mL) as amino acid markers.
3. For two samples/run: Draw a line down the center of the back of the sheet with a laboratory marker parallel to the machine lines (they can be subtle, but always electrophoresis parallel to these lines). Mark two spots 2.5 cm from the end of the sheet and 1.5 cm from centerline on the back of the sheet to mark where to load samples. Marks will show through when you turn the sheet so the cellulose is facing up. For three samples/run: Draw two lines on the back of the sheet (parallel to machine lines) 6.7 and 13.4 cm from left edge of sheet and mark three spots, each 8 cm from the end of the plate and 1 cm to the right of the left edge and each line (*see* Note 16).
4. Use a graduated, 1–5 μL capillary pipet, to spot 2 μL (2×10^5 cpm) if two samples are used, or 1.5 μL (~1.5×10^5 cpm) if three samples are used, 0.5 μL at a time (dry spot with hair dryer each time) to one mark on the sheet. Repeat for each sample on the other mark(s). To verify proper electrophoresis, spot 1 μL of 1% methyl green on the centerline. The methyl green should migrate rapidly toward the cathode in a *straight line*. Veering indicates a problem.
5. Spray the plate with TLE buffer using a laboratory sprayer. Do not overwet. Remove any standing buffer with one paper towel. Always blot TLE plates in exactly the same manner.
6. Place in the electrophoresis chamber with the samples toward the anode. Run the electrophoresis at 1200 V (about 20 W and 20 mA) for 45 min (two samples/run) or 31 min (three samples/run).
7. Remove the sheet from the chamber and immediately dry with a hair dryer. The "varsol" will dry first, then the buffer. Cut the sheet along the lines on the back of the sheet. Score the cellulose 0.5 cm down from top edge of each piece (bottom is the edge closest to the sample) to form a moat.
8. Place the sheets in chromatography chamber so that chromatography can proceed perpendicularly to the electrophoresis. The TLC buffer should be about 0.5 cm deep. Chromatograph until the buffer reaches the moat. Remove and dry with a hair dryer (best done in hood).
9. Spray the sheet with 0.25% ninhydrin in acetone *(do not saturate)* and dry with a hair dryer to locate amino acid markers. Ninhydrin can also be used to locate peptides if larger amounts of sample are separated (10–100 μg). Be sure to run enzyme controls to distinguish sample from enzyme. Markers should migrate identically in all separations.

10. Overlay the sheets with X-ray film, place Lightening Plus intensifying screen over film, and place in cassette. Expose for 16–24 h at –70°C or expose film without a screen for about 4 d at room temperature. More sample can be run but resolution will decrease. Develop the film (*see* Note 17).

Figure 2 is presented to demonstrate the separation of peptides generated by cleavage with trypin by 2D-TLE-TLC. These peptide maps again indicate that the POR protein is structurally unrelated to the 44 kDa proteins, whereas the 44 kDa protein from the sarkosyl insoluble (membrane) extract (44 kDa Mem.) is structurally indistinguishable from the 44 kDa protein from the periplasmic extract (44 kDa Peri.). Note the high resolution of the peptide fragments using this technique.

3.4.3. High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography separation of peptides can be used for comparisons but its main advantage is its ability to recover peptides *(8,11–13)* for further studies. If radioiodinated peptides are to be separated, the Iodogen method of radiolabeling should be used since CT-mediated labeling results in considerable "noise" in HPLC chromatograms. Most peptides can be separated by reverse phase chromatography using a C_{18} column and a linear gradient of H_2O–0.005% trifluoroacetic acid to 100% methanol *(8)*. Different gradients using these solvents, or other solvents such as acetonitrile-0.005% TFA *(11–13)*, isopropanol, and so on, may be needed to achieve adequate separation. An in-line gamma radiation detector (e.g., Beckman Model 170) is helpful but fractions can be collected and counted. Ten to 100 µg of sample should be used if UV absorbance (280_{nm}) is to be used to detect peptides. The sensitivity of UV detection roughly parallels that of CBB staining in gels. Precision of repeated separation should be ±0.005 min retention time for all peaks allowing for direct comparisons of elution profiles of different samples (*see* Note 18).

1. Rehydrate the peptides in about 0.1 mL of H_2O containing 1 mg/mL of Phe, Trp, and Tyr (internal amino acid markers to verify consistent separations).
2. Inject between 1.5×10^5 and 1×10^6 cpm/separation into H_2O–0.005% TFA mobile phase running at 1 mL/min. Excellent separation can be achieved using a 1 h linear gradient (0.05% TFA to 100% methanol).

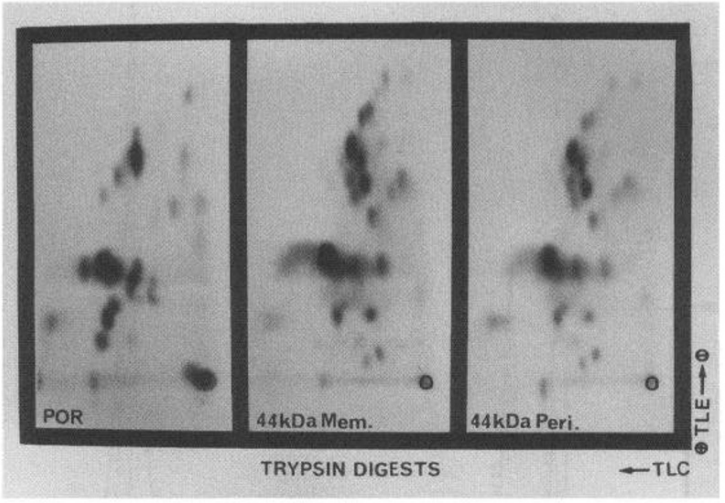

Fig. 2. Example of peptides separated by 2D TLE-TLC. The radiolabeled proteins described in Fig. 1 on NCP strips were cleaved with trypsin as described in Section 3.3.1. The peptides (1×10^5 cpm) were spotted on a thin-layer cellulose sheet and subjected to 2D TLE-TLC as described in Section 3.4.2. The origin (O) is at the lower right of each map. TLE—direction of thin-layer electrophoresis; TLC—direction of thin-layer chromatography. The ^{125}I-labeled peptides were visualized by autoradiography.

3. Monitor amino acid marker elution, or peptide elution if using UV absorbance, using an in-line UV detector at 280 nm. Monitor radiolabeled peptide elution using an in-line gamma radiation detector. Alternatively, fractions can be collected and the marker and peptide elution times monitored manually. Peaks can be collected, washed, dried, and reseparated by SDS-PAGE or 2D TLE-TLC.

Figure 3 is presented to demonstrate the separation of peptides generated by cleavage with themolysin by HPLC. These maps again indicate that the POR protein is structurally unrelated to the 44 kDa proteins, whereas the 44 kDa protein from the sarkosyl (membrane) extract (44 kDa Mem.) is structurally similar to the 44 kDa protein from the periplasmic extract (44 kDa Peri.). Note the somewhat lower resolution of the peptide fragments using this technique. Note also that there appear to be several peptides eluting in a single peak in the 30 min region of the chromatogram (a common problem). The gradient must be modified in this region to adequately resolve these peptides.

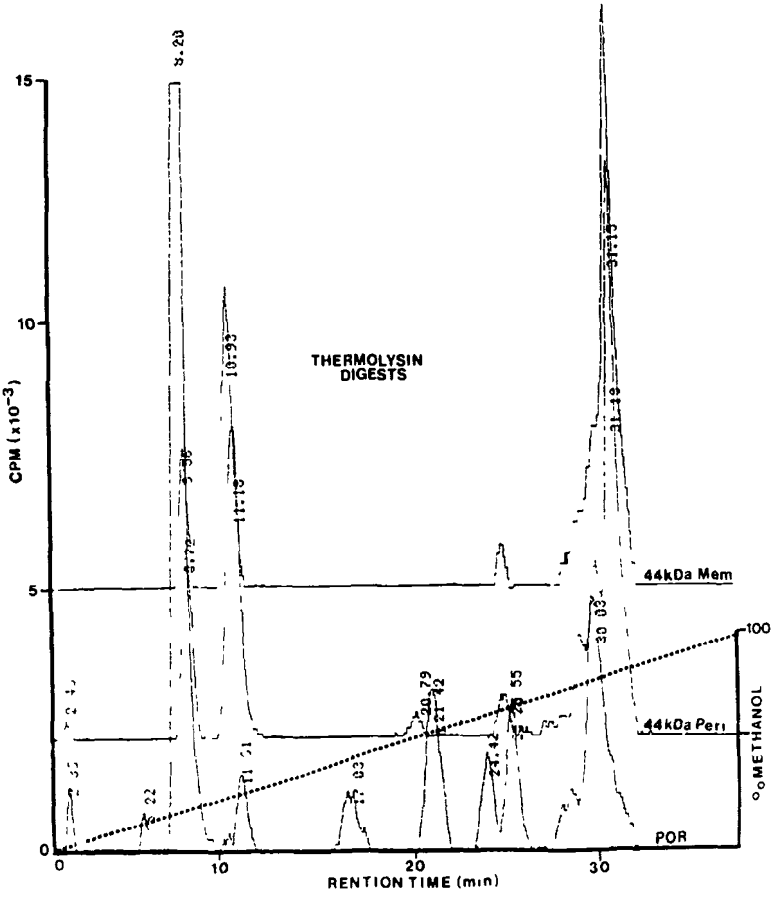

Fig. 3. Example of peptides separated by HPLC. The radiolabeled proteins described in Fig. 1 on NCP strips were cleaved with thermolysin as described in Section 3.3 1. The peptides (2×10^5 cpm) were injected into a mobile phase of 0.05% TFA-H_2O (flowrate of 1 mL/min) and separated over a 40-min linear gradient to 100% methanol as described in Section 3.4.3. The ^{125}-I-labeled peptides were visualized using an in-line gamma radiation detector linked to a computing integrator. Chromatograms were superimposed for comparison.

4. Notes

1. Regardless of the labeling procedure, never use ^{125}I that is over one half-life (60 d) old. Do not increase the amount of older ^{125}I to bring up the activity, it does not work.
2. Ponceau S can be *completely* removed with H_2O; NBB, or India ink cannot.

3. Do not compare proteins stained with Ponceau S with those stained with NBB or India ink. Use the same staining procedure for all proteins to be compared.
4. *Never* use carrier free ^{125}I in acid buffers. The iodine becomes volatile and could be inhaled.
5. It is common to have between 3×10^6 and 6×10^6 cpm for a strip 1×5 mm. This provides enough material to run 15–30 peptide maps/strip.
6. It is common to have between 2×10^6 and 4×10^6 cpm in a gel slice (1×5 mm). This provides enough material to run 10–20 peptide maps/slice.
7. Chemical cleavage reagents are preferred when peptides are not radiolabeled since enzymes cleave themselves resulting in confusing data. **CNBr is extremely toxic—handle with great care in a chemical hood.**
8. Repeated digestions will release about the same percentage of counts. Generally, one or two digestions are adequate. Only 1×10^5 cpm are necessary to produce a peptide map.
9. If >95% of counts are released in the first enzyme digestion, there may be excess, unbound iodine left in sample. This could cause serious problems as the enzyme may become labeled. The resultant peptide maps will all be identical maps of the enzyme and not your sample.
10. If the protein is rich in Cys, up to 10% β-mercaptoethanol may be added to the solubilizing buffer.
11. To insure complete digestion, it is advisable to incubate the samples for increasing periods of time or to digest with increasing amounts of enzyme. Once optimal conditions are established, a single incubation time and enzyme concentration can be used.
12. It is often advisable to solubilize protein in SDS prior to cleavage since some peptides do not bind SDS well.
13. PVDF-nylon membrane is preferable to NCP when blotting small peptides.
14. Between 5×10^4 cpm and 10^5 cpm should be loaded in each lane if autoradiography is to be used. Autoradiography should be performed on unfixed gels. Fixation and staining may wash out small peptides. Place gel in a plastic bag and overlay with XAR-5 film place in a cassette with a Lightening Plus intensifying screen, and expose for 16 h at –70°C
15. CNBr should not be used to generate peptides for use in 2D TLE-TLC since it produces very hydrophobic peptides which tend to compress at the top of the chromatogram. CNBr is excellent for generating peptides to be separated by SDS-PAGE or HPLC.

16. Always spot sample to be compared the same distance from the anode (positive) terminal since peptides migrate more rapidly close to the anode and more slowly far from the anode.
17. Migration of peptides should be consistent enough to directly overlay peptide maps for comparisons. Coordinates of amino acid markers and peptides can be determined and used to compare migration. The labeling procedures described here are precise enough to use emission intensities as a criterion for comparison.
18. CNBr and BNPS-skatole are excellent cleavage reagents to generate peptides to be separated by HPLC.

Acknowledgments

I thank the Public Health Service, NIH, NIAID (grants RO1 AI21236 and KO4 AI00834) and UM Research Program for their continued support.

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Chapter 24

Protein Blotting

G. Brian Wisdom

1. Introduction

Gel electrophoresis and electrofocusing have high resolving power but the separated proteins, particulary in polyacrylamide gels, are difficult to access by macromolecular reagents. Blotting proteins from the gel onto an adsorbent membrane overcame this problem *(1)*. This technique is sometimes called Western blotting (analogous to Southern and Northern blotting of nucleic acids) and the term Eastern blotting has been used for the blots formed from electrofocusing gels; however, protein blotting appears to be the most generally descriptive term.

The blotted proteins form a replica of the gel and they have been utilized in various ways *(2,3)*. Antibodies are very widely used to detect particular proteins (immunoblotting) usually with the aid of a second, enzyme-labeled antibody, or enzyme-labeled Protein A or Protein G (*see* Chapter 25). Biotin labels in combination with enzyme-labeled streptavidin are also frequently used (*see* Chapter 26). Other probes include lectins and nucleic acids. Most complementary binding reactions can be exploited but, for those requiring intact native conformation of the separated protein, the electrophoresis must be carried out under nondenaturing conditions (*see* Note 1). Blotted proteins can function as ligands in the affinity purification of small amounts of proteins such as antibodies, and can act as substrates for various protein-modifying reactions such as phosphorylation. In addition, portions of blots containing a protein of interest may be subjected to gas phase sequencing or be used as an immunogen either in vivo or in vitro.

From. *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
Edited by: J M Walker

There are several methods of moving proteins out of the electrophoresis gels. These are mainly electrophoretic and the protein is moved sideways onto the capturing membrane. This is either done in a tank of buffer containing the gel and membrane sandwiched between foam pads with the electrodes on either side *(1)* or in the so-called semidry mode where the gel and membrane are placed between buffer-impregnated sheets of filter paper with plate electrodes above and below *(4)*. The latter method is more rapid and generally more efficient in transferring proteins to the membrane; it also uses less buffer. Apparatus for both forms of electrophoretic transfer is available from numerous companies. Diffusion and capillary action may be employed; these are not efficient with polyacrylamide gels but work well with agarose gels that have larger pores. This approach is particularly useful when the agarose gels are cast on a plastic backing sheet such as GelBond. Movement of proteins from gels has also been effected by the application of a vacuum *(5)* but this is not widely used.

The most commonly used membrane for protein blotting is nitrocellulose; it has satisfactory capacity, gives low backgrounds but is fragile. This problem is overcome by the use of reinforced versions, which are commercially available. Polyvinylidene difluoride (PVDF) in increasingly used; this is tough, has a high capacity, gives low backgrounds, and is the membrane of choice when the blotted proteins are used for sequencing. The nature of the interaction between the proteins and these membranes is not understood in detail. It is thought to be predominantly hydrophobic and can be reversed by detergents. Other protein-immobilizing membranes include nylon and diazo-modified cellulose; they show very strong binding but have various disadvantages *(2)*.

Before probing blotted proteins with, for example, antibody, it is nearly always essential to block the unused binding sites on the membrane. This is done by incubating with a protein or protein mixture that is inert in the subsequent probing and detection reactions. Fat-free milk powder has proved to be an economical and effective blocker in most systems.

There are many different buffers and conditions used in transferring proteins from gels; the procedures given here are suitable for "average" proteins. It is important to check the efficieny of the blot-

ting in any new application and, where necessary, optimize the transfer conditions (*see* Note 2).

2. Materials

2.1. Semidry Electrophoretic Blotting

1. Transfer apparatus (available from several companies).
2. Power pack (preferably capable of delivering constant current).
3. Nitrocellulose membrane (0.2 μm pore size but others are usually adequate) or PVDF membrane, cut to the same size as the gel, NB. To recollect the correct orientation of the membrane with respect to the gel it is advisable to mark the membrane in some way, e.g., cut off the upper right corner.
4. Filter papers (e.g., Whatman no.1): 16 sheets (cut to the same size as the gel).
5. Transfer buffer: 25 m*M* Tris-192 m*M* glycine buffer, pH 8.3, containing 10% methanol (*see* Note 3).

2.2. Capillary Blotting

1. Cellulose acetate membrane (cut to the same size as the gel).
2. Membrane: as in Section 2.1.
3. Filter paper (e.g., Whatman no. 1): one sheet (cut to the same size as the gel).
4. Blotting paper or thick filter paper (e.g., Whatman 3MM): Six sheets (cut to the same size as the gel).
5. Glass plate.

2.3. Protein Staining

1. Ponceau S: 0.5% (w/v) in 1% acetic acid.
2. Amido black (for nitrocellulose membranes): 0.1% in 25% *iso*-propanol/10% acetic acid. Destain is 25% *iso*-propanol/10% acetic acid.
3. Coomassie brilliant blue R250 (for PVDF membranes): 0.1% in 1% acetic acid/40% methanol. Destain is 50% methanol.

2.4. Blocking

Either milk powder (fat-free), bovine serum albumin, or serum are dissolved (at 3–5%) in an appropriate buffer, e.g., 0.02*M* Tris-HCl buffer, pH 7.4, containing 0.15*M* NaCl (TBS) (*see* Note 4).

3. Methods

Evaluating the transfer of proteins can be done by staining the membrane, or part of it, for protein prior to blocking (*see* Note 2).

However, monitoring of the electrophoresis and transfer is facilitated by the inclusion of Pyronin Y, which adheres to the membranes, in the sample instead of bromophenol blue. The use of colored or prestained proteins as markers is valuable (e.g., prestained mol-wt standards from Bio-Rad and colored isoelectric point standards from BDH).

It is advisable to use gloves and forceps when handling the membranes to minimize contamination with extraneous proteins or other substances.

Good contact between the membrane and the gel is essential and air bubbles can be excluded from the interface and from the filter paper layers by using a glass rod or pipet as a roller.

3.1. Semidry Electrophoretic Blotting

The following provides a general procedure for the transfer of proteins from sodium dodecylsulfate (SDS)-containing polyacrylamide gels (1.5-mm thick). Graphite electrodes are used in most forms of semidry transfer apparatus; these should be rinsed thoroughly and saturated with water before use.

1. Soak eight sheets of filter paper in transfer buffer, drain them, and place them on the lower electrode (anode).
2. Rinse the gel briefly in transfer buffer then place it carefully on top of the stack of filter papers.
3. Prepare the membrane. *Nitrocellulose*: Float the membrane on transfer buffer until it is evenly wet and then submerge it. *PVDF*: Float the membrane on 100% methanol for a few seconds then submerge it in transfer buffer. Drain the membrane, and lay it on top of the gel, being careful to exclude air bubbles.
4. Soak the remaining eight sheets of filter paper in transfer buffer, drain them and place them on top of the membrane (*see* Note 5).
5. Place the upper electrode (cathode) firmly on top of the stack.
6. Connect to a power pack and apply constant current at a density of 0.8–1.0 mA/cm^2 for 0.5–2 h. (Cooling is not usually necessary under these conditions but, if required, a cooling cushion may be placed on top of the apparatus if the design permits it).
7. Disassemble the stack, remove the membrane, rinse it in TBS and store it in the same buffer at 4°C until required (*see* Note 6).

3.2. Capillary Blotting

This protocol is primarily for the transfer of proteins from agarose gels (1-mm thick) on a GelBond backing sheet. It is best carried out on the cooling plate of a horizontal electrophoresis apparatus (with

coolant circulating at 5–10°C); this minimizes the diffusion of proteins during blotting.

1. Blot the gel, on the cooling plate, briefly with a cellulose acetate membrane (soaked in TBS) to remove any surface protein.
2. Prepare the nitrocellulose or PVDF membrane and apply it to the gel as in Section 3.1., step 3.
3. Cover the membrane with a sheet of filter paper (soaked in TBS), six sheets of dry blotting paper (or thick filter paper), a glass plate and a 1 kg weight (for a 260 cm^2 gel).
4. After 1 h disassemble the stack, remove the membrane, rinse it in TBS and store it in the same buffer at 4°C until required (*see* Note 6).

3.3. Protein Staining

Staining and destaining are best done with constant orbital shaking.

3.3.1. Ponceau S

1. Rinse the membrane in water or TBS.
2. Stain for 2 min.
3. Destain in water for 2–3 min. Further destaining removes the dye.

3.3.2. Amido Black

1. Rinse the nitrocellulose membrane in water or TBS.
2. Stain for 1 min.
3. Destain in 25% *iso*-propanol/10% acetic acid for 30 min.
4. Wash in water.

3.3.3. Coomassie Brilliant Blue

1. Rinse the PVDF membrane in water or TBS.
2. Stain for 2 min.
3. Destain in 50% methanol for 30 min.
4. Wash in water.

3.4. Blocking

Individual membranes are incubated in the solution of the blocking protein (about 0.3 mL/cm^2 of membrane) at room temperature in a flat or, better, a ribbed-bottomed container with gentle shaking or rocking for 0.5–1 h (*see* Note 4). Once blocked, various detection systems such as those described in Chapters 25–28 may be used.

4. Notes

1. Some renaturation of proteins separated under denaturating conditions can occur during transfer and during blocking and, for example, blot-

ted enzymes may show some catalytic activity. There is rarely any problem in identifying proteins with polyclonal antiserums but most monoclonal antibodies will probably not recognize the (partially) unfolded structure. Renaturation has been reported to be increased by incubation with nonionic or zwitterionic detergents. In this context it is also worth examining the separation step to determine if reducing conditions, the standard concentrations of SDS and boiling of the sample are essential for the resolution of the proteins of interest.

2. Different conditions may be required for the effective transfer of some proteins especially if they are small, large, or basic. The pH of the buffer is particularly important in relation to the isoelectric points of the proteins under investigation. Transfer can be evaluated by staining the gel for residual protein after blotting and by placing a second membrane behind the first (to capture excess or small proteins) or on the other side of the gel (to capture proteins going the "wrong" way). The primary and secondary membranes can be stained for protein or probed with, for example, antibody. Large proteins (>100 kDa) are difficult to transfer in high yield from polyacrylamide gels and longer running times, higher currents, or other modifications are necessary (*see* Note 3).
3. Methanol is included in many transfer buffers. It prevents polyacrylamide gels swelling, removes SDS from polypeptides and enhances the binding of proteins to the membrane but it reduces the efficacy of transfer of larger proteins (>100 kDa). This problem can be minimized by the addition of 0.1% SDS to the transfer buffer.
4. Solutions containing nonionic detergents have also been used to block or quench binding sites, sometimes in addition to a protein blocker. There is evidence that detergents cause the removal of adsorbed proteins from membranes and they must be used with care. Tween 20 (polyoxyethylenesorbitan monolaurate) at low concentration, typically 0.05 or 0.1%, is effective in miminizing nonspecific binding in many systems. Prolonged incubations in either protein or detergent-containing blocking solutions should be avoided because of the danger of desorption of some of the blotted protein.
5. Several transfers may be carried out simultaneously by placing up to six individual stacks on top of each other with a dialysis membrane between each *(4)*.
6. In many cases dried membranes, stored between filter papers, have retained much of the blotted protein after long periods.

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CHAPTER 25

Detection of Polypeptides on Immunoblots Using Secondary Antibodies or Protein A

Nicholas J. Kruger

1. Introduction

Immunoblotting provides a simple and effective method for identifying specific antigens in a complex mixture of proteins. Initially, the constituent polypeptides are separated using SDS-polyacrylamide gel electrophoresis, or a similar technique, and then are transferred either electrophoretically or by diffusion onto a nitrocellulose filter. Once immobilized on a sheet of nitrocellulose, specific polypeptides can be identified using antibodies that bind to antigens retained on the filter and subsequent visualization of the resulting antibody–antigen complex. This chapter describes conditions suitable for binding antibodies to immobilized proteins and methods for locating these antibody–antigen complexes using appropriately labeled ligands. These methods are based on those of Blake et al. *(1)*, Burnette *(2)*, and Towbin et al. *(3)*.

Although there are several different techniques for visualizing antibodies bound to nitrocellulose, most exploit only two different types of ligand. One is Protein A, which is either radiolabeled or conjugated with a marker enzyme. The other ligand is an antibody raised against IgG from the species used to generate the primary antibody.

From *Methods in Molecular Biology, Vol 32 Basic Protein and Peptide Protocols*
Edited by J M Walker

Usually, this secondary antibody is either conjugated with a marker enzyme or linked to biotin. In the later instance, the biotinylated antibody is subsequently detected using avidin linked to a marker enzyme.

Detection systems based on Protein A are both convenient and sensitive. Protein A specifically binds the Fc region of immunoglobulin G (IgG) from many mammals *(4)*. Thus, this compound provides a general reagent for detecting antibodies from several sources. Using this ligand, as little as 0.1 ng of protein may be detected, although the precise amount will vary with the specific antibody titer *(5)*. The principal disadvantage of Protein A is that it fails to bind effectively to major IgG subclasses from several experimentally important sources, such as rat, goat, and sheep (*see* Table 1). For antibodies raised in such animals, a similar method using Protein G derivatives may be suitable (*see* Note 1). Alternatively, antibody bound to the nitrocellulose filter may be detected using a second antibody raised against IgG (or other class of immunoglobulin) from the species used to generate the primary antibody. The advantage of such secondary antibody systems is that they bind only to antibodies from an individual species. When combined with different marker enzymes, the specificity of secondary antibodies may be exploited to identify multiple polypeptides on a single nitrocellulose membrane *(4)*.

The marker enzymes most commonly used for detection are alkaline phosphatase and horseradish peroxidase. Both enzymes can be linked efficiently to other proteins, such as antibodies, Protein A, and avidin, without interfering with the function of the latter proteins or inactivating the enzyme. Moreover, a broad range of synthetic substrates has been developed for each of these enzymes. Enzyme activity is normally visualized by incubating the membrane with an appropriate chromogenic substrate that is converted to a colored, insoluble product. The latter precipitates onto the membrane in the area of enzyme activity, thus identifying the site of the antibody–antigen complex (*see* Note 2).

Both antigens and antisera can be screened efficiently using immunoblotting. Probing of a crude extract after fractionation by SDS-polyacrylamide gel electrophoresis indicates the specificity of an antiserum. The identity of the antigen can be confirmed using a complementary technique, such as immunoprecipitation of enzyme activity. This information is essential if the antibodies are to be used reliably. Once characterized, an antiserum may be used to identify

Table 1
Binding of Protein A to Immunoglobulins from Various Species

Species	Serum IgG level,[a] mg/mL	Affinity[b]		
		Strong	Weak	Unreactive
Rabbit	5	IgG		
Human	12	IgG_1[c],2,4 IgA_2 IgM (some)		IgG_3
Guinea pig	6	$IgG_{1,2}$[c]		
Mouse	7	$IgG_{2a,2b,3}$	IgG_1[c] IgM	
Pig	18	IgG IgM (some) IgA (some)		
Goat	–	IgG_2	IgG_1	
Sheep	–	IgG_2		IgG_1
Dog	9	IgG IgM (some) IgA (some)		
Rat	16	$IgG_{1,2c}$	IgG_{2b}	IgG_{2a}
Cow	20	IgG_2		IgG_1

[a]The values for serum IgG levels are approximate, and significant variation may occur among individuals

[b]Immunoglobulins failing to bind protein A at pH 8.0 are described as unreactive. Immunoglobulins that bind protein A at pH 8.0, but not at pH 6.0 are considered to have a weak affinity. High affinity indicates binding below pH 6.0. Data from Tijssen *(4)* and references therein.

[c]Denotes the major IgG subclass.

antigenically related proteins in other extracts using the same technique. Examples of the potential of immunoblotting have been described by Towbin and Gordon *(6)*.

2. Materials

1. Electrophoretic blotting system, such as Trans-Blot, supplied by Bio-Rad.
2. Nitrocellulose paper: 0.45-µm pore size.
3. Protein A derivative.
   a. Alkaline phosphatase-conjugated Protein A obtained from Sigma Chemical Co.: Dissolve 0.1 mg in 1 mL of 50% (v/v) glycerol in water. Store at –20°C.

b. Horseradish peroxidase-conjugated Protein A obtained from Sigma Chemical Co.: Dissolve 0.1 mg in 1 mL of 50% (v/v) glycerol in water. Store at –20°C.

c. ^{125}I-labeled Protein A, SA 30 mCi/mg. Affinity-purified Protein A, suitable for blotting, is available commercially (*see* Note 3). **^{125}I emits γ-radiation. Check the procedures for safe handling and disposal of this radioisotope.**

4. Secondary antibody: A wide range of both alkaline phosphatase- and horseradish peroxidase-conjugated antibodies is available commercially. These antibodies are usually supplied as an aqueous solution containing protein stabilizers. The solution should be stored under the conditions recommended by the supplier. Ensure that the enzyme-linked antibody is against IgG of the species in which the primary antibody was raised.

5. Washing solutions: Phosphate-buffered saline (PBS): Make 2 L containing 10 m*M* NaH_2PO_4, 150 m*M* NaCl adjusted to pH 7.2 using NaOH. This solution is stable and may be stored at 4°C. It is susceptible to microbial contamination, however, and is usually made as required.

   The other washing solutions are made by dissolving the appropriate weight of bovine serum albumin or Triton X-100 in PBS. Dissolve bovine serum albumin by rocking the mixture gently in a large, sealed bottle to avoid excessive foaming. The "blocking" and "antibody" solutions containing 8% albumin may be stored at –20°C and reused several times. Microbial contamination can be limited by filter-sterilizing these solutions after use or by adding 0.05% (w/v) NaN_3 (but *see* Note 4). Other solutions are made as required and discarded after use.

6. Alkaline phosphatase substrate mixture:

   a. Diethanolamine buffer. Make up 100 m*M* diethanolamine and adjust to pH 9.8 using HCl. This buffer is usually made up as required, but may be stored at 4°C if care is taken to avoid microbial contamination.

   b. 1*M* $MgCl_2$. This can be stored at 4°C.

   Combine 200 µL of 1*M* $MgCl_2$, 5 mg nitroblue tetrazolium, and 2.5 mg 5-bromo-4-chloroindolyl phosphate (disodium salt—*see* Note 5). Adjust the volume to 50 mL using 100 m*M* diethanolamine buffer. Make up this reaction mixture as required and protect from the light before use.

7. Horseradish peroxidase substrate mixture.

   a. Make up 50 m*M* acetic acid and adjust to pH 5.0 using NaOH. This buffer is usually made up as required, but may be stored at 4°C if care is taken to avoid microbial contamination.

b. Diaminobenzidine stock solution of 1 mg/mL dissolved in acetone. Store in the dark at –20°C. **Caution: Diaminobenzidine is potentially carcinogenic; handle with care.**
c. Hydrogen peroxide at a concentration of 30% (v/v). This compound decomposes, even when stored at 4°C. The precise concentration of the stock solution can be determined by measuring its absorbance at 240 nm. The molar extinction coefficient for H_2O_2 is $43.6M^{-1} \cdot cm^{-1}$ at this wavelength (*see* Note 6).

Combine 50 mL of acetate buffer, 2 mL of diaminobenzidine stock solution, and 30 µL of hydrogen peroxide immediately before use. Mix gently and avoid vigorous shaking to prevent unwanted oxidation of the substrate. Protect the solution from the light.

8. Protein staining solutions: These are stable at room temperature for several weeks and may be reused.
   a. Amido black stain (100 mL): 0.1% (w/v) Amido black in 25% (v/v) propan-2-ol, 10% (v/v) acetic acid.
   b. Ponceau S stain (100 mL): 0.2% (w/v) Ponceau S, 10% (w/v) acetic acid.
   c. Ponceau S destain (400 mL): distilled water.

3. Methods

3.1. Immunodetection of Polypeptides

1. Following SDS-polyacrylamide gel electrophoresis (*see* Chapter 5), electroblot the polypeptides from the gel onto nitrocellulose at 50 V for 3 h using a Bio-Rad Trans-Blot apparatus, or at 100 V for 1 h using a Bio-Rad Mini Trans-Blot system (*see* Chapter 24).
2. After blotting, transfer the nitrocellulose filters individually to plastic trays for the subsequent incubations. Ensure that the nitrocellulose surface that was closest to the gel is uppermost. Do not allow the filter to dry out, since this often increases nonspecific binding and results in heavy, uneven backgrounds. The nitrocellulose filter should be handled sparingly to prevent contamination by grease or foreign proteins. Always wear disposable plastic gloves, and only touch the sides of the filter.
3. If desired, stain the blot for total protein using Ponceau S as described in Section 3.3.2. (*see* Note 7).
4. Rinse the nitrocellulose briefly with 100 mL of PBS. Then incubate the blot at room temperature with the following solutions, shaking gently (*see* Note 8).

a. 50 mL of PBS/8% bovine serum albumin for 30 min. This blocks the remaining protein-binding sites on the nitrocellulose.
b. 50 mL of PBS/8% bovine serum albumin containing 50–500 µL of antiserum for 2–16 h (*see* Note 9).
c. Wash the nitrocellulose at least five times, each time using 100 mL of PBS for 15 min, to remove unbound antibodies.
d. 50 mL of PBS/4% bovine serum albumin containing an appropriate ligand for 2 h (*see* Note 10). This likely to be one of the following:
   1. 1 µCi ^{125}I-labeled Protein A.
   2. 5 µg enzyme-conjugated Protein A.
   3. Enzyme-conjugated secondary antibody at the manufacturer's recommended dilution (normally between 1: 1000 and 1: 10,000).
e. Wash the nitrocellulose at least five times, each time using 100 mL PBS/1% Triton X-100 for 5 min, to remove unbound Protein A or secondary antibody. To ensure effective washing of the filter, pour off each solution from the same corner of the tray, and replace the lid in the same orientation.

3.2. Visualization of Antigen–Antibody Complex

3.2.1. Alkaline Phosphatase-Conjugated Ligand

In this method, the enzyme hydrolyzes 5-bromo-4-chloroindolyl phosphate to the corresponding indoxyl compound. The product tautomerizes to a ketone, oxidizes, and then dimerizes to form an insoluble blue indigo that is deposited on the filter. Hydrogen ions released during the dimerization reduce nitroblue tetrazolium to the corresponding diformazan. The latter compound is an insoluble intense purple compound that is deposited alongside the indigo, enhancing the initial signal.

1. Briefly rinse the filter twice, each time using 50 mL of diethanolamine buffer.
2. Incubate the filter with 50 mL of alkaline phosphatase substrate mixture until the blue-purple products appear, usually after 5–30 min.
3. Prevent further color development by removing the substrate mixture and washing the filter thrice, each time in 100 mL of distilled water. Finally, dry the filter thoroughly before storing (*see* Note 11).

3.2.2. Horseradish Peroxidase-Conjugated Ligand

Peroxidase catalyzes the transfer of hydrogen from a wide range of hydrogen donors to H_2O_2, and it is usually measured indirectly by the oxidation of the second substrate. In this method, soluble 3,3'-

diaminobenzidine is converted to a red-brown insoluble complex that is deposited on the filter. The sensitivity of this technique may be increased up to 100-fold by intensifying the diaminobenzidine-based products using a combination of cobalt and nickel salts that produce a dense black precipitate (*see* Note 12).

1. Briefly rinse the filter twice, each time using 50 mL of sodium acetate buffer.
2. Incubate the filter with 50 mL of horseradish peroxidase substrate mixture until the red-brown insoluble products accumulate. Reaction times longer than about 30 min are unlikely to be effective because of substrate inactivation of peroxidase (*see* Note 6).
3. When sufficient color has developed, remove the substrate mixture, and wash the filter three times with 100 mL of distilled water. Then dry the filter, and store it in the dark (*see* Note 11).

3.2.3. ^{125}I-Labeled Protein A

1. If desired, stain the blot for total protein as described below.
2. Allow the filter to dry. Do not use excessive heat since nitrocellulose is potentially explosive when dry.
3. Mark the nitrocellulose with radioactive ink to allow alignment with exposed and developed X-ray film.
4. Fluorograph the blot using suitable X-ray film and intensifying screens. Expose the film at –70°C for 6–72 h, depending on the intensity of the signal.

3.3. Staining of Total Protein

Either of the following stains is suitable for visualizing polypeptides after transfer onto nitrocellulose. Each can detect bands containing about 1 μg of protein. Coomassie blue is unsuitable, since generally it produces heavy background staining.

3.3.1. Amido Black

Incubate the filter for 2–5 s in 100 mL of stain solution. Transfer immediately to 100 mL of destain solution, and wash with several changes to remove excess dye. Unacceptably dark backgrounds are produced by longer incubation times in the stain solution.

3.3.2. Ponceau S

Incubate the filter with 100 mL of Ponceau S stain solution for 30 min. Wash excess dye off the filter by rinsing in several changes of

distilled water. The proteins may be destained by washing the filter in PBS (*see* Note 7).

4. Notes

1. Protein G, a cell-wall component of group G streptococci, binds to the Fc region of IgG from a wider range of species than does Protein A *(7)*. Therefore, antibodies that react poorly with Protein A, particularly those from sheep, cow, and horse, may be detected by a similar method using protein G derivatives. In principle, the latter are more versatile. At present, however, the limited availability of suitable protein G conjugates means that, where suitable, Protein A derivatives remain preferable.
2. Several visualization systems have been developed for both alkaline phosphatase and horseradish peroxidase. Alternatives to the assay systems provided in this chapter are described by Tijssen *(4)*. Currently, the greatest sensitivity is provided by luminescent detection systems that have been developed recently by several biochemical companies (*see* Chapter 27). The alkaline phosphatase system is based on the light emission that occurs during the hydrolysis of AMPPD (3-[2'-spiroadamantane]-4-methoxy-4-[3"-phosphoryloxy]-phenyl-1,2-dioxetane). The mechanism involves the enzyme-cataylzed formation of the dioxetane anion, followed by fragmentation of the anion to adamantone and the excited state of methyl *meta*-oxybenzoate. This latter anion is the source of light emission. The peroxidase detection system relies on light emitted during the oxidation of luminol (3-aminophthalhydrazine) by peroxide radicles. The latter are formed during the enzyme-catalyzed reduction of hydrogen peroxide or other suitable substrates.
3. Iodination of Protein A using Bolton and Hunter reagent labels the ε-NH_2 group of lysine, which apparently is not involved directly in the binding of Protein A to the Fc region of IgG. This method is preferable to others, such as those using chloramine T or iodogen, which label tyrosine. The only tyrosine residues in Protein A are associated with Fc binding sites, and their iodination may reduce the affinity of Protein A for IgG *(8)*.

   ^{35}S-labeled Protein A may be substituted for iodinated Protein A in the protocol described in this chapter by those researchers not wishing to handle ^{125}I. ^{35}S-labeled Protein A has the additional advantage of producing a far sharper image on X-ray film. However, this radioisotope requires longer exposure times.
4. Many workers include up to 0.05% sodium azide in the antibody and washing buffers to prevent microbial contamination. However, azide

inhibits horseradish peroxidase. Therefore, do not use buffers containing azide when using this enzyme.

5. In the original description of this protocol, 5-bromo-4-chloroindolyl phosphate was made up as a stock solution in dimethylformamide. However, this is not necessary if the disodium salt is used, since this compound dissolves readily in aqueous buffers.
6. Urea peroxide may be used instead of hydrogen peroxide as a substrate for peroxidase. The problems of instability, enzyme inactivation, and possibility of caustic burns associated with hydrogen peroxide are eliminated by using urea peroxide. A 10% (w/v) stock solution of urea peroxide is stable for several months and is used at a final concentration of 0.1% in the peroxidase substrate mixture.
7. If desired, the nitrocellulose filter may be stained with Ponceau S immediately after electroblotting. This staining apparently does not affect the subsequent immunodetection of polypeptides, if the filter is thoroughly destained using PBS before incubation with the antiserum. In addition to confirming that the polypeptides have been transferred successfully onto the filter, initial staining allows tracks from gels to be separated precisely and probed individually. This is useful when screening several antisera.
8. Nonspecific binding is a common problem in immunoblotting. Several factors are important in reducing the resulting background.

   First, the filter is washed in the presence of an "inert" protein to block the unoccupied binding sites. Bovine serum albumin is the most commonly used protein, but others, such as fetal calf serum, hemoglobin, gelatin, and nonfat dried milk, have been used successfully. Economically, the latter two alternatives are particularly attractive. The quality of protein used for blocking is important, since minor contaminants may interfere with either antigen–antibody interactions or the binding of Protein A to IgG. These contaminants may vary between preparations and can be sufficient to inhibit completely the detection of specific polypeptides. Routinely, we use bovine serum albumin (fraction V) from Sigma Chemical Co. (product number A 4503), but no doubt albumin from other sources is equally effective. The suitability of individual batches of protein should be checked using antisera known to react well on immunoblots. Second, the background may be reduced further by including nonionic detergents in the appropriate solutions. These presumably decrease the hydrophobic interactions between antibodies and the nitrocellulose filter. Tween 20, Triton X-100, and Nonidet P-40 at concentrations of 0.1–1.0% have been used. In my experience, such detergents may supplement the blocking agents

described above, but cannot substitute for these proteins. In addition, others have reported that these detergents sometimes remove proteins from nitrocellulose *(9)*.

Third, the nitrocellulose must be washed effectively to limit nonspecific binding. For this, the volumes of the washing solutions should be sufficient to flow gently over the surface of the filter during shaking. The method described in this chapter is suitable for 12 × 7 cm filters incubated in 14 × 9 cm trays. If the size of the filter is significantly different, the volumes of the washing solutions should be adjusted accordingly. Finally, reducing the incubation temperature to 4°C may greatly decrease the extent of nonspecific background binding *(9)*.

9. The exact amount of antibody to use will depend largely on its titer. Generally, it is better to begin by using a small amount of antiserum. Excessive quantities of serum tend to increase the background rather than improve the sensitivity of the technique. Nonspecific binding can often be reduced by decreasing the amount of antibody used to probe the filter.
10. Deciding which form of detection system to use is largely a personal choice. ^{125}I-labeled Protein A is extremely sensitive. This method has the advantage of allowing the polypeptide recognized by the antibody to be precisely identified by aligning the fluorograph with the original filter after staining for total protein. However, many researchers prefer not to work with this radioactive isotope. Comparison between the two enzymic detection systems is difficult because the reported sensitivity limits of both systems vary considerably, and most studies use different antigens, different primary antibodies, and different protocols. Despite these uncertainties, alkaline phosphatase is generally considered more sensitive than horseradish peroxidase. For routine work, I prefer to use alkaline phosphatase-conjugated Protein A.
11. The products of the peroxidase reaction are susceptible to photobleaching and fading. Consequently, the developed filters should be stored in the dark, and the results photographed as soon as possible. The products of the phosphatase reaction are reportedly stable in the light. However, I treat such filters in the same way—just in case!
12. To increase sensitivity of the diaminobenzidine-based staining protocol, replace the standard substrate mixture with the following intensifying solution *(10)*. Dissolve 100 mg diaminobenzidine in 100 mL of 200 m*M* phosphate buffer (pH 7.3). To this solution add, dropwise and with constant stirring, 5 mL of 1% (w/v) cobalt chloride followed by 4 mL of 1% (w/v) nickel ammonium sulfate. Finally add 60 µL of 30% (v/v) hydrogen peroxide just before use.

13. Particular care should be taken when attempting to detect antigens on nitrocellulose using monoclonal antibodies. Certain cell lines may produce antibodies that recognize epitopes that are denatured by detergent. Such "conformational" antibodies may not bind to the antigen after SDS-polyacrylamide gel electrophoresis.
14. Even before immunization, serum may contain antibodies, particularly against bacterial proteins. These antibodies may recognize proteins in an extract bound to the nitrocellulose filter. Therefore, when characterizing an antiserum, control filters should be incubated with an equal amount of preimmune serum to check whether such preexisting antibodies interfere in the immunodetection of specific proteins.
15. Quantitation of specific antigens using this technique is difficult and must be accompanied by adequate evidence that the amount of product or radioactivity bound to the filter is directly related to the amount of antigen in the initial extract. This is important, since polypeptides may vary in the extent to which they are eluted from the gel and retained by the nitrocellulose. Additionally, in some tissues proteins may interfere with the binding of antigen to the filter. Therefore, the reliability of the technique should be checked for each extract.

    Perhaps the best evidence is provided by determining the recovery of a known amount of pure antigen. For this, duplicate samples are prepared, and to one is added a known amount of antigen comparable to that already present in the extract. Ideally, the pure antigen should be identical to that in the extract. The recovery is calculated by comparing the antigen measured in the original and supplemented samples. Such evidence is preferable to that obtained from only measuring known amounts of pure antigen. The latter indicates the detection limits of the assay, but does not test for possible interference by other components in the extract.

    The other major problem in quantifying the level of antigen on immunoblots derives from the technical problems associated with relating densitometric measurements from photographs or fluorographs to the amount of antibody bound to the filter. Recently, a combined radiochemical-color method has been described that circumvents these problems *(11)*. The technique involves challenging the filter sequentially with alkaline phosphatase-conjugated secondary antibody and ^{125}I-labeled Protein A (which binds to the secondary antibody). The color reaction derived from the enzyme conjugate is used to localize the antibody–antigen complex. The appropriate region of the filter is then excised, and the radioactivity derived from the Protein A associated with the band is measured to provide a direct estimate of the amount of antigen.

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CHAPTER 26

Detection of Proteins on Blots Using the Avidin-Biotin System

Michael J. Dunn

1. Introduction

Techniques of one- and two-dimensional polyacrylamide gel electrophoresis are capable of resolving complex mixtures of proteins, allowing characterization of the separated proteins in terms of their size, charge, hydrophobicity, and abundance. However, the further characterization of the separated components is dependent on the availability of other techniques. One powerful approach to this problem is to investigate the interactions of the separated proteins with specific antibodies or other ligands, such as lectins. This is best achieved by the technique of Western blotting *(1)* in which the separated proteins are transferred out of the gels onto the surface of a support matrix, such as nitrocellulose or polyvinylidene difluoride (PVDF). Protein transfer can be achieved by capillary, contact diffusion of vacuum techniques, but more rapid and efficient transfer is achieved if the proteins are electrophoretically removed from the gel to the support by application of an electric field perpendicular to the plane of the gel. This technique, based on the method of Towbin et al. *(2)*, is known as electroblotting and is described in Chapter 24.

After proteins have been transferred to the blotting membrane, all unoccupied protein binding sites remaining on the blot must be blocked before probing with a specific ligand. This has often been achieved by soaking the blot in a solution of bovine serum albumin

From: *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
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(2) or other protein, such as animal sera, ovalbumin, hemoglobin, casein, or gelatin *(1)*. These proteins do not always prove to be unreactive during subsequent probing, particularly if lectins are used as probes, because of the presence of contaminating glycoproteins in the blocking agent. More recently, nonfat dried milk has become a popular blocking agent, resulting in very low backgrounds *(3)*. An alternative strategy is to use a dilute solution of nonionic detergent for the blocking step, and polyethylene sorbitan monolaurate (Tween 20) has been found to be particularly suitable *(4)*. We have found that a combination of 3% (w/v) nonfat dried milk in combination with 0.05% (w/v) Tween 20 results in very low background staining *(5)*. However, it should be remembered that certain proteins can be displaced from the blotting matrix by nonionic detergents.

Once the blot has been blocked, it is then reacted with the specific antibody or ligand. The specifically bound ligand can then be detected and visualized by a variety of methods. It is possible to label the primary ligand directly, but indirect sandwich methods using a secondary reagent specific for the primary ligand are generally used because of the increased sensitivity that can be obtained. The second antibody can be fluorescently labeled (e.g., with fluorescein isothiocyanate, FITC), radiolabeled (usually with ^{125}I, *see* Chapter 45), or conjugated to an enzyme, such as horseradish peroxidase or alkaline phosphatase (*see* Chapter 44). The secondary antibody can be replaced with appropriately labeled Protein A, which specifically binds to the F_c region of IgG (*see* Chapter 25). Another alternative is to use secondary antibodies conjugated with colloidal gold particles (*see* Chapter 27). This approach has the advantage that the bound antibodies are visible (red color) without further development. The sensitivity of this technique can be increased by a silver enhancement procedure (*see* Chapter 28).

A technique that has been found to increase the sensitivity of detection of blots exploits the specificity of the interaction between the low-mol-wt vitamin, biotin (224 Dalton), and the protein avidin. Proteins, such as antibodies and lectins, can be readily conjugated with biotin and used as the secondary detection reagent for probing blots. The blots are then visualized using in a third step, avidin conjugated with a suitable reporter enzyme (e.g., peroxidase, alkaline

phosphatase, β-galactosidase, glucose oxidase). Even greater sensitivity can be obtained at this stage by using preformed complexes of a biotinylated enzyme with avidin since many enzyme molecules are present in these complexes producing an enhanced signal. Egg white avidin (M_r 68 kDa) is often used in these procedures, but has two distinct disadvantages: (1) it is highly charged at neutral pH so that it can bind to proteins nonspecifically, and (2) it is a glycoprotein that can interact with other biomolecules, such as lectins, via the carbohydrate moiety. It is, therefore, advantageous to use streptavidin (M_r 60 kDa), isolated from *Streptomyces avidinii*, which has a p*I* close to neutrality and is not glycosylated.

2. Materials

1. Washing solution (PBS-T): Phosphate-buffered saline (PBS) containing 0.05% (w/v) Tween 20. For 1 L of solution, use 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 0.2 g KCl, 8.0 g NaCl, 0.5 g Tween 20.
2. Blocking solution (PBS-TM): PBS containing 0.05% (w/v) Tween 20 and 3% (w/v) nonfat dried milk. For 100 mL of solution, use 0.02 g KH_2PO_4, 0.115 g Na_2HPO_4, 0.02 g KCl, 0.8 g NaCl, 0.05 g Tween 20, 3 g nonfat dried milk powder.
3. Biotinylated secondary antibody (*see* Note 2): Biotinylated affinity-isolated rabbit immunoglobulins to mouse immunoglobulins (DAKO, High Wycombe, Bucks, UK) diluted 1:200 with PBS-TM.
4. Peroxidase-conjugated streptavidin (DAKO), diluted 1:10,000 with PBS-TM.
5. Diaminobenzidine (DAB) solution: 0.05% (w/v) diaminobenzidine (*see* Note 3) and 0.01% (v/v) hydrogen peroxide (from 30% stock solution) in PBS-T. Prepare fresh when required.

3. Method

Note: Perform all steps at room temperature with gentle agitation.

1. Seal the blot in a plastic bag containing PBS-TM blocking solution, and incubate for 60 min.
2. Drain the blocking solution from the bag, and incubate the blot for 60 min with the primary antibody diluted to an appropriate concentration in PBS-TM. In the example illustrated in Fig. 1A, mouse monoclonal antibody to desmin (Amersham International, Amersham, Bucks, UK) was diluted 1:250 (*see* Note 4).
3. Wash the blot for 3 × 10 min with PBS-T.

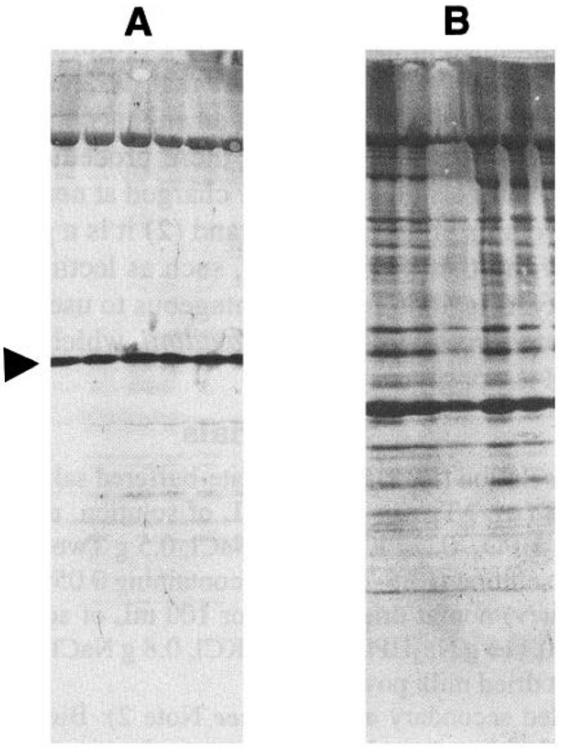

Fig. 1. Human skeletal muscle tissue proteins were separated by SDS-PAGE and transferred to nitrocellulose by electroblotting. The blots were blocked using PBS-TM. **A.** The blot was reacted sequentially with mouse monoclonal antibody to desmin, biotinylated antibody to mouse immunoglobulins, and peroxidase-conjugated streptavidin, and visualized using DAB. The darkly stained band (arrowhead) represents desmin present in the skeletal muscle. **B.** The blot was reacted sequentially with biotinylated Concanavalin A and alkaline phosphatase-conjugated streptavidin, and visualized with Fast red. The stained bands represent skeletal muscle glycoproteins bearing carbohydrate groups reactive with Concanavalin A.

4. Incubate the blot for 60 min with biotinylated secondary antibody diluted in PBS-TM.
5. Repeat washing step 3.
6. Incubate the blot for 60 min with the streptavidin-peroxidase diluted in PBS-TM (*see* Notes 5 and 6).
7. Repeat washing step 3.
8. Visualize the blot by the addition of DAB solution for 10 min. Brown bands of peroxidase activity are detected (*see* Fig. 1A).

4. Notes

1. If lectins are used to detect glycoproteins, a similar method to that described can be used if an antibody specific for the lectin is available. Alternatively, if the lectin is conjugated with biotin (many conjugates are available commercially), it can be used directly so that the secondary antibody procedure (steps 4 and 5 in Section 3.) is omitted. An example of this technique using biotinylated Concanavalin A and alkaline phosphatase-conjugated streptavidin visualized with Fast red is shown in Fig. 1B.
2. The appropriate biotinylated secondary antibody specific for the immunoglobulins of the species in which the primary antibody was raised must be used.
3. Diaminobenzidine has been classified as a suspected carcinogen, but these suspicions appear to have been retracted *(6)*. Nevertheless, it is recommended that the solid compound is dispensed with care (e.g., in a fume cupboard).
4 No general guide can be given to the appropriate dilution for any particular primary antibody. This must be established for each antibody using serial dilutions (starting at a dilution of 1:10), so that a strong specific signal is obtained, with minimal nonspecific and background staining.
5. Other enzymes conjugated with avidin can be used with appropriate substrates at step 6 in Section 3., for example β-galactosidase or alkaline phosphatase (*see* Chapter 44).
6. If it is necessary to use an avidin–biotin complex (ABC) to enhance sensitivity, it is used at step 6 (Section 3.) diluted according to the manufacturer's instructions in PBS-TM. Reagents for these complexes are available from Vector Laboratories (Breton, Peterborough, UK) (avidin with biotinylated peroxidase, alkaline phosphatase, or glucose oxidase), DAKO (High Wycombe, Bucks, UK) (avidin with biotinylated peroxidase or alkaline phosphatase), and Amersham (Amersham, Bucks, UK) (avidin with biotinylated peroxidase or β-galactosidase).

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CHAPTER 27

Detection of Proteins on Protein Blots Using Chemiluminescent Systems

Samantha J. Crisp and Michael J. Dunn

1. Introduction

Western blotting is a powerful technique for the analysis of proteins separated by polyacrylamide gel electrophoresis (PAGE) (*see* Chapter 24). By using specific ligands, such as antibodies, proteins of interest can be readily localized and identified on the blotting membrane. The visualization of these ligands can be achieved using a variety of techniques. The ligand itself may be labeled directly, for example, by radiolabeling (e.g., ^{125}I) or by enzyme conjugation. However, an indirect approach in which the blot is reacted with a secondary reagent specific for the primary ligand is generally more popular. Again visualization can be achieved by radiolabeling, for example, ^{125}I-protein A (*see* Chapter 25). Although radioactive reporter systems offer excellent sensitivity and specificity, their use is limited by the safety considerations associated with radioisotope work and the disposal of radioactive waste. Therefore, techniques that use enzymes, such as horseradish peroxidase or alkaline phosphatase, as reporter molecules are much more popular (*see* Chapter 25). These enzymes are conjugated with the secondary ligand and are visualized using a color reaction. The sensitivity of these methods can be substantially increased using the avidin-biotin system (*see* Chapter 26) or colloidal gold (*see* Chapter 28).

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Recently, visualization techniques have been developed that are based on chemiluminescent reactions, and this approach has been shown to provide a significant improvement in detection sensitivity over existing methods. These reactions are catalyzed by the enzymes previously used for colormetric detection. For example, alkaline phosphatase can be used to catalyze the dephosphorylation of the chemiluminescent substrate, adamantyl-1-2-dioxetane phosphate (AMPDD), resulting in the emission of light *(1–3)*. An alternative approach, and the one described here, is to use horseradish peroxidase to catalyze the oxidation of luminol in the presence of hydrogen peroxide *(4,5)*. Immediately following this reaction, the luminol is in an excited state and may decay to the ground state via a light-emitting pathway. The advantage of this system is that the emission of light may be enhanced by the addition of compounds, such as phenols, increasing the intensity of the reaction up to 1000-fold. This enhanced chemiluminescence system (ECL) is used in conjunction with standard immunodetection protocols that use peroxidase-conjugated secondary antibodies as shown in Fig. 1. The image is visualized by exposing the film to a blue sensitive film for times between 15 s and 1 h. Using this system, <1 pg of protein can be detected, which represents at least a 10-fold increase in sensitivity over other detection methods. An additional advantage of using the ECL system is that the antibodies used to detect the proteins can be stripped from the blot, facilitating the reprobing of the same blot with other specific ligands. A protocol for this procedure is given in Note 6.

2. Materials

1. Washing solution (PBS-T): Phosphate-buffered saline (PBS) containing 0.05% (w/v) Tween 20. For 1 L of solution, use 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 0.2 g KCl, 8.0 g NaCl, 0.5 g Tween 20.
2. Blocking solution (PBS-T M): Phosphate-buffered saline (PBS) containing 0.05% (w/v) Tween 20 and 3% (w/v) nonfat dried milk (e.g., Marvel). For 100 mL of solution, use 0.02 g KH_2PO_4, 0.115 g Na_2HPO_4, 0.02 g KCl, 0.8 g NaCl, 0.05 g Tween 20, 3 g nonfat dried milk.
3. Secondary antibody (*see* Note 1): Peroxidase-conjugated rabbit antimouse immunoglobulins (Amersham International, Amersham, UK) diluted 1:1000 with PBS-T M.
4. ECL Western blotting detection kit (Amersham) (*see* Note 2).

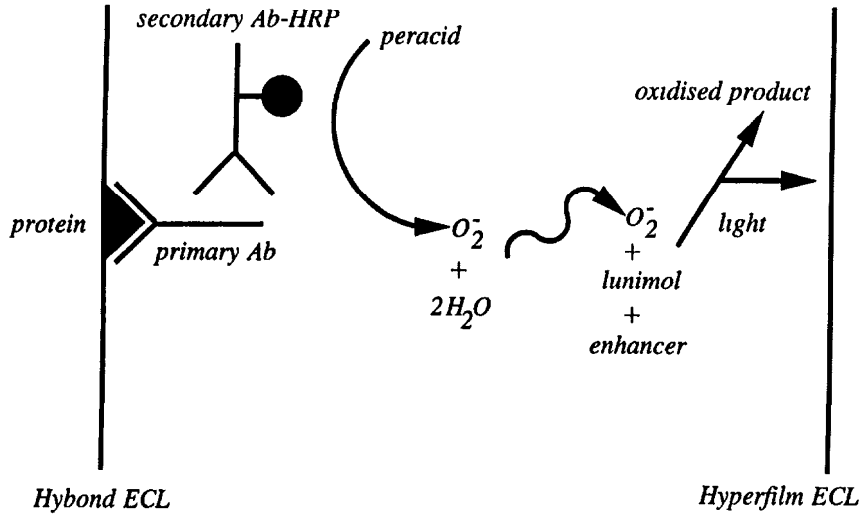

Fig. 1. Diagram showing the mechanism of immunodetection of proteins on Western blots using the ECL system

5. Detection film: Hyperfilm ECL (Amersham) (*see* Note 3).
6. Glass plate and food wrapping film (e.g., cling film, Saran Wrap™).
7. Photographic equipment: Autoradiography cassettes without intensifying screens are required for exposure of blots with ECL film. The film can be developed using an automatic X-ray developing unit or by hand using standard X-ray developer and fixer.

3. Method

Note: All incubations are carried out at room temperature with gentle agitation.

1. Seal the blot in a plastic bag containing PBS-T M, and incubate for 1 h. (This step blocks nonspecific binding sites on the membrane and so helps to reduce background staining.)
2. Drain the blocking solution from the bag, and incubate the blot for 1 h with the primary antibody, diluted at an appropriate concentration in PBS-T M (*see* Note 4).
3. Wash the blot three times in the bag with PBS-T.
4. Incubate the blot for 1 h with the peroxidase-conjugated secondary antibody diluted in PBS-T M (*see* Note 4).
5. Wash the blot in the bag for 5 min with PBS-T and then for a further 30 min in a dish containing 100–200 mL of PBS alone (*see* Note 5).

6. Mix the two ECL reagents together in a ratio of 1:1 to give a final vol of 0.125 mL/cm^2 transfer membrane. Incubate the blot in the ECL reagent mixture for 1 min.
7. Drain the blot of excess reagent, and place it on a glass plate, protein side uppermost. Cover the blot with food wrapping film, and remove all air bubbles and creases by rubbing the surface with a dry tissue. (It is important that the blot is not allowed to dry out at this stage.)
8. Expose the blot to film in the dark for 15 s to 1 h and then develop using an automated processor or by hand. In the example illustrated in Fig. 2, an exposure time of 1 min was used.

4. Notes

1. The appropriate peroxidase-conjugated secondary antibody specific for the species in which the primary antibody was raised must be used.
2. Other manufacturers also produce blot detection kits based on chemiluminescence. For example, Bio-Rad (Hemel Hempstead, Hertfordshire, UK), USB (Cleveland, OH, USA), Tropix (Bedford, MA, USA), and Boehringer Mannheim (Lewes, Sussex, UK).These kits use substrates that emit light when dephosphorylated by alkaline phosphatase and therefore require alkaline phosphatase (rather than horseradish peroxidase) conjugated secondary antibodies.
3. The film used to detect the signal produced by the ECL system must be sensitive to blue light. Hyperfilm ECL has been specifically chosen to give optimum results with the ECL technique.
4. The appropriate dilutions of the primary and secondary antibody reagents and the exposure time required to obtain optimal results will vary for each particular primary antibody. These parameters must be established using serial dilutions (starting at say 1:100) and varying exposure times so that a strong specific signal is obtained with minimal nonspecific background staining.
5. An extreme washing step is required at this stage to minimize the amount of background staining.
6. Stripping protocol: Using the following method, the primary and secondary antibodies can be completely removed from the membrane. The membrane can be stripped and reprobed several times, but it must be stored wet at 4°C after each immunodetection.
   a. Incubate the blot in the stripping solution (62.5 m*M* Tris-HCl, pH 6.7, 100 m*M* β-mercaptoethanol, 2% [w/v] sodium dodecylsulfate) for 30 min at 50°C.
   b. Wash the blot twice for 10 min with PBS-T at room temperature in as large a volume as possible.

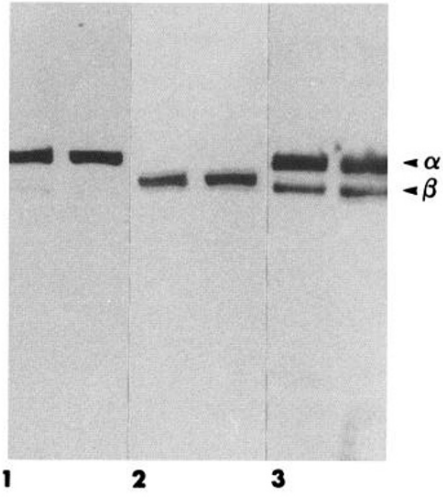

Fig. 2. Result of a Western blotting experiment to detect the α- and β-subunits of spectrin using the ECL system following SDS-PAGE separation of human erythrocyte membrane proteins. 1, Monoclonal antibodies to α-spectrin (diluted 1:10,000); 2, monoclonal antibodies to β-spectrin (diluted 1:1,000); 3, monoclonal antibodies to αβ-spectrin (diluted 1:500).

c. The blot may be reprobed immediately by following the protocol described above starting with the blocking step. Alternatively, it can be dried on filter paper and stored until required.

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CHAPTER 28

The Detection of Proteins on Blots Using Gold or Immunogold

Susan J. Fowler

1. Introduction

Immunogold probes were first used as electron-dense markers in electron microscopy *(1–3)* and as color markers in light microscopy *(4)*. It was not until later that their application to immunoblotting was examined *(5–7)*. Gold-labeled antibodies and protein A were demonstrated to be suitable for the visualization of specific antigens on Western blots and dot blots *(5,6)*. When gold-labeled antibodies are used as probes on immunoblots, the antigen–antibody interaction is seen as a pinkish signal, owing to the optical characteristics of colloidal gold *(5)*. Used on its own, the sensitivity of immunogold detection is equivalent to indirect peroxidase methods, and, hence only suitable for situations where there are higher levels of antigen. In addition, the signal produced is not permanent. In order to overcome this problem and to allow the technique to be used for more demanding applications, a way of amplifying the signal was subseqently developed using the capacity of gold particles to catalyze the reduction of silver ions *(8)*. This reaction results in the growth of the gold particles by silver deposition. A stable dark brown signal is produced on the blot, and sensitivity is increased 10-fold. The sensitivity achieved using immunogold silver staining (IGSS) is similar to that obtained with alkaline phosphatase using colorimetric detection and several times more sensitive than ^{125}I-labeled antibodies. However, unlike colorimetric detection, the result is stable and not prone to

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Edited by J M Walker

fading, and the chemicals used present no hazards. In addition, the signal-to-noise ratio of IGSS is usually very high, and there are none of the handling or disposal problems that are asssociated that ^{125}I-labeled antibodies.

The binding of the gold to antibodies is via electrostatic adsorbtion. It is influenced by many factors, including particle size, ionic concentration, the amount of protein added, and its molecular weight. Most importantly, it is pH dependent *(9)*. A further feature is that the binding of the gold does not appear to alter the biological or immunological properties of the protein to which it is attached. The colloidal gold particles used to label antibodies can be produced in different sizes ranging from 1–40 nm in diameter. For immunoblotting, Amersham International plc. (Amersham, UK) supply AuroProbe™ BLplus antibodies labeled with 10 nm particles and AuroProbe One antibodies labeled with 1 nm gold particles. In the case of the larger 10 nm gold particles there will be several antibodies bound to each gold particle and the probe can be considered to be a gold particle coated with antibodies (*see* Fig. 1). For the small 1 nm particles each antibody has at least one individual gold particle bound to it. Reducing the size of the gold particles allows increased labeling efficiency and can give greater sensitivity than larger particles *(10)*. This may be owing in part to the larger number of intensifiable gold particles per unit of antigen *(11)*. In addition, when prolonged incubations in AuroProbe One are performed, the nonspecific binding that can sometimes occur with larger gold probes is absent, and potential background problems are avoided. However, when using antibodies labeled with AuroProbe One, the small size of the gold particles means that visualization of the antigen–antibody interaction can only be achieved using silver enhancement.

Silver enhancement was first reported by Danscher *(8)* who used silver lactate and hydroquinone at a pH of 3.5. In the presence of the gold particles, which act as catalysts, the silver ions are reduced to metallic silver by the hydroquinone. The silver atoms formed are deposited in layers on the gold surface, resulting in significantly larger particles and a more intense macroscopic signal (*see* Fig. 2). This classical enhancement worked well, but had several disadvantages. The system was sensitive to light and chemical contamination. In

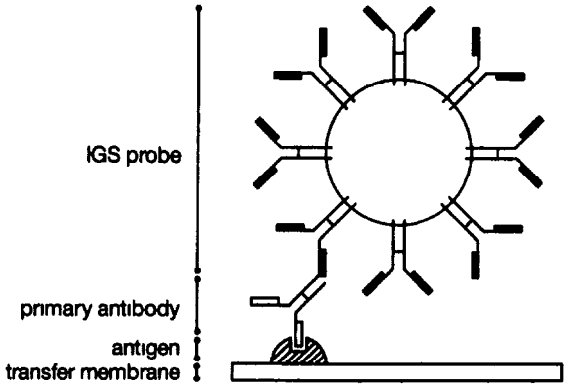

Fig. 1. Principle of the indirect visualization of antigens using immunogold probes. The primary antibodies bind to immobilized antigens on the blot and are in turn recognized by gold-labeled secondary antibodies. The above illustration of the binding pattern of secondary antibodies to gold particles represents the type of conjugate formed with gold particles of 10 nm or larger. The exact configuration adopted by the antibodies on the gold particle is not known.

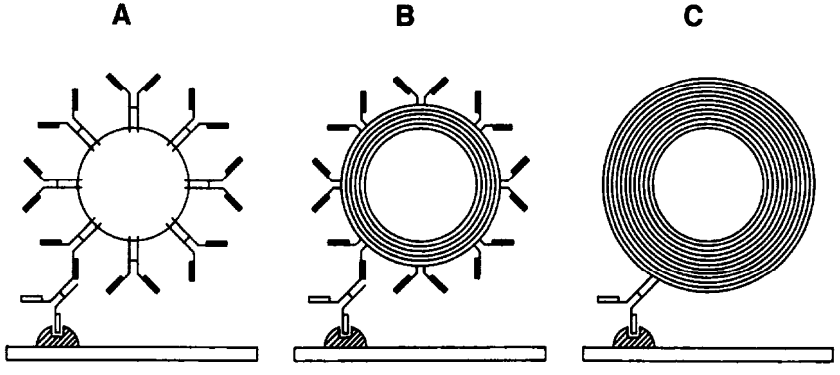

Fig 2 Schematic representation of the silver enhancement process. In the initial phase (a), the gold probe attaches to the primary antibody, which is bound to immobilized antigen. During the silver enhancement process, layers of silver selectively precipitate on the colloidal gold surface (b). The result is a significantly larger particle and a silver surface that generates a more intense macroscopic signal (c)

addition, the components were not stable and were prone to self-nucleation, a phenomenon whereby the reduction of silver ions occurs spontaneously in solution to form silver particles that can be deposited and lead to high background.

More recently, silver enhancement reagents have been developed that overcome these problems. IntenSE™ BL (Amersham International plc.) is light insensitive and has a neutral pH. It also exhibits delayed self-nucleation, which allows a fairly large time margin before it is necessary to stop the reaction (*see* Fig. 3). Using silver enhancement allows sensitivity to be increased 10-fold over immunogold detection.

In addition to the immunological detection of proteins, gold particles can also be used as a general stain for proteins on blots *(12)*. AuroDye™ forte (Amersham International plc.) is a stabilized colloidal gold solution adjusted to a pH of 3. At this pH, the negatively charged gold particles bind very selectively to proteins by hydrophobic and ionic interactions. The proteins thus stained appear as dark red. The sensitivity obtained is comparable to that of silver staining for polyacrylamide gels. Segers and Rabaey *(13)* found it detected more spots on transfers of 2-D gels than silver staining of the gels. For applications demanding very high sensitivity, it is also possible to amplify the signal further by perfoming a silver enhancement step using IntenSE BL. An additional feature of total protein staining with gold is that, as with India ink *(14)*, the immunoreactivity of the proteins is not altered. Thus, it is possible for specific proteins to be immunodetected with chemiluminescent or colorimetric substrates after total protein staining *(15)*. Alternatively, if proteins are omitted from block solutions, total protein staining can be performed after immunodetection *(16)*.

In summary, immunogold silver staining is a highly sensitive detection method that provides a permanent record of results. Protocols are simple to use and provided the silver enhancement step is carefully monitored, background interference is negligible. Results are obtained without the prolonged autoradiography exposures associated with radioactive methods, and the chemicals used do not present any hazards or disposal problems.

2. Materials

2.1. General

1. Nitrocellulose, nylon, or PVDF membranes: AuroProbe BLplus can be used with any of the three types of membrane. AuroProbe One and AuroDye forte are only compatible with nitrocellulose and PVDF membranes.

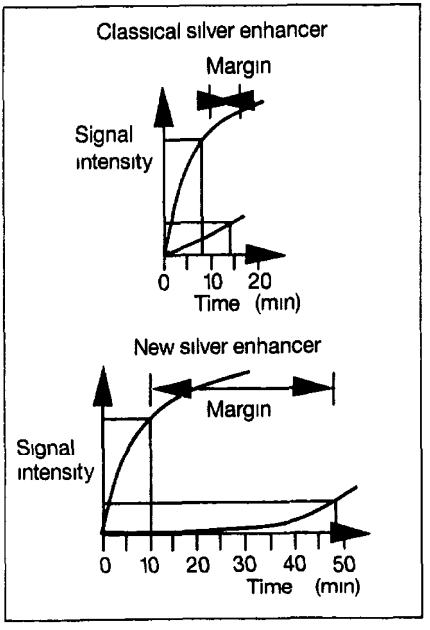

Fig. 3 Silver enhancement time dependency for both the classical and IntenSE BL silver enhancers

2. Phosphate buffered saline (PBS), pH 7.2, containing 0.02% azide: 8 g NaCl, 0.2 g KCl, 1.4 g $Na_2HPO_4 \cdot 2H_2O$, 0.2 g KH_2PO_4, 0.2 g sodium azide, adjust pH to 7.2 and make up to 1000 mL with distilled water.
3. Gelatin: The gelatin used should be of high quality if it is to inhibit nonspecific binding of gold probes effectively. Gelatin of immunogold silver stain (IGSS) quality is supplied as a component of AuroProbe BLplus and AuroProbe One kits.
4. Analytical-grade chemicals should be used throughout, and water should be distilled and deionized. Where silver enhancement is used, it is important the glassware and plastic containers used are scrupulously clean and are not contaminated with heavy metals or their salts.

2.2. Immunogold Silver Staining Using AuroProbe BLplus

The buffer system outlined below gives very clean backgrounds without the use of Tween 20. The use of Tween 20 in blocking, incubation, and/or washing can lead to nonspecific binding of gold probes to blotted proteins from certain types of sample, such as whole cultured cells and isolated nuclei extracts.

1. Wash buffer: 0.1% (w/v) BSA in PBS.
2. Block buffer for nitrocellulose or PVDF membranes: 5% (w/v) BSA in PBS.
3. Block buffer for nylon membranes: 10% (w/v) BSA in PBS.
4. Primary antibody diluent buffer: 1% (v/v) normal serum (from the same species as that in which the secondary antibody was raised) diluted in wash buffer.
5. Gelatin buffer: 1% (v/v) IGSS-quality gelatin in wash buffer, (equivalent to a 1:20 dilution of the gelatin supplied with AuroProbe BLplus).
6. AuroProbe BLplus secondary antibody, 1:100 diluted in gelatin buffer, or, biotinylated secondary antibody 2 µg/mL diluted in gelatin buffer and AuroProbe BLplus streptavidin 1:100 diluted in gelatin buffer.
7. Enhancer solution: ready-to-use component of the IntenSE BL kit.
8. Initiator solution: ready-to-use component of the IntenSE BL kit.

2.3. Immunogold Silver Staining Using AuroProbe One

1. Wash buffer: 0.8% (w/v) BSA, 0.1% (v/v) gelatin in PBS.
2. Block buffer: 4% (w/v) BSA, 0.1% (v/v) gelatin in PBS.
3. Primary and secondary antibody diluent buffer.
   a. For AuroProbe One goat antirabbit and goat antimouse: 1% (v/v) normal goat serum in diluted in wash buffer.
   b. For AuroProbe One streptavidin or antibiotin: 1% (v/v) normal serum (from the same species as that in which the biotinylated secondary antibody was raised) diluted in wash buffer.
4. AuroProbe One secondary antibody: 1:200 to 1:400 diluted in antibody diluent buffer, or, biotinylated antibody 2 µg/mL diluted in antibody diluent buffer and AuroProbe One streptavidin or antibiotin 1:200 to 1:400 diluted in antibody diluent buffer.
5. Enhancer solution: ready-to-use component of the IntenSE BL kit.
6. Initiator solution: ready-to-use component of the IntenSE BL kit.

2.4. General Staining of Blotted Proteins Using AuroDye Forte

1. Tween 20: component of AuroDye forte kit (not all brands of Tween 20 give satisfactory results, it is important to use reagent that has been quality controlled for this purpose).
2. Wash and block buffer: 0.3% (v/v) Tween 20 in PBS.

3. Methods

3.1. General

Avoid skin contact with the transfer membrane. Wear gloves throughout the procedure. Handle blots by their edges using clean plastic forceps. Incubations and washes should be carried out under constant agitation. Plastic containers on an orbital shaker are ideal for this purpose. Alternatively, if it is necessary to conserve antibodies, antibody incubations can be performed in cylindrical containers on roller mixers *(17)*.

3.2. Immunogold Silver Staining Using AuroProbe BLplus

1. After transfer of the proteins to the membrane, incubate the blot in block solution for 30 min at 37°C. A shaking water bath is suitable for this incubation. If nylon membranes are used, this period should be extended to overnight. All subsequent steps are performed at room temperature. It is important that there is enough block solution to cover the blot easily.
2. Remove excess block by washing the blot three times for 5 min in wash buffer. As large a volume of wash buffer as possible should be used each time.
3. Prepare a suitable dilution of primary antibody in diluent buffer. If the antibody is purified, 1–2 µg/mL is a suitable concentration. If unpurified antiserum is used, a dilution greater than 1:500 is recommended.
4. Incubate the blot in this solution for 1–2 h.
5. Wash the blot as described in step 2.
6. Prepare a 1:100 dilution of AuroProbe BLplus antibody in gelatin buffer. If the AuroProbe BLplus streptavidin system is being used, the biotinylated second antibody should be diluted to a concentration of 2 µg/mL in gelatin buffer.
7. Incubate the blot in second antibody for 2 h under constant agitation.
8. Wash blot as described in step 2.
9. If using the AuroProbe BLplus streptavidin system, incubate the blot in a 1:100 dilution of streptavidin gold for 2 h.
10. Wash blot as described in step 2.
11. Wash the gold-stained blot twice for 1 min in distilled water. Do not leave the blot in distilled water for long periods, since this may lead to the release of gold particles from the surface. The result can be reviewed at this stage before going on to perform the silver enhancement step.

12. Pour equal volumes of enhancer and initiator solutions into a plastic container (100 mL are sufficient for a 10 × 15 cm blot). Immediately add the gold-stained blot, and incubate under constant agitation for 15–40 min. The enhancement procedure can be monitored and interrupted or extended as necessary.
13. Wash the blot three times for 10 min in a large volume of distilled water.
14. Remove blot and leave to air-dry on filter paper.

See Fig. 4.

3.3. Immunogold Silver Staining with AuroProbe One

1. After transfer of the proteins to the membrane, incubate the blot in block solution for 30 min at 45°C. A shaking water bath is suitable for this incubation. All subsequent incubations are performed at room temperature.
2. Remove excess block by washing the blot three times for 5 min in wash buffer with constant agitation. As large a volume of wash buffer as possible should be used each time.
3. Prepare a suitable dilution of primary antibody in antibody diluent buffer. If the antibody is pure, 1 µg/mL is a suitable concentration. If unpurified antiserum is used, a dilution >1:500 is recommended.
4. Incubate the blot in this solution for 1 h with constant agitation.
5. Wash the blot as described in step 2.
6. Prepare a 1:200 to 1:400 dilution of AuroProbe One secondary antibody in antibody diluent buffer. If using AuroProbe One streptavidin or AuroProbe One antibiotin, dilute the biotinylated secondary antibody 2 µg/mL in antibody diluent buffer.
7. Incubate blot in second antibody for 1 h
8. Wash blot as described in step 2.
9. If using the AuroProbe One streptavidin or antibiotin, incubate the blot in a 1:200 to 1:400 dilution of streptavidin or antibiotin in antibody diluent buffer for 2 h.
10. Wash blot as described in step 2.
11. Further wash the gold stained blot twice for 5 min in distilled water. Do not leave the blot in distilled water for prolonged periods, since this may lead to the release of gold particles from the blot. The result can be reviewed at this stage before going on to perform the silver enhancement step.
12. Pour equal volumes of enhancer and initiator solutions into a plastic container (100 mL are sufficient for a 10 × 15 cm blot). Immediately

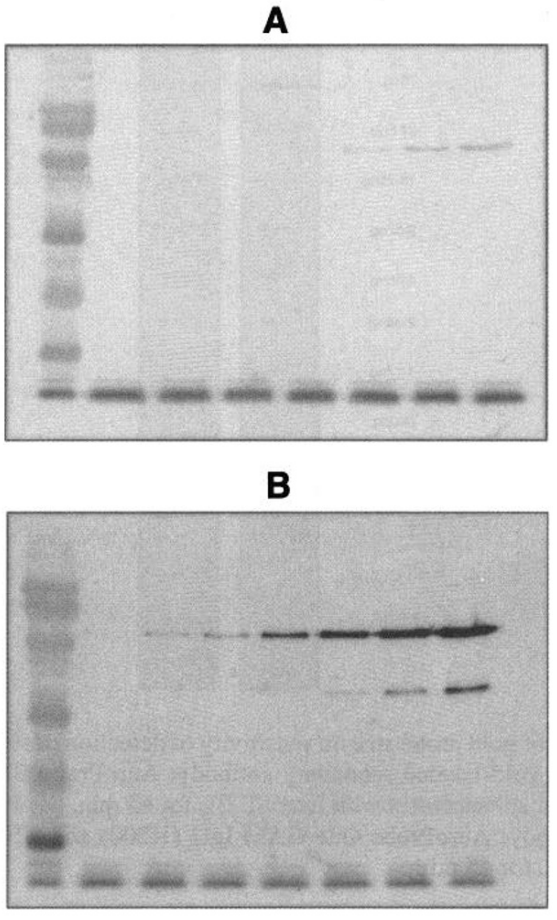

Fig. 4. Detection of bound proteins using colloidal gold-labeled antibodies with (**B**) and without (**A**) silver enhancement. (A) Doubling dilutions of rat brain homogenate separated by 12% SDS-PAGE and transferred to nitrocellulose membrane, followed by immunodetection with mouse monoclonal anti-β-tubulin (1:1000) and AuroProbe BLplus GAM IgG (1:1000) (B) as for (A), but then subjected to silver enhancement with IntenSE BL for 20 min.

add the gold-stained blot and incubate under constant agitation for 15–40 min. The enhancement procedure can be monitored and interrupted or extended as necessary.

13. Wash the blot three times for 10 min in a large volume of distilled water.
14. Remove blot and leave to air-dry on filter paper.

See Fig. 5.

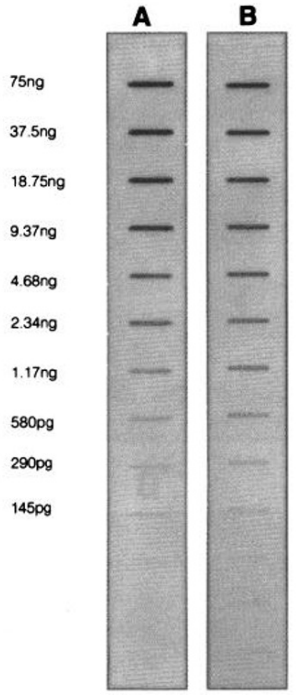

Fig. 5. Effect of gold probe size on sensitivity of detection of slot blots of mouse IgG. (**A**) 10 nm gold-labeled secondary antibody: AuroProbe BLplus GAM IgG (1:100) and silver enhancement with IntenSE BL for 45 min. (**B**) 1 nm gold-labeled secondary antibody: AuroProbe One GAM IgG (1:200) and silver enhancement with IntenSE BL for 45 min.

3.4. General Protein Staining with AuroDye Forte

1. Incubate the blot in an excess of PBS containing 0.3% Tween 20 at 37°C for 30 min. Perform subsequent incubations at room temperature.
2. Further incubate the blot in PBS containing 0.3% Tween 20 three times for 5 min at room temperature.
3. Rinse the blot for 1 min in a large volume of distilled water.
4. Place the blot in AuroDye forte for 2–4 h. The staining can be monitored during this time.
5. When sufficient staining has been obtained, wash the blot in a large volume of distilled water, and leave to air-dry on a piece of filter paper.
6. In cases where extremely high sensitivity or contrast is required, the AuroDye signal can be further amplified with IntenSE BL as described in Section 3. 2., steps 12–14.

4. Notes

4.1. Electrophoresis and Electroblotting

1. During electrophoresis, care should be taken to ensure no extraneous proteins are introduced. Glassware should be thoroughly cleaned, and all solutions should be prepared freshly. Low-ionic-strength transfer buffers are recommended for blotting (i.e., 25 m*M* Tris, 192 m*M* glycine, 20% methanol, pH 8.3).
2. For optimum total protein staining with AuroDye forte, it is important to place a piece of transfer membrane on both sides of the gel. The use of the extra transfer membrane at the cathodic side of the gel, combined with the use of high-quality filter paper, ensures high contrast staining with negligible background. For semidry blotting, the extra piece of transfer membrane is not necessary.

4.2. Immunogold Silver Staining with AuroProbe BLplus and AuroProbe One

1. When using a primary antibody for the first time, it is recommended that its concentration be optimized by performing a dot-blot assay. Antigen dot blots of a suitable concentration are prepared and air-dried. The blocking, washing, and incubation conditions are as outlined in the appropriate method section. A series of primary antibody dilutions is then made, and a dot blot incubated in each. The dilution of the gold-labeled second antibody is kept constant. The primary antibody dilution giving maximum signal with minimum nonspecific binding should be chosen. *See* Table 1 for trouble-shooting tips.
2. It is essential to have gelatin in the incubation with the immunogold reagent to prevent nonspecific binding. The source of gelatin is extremely important. If gelatin other than the one supplied in the AuroProbe kits is used, the inclusion of a negative control is essential.
3. If desired, the incubations in immunogold reagent can be extended to overnight when concentrations of 1:100 to 1:400 are used. If this is necessary, AuroProbe One has been shown to give less nonspecific binding on prolonged incubations than AuroProbe BLplus *(10)*.
4. If, after performing the experiment, there is a complete absence of signal, the reactivity of the immunogold reagent with the primary antibody should be checked by performing a dot-blot assay. Prepare a dilution series of primary antibody, e.g., from 250 ng/µL to 0.5 ng/µL. Spot out 1 µL onto the membrane and allow to air-dry. Proceed with the appropriate immunogold silver staining using the blocking, washing, and incubation conditions as described in Section 3.

Table 1
Trouble-Shooting Immunogold Silver Staining (IGSS)

Observation	Probable cause	Remedy
Precipitation of silver enhancement mixture before indicated time interval	Glassware: Chromic acid was used for rinsing, and it was not washed away with HCl	Rinse glassware several times with 0.1*M* HCl and distilled water
	Glassware: traces of metals originating from metal parts, e.g., cleaning brushes or originating from previous experiments in which metal lic compounds were used, e.g., silver staining	Avoid contact with metallic objects; do not use glassware brush with metallic handle to clean glassware; use disposable plastics instead
High background and nonspecific staining	Microprecipitates that are macroscopically invisible produce a high background when the stability time limit is reached	Incubate for a shorter time in silver enhancement mixture
	Primary antibody is too concentrated	Optimize dilution of primary antibody using dot blot assay
	Sample is too concentrated	Use more diluted sample
	Wrong type of gelatin or no gelatin used during incubation with AuroProbe	Use the type of gelatin as prescribed
No staining	Difficulties regarding the reactivity of the primary antibody with the immunogold reagents	Perform a dot-blot assay (*see* Section 4 2)
	Inefficient transfer from gel to membrane	Optimize blotting conditions, silver stain gel to see what remains
	Error in handling· The steps were not performed in the right order or a step was omitted	Repeat the procedure in the right order
	Excessive dilution of primary antibody	Optimize primary antibody dilution using a dot blot assay
	Nonreactive primary antibody or a primary antibody that was destroyed by inappropriate storage conditions	Use a primary antibody of highest possible quality, and repeat the procedure with a new batch of primary antibody

(continued)

Table 1 *(continued)*

Observation	Probable cause	Remedy
	The gold probe may have been denatured because of wrong storage conditions	Repeat procedure with fresh gold probe
Signal too weak	Excessive dilution of primary antibody	Optimize dilution of primary antibody using a dot blot assay
	Excessive dilution of AuroProbe reagent or too short an incubation time	Use AuroProbe reagent as recommended in the method
	Silver enhancement time too short	Rinse the membrane in distilled water and repeat the silver enhancement in fresh reagent

4.3. Silver Enhancement

1. The enhancement reagents are extremely sensitive to the purity of the water used. Low-quality water results in the formation of precipitates that reduce the reactivity of the enhancement reagent and can lead to high backgrounds. In addition, glassware contaminated with heavy metals in elemental form or as heavy metal salts will decrease the performance of the enhancer reagents.
2. The silver enhancement reagents are prepared by mixing the enhancer and initiator solutions in equal quantities. The mixture is only useable over a defined time period (*see* Table 2), so it is important the components should be combined immediately before use. There is no need to shield the silver enhancer from normal daylight.
3. Both the enhancement time and the stability of the silver enhancement mixture vary considerably with ambient temperature. A typical enhancement time for most blotting experiments of 15–40 min at room temperature (22°C) is recommended. For some applications, it may be necessary to extend the enhancement time and for others to shorten it. At room temperature, there is a comfortable safety margin to enable maximum enhancement before there is any danger of self-nucleation of the enhancer reagent occurring.
4. When a very strong amplification signal is desired, it is possible to perform a second silver enhancement step before self-nucleation starts. In this case the blot is subjected to silver enhancement for 30 min at room temperature (22°C). It is then rinsed with distilled water and

Table 2
Effect of Temperature
on Enhancement Time and Enhancement Reagent Stability

Temperature (°C)	Typical enhancement time, min	Typical stability time, min
16	27–45	>80
18	22–38	>70
20	20–35	>55
22	18–33	>45
24	16–27	>40

immersed in a freshly prepared silver enhancement solution for another 20–30 min. The increase in signal slows down considerably with time. After enhancement, the blot should be washed in distilled water and dried.

4.4. General Protein Staining with AuroDye Forte

1. Because of the high sensitivity of AuroDye forte, special care needs to be taken to avoid background staining. It is important to wear gloves when handling gels and blots. Where possible, handle blots by their edges with forceps since gloves can leave smears. High-quality chemicals should be used throughout. *See* Table 3 for trouble-shooting tips.
2. If large amounts of protein are loaded on the gel, when transferred to membrane, they will not only be heavily stained but will leak off the membrane from saturated sites. Excessive protein leakage will cause an aggregation of the gold particles and destroy the AuroDye forte reagent. The problem is generally more severe with 1-D than 2-D gels, where the proteins are more spread out. For 1-D gels, it is recommended that protein loads should be equivalent to amounts capable of giving resolvable bands after silver staining. Single bands in 1-D gels should not exceed 1000 ng of protein. Molecular-weight standards should be loaded at approx 200 ng/band. In general, the use of lower protein loadings will give better separation, and samples will be conserved.
3. When staining 2-D gels, they should be thoroughly washed in several changes of excess transfer buffer after electrophoresis, to remove any remaining ampholytes.
4. AuroDye forte is a stabilized gold colloid sol, adjusted to a pH of approx 3. At this low pH, the negatively charged gold particles bind very

Table 3
Trouble-Shooting General Protein Staining with AuroDye™ Forte

Observation	Probable cause	Remedy
AuroDye forte turns purplish during staining	Agglutination of gold particles by proteins released from the blot	Wash blot briefly in excess distilled water; replace AuroDye forte; if possible, use lower protein loads; this phenomenon is less frequently observed with PVDF membrane, because it retains proteins better than nitrocellulose.
AuroDye forte turns colorless during staining	Adsorption of all gold particles by excess protein on the blot	Replace AuroDye forte and double the volume used/cm^2 of blot; if possible use lower protein loads
Spotty background	Impurities released from filter paper adsorbed onto blot during transfer	Use high-quality filter paper during electro-blotting procedure
High background	Interference by proteinaceous contaminants	Use extra transfer membrane on cathodic side of the gel; use clean Scotch-Brite pads
	Optional silver enhancement time was too long	Use a shorter silver enhancement time
	Interference by chemical contaminants	Always use high-quality chemicals
Smears on background	Incorrect handling	Handle blots by their edges using clean plastic forceps; avoid contact with gloves

selectively to proteins by hydrophobic and ionic interactions. This may result in different staining intensities depending on the isoelectric point of the proteins being stained. However, this has been reported to be a feature of other protein staining methods, such as silver staining and Coomassie blue *(18)*.

5. AuroDye forte is designed for use on nitrocellulose and PVDF membranes. It cannot be used on nylon membranes where its charge will result in staining of the whole membrane.

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CHAPTER 29

Amino Acid Analysis

G. Brent Irvine

1. Introduction

The method described in this chapter is based on derivatization of amino acids, produced by hydrolysis of peptides or proteins, with phenylisothiocyanate. This forms phenylthiocarbamyl amino acids, which are then separated by reversed-phase high-performance liquid chromatography and quantified from their UV absorbance at 254 nm.

Quantitative amino acid analysis, based on separation by ion-exchange chromatography followed by postcolumn derivatization using ninhydrin for detection, was developed during the 1950s *(1)* and remained the predominant method for 20 years. With the advent of reversed-phase high-performance liquid chromatography, however, rapid separation of amino acid derivatives became possible. Precolumn derivatization also avoids dilution of peaks and so increases sensitivity. Methods involving derivatization with fluorogenic reagents, such as dansyl chloride *(2)* and *o*-phthaldialdehyde *(3)*, were the first to be developed, and these enabled detection of <1 pmol of an amino acid. These methods have some disadvantages, however, including instability of derivatives, reagent interference, and lack of reaction with secondary amino acids. Derivatization using phenyisothiocyanate, the reagent used in the first step of the Edman method for determining the sequence of proteins, avoids many of these problems. The reaction, shown in Fig. 1, is rapid and quantitative with both primary and secondary amino acids.

From *Methods in Molecular Biology, Vol 32. Basic Protein and Peptide Protocols*
Edited by: J M Walker

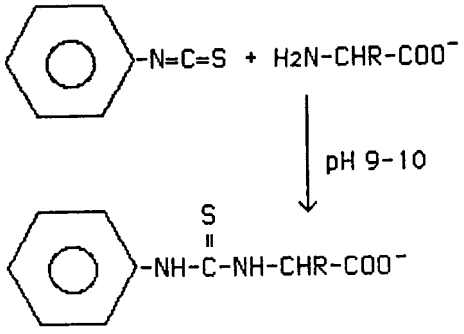

Fig. 1. Reaction of phenylisothiocyanate with an amino acid to form a phenylthiocarbamyl amino acid derivative.

The products are relatively stable, and excess reagent, being volatile, is easily removed. Sensitivity, at about the level of 1 pmol, is more than adequate, since it is difficult to reduce background contamination below this level. Quantitative analysis of phenylthiocarbamyl amino acids was first described by Knoop and coworkers *(4)*. Full details of the application of this method to the analysis of protein hydrolysates were published in 1984 *(5)*. A similar procedure was published *(6)* by employees of the Waters Chromatography Division of Millipore Corporation, and the equipment that they developed is commercially available as the Waters Pico-Tag® system (Millipore, Milford, MA). The method described in this chapter was carried out using Waters' equipment, but equivalent instrumentation could be used. A recent review *(7)* describes the application of the method to the analysis of free amino acids in physiological fluids, amino acids in foodstuffs, and unusual amino acids.

2. Materials

2.1. Apparatus

Two pieces of equipment are required. The first is for hydrolysis of proteins and for derivatization of the resulting amino acids. This can be carried out using the Pico-Tag work station, which comprises an oven that can accommodate up to four vacuum vials, and a vacuum/purge manifold with vacuum gage and a cold trap. Also required are a nitrogen line and a vacuum pump. Up to 12 tubes (6 × 50 mm, Waters), each containing a sample of protein for hydrolysis, can be

inserted into a vacuum vial. The vial can be sealed with an air-tight plastic screw cap that has a drilled-through Teflon™ valve fitted with a slider control that can be pushed to seal or release the vacuum. To the bottom of the vacuum vial is added HCl. The top is screwed on and inserted into the vacuum manifold, and the vial is evacuated using a vacuum pump and cold trap. The valve is then sealed, and the vial is removed and heated in the built-in oven, causing hydrolysis of protein by HCl in the vapor phase. The vial has a bulge in the middle to prevent condensing HCl from running down the inside into the sample tubes. After removal of HCl under vacuum, phenylthiocarbamyl amino acids are formed by addition of phenylisothiocyanate. Excess reagent is then removed under vacuum.

The second piece of equipment is a reversed-phase high-performance liquid chromatography system for separation and quantification of the phenylthiocarbamyl amino acids. This requires two pumps, a gradient controller, an automated injector, a C18 column and column heater, a UV detector set at 254 nm, and an integrator.

2.2. Chemicals (see Note 1)

2.2.1. Hydrolysis and Derivatization

1. HCl/phenol: add melted crystalline phenol (10 µL) to hydrochloric acid (constant boiling at 760 mm) (1.0 mL) (Pierce, Rockford, IL).
2. Redry solution: ethanol:water:triethylamine (2:2:1 by vol).
3. Derivatization solution: ethanol:water:triethylamine:phenylisothiocyanate (7:1:1:1 by vol). Vortex and allow to stand for 5 min before using. Use within 2 h. Phenylisothiocyanate (Pierce): Store at –20°C under nitrogen. After opening an ampule, it can be divided into aliquots that should be resealed under nitrogen. It is important to allow the container to come to room temperature before opening, since this reagent is sensitive to moisture. Each aliquot should be used within 3 wk of opening.
4. Amino Acid Standard H (Pierce): This contains a solution of 17 amino acids (2.5 m*M* each, except cystine, which is 1.25 m*M*) in 0.1*N* HCl.

2.2.2. Chromatography

1. Sample buffer: Dissolve anhydrous sodium dihydrogen phosphate (0.71 g) in water (1 L). Adjust the pH to 7.4 with 1% (v/v) orthophosphoric acid. Add 52.6 mL acetonitrile. Filter through a 0.22-µm filter (Millipore type GV).

2. Eluent A: Dissolve sodium acetate trihydrate (19.0 g) in water (1 L). Add triethylamine (0.5 mL). Adjust the pH to 5.7 with acetic acid (*see* Note 2). Add acetonitrile (63.8 mL). Add 1.07 mL of a solution of ethylenediaminetetra-acetic acid dipotassium salt (100 mg) in water (100 mL). Filter through a 0.22-μm filter (Millipore type GV).
3. Eluent B: Mix acetonitrile (600 mL) with water (400 mL). Filter through a 0.22-μm filter (Millipore type GV).

3. Methods

3.1. Hydrolysis and Derivatization

1. To a pyrex tube (6 × 50 mm), add an aliquot (10 μL) of a solution (2.5 m*M*) of peptide or protein in a volatile solvent, such as methanol or water (*see* Note 3). Volumes in excess of 50 μL should not be used, since solutions may "bump" out of the tube. Place these tubes into the vacuum vial.
2. Dry under vacuum on the work station until the pressure has fallen to 65 mtorr. Drying times using the work station depend on the efficiency of the vacuum pump. With a good pump, this step should take <1 h.
3. To the bottom of the vial (not into the tubes), add HCl/phenol (200 μL).
4. Flush the vial with oxygen-free nitrogen gas, and then evacuate to 1–2 torr. Repeat this twice, sealing the vial after the third evacuation step.
5. Place the vial in the heating block of the work station at 110°C for 22 h (*see* Note 4).
6. Remove the vial, and allow it to cool. Remove excess HCl from the outside of tubes by wiping with a tissue.
7. Add to the vial two tubes, each containing an aliquot (10 μL) of Amino Acid Standard H. Dry under vacuum on the work station until the pressure has fallen to 65 mtorr (about 1 h).
8. Clean out the cold trap to remove any traces of acid that might react with cyanate in the next stages.
9. To each tube, add redry solution (20 μL) and vortex.
10. Dry under vacuum on the work station (about 30 min).
11. To each tube, add derivatization solution (20 μL) and vortex. Leave at room temperature for 20 min.
12. Dry under vacuum on the work station. After the pressure has dropped to 65 mtorr (about 2 h), leave for a further 10 min to ensure complete removal of phenylisothiocyanate.

3.2. Chromatography

1. To each tube, add sample buffer (200 μL) and vortex. The relatively large volume added here facilitates the next (filtration) step. Only a

small proportion of the filtered sample is analyzed. If sample quantity is limited, *see* Note 5.

2. Filter the samples through a 0.45-μm filter (Millipore type HV). Use a 1-mL plastic syringe and needle to withdraw samples. Then place the filter over the syringe and add a fresh needle to ensure that the sample is placed at the bottom of the tube from which it will be injected for chromatography. This should be carried out as soon as possible, and certainly within a few hours if samples are left at room temperature (*see* Note 6).
3. Place the Pico-Tag column in the heating module at 39.0°C, and commence flow of Eluent B (in which the column is stored) at a rate of 1.0 mL/min.
4. Run a linear gradient from 100% Eluent B to 100% Eluent A during 2 min. Allow the column to equilibrate for 30 min in the latter mobile phase.
5. Inject an aliquot (5 μL) of standard using the gradient shown in Table 1. Run time should be set at 21.0 min. The use of an automatic injector, such as the WISP (Waters), is highly recommended (*see* Note 7).
6. The second and third injections should be of the same solution (standard) as the first. Invariably, poor chromatography is obtained with the first injection, but good separation should be achieved with the second and third injections. These chromatograms should be checked to ensure that this is the case before proceeding with further samples.
7. Inject an aliquot (5 μL) of each unknown sample, recording the absorbance at 254 nm.
8. After the run, peaks are integrated, and the amino acid composition of the unknown is determined by comparison with peak areas given by the Amino Acid Standard H.

4. Notes

1. The highest quality of reagents should be used. Suitable water can be obtained using a Milli-Q purification system (Millipore, Bedford, MA) fed by a supply of tap water that has been distilled once. For other chemicals, suppliers of some suitable grades are suggested in Section 2. This is particularly important if high sensitivity is required (*see* Note 5).
2. Other workers often adjust the pH of Eluent A to 6.35 *(6)* or 6.40 *(7)* rather than the lower pH of 5.7 described above. At this lower pH, the resolution of Asp and Glu is increased.
3. Protein samples should be free of salts, amines, and detergents, although it has been reported that salts at concentrations up to 4*M* do not affect derivatization or separation *(8)*.

Table 1
Gradient Table

Time, min	Flow, mL/min	%A	%B	Curve no.[a]
Initial	1.0	100	0	*
10 0	1.0	54	46	5
10.5	1.0	0	100	6
11.5	1.0	0	100	6
12.0	1.5	0	100	6
12.5	1.5	100	0	6
20.0	1.5	100	0	6
20.5	1.0	100	0	6

[a]The column headed "Curve No " refers to the setting used on the Waters gradient controller. Number 6 is linear, whereas number 5 is a shallow convex curve (the rate of change is greater in the earlier part of the period).

4. Rapid hydrolysis may be carried out at 150°C for 1 h *(6)*. However, more satisfactory results are obtained using the lower temperature and longer time described in Section 3.1. Acid hydrolysis of proteins causes conversion of asparagine and glutamine to aspartic acid and glutamic acid, respectively. Serine, threonine, and, to a lesser extent, tyrosine are slowly destroyed (serine suffers about 10% loss during 22 h at 110°C; threonine and tyrosine rather less than this). Accurate quantification of these amino acids requires hydrolysis of replicate samples for 24, 48, and 72 h and extrapolation back to zero time. The longer hydrolysis times can also give more accurate values for alanine, valine, and isoleucine, since hydrolysis of peptide bonds between certain aliphatic amino acids is incomplete after 24 h. Methionine, cysteine, and cystine are partially destroyed by acid hydrolysis with HCl, and for accurate determination, require conversion to more stable derivatives. Tryptophan is totally destroyed by acid hydrolysis with HCl, but may be analyzed after hydrolysis with methanesulfonic acid *(7)*.

   After hydrolysis, in order to prevent condensing HCl from running down the side of the vial into the tubes, keep the tubes upright. With fewer than 12 samples, add blank tubes for support.
5. The amount of each amino acid present in the chromatogram shown in Fig. 2 is 625 pmol. This value is very much higher than the sensitivity limit of the method (about 1 pmol). However, if adequate quantities of

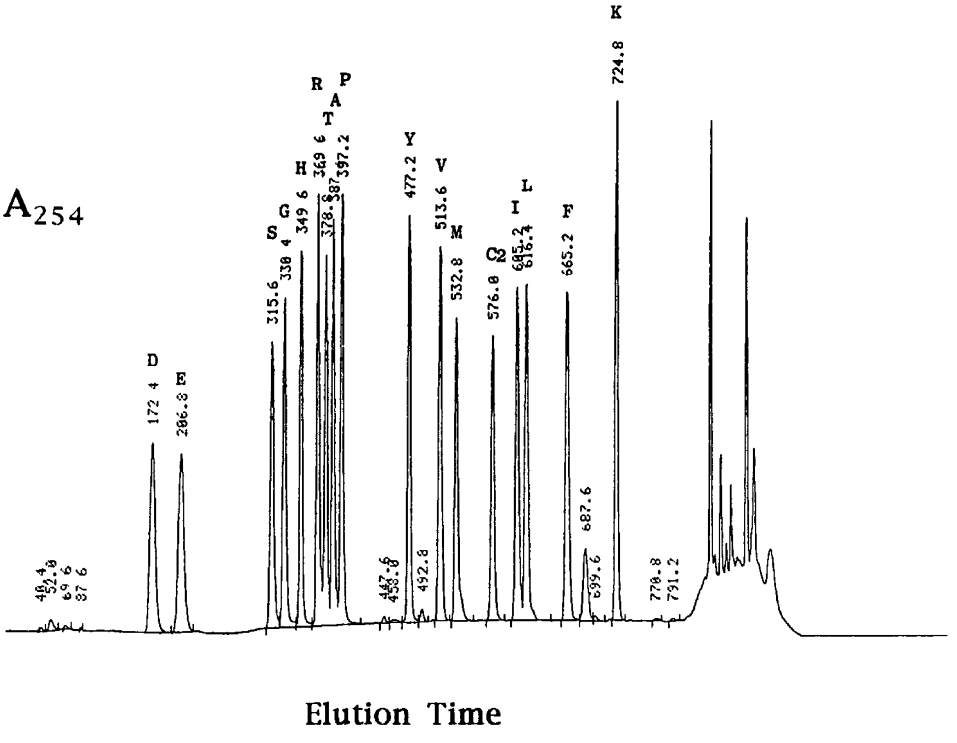

Fig. 2. Separation of a standard mixture of 17 amino acids after derivatization with phenylisothiocyanate. The peak owing to each phenylthiocarbamyl amino acid is identified using the one-letter code for that amino acid, but with C2 being cystine rather than cysteine. The number above each peak is the elution time in seconds. The absorbance at 254 nm of the largest peak (K) was 0.14

Amino acid standard H (10 µL) (Pierce) was treated as described in Section 3.1. The phenylthiocarbamyl derivatives were dissolved in sample buffer (200 µL), and an aliquot (5 µL, containing 625 pmol of each amino acid derivative) was subjected to chromatography as described in Section 3.2. Injections were made using a WISP 712 with two Model 510 pumps and an Automated Gradient Controller onto a Pico-Tag column (3.9 × 150 mm) equilibrated at 39.0°C in a Column Heater controlled by a Temperature Control Module. Peaks were measured by absorbance at 254 nm using a 441 Absorbance Detector (all above equipment from Waters) linked to a Trio Chromatography Computing Integrator (Trivector Inc., West Chester, PA) It can be seen that all the amino acid derivatives have been eluted by 12.1 min. The peaks eluting between F and K and between 13 and 15 min are the result of side reactions of phenylisothiocyanate with nonamino acid material.

protein are available, working at this sensitivity will avoid problems owing to background contamination of samples. If limited quantities of protein are available, say 200 pmol (2 μg of a protein of mol wt 10,000), the procedures described above will need to be modified by redissolving dried, derivatized material in less sample buffer and/or injecting a larger volume (maximum 25 μL). This will ensure that more of the sample goes onto the column. A corresponding adjustment in the treatment of standards will also be required. However, if it is necessary to carry out analyses in the low pmol range, special precautions must be taken, since background contamination (especially of serine and glycine, where it can reach several pmol per sample) is a common problem. These precautions include: pyrolysing glassware at 500°C overnight or steeping in sulfuric acid (250 mL) containing sodium nitrate (1 g); handling with clean gloves (nontalc) and forceps; including a control blank that has been subjected to hydrolysis. Of course, it is good practice to use these procedures in any case, even for lower sensitivity analyses.

6. Phenylthiocarbamyl amino acids in solution at neutral pH are relatively stable, with <10% loss of the least stable derivatives (Leu, Ile) during 10 h at room temperature. Losses are much reduced in the cold, with <5% loss during 48 h at 4°C.
7. The use of automatic injectors, apart from their labor-saving function, gives constant injection volumes and constant intervals between injections (when using the WISP, this interval is actually about 21.7 min for run time set at 21.0 min). An identical interval between injections is an important criterion for obtaining reproducible retention times for each amino acid in different chromatograms, since the column is not given sufficient time to reequilibrate in Eluent A.

Acknowledgment

Thanks are owed to Adrienne Healy for expert technical assistance.

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CHAPTER 30

Molecular-Weight Estimation for Native Proteins Using Size-Exclusion High-Performance Liquid Chromatography

G. Brent Irvine

1. Introduction

The chromatographic separation of proteins from small molecules on the basis of size was first described by Porath and Flodin, who called the process "gel filtration" *(1)*. Moore applied a similar principle to the separation of polymers on crosslinked polystyrene gels in organic solvents, but named this "gel permeation chromatography" *(2)*. Both terms came to be used by manufacturers of such supports for the separation of proteins, leading to some confusion. The name size-exclusion chromatography is more descriptive of the principle on which separation is based and has largely replaced the older names *(3)*.

The original matrices were based on crosslinked polysaccharides, but these are too compressible for use in high-performance liquid chromatography (HPLC). Currently, the most efficient columns are based on surface-modified silica of particle size about 5 μm. These columns (about 1 × 30 cm) have many thousands of theoretical plates. They can be operated at flow rates of about 1 mL/min, giving run times of about 12 min, 10–100 times faster than conventional chromatography on soft gels. The improved peak sharpness and speed have led to a resurgence of interest in the technique.

From: *Methods in Molecular Biology, Vol 32 Basic Protein and Peptide Protocols*
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As well as being a standard chromatographic mode for the purification of proteins, size-exclusion chromatography can be used for estimation of mol wts. For polymers of the same shape, plots of log mol wt against K_d (*see* Note 1) give straight lines within the range $0.1 < K_d < 0.8$ *(3)*. This is true only for ideal size-exclusion chromatography, in which the support does not interact with solute molecules (*see* Note 2). In any case, it must be borne in mind that it is the size, rather than the mol wt, of a solute molecule that determines its elution volume. Hence, calibration curves prepared with globular proteins as standards cannot be used for the assignment of mol wts to proteins with different shapes, such as the rod-like protein, myosin. It has been found that the most reliable measurements of mol wt by size-exclusion HPLC are obtained under denaturing conditions, when all proteins have the same random-coil structure. Disulfide bonds must be reduced, usually with dithiothreitol, in a buffer that destroys secondary and tertiary structure. Buffers containing guanidine hydrochloride *(4,5)* or sodium dodecylsulfate (SDS) *(6)* have been used for this purpose.

However, the use of denaturants has many drawbacks, which are described in Note 3. In any case, polyacrylamide gel electrophoresis in sodium dodecylsulfate-containing buffers is widely used for determining the mol wt of protein subunits. This technique can also accommodate multiple samples in the same run, although each run takes longer than for size exclusion HPLC.

The method described below is for the estimation of mol wts of native proteins. The abilities to measure bioactivity and to recover native protein in high yield make this an important method, even though a number of very basic, acidic or hydrophobic proteins will undergo nonideal size exclusion under these conditions.

2. Materials

2.1. Apparatus

A high-performance liquid chromatography system for isocratic elution is required. This comprises a pump, an injector, a size-exclusion column and guard column (*see* Note 4), a UV detector, and a data recorder. There are many size-exclusion columns based on surface-modified silica on the market. The results described below were obtained using a Zorbax Bioseries GF-250 column (0.94×25 cm), of

particle size 4 µm, sold by Dupont, Wilmington, DE. This column can withstand very high back pressures (up to 380 bar or 5500 psi) and can be run at flow rates up to 2 mL/min with little loss of resolution *(7)*. It has a mol-wt exclusion limit for globular proteins of several hundred thousand. The exclusion limit for the related GF-450 column is about a million.

2.2. Chemicals

1. 0.2*M* Disodium hydrogen orthophosphate (Na_2HPO_4).
2. 0.2*M* Sodium dihydrogen orthophosphate (NaH_2PO_4).
3. 0.2*M* Sodium phosphate buffer, pH 7.0: Mix 610 mL of 0.2*M* Na_2HPO_4 with 390 mL of 0.2*M* NaH_2PO_4. Filter through a 0.22-µm filter (Millipore type GV).
4. Solutions of standard proteins: Dissolve each protein in 0.2*M* sodium phosphate buffer, pH 7.0, at a concentration of about 0.5 mg/mL. Filter the samples through a 0.45-µm filter (Millipore-type HV). Proteins suitable for use as standards are listed in Table 1 (*see* Note 5).
5. Blue dextran, average mol wt 2,000,000 (Sigma), 1 mg/mL in 0.2*M* sodium phosphate buffer, pH 7.0. Filter through a 0.45-µm filter (Millipore-type HV).
6. Glycine, 10 mg/mL in 0.2*M* sodium phosphate buffer, pH 7.0. Filter through a 0.45-µm filter (Millipore type HV).

3. Method

1. Allow the column to equlibrate in the 0.2*M* sodium phosphate buffer, pH 7.0, at a flow rate of 1 mL/min (*see* Note 6) until the absorbance at 214 nm is constant.
2. Inject a solution (20 µL) of a very large molecule, such as blue dextran (*see* Note 7), to determine V_o. Repeat with 20 µL of water (negative absorbance peak) or a solution of a small molecule, such as glycine, to determine V_t. Definitions of V_o, V_e, V_t, and K_d are given in Note 1.
3. Inject a solution (20 µL) of one of the standard proteins, and determine its elution volume, V_e, from the time at which the absorbance peak is at a maximum. Repeat this procedure until all the standards have been injected. A chromatogram showing the separation of seven solutes during a single run on a Zorbax Bio-series GF-250 column is shown in Fig. 1. Calculate K_d (*see* Note 1) for each protein, and plot log mol wt against K_d. A typical plot is shown in Fig. 2.
4. Inject a solution (20 µL) of the protein of unknown mol wt and measure its elution volume, V_e, from the absorbance profile. If the sample

Table 1
Protein Standards

Protein[a]	Mol Wt
Thyroglobulin	669,000
Apoferritin	443,000
β-Amylase	200,000
Immunoglobulin G	160,000
Alcohol dehydrogenase	150,000
Bovine serum albumin	66,000
Ovalbumin	42,700
β-Lactoglobulin	36,800
Carbonic anhydrase	29,000
Trypsinogen	24,000
Soybean trypsin inhibitor	20,100
Myoglobin	16,900
Ribonuclease A	13,690
Insulin	5,900
Glucagon	3,550

[a]All proteins listed were obtained from Sigma, Poole, England.

contains more than one protein and the peaks cannot be assigned with certainty, collect fractions and assay each fraction for the relevant activity.

5. Calculate K_d for the unknown protein and use the calibration plot to obtain an estimate of its mol wt.

4. Notes

1. The support used in size-exclusion chromatography consists of particles containing pores. The molecular size of a solute molecule determines the degree to which it can penetrate these pores. Molecules that are wholly excluded from the packing emerge from the column first, at the void volume, V_o. This represents the volume in the interstitial space (outside the support particles) and is determined by chromatography of very large molecues, such as blue dextran or DNA. Molecules that can enter the pores freely have full access to an additional space, the internal pore volume, V_i. Such molecules emerge at V_t, the total volume available to the mobile phase, which can be determined from the elution volume of small molecules. Hence $V_t = V_o + V_i$. A solute molecule that is partially restricted from the pores will emerge with elution volume, V_e, between the two extremes, V_o and V_t. The distribution coefficient, K_d, for such a molecule represents the fraction of V_i available to it for diffusion. Hence:

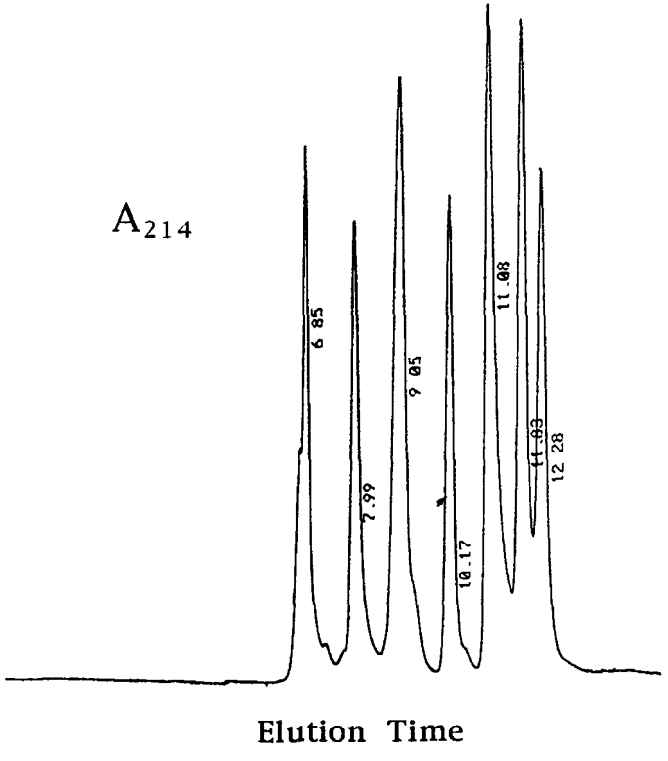

Fig. 1. Separation of a mixture of seven solutes on a Zorbax Bio-series GF-250 column. Twenty microliters of a mixture containing about 1.5 μg of each protein were injected The solutes were, in order of elution, thyroglobulin, alcohol dehydrogenase, ovalbumin, myoglobin, insulin, glucagon, and sodium azide. The number beside each peak is the elution time in minutes. The absorbance of the highest peak, insulin, was 0 105 The equipment was a Model 501 Pump, a 441 Absorbance Detector operating at 214 nm, a 746 Data Module, all from Waters (Millipore, Milford, MA) and a Rheodyne model 7125 injector (Rheodyne, Cotati, CA) with 20 μL loop. The column was a Zorbax Bio-Series GF-250 with guard column (Dupont, Wilmington, DE). The flow rate was 1 mL/min, and the chart speed was 1 cm/min. The attenuation setting on the Data Module was 128.

$$V_e = V_o + K_d \cdot V_t \tag{1}$$

and

$$K_d = (V_e - V_o)/V_t = (V_e - V_o)/(V_t - V_o) \tag{2}$$

2. Silica to which a hydrophilic phase, such as a diol, has been bonded still contains underivatized silanol groups. Above pH 3, these are largely anionic and will interact with ionic solutes, leading to nonideal size-

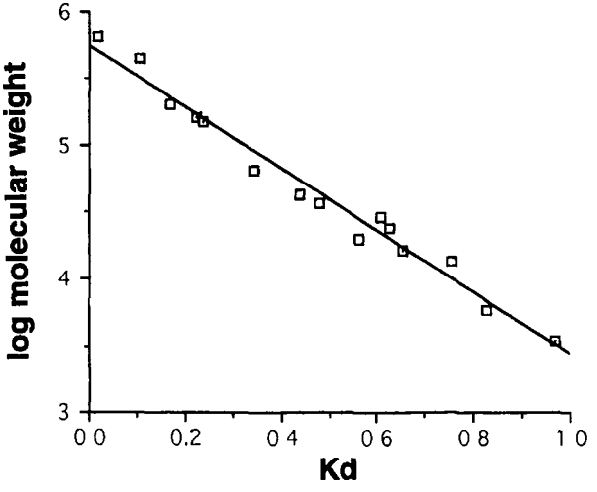

Fig. 2. Plot of log mol wt vs K_d for the proteins listed in Table 1. Chromatography was carried out as described in Fig. 1 V_o was determined to be 6.76 mL from the elution peak of blue dextran. V_t was determined from the elution peak of glycine and from the negative peak given by injecting water, both of which gave a value of 11.99 mL. The regression line ($y = 5\ 751 - 2\ 321x$, $r^2 = 0\ 977$) was computed using all the points shown.

exclusion chromatography. Depending on the value of its isoelectric point, a protein can be cationic or anionic at pH 7. Proteins that are positively charged will undergo ion exchange, causing them to be retarded. Conversely, anionic proteins will experience electrostatic repulsion from the pores, referred to as "ion exclusion," and will be eluted earlier than expected on the basis of size alone. When size-exclusion chromatography is carried out at a low pH, the opposite behavior is found, with highly cationic proteins being eluted early and anionic ones being retarded. To explain this behavior, it has been suggested that at pH 2, the column may have a net positive charge *(8)*. In order to reduce ionic interactions, it is necessary to use a mobile phase of high ionic strength. On the other hand, as ionic strength increases, this promotes the formation of hydrophobic interactions. To minimize both ionic and hydrophobic interactions, the mobile phase should have an ionic strength between 0.2 and 0.5*M (9)*.

3. Problems arising when size-exclusion chromatography is carried out under denaturing conditions include:
   a. For a particular column, the mol-wt range in which separation occurs is reduced. This is because the radius of gyration, and hence the

hydrodynamic size, of a molecule increases when it changes from a sphere to a random coil. For example, the separation range of a TSK G3000SW column operating with denatured proteins is 2000–70,000, compared to 10,000–500,000 for native proteins *(5,10)*. Of course, this may actually be an advantage when working with small proteins or peptides.

b. Proteins are broken down into their constituent subunits and polypeptide chains, so that the mol wt of the intact protein is not obtained.
c. Bioactivity is usually destroyed or reduced, and it is not usually possible to monitor enzyme activity. This can be a serious disadvantage when trying to identify a protein in an impure preparation.
d. The denaturants usually absorb light in the far UV range, so that monitoring the absorbance in the most sensitive region for proteins (200–220 nm) is no longer possible.
e. Manufacturers often advise that once a column has been exposed to a mobile phase containing denaturants, it should be dedicated to applications using that mobile phase, since the properties of the column may be irreversibly altered. In addition, the denaturant, especially if it is sodium dodecylsulfate, may be difficult to remove completely.
f. Since these mobiles phases have high viscosities, flow rates may have to be reduced to avoid high back pressures.
g. High concentrations of salts, especially those containing halide ions, can adversely affect pumps and stainless steel.

4. Most manufacturers sell guard columns appropriate for use with their size-exclusion columns. In order to protect the expensive size-exclusion column it is strongly recommended that a guard column be used.
5. Even in the presence of high-ionic-strength buffers, several proteins show nonideal behavior and are thus unsuitable as standards. For example, the basic proteins cytochrome c ($pI \approx 10$) and lysozyme ($pI \approx 11$) have K_d values >1.0, under the conditions described for Fig. 1, because ion-exchange interactions are not totally suppressed. On the other hand, the very acidic protein pepsin ($pI \approx 1$) emerges earlier than expected on the basis of size, because of ion exclusion. One should be aware that such behavior may also occur when interpreting results for proteins of unknown pI.
6. Columns are often stored in 0.02% sodium azide to prevent bacterial growth. When changing mobile phase, some manufacturers recommend that the flow rate should not be greater than half the maximum flow rate.
7. Although V_o is most commonly measured using blue dextran, Himmel and Squire suggested that it is not a suitable marker for the TSK G3000SW column, because of tailing under nondenaturing conditions,

and measured V_o using glutamic dehydrogenase from bovine liver (Sigma Type II; mol wt 998,000) *(11)*. Calf thymus DNA is also a commonly used marker for V_o.

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CHAPTER 31

Peptide Purification by Reverse-Phase HPLC

Chris Shaw

1. Introduction

The increasing sophistication and sensitivity of microsequencing of peptides and proteins in recent years have presented the biochemist with the feasibility of primary structural determinations on low-picomole quantities of material. This has led to a plethora of structural data in many different areas of interest, which would until now have taken decades of effort to produce. Complementary to the increased sensitivity of analytical technology has been the application of HPLC in the rapid and highly discriminating separation of biomolecules. If such fractionations are interfaced with a specific rapid detection system, such as an immunoassay for the peptide or protein of interest, then purification to homogeneity from a crude extract can be accomplished in a matter of days. Purification to homogeneity is an absolute prerequisite for successful and meaningful structural analyses. Reverse-phase HPLC is usually the technique of choice for isolation procedures, but if complex biological material represents the starting point of an isolation, it is wise to perform a degree of sample clean up and/or concentration initially. Much of what applies to peptide isolation from complex biological material is also relevant to purification of synthetic peptides from resin eluates. In the case of purification of a peptide from a complex biological material, such as the tissue of production, an appropriate quantity of such starting material is essential. Initial pilot studies are required to determine this quantity

From: *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
Edited by J. M. Walker

by employing either immunometric or biometric analysis. Such studies should also determine the extraction medium that produces maximal solubilization and stability of the peptide of interest. Once these factors have been determined, a preparative extraction can be performed. Often a large volume of turbid extracts results, which would be totally unsuitable for HPLC fractionation. Clarification can often be achieved by high-speed centrifugation, and if the extraction medium is organic-solvent-based, concentration can be affected in part by rotary evaporation. Peptides in the remaining solution can be further concentrated by pumping through disposable cartridges of reverse-phase stationary phases arranged in series. Following washing, bound peptides can be eluted with an organic solvent, such as acetonitrile, and the eluate evaporated to near dryness. The peptides that were once contained in perhaps several liters of extraction medium are now contained in a volume of several milliliters. The total peptide concentration in the extract, however, is now very high, remaining unsuitable for HPLC. At this stage, a high-capacity biomolecule separation system such as soft gel permeation chromatography is often employed. This serves several purposes. By choosing an appropriate gel for the peptide of interest, a high degree of purification can be achieved by removal of higher and lower mol-wt contaminants. This fractionation will yield an estimation of molecular mass and may also yield data on peptide heterogeneity. If the gel is eluted with an acid mobile phase, such as acetic acid, fractions containing the peptide of interest can be directly pumped onto a semipreparative HPLC column. Silica is the most commonly employed reverse-phase HPLC support, and this can be modified in many ways with respect to particle size and shape, pore size, and surface chemistry, which is most often C-18, C-8, C-4, or diphenyl. Combining some or all of these attributes in a sequential series of fractionations usually results in purification of the peptide of interest to apparent homogeneity. Column eluates can be monitored simultaneously at various wavelengths. In initial fractionations, fractions can be collected automatically, but as purification proceeds, it is best to collect peaks of absorbance manually. This achieves even higher resolution of peptides with similar retention times. At each stage, fractions or peaks of absorbing material are screened for the peptide of interest until the detection proce-

dure indicates that the material of interest corresponds to a discrete symmetrical peak of absorbance. If the peptide contains tyrosyl or tryptophanyl residues, which absorb strongly at 280 nm, then symmetrical peaks of absorbance at 214 and 280 nm are indicative of a high degree of purity. Structural analysis of an isolated peptide will usually involve an accurate mass determination by some method of mass spectroscopy. This can usually be achieved for 10–50 pmol quantities of peptide. Amino acid composition may be obtained by conventional analysis following acid hydrolysis and derivatization usually with phenylisothiocyanate (PITC) or with phenylthiohydantoin (PTH). These derivatizing reagents react with amino groups and render all amino acids visible in the UV range. Larger quantities of peptide (500–1000 pmol) are usually required for this analysis. Finally, the primary structure of the isolated peptide can be established by automated Edman degradation using a gas-phase sequencer. Sensitivities of such instruments are in the subpicomole range, but usually 200–500 pmol of a peptide are required to establish the full primary structure unequivocally. The quantity of peptide required is often dependent on the mol mass with larger quantities required for longer peptides. Even so, some peptides will require enzymatic fragmentation and sequencing of fragments to determine the full structure. This can be achieved by using one or more highly site-specific endoproteinases, the choice of which is based on preliminary compositional and/or sequence information. By employing such enzymes at 10–50-fold lower molar concentrations than peptide, there is little interference in subsequent structural analyses from amino acids or oligopeptides resulting from enzyme autodigestion. Usually, peptide digests would be repurified by reverse-phase HPLC, and resulting fragments would be separately subjected to structural analyses.

2. Materials

1. All water employed should be of HPLC grade, either commercially available or produced from a purpose-designed HPLC water system. Periodic checks should be made on the quality of all reagents employed by running blank gradients on chromatographic columns to assess for spurious peaks owing to contaminants. (N.B. Some spurious reagent peaks, if different in retention time from peptides of interest, can act as useful internal standards.)

2. Acetonitrile should be of far UV grade or, better still, the even more UV transparent "super gradient" grade as offered by some commercial companies. All HPLC solvents should be filtered and degassed with helium either before daily usage or, as facilitated by some equipment, during chromatography.
3. Several different counterions can be employed, and these should be used in the same concentration (usually 0.1%, v/v) in both aqueous and organic mobile phases. Trifluoroacetic acid, heptafluorobutyric acid, and phosphoric acid are most commonly employed. For peptides of more acidic character, triethylamine can be employed, but must be buffered to a pH consistent with chromatography on silica-based stationary phases (*see* Note 1). All counterions must be of HPLC grade.
4. Avoid the use of glass or polystyrene tubes during chromatography, since these possess charged groups on their surfaces that can result in a high degree of peptide adsorption. This becomes especially evident as the peptide becomes purer. Use uncharged plastic tubes, such as polyethylene or, ideally, polypropylene tubes, throughout.
5. For gel permeation chromatography, a gel with a suitable fractionation range should be chosen. Sephadex G-50 or the Sephacryl equivalent has such a suitable range for most peptides in the range of 1–30 kDa. The elution buffer may vary, but it is important not to add protein since this defeats the purpose of peptide purification. As a consequence, chosen eluants should facilitate high peptide recoveries, and for this reason acetic acid, in the range of 0.5–2*M*; has been used extensively with a high rate of success. The higher molarity solutions inhibit bacterial growth, and once the column has been calibrated with suitable markers, facilitate numerous fractionations.
6. HPLC equipment should include an elution gradient programmer, a solvent delivery system, two detectors, one of which monitors column effluents at 280 nm for detecting peptides containing tyrosine and tryptophan and the other at 214 nm for detecting all peptides. Column effluents should be collected using a programmable fraction collector.
7. HPLC columns can contain C-4, C-8, C-18, or diphenyl-derivatized stationary phases for reverse-phase fractionations. In initial stages, semipreparative columns can be employed (1 × 60 cm or 1 × 30 cm) followed by analytical columns (0.46 × 25 cm) as the peptide becomes purer. For ion-exchange HPLC fractionations, either cation- or anion-exchange columns can be employed, but organic modifiers, such as acetonitrile should be added to both start and elution solvents in the range of 20–30% (v/v).

8. Endoproteinases employed for peptides of more than 30 amino acid residues, which are difficult to sequence directly, should be of sequencing grade. Manufacturer's instructions, supplied with each batch, should be adhered to.

3. Methods

3.1. Preparation of Crude Extract

1. Once suitable pilot experiments have been performed to determine the appropriate extraction medium for a given peptide and the quantity of starting material required, a preparative extraction for peptide purification should be performed. Tissues should be homogenized in extraction medium, and maintaining this at a low temperature (4°C) will aid the inhibition of endogenous peptidase activity. For many peptides, ethanol/0.7*M* HCl (3:1, v/v) is a highly efficient extraction medium, and this should be employed at a ratio of 8 vol/g of tissue or 3 vol/mL of plasma, serum, culture medium, or other biological fluid. Homogenates or other biological fluid extracts should be constantly stirred and kept at 4°C for 12–18 h to ensure efficient solubilization of peptides.
2. The volume of the extract should be reduced by organic solvent evaporation and/or lyophilization where appropriate, and peptides in the remaining solution should be concentrated by pumping through disposable C-18 cartridges arranged in series. Low flow rates of 10–12 mL/h ensure a high degree of adsorption. After washing in 0.1% (v/v) aqueous trifluoroacetic acid, bound peptides can be eluted from the columns with acetonitrile. Eluants can then be evaporated to *near* dryness (*see* Note 2).
3. Peptides generated by chemical or enzymatic cleavage of purified proteins (Chapters 32 and 33) can be subjected to chromatographic fractionation directly.

3.2. Gel Permeation Chromatography

1. Gel permeation chromatography, with a 2*M* acetic acid mobile phase, can be carried out successfully at room temperature with no significant losses in peptides.
2. Prior to loading lyophilized samples onto these columns, reconstitution in mobile phase (2–3 mL) followed by a short high-speed centrifugation step to remove microparticulate matter, is advised since this latter step greatly increases the working life of the column. A 90 × 1.6 cm column should be eluted at a flow rate of 10–12 mL/h to facilitate component partition.

3. Fractions should be collected at 15-min intervals and, after the predetermined total column volume has been collected, a small aliquot of each should be subjected to a suitable detection system, such as bioassay or radioimmunoassay, for the peptide of interest.

3.3. Reverse-Phase HPLC

1. Gel permeation chromatographic fractions containing the peptide of interest should be pooled and, if in an acetic acid mobile phase, can be pumped directly onto a semipreparative reverse-phase HPLC column that has been equilibrated in starting solvent, such as 0.1% (v/v) aqueous TFA. Generally, the size of column chosen at this stage should relate to the mass of starting tissue employed in the extraction, since this reflects the peptide loading, which in turn will affect resolution. Ideally, the column should be at least 1 × 30 cm, preferably 1 × 60 cm, but employment may be affected by the capital cost. For peptides >4 kDa in mol mass, C-18 chemistry is appropriate at this initial stage, because this then permits the subsequent employment of wide-pore (300 Å) lower carbon-loaded analytical columns. These greatly improve the resolution of peptides of this size. The flow rates employed in this semipreparative stage should be appropriate for column dimensions and are usually in the range of 2–4 mL/min. Gradients are usually linear running from TFA/water (0.1:99.9, v/v) to TFA/water/acetonitrile (0.1:29.9:70.0, v/v/v) in 70 min.
2. To obtain a peptide of apparent homogeneity, sequential chromatography on wide-pore C-8, C-4, or diphenyl analytical columns is required. At each stage, fractions containing the peptide of interest should be pooled and diluted 1:4 with initial aqueous mobile phase to remove the eluting potential of acetonitrile.
3. After pumping of this diluted peptide solution onto the column, one should wait until the absorbance returns to baseline (usually 15–20 min) before initiating the elution gradient. Gradients employed for elution will depend on the hydrophobicity of the peptide of interest. Generally, the gradient should rapidly progress to approx 15% of eluting solvent less than that required for peptide elution and then proceed at approx 0.5% or less of eluting solvent/min. These shallow gradients permit resolution of peptides with similar hydrophobic properties.
4. On analytical columns, low flow rates of 1 mL/min or less favor partition of peptides with similar hydrophobic properties.
5. On all analytical runs, column effluents should be monitored at several wavelengths. Although diode array detection would be the ideal, much

information about peptide composition and degree of purity can be obtained by simultaneous monitoring at 214 and 280 nm either by means of a dual-wavelength detector or two fixed-wavelength detectors arranged in series. Although the 214-nm-detector will detect all peptidic material, the 280 nm detector will indicate the degree of aromaticity of the peptide. With the 280-nm detector set at a sensitivity 10 times higher than the 214-nm detector, a single tyrosyl side chain yields a similar deflection to approximately five peptide bonds. A single tryptophanyl side chain on a peptide yields approximately three times this absorbance. Toward the end of a purification scheme, peaks of peptide absorbance should be collected manually. This ensures that closely eluting contaminants are not collected into the same tube as the peptide of interest, which may occur if automatic fraction collection is employed. The delay between detection and elution from the end of the chromatography tubing can be ascertained in any fixed system by calibration with a known peptide standard, which can be detected in manual fractions either immunochemically or by bioassay.

6. For peptides that are difficult to purify to homogeneity by this standard scheme (i.e., using trifluoroacetic acid as counterion), different counterions, such as phosphoric acid or heptafluorobutyric acid, may be employed (*see* Note 3). These counterions render the peptide more hydrophilic and more hydrophobic, respectively, but also alter the selectivity of peptide interaction with the stationary phase often producing baseline resolution of peptides that cannot be separated conventionally. Shaw et al. *(1)* illustrates the resolution obtained by changing counterions. Ion-exchange HPLC may also be employed in troublesome cases, often with remarkable resolving power (*see* Note 4). Leung et al. *(2)* describe an application of cation-exchange HPLC to solve a problem of peptide purification. Many such columns, however, although excellent for protein chromatography, bind small peptides by hydrophobic interaction in small hydrophobic pockets. These may be difficult to recover. Incorporation of 20–30% acetonitrile in both starting and eluting mobile phases often overcomes this effect. However, if salt or buffer concentration gradients are employed, the concentration in the eluting solvent will be reduced by the presence of organic modifier. This may mean that the final concentration employed may not be of sufficient strength to elute the peptide of interest. If the peptide is eluted from this system, it is usually highly pure if the ion-exchange run has been carried out toward the end of a reverse-phase purification scheme. Ideally, a final reverse-phase fractionation should be carried out to facilitate removal of ion-exchange buffer salts.

7. If peptides are forwarded to a core facility for structural analysis, the samples should be sent in a sealed polypropylene tube in the elution solvent and should not be lyophilized. This may result in significant losses. When received for structural analysis, the sample can be evaporated to a volume appropriate for mass spectroscopy or gas-phase sequencing by direct application. The peptide will thus not have been subjected to lyophilization at any stage of the purification procedure, except when present initially in the crude extract.

3.4. Endoproteinase Digestion

1. In some cases, where the peptide of interest is above 30 amino acid residues in size or of unusual structure, direct gas-phase sequencing may not, because of sequential sequencer losses, result in elucidation of the entire primary structure. Mass spectroscopy data will enable estimation of the approximate size of the segment not sequenced by computation of the mass of the primary structure deduced by the gas-phase sequencer. The primary structural information obtained will enable the choice of a suitable endoproteinase to be made. A range of such endoproteinases is available commercially in highly purified sequencing grades, and the choice will depend on aspects of the primary structure of each individual peptide. Trypsin, chymotrypsin, endoproteinases Asp-N, Glu-C, Lys-C, and Arg-C are a few of those available (*see* Chapter 32).
2. After incubation with a suitable specific endoproteinase, the digest is fractionated by reverse-phase HPLC, and peptide fragments are collected manually. Each can then be subjected to structural analyses, when the full primary structure of the peptide of interest can be determined (*see* Note 5). McKay et al. *(3)* and Maule et al. *(4)* describe the use of such endoproteinases to elucidate the full primary structure of peptides.
3. In other circumstances, when there is a high degree of probability that an isolated peptide has an N-terminal pyroglutamate group (caused by cyclization of an N-terminal glutamine in the presence of acid, either within secretory granules or in the isolation procedure), incubation of the peptide with pyroglutamate aminopeptidase is a necessary prerequisite to automated Edman degradation, which will not work if the peptide has this blocked N-terminus. The deblocking reaction proceeds rapidly (1 h at 37°C), after which the digest can be directly injected onto a reverse-phase HPLC column. If the same gradient as used in the final purification is employed, then the elution of the peptide a few

minutes earlier is indicative of successful deprotection. Gas-phase sequencing can then be performed. The primary structures of possum *(1)* and frog *(5)* neurotensins were deduced using this methodology.

4. Notes

1. The matrix of silica-based HPLC columns, as indicated in manufacturer's operating instructions, is damaged by mobile phases whose pH is around or above neutral. When employing basic counterions, such as triethylamine, ensure that mobile-phase pH values are below 7. If pH values above this are required, then reverse-phase columns with an organic polymer matrix can be utilized.
2. When lyophilizing extracts, it is important not to achieve complete dryness since this will often produce a protein/peptide pellet that is difficult to redissolve resulting in significant peptide losses.
3. Because of the suppression of 214-nm absorbance of peptides when heptafluorobutyric acid is employed as a counterion, this should be employed during the early stages of fractionation when absorbances are not of importance. Other counterions, such as trifluoroacetic acid or phosphoric acid, can be employed in later stages. However, it is not recommended to employ phosphoric acid at the final stage, especially if the peptide sample is to be lyophilized prior to structural analysis. Phosphoric acid is not volatile, and the resultant high concentration of acid at the latter stages of drying may hydrolyze the sample.
4. Extreme caution should be exercised when employing ion-exchange HPLC columns. Such columns are normally stored in a high concentration of organic solvent (20–50%) containing a weak solution of appropriate counterion. Always ensure that columns are washed clean of high molarity elution buffers prior to storage, or precipitation in the pumps and mixing chambers may occur. This may also occur on the column and, if the column is in a glass package, explosive shattering may occur.
5. The use of endoproteinases and the methodology for isolation of peptide fragments described in this section can also be employed for primary structural analysis of large proteins. Fragments of large proteins may also be generated by chemical means, such as by cyanogen bromide. For endoproteinase digestion, sequencing grades of appropriate enzymes should be employed to ensure against the risk of aberrant cleavages.
6. A schematic representation of a hypothetical novel neuropeptide isolation and characterization is shown in Figs. 1–4.

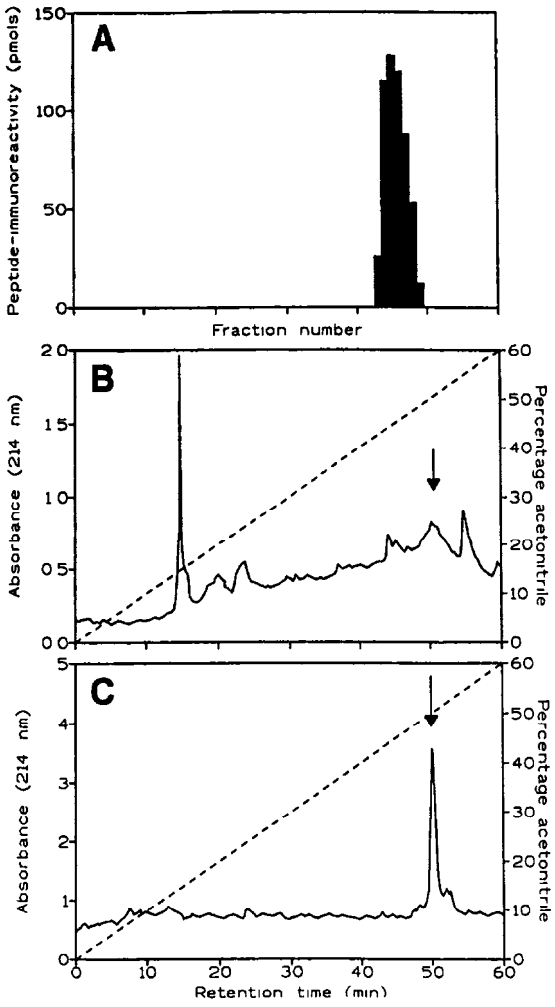

Fig. 1. (A) Gel permeation chromatogram (Sephadex G-50 [fine]; 90 × 1.6 cm; mobile-phase 2*M* acetic acid; flow rate 10 mL/h) of a crude tissue extract containing a novel neuropeptide. Fractions containing the neuropeptide were localized by radioimmunoassay of a small aliquot of each fraction. The antiserum employed for radioimmunoassay was raised to a known peptide and crossreacted with the novel neuropeptide. (B) Semipreparative (C-18) reverse-phase HPLC chromatogram (Whatman Partisil ODS3; 60 × 1 cm) of gel permeation chromatographic fractions containing neuropeptide immunoreactivity. Aliquots of fractions were screened by radioimmunoassay as before, and the retention time of the novel neuropeptide is indicated by an arrow. (C) Analytical (C-8) reverse-phase HPLC chromatogram (Vydac 208TP54; 25 × 0.46 cm) of the novel neuropeptide resolved by semipreparative chromatography Immunoreactivity is indicated by an arrow. HPLC elution gradients are indicated by hatched lines, and the starting solvent in each case was 0 1% (v/v) aqueous TFA.

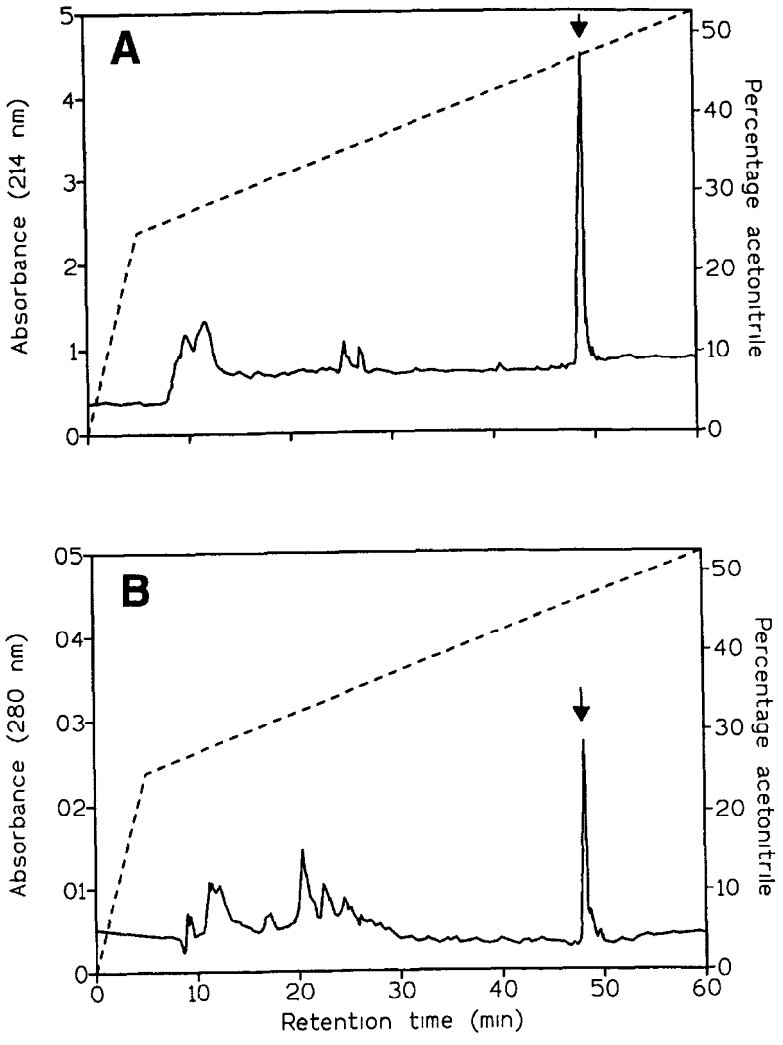

Fig 2. Analytical (C-4) reverse-phase HPLC chromatogram (Vydac 214TP54; 25 × 0.46 cm) of the final fractionation of the novel neuropeptide. The column effluent was monitored at 214 nm (**A**) and 280 nm (**B**). Note the 10-fold difference in sensitivity of both wavelengths. Peaks of absorbing material were collected manually, and immunoreactivity corresponded to the major peak of absorbance, which is marked by an arrow. The 280-nm absorbance of the peptide indicates the presence of aromatic amino acid residues, and the symmetry of absorbance peaks at both wavelengths indicates a high degree of purity The elution gradient is indicated by the hatched line, and the starting solvent was 0.1% (v/v) aqueous TFA

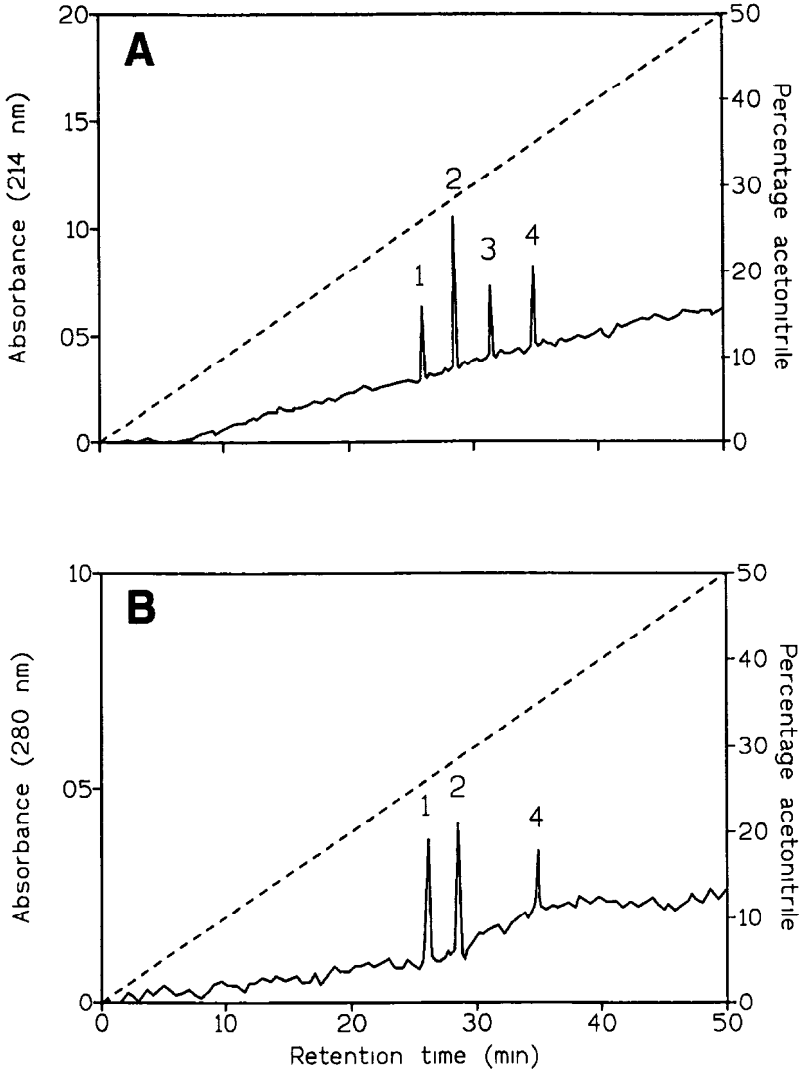

Fig 3. Analytical (C-8) reverse-phase HPLC chromatogram (Vydac 208TP54; 25 × 0.46 cm) of an endoproteinase Glu-C digest of the novel neuropeptide purified to apparent homogeneity as illustrated in the previous figure. The absorbance of the column effluent was monitored at 214 nm **(A)** and 280 nm **(B)** Four fragments (F1–F4) of the novel neuropeptide were baseline-resolved, and note that F3 has no absorbance at 280 nm, indicating that it does not contain aromatic amino acid residues The structural characterization of these fragments is schematically represented in Fig. 4. The elution gradient is indicated by a hatched line, and the starting solvent was 0 1% (v/v) aqueous TFA.

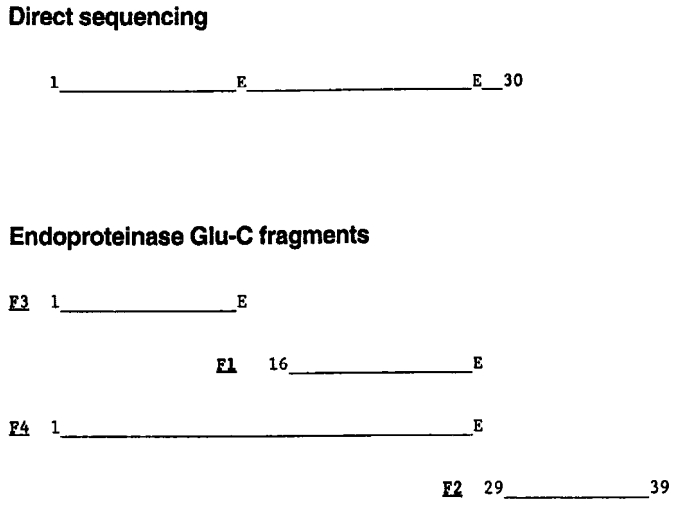

Fig. 4. Residues 1–30 of a novel neuropeptide were assigned following a single direct gas-phase sequencing run The computed molecular mass of this peptide was less than that derived by mass spectroscopy, indicating that 9–12 amino acid residues at the C-terminal end of the peptide had not been obtained. Two glutamic acid residues (E in single-letter notation) were present at positions 15 and 29, respectively. A second batch of peptide was digested with endoproteinase Glu-C, and the profile of peptide fragments obtained is shown in Fig. 3. Sequencing of the peptide fragments F1–F4, as indicated schematically, resulted in the deduction of the full primary structure of a 39 amino acid residue peptide whose computed mol mass was in agreement with that obtained by mass spectroscopy.

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CHAPTER 32

Enzymatic Methods for Cleaving Proteins

Bryan John Smith

1. Introduction

There could be various reasons why a protein chemist may want to break a protein of interest into fragments, but foremost among them must be the purposes of peptide mapping, primary structure determination and preparation of particular fragments for studies relating structure and function. Proteinases (or more specifically endproteinases) are commonly used to generate peptides for these purposes, but the number of particularly useful and commercially available proteinases is not great. This is because enzyme specificity is an important consideration—the laboratory worker generally requires good yields of clean, pure peptides, but significantly less than 100% cleavage at some or all sites of action gives rise to a complex mixture of a large number of polypeptides.

One of the best characterized and widely used proteinases is trypsin (EC 3.4.21.4), and it is the use of this enzyme that is described below. Other useful enzymes for cleaving proteins are described in Section 4. Trypsin is synthesized as trypsinogen by the vertebrate pancreas, but rapidly becomes converted by the removal of the amino-terminal hexapeptide to the active enzyme of approx 23,500 Dalton. It has a serine at its active site, and therefore belongs to the serine proteinase family. Trypsin displays good specificity, catalyzing the hydrolysis of the peptide bond to the COOH side of lysyl and arginyl residues. If an acidic residue occurs to either side of the basic residue, hydrolysis

From: *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
Edited by: J. M. Walker

is slower, and if the residue to the COOH side is proline, hydrolysis is very slow. Polylysine sequences may also be fully cleaved with difficulty. Trypsin is optimally active at about pH 8.

2. Materials (for Trypsin)

1. Trypsin (EC 3.4.21.4) is available from various commercial suppliers. It is stable for periods of years as a dry solid at –20°C.
2. Stock solutions:
   a. Trypsin, 1 mg/mL in distilled water. Use fresh or divide into aliquots and store frozen. A stock solution may be thawed and refrozen several times, but for consistent results, thaw once only.
   b. Ammonium bicarbonate (0.4*M*) in distilled water, pH ≈8.5. May be stored refrigerated or frozen for long periods

3. Method (for Trypsin)

1. Dissolve the substrate in water to 2 mg/mL. Add an equal volume of 0.4*M* ammonium bicarbonate solution. Add trypsin solution to an enzyme/substrate ratio of 1/50 (w/w), i.e., to 1 mL of 1 mg/mL substrate solution add 20 µL of 1 mg/mL of trypsin solution.
2. Incubate at 37°C for 24 h.
3. Terminate digestion by immediate submission to peptide mapping or isolation techniques, acidification, freeze-drying, or addition of specific inhibitor (e.g., Nα-tosyl-L-lysyl chloromethyl ketone, or TLCK, in molar excess to the trypsin used). As with other serine proteinases, trypsin may be inactivated by reaction of the serine residue at the active site with phenylmethylsulfonyl fluoride (PMSF). Prepare this agent just before use by dissolving in propan-2-ol to a 1*M* solution. Dilute 1000-fold to 1 m*M* in the reaction mixture. It is effective within a few minutes. Beware of the toxic nature of PMSF.

4. Notes

1. Various grades of trypsin are available commercially. Most are from bovine pancreas. That which has been treated with L-1-chloro-3-tosylamido-4-phenylbutan-2-one (or TPCK), or similar, is recommended since this treatment specifically inhibits chymotrypsin, which may contaminate trypsin preparations. Enzyme activity may vary, in detail, from source to source or batch to batch. Attention to this is recommended if reproducibility is important (e.g., for peptide mapping).
2. During incubation with substrate, trypsin will also undergo autolysis to produce (among other products) ψ-trypsin, which has chymotrypsin-like activity. This may contribute low-frequency "nontryptic" cleav-

ages at some tyrosyl, phenylalanyl, or tryptophanyl bonds. The literature claims that low levels of Ca^{2+} (0.1 m*M* $CaCl_2$) added to digestion buffers may reduce production of ψ-trypsin by autolysis.

3. The buffer described above is a simple volatile buffer that gives an appropriate pH of approx 8. Other buffers of pH 8 may be readily substituted, e.g., 0.05*M* Tris-HCl, pH 8.
4. The method described above is the basic procedure and is subject to great change, according to the requirements of the worker and protein in question. Any one set of experimental conditions will give different results with different substrates, and the reader is encouraged to discover optimal conditions for digestion of a substrate empirically.

   For reproducible peptide mapping, when complete digestion is desirable, prolonged digestion may be required (e.g., 48 h at 37°C, pH 8, with a second addition of trypsin, similar to the first at 24 h). On the other hand, for preferential cleavage of particularly sensitive bonds or for production of partial cleavage products (e.g., overlapping peptides for sequencing purposes or production of folded domains), digestion can be limited by use of a low enzyme/substrate ratio, shorter digestion times, and lower incubation temperatures, and/or the buffer may be adjusted to suboptimal pH. High salt conditions (e.g., 0.5*M* NaCl, in buffer), which favor compact folding of a structured polypeptide chain, may also be used. Such a tightly folded sequence will be more resistant to proteolytic attack than nonstructured regions, though not necessarily completely or indefinitely resistant. Thus, by careful adjustment of buffer and incubation conditions, these partially resistant regions may be prepared and used, for example, in studies on their function.
5. The condition of the substrate is important. First, the substrate should be soluble, or as finely divided as possible, in the digestion buffer. If a sample is not readily soluble in water or ammonium bicarbonate solutions, suitable solvents can be used initially and then adjusted by dilution or titration of pH to allow for trypsin action. If the polypeptide remains insoluble, the precipitate should be kept in suspension by stirring. Thus, 8*M* urea may be used to solubilize a protein or disrupt a tightly folded structure, and then diluted to 2*M* urea for digestion by trypsin. Trypsin will also function in 2*M* guanidinium chloride, in the presence of sodium dodecylsulfate (SDS, e.g., 0.1% [w/v]), or in the presence of acetonitrile (up to about 50% [v/v]), so that fractions from reverse-phase HPLC (acetonitrile gradients in water/trifluoroacetic acid, 0.1% [v/v]) may be readily digested after simple addition of ammonium bicarbonate or other neutralization, and dilution (if necessary to lower the acetonitrile concentration).

Second, a native protein may be tightly folded, such as to markedly slow up or inhibit proteolytic attack. To remedy this, the substrate may be denatured and the structure opened out to allow for access of the proteinase. This may be done by boiling in neutral pH solution, or by use of such agents as urea, SDS, or organic solvent, as described above. Low concentrations of these agents (e.g., 5–10% [v/v] acetonitrile) may give more rapid digestion than will a buffer without them, but high concentrations (e.g., 50% [v/v] acetonitrile) will slow the digestion.

An additional, and very common, technique is reduction and carboxymethylation, i.e., permanent disruption of disulfide bonds. This treatment opens out the protein structure to allow for ready digestion and to minimize complications in peptide separation that are caused by pairs (or greater) of peptides remaining connected by S—S bonds. This treatment is carried out as described in Chapter 34. Alternatively, if a sample has already been digested, S—S bonds may be reduced by simple addition of small amounts of solid dithiothreitol and incubation at room temperature (and pH 8) for 30 min or so. This treatment is followed immediately by HPLC to separate the various peptides. The amount of dithiothreitol required (i.e., slight molar excess over S—S bonds) may be calculated accurately if the cystine content of the protein concerned is known.

6. The result of proteolytic cleavage may be monitored, and the resulting peptides purified, by various forms of electrophoresis and chromatography, as described in other chapters in this series. Typical HPLC conditions might be, for example, reverse-phase chromatography on a 4 mm id C-18 or C-8 column, using a gradient of 0–100% (v/v) acetonitrile in water, 0.1% (v/v) trifluoroacetic acid, at a flow rate of 1 mL/min at room temperature (*see* Chapter 31).
7. During digestion, autolysis may produce a low background of peptides from the proteinase itself. If this is to prove a problem, enzyme-only controls are recommended. The primary structure of trypsin is known (e.g., *see* the structure of pig trypsinogen given in ref. *1*), so the products of autolysis should not prove misleading to the protein sequencing fraternity! A form of trypsin is now available which has been alkylated rendering it less susceptible to autolysis and providing it with greater stability.
8. Lysine is a fairly common constituent of proteins, and digestion with trypsin can generate a large number of peptides of small average size. This is a good point when peptide mapping, but some purposes, such as sequencing, may require longer peptides. The action of trypsin may

be modified in an attempt to achieve this. It is done by modification of the side chains of lysyl or arginyl residues in the substrate as described in ref. *2* and Chapter 34, such that cleavage only occurs at unmodified residues. Perhaps the most common such method is succinylation of lysyl side chains, leading to tryptic cleavage at arginyl (and any remaining unmodified lysyl) residues (*see* Chapter 34).

Introduction of additional sites of cleavage by trypsin may be achieved by conversion of cysteinyl residues. to aminoethyl cysteinyl residues by reaction with ethyleneimine as described in Chapter 34.

9. The need for substrate modification as described above has been reduced by the commercial advent of proteinases with good specificity for either lysyl or arginyl residues, namely endoproteinases Lys-C and Arg-C (*see* Table 1).

   Clostripain is also reported to cleave to the COOH side of arginyl residues, but also may cleave (less frequently) to the COOH side of lysyl residues. It will, however, cleave Arg-Pro bonds.

10. There are a large number of proteinases available commercially. Data on some of the more useful ones are given in Table 1, summarized from personal observation, published data, and suppliers' information. They may be used in much the same manner as is trypsin, with due attention paid to buffer (pH) conditions and methods of termination of digestion.

    Thus, for pepsin (active in the range pH 1–5), the volatile buffer 5% (v/v) acetic acid in water may be used. To stop digestion, raise the pH beyond pH 5 and/or freeze dry.

    Proteinases such as clostripain containing a sulfhydryl group in the active site require activation by inclusion in the buffer of a sulfhydryl agent, such as dithothreitol (1–10 m*M*) and may be inactivated by addition of iodoacetic acid in molar excess to the sulfhydryl agent present.

    Metalloproteinases require the presence of metal ions. Thus, thermolysin requires Ca^{2+}. Preparations of this enzyme, or of a substrate, may themselves contain sufficient metals ions, but otherwise the Ca^{2+} should be added to the buffer to a concentration of at least 5 m*M*. Reaction may be ended by addition of EDTA in excess to the metal ion concentration (remembering that sample and enzyme may contribute metal also).

    Most of the common proteinases are serine proteinases (*see* Table 1) and may be inactivated by reaction with PMSF as described above for trypsin. In addition to these general methods for inhibiting digestion, inhibitors specific for various proteinases are available from commercial sources (*see* suppliers' literature).

Table 1
Summarized Data on Some Commercially Available Endoproteinases
Suitable for the General of Peptides from Proteins

Endoproteinase	EC no.	Type of proteinase	Apparent mol wt	pH optimum	Specificity[a]
Chymotrypsin	3.4.21.1	Serine	25,000	7–9	-X ↓Y- (X = aromatic)
Clostripain	3.4.22.8	-SH	50,000	7.7	-Arg↓Y- (Lys↓Y-, less frequently)
Elastase	3.4.21.36	Serine	25,900	7–9	-X↓Y-(X = uncharged aliphatic)
Endoproteinase Arg-C ("submaxillary gland protease")	3.4.21.40	Serine	30,000	7.5–8.5	-Arg↓Y-; -Lys-Lys↓Y-
Endoproteinase Glu-C	3.4.21.9	Serine	27,000	4 7.8	-Glu↓Y- (not if Glu within two or -Glu↓Y; -Asp↓Y- three residues of the N-Terminus)
Endoproteinase Lys-C (nonreduced) (reduced)	3.4.99.30	Serine	30,000 33,000	8.5–8.8	-Lys↓y
Kallikrein (α Form) (β-Form)	3.4.21.8	Serine	27,000 28,900	7–8	-X-Arg↓Y- (preferentially if X = Phe, Leu)
Papain	3.4.22.2	-SH	23,000	6–7	-X ↓Y- (preferentially if X = Arg, Lys, Gln, His, Gly, Tyr)
Pepsin	3.4.23.1	Acid	34,500	1.8–2.2	-X ↓Y- (Y = Hydrophobic; preferentially if X = Phe, met, Leu, Tryp)
Plasmin	3.4.21.7	Serine	85,000	8.9	-Arg↓Y-; Lys↓Y-
Thermolysin	3.4.24.4	Metallo-(Ca^{2+})	37,500	7–9	-Y↓X- (X = Hydrophobic; also less frequently if Gly, Ser, Thre; Not if -Y-X-Pro-)
Thrombin (α Form) (Factor IIa) (β Form)	3 4.21.5	Serine	36,000 38,000	8.2–9	-Arg↓Y
Trypsin	3.4.21.4	Serine	23,500	8	-Arg↓Y-; Lys↓Y-; Aminoethylcysteine↓Y-

[a]Y, unspecific. NB, Frequently, susceptibility to proteolysis is reduced or lost if either the potentially labile bond is linked to proline (e.g., -X-Pro- for chymotrypsin) or is between two like residues (e.g., -Glu-Glu- for endoproteinase Glu-C)

11. The specificities of various enzymes are indicated in Table 1, but in addition, two common (but not generally applicable) rules may be applied:
    a. Bonds involving prolyl residues may not be cleaved, e.g., endoproteinases Glu-C will commonly not cleave Glu-Pro bonds.
    b. Potentially labile bonds in a multiple sequence may not each be cleaved in good yield, e.g., Glu-Glu-X for endoproteinase Glu-C.
12. One of the most useul proteinases is endoproteinase Glu-C (or "V8 protease") isolated from *Staphylococcus aureus* strain V8, since like trypsin and endoproteinases Lys-C and Arg-C, it shows good specificity. It cuts to the COOH side of glutamyl residue. A lower frequency of cleavage to the COOH side of aspartyl residues may also occur at neutral pH, although at pH 4 this may not occur. Endoproteinase Glu-C functions in buffers containing 0.2% (w/v) SDS or 4*M* urea. Its sequence is known *(3)*.
13. Other proteinases are of broader specificity and may be affected by surrounding sequences. Their action is therefore difficult to predict. In particular instances, however, their observed action may prove beneficial perhaps cleaving at one or a few particularly sensitive sites when incubated in suboptimal conditions (e.g., short duration digestion or nondenatured substrate).

    Good examples of this come from work on preparation of $F(ab)_2$, antigen-binding fragments of immunoglobulin IgG, that are bivalent and lack the constant, Fc region of the molecule. Incubation of nondenatured IgG molecules with a proteinase of broad specificity can lead to proteolytic cleavage at a few sites or a single site in good yield. Pepsin has been used for this purpose (e.g., pH 4.2–4.5; enzyme/substrate, 1/33 [w/w]; 37°C *[4]*). Different subclasses of mouse IgG were found to be digested at different rates, however, in the order IgG3 > IgG2a > IgG1. Different antibodies of the same subclass may also be degraded differently, some rapidly and without formation of $F(ab')_2$ *(4)*. Papain has been used to prepare $F(ab')_2$ fragments from the IgG1 subclass, which is the subclass that is most resistant to pepsin. The method described by Parham et al. *(5)* uses papain (which has been activated just before use by reaction with cysteine), at pH 5.5 (0.1*M* acetate, 3 m*M* EDTA) 37°C, with an IgG concentration of about 10 mg/mL. The enzyme is added at time 0, and again later (e.g., at 9 h) to an enzyme/substrate ratio of 1/20 (w/w). Digestion can be halted by addition of iodoacetamide (30 m*M*) *(5)*. Rousseaux et al. *(6)* also describe conditions for generating rat $F(ab')_2$, using papain (in the presence of 10 m*M* cysteine), pepsin, or endoproteinase Glu-C (V8 protease). Incubation of the IgG1 and 2a subclasses at pH 2.8 prior to

digestion with pepsin improved the yields of $F(ab')_2$ fragments, presumably because the proteins thus denatured were effectively better substrates.

14. Two enzymes of broader specificities are worthy of further mention. The first is thermolysin, for its good thermostability, which may prove useful when keeping awkward substrates in solution. Thermolysin remains active at 80°C or in 8*M* urea. The second enzyme is pepsin, which acts at low pH. Disulfide bonds rearrange less frequently in acid than in alkaline conditions, so use of low pH buffers may not only help solubilize a substrate, but may also help preserve naturally disulfide-bonded pairs of peptides. Endoproteinase Glu-C may also be used at low pH, having an optimum at pH 4.
15. For more information on the various proteinases available, the reader is guided to works such as those by Allen *(7)*, Wilkinson *(8)* (and references therein), as well as to the commercial suppliers of the enzymes.
16. Exoproteinases are not discussed here, since their use generally does not generate clean peptides.
17. Further details on the characteristics and use of proteolytic enzymes can be found in ref. *9*.

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Chapter 33

Chemical Cleavage of Proteins

Bryan John Smith

1. Introduction

There is a variety of chemical reactions known to result in the cleavage of the peptide bond. Some are nonspecific—for instance, 6*M* hydrochloric acid at 110°C for 24 h hydrolyzes a polypeptide to a mixture of single amino acids. Others show some discrimination, however, as to the precise nature of the amino acid residues around the bond to be broken. Some of these methods are sufficiently specific to be of use, for instance, for generating peptides for primary structure determination. These methods usefully augment those that use proteolytic enzymes (*see* Chapter 32), especially since they tend to act at positions occupied by less common amino acids and so generate large peptides.

The efficiency of the cleavage reaction is an important factor when considering which method to use for generating peptides from proteins. It is an unfortunate aspect of chemical methods of proteolysis that, in the main, they afford significantly less than 100% yields and commonly exhibit undesirable side reactions. One of the best current methods is cleavage of the bond to the COOH-side of methionyl residues by treatment with cyanogen bromide; yields are 90–100% with few side reactions. It is a well-established and well-used method. A newer technique for cleavage of the bond to the COOH side of tryptophanyl residues also involves treatment with cyanogen bromide, after an oxidation step using dimethylsulfoxide in acid. This method too is highly specific and efficient. Most other methods are less effi-

From: *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
Edited by: J. M Walker

cient, but still may be of some use for cleavage of small substrates in which problems of poor yields and side reactions are minimized. One such method is limited hydrolysis in dilute acid. This method, together with the two mentioned above, has the advantage of requiring reagents that are stable and pure enough as supplied commercially. The methods are also relatively simple and adaptable to large- and small-scale cleavages. These three methods are described below in some detail. Other available methods are described in Section 4.4.

2. Materials

2.1. Cleavage at Met-X Bonds

1. Ammonium bicarbonate (0.4*M*) solution in distilled water. Stable for weeks in refrigerated stoppered bottle.
2. β-Mercaptoethanol. Stable for months in dark, stoppered, refrigerated bottle.
3. Formic acid, minimum assay 98%. Aristar grade.
4. Cyanogen bromide. Stable for months in dry, dark, refrigerated storage. Warm to room temperature before opening. Use only white crystals, not yellow ones. **Beware of the toxic nature of this reagent.**
5. Sodium hypochlorite solution (domestic bleach).
6. Equipment includes a nitrogen supply, fume hood, and suitably sized and capped tubes (e.g., Eppendorf microcentrifuge tubes).

2.2. Cleavage at Tryp-X Bonds

1. Oxidizing solution: 30 vol glacial acetic acid, 15 vol 9*M* HCl, and 4 vol dimethylsulfoxide. Use best grade reagents. Though each of the constituents is stable separately, mix and use the oxidizing solution when fresh.
2. Ammonium hydroxide (15*M*).
3. Cyanogen bromide solution in formic acid (60%): Formic acid (minimum assay 98%, Aristar grade). Make to 10 mL with distilled water. Use fresh. Add cyanogen bromide to a concentration of 0.3 g/mL. Use fresh.

   Store cyanogen bromide refrigerated in the dry and dark, where it is stable for months. Use only white crystals. **Beware of the toxic nature of this reagent.**
4. Sodium hypochlorite solution (domestic bleach).
5. Equipment includes a fume hood and suitably sized capped tubes (e.g., Eppendorf microcentrifuge tubes).

2.3. Cleavage of Asp-X Bonds

1. Dilute hydrochloric acid (approx 0.013*M*) pH 2 ± 0.04: Dilute constant boiling (6*M*) HCl (220 µL) to 100 mL with distilled water.
2. Pyrex glass hydrolysis tubes.
3. Equipment includes a blowtorch suitable for sealing the hydrolysis tubes, a vacuum line, and an oven for incubation of samples at 108°C.

3. Methods

3.1. Cleavage at Met-X Bonds (see Notes 1–6)

1. Reduction:
   a. Dissolve the polypeptide in water to between 1 and 5 mg/mL, in a suitable tube. Add 1 vol of ammonium bicarbonate solution, and add β-mercaptoethanol to between 1 and 5% (v/v).
   b. Blow nitrogen over the solution to displace oxygen, seal the tube, and incubate at room temperature for approx 18 h.
2. Cleavage:
   a. Dry down the sample under vacuum, warming if necessary to help drive off all of the bicarbonate. Any remaining ammonium bicarbonate will form a nonvolatile salt on subsequent reaction with formic acid.
   b. Redissolve the dried sample in formic acid (98%) to 1–5 mg/mL. Add water to make the acid 70% (v/v) finally.
   c. Add excess white crystalline cyanogen bromide to the sample solution, to between 2 and 100-fold molar excess over methionyl residues. Practically, this amounts to approximately equal weights of protein and cyanogen bromide. To very small amounts of protein, add one small crystal of reagent. Carry out this stage in the fume hood.
   d. Seal the tube and incubate at room temperature for 24 h.
   e. Terminate the reaction by drying down under vacuum. Store samples at –10°C or use immediately.
   f. Immediately after use, decontaminate spatulas, tubes, and so on, that have contacted cyanogen bromide, by immersion in hypochlorite solution (bleach) until effervescence stops (a few minutes).

3.2. Cleavage at Tryp-X Bonds (see Notes 7–14)

1. Oxidation: Dissolve the sample to approx 0.5 nmol/µL in oxidizing solution (e.g., 2–3 nmol in 4.9 µL oxidizing solution). Incubate at 4°C for 2 h.
2. Partial neutralization. To the cold sample, add 0.9 vol of ice cold NH_4OH (e.g., 4.4 µL of NH_4OH to 4.9 µL oxidized sample solution). Make this addition carefully so as to maintain a low temperature.

3. Cleavage. Add 8 vol of cyanogen bromide solution. Incubate at 4°C for 30 h in the dark. Carry out this step in a fume hood.
4. To terminate the reaction, lyophilize the sample (all reagents are volatile).
5. Decontaminate equipment, such as spatulas, that have contacted cyanogen bromide, by immersion in bleach until the effervescence stops (a few minutes).

3.3. Cleavage at Asp-X Bonds (see Notes 15–18)

1. Dissolve the protein or peptide in the dilute acid to a concentration of 1–2 mg/mL in a hydrolysis tube.
2. Seal the hydrolysis tube under vacuum—i.e., with the hydrolysis (sample) tube connected to a vacuum line, using a suitably hot flame, draw out and finally seal the neck of the tube.
3. Incubate at 108°C for 2 h.
4. To terminate the reaction, cool and open the hydrolysis tube, dilute the sample with water, and lyophilize.

4. Notes

4.1. Cleavage at Met-X Bonds

1. The mechanism of the action of cyanogen bromide on methionine-containing peptides is shown in Fig. 1. For further details, see the review by Fontana and Gross *(1)*. Methionine sulfoxide does not take part in this reaction. An acid environment is required to protonate basic groups and so prevent reaction there and maintain a high degree of specificity. Met-Ser and Met-Thr bonds may give less than 100% yields of cleavage because of the involvement of the β-hydroxyl groups of seryl and threonyl residues in alternative reactions, which do not result in cleavage *(1)*.
2. Although the specificity of this reaction is excellent, some side reactions may occur. This is particularly so if colored (yellow or orange) cyanogen bromide crystals are used, when destruction of tyrosyl and tryptophanyl residues may occur.

   The acid conditions employed for the reaction may lead to small degrees of deamidation of side chains and cleavage of acid-labile bonds, e.g., Asp-Pro. A small amount of oxidation of cysteine to cysteic acid may occur, if these residues have not previously been reduced and carboxymethylated (*see* Chapter 34).

   In great excess of cyanogen bromide (1000-fold or more) oxidation of methionine can occur. This phenomenon can be used to effect cleavage at tryptophanyl (but not methionyl sulfoxide residues). To promote this reaction, strong acid (heptafluorobutyric acid) is used, but the yield from this method is poor (*see* Table 1), and tyrosyl residues become brominated.

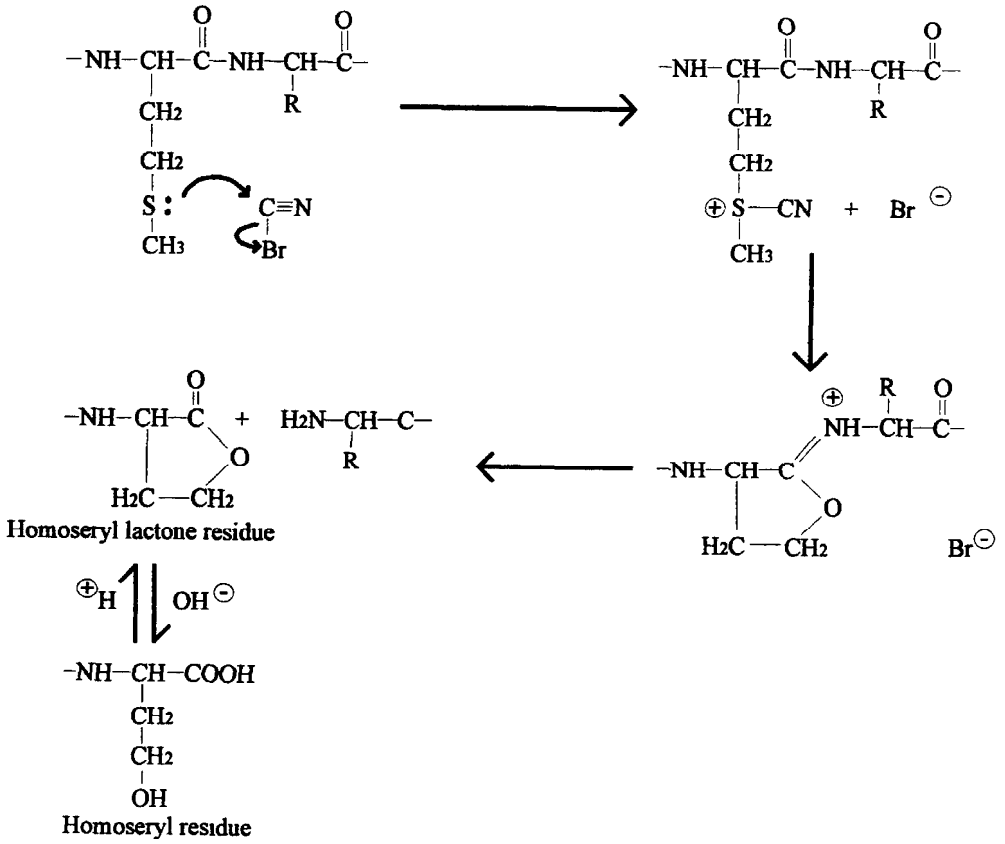

Fig. 1 Mechanism of cleavage of Met-X bonds by cyanogen bromide

Table 1
Summary of Chemical Method for Specific Cleavage of Peptide Bonds

Site of cleavage	Brief method details	Comments	Example Ref.
Asp↓X	Incubation in dilute acid (pH2), high temperature	Some X-Asp bonds cleaved in lesser yield. Rate of cleavage dependent on various factors (e.g , identity of X). Side reactions include deamidation. Yield moderate to good, but unpredictable.	*4*
Asp↓Pro	Incubation in 7*M* guanidine HCl, dilute acid (e g , 10% acetic acid adjusted to 2.5 with pyridine), moderate temperature (e.g., 37°C), prolonged incubation (e.g , 24–h).	Some Asp-Pro may remain resistant despite inclusion of guanidine HCl Deamidation may occur. Yields moderate to good.	*6*
Asn↓Gly	Incubation in 2*M* hydroxylamine, 6*M* guanidine HCl (brought to pH 9 with LiOH) 45°C, 4 h.	Some Asn-Gly bonds may remain resistant despite inclusion of guanidine HCl. Specificity generally good, although some Asn-X bonds may cleave.	*7*
X↓Cys	(a) Incubation in 6*M* guanidine HCl, 0.2*M* Tris-acetate, pH8, with excess dithiothreitol. (b) Add 2-nitro-5-thiocyanobenzoic acid (5-fold molar excess over thiols). Adjust pH to 8 *See also* Chapter 38 of this volume. (c) Acidify to pH 4 Dialyze. Lyophilize. (d) To cleave, dissolve in 6*M* guanidine HCl, 0.1*M* sodium borate, pH 9, 12–16 h, 37°C.	For cleavage at cysteine only, not cystine, delete DTT (a) Cysteinyl residue converted to iminothiazolidinyl which blocks sequencing unless converted to residue, alanyl residues by treatment as in ref. *9*. Yield good. *See also* Chapter 38 of this volume.	*8*
His↓X	Incubation in *N*-bromosuccinimide (3-fold molar excess over His, Trp, and Tyr) pH 3–4 (e.g., pyridine-acetic acid, pH 3.3) 1 h, 100°C.	Cleavage at Trp-X and Tyr-X occurs preferentially (*q.v.*) Met and Cys may be oxidized *N*-Bromosuccinimide unstable in storage. Yields moderate to poor. His converted to lactone derivative.	*10*
Met↓X	Incubation in cyanogen bromide (20–100-fold molar excess over Met) in acid (e.g., 70% formic acid), 18–20 h, room temperature in the dark.	Specificity and yield excellent Cys may be slowly oxidized, and Asp-Pro bonds cleaved. Excess reagent can cause degradation of Trp and Tyr side chains. Cyanogen bromide is toxic.	*1*
X↓Ser	(a) Incubation in conc. anhydrous acid (e.g., H_2SO_4, HF or HCOOH) room temperature, few days. (b) Controlled acid hydrolysis (e g , 6*M* HCl, 18°C, 6 h)	Mechanism involved (reversible) N → O peptidyl rearrangement in Ser. Yield moderate. Thr-X bonds cleaved at lower yield, and others nonspecifically at low yield Tyr sulfonation (in H_2SO_4) and Trp destruction may occur.	*11*

X↓Thr	As for X-Ser (N→ O shift mechanism)—*q v.*	*See* Ser-X (N→ O shift mechanism). Yields moderate to poor.	*11*
Tyr↓X	As for His-X (*N*-bromosuccinimide)—*q.v*	Tyr-X cleaved more quickly than His-X, more slowly than Trp-X (*q.v.*). Yields moderate to poor, or zero if Tyr is at the N-Terminus. Met and Cys may undergo oxidation Tyr converted to lactone derivitive.	*10*
Tyr↓X	Electrolytic oxidation at Pt electrodes in pH 2 2–2 4 (e.g., 10% acetic acid), 4–5 h.	Oxidation of other groups may occur Yields moderate to poor.	*12*
Trp↓X	Incubation in BNPS-skatole (100-fold molar excess over Trp) in 50% acetic acid (v/v) room temperature, 48 h, in the dark.	Reagent is unstable—fresh reagent required to minimize side reactions. Some reaction with Tyr may occur. Met and Cys may be oxidized. Yields moderate to good.	*13*
Trp↓X	As for His-X (*N*-bromosuccinimide)—*q.v.*	Also less rapid cleavage at His -X and Tyr-X (*q.v.*). Yields moderate to poor. Trp converted to lactone derivative.	*10*
Trp↓X	Incubation in very large (up to 10,000-fold) molar excess of cyanogen bromide over Met, in heptafluorobutyric acid/88% HCOOH (1/1 [v/v]), room temperature, 24 h, in the dark	To inhibit Met-X cleavage, Met is photoxidized irreversibly to Met sulfone Yield poor.	*14*
Trp↓X	(a) Incubation in glacial acetic acid. 12*M* HCl (2/1 [v/v]) with phenol and dimethylsulfoxide, room temperature, 30 min. (b) Add HBr and dimethylsulfoxide, room temperature, 30 min	Cys and Met oxidized. Some hydrolysis of acid-labile bonds around Asp may occur, as may deamidation. Fresh (colorless) HBr required Yields moderate. Trp converted to dioxindolalanyl lactone.	*15*
Trp↓X	(a) Incubation in glacial acetic acid 9*M* HCl (2/1 [v/v]) with DMSO, room temperature, 30 min. (b) Neutralization. (c) Incubation with cyanogen bromide in acid (e g , 60% formic acid) 4°C, 30 h in the dark.	Yields and specificity excellent. Met oxidized to sulfoxide	*3*
Trp↓X	Incubation in 80% acetic acid containing 4*M* guanidine HCl, 13 mg/mL iodosobenzoic acid, 20 μL/mL *p*-cresol, for 20 h room temperature, in the dark.	Specificity and yields good. The *p*-cresol is used to prevent cleavage at Tyr Trp converted to lactone derivative.	*16*

3. Acid conditions are required for this reaction. Formic acid is commonly used because it is a good protein solvent and denaturant, but other acids may be used instead if desired, e.g., 0.1*M* HCl; 70% HF. Stronger acid (e.g., 75% TFA; HFBA) may cause increased acid hydrolysis, and so on, as described above.
4. It is possible to cleave Met-X bonds of protein samples that have been applied to glass fiber discs, such as are used in automated protein sequencers of the gas-phase type. The filter is removed from the sequencer and saturated with a fresh solution of cyanogen bromide in 70% formic acid. After 24 h incubation in the dark at room temperature, the filter can be dried under vacuum and then replaced in the sequencer. Yields may be low (>50%), but may prove useful for obtaining protein sequence data from samples that have proven to be blocked, i.e., do not have a free NH_2 group at their N-terminus.
5. As described in Note 1 above, the peptide to the N-terminal side of the point of cleavage has at its C-terminus a homoserine or homoserine lactone residue. The lactone derivative of methionine can be coupled selectively and in good yield *(2)* to solid supports of the amino type, e.g., 3-amino propyl glass. This is a useful technique for sequencing peptides on solid supports. The peptide from the C-terminus of the cleaved protein will, of course, not end in homoserine lactone (unless the C-terminal residue was methionine!) and so cannot be so readily coupled.
6. The reagents used are removed by lyophilization, unless salt has formed following failure to remove all of the ammonium bicarbonate. The products of cleavage may be fractionated by the various forms of electrophoresis and chromatography currently available, as described elsewhere in volumes of this series. It should be remembered, however, that since methionyl residues are among the less common residues, peptides resulting from cleavage at Met-X may be large. Accordingly, in HPLC, for instance, use of wide-pore column materials may be advisable (e.g., 30-μm pore size reverse-phase columns, using gradients of acetonitrile in 0.1% TFA in water).

4.2. Cleavage at Tryp-X Bonds

7. The method described is that of Huang et al. *(3)*. Although full details of the mechanism of this reaction are not clear, it is apparent that tryptophanyl residues are converted to oxindolylalanyl residues in the oxidation step, and the bond to the COOH side at each of these is readily cleaved, in excellent yield (approaching 100% in ref. *3*) by the subsequent cyanogen bromide treatment. The result is seemingly unaffected by the nature of the residues surrounding the cleavage site.

During the oxidation step, methionyl residues are converted to sulfoxides, so bonds at these residues are not cleaved by the cyanogen bromide treatment. Cysteinyl residues will also suffer oxidation if they have not been reduced and alkylated beforehand (*see* Chapter 34).

The peptide to the C-terminal side of the cleavage point has a free N-terminus and so is suitable for sequencing.

8. Methionyl sulfoxide residues in the peptides produced may be converted back to methionyl residues by the action, in aqueous solution, of thiols (e.g., dithiothreitol, as described in ref. *3*, or see use of β-mercaptoethanol above).
9. The acid conditions used for this reaction seem to cause little deamidation *(3)*, but one side reaction that can occur is hydrolysis of acid-labile bonds. The use of low temperature minimizes this problem. If a greater degree of such acid hydrolysis is not unacceptable, speedier and warmer alternatives to the reaction conditions described above can be used as follows:
   a. Oxidation at room temperature for 30 min, but cool to 4°C before neutralization.
   b. Cleavage at room temperature for 12–15 h.
10. As alternatives to the volatile base, NH_4OH, other bases may be used (e.g., the nonvolatile potassium hydroxide or Tris base).
11. As an alternative to formic acid (used in the cleavage step), *5M* acetic acid may be used.
12. Samples eluted from sodium dodecylsulfate (SDS) gels may be treated as described, but for good yields of cleavage, Huang et al. *(3)* recommended that the sample solutions are acidified to pH 1.5 before lyophilization in preparation for dissolution in the oxidizing solution. Any SDS present may help to solubilize the substrate and, in small amounts at least, does not interfere with the reaction. However, nonionic detergents that are phenolic or contain unsaturated hydrocarbon chains (e.g., Triton, Nonidet P-40), and reducing agents, are to be avoided.
13. The method is suitable for large-scale protein cleavage, this requiring simple scaling up. Huang et al. *(3)* made two points, however:
   a. The neutralization reaction generates heat. Since this might lead to protein or peptide aggregation, cooling is important at this stage. Ensure that the reagents are cold and are mixed together slowly and with cooling. A transient precipitate is seen at this stage. If the precipitate is insoluble, addition of SDS may solubilize it (but will not interfere with the subsequent treatment.)
   b The neutralization reaction generates gases. Allow for this when choosing a reaction vessel.

14. The results of cleavage may be inspected by polyacrylamide gel electrophoresis, HPLC, and so on, as after cleavage at Met-X bonds *(see above)*. All reagents in the described method are readily removed during lyophilization.

4.3. Cleavage at Asp-X Bonds

15. The bond most readily cleaved in dilute acid is the Asp-X bond, by the mechanism outlined in Fig. 2(i). The bond X-Asp may also be cleaved, in lesser yields *see* Fig. 2(ii). Thus, either of the peptides resulting from any one cleavage may keep the aspartyl residue at the point of cleavage, or neither might, if free aspartic acid is generated by a double cleavage event. Any of these peptides is suitable for sequencing.

    The amino acid sequence of the protein can affect the lability of the affected bond. Thus, the Asp-Pro bond is particularly labile in acid conditions (*see* Table 1). Again, ionic interaction between the aspartic acid side chains and basic residue side chains elsewhere in the molecule can adversely affect the rate of cleavage at the labile bond. Such problems as these make prediction of cleavage points somewhat problematical, particularly if the protein is folded up (e.g., a native protein). The method may well prove suitable, however, for use in cleaving small proteins or peptides.
16. The conditions of low pH can be expected to cause a number of side reactions: cleavage at glutamyl residues; deamidation of (and subsequent cleavage at) glutaminyl and asparginyl residues; cyclization of N-terminal glutaminyl residues to residues of pyrolidone carboxylic acid; α-β shift at aspartyl residues, creating a blockage to Edman degradation; partial destruction of tryptophan. The short reaction time of 2 h is intended to minimize these side reactions. Loss of formyl and acetyl groups from N-termini *(4)* are other possible side reactions, but these may be advantageous in deblocking otherwise unsequenceable polypeptides.
17. A substrate that is insoluble in cold dilute HCl may dissolve during incubation at 108°C. Alternatively, formic acid, a good protein denaturant and solvent, may be used, as follows: Dissolve the sample in formic acid, 98%, then dilute 50-fold to pH 2.
18. The comments above concerning the effect of the amino acid sequence and of the environment around potentially labile bonds, and the various side reactions that can occur, indicate that the consequences of incubation of a protein in dilute acid are difficult to predict—they are best investigated empirically by monitoring production of peptides by electrophoresis, HPLC, and so on (as suggested for cleavage of Met-X, above).

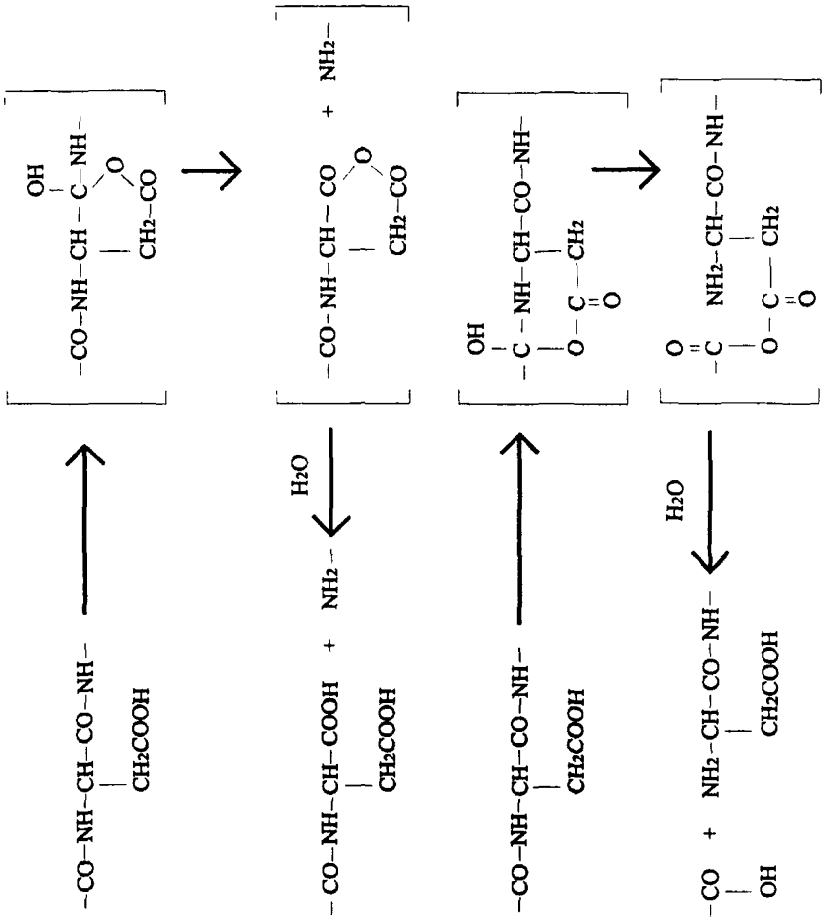

Fig. 2 Mechanisms of the cleavage of bonds to the COOH side (scheme i) and to the NH_2 side (scheme ii) of aspartyl residues in dilute acid

Yields of cleavage of Asp-X bonds of up to about 70% have been reported *(4)*. Such partial hydrolysis at just a few sites can give rise to a complicated mixture of peptides. For this reason this method may not be the one of choice for proteins, but may be suitable for small proteins or peptides containing few aspartyl residues, while also having the advantage of speed and simplicity. It is also readily adapted for large or small (subnanomolar) amounts of substrate.

4.4. Other Methods of Chemical Cleavage

19. A summary of various of common chemical methods of protein cleavage is given in Table 1. The literature contains descriptions of many other, less frequently used techniques (e.g., *see* ref. *1*). Reaction mechanisms are not known for all methods. Yields are generally less than 100% and often vary according to the nature of the substrate, surrounding amino acid sequence, exact conditions of reaction, and so on, but for guidance are put into broad classes in Table 1.
20. The most useful and widely used techniques provide good specificity at good yield, but some of the less likely techniques may also prove useful in particular instances.

    The simplicity of a method is also worthy of consideration. Thus cleavage at Met-X can be conducted in one vessel, and losses experienced in manipulation of the sample are minimized. On the other hand, cleavage at X-Cys bonds, as outlined in Table 1, involves dialysis and further treatment to deblock peptides for sequencing. Accumulative losses may limit the minimum size of samples required for this method.
21. Various of the techniques generate a lactone derivative at the C-terminus of the peptide to the NH_2 side of the cleavage point (*see Table* 1) This may be useful for the selective attachment of these peptides to amino supports such as 3-amino propyl glass *(5)*, for the purposes of solid phase protein sequencing.
22. Because of the variety of chemical techniques available, it is not easy to generate general rules. One point for consideration, however, is denaturation of the substrate. It is as well to reduce and alkylate cystinyl residues (*see* Chapter 34) so as to minimize the problems caused by aggregation of proteins or peptides. This is obviously not recommended if positions of disulfide bonds are of interest. If this is the case, be wary of strong acid (e.g., concentrated HCl) or alkaline conditions in which disulfide interchange may occur. Again, folding of the polypeptide may affect reactivity by allowing interaction of side chains. Denaturation by inclusion of agents such as guanidine HCl can help to overcome this and to solubilize the substrate and peptides from it.

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Chapter 34

Chemical Modification of Proteins

Alex F. Carne

1. Introduction

The two most widely used applications for chemical modification are in primary structure analysis and in the identification of essential groups involved in the binding and catalytic sites of proteins. The methods discussed here are those used frequently in primary structure analysis. Chemical modifications involving protein "active center" identification are the subject of a review by Pfleiderer *(1)*.

With the advent of the gas-phase sequencer *(2)*, the amounts of protein necessary for sequence analysis are in the low-picomole range, and it is therefore necessary to modify some of the more well-known methods to suit the smaller amounts of protein.

Cysteine presents a problem both in amino acid and sequence analysis. It is subject to auto-oxidation leading to a variety of products, such as mixed disulfides *(3)* and is involved in the formation of protein disulfide bridges. The presence of disulfide bridges within a polypeptide chain leads to difficulties in peptide isolation after enzymic digestion and in sequence determination. Reductive alkylation with iodoacetic acid yields the more stable carboxymethyl derivative and also introduces a charged group that tends to enhance the solubility of the polypeptide in aqueous buffers at alkaline pH. This makes the protein more susceptible to digestion by proteases, such as trypsin, chymotrypsin, and so forth. Also, using ^{14}C-iodoacetic acid as the alkylating agent enables a radioactive label to be introduced into the polypeptide chain.

From: *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
Edited by J M Walker

A further useful modification is the succinylation of the ε-amino sidechain of lysine. This is an irreversible reaction and hinders tryptic attack at lysine residues, thus giving rise to peptides with arginine at the carboxyterminus. As with carboxymethylation, the use of ^{14}C-succinic anhydride allows the incorporation of a radiolabel. Also proteins modified by dicarboxylic anhydrides are generally more soluble at pH 8, and the polypeptide chains are unfolded, allowing ready digestion with proteases.

For quantitative amino acid analysis, involving cystine or cysteine residues, carboxymethylation is not ideal, since it is difficult to assess the completeness of the reaction. A better method is to oxidize the S–S or SH groups using performic acid *(3)*, hydrolyze the polypeptide, and identify the cystine/cysteine as cysteic acid. Pyridylethylation is the preferred method of cystine/cysteine modification for both sequence analysis and for those amino acid analyzers that utilize phenylisothiocyanate as an amino-terminal derivatizing agent. The reaction is best carried out in the vapor-phase where the sample has been transferred to an inert support, e.g., glass fiber or polyvinylidene difluoride (PVDF). The vapor-phase reaction circumvents the need to remove excess reagents by microbore reverse-phase high-performance liquid chromatography (HPLC), and, hence, partial loss of sample on the column.

The method may also be used to identify cysteine containing peptides in protein digests by HPLC *(4)*. In this case, the reaction is carried out in the liquid-phase, the excess reagents being removed by reverse-phase HPLC.

The loading of the sample onto the membrane of a ProSpin™ sample preparation cartridge also permits removal of all contaminants with minimal sample loss *(5)* The membrane then serves as an inert support for subsequent N-terminal sequence analysis. This cartridge facilitates the method described by Sheer *(6)* for recovery of proteins from solution to a PVDF membrane.

2. Materials (*see* Note 1)

2.1. Reductive Alkylation

1. Denaturing buffer: 6*M* guanidinium chloride, 0.1*M* Tris-HCl, pH 8.5.
2. Source of oxygen-free nitrogen.

3. Dithiothreitol (DTT): 4 m*M* in distilled water. This solution should be freshly made.
4. Iodoacetic acid: 500 m*M* in distilled water, pH adjusted to 8.5 with sodium hydroxide. Iodoacetic acid is light-sensitive and it is preferable to make up a fresh solution each time, although the solution can be stored in the dark at –20°C. (*See* Notes 2, 3, and 4.)
5. Ammonium bicarbonate: 50 m*M* in distilled water, pH approx 8.0. (*See* Note 5.)
6. Microdialysis equipment (as supplied by BRL).
7. HPLC system with a Brownlee RP-300 "cartridge"-type column fitted (30 × 2.1 mm).
8. 0.1% TFA (HPLC grade).
9. Acetonitrile (HPLC, Far UV grade).

2.2. Performic Acid Oxidation

1. Hydrogen peroxide (30% w/v). **Care:** Strong oxidizing agent.
2. Formic acid (88% w/v).
3. Hydrobromic acid (48% w/v). **Care:** this acid gives off a caustic, irritating vapor and its use should be confined to a fume hood.

2.3. Succinylation

1. Guanidinium chloride: 6*M* in distilled water.
2. Succinic anhydride.
3. Sodium hydroxide: 1*M* in distilled water.
4. Micro pH probe.
5. Ammonium bicarbonate: 50 m*M* in distilled water, pH approx 8.0. (*See* Note 5.)
6. Microdialysis or HPLC equipment.

2.4. Pyridylethylation

2.4.1. Vapor-Phase Derivitization

1. 4-Vinylpyridine: Store at –20°C.
2. Tri-*n*-butylphosphine: Store at –20°C.
3. Pyridine: Store in the dark at 4°C.
4. Sequencer-grade *n*-heptane.
5. Sequencer-grade ethyl acetate.
6. TFA-washed glass-fiber disks (Applied Biosystems).
7. PVDF membrane.
8. Pyrex™ glass reaction vessel (*see* Fig. 1).
9. Vacuum source.

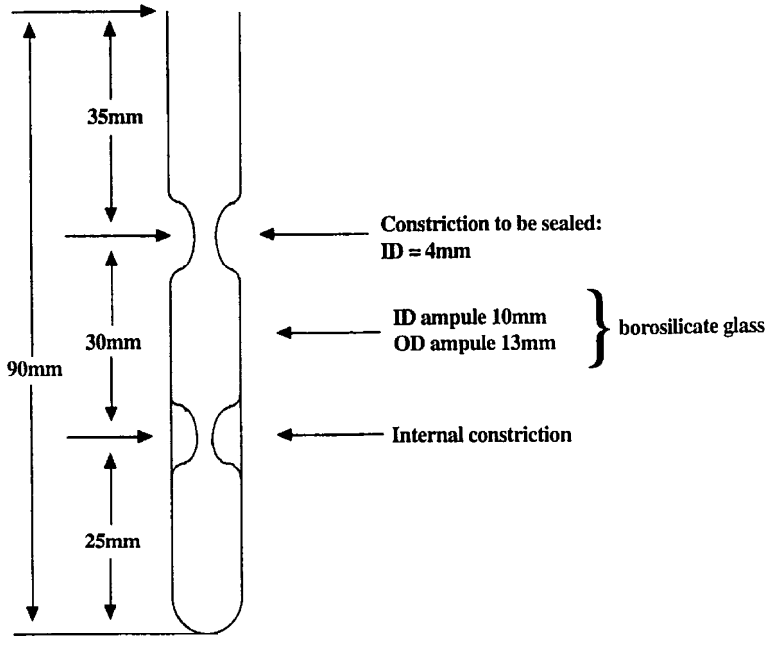

Fig. 1. Ampule used for vapor-phase *S*-pyridylethylation.

10. Glass-blowing torch with gas and oxygen flame.

2.4.2. Liquid-Phase Derivatization

1. 4-Vinylpyridine.
2. *N*-Ethylmorpholine.
3. Acetic acid (Aristar).
4. β-Mercaptoethanol.
5. HPLC with variable wavelength detector and suitable column *(4)*, for peptide mapping.
6. 0.1% TFA (HPLC-grade reagents).
7. Acetonitrile (HPLC, far UV grade).
8. ProSpin™ Cartridge (Applied Biosystems).
9. 20% Methanol (HPLC-grade reagents) for washing the PVDF membrane of the ProSpin cartridge.

3. Methods

3.1. Reductive Carboxymethylation

1. Dissolve the protein in 50 µL of denaturing buffer in a 1.5-mL Eppendorf tube.

2. Gently blow N_2 over the top of the solution for 15 min.
3. Add an equal volume of DTT solution to a concentration of 2 m*M*.
4. Blow N_2 over the solution for 60 min.
5. Wrap the Eppendorf tube in aluminum foil before the addition of iodoacetic acid.
6. Add 40 µL of iodoacetic acid solution dropwise using a micropipet. During this addition, the solution should be stirred. (It is possible to obtain microstirrer bars that will fit Eppendorf tubes; *see* Note 6.)
7. Again blow N_2 over the surface of the solution and seal the reaction tube.
8. Incubate in the dark at 37°C for 30 min. The reaction is carried out in the dark to prevent the formation of iodine, from the iodoacetate, thus preventing the iodination of tyrosine residues.
9. Removal of excess reagents: This may be performed in one of two ways:
   a. Microdialysis—pipet the solution into a 1–2 mL perspex chamber under which there is a sheet of dialysis membrane (mol wt cutoff 6000–8000). The underside of the membrane is in contact with the dialysis buffer, usually 50 m*M* ammonium bicarbonate. Carry out dialysis for about 24 h. (*See* Note 7.)
   b. HPLC—the protein can be separated from the reaction byproducts by reverse-phase HPLC. To minimize product loss, a 30 × 2.1 mm microbore "cartridge" column is employed. For proteins, a C_3 or C_8 matrix with a 10-µm particle diameter and a 30-nm pore size can be used (e.g., Aquapore octyl RP-300). Equilibrate the column in 0.1% aqueous trifluoroacetic acid. Inject the sample onto the column, and elute using an acetonitrile gradient (1% min, flow rate 0.2 mL/min). The excess reagents and reaction byproducts all appear in the breakthrough, i.e., they are not retained by the column packing material.

3.2. Performic Acid Oxidation

1. Add 100 µL of hydrogen peroxide to 900 µL of formic acid, and allow to stand at room temperature for 1 h. This produces performic acid (HCOOOH).
2. Cool the performic acid on ice to approx 0°C.
3. Dissolve the protein in 50 µL of performic acid, (about 400–500 µg/mL) in a precooled tube.
4. Keep at 0°C for 4 h. (*See* Note 8.)
5. Add 7.5 µL of *cold* HBr to neutralize the performic acid. Care must be taken at this stage, since bromine will be liberated, although a small amount.

6. Dry the sample thoroughly *in vacuo* over NaOH to remove bromine and formic acid.
7. Hydrolyze the sample, and subject to amino acid analysis. (*See* Notes 9–13.)

3.3. Succinylation (see Notes 14 and 15)

1. Dissolve the protein or protein derivative in 200 µL of 6*M* guanidinium chloride, and use a micro-pH probe to adjust the pH to 9.0 with the sodium hydroxide solution.
2. Crush one to two crystals (1–2 mg) of solid succinic anhydride, and add to the stirred solution over a period of about 15 min, while maintaining the pH of the solution at 9.0 with 1.0*M* NaOH. (*See* Note 16.)
3. Remove excess reagents by microdialysis or by HPLC. (*See* Note 17.)

3.4. Pyridylethylation

3.4.1. Vapor-Phase

Pyridylethylation may be carried out in the vapor phase according to the method of Amons *(7)* except that modifications were made to the glass ampule, and PVDF membrane can be used as an alternative to the glass-fiber disks.

1. Dissolve 1–100 µg protein in a minimum volume of distilled water or other appropriate solvent, and spot in small aliquots onto a glass-fiber disk. If PVDF is used, wet the membrane with methanol before the application of the sample.
2. Allow the sample to dry on the matrix.
3. Carefully fill the lower chamber of the glass ampule (*see* Fig. 1) with a freshly prepared mixture of:
   a. 100 µL H_2O.
   b. 100 µL pyridine.
   c. 20 µL 4-vinylpyridine.
   d. 20 µL tributylphosphine.
4. Place the disk/PVDF in the central part of the ampule.
5. Evacuate the ampule and seal at the top constriction using a gas/oxygen flame.
6. Allow the reaction to proceed at 60°C for 2 h.
7. Open the ampule and remove the disk.
8. Wash three times with each of the following:
   a. *n*-Heptane.
   b. *n*-Heptane:ethyl acetate (2:1 v/v).
   c. Ethyl acetate.

9. Air-dry the disk/PVDF, which is now ready for further analysis, e.g., N-terminal sequencing.

3.4.2. Liquid-Phase (see *Note 19*)

This method is similar to that used by Fullmer for chick calcium-binding protein *(4)*.

1. Dissolve the protein at an approximate concentration of 1 mg/mL in 0.2*N* *N*-ethylmorpholine acetate buffer, pH 8.0. The buffer is prepared by adding 230 µL of *N*-ethylmorpholine to approx 8.0 mL of distilled water. Acetic acid is added to give a pH of 8.0, and the volume adjusted to a total of 10.0 mL.
2. Add 1 µL of β-mercaptoethanol.
3. Add 2 µL of 4-vinylpyridine.
4. Allow to react for 90 min at room temperature.
5. Desalt the *S*-pyridylethylated protein by reverse-phase HPLC using the method described earlier in this chapter.
6. Remove the solvent under a stream of nitrogen, if the HPLC method is used.
7. The sample is now ready for enzymic digestion and peptide mapping. (*See* Chapter 23)
8. If the sample is to be used for sequencing directly and the vapor-phase method cannot be used, then it is possible to remove the reaction byproducts by centrifugation onto PVDF membrane using a ProSpin™ Cartridge *(5,6)*. The membrane is centrifuged until dry and then washed with 20% methanol to remove any further contaminants. The membrane is allowed to dry, and can then be used for sequence or amino acid analysis. (*See* Notes 20 and 21.)

4. Notes

1. Reagents for all the methods should be of the highest quality available.
2. The iodoacetic acid used must be colorless; any iodine present, revealed by a yellow color, causes rapid oxidation of thiol groups, preventing alkylation and possibly modifying tyrosine residues.
3. If good quality iodoacetic acid is not available, it may be recrystallized from hexane.
4. The method described for reductive alkylation does not include the use of iodo [2-^{14}C] acetic acid. The radiolabeled iodoacetic acid should be diluted before use with carrier iodoacetic acid to the required final specific activity according to the requirements of the worker.
5. Ammonium bicarbonate (50 m*M*) gives a pH of approx 8.0. No pH adjustment is necessary.

6. Continuous stirring during the addition of the iodoacetic acid is necessary to keep the pH constant throughout the solution, especially when dealing with small volumes.
7. Dialysis tubing should be avoided, since the losses involved when handling small amounts of protein can be high. The microdialysis system, which uses a sheet of dialysis membrane is preferable, but losses can still occur. If the reader has access to an HPLC system, then I would recommend that this should be the method of choice.
8. During performic acid oxidation, the temperature of all the reactants must be at or near 0°C to minimize any side reactions, such as the oxidation of phenolic groups and the hydroxyl groups of serine and threonine.
9. Oxidation of methionine occurs producing methionine sulfone, which can be quantified by amino acid analysis.
10. The addition of HBr causes bromination of tyrosine residues producing a mixture of mono- and di-substituted bromotyrosines. These derivatives have lower color values than tyrosine, and quantitation is generally not good after acid hydrolysis.
11. Since several residues are modified, this method is best used in conjunction with amino acid analysis. It is not recommended to use performic acid-oxidized samples for sequence analysis.
12. For the estimation of S–S or S–H groups by amino acid analysis, the most efficient method is still performic acid oxidation.
13. Instruments using RP-HPLC for the analysis of derivitized amino acids may need gradient modification to separate cysteic acid from aspartate.
14. Succinylation: This is best carried out in proteins that have been carboxymethylated to prevent S–S or SH groups from reacting. This gives complete selective modification.
15. Succinylation is a better method than maleylation or citraconylation, for the modification of lysine residues, since it avoids problems arising from the still reactive ethylenic groups. However, if there is a need for the removal of the blocking groups, for subsequent tryptic cleavage at lysine residues, maleylation or citraconylation should be used since the groups introduced by citraconic or maleic anhydride can be removed by treatment with dilute acid.
16. The addition of small amounts of succinic anhydride over a 15-min time period enables the worker to control the pH so that it can be maintained at or near pH 9.0.
17. For the removal of excess reagents, the same criteria apply as for carboxymethylation.

18. Both clostripain (EC 3.4.22.8) and Endoproteinase Arg-C *(8)* can be used for cleavage at arginine residues instead of succinylation followed by trypsin digestion. However, clostripain may cleave at other sites *(9)*, and endoproteinase Arg-C, although highly specific for the C-terminal side of arginine residues, does cleave some arginine peptide bonds slowly *(10)*. Thus, succinylation is still of use to the protein chemist trying to achieve specific cleavages coupled with the property of enhanced solubility.
19. The liquid-phase method of pyridylethylation is used to introduce a reporter group into cysteine containing peptides derived from digests of derivitized proteins and facilitates detection of these peptides at 254 nm.
20. Pyridylethylation on PVDF membrane enables the sample to be hydrolyzed, since *S*-β-(4-Pyridylethyl) cysteine (PE-cysteine) is acid-stable *(11)*.
21. The use of a ProSpin cartridge enables better yields of pyridylethylated material with subsequent removal of excess reagents. For small quantities of protein (<1 nmol), this would be the preferred method for sequence or amino acid analysis.

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CHAPTER 35

The Dansyl Method for Identifying N-Terminal Amino Acids

John M. Walker

1. Introduction

The reagent 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride, DNS-Cl) reacts with the free amino groups of peptides and proteins as shown in Fig. 1. Total acid hydrolysis of the substituted peptide or protein yields a mixture of free amino acids plus the dansyl derivative of the N-terminal amino acid, the bond between the dansyl group and the N-terminal amino acid being resistant to acid hydrolysis. The dansyl amino acid is fluorescent under UV light and is identified by thin-layer chromatography on polyamide sheets. This is an extremely sensitive method for identifying amino acids and in particular has found considerable use in peptide sequence determination when used in conjunction with the Edman degradation (*see* Chapter 36). The dansyl technique was originally introduced by Gray and Hartley *(1)*, and was developed essentially for use with peptides. However, the method can also be applied to proteins (*see* Note 1).

2. Materials

1. Dansyl chloride solution (2.5 mg/mL in acetone). Store at 4°C in the dark. This sample is stable for many months. The solution should be prepared from concentrated dansyl chloride solutions (in acetone) that are commercially available. Dansyl chloride available as a solid invariably contains some hydrolyzed material (dansyl hydroxide).

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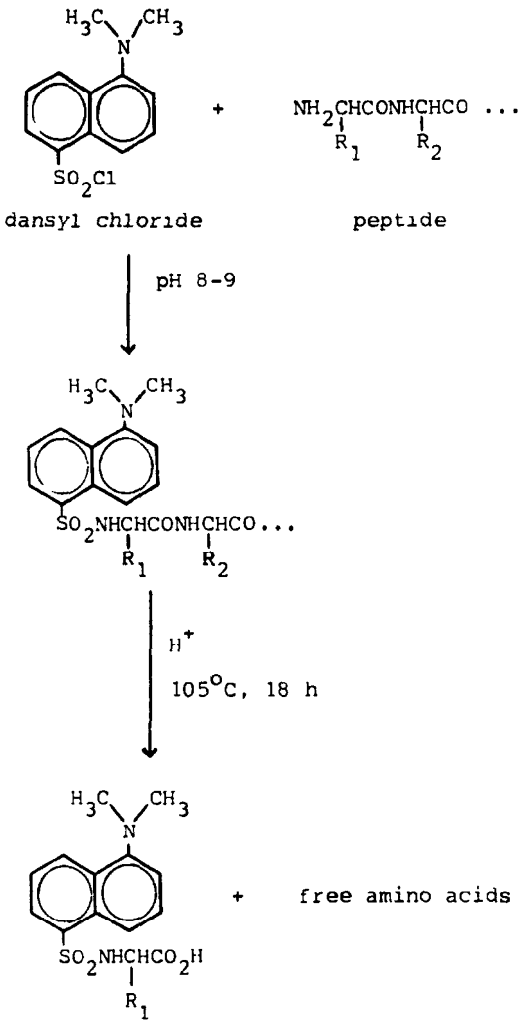

Fig. 1. Reaction sequence for the labeling of N-terminal amino acids with dansyl chloride.

2. Sodium bicarbonate solution (0.2*M*, aqueous). Store at 4°C. Stable indefinitely, but check periodically for signs of microbial growth.
3. 5*N* HCl (aqueous).
4. Test tubes (50 × 6 mm) referred to as "dansyl tubes."
5. Polyamide thin layer plates (7.5 × 7.5 cm). These plates are coated on both sides, and referred to as "dansyl plates." Each plate should be

numbered with a pencil in the top corner of the plate. The origin for loading should be marked with a pencil 1 cm in from each edge in the lower left-hand corner of the numbered side of the plate. The origin for loading on the reverse side of the plate should be immediately behind the loading position for the front of the plate, i.e., 1 cm in from each edge in the lower right-hand corner.

6. Three chromatography solvents are used in this method.
   Solvent 1: Formic acid:water, 1.5:100 (v/v);
   Solvent 2: Toluene:acetic acid, 9:1 (v/v);
   Solvent 3: Ethyl acetate:methanol:actic acid, 2:1:1 (v/v/v).
7. An acetone solution containing the following standard dansyl amino acids. Pro, Leu, Phe, Thr, Glu, Arg (each approx 50 µg/mL).
8. A UV source, either long wave (265 µm) or short wave (254 µm).

3. Method

1. Dissolve the sample to be analyzed in an appropriate volume of water, transfer to a dansyl tube, and dry *in vacuo* to leave a film of peptide (1–5 nmol) in the bottom of the tube.
2. Dissolve the dried peptide in sodium bicarbonate (0.2*M*, 10 µL) and then add dansyl chloride solution (10 µL) and mix (*see* Note 2).
3. Seal the tube with parafilm and incubate at 37°C for 1 h, or at room temperature for 3 h.
4. Dry the sample *in vacuo*. Because of the small volume of liquid present, this will only take about 5 min.
5. Add 6*N* HCl (50 µL) to the sample, seal the tube in an oxygen flame, and place at 105°C overnight (18 h).
6. When the tube has cooled, open the top of the tube using a glass knife, and dry the sample *in vacuo*. If phosphorus pentoxide is present in the desiccator as a drying agent, and the desiccator is placed in a water bath at 50–60°C, drying should take about 30 min.
7. Dissolve the dried sample in 50% pyridine (10 µL) and, using a microsyringe, load 1-µL aliquots at the origin on each side of a polyamide plate. This is best done in a stream of warm air. Do not allow the diameter of the spot to exceed 3–4 mm (*see* Note 3).
8. On the *reverse side only*, also load 0.5 µL of the standard mixture at the origin.
9. When the loaded samples are completely dry, the plate is placed in the first chromatography solvent and allowed to develop until the solvent front is about 1 cm from the top of the plate. This takes about 10 min, but can vary depending on room temperature.

10. Dry both sides of the plate by placing it in a stream of warm air. This can take 5–10 min since one is evaporating an aqueous solvent.
11. If the plate is now viewed under UV light, a blue fluorescent streak will be seen spreading up the plate from the origin, and also some green fluorescent spots may be seen within this streak. However, no interpretations can be made at this stage (*see* Note 4).
12. The dansyl plate is now developed in the second solvent, at right angles to the direction of development in the first solvent. The plate is therefore placed in the chromatography solvent so that the blue "streak" runs along the bottom edge of the plate.
13. The plate is now developed in the second solvent until the solvent front is about 1 cm from the top of the plate. This takes 10–15 min.
14. The plate is then dried in a stream of warm air. This will only take 2–3 min since the solvent is essentially organic. However, since toluene is involved, drying **must** be done in a fume cupboard.
15. The side of the plate containing the sample only should now be viewed under UV light. Three major fluorescent areas should be identified. Dansyl hydroxide (produced by hydrolysis of dansyl chloride) is seen as a blue fluorescent area at the bottom of the plate. Dansyl amide (produced by side reactions of dansyl chloride) has a blue–green fluorescence and is about one-third of the way up the plate. These two spots will be seen on all dansyl plates and seen as useful internal markers. Occasionally other marker spots are seen and these are described in the Notes section below. The third spot, which normally fluoresces green, will correspond to the dansyl derivative of the N-terminal amino acid of the peptide or protein. However, if the peptide is not pure, further dansyl derivatives will of course be seen. The separation of dansyl derivatives after solvent 2 is shown in Fig. 2. Solvent 2 essentially causes separation of the dansyl derivatives of hydrophobic and some neutral amino acids, whereas derivatives of charged and other neutral amino acids remain at the lower end of the chromatogram.
16. A reasonable identification of any faster-moving dansyl derivatives can be made after solvent 2 by comparing their positions, relative to the internal marker spots, with the diagram shown in Fig. 2. Unambiguous identification is made by turning the plate over and comparing the position of the derivative on this side with the standard samples that were also loaded on this side.

    Note that both sides of the plate are totally independent chromatograms. There is no suggestion that fluorescent spots can be seen through the plate from one side to the other.

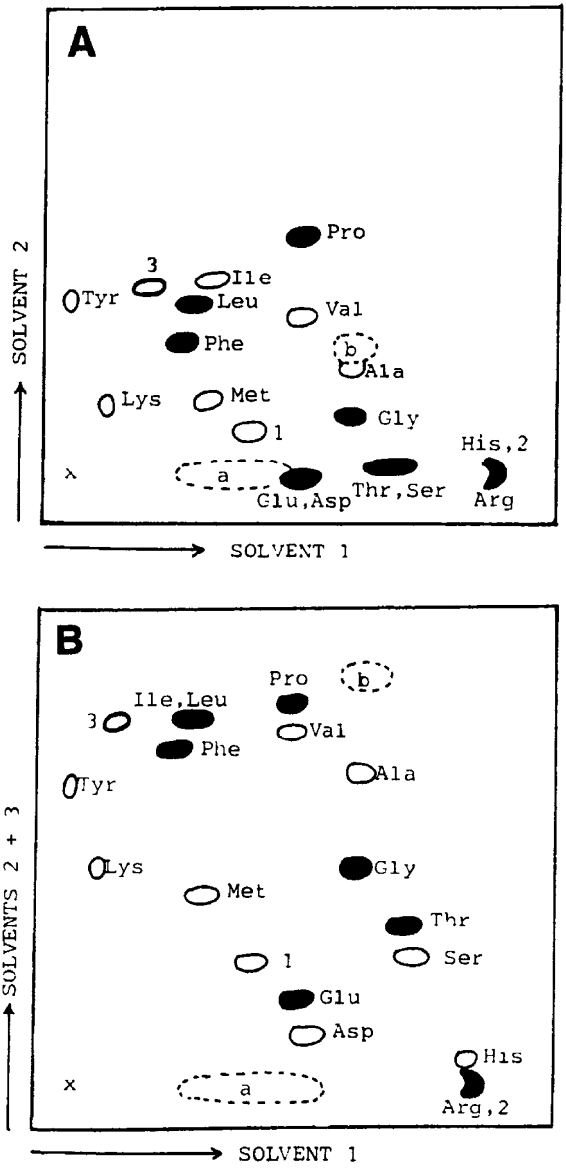

Fig. 2. Diagrams showing the separation of dansyl amino acids on polyamide plates after two solvents (**A**), and after three solvents (**B**): a = dansyl hydroxide; b = dansyl amide; 1 = tyrosine (*o*-DNS-derivative); 2 = lysine (ε-DNS-derivative); 3 = histidine (*bis*-DNS-derivative). The standard dansyl amino acids that are used are indicated as black spots.

17. Having recorded one's observations after the second solvent, the plate is now run in solvent 3 in the same direction as solvent 2. The plate is run until the solvent is 1 cm from the top, and this again takes 10–15 min.
18. After drying the plate in a stream of warm air (1–2 min), the plate is again viewed under UV light. The fast-running derivatives seen in solvent 2 have now run to the top of the plate and are generally indistinguishable (hence the need to record one's observations after solvent 2). However, the slow-moving derivatives in solvent 2 have now been separated by solvent 3 and can be identified if present. The separation obtained after solvent 3 is also shown in Fig. 2. The sum of the observations made after solvents 2 and 3 should identify the number and relative intensities of N-terminal amino acids present in the original sample (*see* Notes 5–12).

4. Notes

1. The dansylation method described here was developed for use with peptides. However, this method can also be applied quite successfully to proteins, although some difficulties arise. These are caused mainly by insolubility problems, which can limit the amount of reaction between the dansyl chloride and protein thus resulting in a lower yield of dansyl derivative, and the presence of large amounts of *o*-DNS-Tyr and ε-DNS-Lys on the chromatogram that can mask DNS-Asp and DNS-Glu. A modification of the basic procedure described here for the dansylation of proteins is described in ref. *3*.
2. It is important that the initial coupling reaction between dansyl chloride and the peptide occurs in the pH range 9.5–10.5. This pH provides a compromise between the unwanted effect of the aqueous hydrolysis of dansyl chloride and the necessity for the N-terminal amino group to be unprotonated for reaction with dansyl chloride. The condition used, 50% acetone in bicarbonate buffer, provides the necessary environment. The presence of buffer or salts in the peptide (or protein) sample should therefore be avoided to prevent altering the pH to a value outside the required range.
3. Because of the unpleasant and irritant nature of pyridine vapor, loading of samples onto the dansyl plates should preferably be carried out in a fume cupboard.
4. The viewing of dansyl plates under UV light should always be done wearing protective glasses or goggles. Failure to do so will result in a most painful and potentially damaging conjunctivitis.
5. Most dansyl derivatives are recovered in high (>90%) yield. However, some destruction of proline, serine, and threonine residues occurs dur-

ing acid hydrolysis, resulting in yields of approx 25, 65, and 70%, respectively. When viewing these derivatives, therefore, their apparent intensities should be visually "scaled-up" accordingly.

6. The sensitivity of the dansyl method is such that as little as 1–5 ng of a dansylated amino acid can be visualized on a chromatogram.
7. The side chains of both tyrosine and lysine residues also react with dansyl chloride. When these residues are present in a peptide (or protein), the chromatogram will show the additional spots, *o*-DNS-Tyr and ε-DNS-Lys, which can be regarded as additional internal marker spots. The positions of these residues are shown in Fig. 2. These spots should not be confused with *bis*-DNS-Lys and *bis*-DNS-Tyr, which are produced when either lysine or tyrosine is the N-terminal amino acid.
8. At the overnight hydrolysis step, dansyl derivatives of asparagine or glutamine are hydrolyzed to the corresponding aspartic or glutamic acid derivatives. Residues identified as DNS-Asp or DNS-Glu are therefore generally referred to as Asx or Glx, since the original nature of this residue (acid or amide) is not known. This is of little consequence if one is looking for a single N-terminal residue to confirm the purity of a peptide or protein. It does, however, cause difficulties in the dansyl-Edman method for peptide sequencing (*see* Chapter 36) where the residue has to be identified unambiguously.
9. When the first two residues in the peptide or protein are hydrophobic residues, a complication can occur. The peptide bond between these two residues is particularly (although not totally) resistant to acid hydrolysis. Under normal conditions, therefore, some dansyl derivative of the first amino acid is produced, together with some dansyl derivative of the N-terminal dipeptide. Such dipeptide derivatives generally run on chromatograms in the region of phenylalanine and valine. However, their behavior in solvents 2 and 3, and their positions relative to the marker derivatives should prevent misidentification as phenylalanine or valine. Such dipeptide spots are also produced when the first residue is hydrophobic and the second residue is proline, and these dipeptide derivatives run in the region of proline. However, since some of the N-terminal derivative is always produced, there is no problem in identifying the N-terminal residue when this situation arises. A comprehensive description of the chromatographic behavior of dansyl-dipeptide derivatives has been produced *(2)*.
10. Three residues are difficult to identify in the three solvent system described in the methods section; DNS-Arg and DNS-His because they are masked by the ε-DNS-Lys spot, and DNS-Cys because it is masked by DNS-hydroxide. If these residues are suspected, a fourth solvent is

used. For arginine and histidine, the solvent is 0.05*M* trisodium phosphate:ethanol (3:1 [v/v]). For cysteine the solvent is 1*M* ammonia:ethanol (1:1 [v/v]) Both solvents are run in the same direction as solvents 2 and 3, and the residues are identified by comparison with relevant standards loaded on the reverse side of the plate.

11. When working with small peptides it is often of use also to carry out the procedure known as "double dansylation." Having identified the N-terminal residue, the remaining material in the dansyl tube is dried down and the dansylation process (steps 2–4) repeated. The sample is then redissolved in 50% pyridine (10 µL) and a 1-µL aliquot is examined chromatographically. The chromtogram will now reveal the dansyl derivative of each amino acid present in the peptide. Therefore for relatively small peptides (<10 residues) a quantitative estimation of the amino acid composition of the peptide can be obtained. This method is not suitable for larger peptides or proteins since most residues will be present more than once in this case, and it is not possible to quantitatively differentiate spots of differing intensity.
12. Although the side chain DNS-derivative will be formed during dansylation if histidine is present in the peptide or protein sequence, this derivative is unstable to acid and is not seen during N-terminal analysis. Consequently, N-terminal histidine yields only the α-DNS derivative and not the *bis*-DNS compound as might be expected. The *bis*-DNS derivative is observed, however, if the mixture of free amino acids formed by acid hydrolysis of a histidine-containing peptide is dansylated and subsequently analyzed chromatographically (i.e., during "double dansylation").

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CHAPTER 36

The Dansyl-Edman Method for Peptide Sequencing

John M. Walker

1. Introduction

The Edman degradation is a series of chemical reactions that sequentially removes N-terminal amino acids from a peptide or protein. The overall reaction sequence is shown in Fig. 1. In the first step (the coupling reaction) phenylisothocyanate (PITC) reacts with the N-terminal amino group of the peptide or protein. The sample is then dried and treated with an anhydrous acid (e.g., trifluoracetic acid), which results in cleavage of the peptide bond between the first and second amino acid. The N-terminal amino acids is therefore released as a derivative (the thiazolinone). The thiazolinone is extracted into an organic solvent, dried down, and then converted to the more stable phenylthiohydantoin (PTH) derivative (the conversion step). The PTH amino acid is then identified, normally by reverse-phase HPLC. This is known as the direct Edman degradation and is, for example, the method used in an automated sequencing machine. The dansyl-Edman method for peptide sequencing described here is based on the Edman degradation, but with the following modifications. Following the cleavage step the thiazolinone is extracted, but rather than being converted to the PTH derivative it is discarded. Instead, a small fraction (5%) of the remaining peptide is taken and the newly liberated N-terminal amino acid determined in this sample by the dansyl method (*see* Chapter 35). Although the dansyl-Edman method results in successively less peptide being present at each cycle of the Edman deg-

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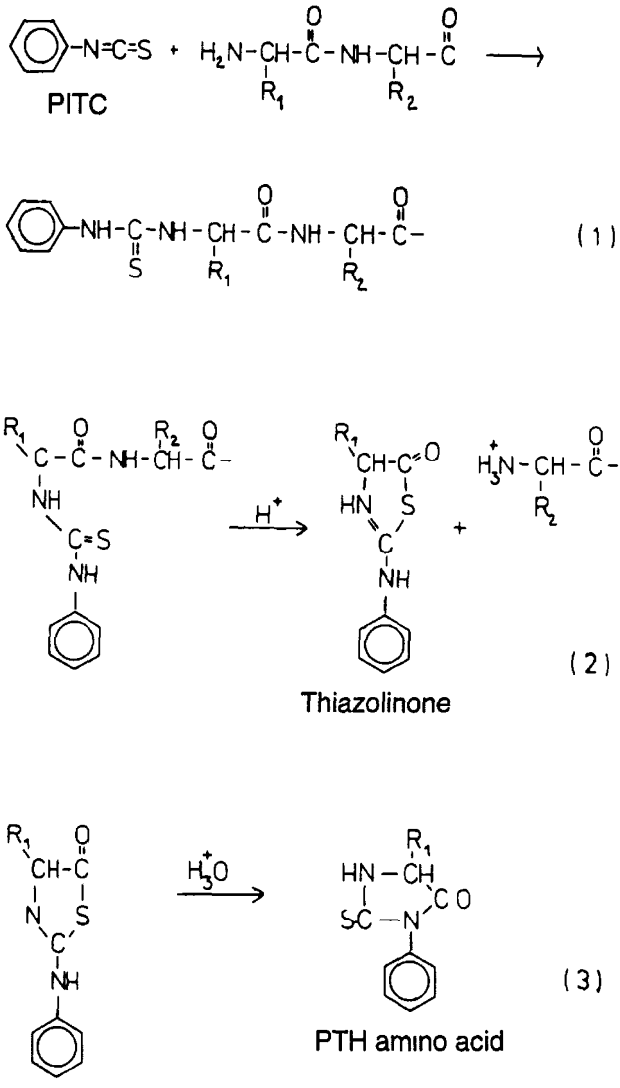

Fig. 1. The Edman degradation reactions and conversion step: (1) the coupling reaction, (2) the cleavage reaction; (3) the conversion step.

radation, this loss of material is compensated for by the considerable sensitivity of the dansyl method for identifying N-terminal amino acids. The dansyl-Edman method described here was originally introduced by Hartley *(1)*.

2. Materials

1. Ground glass stoppered test tubes (approx 65 × 10 mm, e.g., Quickfit MF 24/0). All reactions are carried out in this "sequencing" tube.
2. 50% Pyridine (aqueous, made with AR pyridine). Store under nitrogen at 4°C in the dark. Some discoloration will occur with time, but this will not affect results.
3. Phenylisothiocyanate (5% [v/v]) in pyridine (AR). Store under nitrogen at 4°C in the dark. Some discoloration will occur with time, but this will not affect results. The phenylisothiocyanate should be of high purity and is best purchased as "sequenator grade." Make up fresh about once a month.
4. Water-saturated *n*-butyl acetate. Store at room temperature.
5. Anhydrous trifluoracetic acid (TFA). Store at room temperature under nitrogen.

3. Method

1. Dissolve the peptide to be sequenced in an appropriate volume of water, transfer to a sequencing tube, and dry *in vacuo* to leave a film of peptide in the bottom of the tube (*see* Notes 1 and 2).
2. Dissolve the peptide in 50% pyridine (200 µL) and remove an aliquot (5 µL) for N-terminal analysis by the dansyl method (*see* Chapter 35 and Note 3).
3. Add 5% phenylisothiocyanate (100 µL) to the sequencing tube, mix gently, flush with nitrogen and incubate the stoppered tube at 50°C for 45 min.
4. Following this incubation, unstopper the tube and place it *in vacuo* for 30–40 min. The desiccator should contain a beaker of phosphorus pentoxide to act a drying agent, and if possible the desiccator should be placed in a water bath at 50–60°C. When dry, a white "crust" will be seen in the bottom of the tube. This completes the coupling reaction (*see* Notes 4–6).
5. Add TFA (200 µL) to the test-tube, flush with nitrogen, and incubate the stoppered tube at 50°C for 15 min.
6. Following incubation, place the test tube *in vacuo* for 5 min. TFA is a very volatile acid and evaporates rapidly. This completes the cleavage reaction.
7. Dissolve the contents of the tube in water (200 µL). Do not worry if the material in the tube does not all appear to dissolve. Many of the side-products produced in the previous reactions will not in fact be soluble.
8. Add *n*-butyl acetate (1.5 mL) to the tube, mix vigorously for 10 s, and then centrifuge in a bench centrifuge for 3 min.
9. Taking care not to disturb the lower aqueous layer, carefully remove the upper organic layer and discard (*see* Note 7).

10. Repeat this butyl acetate extraction procedure once more and then place the test-tube containing the aqueous layer *in vacuo* (with the desiccator standing in a 60°C water bath if possible) until dry (30–40 min).
11. Redissolve the dried material in the test tube in 50% pyridine (200 µL) and remove an aliquot (5 µL) to determine the newly liberated N-terminal amino acid (the second one in the peptide sequence) by the dansyl method (*see* Chapter 35).
12. A further cycle of the Edman degradation can now be carried out by returning to step 3. Proceed in this manner until the peptide has been completely sequenced (*see* Notes 8 and 9).
13. The identification of N-terminal amino acids by the dansyl method is essentially as described in Chapter 35. However, certain observations peculiar to the dansyl-Edman method are described in Notes 10–15.

4. Notes

1. Manual sequencing is normally carried out on peptides between 2 and 30 residues in length and requires 1–5 nmol of peptide.
2. Since the manipulative procedures are relatively simple it is quite normal to carry out the sequencing procedure on 8 or 12 peptides at one time.
3. As sequencing proceeds, it will generally be necessary to increase the amount of aliquot taken for dansylation at the beginning of each cycle, since the amount of peptide being sequenced is reduced at each cycle by this method. The amount to be taken should be determined by examination of the intensity of spots being seen on the dansyl plates.
4. It is most important that the sample is completely dry following the coupling reaction. Any traces of water present at the cleavage reaction step will introduce hydrolytic conditions that will cause internal cleavages in the peptide and a corresponding increase in the background of N-terminal amino acids.
5. Very occasionally it will prove difficult to completely dry the peptide following the coupling reaction and the peptide appears oily. If this happens, add ethanol (100 µL) to the sample, mix, and place under vacuum. This should result in a dry sample.
6. Vacuum pumps used for this work should be protected by cold traps. Considerable quantities of volatile organic compounds and acids will be drawn into the pump if suitable precautions are not taken.
7. When removing butyl acetate at the organic extraction step, take great care not to remove any of the aqueous layer as this will considerably reduce the amount of peptide available for sequencing. Leave a small layer of butyl acetate above the aqueous phase. This will quickly evaporate at the drying step.

8. A repetitive yield of 90–95% is generally obtained for the dansyl-Edman degradation. Such repetitive yields usually allow the determination of sequences up to 15 residues in length, but in favorable circumstances somewhat longer sequences can be determined.
9. A single cycle takes approx 2.5 h to complete. When this method is being used routinely, it is quite easy to carry out three or four cycles on eight or more peptides in a normal day's work. During the incubation and drying steps the dansyl samples from the previous days sequencing can be identified.
10. Tryptophan cannot be identified by the dansyl method as it is destroyed at the acid hydrolysis step. However, where there is tryptophan present in the sequence, an intense purple color is seen at the cleavage (TFA) step involving the tryptophan residue that unambiguously identifies the tryptophan residue.
11. If there is a lysine residue present in the peptide, a strong ε-DNS-lysine spot will be seen when the dansyl derivative of the N-terminal amino acid is studied. However, when later residues are investigated the ε-DNS-lysine will be dramatically reduced in intensity or absent. This is because the amino groups on the lysine side chains are progressively blocked by reaction with phenylisothiocyanate at each coupling step of the Edman degradation.
12. The reactions of the lysine side chains with phenylisothiocyanate causes some confusion when identifying lysine residues. With a lysine residue as the N-terminal residue of the peptide it will be identified as *bis*-DNS-lysine. However, lysine residues further down the chain will be identified as the α-DNS-ε–phenylthiocarbamyl derivative because of the side chain reaction with phenylisothiocyanate. This derivative runs in the same position as DNS-phenylalanine in the second solvent, but moves to between DNS-leucine and DNS-isoleucine in the third solvent. Care must therefore be taken not to misidentify a lysine residue as a phenylalanine residue.
13. When glutamine is exposed as the new N-terminal amino acid during the Edman degradation, this residue will sometimes cyclize to form the pyroglutamyl derivative. This does not have a free amino group, and therefore effectively blocks the Edman degradation. If this happens, a weak DNS-glutamic acid residue is usually seen at this step, and then no other residues are detected on further cycles. There is little one can do to overcome this problem once it has occurred, although the enzyme that cleaves off pyroglutamyl derivatives (pyroglutamate aminopeptidase) is commercially available (Boehringer, Lewes, UK).

14. The amino acid sequence of the peptide is easily determined by identifying the new N-terminal amino acid produced after each cycle of the Edman degradation. However, because the Edman degradation does not result in 100% cleavage at each step, a background of N-terminal amino acids builds up as the number of cycles increases. Also, as sequencing proceeds, some fluorescent spots reflecting an accumulation of side products can be seen toward the top of the plates. For longer runs (10–20 cycles) this can cause some difficulty in identifying the newly liberated N-terminal amino acid. This problem is best overcome by placing the dansyl plates from consecutive cycles adjacent to one another and viewing them at the same time. By comparison with the previous plate, the increase of the new residue at each cycle, over and above the background spots, should be apparent.
15. The main disadvantage of the dansyl-Edman method compared to the direct Edman method is the fact that the dansyl method cannot differentiate acid and amide residues. Sequences determined by the dansyl-Edman method therefore usually include residues identified as Asx and Glx. This is most unsatisfactory since it means the residue has not been unambiguously identified, but often the acid or amide nature of an Asx or Glx residue can be deduced from the electrophoretic mobility of the peptide *(3)*.
16. Having identified any given residue it can prove particularly useful to carry out the procedure referred to as "double dansylation" on this sample (*see* Chapter 35). This double-dansylated sample will identify the amino acids remaining beyond this residue. Double dansylation at each step should reveal a progressive decrease in the residues remaining in the peptide, and give an excellent indication of the amount of residues remaining to be sequenced at any given cycle.

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CHAPTER 37

C-Terminal Sequencing of Peptides

The Thiocyanate Degradation Method

Franca Casagranda and John F. K. Wilshire

1. Introduction

The possibility of sequencing peptides or proteins from their N-terminal ends became a reality with the discovery and the application of the Edman stepwise degradation *(1,2; also see* Chapter 36). The procedure developed was automated in 1967 *(2)*, and it is now possible to obtain unequivocal sequence information for up to 35–70 residues for a wide variety of peptides and proteins before the build-up of background material makes assignments difficult, if not impossible. In practice, the complete sequence often has to be derived by establishing the sequences of smaller fragments (i.e., peptides) obtained from the peptide by the action of either enzymes or chemical reagents *(3)*.

N-terminal sequencing by itself is often not sufficient to establish the complete sequence of a lengthy peptide, because uncertainty can exist concerning the identification of the last few amino acids in the chain. Furthermore, if the N-terminus is blocked, e.g., if the amino group is acetylated or formylated *(4)*, as occasionally occurs, then the sequence is clearly not determinable by the Edman degradation. Other frequently encountered blocking reactions are: (1) the cyclization of N-terminal glutamine residues to form pyroglutamyl residues, and (2) the migration of the *O*-acyl groups of serine or threonine resi-

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dues to the N-terminus. Moreover, C-terminal sequencing information would be of great value in the area of molecular biology, particularly for:

1. Detecting posttranslational modifications at the carboxy terminus of the expressed gene products obtained from known DNA sequences;
2. Confirming the correct placement of initiation codons and reading frames; and
3. Providing a basis for the design of oligonucleotide probes capable of screening cDNA libraries.

For these reasons, a viable C-terminal sequencing method would become a very important tool in protein chemistry. Although both mass spectroscopy (*see* Note 1) and the specific hydrolytic capability of certain enzymes (*see* Note 2) have been used to a limited extent, for C-terminal sequencing, the present account will be devoted to a description of the thiocyanate degradation procedure, the most successful C-terminal sequencing method yet devised.

1.1. Thiocyanate Degradation Procedure

The thiocyanate degradation procedure is a chemical method based on reactions discovered by Schlack and Kumpf *(5)* (for discussion of alternative but less successful chemical methods, *see* Ward *[6]*). This procedure (*see* Fig. 1), which has recently been critically reviewed by Inglis *(7)*, involves the conversion of the C-terminal amino acid of a peptide into the corresponding peptidyl thiohydantoin by reaction of the peptides with acetic anhydride/acetic acid (the activation reaction), followed by treatment with either a thiocyanate salt or a solution of thiocyanic acid in acetone (the coupling reaction). Subsequent treatment with base (the cleavage reaction) releases the C-terminal amino acid (as its thiohydantoin derivative) together with a shortened (by one residue) peptide possessing a free carboxyl group available for repetition of the procedure. This method has proved successful for the sequencing of peptides containing hydrophobic residues, and also tyr and trp residues, but not of peptides containing sensitive side chains (*see* Note 3). Degradations can be performed either with a solution of the peptide or (preferably) with the peptide attached to a solid support.

Degradations performed with the peptide in solution can provide a rapid procedure for determining the C-terminal residue, but the method is limited to those peptides that are soluble in the coupling solution.

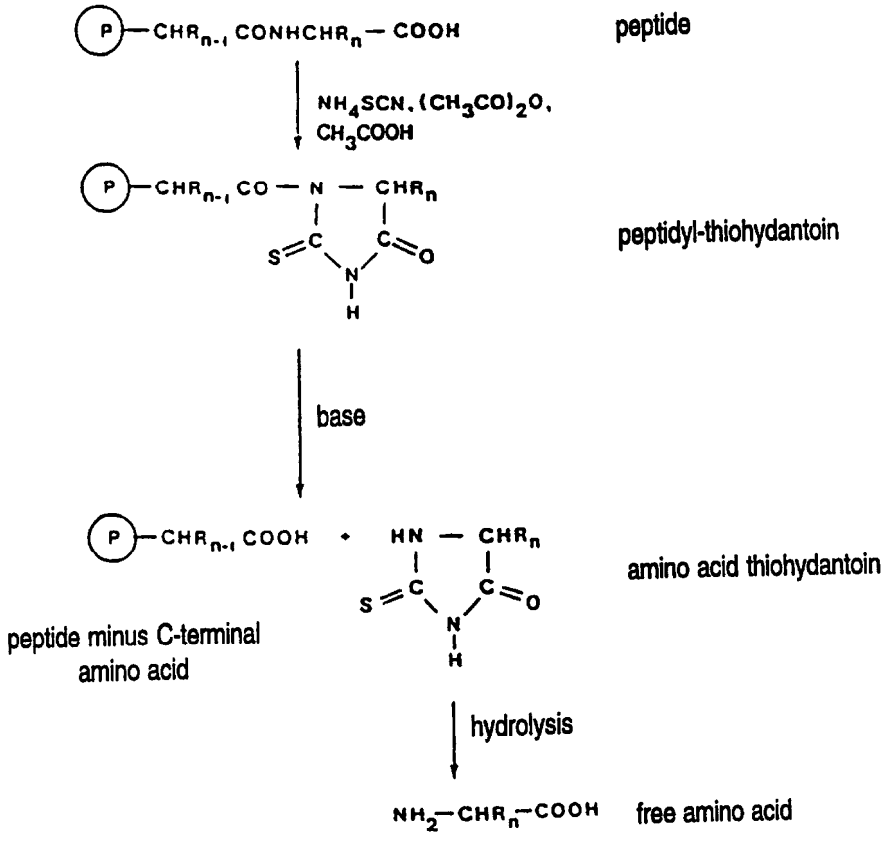

Fig. 1 Reactions involved in the Schlack-Kumpf degradation procedure.

Furthermore, because removal of the cleavage and other reagents from the peptide (gel filtration *[8]* or filtration followed by freeze-drying *[9]* has been used) can be time-consuming and incomplete, only a single cycle of reactions is usually obtained. In order to obtain more extensive sequencing information, therefore, procedures have been developed for performing degradation cycles on peptides that have been immobilized on a solid support by covalent attachment through their amino groups *(10)*. The use of this technology, together with (1) the identification of the thiohydantoin derivatives by means of high-performance liquid chromatography (HPLC) *(11–13)*, and (2) the introduction *(13)* of antioxidants (e.g., dithioerythritol) for the protection of the somewhat unstable thiohydantoin derivatives (*see* Note 4), has

simplified the procedure and extended the number of C-terminal residues that can be sequenced (some of the sequences that have been determined are shown in Table 1).

2. Materials

High-purity reagents are necessary for optimal results when sequencing. Recommended purification procedures are given in this section.

1. Controlled-pore glass beads (CPG 10Å pore size, 200–400 mesh [Electro-Nucleonics]) (1 g) are washed by suspending them in 10% (v/v) hydrofluoric acid for 2 min. The beads are then rinsed thoroughly with water and dried overnight in an oven at 100°C.
2. γ-Aminopropyltriethoxysilane in acetone (5% w/v).
3. Triethylamine (TEA).
4. Succinic anhydride.
5. Dimethylformamide (DMF), redistilled.
6. Trifluoroacetic acid (TFA), anhydrous.
7. Sodium hydrogen phosphate (Na_2HPO_4, 0.5*M*, pH 9) containing sodium chloride (0.2*M*).
8. Hydrochloric acid (5.8*M*).
9. Trisodium citrate buffer: trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) (19.6 g), concentrated nitric acid (10 mL), methanol (40 mL), and phenol (1 g) are mixed cautiously together; the resultant mixture is then made up to 1 L, and the solution adjusted to pH 2.0 with nitric acid.
10. Dowex 50X8 (H^+ form) resin is prepared as follows. Soak commercial Dowex 50X8 (H^+ form) resin (Bio-Rad) in NaOH (2*M*) for 2 h, wash with water, and finally soak in aqueous EDTA solution (0.1*M*) overnight. Then wash the resin with water, soak in HCl (2*M*) for several hours, wash with water again, and finally rinse the resin with acetone several times (to remove any water).
11. Ammonium thiocyanate.
12. Acetic anhydride.
13. Acetic acid.
14. Dithioerythritol (DTE).
15. Activation solution: acetic anhydride, acetic acid (4:1).
16. Coupling solution: thiocyanic acid in acetone solution (~1.8*M*) (*see* Sections 3.1.4. and 3.1.5.).
17. Cleavage solution: 0.5*M* KOH in 33% MeOH/water containing DTE (~1 mg/10 mL).
18. HPLC buffers: buffer A: ammonium acetate (0.25 g/L; pH 4.76); buffer B: acetonitrile.

Table 1
Some C-Terminal Sequences Determined
Using the Thiocyanate Degradation

Researchers	Sequence determined
Stark (1968)* *(8)*	-Leu-Val-Cy(SO_3H)-Gly-Glu-Arg
Yamashita (1971)* *(30)*	-Ala-Ser-Val
Williams and Kassell (1975) *(10)*	-Asn-Ala-Lys
Rangarajan and Darbre (1976) *(9)*	-His-Phe-Asp-Ala-Ser-Val
Meuth et al. (1982) *(12)*	Leu-Gly-Tyr-Gln-Gly
Hawke et al. (1987) *(16)*	Tyr-Gln-Gly
Miller and Shively (1989) *(15)*	-Leu-Ala
Inglis et al. (1989) *(13)*	-Leu-Ala-Ile-Tyr-Val-Met-Ala-Phe-Val
Bailey and Shively (1990) *(17)*	-Glu-Leu
Boyd et al (1992) *(18)*	-Leu-Tyr-Phe-Gly-Leu-Tyr-Gln-Phe-Gly

*Degradations performed in solution All other sequences were obtained using a solid-phase support.

19. HPLC column: Waters Pico-Tag ODS, 300 × 2.1 mm, 5-μm pore size.

3. Methods

3.1. Manual C-Terminal Sequencing

The following section describes an experimental procedure (*see* Note 5) with which the authors are familiar *(13,19)*; this procedure is capable of giving useful sequence determination for peptides (*see* Note 6) containing all the common hydrophobic amino acids, as well as Tyr and Trp. We do not wish to imply that this procedure, which has recently been automated (*see* Note 7), is superior to procedures reported recently by other workers in this field *(16,18,21,22)*, who have all made significant contributions to C-terminal sequencing technology based on the thiocyanate degradation reaction. It remains to be seen, however, whether this recent work, which has described interesting new chemistry, will eventually lead to a C-terminal sequencing procedure routinely applicable to *all* the common amino acids.

3.1.1. Preparation of Carbonyldiimidazole-Activated Glass Beads

Several steps are involved in the preparation of the activated *N,N*-carbonyldiimidazole (CDI) glass beads, which are used as the solid support *(12)*. We have modified the literature method, however, in

the way in which the glass beads are washed. The preparation of the activated glass beads is carried out as follows:

3.1.1.1. Preparation of Aminoalkylsilyl Beads

1. Suspend the glass beads (0.5 g) in a solution of (20 mL) of γ-aminopropyltriethoxysilane in acetone (5% w/v); degas the mixture and shake for 20 h at 45–50°C.
2. Filter the aminoalkylsilyl beads, which will have become yellow, wash with acetone (4 × ~2 mL), dry in a vacuum, and store in a desiccator at –4°C.

3.1.1.2. Preparation of Succinyl Beads

1. Suspend aminoalkylsilyl glass beads (0.5 g) in a solution of acetone (12.5 mL) containing triethylamine (0.75 mL) and succinic anhydride (0.5 g). Degas the mixture, and shake for 3 h at room temperature.
2. Filter the resultant succinyl glass beads, wash with water and finally with acetone, dry in a vacuum, and store in a desiccator at –4°C.

3.1.1.3. Activation of Succinyl Beads with Carbonyldiimidazole (CDI)

1. Suspend succinyl glass beads (0.5 g) in dimethylformamide (DMF) (3 mL). Add CDI (2.4 g), degas the mixture, and shake overnight at room temperature.
2. Filter the beads, and thoroughly wash them with DMF and finally with dichloromethane. Dry the CDI-activated glass beads under vacuum, and store in a desiccator at –4°C.

3.1.2. Attachment of Peptide to CDI-Activated Glass Beads (see *Notes 8 and 9)*

1. Dissolve the peptide (~1 μmol) in anhydrous trifluoroacetic acid (TFA) (1 mL), and allow the resultant solution to stand at room temperature for 1 h under an atmosphere of nitrogen.
2. Remove TFA on a rotary evaporator using a nitrogen flush, and wash the dried residue with dichloromethane (2 × 3 mL).
3. Dissolve the resultant residue in DMF (1 mL), and add triethylamine (30 μL).
4. Add CDI-activated glass beads (100 mg) to the mixture, degas the resultant suspension, and cover the suspension with a nitrogen atmosphere.
5. Gently shake the suspension at room temperature overnight in order to ensure maximum covalent binding of the peptide to the beads.

6. Filter the beads, and wash successively with water, Na_2HPO_4 solution (0.5*M*, pH 9) containing NaCl (0.2*M*), water again, and finally with acetone. Dry the peptide-bound glass beads in a vacuum and store in a desiccator at –4°C.

3.1.3. Amino Acid Analysis of Peptide-Bound Glass Beads

1. To determine the amount of peptide bound to the glass beads, treat the beads (1–2 mg) with 5.8*M* HCl (200 µL) at 108°C for 24 h under vacuum in a Waters Pico Tag Work Station.
2. Dry the beads under vacuum, add trisodium citrate buffer (0.2*M*, pH 2, 200 µL), briefly stir the mixture, remove the supernatant liquor, and analyze the liquor on a Waters Amino Acid Analyzer.

3.1.4. Preparation of Thiocyanic Acid (HSCN) in Acetone Solution

A modified procedure of the literature Method II *(23)* is adopted as follows:

1. Suspend Dowex H^+ resin (*see* Section 2.) in acetone; degas the suspension under vacuum and pour into a glass column (2 × 30 cm). After the resin particles have settled, wash the column through with acetone.
2. Adjust the flow rate to 1 drop/8 s, and add a solution of ammonium thiocyanate (6 g) in acetone (20 mL). When the eluate begins to produce a deep red color with dilute (~1%) aqueous ferric chloride solution, collect fractions. When stored in a freezer at –20°C, HSCN/acetone solutions are stable for long periods (upwards of 1 yr or longer).

3.1.5. Determination of HSCN/Acetone Concentration

A modification of the Volhard method (cf *[12]*) is used as follows:

1. To an Erlenmeyer flask, add water (20 mL), concentrated nitric acid (2 mL), saturated ferric ammonium sulfate solution in water (10 drops), a known volume (100 µL) of an HSCN/acetone solution, and 0.1*M* silver nitrate solution (3 mL).

   Cover the resultant mixture with toluene (3 mL), allow to react for a few mintues, and then titrate with 0.1*M* ammonium thiocyanate standard solution; the endpoint is reached when the solution turns a pale orange color. The concentrations of HSCN solutions in acetone vary from 0.2*M* to 2.5*M*. In general, 1.5–2.0*M* solutions are used for the coupling reactions.

3.1.6. C-Terminal Amino Acid Sequencing (Solid-Phase Method)

1. Place glass beads (~2–10 mg) coupled with peptide or protein (~15–30 nmol) in a screw-cap Eppendorf vial (1.5 mL). Add a solution of acetic anhydride and acetic acid (4:1, 120 µL), vortex the mixture and heat at 80°C (water bath) for 5 min.
2. Add HSCN/acetone solution (~1.8*M*, 30 µL), stir the resultant mixture, and heat for a further 30 min at 80°C.
3. Remove the vial from the water bath, and carefully remove the supernatant liquid by pipet and discard it.
4. Wash the glass beads with DMF (2 × 200 µL) and then 70% acetonitrile/water (3 × 200 µL).
5. Cover the beads with a solution (50 µL) of 0.5*M* KOH in 33% MeOH/water containing dithioerythritol (DTE, 0.01%); stir the suspension at room temperature for 3 min.
6. Remove the supernatant alkaline liquor by pipet, neutralize with 5% acetic acid in 20% acetonitrile (50 µL), and analyze (HPLC) the resultant solution for thiohydantoin content.
7. Immediately wash the glass beads successively (200-µL aliquots) with 5% acetic acid in 70% acetonitrile (two times), 70% acetonitrile (two times), acetonitrile (two times), and finally rinse twice with activating solution (acetic anhydride/acetic acid; 4:1) before commencing the next cycle.

3.2. Thiohydantoins: HPLC Identification and Analysis

3.2.1. Preparation of Standard Amino Acid Thiohydantoins (Crystalline)

1. Add acetylamino acid (15 mmol) and ammonium thiocyanate (1.5 g) to a stirred mixture of acetic anhydride (9 mL) and acetic acid (1 mL). Stir the suspension at 80–85°C (water bath) for 60 min, by which time the reagents will have dissolved.
2. Evaporate the solution to dryness with hydrochloric acid (1*M*, 40 mL) in an evaporating dish.
3. Dissolve the residue in water (40 mL), and again evaporate the solution to dryness. Wash the resultant solid from the evaporating dish with ice water, filter, wash with more ice water, and dry at 50°C. If desired, the thiohydantoin derivative can be crystallized either from water or from aqueous ethanol.

3.2.2. Preparation of Standard Amino Acid Thiohydantoins (In Situ)

1. Add acetylamino acid (100 nmol) to a mixture of acetic anhydride/acetic acid (4:1, 120 µL) in a screw-cap Eppendorf vial, and heat the resultant mixture for 5 min at 80°C.
2. Add HSCN/acetone solution (~1.8*M*, 30 µL), and heat the mixture at 80°C for 30 min; finally evaporate the mixture to dryness under vacuum.
3. Treat the residue at room temperature with cleavage reagent (0.5*M* KOH in 33% methanol/water), stir the mixture, immediately remove the reagent and analyze for the corresponding thiohydantoin by HPLC.

3.2.3. Analysis of Standard Amino Acid Thiohydantoins

1. All the crystalline 2-thiohydantoins are resolved by gradient elution using a Waters Pico-Tag ODS column *(13,19,20)*. Several amino acid 2-thiohydantoins have not yet been obtained in a crystalline form; consequently, the HPLC peaks obtained by *in situ* reactions with the respective amino acids (*see* Fig. 2) may not necessarily be the corresponding thiohydantoins.
2. The following buffers are used: buffer A: ammonium acetate (0.25 g/L, pH 4.76); buffer B: acetonitrile. Both buffers contain DTE (~10 mg/L). A column temperature of 40°C and a buffer flow rate of 1.0 mL/min are used. A typical buffer gradient is shown in Fig. 2. All thiohydantoins are eluted within 16 min (*see* Fig. 2).

4. Notes

4.1. Other C-Terminal Sequencing Methods

1. Mass spectrometry has proven useful for the sequencing of certain peptides because peptides tend to fragment in the ion source of the mass spectrometer at the peptide bond *(24–27)*. Disadvantages in the use of mass spectrometry are the need for:
   a. Large amounts of peptide (>100 nmol);
   b. Extremely high-resolution (and expensive) spectrometers in order to identify residues of similar or identical molecular weight, e.g., lys and gln, and leu and ile; and
   c. Sophisticated operator expertise for the interpretation of the complex mass spectra produced.
2. The use of some carboxypeptidases has aroused interest because they are capable of removing amino acids one at a time from the C-terminal

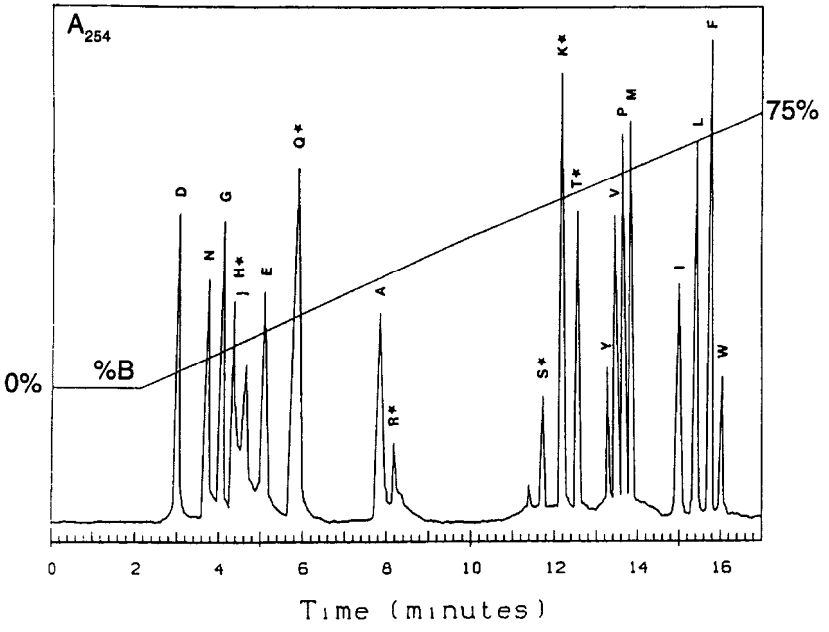

Fig. 2. Gradient elution profile of common amino acid thiohydantoins separated on a Waters Pico Tag ODS column (flow rate = 1 mL/min, column temperature = 40°C) and detected at 254 nm. The elution gradient used is shown in the figure. * Indicates amino acid "thiohydantoins" prepared *in situ*.

end of a peptide *(6,28,29)*. In practice, however, interpretation of the results obtained is not clear-cut, because the rates of cleavage are dependent on the nature of the side chain of the amino acid being removed and, to a lesser extent, on the nature of the adjacent residues. Furthermore, many carboxypeptidases undergo autodigestion; "foreign" amino acids are thereby produced, and problems in interpretation of the results therefore arise *(6)*.

4.2. Limitations to the Thiocyanate Degradation Procedure

3. At present, this procedure is limited to the sequencing of peptides containing the hydrophobic amino acids, and Trp and Tyr. This limitation arises because only the thiohydantoin derivatives of Gly, Val, Ala, Leu, Ile, Phe, Tyr, Lys, Met, His, Trp, Asn, and Gln are readily obtainable (and unequivocally characterized as such) by the application of the original thiocyanate degradation procedure (or minor variations thereof) to the

parent amino acids *(8,12,19)*. Much confusion still exists as to whether or not the thiohydantoins of Asp, Glu, Ser, Thr, Arg, and Pro (the so-called 'difficult' amino acids) can similarly be prepared *(14,18,30–32)*, (in any event, sequencing of peptides containing these amino acids has not yet been achieved routinely, if at all). Recently, it has been reported *(33)* that many of the previously unknown thiohydantoins can be prepared by the judicious use of appropriate protecting groups. Full details of this work, however, have yet to appear. It is relevant to note, however, that the thiohydantoins of His, Arg, and Lys (as their hydrochlorides) and of pro were prepared more than 40 yr ago by *indirect* routes *(34)* not involving the Schlack-Kumpf reaction. More recently, a detailed investigation *(35)* of the thiocyanate degradation with these "difficult" amino acids has been carried out. The thiohydantoin derivatives of Asp and Glu could not be prepared *(36)*, although both derivatives are obtainable by indirect methods *(37,38)*. Curiously, Asp thiohydantoin and Glu thiohydantoin have been detected during the solid-phase sequencing of certain Asp and Glu peptides *(13,20)*; the reasons for this unexpected result are unknown. Although Arg thiohydantoin has not yet been prepared in a pure state, an apparently pure (HPLC analysis) product has been obtained (by an *in situ* reaction), which is expected to be a useful indicator for the presence of Arg in a peptide. The reaction of Pro is very slow, and yields of no more than 3% have been obtained *(30,35)*; consequently, the C-terminal sequencing of peptides containing proline is expected to be very difficult. The Schlack-Kumpf reaction with Ser and Thr gives thiohydantoin derivatives of unusual structure *(39)*, that do not survive the cleavage reaction (decomposition to give the corresponding olefinic thiohydantoins occurs to a significant extent).

4.3. Manual Sequencing

4. The addition of a thiol antioxidant, e.g., dithioerythritol (DTE), retards decomposition of the amino acid thiohydantoin released from the C-terminus by the cleavage reagent. All washing solvents (except for the final acetonitrile rinse) and the cleavage reagent therefore contained DTE (0.01%).
5. With our procedure, 3 d are required for the preparation of the insoluble glass beads support. Each cycle time requires 48 min, and therefore, the procedure is comparable in speed with that used in current automated N-terminal sequencing methodology. The method does not require any drying steps and is simple to carry out. Manual solid-phase sequencing from the C-terminal end has been used to sequence the last

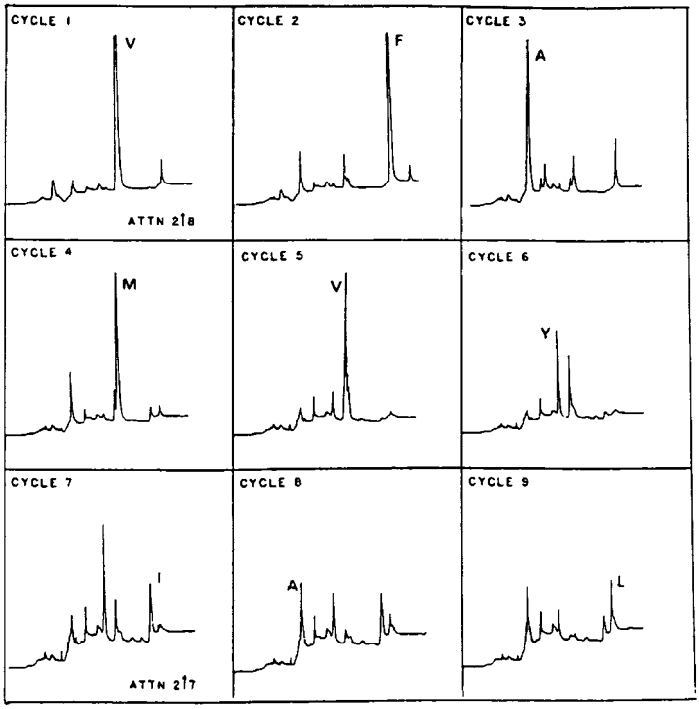

Fig. 3 C-terminal sequence (nine residues) of a synthetic decapeptide [Ⓖ-YLAIYVMAFV]

nine residues of a hydrophobic decapeptide (Tyr-Leu-Ala-Ile-Tyr-Val-Met-Ala-Phe-Val) (*see* Fig. 3).

6. The C-terminal sequencing of proteins by the thiocyanate degradation method has been less well studied; nevertheless, the C-terminal amino acids of lysozyme and ribonuclease (Leu and Val, respectively) were readily identified *(20)*.
7. Automation of the thiocyanate degradation procedure would enable:
   a. The acquisition of sequence data to be accelerated;
   b. Repetitive yields and the sequencing capability to be improved;
   c. Much smaller amounts of peptide to be sequenced; and
   d. The C-terminal sequencing procedure to be simplified.

   Recently, modified N-terminal sequenators have been successfully used for automated C-terminal sequencing *(20,40)*. With these sequenators, suitable programs have been developed for the sequencing of peptides covalently attached to activated glass beads and to PVDF membranes (the sequencing protocol is similar to that described above for the manual method).

4.4. Sequencing of Peptides Covalently Attached to Other Solid Supports

8. In our experience, only limited sequencing information can be obtained by application of thiocyanate degradation procedure to peptides (e.g., met-enkephalin) covalently bound to other solid (membrane) supports, e.g., Sequelon (DITC disks, MilliGen/Biosearch). Only two or three of the hydrophobic amino acid residues can be sequenced, an observation that is probably owing to a combination of factors, e.g., some of the peptide is held electrostatically and therefore not covalently bound. Furthermore, it is probable that the basic cleavage conditions used (*see* Section 3.1.6.) also remove the peptide. If less concentrated base is used or a shorter cleavage time is employed in order to overcome this problem, then not all the C-terminal amino acid thiohydantoin is removed in the first cycle and, therefore, thiohydantoin overlap occurs in the next cycle.
9. A new support (a carboxylic acid-modified polyethylene film) has been developed *(22)* that exhibits increased stability to base and high temperatures, and possesses a higher capacity for coupling peptides. As yet, we have been unable to investigate the use of this support with our thiocyanate degradation procedure.

Acknowledgments

We wish to thank A. Kirkpatrick and Auspep Pty. Ltd. (Australia) for making available to us the model peptides used in our studies.

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Chapter 38

Analysis of Cysteine Residues and Disulfide Bonds

Alastair Aitken

1. Introduction

1.1. Amino Acid Analysis

If cysteine and cystine are identified in proteins, they will require modification before they can be quantified. Oxidation to cysteic acid is still commonly used with postcolumn amino acid analyzers employing ion-exchange resins and ninhydrin detection. Oxidation with performic acid converts cysteine and cystine to cysteic acid and methionine to methionine sulfone. Tryptophan is also modified during the oxidation procedure by indole ring opening to form *N*-formylkynurenine and other products. Tyrosine may become halogenated if, for example, traces of bromide or chloride ions are present. The yield of cysteic acid is 90%, whereas 100% oxidation of Met to the sulfone is achieved.

A number of commercial high-sensitivity amino acid analyzers employ precolumn derivatization with PITC and HPLC separation of the resulting PTC-amino acids (*see* Chapter 29). Determination of cysteine poses problems, since several commonly used derivatives are difficult to resolve in most separation systems. PTC-cysteic acid migrates with the solvent front on C_{18} columns, and carboxymethyl cysteine migrates very closely to other polar PTC-amino acids. However, the 4-pyridylethyl derivative is an acid stable derivative of cysteine, which is well resolved in all separation systems. Cysteine-con-

From: *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
Edited by J M Walker

taining peptides can be detected by the absorbance of this group at 256 nm during HPLC.

The identification of cysteinyl residues in proteins and peptides is also a major problem in microsequencing, and 4-vinylpyridine also offers several advantages for the gas-phase sequencer. The PTH derivative (PTH-*S*-pyridylethylcysteine) is well resolved in sequence analysis (*see* Note 1). A vapor-phase method for pyridylethylation of peptides and proteins prior to microsequencing has been published by Amons *(1)* (*see also* Chapter 34).

1.2. Chemical Cleavage

The presence of cysteine residues in a protein may also be established if these are the target of specific cleavage methods. The best chemical method utilizes conversion of the —SH moiety to the thiocyano group *(2)*. This can cyclize to an acyliminothiazolidine followed by rapid hydrolysis to give fragments whose first residue contains the 2-iminothiazolidinyl moiety. Raney nickel has been used to remove this group to enable Edman degradation.

1.3. Enzymatic Cleavage

The following proteases will cleave at cysteine residues if these are first converted to *S*-aminoethylcysteine. Trypsin will cleave at the C-terminal side of arginine, lysine, and *S*-aminoethylcysteine bonds. Some chymotryptic-like anomalous cleavages may be seen, especially cleavage at tyrosine. This may be because of inherent specificity of trypsin. Since the rate of cleavage of *S*-aminoethylcysteine residues with trypsin is slower, the extended incubation time may increase these additional cleavages. Incidentally, C-terminal aminoethylcysteine residues are, like lysine and arginine, susceptible to cleavage with carboxypeptidase B. Lysine proteinase from *Armillaria mellea,* which cleaves at the N-terminal side of lysine residues (Xaa-Lys), will also cleave 2-aminoethylcysteine bonds. Combined with trifluoracetylation to block lysine side chains, this can be a very useful method of obtaining specific cleavage at modified cysteine residues. There may, however, be ineffective trifluoroacetylation if cysteine residues are adjacent to lysine, due to a catalytic deblocking mechanism *(3)*. Endoproteinase Asp-N (commercially available from Boehringer) cleaves peptide bonds N-terminal to aspartic and cysteic acid residues *(4)*. The cleavage may be restricted to cysteine residues

if carboxyl groups are blocked by a glycinamide-carbodimide coupling procedure *(5)*.

1.4. Are Disulfide Bonds Present?

The presence of disulfide bonds in a protein is most easily determined by comparing electrophoretic mobility in acrylamide gels with and without reduction. Free thiols should first be blocked with the neutral reagent, iodoacetamide (which does not introduce charge changes). The electrophoresis system may be in SDS, or acid urea gels may be run. If different ratios of iodoacetamide and iodoacetate are used to derivatize the protein (with or without prior reduction of the disulfide bonds), the number of cysteine and/or cystine residues can be calculated from the resulting mobility "ladder" on acid-urea gels *(6)*.

1.5. Titration with Ellman's Reagent

In the past, Ellman's reagent (DTNB) has been widely used to assay the number of free thiols by measuring the nitrothiobenzoate released *(7)*. Alternatively, half the protein sample may be reacted with iodoacetic acid, iodoacetamide, or 4-vinylpyridine to modify free-SH groups. The remainder is then reduced under denaturing conditions and alkylated. Hydrolysis and analysis of the two samples will give free sulfhydryl and total cysteine compositions, respectively.

1.6. Diagonal Electrophoresis

Classical techniques for determining disulfide bond patterns usually require the fragmentation of proteins into peptides under low-pH conditions to prevent disulfide exchange. Pepsin or cyanogen bromide are particularly useful (*see* Note 2). The diagonal techniques were developed by Brown and Hartley *(8)* still find application today (*see also* vol. 1 of this series). After paper or thin-layer electrophoresis at pH 6.5, the TLC plate or paper was exposed to performic acid, which oxidizes cystine residues to cysteic acid. Electrophoresis in the second dimension then produced off-diagonal spots, which represented two peptides previously covalently linked. A modern micromethod employing reverse-phase HPLC is given below.

1.7. Electrochemical Detection

A dual-electrode electrochemical (EC) HPLC detector has been used to identify peptides that contain cysteine residues and/or disul-

fide bonds *(9)*. The principle of this, highly specific, detector is based on the redox reaction that occurs at an amalgam electrode (+0.15 V) relative to a silver/silver chloride reference electrode. If a second mercury electrode (which is –1.0 V relative to the Ag/AgCl electrode) is placed upstream from the thiol-detecting electrode, some of the disulfides will be reduced to thiols, which will then be detected at the downstream electrode. In brief, when operated in the two-electrode mode this detects both disulfide and thiol-containing peptides, whereas, in the one-electrode mode, only thiol-containing peptides will cause a response.

1.8. Mass Spectrometry

Mass spectrometry, particularly fast atom bombardment (FABMS), is playing a rapidly increasingly role in protein chemistry and sequencing *(10)*. This is particularly useful in determining sites of co- and posttranslational modification. The use of FABMS in locating disulfide bonds is no exception. Like the diagonal methods outlined above, mass spectrometry can be used to identify peptides linked in pairs by —S—S— bonds most readily when cleavage methods are employed that minimize disulfide exchange (*see* Note 2). FABMS can of course readily analyze peptide mixtures. Therefore, it is not always necessary to isolate the constituent peptides. Partial acid hydrolysis, although nonspecific, has been successfully used in a number of instances *(11)*. Combined with computer programs that will predict the cleavage position of any particular proteinase or chemical reagent, simple knowledge of the mass of the fragment will, in most instances, give unequivocal answers as to which segments of the polypeptide chain are disulfide-linked. If necessary, one cycle of Edman degradation can be carried out on the peptide mixture, and the FABMS analysis repeated. The shift in mass(es) that correlate with loss of specific residues will confirm the assignment.

It may be possible to estimate the number of intramolecular disulfide bonds by mass spectrometry by measuring the mass of the intact polypeptide before or after reduction. Electrospray mass spectrometers will give accurate estimates of mol wt above 100,000 Dalton, but the increased mass of 2 Dalton for each disulfide bond will in all probability be too small to obtain an accurate estimate for polypep-

tide of mass greater than ca. 10,000 (accuracy obtainable is >0.01%). It is unlikely that the lower resolution obtained with laser desorption time-of-flight mass spectrometers (ca. 0.1% up to 20,000 Dalton) would permit a meaningful analysis. On the other hand, oxidation with performic acid *(12)* will cause a mass increase of 48 Dalton for each cysteine and 49 Dalton for each half-cystine residue. (Remember that Met and Trp will also be oxidized.)

It is clear from the above that a range of techniques is available for the identification of cysteine residues and the determination of disulfide bridges. This chapter will describe three methods commonly used in laboratories, namely chemical cleavage at cysteine residues with NTCB, detection of disulfide-linked peptides by HPLC, and mass spectrometry methods.

2. Materials

2.1. Chemical Cleavage at Cysteine

1. 6*M* Guanidinium—HCl, in 0.2*M* Tris-acetate, pH 8.
2. 1 m*M* and 10 m*M* Dithiothreitol (DTT).
3. 0.2*M* Tris-acetate, pH 8.
4. 2-Nitro-5-thiocyanobenzoic acid (NTCB).
5. 0.2*M* Sodium borate, pH 9.
6. Raney nickel.
7. 50 m*M* Tris-HCl, pH 7.0.

2.2. Analysis of Disulfide-Linked Peptides by HPLC

1. Tri-*n*-butyl-phosphine: 1% in isopropanol.
2. 4-Vinylpyridine. Stored at –20°C under argon or nitrogen to minimize polymerization.
3. 100 m*M* Tris-HCl, pH 8.5.
4. Isopropanol.
5. 1*M* Triethylamine-acetic acid, pH 10.0.
6. Trifluoracetic acid.
7. HPLC-grade water (purchased bottled or purified by "Elgastat").
8. Acetonitrile (far-UV HPLC grade).
9. Vydac reverse-phase HPLC columns.

2.3. Mass Spectrometry

Thioglycerol (Fluka).

3. Methods

3.1. Chemical Cleavage at Cysteine

1. Dissolve the protein in 6*M* guanidinium-HCl, 0.2*M* Tris-acetate, pH 8.0, containing 1 m*M* DTT (if disulfides are present add 10 m*M* DTT and incubate at 37°C for 1 h or room temperature for 2 h).
2. Add a fivefold molar excess (over total thiol) of NTCB (*see* Note 3), readjust pH to 8.0 with 1*M* NaOH if necessary, and incubate at 37°C for 30 min or a few hours.
3. Acidify the reaction mixture to pH 4.0 with acetic acid, cool to 4°C, remove the protein by dialysis or gel filtration, and freeze-dry.
4. Dissolve the derivatized protein in 6*M* guanidinium-HCl, sodium borate, or Tris-acetate (0.2*M*) pH 9.0 and incubate for 18–72 h at 37°C. This will cleave the peptide bonds containing the modified cysteines (*see* Note 4).
5. Following cleavage, the liberated peptides (containing the iminothiazolidine derivative of cysteine) can be unblocked by incubation with Raney nickel (10-fold excess by weight over total fragments of protein to be desulfurized) in 50 m*M* Tris-HCl, pH 7.0 at 50°C for 7 h in a nitrogen atmosphere (*see* Note 5).
6. Cool the mixture to 4°C and centrifuge (2000 rpm for 20 min or similar in a microfuge) to remove the Raney nickel.

3.2. Detection of Disulfide-Linked Peptides by HPLC

1. Alkylate the protein without reduction to prevent possible disulfide exchange by dissolving in 100 m*M* Tris-HCl pH 8.5 and adding 1 µL of 4-vinylpyridine. Incubate for 1 h at room temperature, and desalt by precipitation with 95% ice-cold ethanol (*see* Note 6).
2. Fragment the protein under conditions of low pH (*see* Note 2), and subject the peptides from half the digest to reverse-phase HPLC. A combination of different chemical and enzymatic cleavages may be required if fragments are large.
3. For reverse-phase HPLC of polypeptides, gradients of water to acetonitrile (containing TFA) are most commonly used (*see also* Chapter 31). Optimal separation conditions are found with a pilot run using 5 or 10% of the sample with the absorbance set at an appropriately higher sensitivity. The UV detector is set at 210–215 nm, which is the absorbance maximum of the peptide backbone. (The exact wavelength selected is dependent on the particular UV detector and how well it can be "backed off.") The ion-suppression agent, TFA, and the acetonitrile may have some absorbance at this wavelength, and the exact

percentage of TFA required in each buffer is determined by measuring the absorbance of each. Typically, 0.1% TFA in buffer A (water) and 0.088% TFA in buffer B (acetonitrile) using the sources and quality of reagents listed in "Section 2" will result in a much smoother baseline that does not rise across the gradient. A gradient from 0% buffer B to 60% buffer B over 60 min at a flow rate of 1 ml min^{-1} is a good initial condition to test. Vydac C_4, C_8, or C_{18} columns give particularly good resolution depending on the size range of fragments produced. With a particularly complex mixture of polypeptides, an additional HPLC separation using higher pH in the same solvents (but replacing TFA with ammonium or sodium acetate, 50 m*M*, pH 4 to 7 or 50 m*M* sodium phosphate) may be required to obtain complete separation of the fragments of interest. In this case, it is better to use this separation system first, followed by the separation in TFA, since this will avoid problems with the presence of these salts in subsequent microsequencing or mass spectrometry (*see* Note 7).

4. To the other half of the digest (dried and resuspended in 10 µL of isopropanol), add 5 µL of 1*M* triethylamine-acetic acid, pH 10, 5 µL of tri-*n*-butyl-phosphine (1% in isopropanol) and 5 µL of 4-vinylpyridine. Incubate for 30 min at 37°C, and dry *in vacuo*, resuspending in 30 µL of isopropanol twice. This procedure cleaves the disulfides and modifies the resultant SH groups. (The reduced material may alternatively be reacted with radioactive iodoacetamide to facilitate detection of the disulfide-linked cysteines.)
5. Cysteine-linked peptides are identified by the differences between elution of peaks from reduced and unreduced samples on reverse-phase HPLC. Collection of the alkylated peptides (which can be identified by rechromatography with detection at 254 nm) and a combination of sequence analysis and mass spectrometry should allow disulfide assignments to be made.

3.3. Mass Spectrometry Methods

1. Peptides generated by any suitable proteolytic or chemical method (*see* Note 8) are analyzed by FABMS by standard techniques *(13)*. However, the use of thioglycerol and mixtures of related compounds in the matrix should be avoided for obvious reasons. Despite this, even with glycerol alone, as the matrix compounds, disulfide bonds will be partially reduced during the analysis and peaks corresponding to the individual components of the disulfide-linked peptides will also be observed.
2. The peptide mixture is incubated with reducing agents, such as mercaptoethanol and DTT, and reanalyzed as before. Control samples

with the above reagents (employing the same matrix compounds) are essential to avoid misleading results owing to additional matrix-derived peaks. Peptides that were disulfide-linked disappear from the spectrum and reappear at the appropriate positions for the individual components. For example, in the positive ion mode, the mass (M) of disulfide-linked peptides (of individual masses A and B) will be detected as the pseudomolecular ion at $(M + H)^+$, and after reduction, this will be replaced by two additional peaks for each disulfide bond in the polypeptide at masses $(A + H)^+$ and $(B + H)^+$. Remember that A + B equals M + 2 because of the conversion of cystine to two cysteine residues, i.e., —S—S— → —SH + HS—.

3. Peptides containing an intramolecular disulfide bond will appear at 2 amu higher. Such peptides, if they are in the reduced state, can normally be readily reoxidized to form an intramolecular disulfide bond by bubbling a stream of air through a solution of the peptide for a few minutes.

4. Notes

1. The relative elution position of PTH-pyridylethylcysteine from the on-line HPLC system of the Applied Biosystems gas-phase sequencer is between PTH-valine and diphenylthiourea. On the PTC-amino acid analyzer, PTC-pyridylethylcysteine elutes between the derivatives of methionine and isoleucine, in approx 15 min (in contrast, PTC-carboxymethylcysteine elutes between PTC-Glu and PTC-Ser).
2. Pepsin will cleave at the N- and C-terminal side of hydrophobic and neutral amino acids. It would appear to have a particular preference for leucine and phenylalanine (and, to a lesser extent, methionine and tryptophan). Glutamate-containing bonds may also be cleaved. Isoleucine-containing and Tyr-X bonds are relatively resistant. Although it has this broad specificity, pepsin is a very useful proteinase in the study of disulfide bonds. At the pH optimum, there is little disulfide exchange. Normal digestion conditions are incubation at 25°C for 1–2 h at pH 2–3 (10 m*M* HCl or 5% formic acid) with an enzyme substrate ratio of 1.50.

   Endoproteinase Glu-C has two pH optima, 8 and 4. At this latter, acidic pH (in ammonium acetate) disulfide bond rearrangement would be limited. It may be useful to note that the pH optimum of thermolysin is 1 U lower (at pH 7) than most serine proteinases. Thermolysin can be used above 60°C (when 2–3 m*M* calcium should be added to enhance thermostability), and both proteases are active in urea (4*M* for Glu-C; 6*M* for thermolysin) and SDS (0.2 and 0.5%, respectively).

   The most widely applicable chemical method for specific chemical

cleavage is undoubtedly with cyanogen bromide. It has been suggested that guanidinium hydrochloride (6*M*) in 0.1–0.2*M* HCl may be a more suitable acid medium for CNBr cleavage during the analysis of disulfide bonds because of the inherent reducing redox potential of formic acid, which is the most commonly used protein solvent *(14)*.

3. 2-Nitro-5-thiocyanobenzoic acid (NTCB) can be synthesized from 5,5'-dithionitrobenzoic acid (DTNB) using NaCN, and an alternative two-step procedure for *S*-cyanylation is also available. This involves treatment of the protein with DTNB followed by generation of the thiocyano derivative with KCN *(15)*.
4. This process will generate peptides blocked at their N-termini by the modified cysteine derivative (the iminothiazolidine). Without generation of a free amino group, therefore, the Edman degradation procedure will not proceed. The blocked amino terminus would not pose a problem during sequencing by mass spectrometric techniques.
5. W-6 Raney Nickel catalyst, prepared by addition of NaOH to Raney Nickel-aluminum alloy *(16)* was found to be much more effective than the commercial catalyst. Cysteine derivatives and methionine are converted to alanine and β-aminobutyric acid, respectively.
6. Cysteine-containing peptides generally behave badly on reverse-phase HPLC, being very hydrophobic and eluting, if at all, as broad peaks. It is advisable, therefore, to derivatize this residue before enzymatic or chemical cleavage. Classically, the reagent used has been iodoacetic acid or iodoacetamide.
7. If microbore (1 mm internal diameter) or narrow bore (2.1 mm) columns are used, the conditions are scaled down accordingly. For large polypeptides, columns with larger pore size particles (300 Å) may result in better recovery. An alternative ion-suppression agent, heptafluorobutyric acid, or an alternative organic solvent, propanol, may give suitably different selectivity of retention on any particular column. At higher pH, using the salt buffers mentioned, the side chain and C-terminal carboxyl group ionization will not be suppressed. Hence, different modes of interaction with the reverse-phase support (usually silica-based) will be possible, resulting in very different retention properties for some particular peptides.
8. When analyzing proteins that contain multiple disulfide bonds it may be appropriate to carry out an initial chemical cleavage (CNBr, is particularly useful) followed by a suitable proteolytical digestion. The initial acid chemical treatment will cause sufficient denaturation and unfolding as well as peptide bond cleavage to assist the protease in digestion to completion. If a protein has two adjacent cysteine residues, this peptide bond will not be readily cleaved by specific endopeptidases. For example, this problem was overcome during mass spectrometric analysis of the

disulfide bonds in insulin by using a combination of an acid proteinase (pepsin) and carboxypeptidase A as well as Edman degradation *(11)*.

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CHAPTER 39

Preparation and Characterization of Monoclonal Antibodies to Proteins and Other Cellular Components

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1. Introduction

Monoclonal antibodies (MAbs) have provided cell and molecular biologists with a key that has unlocked the door behind which the function of many proteins and other cellular molecules would have lain hidden. Indeed, monoclonal antibodies are essential reagents for the isolation, identification, and cellular localization of specific gene products, and for aiding in the determination of their macromolecular structure. They can also help in identifying the function of the protein. Although the ability to clone and sequence specific genes has revolutionized our understanding of cellular structure and function, the preparation of recombinant proteins and the synthesis of peptides based on protein sequence derived from cDNA clones have provided sufficient material for generating specific antibodies. The proteins may be isolated and purified directly from cells, or recombinant proteins may be derived from prokaryotic systems, such as *E. coli*, or from eukaryotic expression systems, such as Chinese hamster ovary cells (CHO) or insect cells expressing constructs in baculovirus. The eukaryotic system are being used increasingly for expression of glycoproteins because the recombinant material is glycosylated. It should be remembered, however, that glycosylation

From: *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
Edited by: J. M. Walker

may be species-specific, and if one of the functions of the protein depends on carbohydrate, then the function of a recombinant glycoprotein may be altered depending on the species used for expression.

A number of protocols will be described here that we have used successfully with both rat (Y3 and IR983F) and mouse (SP2/0) myelomas to generate MAbs to cellular proteins, recombinant proteins, or peptides based on cDNA sequences. Successful hybridoma production relies on the ability to:

1. Generate specific B-cells;
2. Fuse them with a myeloma cell line;
3. Identify the antibodies that are sought in culture supernatants; and
4. Isolate and clone the specific hybridoma.

Of particular importance is the elicitation of the specific B-cells required for fusion, and several protocols to achieve this aim will be described. It should be remembered that the presence of specific antibody in serum is not necessarily a guarantee of success, nor is its absence a surety for failure.

The second important requirement is for a quick, reliable assay(s) for the specific antibody that can be applied to the large number of culture supernatants (≥96) that may be generated. usually, the assays make use of a labeled second antibody (e.g., rabbit, sheep, or goat antibodies directed against the $F(ab')_2$ of mouse or rat immunoglobulins) to identify the binding of monoclonal antibody to antigen. The second antibody can be detected because it is conjugated to a fluorescent marker e.g., fluorescein, or a radiolabel, such as Iodine-125. Alternatively, conjugates of second antibody with enzymes such as alkaline phosphatase, peroxidase, or β-galactosidase may be employed.

Persistence is an absolute requirement for the hybridoma producer; fusions can fail for many reasons, and it is essential not to give up because of early failures. The methods described in this chapter include techniques for:

1. Preparation of antigen;
2. Immunization;
3. Hybridoma production; and
4. Assaying the MAb-producing hybridomas.

2. Materials

2.1. Generation of Immune Spleen or Lymph Node Cells

1. Rats of any strain aged 10–12 wk, BALB/c mice aged 6–8 wk (*see* Notes 1 and 2).
2. Phosphate buffered saline (PBS): Dissolve in water 1.15 g of Na_2HPO_4, 0.2 g of KH_2PO_4, 0.2 g KCl, 8.0 g of NaCl and make up to 1 L. The pH should be 7.4.
3. Antigen: One of the following sources of antigen can be used to raise MAbs.
   a. Cells, e.g., mammalian cells overexpressing a specific protein or mouse 3T3-cells expressing recombinant human membrane protein (*see* Note 3).
   b. Soluble protein dissolved in PBS at a concentration of 1.0–4.0 mg/mL.
   c. Soluble recombinant proteins extracted from cells or supernatants of eukaryotic cells (e.g., Chinese hamster ovary cells or insect cells expressing recombinant baculovirus) or bacteria, such as *E. coli* harboring plasmid or recombinant viruses. In *E. coli,* recombinant material is often generated as a fusion protein with β-galactosidase (*see* Notes 3 and 4).
   d. Proteins separated in sodium dodecylsulfate containing polyacrylamide gels (SDS-PAGE) and eluted electrophoretically from gel slices (*see* Chapter 21). Often, β-galactosidase fusion proteins are prepared in this way because of their poor solubility (*see* Note 4).
   e. Peptide conjugated to a protein carrier and dissolved in PBS.
4. Freund's complete adjuvant (CFA).
5. Freund's incomplete adjuvant (IFA).

2.1.1. Conjugation to Carriers

1. Ovalbumin, bovine serum albumin (BSA), or Keyhole lympet hemocyanin (KLH), at 20 mg/mL in PBS.
2. PPD-kit (tuberculin-purified protein derivative, Cambridge Research Biochemicals Ltd., Cheshire, UK).
3. Glutaraldehyde (specially purified grade 1): 25% solution in distilled water.
4. 1*M* glycine-HCl, pH 6.6.
5. Phosphate-buffered saline (PBS).

2.2. Hybridoma Production

1. Dulbecco's Modified Eagle's Medium (DMEM): Containing glucose (1 g/L) bicarbonate (3.7 g/L), glutamine ($4 \times 10^{-3}M$), penicillin (50 U/mL), streptomycin (50 µg/mL) and neomycin (100 µg/mL) stored at 5–6°C and used within 2 wk of preparation.
2. Fetal calf serum: inactivated by heating for 30 min at 56°C and tested for ability to support growth of hybridomas (*see* Note 5).
3. HAT selection medium: Prepare 100X HT by dissolving 136 mg of hypoxanthine and 38.75 mg of thymidine in 100 mL of 0.02*M* NaOH prewarmed to 60°C. Cool, filter-sterilize, and store at –20°C in 1- and 2-mL aliquots. Prepare 100X A by dissolving 1.9 mg aminopterin in 100 mL 0.01*N* NaOH. Then filter sterilize and store at –20°C in 2 mL aliquots. HAT medium is prepared by adding 2 mL of HT and 2 mL of A to 200 mL of DMEM containing 20% fetal calf serum.
4. HT medium: Add 1 mL of HT to 100 mL DMEM containing 10% FCS.
5. Feeder cell for fusion cultures (*see* Note 6) essential for fusions employing rat myelomas: Three hours to 1 d before fusion, $2–4 \times 10^6$ rat fibroblasts in 10 mL DMEN and contained in a 30 mL plastic universal are irradiated with about 30 grays (3000 rad) of γ or X-rays. Dilute with 90 mL of HT (3 h before fusion) or DMEM containing 10% FCS (1 d before fusion), and plate 1-mL aliquots into four 24-well plates or 0.2-mL aliquots into five 96-well plates. Alternatively, use thymocytes from spleen donors (mouse).
6. PEG solution: Weigh 50 g of polyethyleneglycol (1500 mol wt) into a capped 200 mL bottle, add 1 mL water, and then autoclave for 30 min at 120°C. Cool to about 70°C, add 50 mL of DMEM, mix, and after cooling to ambient temperature, adjust the pH to about 7.2 with NaOH (the mixture should be colored orange). Store a 1-mL aliquots at –20°C.
7. Freezer medium: Freshly prepared 5% dimethyl sulfoxide–95% FCS.
8. Myeloma cell line: Mouse SP2/0-Ag14 or rat IR 983F cells growing exponentially in 25- or 75-cm^2 flasks containing DMEM-10% FCS (dilute to 2–3 × 105 cells/mL the day before fusion). The rat myeloma Y3 Ag1.2.3. has to be grown in spinner culture to fuse well. Seven to 10 d before cells are required, about 5×10^6 cells, stored frozen in liquid nitrogen a 1-mL aliquots in 5% dimethylsulfoxide/95% FCS, are thawed quickly at 37°C, diluted with 10 mL DMEM-10% FCS, centrifuged (500*g* × 2 min), and then resuspended in 100 mL of the same medium and placed in a 200-mL spinner flask. Stand for 2 d at 37°C to allow cells to attach to the base of the vessel. Then place the spinner flask on a Bellco magnetic stirrer running at about 160 rpm.

The Y3 myeloma has a generation time of about 10 h and exponentially growing cultures require feeding daily by fourfold dilution with fresh medium.

2.3. Screening Culture Supernatants

1. PBSA: PBS containing 0.05% NaN_3 (NaN_3 should be handled with care. It is an inhibitor of cytochrome oxidase, and is highly toxic and mutagenic. Aqueous solutions release HN_3 at 37°C).
2. PBST: PBSA containing 0.4% Tween 20.
3. PBS-BSA: PBSA containing 0.5% BSA.
4. PBS-Marvel: PBSA containing 3% Marvel (skimmed milk powder). Centrifuge or filter through Whatman No. 1 paper to remove undissolved solids. This is cheaper to use and just as effective as PBS-BSA.
5. PCB: Plate coating buffer, pH 8.2, containing 0.01*M* Na_2HPO_4/KH_2PO_4 and 0.14*M* NaCl.
6. Alkaline sarkosyl: 1% sodium dodecyl sarkosinate in 0.5*M* NaOH.
7. Plates containing cell-bound antigens: Monolayers of cells grown in 96-well polystyrene plates (*see* vol. 5, Chapter 54 for detailed instructions).
   a. Rodent cells expressing a recombinant protein, e.g., CHO or 3T3 cells, transfected with genes for human transmembrane proteins;
   b. Tumor cell lines overexpressing transmembrane proteins, e.g., the receptor for EGF or the product of the c-*erb*B-2 gene;
   c. Adherent cell line expressing high cytoplasmic levels of the specific antigen that can be accessed following fixation and permeabilization with methanol. Wash the cell with ice-cold medium and then add to each well 200 µL of methanol that has been precooled to –70°C by standing in cardice-ethanol. Leave at ambient temperature for 5 min, "flick" off the methanol, and wash twice with either medium containing 5% FCS (live cells) or PBS-Marvel (fixed cells); and
   d. Control cell line that either does not express the specific antigen (e.g., normal 3T3 cells) or in which expression is at the normal one gene copy level. This will act as the negative control.
8. Plate coated with purified soluble antigen: 96-well polystyrene (PS) or polyvinyl chloride (PVC) plate are coated with protein, recombinant protein, peptide, or peptide conjugated to a carrier protein as follows:
   a. Dissolve the antigen at 1 µg/mL in PCB.
   b. Coat by incubation with 50 µL of antigen/well for 2 h at 37°C or overnight at 4–6°C.
   c. Block the remaining reactive site by incubation for 2 h at 37°C with 200 µL/well of PBS-Marvel.

d. Wash the plates with PBST before use. In many cases, the coated plates can be stored at 4–6°C for several week (fill well with PBST), but it is wise to check their antibody-binding capacity before use.

9. Plates for antibody capture assay: Where a specific antibody is available this may be a better assay to use than 8 above, because the antigen is less likely to be subject to denaturation.
   a. Coat the well first by incubating for either 2 h at 37°C or overnight at 4–6°C with 50 µL (1–5 µg/mL in PCB) of a polyclonal or monoclonal antibody to the specific antigen. Where peptides coupled to BSA are used the plate can be coated first with rabbit antibodies to BSA.
   b. Block the well with PBSA-Marvel for 2 h at 37°C.
   c. Incubate with the specific antigen (50 µL/well of a 0.1–1 µg/mL solution in PBST. For antigens that are not readily soluble, e.g., membrane proteins extracted in nonionic detergents, the extract in 0.5–1% Triton X-100 or Nonidet-P40 can be used after suitable dilution with PBSA containing 0.5% of the detergent. All subsequent procedures should be carried out using buffers containing detergent at 0.1–0.5%. These plate are best prepared within a day of use.

2.3.1. Immunoprecipitations

1. Protein A, or specific antiimmunoglobulin covalently linked to Sepharose 4B or similar bead support for preparing immunoprecipitates.
2. CNBr-activated Sepharose 4B.
3. Radiolabeled (H^3, C^{14}, S^{35}, or I^{125}) protein or cell extract prepared in PBSA containing $10^{-3}M$ phenyl methyl sulfonyl fluoride (PMSF) as proteinase inhibitor and 0.5–1.0% nonionic detergent.
4. Purified specific monoclonal antibody.
5. Polyclonal antibody to mouse or rat immunoglobulins depending on the species in which the MAb was raised.

2.3.2. Second Antibodies

1. Sheep, rabbit, or goat antibodies to rat or mouse $F(ab')_2$, IgG, IgA, and IgM for labeling with iodine-125 to carry out radioimmunoassay (RIA), or conjugated to alkaline phosphatase or biotin for an ELISA.
2. 1.5-mL Polypropylene microcentrifuge tubes coated with 10 µg of IODOGEN (Pierce Chemical Co.) by evaporation, under a stream of nitrogen, from a 100 µg/mL solution in methylene chloride (*see* Note 7).
3. Carrier-free Iodine-125, radioactive concentration 100 mCi/mL (e.g., code IMS.30, Amersham International).
4. Gamma counter.

5. UB:100 m*M* phosphate buffer (Na_2HPO_4/KH_2PO_4) pH 7.4 containing 0.5*M* NaCl and 0.02% NaN_3.
6. 30 × 0.7 cm Disposocolumn (Bio-Rad) containing Sephadex G25, equilibrated before use with UB and pretreated with 100 µL of FCS to block sites that bind protein nonspecifically.
7. 3-mL tubes for collection of sample.
8. Lead pot for storage of ^{125}I-labeled antibodies.
9. Streptavidin labeled with ^{125}I or conjugated to alkaline phosphatase or to fluorescein.

2.3.3. Buffers and Substrates for Alkaline Phosphatase Used in ELISA

1. 10 m*M* Diethanolamine, pH 9.5, containing 0.5*M* NaCl.
2. 100 m*M* Diethanolamine, pH 9.5, containing 100 m*M* NaCl and 5 m*M* $MgCl_2$.
3. Substrate to give a soluble product (plate assays): 0.1% *p*-nitrophenyl phosphate in 10 m*M* diethanolamine, pH 9.5, containing 0.5 m*M* $MgCl_2$.
4. Substrate to give an insoluble product (Western blots): NBT stock-5% nitroblue tetrazolium in 70% dimethyl formamide. BCIP stock-5% disodium bromochloroindolyl phosphate in dimethyl formamide. Alkaline phosphatase buffer—100 m*M* diethanolamine, pH 9.5, containing 100 m*M* NaCl and 5 m*M* $MgCl_2$. Just before use, add 66 µL NBT stock solution to 10 mL of alkaline phosphatase buffer, mix well and add 33 µL of BCIP stock solution.

3. Methods

3.1. Conjugation of Peptides to Carriers

Peptides that do not bear epitopes recognized by T-cells are poor immunogens, and must be conjugated to carrier protein or PPD to elicit good immune responses.

1. Protein carrier, such as ovalbumin, BSA, or KLH: Mix the peptide and carrier in a 1:1 ratio (w:w), e.g., pipet 250 µL of each into a 5-mL glass beaker on a magnetic stirrer. Small fleas can be made from pieces of paper clip sealed in polythene tubing by heating. Add 5 µL 25% glutaraldehyde and continue stirring for 15 min at room temperature. Block excess glutaraldehyde by adding 100 µL 1*M* glycine and stirring for a further 15 min. Use directly or dialyze overnight against PBS and store at –20°C.
2. PPD kit: **Read instructions supplied with the kit very carefully.** Inhalation of the ether-dried tuberculin PPD is dangerous for tuberculin-

sensitive people to handle. Follow specific instruction to couple 2 mg of peptide to 10 mg PPD and, after dialysis, store at –20°C.

3.2. Antigens for Immunization

1. Suspend cells in PBS or DMEM at 5×10^6–10^7 cell/mL; or
2. Mix protein, peptide conjugates or eluates from polyacrylamide gels in PBS 1:1 with adjuvant (FCA for the first immunization, subsequently with IFA) in a capped plastic tube (LP3, Bijou or 30-mL universal) by vortexing until a stable emulsion is formed. Check that phase separation does not occur on standing at 4°C for >2 h. Alternatively, allow drop to fall from a Pasteur pipet onto a water surface, the drop should contract and remain a droplet and not disperse.

3.3. Immunization Procedures

1. Anesthetize animals (*see* Note 1), and take a blood sample from the jugular or tail vein into a capped 0.5- or 1.5-mL microcentrifuge tube to act as a preimmune sample. Allow it to clot, centrifuge (1500*g*), remove the serum, and store at –20°C.
2. For fusions that will use spleen cells, immunize at five sites (four times sc and one time ip) with a total of 50–500 µg of antigen in FCA/animal. Test bleed 14 d later and reimmunize using the same protocol but with antigen in FIA. Test bleed and reimmunize at monthly intervals until sera are positive for antibodies to the antigen (*see* Section 3.5. *below*). Three days before the fusions are done, rechallenge the animals iv with antigen in PBS alone.
3. For fusions using mesenteric lymph nodes of rats, the antigens are injected into the Peyer's patches that lie along the small intestine. The surgical procedures are described in vol. 5, p. 673. Again, test bleed the rats, then immunize twice or three times at 1-mo intervals, and use the mesenteric nodes 3 d after the final immunization. This protocol has resulted in good yields of specific IgG-and IgA-producing hybridomas (*see* refs. *1,2*).

3.4. Hybridoma Production

3.4.1. Preparation of Cells for Fusion

1. Centrifuge exponentially growing rat or mouse myeloma cells in 50-mL aliquots for 3 min at 400*g*, wash twice by resuspension in serum-free DMEM, count in a hemocytometer, and resuspend in this medium to $1–2 \times 10^7$ cells/mL.
2. Kill immune animals by cervical dislocation or CO_2 inhalation, test bleed, and open abdominal cavity. Remove spleens or mesenteric nodes by blunt dissection.

3. Disaggregate spleens or nodes by forcing through a fine stainless-steel mesh (e.g., tea strainer) into 10 mL of serum-free DMEM using a spoon-head spatula (dipped into ethanol and flamed to sterilize it).
4. Centrifuge cells for 5 min at 400*g*, wash twice in serum-free DMEM, and resuspend in 10 mL of the same medium.
5. Count viable lymphoid cells in a hemocytometer. Spleens from immune mice yield about 10^8 cells, from rats $3–5 \times 10^8$ cells, and the mesenteric nodes of rats up to 2×10^8 cells.

3.4.2. Fusion Protocol

1. Mix 10^8 viable lymphocytes with 5×10^7 rat myeloma cells or 2×10^7 mouse myeloma cells in a 10-mL sterile capped tube, and centrifuge for 3 min at 400*g*.
2. Pour off the supernatant, drain carefully with a Pasteur pipet, and then release the cell pellet by gently tapping tube on bench.
3. Stir 1-mL of PEG solution, prewarmed to 37°C, into the pellet over a period of 1 min. Continue mixing for a further minute by gently rocking the tube.
4. Dilute the fusion mixture with DMEM (2 mL over a period of 2 min and then 5 mL over 1 min).
5. Centrifuge for 3 min at 400*g*, then resuspend the cells in 200-mL HAT selection medium, and plate 2-mL aliquot into four 24-well plates seeded with irradiated fibroblasts or, if necessary, five 96-well plates (fusions with SP2/0 myeloma). (*See* Note 6 re: feeder cells.)
6. Screen culture supernatants for specific antibody 6–14 d after commencement of incubation at 37°C in 5% CO_2-95% air (*see* Section 3.5. *below*).
7. With a Pasteur pipet, pick individual colonies into 1 mL of HT medium contained in 24-well plates. Feed with 1 mL of HT medium and split when good growth commences. Freeze samples in liquid N_2.
8. Rescreen the picked colonies, and expand positive cultures. Freeze samples of these in liquid nitrogen and clone twice.

3.4.3. Cloning of Hybridomas

1. Prepare a suspension of mouse thymocytes (2.5×10^4/mL of DMEM-10% FCS), and use directly or seed irradiated rat fibroblasts in DMEM-10% FCS at 5×10^3 cells/well into 96-well plates for use the next day.
2. Centrifuge cells from at least two wells of a 24-well plate that contain confluent layers of hybridoma cells. Count the number of cells, and then dilute to give about 50 cells in 20 mL of HT or DMEM-10% FCS.
3. Carefully "flick off" the supernatant medium from the rat feeder cells, and plate 0.2-mL aliquots of hybridoma cell into each of the 96-wells.

4. Examine the plates 5-10 d later, and screen those wells that contain only single colonies.
5. Pick cells from positive wells into 24-well plates, expand, and freeze in liquid nitrogen.
6. Reclone the best antibody-producing colonies (*see* Section 3.5. *below*).

3.5. Assays for Specific Monoclonal Antibodies

In most of the assays described, the detection of rat or mouse MAb depends on the use of a second antibody reagent specific for $F(ab')_2$ or heavy-chain isotype. The second antibodies are detected because they have either a radiolabel (Iodine-125) fluorescent tag (fluorescein), or are conjugated to an enzyme (e.g., alkaline phosphatase, peroxidase, or β-galactosidase) either directly or indirectly via a biotin-streptavidin bridge. As examples of these procedures, two alternative types of methodology, i.e., radioimmunoassay (RIA) using ^{125}I-labeled antibodies and enzyme-linked immunosorbant assay (ELISA), using alkaline phosphatase conjugates will be described. It is assumed that in most cases, the second antibodies will be bought in either as purified material for radiolabeling or already conjugated to fluorescein, biotin, or the enzyme of choice (*see* ref. *3* for additional methods).

3.5.1. Radiolabeling with Iodine-125

All manipulations should be carried out in a Class I fume hood according to local safety regulations.

1. Add 50 µg purified antibody in 0.1-mL PBS to an IODO-GEN-coated tube followed by 500 µCi of ^{125}I (e.g., 5 µL of IMS-30, Amersham International). Cap and mix immediately by "flicking," and keep on ice with occasional shaking (*see* Note 8).
2. After 5 min, transfer the contents of the tube with a polythene, capillary ended, Pasteur pipet to a prepared Sephadex G-25 column, and then wash in and elute with UB.
3. Collect 1-mL fractions by hand, count 10-µL aliquots, and pool the fractions containing the first peak of radioactivity.
4. Store at 4°C in a lidded lead pot (e.g., Iodine-125 container).

3.5.2. RIA Using Antigens Bound Directly to PVC Multiwell Plates

1. Add 50 µL of antibody containing culture supernatant, purified antibody, or ascites, diluted to 1–10 µg/mL in PBS-Marvel to each of the antigen-coated wells.

2. Incubate for 1 h at ambient temperature. Then wash three times with 200 µL of PBST/well.
3. Add 50 µL of ^{125}I-labeled second antibody (10^5 cpm/50 µL in PBS-Marvel) to each well, and incubate for a further 1 h at ambient temperature.
4. Carefully discard the radioactive supernatant by inverting the plate over a sink, designated for aqueous radioactive waste, and then wash the wells three times with PBST.
5. Cut the plates into individual wells with scissors, and determine the ^{125}I-bound in a gamma counter.

3.5.3. RIA Using Antigens Bound via Antibody to PVC Multiwell Plates

The assays are done as described in Section 3.5.2. above with the modification that, if the antigens require the presence of a nonionic detergent to retain their solubility or native conformation, the detergent should be added to the PBS-Marvel diluent/wash solution.

3.5.4. Assay Using Live Adherent Cells Grown in Multiwell PS-Plates

All solutions should be prepared in DMEM or other suitable growth medium containing 5% FCS—new born calf serum (NCS), tested for nontoxicity, is a cheaper alternative. If the effect of antibody binding on the behavior of the membrane protein is unknown, then all incubations should be carried out at 4°C (float plates on ice bath, and precool diluents). Monolayers of cells vary widely in their adhesion to plastic, and also, they may roundup after prolonged incubation at 4°C because of depolymerization of microtubules. *See* Note 7 regarding use of fixed cells.

1. Wash cell monolayer with DMEM-5% FCS or NBS to remove nonadherent/dead cells then proceed as described for antigens bound to PVC plates (Section 3.5.2. above), but using DMEM-5% FCS/NBS for all diluents and washings.
2. After the final wash, lyse the cells by incubating for 15 min at room temperature with 200 µL/well of alkaline sarkosyl.
3. Transfer lysates to LP2 tubes, and determine the amount of ^{125}I present. Depending on the cells used, the background binding will vary from 50–200 cpm/well, whereas positive wells will be at least five times this value.

3.5.5. Competitive Assays

These are useful for mapping epitopes, determining whether or not two antibodies crossreact, and for comparing antibody affinities. They are the basis of quantitative assays (RIA), and use multiwell plates coated either with antigen or antibody as described in Section 2.3. above. The principle is either to compete the test antibody with ^{125}I-labeled specific monoclonal (*see* Section 3.5.1. for preparation) or polyclonal antibody for binding to antigen bound to a plastic surface (antibody capture can be used to secure antigen to the plastic well, and this form of assay is useful where it is necessary to retain the antigen in its native conformation), or to compete test antibody with ^{125}I-labeled antigen for binding to a specific antibody bound to a plastic surface.

1. Make doubling dilutions in PBS-Marvel of culture supernatant or of purified antibody starting at 20 µg/mL to give a 50-µL final vol.
2. Add to each well 50 µL ^{125}I-labeled specific antibody or antigen (2–4 $\times 10^4$ cpm/mL in PBS-Marvel).
3. Transfer 50-µL aliquots of the mixtures to the antibody or antigen coated PVC multiwell plate so that each well contains 1–4 $\times 10^4$ cpm of radiolabel.
4. After 1–4 h at ambient temperature, wash the plates three times with PBST, and determine the ^{125}I bound.

Controls to determine maximum binding should be included together with a standard curve prepared from dilutions of unlabeled specific antibody or antigen. Comparison of the inhibition curve produced with the test antibody with that of the control yields information on the crossreactivity of the two antibodies and their relative affinities for antigen. Expect to get a maximum binding of between 1–5 $\times 10^3$ cpm/well, depending on the purity and quality of the labeled antigen or antibody. Hybridoma supernatant containing good competing antibodies (1–10 µg/mL) will reduce binding to the background (50–100 cpm/well).

3.5.6. Immunoprecipitation

This is an essential procedure for the isolation of antigens from complex sources (e.g., cells) and for their subsequent separation by electrophoresis in SDS-containing polyacrylamide gel and analysis by Western blotting.

1. Label cellular proteins metabolically by incubating cultures for 4–24 h with ^{35}S-methionine, ^{3}H-lysine or ^{14}C-amino acids in medium deficient in the relevant amino acid (*see* Chapter 10).
2. Wash cell three times with complete medium, and then incubate for a further hour in the same medium.
3. Wash the cells in ice-cold PBSA containing $10^{-3}M$ PMSF then lyse with the minimum volume (1–2 mL/25 cm^2 flask) of PBSA containing $10^{-3}M$ PMSF and 0.5% Triton-X 100 by incubating for 30 min on ice.
4. Transfer the lysate to a centrifuge tube, and spin at 30,000*g* for 30 min to remove cell debris.
5. Prepare immunoabsorbant beads by linking 5–15 mg of purified MAb or polyclonal antibody to mouse or rat $F(ab')_2$ to Sepharose 4B (about 3 mL of swollen gel following manufacturer's instructions). Alternatively, use Protein A-beads for mouse antibodies.
6. Incubate 1 mL of cell lysate (ca 3×10^6 cells) with either 10 µg of MAb or 100 µL (packed vol) Ab-beads overnight at 4°C.
7. When using soluble MAb add 50 µL (packed vol) of Protein A-beads (mouse) or anti-Ig beads (rat), and incubate for a further 1 h at 4°C.
8. Wash the beads three times with PBSA containing 0.5% Triton-X 100 and PMSF, and pelleting beads by centrifugation.
9. Elute the antigen (± MAb) by heating beads (5 min, 95°C) with equal volume of SDS-sample buffer, and apply to an SDS-containing polyacrylamide gel (*see* Chapter 5). Run prestained markers on these gels because they will transfer to blots, and assist in determining the molecular size of the proteins.

3.5.7. Western Blotting

This procedure is particularly useful where an antibody recognizes an amino acid sequence or carbohydrate moiety, but may be unsuitable for antibodies that bind to a conformational epitope on the protein. The proteins are electrophoretically transferred from the SDS-containing polyacrylamide gels to nitrocellulose or PVDF membranes and then probed with antibody (*see* Chapter 24 for detailed protocol).

1. Follow the instructions of the suppliers of the blotting equipment.
2. Carefully separate the membrane from the polyacrylamide gel. Block the blot by placing it in a polythene bag, add 20 mL PBS-Marvel ± 3% BSA/180 × 150 cm blot, and then, after sealing the bag, incubate for 2 h at 37°C on a rocking platform.

3. Wash the blot twice with PBST, and then cut into strips when necessary (after labeling the individual strip in pencil), using the gel comb as a guide. Alternatively, use a proprietory manifold that allows staining of individual tracks without the need for cutting the blot into individual strips.
4. Place each blot strip in a suitably-sized polythene bag, and add 1 mL of neat culture supernatant or purified antibody (10 µg/mL in PBS-Marvel) for every 20 cm^2 of membrane.
5. Seal the bags, and incubate for 1 h at room temperature on a rocking platform.
6. Cut off one end of each bag, discard the contents, and wash the blot strips three times with PBST. Transfer the strips to "communal" bags, and add (1 mL/20 cm^2 blot) of I-125-labeled antibodies to rat or mouse $F(ab')_2$ (10^6 cpm/mL in PBS-Marvel containing 3% normal rabbit, sheep, or goat serum).
7. After rocking for 1 h at room temperature, open the bags, dispose of the radioactive supernatant, then wash the blots four times with PBST, and dry at 37°C.
8. Secure the blot strips to a sheet of Whatman 3 MM paper using Photomount or other spray-on adhesive. Then autoradiograph at –70°C using prefogged X-ray film and an intensifying screen (*see* ref. *4* for details).

3.5.8. Enzyme-Linked Immunosorbent Assays (ELISA)

As an alternative to the use of ^{125}I, most, if not all of the procedures described in the preceding section can be done using antibodies conjugated to an enzyme that converts a substrate into a colored or fluorescent product that is either soluble (plate assays) or insoluble (Western blots) (*see* Note 11). The protocols for the use of either radiolabeled or enzyme-conjugated antibodies are the same until the completion of washing following treatment with the second antibody or, in the case of competitive assays, the specific antibody. At this time, the substrate for the enzyme is added, the samples are incubated at room temperature or 37°C for a suitable time, and then the reaction is terminated. Plates coated with proteins, peptides, or conjugated peptides and those used in antibody capture assay can usually be read directly in a multiwell plate reader (e.g., Titertek multiscan). For this reason, it is better to use flat-bottom PS multiwell plates because of their better optical qualities. Alternatively, as with tests using cell monolayers, the supernatants can be transferred to a

new plate for reading. The final steps for use with alkaline phosphatase conjugates are given below (*see* Note 10).

Plate assays:

1. Wash the plate once with PBS, and then twice with 10 m*M* diethanolamine, pH 9.5, containing 500 m*M* NaCl.
2. Add 50 µL of *p*-nitrophenyl phosphate substrate solution to each well and incubate at room temperature for 10–30 min.
3. Stop the reaction by addition of 50 µL of 100 m*M* EDTA/well.
4. Read at 405 nm (positives are bright yellow).

Western blots:

1. Wash the blots twice with 100 m*M* diethanolamine, pH 9.5, containing 100 m*M* NaCl and 5 m*M* $MgCl_2$.
2. Add 1 mL of BCIP-NBT substrate/20 cm^2 of membrane and incubate at room temperature on a rocking platform until the purplish-black band/spots are suitably developed.
3. Rinse the membrane in PBS containing 20 m*M* EDTA to stop the reaction.

3.6. Isolation and Purification of Monoclonal Antibodies

MAbs can be prepared in bulk either as culture supernatant or as ascitic fluid. Supernatant should be obtained from cultures grown in DMEM containing as low a concentration of FCS as possible (e.g., rat hybridomas will grow at 2–3% FCS) and tested for maximum level of MAb. Roller cultures can yield milligram quantities (10–20 mg/L) of either rat or mouse antibodies. To obtain ascites from BALB/c mice or athymic rats, grow hybridomas in DMEM-10% FCS in roller culture (essential for rat hybridomas), and inject about 3×10^6 cells/mouse and 10^7 cells/rat into the peritoneal cavity. Harvest ascitic fluid 7–10 d later when tumor growth is visible by swelling of the abdomen. If the hybridomas grow as solid tumors, then use animals that have been injected ip with 0.5 mL (mouse) or 1 mL (rat) of pristane 7–10 d previously (*see* Note 9).

Antibodies sufficiently pure for radiolabeling, conjugation to fluorescein, or preparation of immunoaffinity columns can be obtained from ascitic fluids or culture supernatant by simple procedures using salt fractionation followed by either ion-exchange chromatography or immunoaffinity chromatography on columns consisting of immo-

bilized antigen, Protein A, or antibodies to mouse or rat immunoglobulins (*see* Chapter 43 for detailed protocols).

Salt fractionation is useful as a first step in all procedures, since it gives a partial purification and reduces bulk. Ion-exchange chromatography is a useful method for preparation of mouse and rat antibodies of the IgG_{2a} and IgG_{2b} subclasses, and can yield preparations of >90% purity. Where sufficient antigen is available, e.g., a synthetic peptide or recombinant protein, the monoclonal antibodies can be purified easily and quickly by affinity chromatography. Alternatively, purified preparations of monoclonal (e.g., MARK-1; ref. *5*) or polyclonal antibodies to rat or mouse $F(ab')_2$ can be used following their immobilization to crosslinked agarose or polyacrylamide bead supports. Mouse antibodies of the IgG_{2a} and IgG_{2b} subclasses bind well to Protein A, but antibodies of the IgG_1 subclass bind less well. Only rat antibodies of the IgG_{2c} subclass bind with any affinity to Protein A, and this is a useful procedure for their purification. Protein G is better because it binds a wider range of rodent IgG subclasses with greater affinity than Protein A and can be used for the isolation of rat IgGs other than IgG_{2c}.

It is essential to monitor the isolated antibodies for purity by gel electrophoresis. When analyzed by SDS-PAGE under nonreducing conditions, IgG antibodies should give a single protein band of about 160–170 kDa. On reduction with DTT, two or more bands will be seen corresponding to the individual heavy chains (45–50 kDa) or light chains (25–30 kDa). Where the myeloma parent produces light chains (e.g., the rat Y3), these can be produced also by the hybridoma and may be distinguished from those of the lymphoid parent owing to differences in size. Other protein band that are visible only on reduction may point to discrete attack on the individual chains by proteases. It may help to prevent such degradation by treating the culture supernatants or ascitic fluid with PMSF (make $10^{-3}M$) before storage or processing. If the preparations are contaminated with proteins of different molecular size to the intact IgG, try fractionation by size-exclusion chromatography using one of the proprietory gel permeation media.

Iodination reactions employing either chloramine T or IODO-GEN are particularly sensitive to inhibition by low concentrations of thiocyanate ions and, if antibodies are eluted from affinity columns or

Protein A using this chaotropic ion, it is essential that they be dialyzed thoroughly after elution to remove thiocyanate ions.

3.7. Isotyping of Monoclonal Antibodies

Antibodies can be separated into different classes (IgM, IgG, IgA, and IgE) and subclasses (IgG_1, IgG_{2a} IgG_{2b}, and so on) on the basis of the structure of their heavy chains. This separation into different isotypes can be achieved using antisera directed against antigens on the heavy chains that are specific for the particular isotype. Members of an isotype share important properties, such as the ability to bind and activate components of the complement system and to bind to Fc-receptors present on certain effector cells of the immune system.

1. Coat PVC plates with monoclonal or polyclonal antibodies (5 µg/mL of PBS, pH 8.0) specific for rat or mouse heavy-chain isotype using the protocol described in Section 2.3.
2. Block with PBS-Marvel.
3. Add 50 µL of test MAb to each well at 1 µg/mL in PBS-Marvel or as neat supernatant, and incubate for 1–4 h at room temperature or overnight at 4°C.
4. Wash three times with PBS-Marvel.
5. Add 50 µL per well (10^5 cpm in PBS-Marvel) of ^{125}I-labeled antibodies to rat or mouse $F(ab')_2$, and incubate 1 h at room temperature.
6. Wash three times with PBST, and determine the amount of radioactivity bound. Include MAbs of known isotype as controls (*see* Note 12).

4. Notes

1. The use of animals for experimental purposes is under strict control in many countries, and licenses are necessary before surgical procedures can be performed.
2. For growth of rat hybridomas as ascites, we recommend the use of nude rats, since these have low levels of endogenous immunoglobulins (<1 mg/mL) and can yield up to 30 mL of ascitic fluid/rat (1–5 mg/mL specific antibody).
3. Problems may be encountered when using β-galactosidase fusion proteins (noncleaved) as immunogens, because the response is directed predominantly against the bacterial enzyme. Also with transfected cell lines, it may be desirable to eliminate responses against cell components other than the recombinant antigen. A protocol has been described (*see* ref. *6*) in which animals were stimulated first with unwanted antigen (e.g., normal 3T3-cells). Then the responding lymphocytes were

killed by treatment of the animals with cyclophosphamide. Subsequent immunization of these animals with 3T3 cells expressing the desired recombinant antigen led to successful MAb production.

4. The concentration of protein used for immunization can be critical. In general, we find that good antibody responses are obtained when rats or mice are immunized with doses of antigen of 20–100 µg/challenge. When immunizing via the Peyer's patches of rats, the maximum volume that can be injected is between 8–10 µL/Patch, and the number of accessible Peyer's patches varies from 5–10 depending on the strain of rat. Usually we aim to start with a protein or peptide concentration of 4–5 mg/mL, which after emulsifying with Freund's adjuvant, will yield a concentration of half this value.
5. Some batches of FCS are toxic to hybridomas, and it is important to test all new batches for their ability to support hybridoma growth. Alternatively, obtain samples of FCS that have been tested by the supplier.
6. Feeders are essential when the Y3 myeloma is the fusion partner. We use cell lines derived by trypsinization of the xiphoid cartilage that terminates the xiphisternum of adult rats. Chop the cartilage from 6–8 adult rats into 2–3-mm pieces with a scalpel. Then transfer into 15 mL of DMEM containing 0.5% trypsin (bovine pancreas type III) and 1% collagenase (type II), and stir for 45 min at room temperature. Add FCS to 10%, and filter through sterile gauze to remove debris. Wash the cells in DMEM-10% FCS, and plate into the same medium. Passage the cells in DMEM-10% FCS after removing the cells by incubation for 2–3 min in PBS-0.05% Na_2EDTA containing 0.2% trypsin. Store cells in liquid nitrogen as aliquots of 10^6 cells in 5% DMSO-95% FCS.
7. Cell monolayers fixed with glutaraldehyde or paraformaldehyde can overcome the problem of loss of cells from the wells, but the effect of fixative on the binding of antigen should be determined. Wash cells in PBS, then add to each well 50 µL of freshly prepared 0.5% glutaraldehyde in PBS, containing 2 m*M* $CaCl_2$, 2 m*M* $MgCl_2$ and 300 m*M* sucrose. After incubating on ice for 30 min, "flick off" the fixative and replace with 200 µL PBS containing 0.1*M* glycine to block excess glutaraldehyde, leave for 30 min and then wash twice with PBS-Marvel. Fixed cells can be handled in the same way as antigens bound to PVC plates.
8. IODO-GEN (1,3,4,6-Tetrachloro-3-6-diphenylglycouril) is a better reagent for the iodination of proteins than chloramine-T, and is less damaging and has fewer side reactions than the latter. The insolubility of IODO-GEN in water means that tubes can be precoated with the reagent dissolved in methylene chloride or chloroform. Then the tubes are stored in the dark until required. The reaction is started by adding

the protein and radioiodide, and terminated by removing the sample from the reaction vessel.

9. Pristane (2,6,10,14-tetramethylenedecanoic acid) acts as an irritant, and as a result, macrophages and monocytes are recruited into the peritoneal cavity. The nutrients secreted by these cells provide a good environment for the growth of hybridomas in suspension.
10. When unfixed cells or frozen sections are used for ELISA, it may be necessary to block endogenous alkaline phoshatase activity and, in this case, include 0.1 m*M* levamisole in the substrate solution.
11. The use of biotinylated antibodies provides perhaps the greatest versatility and sensitivity of all methods. The afinity of biotin for avidin or the more usually used streptavidin is very high, and the latter can be conjugated to radioisotope, fluorescent moiety, or enzyme. Again, the basic procedures are the same a outlined for ^{125}I-labeled antibodies with the additional steps required for streptavidin binding and subsequent incubation with enzyme substrate.
12. Very occasionally, a MAb will show reactivity with more than one antiisotypic antibody in this capture assay. In this case, try immunoprecipitation in agarose gels (Ouchterlony procedure; *see* ref. 7 and Chapter 40) using polyclonal reagents. Because a crosslinked lattice must be formed among several different epitopes on the antigen and several antibodies to give an immunoprecipitate, the Ouchterlony procedure gives few false positives.

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CHAPTER 40

The Raising of a Polyclonal Antiserum to a Protein

Graham S. Bailey

1. Introduction

Suitable antisera are essential for use in all immunochemical procedures. Three important properties of an antiserum are avidity, specificity, and titer. The avidity of an antiserum is a measure of the strength of the interactions of its antibodies with an antigen. The specificity of an antiserum is a measure of the ability of its antibodies to distinguish the immunogen from related antigens. The titer of an antiserum is the final (optimal) dilution at which it is employed in the procedure; it depends on the concentrations of the antibodies present and on their affinities for the antigen. The values of those parameters required for a particular antiserum very much depend on the usage to which the antiserum will be put. For example, for use in radioimmunoassay, it is best to have a monospecific antiserum of high avidity, whereas for use in immunoaffinity chromatography, the monospecific antiserum should not possess too high an avidity. Otherwise it may prove impossible to elute the desired antigen without extensive denaturation.

A substance that, when injected into a suitable animal, gives rise to an immune response is called an immunogen. The immunogenicity of a substance is dependent on many factors, such as its size, shape, chemical composition, and structural difference from any related molecular species indigenous to the injected animal. Normally, cellular (particulate) materials are very immunogenic and induce a rapid immune response. However, the resultant antisera do not usually possess a high degree of specificity and do not store well *(1)*.

From *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
Edited by J M Walker

Soluble immunogens differ widely in their ability to produce an immune response. In general, polypeptides and proteins of mol wt above about 5000 and certain large polysacchardies can be effective immunogens. Smaller molecules, such as peptides, oligosaccharides, and steroids, can often be rendered immunogenic by chemical coupling to a protein that by itself will produce an immune response *(2)*. For most situations it is best to use the most highly purified sample available for injecting into the animal. Furthermore, it is usual to inject a mixture of the potential immunogen and an adjuvant that will stimulate antibody production.

Classically, the immune response is described as occurring in two phases. Initial administration of the immunogen induces the primary phase (response) during which only small amounts of antibody molecules are produced as the antibody producing system is primed. Further administration of the immunogen results in the secondary phase (response) during which large amounts of antibody molecules are produced by the large number of specifically programmed lymphocytes. In practice, the time scale and nature of immunization process do not lead to a clear recognition of two distinct phases.

All of the factors that influence antibody production have not yet been elucidated. Thus, the raising of a polyclonal antiserum is, to some extent, a hit or miss affair. Individual animals can respond quite differently to the same process of immunization, and thus, it is best to use a number of animals. The species of animal chosen for immunization will depend on particular circumstances, but, in general, rabbits are often used.

Many different methods of producing polyclonal antisera have been described, varying in amount of immunogen required, route of injection, and frequency of injection *(3)*. Monoclonal antibodies can be used in place of polyclonal antisera in immunological assays. However, monoclonal antibodies are expensive to produce, and their use in assays employing labeled ligand is reported to offer little, if any, advantage over polyclonal antisera *(4)*. This chapter will describe a method of antiserum production *(5)* that has been successfully utilized in the author's laboratory using small doses (microgram quantities) of soluble protein as immunogen *(6)*. A procedure for testing the specificity of the antiserum, Ouchterlony double immunodiffusion, is also described.

2. Materials

2.1. Raising the Antiserum

1. Six rabbits each of 2 kg body wt: Various types can be used, e.g., New Zealand whites, Dutch, and so forth.
2. Solution of the purified immunogen in an appropriate buffer to maintain its stability.
3. Complete and incomplete Freund's adjuvant, available from various commercial sources.
4. Heat lamp.

2.2. Ouchterlony Double Immunodiffusion

1. Agarose.
2. 0.07*M* Barbitone buffer, pH 8.6, containing 0.01% thimerosal as antibacterial agent: The buffer is prepared by dissolving sodium barbitone (14.5 g), disodium hydrogen phosphate decahydrate (7.16 g), boric acid (6.2 g) and sodium ethyl mercurithiosalicylate (0.1 g) in distilled water, and making the final volume 1 L.
3. Solution of antigen and antiserum.
4. Plastic or glass Petri dishes or rectangular plates.
5. Gel punch and template. Suitable gel punches of various sizes and templates of various designs can be obtained from commercial sources.
6. Flat level surface.
7. Humidity chamber at constant temperature.
8. 0.1*M* Sodium chloride in distilled water.
9. Staining solution: The solution is prepared by mixing ethanol (90 mL), glacial acetic acid (20 mL), distilled water (90 mL), and adding Coomassie brilliant blue R-250 (1 g). The solution can be reused several times.
10. Destaining solution: The composition is the same as that of the staining solution but without the dye.

3. Practical Procedure

3.1. Raising the Antiserum

The following procedure is carried out for each rabbit in turn (*see* Note 2).

1. Thoroughly mix 1 vol of the immunogen solution with 3 vol of complete Freund's adjuvant with the aid of a glass pestle and mortar. Initially, the mixture is very viscous, but after about 5 min the viscosity becomes less. The mixture can then be transferred to a glass syringe. The syringe is emptied and refilled with the mixture a number of times,

resulting in the formation of a stable emulsion that can be injected intradermally into the prepared rabbit. The emulsion should be used within 1 h of preparation.

2. The rabbit is prepared by cutting away the long hair along the center of its back. The short hair is removed by shaving. The emulsion of immunogen and complete Freund's adjuvant is injected via a 1-mL glass syringe plus 21-gage needle into two rows of five sites equidistantly spaced along the rabbit's back, each row being about 2 cm from the backbone, such that each site receives 0.1 mL of emulsion containing 1–10 μg immunogen, i.e., a total dose of 10–100 μg immunogen/rabbit (*see* Note 4).
3. After 8–10 wk, a test bleeding is carried out on the rabbit. The fur on the back of one ear is removed by shaving. The eyes of the rabbit are protected while the shaven ear is heated for <1 min with a heat lamp to expand the vein. The expanded vein just below the surface of the back of the ear is nicked with a scalpel blade. Blood is collected into a glass vessel (up to 20 mL can be collected in 10 min), removing the clot from the puncture wound by rubbing the ear with cotton wool from time to time. The blood is allowed to clot standing at room temperature for a few hours. The serum is separated from the clot by centrifugation, and can be stored at 4°C in the presence of 0.1% sodium azide or 0.01% thermerosal as antibacterial agent until tested (*see* Notes 1 and 3).
4. If on testing (*see* Note 10) the serum shows the characteristics required for its particular usage, then further bleeding can be carried out. Up to three bleedings can be made on successive days. After that, it is best to allow the rabbit to rest for about a month before further bleeding.
5. If the original antiserum is unsatisfactory, or if the quality of the bleedings taken over a period of several weeks or months starts to become unsatisfactory, then the rabbit can be boostered, i.e., receive a second injection of immunogen.
6. For the booster injection, half the original dose of immunogen is administered in incomplete Freund's adjuvant. The emulsion is prepared as detailed before, but is injected subcutaneously into the rabbit (for example, into the fold of skin of the neck).
7. After 10 d, a test bleeding is obtained from the rabbit, and the serum so produced is analyzed.
8. Further bleedings can be carried out over a period of time if the boostered antiserum is satisfactory.
9. If after boostering the antiserum is not of the desired quality, it is best to disregard that rabbit. Hopefully, one or more of the other rabbits in the group will have produced good antisera either directly or after boostering. However, in some cases, particularly with weak immuno-

gens, it may be necessary to repeat the process of immunization with a new group of rabbits or other animals.

10. Each sample of antiserum can be tested for its ability to form an immune precipitate with the immunogen by carrying out immunodiffusion and immunoelectrophoresis (*see* Note 5). For example, the titer and a measure of the avidity of the antiserum can be obtained by radioimmunoassay (*see* Chapter 46). The specificity of the antibodies can be determined by running the antiserum against the immunogen and related antigens in Ouchterlony double immunodiffusion in the manner described in the following section.

3.2. Ouchterlony Double Immunodiffusion

1. Agarose (1 g) is dissolved in the barbitone buffer (100 mL) by heating to 90°C on a water bath with constant stirring.
2. The agarose solution is poured to a depth of 1–2 mm into the Petri dishes or on to the rectangular plates that had previously been set on a horizontal level surface. The gels are allowed to form on cooling and when set (5–10 min) can be stored at 4°C in a moist atmosphere for at least one week.
3. A template of the desired pattern, according to the number of samples to be analyzed, is positioned on top of the gel. Commonly used patterns are shown in Fig. 1. The gel punch, which is connected to a water vacuum pump, is inserted into the gel in turn through each hole of the template so that the wells are cleaning formed as the resultant agarose plugs are sucked out.
4. The wells are filled with the solutions of antigen and antisera until the meniscus just disappears. The concentrations of antigen solutions and the dilution of the antiserum to be used have to be established largely by trial and error by running pilot experiments with solutions of different dilutions. However, as a rough guide, for the analysis of antigens, the following concentrations of antigen solutions can be run against undiluted antiserum: for a pure antigen, 1 mg/mL; for a partially pure antigen, 50 mg/mL; for a very impure antigen, 500 mg/mL, using 5-µL samples of antigen solutions and antiserum.
5. The gel plate is then left in a moist atmosphere, e.g., in a humidity chamber at a constant temperature of 20°C for 24 h.
6. The precipitin lines can then be recorded either directly by photography with dark-field illumination, or by drawing the naked eye observation on suitable oblique illumination of the plate against a dark background or after staining. Prior to staining, excess moisture is removed from the gel plate by application of a 1 kg weight (e.g., liter

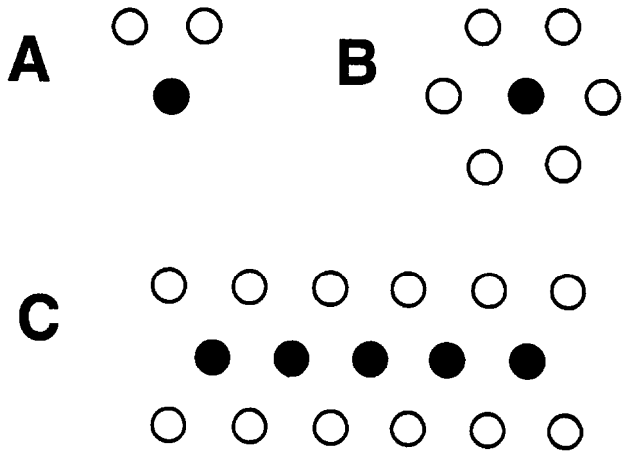

Fig. 1 Patterns of wells often used in double immunodiffusion. ○, well contains the antigen; ●, well contains the antiserum.

beaker full of water) to a wad of filter paper placed on top of the gel for 15 min. Soluble protein is removed by washing the gel (3 × 15 min) in 0.1*M* sodium chloride solution followed by further pressing. The gel is dried using cold air from a hair dryer and is then placed in the staining solution for 5 min. The plate is finally washed with distilled water and placed in the destaining solution for about 10 min (*see* Note 6).

4. Notes

1. Many antisera can be satisfactorily stored at 4°C in the presence of an antibacterial agent for many months. After some time, the antiserum solution may become turbid and even contain a precipitate (mostly of denatured lipoprotein). Even so, there should be no significant reduction in quality of the antiserum. If necessary, the solution can be clarified by membrane filtration. For prolonged storage, the antiserum can be kept at –20°C in small quantities to avoid repeated thawing and freezing.
2. Animals other than rabbits can be used for immunization, e.g., mice, rats, guinea-pigs, sheep, goat, and so forth. The rabbit is often a good initial choice, but if results are unsatisfactory, other species can be tried. Obviously only small volumes of the antisera can be generated in the smaller species, whereas large volumes can be obtained from the larger species. The latter though do require more immunogen and are more expensive to maintain.
3. A clean sample of antiserum should be straw-colored. Pink coloration is the result of partial hemolysis, but should not affect the antiserum's properties.

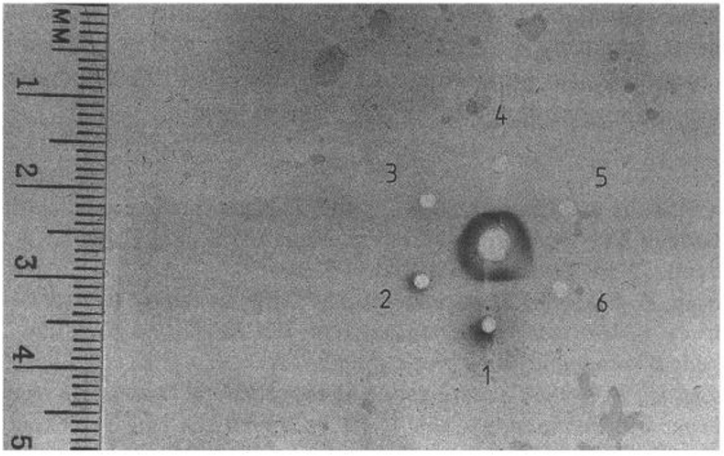

Fig. 2. An immunodiffusion pattern of antiserum to bovine pancreatic prokallikrein. The middle well contains the antiserum. The wells numbered 1–5 contain 5-μL aliquots of fractions containing prokallikrein of increasing purity at the following overall protein concentrations: well 1, 8.0 mg/mL; well 2, 6.3 mg/mL; well 3, 10.2 mg/mL; well 4, 5.5 mg/mL; well 5, pure prokallikrein, 0.44 mg/mL. Well number 6 contains a 5-μL aliquot of a chromatographic side fraction of protein concentration 12 mg/mL that does not contain prokallikrein.

4. The dose of immunogen employed can be of crucial importance in many procedures for antibody production. A state of tolerance can be induced in the animal with little or no production of antibody if too much or too little immunogen is repeatedly given over a relatively short period of time. The method described in this chapter should not suffer from that effect since there is a gap of at least 10 wk between the initial and booster injections. In general, the lower the dose of antigen, the greater is the avidity of the antiserum.
5. Since the antisera produced by conventional methods (polyclonal antisera) consist of mixtures of different antibody molecules, it is to be expected that the properties of the antisera collected during the prolonged period of immunization may change. Thus, each bleeding should be tested for specificity, titer, and avidity.
6. Ouchterlony double immunodiffusion is frequently used to check the specificity of a polyconal antiserum. If the antiserum contains antibody molecules that can react with different antigens, then multiple lines of precipitation will be seen on double immunodiffusion. If, however, the antiserum is monospecific for a particular antigen (*see* Fig. 2), only a single line of precipitation will be seen on immunodiffusion

whenever that antiserum is tested against samples containing the crossreacting antigen. No line of precipitation will be formed between the monospecific antiserum and a sample that does not contain the crossreacting antigen.

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CHAPTER 41

Production of Antisera to Synthetic Peptides

William J. Gullick

1. Introduction

The objective of immunization is to produce polyclonal or monoclonal antibodies that react with a chosen molecule. Immunization may be in vivo by injecting antigen into animals to produce antisera or to provide B-lymphocytes for immortalization as hybridomas, or alternatively, in vitro using splenocytes from untreated animals *(1)* for subsequent fusion with myeloma cells to produce monoclonal antibodies (MAbs). The latter approach, despite showing initial promise, is now only rarely used.

This chapter will be confined to the production of antibodies to proteins using synthetic peptides as immunogens. Conventional immunization may involve the injection of impure, semipure, or pure proteins to produce polyspecific or monospecific antisera. Since monospecific antisera are generally more useful, having a pure antigen is desirable. The problem of impure antigen can, however, be overcome by producing MAbs. This method relies on having an unambiguous screen in order to select the appropriate hybridomas. Frequently, this means a rather complicated analysis such as immunoprecipitation rather than by ELISA, which, when using impure immunogen, again requires purified protein for screening. Additional disadvantages are potential immunodominance by other antigens in the mixture over the desired immune response, and, importantly, there may be an immunodominant epitope on the antigen itself. If MAbs

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Edited by J. M Walker Copyright ©1994 Humana Press Inc , Totowa, NJ

are required that recognize other regions of the molecule, this may involve time-consuming elimination of undesired MAbs of the same specificity produced against the dominant epitope. An example of this problem is work involving the acetylcholine receptor in which one region of the native molecule elicited many different MAbs and was subsequently called the main immunogenic region *(2)*. In addition, mapping the binding sites of such MAbs on the surface or sequence of a protein is time-consuming and complicated *(3)*. If the antibody can recognize the denatured protein, it is now possible in some cases to determine its binding site by epitope scanning using a large series of overlapping synthetic peptides *(4)*. Even this approach, however, is not trivial.

Within the last few years, a different approach to generating protein-reactive antibodies has been developed that has several advantages over conventional immunization. This involves synthesizing short peptide sequences, coupling them to immunogenic carrier molecules, and immunizing animals with the conjugates. An immune response is usually generated both against the carrier and to the peptide acting as a hapten. The antipeptide antibodies produced, frequently, but by no means always, react with the protein from which the peptide sequence was selected. This technique is of course dependent upon having primary sequence information, either derived directly by sequencing a purified protein or quite commonly by using an amino acid sequence predicted from an open reading frame of cloned genomic or cDNA molecules. The latter situation does not require that any protein be purified, and in fact, in most cases the protein may never have been identified or characterized at all.

Immunization with synthetic peptides has the advantages that pure immunogens are used and an unlimited quantity of pure stable antigen is available for screening, often by ELISA, which is particularly useful in the production of MAbs when large numbers of hybridoma supernatants need to be screened quickly. Perhaps more importantly, however, the region within the primary structure of the protein of interest to which the antibody binds is dearly defined with high resolution. The antibodies are, in fact, "site-directed" and can be generated against regions of proteins that are not normally immunogenic when the whole protein is employed as an antigen. The main problem

of synthetic peptide immunogens is that although one almost always can make antipeptide antibodies, perhaps on average only one in three or four of these sequences generates antibodies that react with this structure in the context of the whole molecule. It is therefore very important to choose a peptide immunogen carefully so as to improve the success rate, thereby minimizing the cost and the number of animals employed, and several techniques have now been developed for this end.

1.2. Choosing the Right Peptide

If, for instance, one only has the sequence of the 10 amino-terminal amino acids of a protein, or if two protein sequences are highly related except for a short sequence and one wishes to make antibodies that discriminate between them, the choice is already made. If, however, a longer sequence is available and you have no predisposition to select a particular region, there are several predictive methods that are considered helpful but not infallible.

1.2.1. Hydropathy Plots

Hydropathy is a measure of the polarity of a molecule. Each amino acid is assigned a hydropathy value, defined by its propensity to dissolve in the organic or aqueous phase of a two-phase solvent system. A plot of the local hydropathy of the sequence is generated by selecting groups of usually six or seven contiguous amino acids (the "window") and averaging their hydropathy value. By starting at the N-terminus of the protein and iteratively moving the window by one residue each time and performing the same calculation, one obtains a plot of the varying hydropathy along the sequence. The immunogenic epitopes of some proteins have been defined experimentally and are quite often composed of charged or hydrophilic residues that appear as peaks of local hydrophilicity on the plot *(5,6)*. For a detailed discussion of this method, *see* ref. *7*.

1.2.2. Protrusion Index

Water-soluble proteins are folded such that amino acids with hydrophilic side chains are generally disposed on the surface of the protein and are thus sterically available to interact with antibody combining sites. A different method of calculation called the "protrusion index" has been developed *(8)* that attempts to predict the relative

protrusion of a sequence from the protein's globular surface. This method predicts that the more superficial the region, the more likely it is to be a good candidate as a synthetic peptide.

1.2.3. Secondary Structural Predictions

There is some evidence that turns in proteins are immunogenic, and the position of these within a sequence can be predicted mathematically *(9)*. The great advantages of the above methods are that they only require primary sequence information and that the calculations involved are fairly simple, particularly that for hydropathy plots. Biochemical computer software is now commercially available that can perform one or more of these analyses.

1.2.4. Sequence Mobility

Recently two papers have suggested that the more flexible a stretch of amino acids (the atomic or segmental mobility), the more likely it is to be immunogenic *(10,11)*. This analysis suffers from the fact that a quite detailed X-ray crystallographic structure of the protein is required, and this consequently limits its general usefulness.

All these methods are tested for their predictive ability by comparison with empirically determined antigenic sites of whole proteins. It should be emphasized that this is not the same as immunizing with short haptenic peptides that are attached to a carrier protein, usually at many different sites, and whose conformation will be influenced by their local environment on the surface of the carrier. By using these methods, however, one can hope to improve the chance of producing antiprotein reactive antibodies, and since all essentially lead to the choice of hydrophilic sequences, this produces the more practical advantage that the peptides are likely to be water-soluble and thus easier to use in aqueous coupling reactions (*see* Section 2.1.). One good bet if in doubt is to try the N- or C-termini of the protein, which are frequently, but not always, successful, possibly because they may more closely resemble the peptide immunogen than an internal, more conformationally constrained, sequence.

The choice of immunizing peptide may be influenced by other considerations. Functional significance of a particular region of a protein can sometimes be predicted from primary sequence motifs. An example of this is the sequence contributing to the nucleotide bind-

ing site of various protein kinases that can be rather well defined based on conserved residues with characteristic spacing *(12)*. Antibodies to such regions have been produced that are specific inhibitors of enzyme activity *(13)*. Sometimes antibodies have been successfully raised to proteins homologous to that under study. Choosing the cognate region for synthesis may favor success, since, assuming the two proteins are folded in the same way, it is likely that the chosen region is superficial and available for antibody binding.

The length of peptides that have been synthesized has varied considerably, although 12–15 residues is most common. Longer sequences are more costly and do not seem to be more effective at generating antiprotein reactive antibodies, perhaps because they possess tertiary structure of their own that may not resemble that of the same sequence in the whole protein. Shorter peptides sometimes fail to stimulate a sufficiently high affinity and specific immune response for the native protein. One final point is that it is not wise to select a sequence that may be posttranslationally modified in the mature protein, such as a site of N-linked glycosylation or addition of lipid. Phosphorylation of serine, threonine, or tyrosine residues, however, does not seem to significantly influence the binding of polyclonal antibodies *(14)*, although it might radically affect the binding of some MAbs.

1.3. Peptide Synthesis

There are two ways to obtain synthetic peptides; by synthesizing them oneself or by buying them. Several companies now perform custom synthesis, and some will also couple peptides to carrier molecules. The main disadvantage of this is that it is expensive, although the cost has come down dramatically over the last few years. At the time of writing, a rough estimate of the cost of purchasing peptides is about $25–50 per residue for a partially purified product. The more peptides that are used, the more likely one is to obtain antiprotein reactive sera, but the cost increases. The average delivery time is 4–6 wk, and this may represent an undesirable delay. The great advantage of purchase is that no skill or effort is required from the experimenter, and the product is reliable.

Peptide synthesis, however, is not too daunting for the nonorganic chemist. There are two basic chemistries in use. The traditional one is that developed by Merrifield (for which he won the Nobel prize)

which employs t-BOC amino acid derivatives, and a newer approach, developed by Sheppard, using f-MOC amino acid derivatives, which is now more commonly used. For references to the detailed procedure *see* Chapters 22 and 23 in vol. 3 and refs. *15* and *16*. One very considerable advantage of the new f-MOC chemistry is the avoidance of liquid hydrogen fluoride, which is used in t-BOC syntheses to cleave off protecting groups and to remove the peptide from the solid support after synthesis is complete. This compound is potentially very hazardous, and although contained reaction vessels are available, great care must still be taken when using it.

Synthesis can be performed in instruments varying in cost from about $5–10 for a simple glass tube consisting of a scinter and two taps, to an essentially fully automated machine at about $100,000. There are now, however, more semiautomated machines appearing on the market in the $15,000–75,000 range. The advantages of the more automated machines are that, obviously, less time is required from the operator and, perhaps, a greater degree of reproducibility and purity of product. Clearly, however, the purchase of the more automated systems can only be justified over purchase of the peptides if many sequences are required. Peptide synthesis is described in detail in Chapters 22 and 23 of vol. 3 of this series.

2. Materials

2.1. Glutaraldehyde Coupling of the Peptide to the Carrier Protein

1. Keyhole limpet hemocyanin. Use as a slurry in 65% ammonium sulfate from Calbiochem (La Jolla, CA, catalog no. 374811). It usually comes at about 30–40 mg/mL protein concentration.
2. Phosphate buffered saline (PBS): 0.14*M* NaCl, 20 m*M* sodium phosphate buffer, pH 7.2.
3. 50 m*M* Sodium borate/HCl buffer, pH 9.0.
4. 100 m*M* Sodium phosphate buffer, pH 8.0.
5. 25% Solution of glutaraldehyde. It is not critical to use high-quality material such as EM grade, but this is quite cheap anyway. I have found Sigma (Poole, UK, catalog no. G6257) satisfactory, but almost any variety would do.
6. 1*M* Glycine/HCl buffer, pH 6.0.

2.2. Immunization

1. Complete Freunds adjuvant.
2. Incomplete Freunds adjuvant.

3. Methods

3.1. Coupling of Peptides to Carrier Proteins

Several carrier proteins have been used successfully including bovine serum albumin, ovalbumin, thyroglobulin, and keyhole limpet hemocyanin (KH). A recent review of antipeptide antibodies *(17)* lists almost 200 examples, and one carrier clearly predominates. Keyhole limpet hemocyanin has long been the immunologist's choice as a general antigen to produce humoral antibodies, and it is frequently favored as a carrier for synthetic peptides. Hemocyanins are giant molecules, up to 9,000,000 mol wt, with quite repetitious structures. Many moles of peptide can be reacted per mole of KLH. All the other carriers have, however, been used with good results.

The methods available for coupling synthetic peptides to carriers are also quite varied, but can be classified depending on the functional group of the peptide that is employed for the reaction. Most, although not all, peptides used as immunogens are synthesized with free amino and carboxy termini, and these are most commonly employed for coupling. This is not only because there are several methods worked out for primary amines and carboxylic groups, but also because it is probably not a good thing to modify amino acids within a sequence since it will make the structure less like that of the parent molecule. Glutaraldehyde is the most commonly used reagent for coupling peptides via amine groups. Most papers refer to the method reported by Kagan and Glick *(18)*, but I have found this reference rather difficult to find, so I present my method below, which is a minor modification of theirs.

This makes enough immunogen for a series of three injections into two rabbits.

1. Dialyze the KLH against PBS (normally 1 mL of KLH solution against 2 L of PBS for at least 2 h) without sodium azide, and then measure the protein concentration. A quick way of doing this is to dilute a small aliquot into 50 m*M* sodium borate buffer, pH 9.0, and measure the

optical density of the solution at 280 nm. A 1.0 mg/mL solution of hemocyanin in this buffer gives 1.4 AU. Do not measure at neutral pH, since hemocyanin gives significant light scattering unless it is dissociated by acid or alkaline buffers.

2. Dissolve 7.5 mg of peptide in 100 m*M* sodium phosphate buffer, pH 8.0, mix with 7.5 mg of KLH, and make up to 0.5 mL with buffer.
3. Add 5 µL of 25% glutaraldehyde, mix well, and leave for 15 min at room temperature. The mixture may appear cloudy during this period.
4. Add a further 2.5 µL of glutaraldehyde and leave for 15 min.
5. Add 100 µL of 1*M* glycine, pH 6.0, and leave for 10 min to quench the glutaraldehyde. The reaction mixture will change to a yellow to brown color. Store in aliquots at –20°C until use.

Other types of coupling reactions employ EDIC (1-ethyl-3-(-3-dimethylaminopropyl)carbodiimide), which reacts with amine and carboxylic acid groups *(19)*, MBS (*m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester), which reacts with free sulfhydryl groups *(20)*, and *bis*-diazobenzidine, which reacts with tyrosine residues *(21)*.

3.2. Immunization

1. Immunization protocols are very much the acquired preference of the experimenter. I suggest the following (for rabbits), since I have found it simple and reliable. I also use the same schedule for mice and rats, but employ about one tenth the amount of immunogen for mice and one fifth for rats.
2. First take a preimmune bleed. From a medium-sized rabbit (3–4 kg) about 10–15 mL of serum and from a mouse 50–150 µL would be ideal. Do not forget that this is the one thing that you will never be able to obtain again once you have immunized.
3. Day 1. Mix one third of the immunogen (200 µL from the glutaraldehyde protocol) with 0.8 mL of PBS and 1.0 mL of complete Freunds adjuvant (for a pair of animals) to a stiff paste and inject each animal at multiple sites (about 2–4) subcutaneously with a total of 1.0 mL of the mixture.
4. Day 14. Repeat as for day 1, but use incomplete Freunds adjuvant.
5. Day 28. Repeat as for day 14.
6. Day 35. Test bleed. 10–20 mL of serum for rabbits, 50–100 µL for mice.
7. Day 49. Test bleed.

4. Notes

1. I recommend ELISA for screening for antipeptide antibodies, since the plates can be coated with peptide alone, thereby allowing the antipeptide titer to be measured without any contribution from anticarrier (usually a lot), or anticrosslinker (sometimes present) response. The general procedure as applied to screening for hybridomas has been published (*see* Chapter 39 and ref. *22*). For measuring serum titers, I normally measure in quadruplicate, starting at a dilution of serum of 1/50 and going down in five steps of fourfold dilution to 1/12,800. This will be a good range for most antisera, although high titer ones may need further dilutions. With most peptides, 50% of color development should be reached at a dilution of about 1/3200, although this is a considerable generalization. The antipeptide antibodies can now be tested for their ability to recognize the protein of interest by immunoprecipitation or western blotting (*see* Chapters 24 and 40). Some will work in one or both assays, some in neither. The more peptides that have been used, the more likely it is that antiprotein reactive antibodies will be obtained.
2. Beware that complete Freunds adjuvant will cause just as good immune response in humans as it will in mice or rabbits. Wear gloves and eye protection whenever handling it.
3. Several companies sell KLH as a lyophilized powder. I have heard from others that this is virtually impossible to dissolve without obvious large aggregates. To avoid is problem, I would recommend buying it as a solution or slurry.

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CHAPTER 42

Production of Antibodies Using Proteins in Gel Bands

Sally Ann Amero, Tharappel C. James, and Sarah C. R. Elgin

1. Introduction

A number of methods for preparing proteins as antigens have been described *(1)*. These include solubilization of protein samples in buffered solutions *(2)*, solubilization of nitrocellulose filters to which proteins have been adsorbed *(3)*, and emulsification of protein bands in polyacrylamide gels for direct injections *(4–8)*. The latter technique can be used to immunize mice or rabbits for production of antisera or to immunize mice for production of monoclonal antibodies *(9–11)*. This approach is particularly advantageous when protein purification by other means is not practical, as in the case of proteins insoluble without detergent. A further advantage of this method is an enhancement of the immune response, since polyacrylamide helps to retain the antigen in the animal and so acts as an adjuvant *(7)*. The use of the protein directly in the gel band (without elution) is also helpful when only small amounts of protein are available. For instance, in this laboratory, we routinely immunize mice with 5–10 μg total protein using this method; we have not determined the lower limit of total protein that can be used to immunize rabbits. Since polyacrylamide is also highly immunogenic, however, it is necessary in some cases to affinity-purify the desired antibodies from the resulting antiserum or to produce hybridomas that can be screened selectively for the production of specific antibodies, to obtain the desired reagent.

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2. Materials

1. Gel electrophoresis apparatus; acid-urea polyacrylamide gel or SDS-polyacrylamide gel.
2. Staining solution: 0.1% Coomassie brilliant blue-R (Sigma, St. Louis, MO, B-0630) in 50% (v/v) methanol/10% (v/v) acetic acid.
3. Destaining solution: 5%-(v/v) methanol/7% (v/v) acetic acid.
4. 2% (v/v) glutaraldehyde (Sigma G-6257).
5. Transilluminator.
6. Sharp razor blades.
7. Conical plastic centrifuge tubes and ethanol.
8. Lyophilizer and dry ice.
9. Plastic, disposable syringes (3- and 1-mL).
10. 18-g needles.
11. Spatula and weighing paper.
12. Freund's complete and Freund's incomplete adjuvants (Gibco Laboratories, Grand Island, NY).
13. Phosphate-buffered saline solution (PBS): 50 m*M* sodium phosphate, pH 7.25/150 m*M* sodium chloride.
14. Microemulsifying needle, 18-g (Becton Dickinson, Rutherford, NJ).
15. Female Balb-c mice, 7–8 wk old, or New Zealand white rabbits.

3. Method

1. Following electrophoresis (*see* Note 1), the gel is stained by gentle agitation in several volumes of staining solution for 30 min. The gel is partially destained by gentle agitation in several changes of destaining solution for 30–45 min. Proteins in the gel are then crosslinked by immersing the gel with gentle shaking in 2% glutaraldehyde for 45–60 min *(12)*. This step minimizes loss of proteins during subsequent destaining steps and enhances the immunological response by polymerizing the proteins. The gel is then completely destained, usually overnight (*see* Note 2).
2. The gel is viewed on a transilluminator, and the bands of interest are cut out with a razor blade. The gel pieces are pushed to the bottom of a conical plastic centrifuge tube with a spatula and pulverized. The samples in the tubes are frozen in dry ice and lyophilized.
3. To prepare the dried polyacrylamide pieces for injection, a small portion of the dried material is lifted out of the tube with a spatula and placed on a small square of weighing paper. In dry climates it is useful to first wipe the outside of the tube with ethanol to reduce static electricity. The material is then gently tapped into the top of a 3-mL syringe to which is attached the microemulsifying needle (Fig. 1A). Keeping

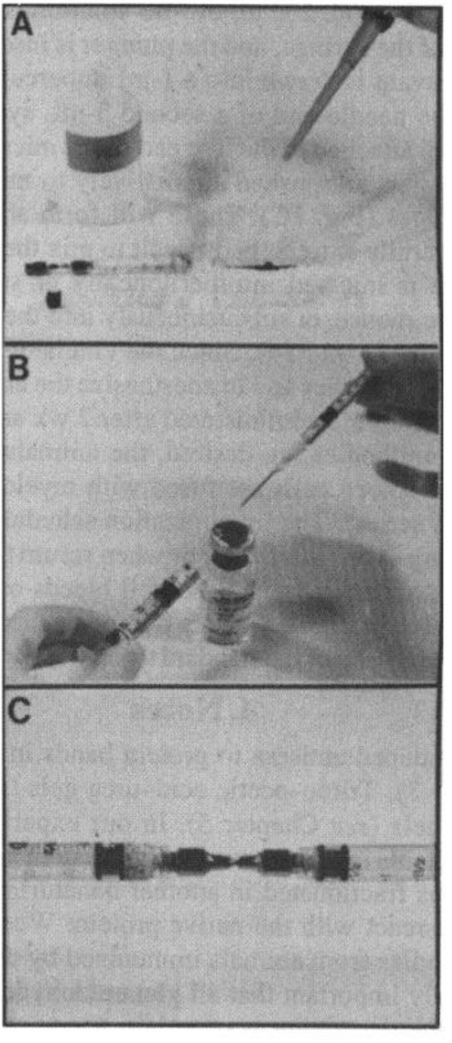

Fig. 1. Preparation of emulsion for immunizations. To prepare proteins in gel bands for injections, an emulsion of Freund's adjuvant and dried polyacrylamide pieces is prepared. **(A)** Dried polyacrylamide is resuspended in 200 µL of PBS solution in the barrel of a 3-mL syringe to which is attached a microemulsifying needle. **(B)** Freund's adjuvant is transferred into the barrel of a second 3-mL syringe. **(C)** An emulsion is formed by mixing the contents of the two syringes through the microemulsifying needle.

the syringe horizontal, 200 µL of PBS solution is carefully introduced to the barrel of the syringe, and the plunger is inserted. Next, 200 µL of Freund's adjuvant is drawn into a 1-mL tuberculin syringe and transferred into the needle end of a second 3-mL syringe (Fig. 1B). This syringe is then attached to the free end of the microemulsifying needle. The two plungers are pushed alternatively to mix the components of the two syringes (Fig. 1C). These will form an emulsion within 15 min; it is generally extremely difficult to mix the material any further.

4. This mixture is injected intraperitoneally or subcutaneously into a female Balb-c mouse, or subcutaneously into the back of the neck of a rabbit (*see* refs. *13* and *14*). Since the emulsion is very viscous, it is best to use 18–g needles and to anesthesize the animals. For mice, subsequent injections are administered after 2 wk and after 3 more wk. If monoclonal antibodies are desired, the animals are sacrificed 3–4 d later, and the spleen cells are fused with myeloma cells (*13* and *see* vol. 1 of this series). The immunization schedule for rabbits calls for subsequent injections after 1 mo or when serum titers start to diminish. Antiserum is obtained from either tail bleeds or eye bleeds from the immunized mice, or from ear bleeds from immunized rabbits. The antibodies are assayed by any of the standard techniques (*see* vol. 1 in this series).

4. Notes

1. We have produced antisera to protein bands in acetic acid–urea gels (*see* Chapter 7), Triton–acetic acid–urea gels *(15,16)*, or SDS–polyacrylamide gels (*see* Chapter 5). In our experience, antibodies produced to proteins in one denaturing gel system will crossreact to those same proteins fractionated in another denaturing gel system and will usually crossreact with the native protein. We have consistently obtained antibodies from animals immunized by these procedures.
2. It is extremely important that all glutaraldehyde be removed from the gel during the destaining washes, since any residual glutaraldehyde will be toxic to the animal. Residual glutaraldehyde can easily be detected by smell. It is equally important to remove all acetic acid during lyophilization. Monoacrylamide is also toxic, whereas polyacrylamide is not. We do observe, however, that approx 50 mm^2 of polyacrylamide per injection is the maximum that a mouse can tolerate.
3. Freund's complete adjuvant is used for the initial immunization; Freund's incomplete adjuvant is used for all subsequent injections. The mycobacteria in complete adjuvant enhance the immune response by provoking a local inflammation. Additional doses of mycobacteria may be toxic.

4. High-titer antibodies have been produced from proteins in polyacrylamide gel by injecting the gel/protein mixture into the lumen of a perforated plastic golf ball implanted subcutaneously in rabbits *(17)*. This approach places less stress on the animal, as complete adjuvants need not be used, and bleeding is eliminated. The technique has also been used in rats.

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CHAPTER 43

Preparation of Purified Immunoglobulin G (IgG)

Mark Page, Michael G. Baines, and Robin Thorpe

1. Introduction

Several immunological procedures can be successfully carried out using nonpurified antibodies, such as unfractionated antisera or ascitic fluid/culture supernatant containing monoclonal antibodies (MAbs). However, a much "cleaner" result can often be obtained if some form of enrichment or isolation of immunoglobulin is employed. Some procedures, such as conjugation with isotopes, fluorochromes, or enzymes, and preparation of immunoaffinity columns cannot usually be efficiently performed with nonpurified immunoglobulin, and some procedures may yield artifactual results if whole antiserum or ascitic fluid is used as a source of antibody. Purification of immunoglobulins is therefore essential or at least useful for a range of immunological methods. This process may consist of purification of total IgG or subpopulations (e.g., subclasses) of IgG from antisera/ascitic fluid/culture supernatant or the isolation of a particular antigen-binding fraction from such fluids. The former can be achieved by biochemical procedures, whereas the latter usually requires some form of affinity purification.

Many biochemical methods can be used for immunoglobulin purification. They range from simple precipitation techniques yielding an immunoglobulin enriched preparation, to more complex chromatographic techniques for the production of "pure" immunoglobulin. Most

From: *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
Edited by: J. M. Walker

of these procedures can be applied to the purification of immunoglobulins from the more commonly used species; however, mouse and rat IgGs are relatively less stable than immunoglobulins from higher mammals, and are generally less easily purified. Each MAb has unique characteristics, and some procedures need to be "tailored" for individual monoclonals. Avian antibodies may require special conditions for efficient purification.

The procedures outlined in this chapter are mostly confined to purification of IgG. For isolation of other immunoglobulin classes, the reader is referred to a more detailed text (*see* ref. *1*).

2. Materials

2.1. Precipitation Techniques

1. Saturated ammonium sulfate solution: Add excess $(NH_4)_2\ SO_4$ to distilled water (about 950 g to 1 L) and stir overnight at room temperature. Chill at 4°C, and store at this temperature. This solution (in contact with solid salt) is stored at 4°C.
2. Ammonium sulfate.
3. PBS: 0.14*M* NaCl, 2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4. Store at 4°C.
4. Sodium sulfate.
5. PEG solution: 20% (w/v) PEG 6000 in PBS.
6. Sodium acetate buffer: 0.06*M* CH_3COONa, pH 4.6. Adjust pH with acetic acid.
7. Caprylic acid.

2.2. Chromatography Techniques

1. HCl: 0.5 and 1*M*.
2. Whatman No. 54 filter paper.
3. NaOH: 0.5, 1, 2, and 4*M*.
4. Tris-HCl buffers: 0.05*M*, pH 8.0; 1*M*, pH 8.8. Adjust pH with 1*M* HCl or concentrated HCl as appropriate. Store at 4°C.
5. Depending on the methods being used, a variety of phosphate buffers of different molarities and pH values are needed. For each buffer, prepare solutions of both Na_2HPO_4 and NaH_2PO_4 of the required molarity, and then add the dihydrogen salt solution to the disodium salt solution until the required pH is obtained. Phosphate buffer is also used containing 1*M* NaCl for "purging" ion-exchange columns, and containing 0.1% NaN_3 for storage of ion-exchange columns. Store at 4°C.
6. DEAE Sepharose CL-6B.

7. NaCl: 0.5, 1, and 2*M*.
8. Methanol: 50 and 10% (v/v).
9. Triethanolamine buffer: 0.02*M* triethanolamine, pH 7.7. For ionic gradient separations by FPLC, use triethanolamine buffer containing 1*M* NaCl. Store at 4°C.
10. Sodium azide solution: 0.02% NaN_3 in distilled water.
11. Sepharose 4B.
12. Sodium carbonate buffer: 0.5*M* Na_2CO_3, pH 10.5. Adjust pH with 0.1*M* NaOH.
13. Cyanogen bromide.
14. Glycine-HCl buffer: 0.1*M* glycine, pH 2.5. Adjust pH with 1*M* HCl.
15. Sodium citrate buffer: 0.1*M* trisodium citrate, pH 6.5. Adjust pH with 0.1*M* citric acid.
16. Ethanolamine buffer: 2*M* ethanolamine.

3. Methods

3.1. Prepurification Techniques

3.1.1. Separation of Serum from Whole Blood

It is obviously necessary to separate the IgG-containing serum from cells and other insoluble components of whole blood. This can be simply achieved by allowing the blood to clot and then centrifuging to yield serum as a supernatant. This should be carried out as soon as possible after collection to avoid hemolysis and degradation of IgG (*see* Note 1).

1. Allow blood to clot at room temperature (this takes approx 1 h). Leave overnight at 4°C—this allows the clot to contract (*see* Notes 2 and 3).
2. Detach the clot from the walls of the container using a wooden or plastic rod, and pour off all liquid into a centrifuge tube or vessel (leave the clot and adhering substances behind).
3. Pour the clot into a separate centrifuge tube, and centrifuge for 30 min at 2500*g* at 4°C. Remove any expressed liquid, and add this to the previously aspirated clot-free liquid.
4. Centrifuge the pooled liquid for 15–20 min at 1500*g* at 4°C. Aspirate the clear serum, and store in aliquots at –20°C (or –70°C). Alternatively, serum can be stored at 4°C if an antibacterial agent (e.g., 0.2% NaN_3) is added. Do not freeze chicken IgG or serum, because the immunoglobulins are particularly subject to damage.

3.1.2. Clarification of Ascitic Fluid

Ascitic fluid derived from the peritoneal cavity of mice or rats that have been injected with hybridomas contains high concentrations of

MAb, but this is usually mixed with variable amounts of blood cells, serum proteins, and fatty materials. It is therefore necessary to separate these components from the ascitic fluid before attempting purification of the MAb.

1. Allow the ascitic fluid to clot at room temperature (some samples will not clot; in this case, proceed as in step 2 below). Detach the clot from the sides of the container using a wooden or plastic spatula.
2. Pour the ascitic fluid into a centrifuge tube, and centrifuge for 10–15 min at 2500*g* at 4°C. Aspirate the clear supernatant, and store at –20°C or –40°C.

3.2. Preliminary Purification (Precipitation) Techniques

Addition of appropriate amounts of salts, such as ammonium or sodium sulfate, or other chemicals, such as rivanol, polyethylene glycol, or caprylic acid, causes precipitation of IgG from serum, hybridoma culture supernatants, or ascitic fluid. Although such IgG is usually contaminated with other proteins, the ease of these precipitation procedures coupled with the high yield of IgG produced has led to them being very widely used to produce enriched IgG preparations suitable for some immunochemical procedures, e.g., production of immunoaffinity columns, and as a starting point for further purification. The precipitated IgG is usually very stable, and such preparations are ideally suited for long-term storage or distribution and exchange between laboratories. Ammonium sulfate precipitation is the most widely used and adaptable procedure, but other precipitation techniques can be the method of choice for some antibodies or purposes.

3.2.1. Ammonium Sulfate Precipitation

1. Prepare saturated ammonium sulfate at least 24 h before the solution is required for fractionation.
2. Centrifuge serum/ascitic fluid/culture supernatant for 20–30 min at 10,000*g* at 4°C. Discard the pellet (*see* Note 4).
3. Cool the serum/ascitic fluid/culture supernatant to 4°C and stir slowly. Add saturated ammonium sulfate solution dropwise to produce 35–45% final saturation (*see* Note 5). Alternatively, add solid ammonium sulfate to give the desired saturation (2.7 g of ammonium sulfate/10 mL of fluid = 45% saturation). Stir at 4°C for 1–4 h or overnight.

4. Centrifuge at 2000–4000*g* for 15–20 min at 4 °C (alternatively for small volumes of 1–5 mL, microfuge for 1–2 min). Discard the supernatant, and drain the pellet (carefully invert the tube over a paper tissue).
5. Dissolve the precipitate in 10–20% of the original volume of PBS or other buffer by careful mixing with a spatula or drawing repeatedly into a wide-gage Pasteur pipet. When fully dispersed, add more buffer to give 25–50% of the original volume, and dialyze against the required buffer (e.g., PBS) at 4°C overnight with two to three buffer changes. Alternatively, the precipitate can be stored at 4°C or –20°C if not required immediately.

3.2.2. Sodium Sulfate Precipitation

Sodium sulfate may be used for precipitation of IgG instead of ammonium sulfate. The advantage of the former salt is that a purer preparation of IgG can be obtained in some cases. The disadvantages are that yield may be reduced and that fractionation must be carried out at a precise temperature (usually 25°C), as the solubility of Na_2SO_4 is very temperature dependent. Sodium sulfate is usually employed only for the purification of rabbit or human IgG, but it can be used for other species. Sodium sulfate is not recommended for precipitation of most murine MAbs.

1. Centrifuge the serum at 10,000*g* for 20–30 min. Discard the pellet, and warm the serum to 25°C. Stir.
2. Add solid Na_2SO_4 to produce an 18% (w/v) solution (i.e., add 1.8 g per 10 mL) and stir at 25°C for 30 min to 1 h.
3. Centrifuge at 2000–4000*g* for 30 min at 25°C.
4. Discard the supernatant, and drain the pellet. Redissolve in the appropriate buffer as described for ammonium sulfate precipitation in Section 3.2.1., step 5.

3.2.3. Precipitation with Polyethylene Glycol (PEG)

This procedure is applicable to both polyclonal antisera and most MAb-containing fluids.

1. Cool 20% (w/v) PEG 6000 solution to 4°C.
2. Prepare serum/ascitic fluid, and so forth, for fractionation by centrifugation at 10,000*g* for 20–30 min at 4°C. Discard the pellet. Cool to 4°C.
3. Slowly stir the antibody-containing-fluid, and add an equal vol of 20% PEG dropwise (*see* Note 6). Continue stirring for 20–30 min.

4. Centrifuge at 2000–4000*g* for 30 min at 4°C. Discard the supernatant, and drain the pellet. Resuspend in PBS or other buffer as described for ammonium sulfate precipitation in Section 3.2.1., step 5 (*see* Note 6).

3.2.4. Caprylic Acid Precipitation

Caprylic acid can be used to purify IgG, but the concentration required varies according to species. For monoclonal antibodies, it is usually necessary to determine experimentally the quantity required to produce the desired purity/yield.

1. Centrifuge the serum at 10,000*g* for 20–30 min. Discard the pellet, and add twice the volume of 0.06*M* sodium acetate buffer, pH 4.6.
2. Add caprylic acid dropwise while stirring at room temperature. For each 25 mL of serum, use the following amounts of caprylic acid: human and horse, 1.52 mL; goat, 2.0 mL; rabbit, 2.05 mL; cow, 1.7 mL. Stir for 30 min at room temperature.
3. Centrifuge 4000*g* for 20–30 min. Retain the supernatant and discard the pellet. Dialyze against the required buffer (e.g., PBS) at 4°C overnight with two or three buffer changes.

3.3. Chromatography Techniques Based on Charge or Size Separation

3.3.1. Ion-Exchange Chromatography (see *Notes 7–9)*

Ion-exchange chromatography is a widely used method for the fractionation of IgG from both polyclonal antisera and preparations containing high concentrations of MAb. The separation of molecules in ion exchange is determined by the charges carried by solute molecules, and it is a technique of high resolving power. Ion-exchange groups are of two types, anion-exchange groups, e.g., diethylaminoethyl (DEAE), and cation-exchange groups, e.g., carboxymethyl (CM). The ion-exchange groups are covalently bound to an insoluble matrix, for example, Sephadex (crosslinked dextran), Sepharose (crosslinked agarose), or Sephacel (beaded cellulose), to form the ion-exchange resin. After proteins have been adsorbed onto the ion-exchange resin, they can be selectively eluted by slowly increasing ionic strength (this disrupts ionic interactions competitively) or by altering the pH (the reactive groups on the proteins lose their charge). This is the basis of ion-exchange chromatography, and the technique can be used to purify IgG from sera of most species.

3.3.1.1. Preparation and Equilibration of Anion Exchanger

1. Gently stir the ion-exchange resin into approximately five times its swollen volume of 0.5*M* HCl. Leave at room temperature with occasional swirling for 30 min.
2. Filter the resin by suction through a Buchner funnel using a Whatman No. 54 filter paper. Then wash the resin cake with distilled water until the pH of the filtrate is >4.
3. Gently stir the ion-exchange resin into approximately five times its swollen volume of 0.5*M* NaOH. Leave at room temperature with occasional swirling for 30 min.
4. Repeat step 2.
5. Add the ion-exchange resin to an equal volume of 10X starting buffer (starting buffer is 0.05*M*, Tris-HCl, pH 8.0, or any other appropriate buffer). Mix thoroughly and leave at room temperature for 30 min.
6. The ion exchanger will adsorb some buffer ions in exchange for protons or hydroxyl ions, and hence alter the pH. Restore the pH to its original value by gently stirring the slurry and adding 1*M* HCl.
7. Leave the slurry at room temperature for 30 min, and then recheck the pH. Adjust if required. The ion exchanger is now at the correct pH with the counterion bound, but the ionic strength will be too high.
8. Wash the ion-exchange resin with five times its vol of starting buffer through a Whatman No. 54 filter paper.
9. Degas the slurry using a Buchner side-arm flask under vacuum for 1 h with periodic swirling.
10. Resuspend the slurry in approximately five times its volume of starting buffer, and leave to stand until most of the beads have settled. Remove the fines by aspirating the supernatant down to about two times the settled slurry vol.
11. Carefully pack a clean column by first filling it with 10 mL of starting buffer and then by pouring the resuspended slurry down a glass rod onto the side wall until the column is filled. Allow to settle under gravity.
12. Wash the column with starting buffer at the operating temperature until the pH and conductivity of the eluent are exactly the same as the starting buffer.

3.3.1.2. Sample Application and Elution

1. Dialyze the serum (ammonium sulfate fractionated) against 0.07*M* sodium phosphate buffer, pH 6.3, exhaustively (at least two changes over a 24-h period) at a ratio of at least 1 vol of sample to 100 vol of buffer.

2. Apply the sample to the column (e.g., DEAE Sepharose CL-6B), and elute with sodium phosphate buffer. Collect 2-mL fractions, and monitor the absorbance at 280 nm (A_{280}).
3. Collect the first protein peak, and stop collecting fractions when the A_{280} falls to baseline.
4. Regenerate the column by passing through 2–3 column vol of phosphate buffer containing 1*M* NaCl.
5. Wash thoroughly in phosphate buffer (2–3 column vol), and store in buffer containing 0.1% NaN_3.
6. Pool the fractions from step 3, and measure the A_{280} (*see* Note 10).

3.3.2. Gel Filtration (see *Notes 11–17)*

In gel filtration, a protein mixture (the mobile phase) is applied to a column of small beads with pores of carefully controlled size (the stationary phase). The movement of the solute is dependent on the flow of the mobile phase and the Brownian motion of the solute molecules causing their diffusion into and out of the chromatographic bed. Large proteins, above the "exclusion limit" of the gel cannot enter the pore and are hence eluted in the "void volume" of the column. Small proteins enter the pores and are therefore eluted in the "total volume" of the column and intermediate size proteins are eluted between the void and total volumes. Proteins are therefore eluted in order of decreasing molecular size.

Gel filtration is not especially effective for the purification of IgG, which tends to elute in a broad peak and is usually contaminated with albumin (derived from dimeric albumin, 135 kDa). The technique is more useful for the purification of IgM. Some IgGs (monoclonal), however, possess pIs that make them unsuitable for fractionation using ion-exchange chromatography. In such cases, it may be desirable to use gel filtration as a method of fractionation.

3.3.2.1. Preparation and Equilibration of Gel Filtration Column (*see* Note 14)

1. Gently stir the filtration medium (enough to fill the column plus 10%) into two times the column volume of buffer.
2. Degas the slurry using a Buchner side-arm flask under vacuum for 1–2 h with periodic swirling. Do not use a magnetic stirrer, since this may damage the beads.
3. Resuspend the slurry in approximately five times its volume of buffer, and leave to stand until most of the beads have settled. Remove the fines by aspirating the supernatant down to about 1.5 times the settled slurry volume.

4. Carefully pack a clean column by first filling it to 10–20 cm height with buffer. Swirl the slurry to resuspend it evenly, and pour it down a glass rod onto the inside wall to fill the column. Allow to settle under gravity for 0.5–1 h to let air bubbles escape.
5. Adjust the height of the outlet end of the column so that the vertical distance between it and the top of the column is less than the maximum operating pressure for the gel. Unclamp the bottom of the column, and allow the gel to pack under this pressure.
6. Top up the column periodically by siphoning off excess supernatant, stirring the top of the gel (if it has settled completely), and filling the column up to the top with resuspended slurry.
7. Once the column is packed (gel bed just runs dry), connect the top of the column to a buffer reservoir, remove any air bubbles in the tube, and allow 1 column vol of buffer to run through the column.

3.3.2.2. Sample Application and Elution (*see* Note 15)

1. Disconnect the top of the column from the buffer reservoir and allow the gel to *just* run dry.
2. Apply the IgG sample carefully by running it down the inside wall of the column so that the gel bed is not disturbed.
3. When the sample has entered the bed, gently overlay the gel with buffer, and reconnect the column to the buffer reservoir.
4. Collect fractions (4–6 mL), and monitor the absorbance at 280 nm (*see* Note 10).

3.3.3. High-Performance Liquid Chromatography (HPLC) and Related Techniques (see *Note 18 and Refs. 2 and 3)*

Fractionation of immunoglobulins by HPLC utilizes the same principles as chromatography in standard columns by gel filtration, ion-exchange, or affinity chromatography. In HPLC, protein separation occurs in a column of small cross-sectional area containing the chromatography matrix in the form of very fine particles (the stationary phase). The solvent (buffer) or mobile phase is pumped through the column using medium to high pressure pumps. This allows the sample molecules in the mobile phase to interact reversibly with the stationary phase in a continuous fashion. The advantages of HPLC over conventional chromatography techniques are speed because of the small, high-capacity columns, improved reproducibility because of the sophisticated pumps and accurate timers, and in many cases, increased resolution because of the fine resins and control systems.

3.3.3.1. HPLC Purification of IgG

1. Prepare serum/ascitic fluid by ammonium sulfate precipitation (45% final concentration). Dialyze sample against appropriate buffer as recommended by the column manufacturers (usually 0.1*M* sodium phosphate, pH range 6.5–7.5). Dilute sample at least 1:1 in sample buffer and filter before use (0.2 μm).
2. Assemble the HPLC system with either a size-exclusion (Anagel TSK) or anion-exchange column (Hydropore SAX).

3.3.3.2. Size-Exclusion HPLC

1. Equilibrate the column using recommended buffer (e.g., 0.15*M* sodium phosphate buffer, pH 6.8) at optimal flow rate until absorbance (280 nm) baseline is stable.
2. Add sample manually using large loop (usually 1–5 mL; *see* Note 17).
3. Monitor absorbance at 280 nm and collect IgG fractions either manually or automatically. A purified standard of the derived IgG should be run initially to determine the sample retention time under the specified set of conditions. This allows the identification of the specific monomeric IgG peak in subsequent runs (*see* Fig. 1).
4. Wash column in water until absorbance is stable, and finally store column in 50% methanol.

3.3.3.3. Ion-Exchange HPLC

1. Wash column in buffer A (e.g., 0.02*M* sodium phosphate buffer, pH 7.4) at optimal flow rate (1.0 mL/min) until absorbance at 280 nm is stable.
2. Run a blank salt gradient at the desired flow rate from 0 to 100% buffer B (0.5*M* NaCl) over 20 min to complete preparation of the matrix. Finally, reequilibrate the column with buffer A prior to sample application.
3. Add sample manually using 1–5 mL loop, and allow at least 10 min before applying salt gradient.
4. Apply salt gradient from 0–40% (buffer B) over 45 min collecting protein peaks. The length of the run will depend on column size. A larger column will take longer to apply the salt gradient. IgG should elute at between 9–20%.
5. Purge the column with 100% buffer B (0.5*M* NaCl) for 10 min.
6. Wash column with sample buffer until absorbance is stable, and then flush with 10 column vol of HPLC-grade water followed by 5–10 column vol of 50% methanol, and store.

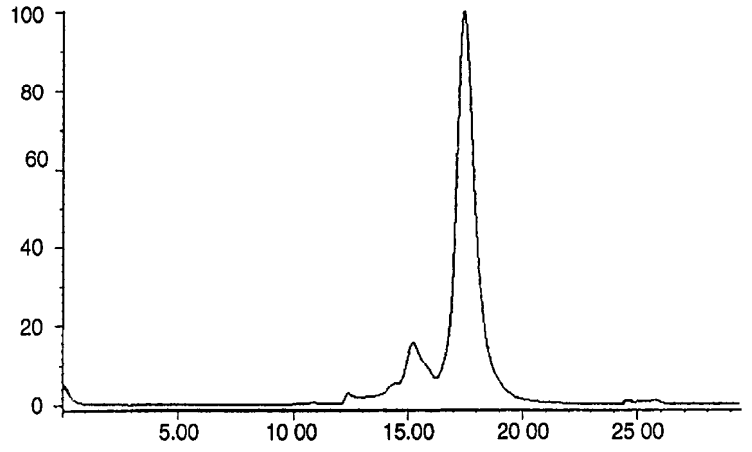

Fig. 1. HPLC gel permeation analysis of human immunoglobulin G preparation. The sample was fractionated using a TSK G3000SW size-exclusion column (300 × 7 8 mm) eluted with 0.2*M* sodium phosphate, pH 6 8, at a flow rate of 0.5 mL/min. Absorbance was measured at 214 nm. The major peak represents monomeric IgG having a mean retention time of 18 min. The small peaks with retention times of 12.5 min and 15 min are aggregated and dimeric IgG, respectively

3.3.3.4. FPLC Purification of Sheep IgG (*see* Fig. 2)

Fast protein liquid chromatography (FPLC) is a variant of HPLC that has proven especially useful in the purification of murine monoclonal antibodies, although the technique is applicable to IgG preparations from all species.

1. Prepare serum by ammonium sulfate precipitation (45% final concentration). Redissolve the precipitate in 0.02*M* triethanolamine buffer, pH 7.7, and dialyze overnight against this buffer at 4°C. Filter the sample before use (0.2 μm).
2. Assemble the FPLC system according to the manufacturer's (Pharmacia, Uppsala, Sweden) instructions for use with the Mono-Q ion-exchange column.
3. Equilibrate the column (method programmed).
4. Assemble the "superloop" according to the manufacturer's instructions.
5. Load the sample.
6. Set the sensitivity in the UV monitor control unit, and zero the chart recorder.

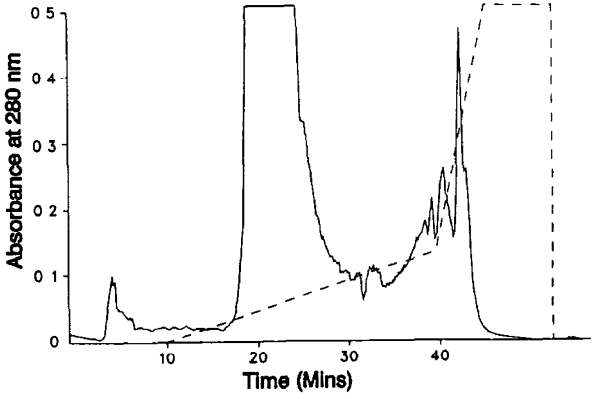

Fig. 2. Elution profile of FPLC-purified sheep antiserum This profile represents the purification of an anti-p27 (core protein of simian immunodeficiency virus) polyclonal serum by the authors. The major peak is IgG, which elutes after about 18 min. The dotted line represents the profile of the ionic gradient used for elution of the IgG.

7. Run a Mono-Q ion-exchange purification method (method programmed). Collect fractions (automatic). Elution buffers are 0.02*M* triethanolamine, pH 7.7, and 0.02*M* triethanolamine, pH 7.7, containing 1*M* NaCl.
8. Wash the Mono-Q ion-exchange with 2*M* NaOH followed by 2*M* NaCl (method programmed).
9. Store the Mono-Q ion-exchange column in distilled water containing 0.02% NaN_3 (method programmed).

3.4. Affinity Chromatography

Affinity chromatography is a particularly powerful procedure that can be used to purify IgG, subpopulations of IgG, or the antigen-binding fraction of IgG present in serum/ascitic fluid/ hybridoma culture supernatant. This technique requires the production of a solid matrix to which a ligand that either has affinity for the relevant IgG or vice versa has been bound. Examples of ligands useful in this context are:

1. The antigen recognized by the IgG (for isolation of the antigen-specific fraction of the serum/ascitic fluid, and so forth).
2. IgG prepared from an antiimmunoglobulin serum, e.g., rabbit antihuman IgG serum, or murine antihuman IgG monoclonal antibody for the purification of human IgG (*see* Note 19).

3. IgG-binding proteins derived from bacteria, e.g., Protein A (from *Staphylococcus aureus* Cowan 1 strain) or proteins G or C (from *Streptococcus; see* Note 20).

The methods for production of such immobilized ligands and for carrying out affinity purification of IgG are essentially similar regardless of which ligand is used. Sepharose 4B is probably the most-widely used matrix for affinity chromatography, but there are other materials available. Activation of Sepharose 4B is usually carried out by reaction with cyanogen bromide (CNBr); this can be carried out in the laboratory prior to coupling, or ready-activated lyophilized Sepharose can be purchased. The commercial product is obviously more convenient than "homemade" activated Sepharose, but it is more expensive and may be slightly less active.

3.4.1. Activation of Sepharose with Cyanogen Bromide and Preparation of Immobilized Ligand

Activation of Sepharose with CNBr requires the availability of a fume hood and careful control of the pH of the reaction—failure to do this may lead to the production of dangerous quantities of HCN as well as compromising the quality of the activated Sepharose. CNBr is toxic and volatile. All equipment that has been in contact with CNBr and residual reagents should be soaked in 1*M* NaOH overnight in a fume hood and washed prior to discarding/returning to the equipment pool. Manufacturers of ready-activated Sepharose provide instructions for coupling (*see* Note 21).

1. Wash 10 mL (settled volume) of Sepharose 4B with 1 L of water by vacuum filtration. Resuspend in 18 mL of water (do not allow the Sepharose to dry out).
2. Add 2 mL of 0.5*M* sodium carbonate buffer pH 10.5 and stir slowly. Place in a fume hood, and immerse the glass pH electrode in the solution.
3. **Carefully** weigh 1.5 g of CNBr into an air-tight container (**weigh in a fume hood; wear gloves**)—remember to decontaminate equipment which has contacted CNBr in 1*M* NaOH overnight.
4. Add the CNBr to the stirred Sepharose. **Maintain** the pH between 10.5 and 11.0 by dropwise addition of 4*M* NaOH until the pH stabilizes and all the CNBr has dissolved. If the pH rises above 11.5, activation will be inefficient and the Sepharose should be discarded.

5. Filter the slurry using a sintered glass or Buchner funnel, and wash the Sepharose with 2 L of cold 0.1*M* sodium citrate buffer pH 6.5—do not allow the Sepharose to dry out. Carefully discard the filtrate (**Care:** this contains CNBr).
6. Quickly add the filtered washed Sepharose to the ligand solution (2–10 mg/mL in 0.1*M* sodium citrate, pH 6.5), and gently mix on a rotator ("windmill") at 4°C overnight (*see* Note 22).
7. Add 1 mL of 2*M* ethanolamine solution, and mix at 4°C for a further 1 h—this blocks unreacted active groups.
8. Pack the Sepharose into a suitable chromatography column (e.g., a syringe barrel fitted with a sintered disk), and wash with 50 mL of PBS. Store at 4°C in PBS containing 0.1% sodium azide.

3.4.2. Purification of IgG Using Affinity Chromatography on Antigen-Ligand Columns (see *Fig. 3)*

Isolation of IgG by affinity chromatography involves application of serum, and so forth, to a column of matrix-bound ligand, washing to remove non-IgG components and elution of IgG by changing the conditions such that the ligand–IgG interaction is disrupted. Affinity isolation of IgG can also be carried out using a batch procedure rather than on a column—this is particularly useful for large volumes containing a relatively small amount of IgG, e.g., cell culture supernatants, especially those produced by MAb secreting human cells.

1. Wash the affinity column with PBS. "Pre-elute" with dissociating buffer, e.g., 0.1*M* glycine-HCl, pH 2.5. Wash with PBS; check that the pH of the eluate is the same as the pH of the PBS (*see* Note 23).
2. Apply the sample to the column, close the column exit, and incubate at room temperature for 15–30 min (*see* Note 24).
3. Wash non-IgG material from the column with PBS; monitor the A_{280} as an indicator of protein content.
4. When the A_{280} reaches a low value (approx 0.02), disrupt the ligand–IgG interaction by eluting with dissociating buffer (*see* Note 23). Monitor the A_{280}, and collect the protein peak into tubes containing 1*M* Tris-HCl, pH 8.8, 120 µL/1 mL fraction (*see* Note 10).
5. Wash the column with PBS until the eluate is at pH 7.4. Store the column in PBS containing 0.1% azide. Dialyze the IgG preparation against a suitable buffer (e.g., PBS) to remove glycine/Tris.

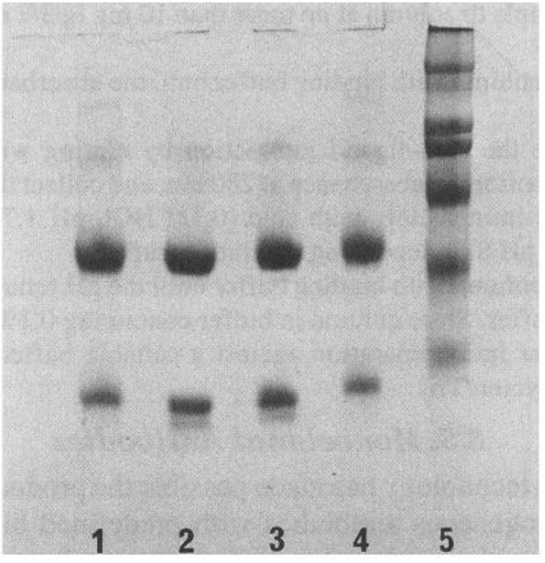

Fig. 3. SDS PAGE of affinity-purified Abs specific for human IL-3. Track 5 shows standard mol-wt markers (from top to bottom: myosin heavy chain M_r 200 kDa; β-galactosidase M_r 116 kDa; phosphorylase b M_r 97.4 kDa; bovine albumin M_r 66 kDa; egg albumin M_r 45 kDa, and carbonic anhydrase M_r 29 kDa). Track 1 shows Cohn fractionated human IgG. The remaining three tracks show three different IgG antibodies purified using a column of human recombinant DNA derived IL-3 coupled to Sepharose 4B. The major bands are κ and γ chains; the faint higher M_r bands are owing to incomplete dissociation of heavy and light chains.

3.4.3. Purification of Immunoglobulin G Using Protein A, Protein C, or Protein G (see *Note 20)*

1. Wash the protein A/C/G column with an appropriate binding buffer. Optimal binding performance occurs using a buffer system between pH 7.5 and 8.0. Suggested buffers include 0.1*M* Tris-HCl, 0.15*M* NaCl, pH 7.5; 0.05*M* sodium borate, 0.15*M* NaCl, pH 8.0; 0.1*M* sodium phosphate, 0.15*M* NaCl, pH 7.5.
2. Pre-elute the column with dissociating buffer, e.g., 0.1*M* glycine-HCl, pH 2.5.
3. Equilibrate the column with binding buffer.
4. Prepare IgG sample by diluting it at least 1:1 in binding buffer and filter through 0.2 μm millipore.

5. Apply sample to column at no more than 10 mg IgG/2 mL column (*see* Note 10).
6. Wash the column with binding buffer until the absorbance at 280 nm is <0.02.
7. Dissociate the IgG–ligand interaction by eluting with dissociating buffer. Monitor the absorbance at 280 nm, and collect the protein peak. Neutralize immediately with acid (0.1*M* HCl, pH 1.7) or alkali (1*M* Tris-HCl, pH 8.8) depending on eluting buffer.
8. Wash the column with binding buffer until the pH returns to that of the binding buffer. Store column in buffer containing 0.1% sodium azide.
9. Dialyze the IgG preparation against a suitable buffer (e.g., PBS) to remove glycine/Tris.

3.5. Monoclonal Antibodies

Hybridoma technology has made possible the production of highly specific, homogeneous antibodies with predefined binding characteristics that can be produced in large amounts, from immortal cell lines (*see* Chapter 39). MAbs can be exquisitely specific, but they are far from pure, being contaminated with tissue-culture additives and nonimmunoglobulin secretion products when grown in vitro, and by host animal proteins when grown as an ascitic fluid.

MAbs have been routinely produced in mice and less easily in rats. There has also been considerable success in the production of human MAbs. Rodent MAbs are often not easily purified using "conventional" ion-exchange chromatography, and as discussed, gel filtration is not a particularly suitable method for the purification of IgG. HPLC or FPLC are ideal methods for the purification of rodent MAbs; the techniques have been described in Section 3.3.3. and with a few minor changes (*see* Note 25), remain in principle the same.

3.6. Analysis of IgG Fractions

After the purification procedure, it is then necessary to obtain some index of sample purity. There are several methods available for this, and two of the most useful are isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

3.6.1. Analysis of IgG by Isoelectric Focusing (see Ref. 4 and Chapter 9)

The very high resolving power of isoelectric focusing enables the technique to be used to demonstrate the charge-dependent heteroge-

neity of IgG. Polyacrylamide gel is the anticonvection medium/support most usually employed for isoelectric focusing of IgG—this enables several samples to be analyzed on the same slab gel (*see* Fig. 4). The charge dependent heterogeneity of IgG is the result of differences in charged amino acids and, particularly, carbohydrate residues present on the individual IgG species—sialic acid residues are especially important for this. Polyclonal antisera usually focus as a fairly broad "smear" with the major components superimposed as stronger bands, whereas oligoclonal IgGs focus as a series of intense bands. MAbs usually focus as three to six closely spaced bands. The pI of different murine MAbs varies very considerably between 5.5 and 9.5. The characteristic pI and banding pattern should be consistent for a given MAb and can be used as an identity test. This pattern is sometimes referred to as the "spectrotype" of the MAb.

Gels for focusing can be purchased ready for use or can be prepared in the lab. Dialyze samples against two changes of 100 vol of glycine solution overnight.

3.6.2. SDS-PAGE

One of the simplest methods for assessing purity of an IgG fraction is by SDS-PAGE (Fig. 5). Although "full size" slab gels can be used with discontinuous buffer systems and stacking gels, the use of a "mini-gel" procedure (*see* Note 26) is much easier, quicker, and is perfectly adequate for assessing purity, monitoring column fractions, and so on (*see* vol. 5, Chapter 53). Samples should be loaded at concentrations of 1 mg/mL.

4. Notes

1. Mouse and rat IgGs are less stable than IgG from higher mammals; it is best to separate mouse/rat serum from whole blood as soon as clotting has occurred.
2. If an anticoagulant has been added to the blood and the plasma isolated, it may be advisable to defibrinate the plasma to yield a serum analog. The method for this varies according to the anticoagulant; for citrate add 1/100 vol of thrombin solution (100 IU/mL in 1*M* $CaCl_2$), to warmed (37°C) plasma, stir vigorously, and incubate for 10 min at 37°C followed by 1 h at room temperature to ensure completion. For heparin, add 1/100 vol of 5 mg/mL protamine sulfate solution and thrombin (as above) to warmed plasma.

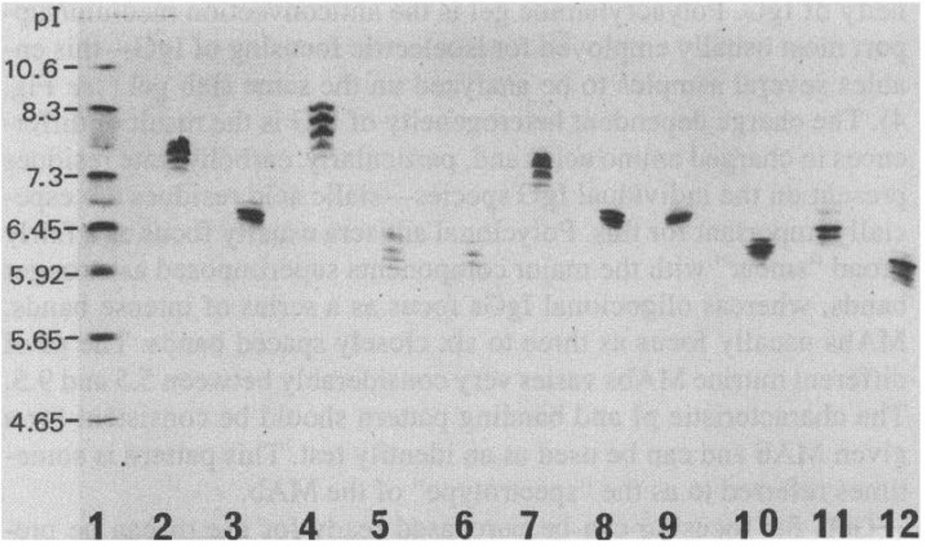

Fig. 4. Isoelectric focusing in polyacrylamide gel of different purified IgG mouse monoclonal antibodies. Track 1 shows marker proteins of known p*I*.

3. Blood clots more quickly in glass than in plastic containers and contracts more readily.
4. If lipid contamination is a particular problem, add silicone dioxide powder (15 mg/mL) and centrifuge for 20 min at 2000*g*.
5. The use of 35% saturation will produce a fairly pure IgG preparation, but will not precipitate all the IgG present in serum/ascitic fluid and so forth. Increasing saturation to 45% causes precipitation of nearly all IgG, but this will be contaminated with other proteins, including some albumin. Purification using $(NH_4)_2SO_4$ can be improved by repeating the precipitation, but this may cause some denaturation, especially of MAbs. Precipitation with 45% $(NH_4)_2SO_4$ is an ideal starting point for further purification steps, e.g., ion-exchange or affinity chromatography and FPLC purification.
6. Although the procedure works well for most antibodies it may produce a fairly heavy contamination with non-IgG proteins with some samples; if this is the case, reduce the concentration of PEG. For this reason, it is best to carry out a pilot-scale experiment before fractionating all of the sample. PEG precipitation is a very mild procedure that results in little denaturation of antibody.

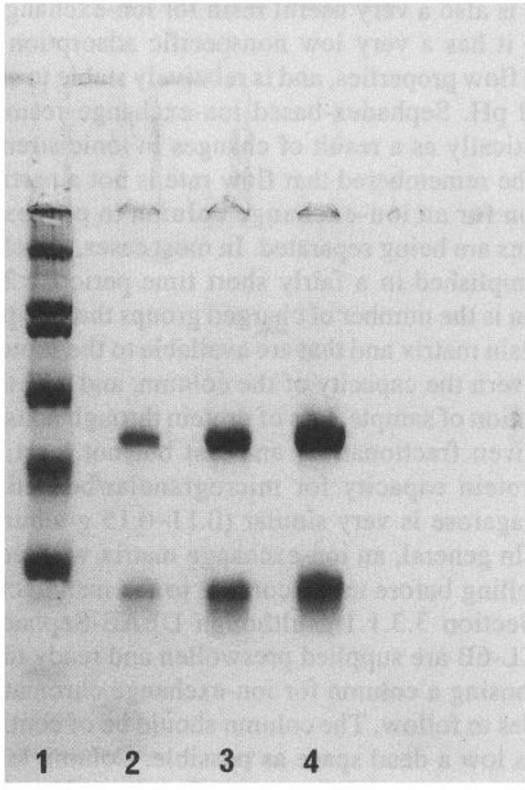

Fig. 5. SDS-PAGE of FPLC-purified MAbs. SDS "mini-gel" electrophoresis analysis of an FPLC-purified mouse MAb stained with Coomassie blue R. Track 1 shows standard mol-wt markers (for details, *see* Fig. 2). The remaining tracks show different loadings of the same MAb. Characteristic heavy chains (50,000) and light chains (22,000) are clearly seen to be free of contamination by other proteins.

7. There are a number of criteria to consider when setting up an ion-exchange chromatography system, e.g., choice of ion exchanger, matrix support, column, and buffer. The most useful ion-exchange resin for the purification of IgG is the anion-exchanger DEAE cellulose, of which there are several forms. The DEAE reactive moiety was originally coupled to fibrous cellulose (Whatman or Bio-Rad, UK), which resulted in poor flow rates and clogging of the matrix. It appears that coupling the DEAE to the newer physical forms of cellulose, microgranules, and beads (DEAE-Sephacel, Pharmacia) results in matrices with more reproducibility, higher capacity, and greater resolving potential than the older fibrous forms. The Sepharose-based DEAE CL-6B

(Pharmacia) is also a very useful resin for ion-exchange of high-mol-wt proteins; it has a very low nonspecific adsorption of macromolecules, good flow properties, and is relatively stable to changes in ionic strength and pH. Sephadex-based ion-exchange resins tend to alter volume drastically as a result of changes in ionic strength and pH. It should also be remembered that flow rate is not a particularly important criterion for an ion-exchange column to possess, unless very labile proteins are being separated. In most cases, the chromatography step is accomplished in a fairly short time period, <24 h. A further consideration is the number of charged groups that are present in a unit volume of resin matrix and that are available to the protein for binding. This will govern the capacity of the column, and will therefore determine the dilution of sample, loss of protein through nonspecific adsorption for a given fractionation, and last but not least, expense! The available protein capacity for microgranular/beaded cellulose and crosslinked agarose is very similar (0.11–0.15 g albumin/mL DEAE derivative). In general, an ion-exchange matrix will require regeneration and swelling before use according to the manufacturer's instructions (*see* Section 3.3.1.1.), although DEAE-Sephacel and DEAE Sepharose CL-6B are supplied preswollen and ready to use.

When choosing a column for ion-exchange chromatography, there are a few rules to follow. The column should be of controlled diameter glass with as low a dead space as possible. Column length should be 20–30 cm, and the internal diameter 1.5–1.6 cm. Homemade columns are quite acceptable for most purposes, although commercial columns are available from several manufacturers.

8. An unusual and useful feature of IgG from many species is that they possess an "abnormal" p*I* (proteins are amphoteric, and their net charge is zero at their p*I*). In such cases the antiserum (fractionated) is simply passed through the anion (DEAE) exchange column under conditions that allow the IgG to pass straight through the column whereas contaminating proteins, including albumin, bind to the column matrix. In these circumstances, there is no requirement for a pH or ionic gradient, although conventional adsorption/elution ion-exchange chromatography can be used for the purification of IgG. The procedure works well with antisera from humans, apes, monkeys, horses, goats, sheep, and rabbits. It does not, however, work for rodent sera or preparations containing mouse or rat monoclonal antibodies. One possible problem is that some immunoglobulins are unstable at low ionic strength, e.g., mouse IgG_3, and precipitation may occur during the ion-exchange pro-

cedure. Furthermore, in preparations of murine monoclonal antibodies, it appears that DEAE cellulose chromatography after ammonium sulfate precipitation does not remove protease and nuclease activity, and does not adequately separate transferrin. Conventional ion-exchange chromatography cannot therefore be considered to be a rapid and efficient general method for the purification of mouse or rat antibodies (especially monoclonal antibodies).

9. If conventional adsorption/elution ion-exchange chromatography is used for the purification of IgG then there are a number of other technical points to consider. Once the sample has been applied to the column, the eluent should be monitored at A_{280}, ideally by passing the eluent through the flow cell of a UV absorbance monitor. If there is a high concentration of proteins in the eluent, then the ion exchanger or sample is not fully equilibrated or the absorbing capacity of the ion exchanger matrix is exceeded. After the sample has been loaded onto the column, the ion exchanger should be washed with two column volumes of starting buffer to ensure complete elution of any unbound protein. The bound proteins are then eluted by increasing the ionic strength (preferable to variation of pH since it is easier to control). The ionic strength is best altered by increasing the concentration of other ions, e.g., NaCl, in which case the pH and buffering capacity are kept constant throughout the separation procedure. The entire process can be automated by using a commercial/homemade gradient maker and fraction collector. As a general rule of thumb, the total volume of the gradient should be between five and ten times the column volume, and the size of the collected fractions should be 10–20% of the total column volume. The elution profile can be plotted by recording the optical density at A_{280} of the eluted fraction and the first major peak contains the IgG.
10. The extinction coefficient ($E_{280}^{1\%}$) of human IgG is 13.6 (i.e., a 1 mg/mL solution will have an A_{280} of 1.36).
11. The "void volume" of the column is the volume of liquid between the beads of the gel matrix and usually amounts to about $^1/_3$ of the total column volume.
12. The operating pressure of the gel is the vertical distance between the top of the buffer in the reservoir and the outlet end of the tube; this should never exceed the manufacturer's recommended maximum for the gel.
13. There are a number of criteria to consider when setting up a gel filtration system, e.g., choice of gel, column, and buffer. The gel of choice

may be composed of beads of carbohydrate or polyacrylamide, and is available in a wide variety of pore sizes and hence fractionation ranges. Useful gels for the separation of IgG include Ultrogel AcA 44 (mixtures of dextran and polyacrylamide) and Bio-Gel P 200 (polyacrylamide). For IgM fractionation, Sephacel S-300 (crosslinked dextran) is most useful. When selecting a column for gel filtration, the column should be of controlled diameter glass with as low a dead space as possible at the outflow. Column tubing should be about 1 mm in diameter, which helps reduce dead space volume. A useful length for a gel filtration column is about 100 cm, and the choice of cross-sectional area is governed by sample size, both in terms of volume and amount of protein. As a rough rule of thumb, the sample volume should not be >5% of the total column volume (1–2% gives better resolution), and between 10–30 mg of protein/cm^2 cross-sectional area is a satisfactory loading. More protein will increase yield, but decrease resolution and hence purity, whereas less protein loaded improves resolution, but cuts back on yield. In general, homemade columns will suffice for gel filtration procedures, although commercial columns are available from a number of manufacturers. Ensure columns are clean before use by washing with a weak detergent solution and rinsing thoroughly with water. Gel filtration can be performed using a wide variety of buffers generally of physiological specification, i.e., pH 7.2, 0.1*M*.

14. Gel filtration columns may also be packed by using an extension reservoir attached directly to the top of the column. In this case, the total volume of gel and buffer can be poured and allowed to settle without continual topping up. When using an extension reservoir, leave the column to pack until the gel bed just runs dry, and then remove excess gel.
15. When loading commercial columns with flow adaptors touching the gel bed surface, the most satisfactory way to apply the sample is by transferring the inlet tube from the buffer reservoir to the sample. The sample enters the tube and gel under operating pressure, and then the tube must be returned to the buffer reservoir. Ensure no air bubbles enter the tube. In the case of homemade columns, it is also possible to load the sample directly onto the gel bed by making it up in 5% (w/v) sucrose. Elution of samples is either by pressure from the reservoir (operating pressure), or by a peristaltic pump between the reservoir and the top of the column. In general the flow rate should be the volume contained in 2–4 cm height of column/h. In order to prevent columns without pumps from running dry, the inlet tube should be arranged so that part of it is below the outlet point of the column. Eluted fractions should be collected by measured volume rather than time (this

prevents fluctuations in flow rate from altering fraction size). In general, for a 100-cm column, a column volume of eluent should be collected in about 100 fractions; the void volume is eluted at about fraction 30–35.

16. The protein yield in gel filtration should be >80 and is often as high as 95%. Yield will improve after the first use of a gel, because new gel adsorbs protein nonspecifically in a saturable fashion. When very small amounts of protein are being fractionated, the column should be saturated with albumin prior to use.
17. Columns should be stored at room temperature or 4°C, usually in the presence of a bacteriostatic agent, e.g., 0.02% sodium azide.
18. Briefly, HPLC and FPLC systems consist of two high-precision pumps connected to a mixer for gradient formation, an automatic motor valve for injection of samples, a sensitive UV monitor and control unit, and a programmable controller (most modern systems are now controlled by computerized software). A fraction collector and printer or chart recorder complete the hardware requirements, together with the column of choice, e.g., ion exchange, gel filtration.

    For FPLC, rapid purification of IgG can be achieved using the Mono Q HR5/5 or HR10/10 anion-exchange columns and the Superose 12 HR10/30 gel filtration columns. The HR5/5 and HR10/10 columns are identical except in size and, thus, capacity. When using the HR10/10 column, it is necessary to use a "superloop" into which the sample must be manually injected. The superloop has a maximum capacity of 10 mL, although any smaller volume may be delivered. The loop size for the smaller column has a maximum volume of 0.5 mL.

    The gel filtration columns may be used to purify IgG, but the Superose 12 HR10/30 column can handle only small sample quantities (<200 µL). Alternatively, preparative scale-up can be achieved by purchasing "prep-grade" Superose 12 and packing the column in house.

    For HPLC, IgG purification can be readily achieved using anion-exchange columns, such as Hydropore SAX, and gel filtration columns, such as Anagel-TSK G3000SW. Both types of column should be protected by guard columns. The Hydropore columns are available in a range of internal diameters for different application needs; the larger columns (≥10 cm internal diameter) would be appropriate for larger-scale purification (≥100 mg IgG), although smaller columns (4.6 cm id) have a nominal capacity of 20 mg and would be suitable for most laboratory needs. The Anagel columns, like the Superose 12 columns for FPLC, separate only small quantities (0.5–1.0 mg) of IgG and are therefore only suitable for analytical purposes (*see* Fig. 1). Preparative

columns are available having slightly larger silica gel particle sizes allowing faster flow rates (3–6 mL/min) and sample loading up to a maximum of 200 mg. It is necessary to use a large-sample injection loop for preparative scale purification. A 5-mL loop would be appropriate for most applications, although smaller sizes are available.

Several criteria require further consideration. It is essential that the sample to be loaded and all buffers used are filtered using 0.2 μm millipore. All the methods for column equilibration, sample elution, and column washing are programmed either into the liquid chromatography controller or via the computer software. The programs have to be entered by the user, and the parameters of each program can be varied (e.g., flow rate, gradient times). In practice, for purification methods using the HR10/10 column, we have found that a flow rate of 4.0–6.0 mL/min, with a salt gradient from 0 to 28% (where buffer B = 1*M* NaCl) over about 30 min is adequate for single-step purification of IgG from serum. This is followed by a salt gradient of 100% buffer B for about 15 min to purge the columns of remaining proteins. IgG elutes between 10 and 25% in the salt gradient, usually around 15%. When IgG elutes at 25% (dependent on pI), then it will tend to coelute with albumin, which elutes at around 27%. When this occurs, an alternative method of purification should be employed, e.g., gel filtration. A typical elution profile of sheep IgG is shown in Fig. 2.

19. The use of subclass specific antibodies or MAbs allows the immunoaffinity isolation of individual subclasses of IgG.
20. Protein A does not bind all subclasses of IgG, e.g., human IgG_3, mouse IgG_3, sheep IgG_1, and some subclasses bind only weakly, e.g., mouse IgG_1. For some species, IgG does not bind to protein A well at all; e.g., rat, chicken, goat and some MAbs show abnormal affinity for the protein. Protein A can be used to separate IgG subclasses from mouse serum (see ref. *1*). Proteins C and G bind to IgG from most species, including rat and goat, and recognize most subclasses (including human IgG_3 and mouse IgG_1 and IgG_3), but have a lower binding capacity. However, they also contain an albumin-binding site, which makes the natural proteins unsuitable for affinity purification. Recombinant DNA technology has enabled mutant proteins to be produced in which the albumin binding site has been spliced out. These are very good for affinity chromatography, but are expensive.
21. Coupling at pH 6.5 is less efficient than at higher pH, but is less likely to compromise the binding ability of immobilized ligands (especially antibodies).

22. Check the efficiency of coupling by measuring the A_{280} of the ligand before and after coupling. Usually at least 95% of the ligand is bound to the matrix.
23. Elution of bound substances is usually achieved by the use of a reagent that disrupts noncovalent bonds. These vary from "mild" procedures, such as the use of high salt or high or low pH, to more drastic agents, such as 8*M* Urea, 1% SDS, or 5*M* guanidine hydrochloride. Chaotropic agents, such as thiocyanate or pyrophosphate, may also be used. Usually an eluting agent is selected that is efficient, but does not appreciably denature the purified molecule; this is often a compromise between the two ideals. In view of this, highly avid polyclonal antisera obtained from hyperimmune animals are often not the best reagents for immunoaffinity purification, since it may be impossible to elute the IgG in a useful form. The 0.1*M* glycine-HCl buffer, pH 2.5, will elute most IgG from immunoaffinity and protein A and G columns, but may denature some MAbs. Most IgGs can be eluted from protein A by using pH 3.5 buffer. "Pre-elution" of the column with dissociating reagent just prior to affinity chromatography ensures that the isolated immunoglobulin is minimally contaminated with ligand.
24. Incubation of the IgG containing sample with the ligand matrix is not always necessary, but will allow maximal binding to occur. Alternatively, slowly pump the sample through the column. Flow rate depends on the IgG concentration in the sample, and the binding capacity and size of the affinity column.
25. The technique described for the purification of sheep polyclonal IgG by FPLC is easily applied to the purification of rodent monoclonal antibodies. Some workers have used hydroxyapatite (hydroxylapatite) for the purification of murine monoclonal antibodies, but the authors have not found this to be very effective in most cases.
26. The "mini-gel" is easily and quickly prepared, consisting only of a resolving gel, and takes approx 1.5 h to run once set up. The sample (IgG) is prepared for electrophoresis under reduced conditions and is run in parallel with standard molecular-weight markers. The sample is loaded onto the "mini-gel" and run at 150 V for approx 1.5 h. The gel is then stained using Coomassie blue R to detect protein bands, followed by destaining, after which the gel can be photographed and/or dried onto filter paper. A typical gel showing three different loadings of an FPLC-purified mouse IgG MAb is shown in Fig. 5. Run under the reduced conditions described, the heavy chains have a characteristic mol wt of approx 50,000 and the light chains a mol wt of approx 22,000.

Acknowledgment

We would like to thank Maryvonne Brasher for her part in the section on isoelectric focusing, Chris Ling for providing the sheep anti-p27 serum, and Chris Bird for his part in the affinity purification of anti-IL-3 MAbs. We are also grateful to Lisa Hudson and Deborah Kirk for typing the manuscript.

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CHAPTER 44

Enzyme and Biotin Labeling of Antibody

G. Brian Wisdom

1. Introduction

Labeled antibodies are key reagents in many procedures, particularly immunoassays and the detection of antigens in tissue sections and on membrane blots. The ideal labeled antibody retains all the immunoreactivity of the antibody and all the signal-generating capacity of the label. In addition, these conjugates should be stable, and have defined and appropriate proportions of the two components.

1.1. Enzyme Labeling

Enzymes are useful labels because they act as amplifiers; this allows the target antigen to be detected at very low concentration. There are many methods of attaching enzymes to immunoglobulins *(1,2)*. Nonessential functional groups in the protein, such as amino, thiol or saccharide, are exploited and, in most cases, bifunctional reagents are used to link the enzyme to immunoglobulin G (IgG). The three most commonly used methods are presented here with minor modifications to the originally described procedures.

1.1.1. Alkaline Phosphatase Labeling Using Glutaraldehyde

The chemistry of the homobifunctional reagent, glutaraldehyde, is complex. It reacts with amino groups of proteins, and when two proteins are mixed in its presence, stable conjugates are obtained with-

From: *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
Edited by: J. M. Walker

out the formation of Schiff bases. This method *(3)* can be applied to most proteins, but self-coupling is a problem unless the proteins are at appropriate concentrations.

1.1.2. Horseradish Peroxidase Labeling Using Periodate Oxidation

This method *(4)* is unusual in that an exogenous cross-linking reagent is not used. The peroxidase is a glycoprotein, and oxidation of its saccharide residues with sodium periodate results in the formation of aldehyde groups. When IgG is added, the modified enzyme reacts with the immunoglobulin's amino groups to form Schiff bases. These are then reduced to give a stable conjugate. (The enzyme has few free amino groups, and self-coupling is not a problem.)

1.1.3. β-Galactosidase Labeling Using m-Maleimidobenzoyl-N-Hydroxysuccinimide Ester (MBS)

MBS is a heterobifunctional reagent, and it is of particular value when one of the proteins involved has no free thiol groups, e.g., IgG. In this method *(5)* the IgG is first modified by allowing the *N*-hydroxysuccinimide ester group of MBS to react with amino groups in the protein. After removal of unused reagent, the enzyme is added, and the modified IgG reacts with thiols in the β-galactosidase via its maleimide groups to form thioether links. This procedure may also be used with enzymes lacking free thiols. This group may be introduced into proteins by several reagents; one of the most useful is *N*-succinimidyl 3-(2-pyridyldithio)propionate *(6)*.

1.1.4. General Comments

The method employing alkaline phosphatase and glutaraldehyde is the easiest to carry out and gives useful conjugates, although the yields are relatively low; the other two methods give active conjugates with similar levels of activity *(7)*. The strength of the signal produced by a conjugate, however, will depend on the substrate and detection system used, as well as the specific activities of its components and the method of preparation. In all cases the conjugates are somewhat heterogeneous, and their molecular weights may exceed 10^6. Removal of unconjugated IgG is important in most applications; this is usually achieved by gel filtration.

1.2. Biotin Labeling

Biotin-labeled antibody is a widely used reagent *(8)*. It can be easily detected using the biotin-binding protein streptavidin; this is usually labeled with an enzyme. The relatively small size of the biotin label and its tight binding to streptavidin ($K_d = 10^{-15}$) can be advantageous in some situations, and additional amplification is possible by using the tetravalent streptavidin to form a bridge between the biotinylated IgG and a biotinylated enzyme, e.g., alkaline phosphatase. Several biotin derivatives are available for labeling proteins; these include malaimide and hydrazide derivatives, but the most commonly used one is the *N*-hydroxysuccinimide ester, which reacts with unprotonated amino groups in the IgG *(9,10)*. The derivatives are also available with extended side chains, which allow better reaction with streptavidin. Usually about 10 biotins are attached to each IgG molecule.

2. Materials

Ideally, the antibody used should be purified by immunoaffinity chromatography (*see* Chapter 43), unless it is derived from a hybridoma and contamination by other proteins is minimal. For many purposes, however, the total IgG fraction of an antiserum may be used. The IgG must be in the appropriate solution; changes can be effected by dialysis (*see* Note 1) or by gel filtration in a small column of, for example, Sephadex G-25.

The enzymes should be free of other proteins and interfering substances. Several manufacturers provide enzymes of very high specific activities for labeling purposes.

Hazards: Protein-modifying reagents are intrinsically hazardous, and precautions must be taken. Carry out the manipulations in a fume hood, and use gloves.

2.1. Alkaline Phosphatase Labeling Using Glutaraldehyde

1. Alkaline phosphatase from calf intestinal mucosa, 1000 U/mg or greater (with 4-nitrophenyl phosphate as substrate). If the enzyme is in an ammonium sulfate suspension, then this salt must be removed (*see* Note 1).

2. PBS: 20 m*M* Sodium phosphate buffer, pH 7.2, containing 0.15*M* NaCl.
3. Glutaraldehyde.
4. 50 m*M* Tris-HCl buffer, pH 7.5, containing 1 m*M* $MgCl_2$, 0.02% NaN_3, and 2% bovine serum albumin (BSA). (See Note 2.)

2.2. Horseradish Peroxidase Labeling Using Periodate Oxidation

1. Horseradish peroxidase, 1000 U/mg or greater (with 2,2'azino-*bis*-[3-ethylbenzthiazoline-6-sulfonic acid] as substrate). (*See* Note 3.)
2. 0.1*M* Sodium periodate.
3. 1 m*M* Sodium acetate buffer, pH 4.4.
4. 10 m*M* Sodium carbonate buffer, pH 9.5.
5. 0.2*M* Sodium carbonate buffer, pH 9.5.
6. Sodium borohydride, 4 mg/mL.
7. Ultrogel AcA34 (IBF Biotechnics, Villeneuve la Garenne, France) or similar gel filtration medium.
8. PBS.
9. BSA. (*See* Note 2.)

2.3. β-Galactosidase Labeling Using MBS

1. β-Galactosidase from *Escherichia coli*, 600 U/mg or greater (with 2-nitrophenyl-β-D-galactopyranoside as substrate). (*See* Note 4.)
2. 0.1*M* Sodium phosphate buffer, pH 7.0, containing 50 m*M* NaCl.
3. MBS.
4. Dioxan.
5. Sephadex G-25 (Pharmacia LKB Biotechnology, Uppsala, Sweden) or equivalent gel filtration medium.
6. 10 m*M* Sodium phosphate buffer, pH 7.0, containing 50 m*M* NaCl and 10 m*M* $MgCl_2$
7. β-Mercaptoethanol.
8. DEAE-Sepharose (Pharmacia) or equivalent ion-exchange medium.
9. 10 mM Tris-HCl buffer, pH 7.0, containing 10 m*M* $MgCl_2$ and 10 m*M* β-mercaptoethanol.
10. Item 9 containing 0.5*M* NaCl.
11. Item 9 containing 3% BSA and 0.6% NaN_3. (*See* Note 2.)

2.4. Biotin Labeling

1. Biotin N-hydroxysuccinimide ester, 2 mg/mL in dimethylformamide.
2. PBS.

3. Methods

3.1. Alkaline Phosphatase Labeling Using Glutaraldehyde

1. Add 0.5 mg of IgG in 100 µL of PBS to 1.5 mg of enzyme.
2. Add 5% glutaraldehyde (about 10 µL) to give a final concentration of 0.2% (v/v), and stir the mixture for 2 h at room temperature.
3. Dilute the mixture to 1 mL, and dialyze against PBS overnight at 4°C.
4. Dilute the dialyzed solution to 10 mL with 50 m*M* Tris-HCl buffer, pH 7.5 containing 1 m*M* $MgCl_2$, 0.02% NaN_3 and 2% BSA; store at 4°C. (See Note 5.)

3.2. Horseradish Peroxidase Labeling Using Periodate Oxidation

1. Dissolve 2 mg of enzyme in 500 µL of water.
2. Add 100 µL of freshly prepared 0.1*M* sodium periodate, and stir for 20 min at room temperature. (*See* Note 6.)
3. Dialyze the modified enzyme against 1 m*M* sodium acetate buffer, pH 4.4, overnight at 4°C. (*See* Note 1.)
4. Dissolve 4 mg of IgG in 500 µL of 10 m*M* sodium carbonate buffer, pH 9.5.
5. Adjust the pH of the dialyzed enzyme solution to 9.0–9.5 by adding 10 µL of 0.2*M* sodium carbonate buffer, pH 9.5, and immediately add the IgG solution. Stir the mixture for 2 h at room temperature.
6. Add 50 µL of freshly prepared sodium borohydride solution (4 mg/mL), and stir the mixture occasionally over a period of 2 h at 4°C.
7. Fractionate the mixture on a column of Ultrogel AcA34 (1.5 × 85 cm) in PBS. Determine the A_{280} and A_{403}. (*See* Note 7.)
8. Pool the fractions in the first peak (both A_{280} and A_{403} peaks coincide), add BSA to a final concentration of 5 mg/mL, and store aliquots at –20°C. (*See* Note 8.)

3.3. β-Galactosidase Labeling Using MBS

1. Dissolve 1.5 mg of IgG in 1.5 mL of 0.1*M* sodium phosphate buffer, pH 7.0, containing 50 m*M* NaCl.
2. Add 0.32 mg of MBS in 15 µL of dioxan, mix, and incubate for 1 h at 30°C.
3. Fractionate the mixture on a column of Sephadex G-25 (0.9 × 30 cm) equilibrated with 10 m*M* sodium phosphate buffer, pH 7.0, containing 50 m*M* NaCl and 10 m*M* $MgCl_2$, and elute with the same buffer. Col-

lect 0.5-mL fractions, measure the A_{280} and pool the fractions in the first peak (about 3 mL in volume).

4. Add 1.5 mg of enzyme to the solution of modified IgG, mix, and incubate for 1 h at 30°C.
5. Stop the reaction by adding 1*M* β-mercaptoethanol to give a final concentration of 10 m*M* (about 30 µL).
6. Fractionate the mixture on a column of DEAE-Sepharose (0.9 × 15 cm) equilibrated with 10 m*M* Tris-HCl buffer, pH 7.0, containing 10 m*M* $MgCl_2$ and 10 m*M* β-mercaptoethanol; elute with the Tris buffer (50 mL) followed by Tris buffer containing 0.5*M* NaCl (50 mL). Collect 3-mL fractions in tubes containing 0.1 mL of Tris buffer containing 3% BSA and 0.6% NaN_3 Pool the major peak (this is eluted with NaCl), and store at 4°C. (*See* Note 9.)

3.4. Biotin Labeling

1. Add 25 µL of biotin *N*-hydroxysuccinimide ester solution to 2 mg of IgG in 1 mL of 0.1*M* $NaHCO_3$, pH 8.0.
2. Incubate for 2 h at room temperature.
3. Dialyze against PBS overnight at 4°C with several buffer changes (*see* Note 1), and store aliquots at –20°C.

3.5. Testing Conjugates

The efficacy of an enzyme-labeled antibody may be tested by immobilizing the appropriate antigen on the wells of a microtiter plate or strip, incubating various dilutions of the conjugate for a few hours, washing, adding the substrate, and measuring the amount of product formed. For biotin-labeled antibody, the amount of antigen-bound antibody is detected by incubating with enzyme-labeled streptavidin prior to the addition of substrate. This procedure may also be used to monitor the purification of conjugates by chromatography. Appropriate substrates are described in Chapters 25–27.

4. Notes

1. Dialysis of small volumes can be conveniently done in narrow dialysis tubing by placing a short glass tube, sealed at both ends, in the tubing so that the space available to the sample is reduced. Transfer losses are minimized by carrying out subsequent steps in the same dialysis bag. There are also various microdialysis cells available commercially.
2. BSA is routinely added to conjugate solutions to improve stability and minimize adsorption to container walls.

3. Preparations of horseradish peroxidase vary in their carbohydrate content, and this can affect the oxidation reaction. Free carbohydrate can be removed by gel filtration. Increasing the sodium periodate concentration to 0.2*M* can also help, but further increases lead to excess oxidation, which damages the activity of the peroxidase.
4. The thiol groups of β-galactosidase may become oxidized during purification and storage. Satisfactory conjugates can be made from preparations with about 10 free thiol groups/molecule. It is relatively easy to measure these groups *(11)*.
5. We have found that purification of these conjugates by gel filtration does not improve their activity.
6. During the course of the sodium periodate treatment, the mixture changes color from orange to green; this is reversed during dialysis.
7. The absorbance at 403 nm is caused by the enzyme's heme group. Horseradish peroxidase is often specified in terms of its RZ; this is the ratio of the A_{403} to the A_{280}, and it provides a measure of the heme content of the preparation. A highly purified sample of enzyme will have a value of about 3. Conjugates with an RZ of 0.4 perform satisfactorily.
8. NaN_3 should not be used with peroxidase conjugates, because it inhibits the enzyme. If an antimicrobial agent is required, 0.02% merthiolate can be used.
9. These conjugates can also be purified by gel filtration in Ultrogel AcA34 (or equivalent medium) in 10 m*M* Tris-HCl buffer, pH 7.0, containing 10 m*M* $MgCl_2$, 10 m*M* β-mercaptoethanol, and 50 m*M* NaCl.

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CHAPTER 45

Labeling of Peptides and Proteins by Radioiodination

Graham S. Bailey

1. Introduction

Many different substances can be labeled by radioiodination. Such labeled molecules are of major importance in a variety of investigations, e.g., studies of intermediary metabolism, determinations of agonist and antagonist binding to receptors, quantitative measurements of physiologically active molecules in tissues and biological fluids, and so forth. In most of those studies, it is necessary to measure very low concentrations of the particular substance, and that in turn implies that it is essential to produce a radioactively labeled tracer molecule of high specific radioactivity. Such tracers, particularly in the case of peptides and proteins, can often be conveniently produced by radioiodination.

Two γ-emitting radioisotopes of iodine are widely available, ^{125}I and ^{131}I. As γ-emitters, they can be counted directly in a well-type crystal scintillation counter (commonly referred to as a γ counter) without the need for sample preparation in direct contrast to β-emitting radionuclides, such as ^{3}H and ^{14}C. Furthermore, the count rate produced by 1-g atom of ^{125}I is approx 75 times and 35,000 times greater than that produced by 1-g atom of ^{3}H and ^{14}C, respectively. In theory, the use of ^{131}I would result in a further sevenfold increase in specific radioactivity. However, the isotopic abundance of commercially available ^{131}I rarely exceeds 20% because of contaminants of ^{127}I, and its half-life is only 8 d. In contrast, the isotopic abundance

From: *Methods in Molecular Biology, Vol 32: Basic Protein and Peptide Protocols*
Edited by: J. M. Walker

of ^{125}I on receipt in the laboratory is normally at least 90% and its half-life is 60 d. Also, the counting efficiency of a typical well-type crystal scintillation counter for ^{125}I is approx twice that for ^{131}I. Thus, in most circumstances, ^{125}I is the radionuclide of choice for radioiodination.

Several different methods of radioiodination of peptides and proteins have been developed *(1,2)*. They differ in, among other respects, the nature of the oxidizing agent for converting $^{125}I^-$ into the reactive species $^{125}I_2$ or $^{125}I^+$. In the main, those reactive species substitute into tyrosine residues of the protein, but substitution into other residues, such as histidine, cysteine, and tryptophan, can occur in certain circumstances. If the native peptide lacks a tyrosine residue, it should be possible to replace one of its amino acid residues, particularly phenylalanine, with tyrosine or to add a tyrosine residue to its N or C terminus by the standard techniques of peptide synthesis. Indeed, such modified peptides may be commercially available. Direct radioiodination of the modified, tyrosine-containing peptide can then be carried out. However, if the modified peptide differs signficantly from the native peptide in the reaction with antibody, then an indirect, conjugation method of radioiodination will have to be employed *(2)*. In this chapter, two direct methods of radioiodination for peptides and proteins will be described, and from here on, the word protein will be used synonymously for peptides and proteins.

1.1. The Chloramine-T Method

This method, developed by Hunter and Greenwood *(3)*, is probably the most widely used of all techniques of protein radioiodination. It is a very simple method in which the radioactive iodide is oxidized by chloramine-T in aqueous solution. The oxidation is stopped after a brief period of time by addition of excess reductant. Unfortunately, some proteins are denatured under the relatively strong oxidizing conditions, and so other methods of radioiodination that employ more gentle conditions have been devised, e.g., the lactoperoxidase method.

1.2. The Lactoperoxidase Method

This method, introduced by Marchalonis *(4)*, employs lactoperoxidase in the presence of a trace of hydrogen peroxide to oxidize the radioactive iodide. The oxidation can be stopped by simple dilution.

Although the technique should result in less chance of denaturation of susceptible proteins than the chloramine-T method, it is more technically demanding and is subject to a more marked variation in optimum reaction conditions.

2. Materials

2.1. The Chloramine-T Method

1. Na ^{125}I: 37 MBq (1 mCi) concentration 3.7 GBq/mL (100 mCi/mL)
2. Buffer A: 0.5*M* sodium phosphate buffer, pH 7.4 (*see* Note 1).
3. Buffer B: 0.05*M* sodium phosphate buffer, pH 7.4.
4. Buffer C: 0.01*M* sodium phosphate buffer containing 1*M* sodium chloride, 0.1% bovine serum albumin, and 1% potassium iodide, final pH 7.4.
5. Chloramine-T solution: A 2 mg/mL solution in buffer B is made just prior to use (*see* Note 4).
6. Reductant: A 1 mg/mL solution of sodium metabisulfite in buffer C is made just prior to use.
7. Protein to be iodinated: A 0.5–2.5 mg/mL solution is made in buffer B.

2.2. The Lactoperoxidase Method

1. Na ^{125}I: 37 MBq (1 mCi) concentration 3.7 GBq/mL (100 mCi/mL).
2. Lactoperoxidase: Available from various commercial sources. A stock solution of 10 mg/mL in 0.1*M* sodium acetate buffer, pH 5.6, can be made and stored at –20°C in small aliquots. A working solution of 20 µg/mL is made by dilution in buffer just prior to use.
3. Buffer A: 0.4*M* sodium acetate buffer, pH 5.6 (*see* Note 6).
4. Buffer B: 0.05*M* sodium phosphate buffer, pH 7.4.
5. Hydrogen peroxide: A solution of 10 µg/mL is made by dilution just prior to use.
6. Protein to be iodinated: A 0.5–2.5 mg/mL solution is made in buffer B.

It is essential that none of the solutions contain sodium azide as antibacterial agent, since it inhibits lactoperoxidase.

3. Practical Procedure

3.1. The Chloramine-T Method

1. Into a small plastic test tube (1 × 5.5 cm) are added successively the protein to be iodinated (10 µg), radioactive iodide (5 µL), buffer A (50 µL), and chloramine-T solution (25 µL) (*see* Notes 2 and 5).
2. After mixing by gentle shaking, the solution is allowed to stand for 30 s to allow radioiodination to take place (*see* Note 3).

3. Sodium metabisulfite solution (500 µL) is added to stop the radioiodination, and the resultant solution is mixed. It is then ready for purification.

3.2. The Lactoperoxidase Method

1. Into a small plastic test tube (1 × 5.5 cm) are added in turn the protein to be iodinated (5 µg), radioactive iodide (5 µL) lactoperoxidase solution (5 µL) and buffer A (45 µL) (*see* Note 7).
2. The reaction is started by the addition of the hydrogen peroxide solution (10 µL) with mixing.
3. The reaction is stopped after 20 min (*see* Note 8) by the addition of buffer B (0.9 mL) with mixing. The resultant solution is then ready for purification (*see* Notes 9 and 10).

3.3. Purification of Radioiodinated Protein

At the end of the radioiodination, the reaction mixture will contain the labeled protein, unlabeled protein, radioiodide, mineral salts, enzyme (in the case of the lactoperoxidase method), and possibly some protein that has been damaged during the oxidation. For most uses of radioiodinated proteins, it is essential to have the labeled species as pure as possible with the constraint, however, that the purification is achieved as rapidly as possible. For that purpose, the most widely used of all separation techniques is gel filtration. Various types of Sephadex resin can be employed, e.g., G-25, G-75, or G-100, depending on the differences in sizes of the molecules present in the mixture.

Typically the mixture is applied to a column (1 × 25 cm) of Sephadex resin and is eluted with 0.05*M* sodium phosphate buffer of pH 7.4 containing 0.15*M* sodium chloride and 0.1% bovine serum albumin. Fractions (0.5–1.0 mL) are collected in plastic tubes, and aliquots (10 µL) are counted. Using those results, an elution profile, such as that shown in Fig. 1, is drawn.

3.4. Assessment of the Quality of the Radioiodinated Protein

Several parameters can be used to assess the quality of the labeled protein. The specific radioactivity of the protein is the amount of radioactivity incorporated per unit mass of protein. It can be calculated in terms of the total radioactivity employed, the amount of the iodination mixture transferred to the gel filtration column, and the

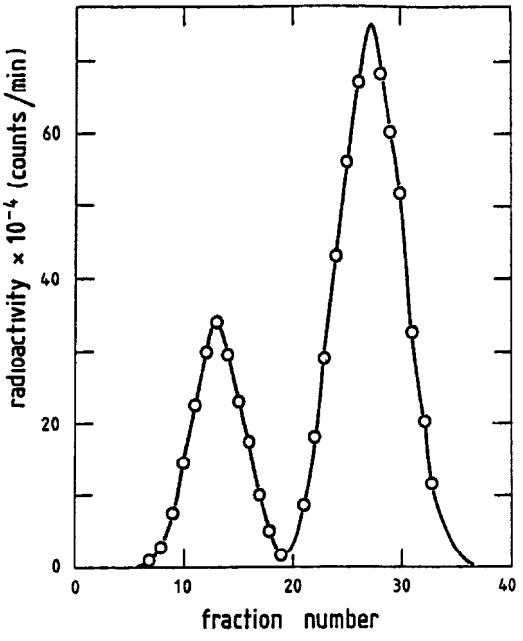

Fig. 1. Gel filtration of radioiodinated kallikrein of rat submandibular gland. The pure enzyme (10 μg) was iodinated with ^{125}I (18.5 MBq) by the chloramine-T method. It was then purified on a column (1 × 20 cm) of Sephadex G-75 resin at a flow rate of 20 mL/h and fractions of 0.6 mL were collected. Aliquots (10 μL) of each fraction were measured for radioactivity. By radioimmunoassay, immunoreactive protein was found only in the first peak, and more than 90% of that radioactivity was bound by the antiserum to kallikrein from rat submandibular gland.

amount of radioactivity present in the labeled protein, in the damaged components, and in the residual radioiodine. However, in practice, the calculation does not usually take into account damaged and undamaged protein. The specific activity is thus calculated from the yield of the radioiodination procedure, the amount of radioiodide, and the amount of protein used, assuming that there are no significant losses of those two reactants. The yield of the reaction is simply the percentage incorporation of the radionuclide into the protein.

For example, consider the results shown in Fig. 1. In terms of the elution volumes, it is to be expected that the first peak of radioactivity represents the labeled protein and that the second peak represents unreacted radioiodide. For this case and, more importantly, for more

complicated elution patterns, the nature of the materials giving rise to those peaks can be checked by employing a specific antiserum to the protein being radioiodinated. Aliquots (10 µL) of different fractions making up the two peaks are diluted so that each gives the same number of counts (e.g., 5000–10,000 counts/min) per 100 µL. Those samples are incubated with an excess of the antiserum. Only samples containing immunoreactive protein will react with the antiserum. The amount of radioactive protein associated with the antibody molecules can then be measured by radioimmunoassay (*see* Chapter 46).

Having identified the peak or peaks containing the radioiodinated protein, the yield of the radioiodination can be calculated in terms of the ratio of the total counts associated with the protein peak to the sum of the total counts associated with the protein peak and total counts associated with the iodide peak.

It is obviously important that the radioiodinated protein should, as far as possible, have the same properties as the unlabeled species. Thus, the behavior of both molecules can be checked on electrophoresis or ion-exchange chromatography. The ability of the two species to bind to specific antibodies can be assessed by radioimmunoassay.

3.5. Storage of the Radioiodinated Protein

Immediately after purification, the radioiodinated protein is split into small aliquots that are then rapidly frozen and stored at –20°C. Alternatively, the aliquots can be freeze-dried. Each aliquot should be thawed and used only once. Radioiodinated proteins differ markedly in their stability. Some can be stored for several weeks (although it must be borne in mind that the half-life of ^{125}I is about 60 d), whereas others can only be kept for several days. If necessary, the labeled protein can be repurified by gel filtration or ion-exchange chromatography prior to use.

4. Notes

1. The pH optimum for the iodination of tyrosine residues of a protein by this method is about 7.4. Lower yields of iodinated protein are obtained at pH values below about 6.5 and above about 8.5. Indeed above pH 8.5, the iodination of histidine residues appears to be favored.
2. The total volume of the reaction should be as low as practically possible to achieve a rapid and efficient incorporation of the radioactive iodine into the protein.

3. Because of the small volumes of reactants that are employed, it is essential to ensure adequate mixing at the outset of the reaction. Inadequate mixing is one of the most common reasons for a poor yield of radioiodinated protein by this procedure.
4. If the protein has been seriously damaged by the use of 50 µg of chloramine-T, it may be worthwhile repeating the radioiodination using much less oxidant (10 µg or less). Obviously, the minimum amount of chloramine-T that can be used will depend on, among other factors, the nature and amount of protein to be iododinated.
5. It is normal to carry out the method at room temperature. However, if the protein is especially labile, it may be beneficial to run the procedure at a lower temperature.
6. The exact nature of buffer A will depend on the properties of the protein to be radioiodinated. Proteins differ markedly in their pH optima for radioiodination by this method *(5)*. Obviously, the pH to be used will also depend on the stability of the protein, and the optimum pH can be established by trial and error.
7. Other reaction conditions, such as amount of lactoperoxidase, amount and frequency of addition of hydrogen peroxide, and so forth, also markedly affect the yield and quality of the radioiodinated protein. Optimum conditions can be found by trial and error.
8. The longer the time of the incubation, the greater the risk of potential damage to the protein by the radioactive iodide. Thus, it is best to keep the time of exposure of the protein to the radioactive iodide as short as possible, but commensurate with a good yield of radioactive product.
9. Some of the lactoperoxidase itself may become radioiodinated, which may result in difficulties in purification if the enzyme is of a similar size to the protein being labeled. Thus, it is best to keep the ratio of the amount of protein being labeled to the amount of lactoperoxidase used as high as possible.
10. Some of the problems may be overcome by the use of solid-phase lactoperoxidase systems. Such a system is commercially available in which immobilized glucose oxidase is used to generate a small, steady stream of hydrogen peroxide from added glucose. The hydrogen peroxide is utilized by immobilized lactoperoxidase to oxidize the radioactive iodide.

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CHAPTER 46

Radioimmunoassay of Peptides and Proteins

Graham S. Bailey

1. Introduction

Radioimmunoassay is often described in terms of the competition between a radiolabeled antigen (Ag*) and its unlabeled counterpart (Ag) for binding to a limited amount of specific antibody (Ab) *(1)*. In most radioimmunoassays, the reaction is allowed to proceed to equilibrium and thus can be represented by Eq. 1.

$$Ag^* + Ag + 2Ab \rightleftharpoons Ag^*Ab + AgAb \quad (1)$$

The concentration of the antibody is limited such that the labeled antigen, although present in a trace amount, is in relative excess over the antibody. So, even in the absence of unlabeled antigen, only some of the radioactive antigen will be associated with the antigen–antibody complex, whereas the remainder will be free in solution. In the radioimmunoassay, the total amounts of antibody and radiolabeled antigen are kept constant. The presence of unlabeled antigen will result in less of the labeled species being able to bind to the antibody. The greater the amount of unlabeled antigen (Ag) present, the lower the amount of radiolabeled antigen combined to the antibody (Ag*Ab). Thus, on suitable calibration, the amount of the unlabeled species can be accurately measured in terms of the amount of radioactivity associated with the antigen–antibody complex.

From: *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
Edited by: J M Walker

The widespread use of radioimmunoassays is the result of several significant advantages that the method has compared to many other quantitative assays, particularly for substances that are otherwise measured by pharmacological assays. Those advantages include very high sensitivity and high specificity.

A wide variety of exogenous and endogenous substances, including peptides, polypeptides, and proteins, can be measured in biological tissues and fluids at concentrations of pg/mL by the use of suitable radioimmunoassays. Such high sensitivity requires the use of antisera of high avidity and the use, as tracers, of antigens labeled to high specific radioactivities. The raising of antisera, and the radioiodination of peptides and proteins are dealt with in other chapters of this book. This chapter will concentrate on the other essential aspects of radioimmunoassay.

1.1. Incubation of Antigens and Antiserum

The incubation conditions have to ensure the stability of all reagents as antigen binds to antibody and allow equilibrium to be reached.

1.2. Separation of Free and Bound Antigen

When equilibrium has been achieved, the antigen bound to antibody is quickly and efficiently separated from free antigen, so that the radioactivity associated with either or both components can be counted. Many different separation procedures have been reported *(2)*. This chapter will deal with two that are widely used.

1.2.1. Fractional Precipitation with Polyethylene Glycol

The basis of the method is the ability of relatively low concentrations of polyethylene glycol to bring about the precipitation of antibody molecules, presumably by removal of the attendant hydration shell of water molecules, without the precipitation of the smaller antigen molecules *(3)*. The method is not efficient in all cases, but is worth trying because of its simplicity and very low cost. The procedure has been successfully used for many years in the author's laboratory in radioimmunoassays of both peptides, such as bradykinin *(4)* and enzymes, such as tissue kallikrein *(5)*.

1.2.2. Double (Second) Antibody Method

This procedure is very widely used and can achieve an efficient separation of free and bound antigen in more or less all radioimmuno-

assays. The basis of the most common type of this method is to use an antiserum (the second antibody), raised to antibodies of the antiserum (first antibody) employed in the incubation, to precipitate the antigen–antibody complex *(6)*. The addition of nonimmune γ globulin of the species in which the first antiserum was raised increases the bulk of material that can interact with the second antiserum and so enables a precipitate to be formed.

2. Materials

2.1. Incubation of Antigens and Antiserum

1. Antiserum: The antiserum to be used in the assay should be of high avidity, high titer, and high specificity for the antigen to be measured.
2. Radiolabeled antigen: Pure antigen must be used for labeling. Purification of the labeled species must also be carried out. Radioiodination is the method of choice for labeling peptides and proteins to high specific radioactivity (*see* Chapter 45).
3. Unlabeled antigen: Pure antigen is used as the standard in the assay. It is essential that the standard and the antigen to be measured show identical behavior toward the antiserum.
4. Buffer: Many different buffers can be used for radioimmunoassay. Whatever buffer is chosen must ensure the stability of all the reagents. In practice, in order to achieve equilibrium in a reasonable time, as well as maintain stability, the buffer normally employed has a pH within the range 6–8 and a molarity within the range 0.01–0.1*M*. For convenience, the same buffer can be used in both the incubation and the separation stages of the radioimmunoassay. A preservative such as sodium azide or thimerosal (0.01–0.1%) has to be included. Such proteins as bovine serum albumin or ovalbumin (0.1–1.0%) are normally added as a carrier.
5. Disposable plastic tubes: Tubes of various shapes and sizes can be used depending on the volume of the incubation mixture and the separation procedure employed.

2.2. Separation of Free and Bound Antigen

2.2.1. Fractional Precipitation using Polyethylene Glycol

1. Polyethylene glycol of mol wt 6000.
2. Bovine γ globulin (Cohn Fraction II).

2.2.2. Double (Second) Antibody Method

1. Antiserum to γ globulins of the species in which the first antiserum (i.e., that used in the incubation) was raised. Such antisera are available from several commercial sources.

2. Nonimmune γ globulin (Cohn Fraction II) or serum of the species in which the first antiserum was raised.

3. Practical Procedure

3.1. Incubation of Antigens and Antiserum

1. At the outset, the amount of labeled antigen to be employed in the incubation is chosen to be of the same order of magnitude as the smallest amount of unlabeled antigen that is required to be measured. In practice, the amount of tracer used is often simply that which gives a specified number of counts per min per unit volume (usually 5000–10,000 counts/min/100 μL).
2. The optimal dilution (titer) of the antiserum to be used in the assay is that which will bind 30–50% of the labeled antigen. That dilution is chosen with the aid of an antiserum dilution curve. The curve is constructed from the results of incubations, carried out at least in duplicate, of serial dilutions of the antiserum with the chosen amount of tracer. The incubations, for example, consisting of diluted antiserum (100 μL), tracer (100 μL), and buffer (200 μL), are allowed to proceed to equilibrium. The time required to reach equilibrium obviously depends on the particular circumstances of the assay. For a completely new radioimmunoassay, it may be necessary to run pilot experiments varying temperature from 4 to 37°C and time of incubation from 4 to 72 h to establish the optimal conditions. The bound and free forms of the radioactive labeled tracer are then separated and counted. A typical antiserum dilution curve is shown in Fig. 1. In that particular case, the dilution of antiserum to be used in the assay was chosen as 1:60,000 (i.e., final dilution 1:240,000), corresponding to 37% binding of the tracer. The slope of the dilution curve in its descending portion can be used as a measure of the avidity of the antiserum; the greater the slope, the greater the avidity.
3. Having chosen the amount of tracer and dilution of antiserum to be used, the radioimmunoassay can then be set up to measure the amount of antigen in unknown solutions with the aid of standard solutions of antigen.
4. Standard solutions of antigen are made by dilution of a master solution of accurately known concentration. The working solutions are made just prior to use and are kept at 4°C until required.
5. The tubes to be used in the incubation are numbered.
6. Buffer is added to all of the tubes apart from the first three tubes, which will used to measure the total counts in the assay. To each of those three tubes, which will be used to measure nonspecific binding, are added 300 μL of buffer. To each of the three tubes that will represent

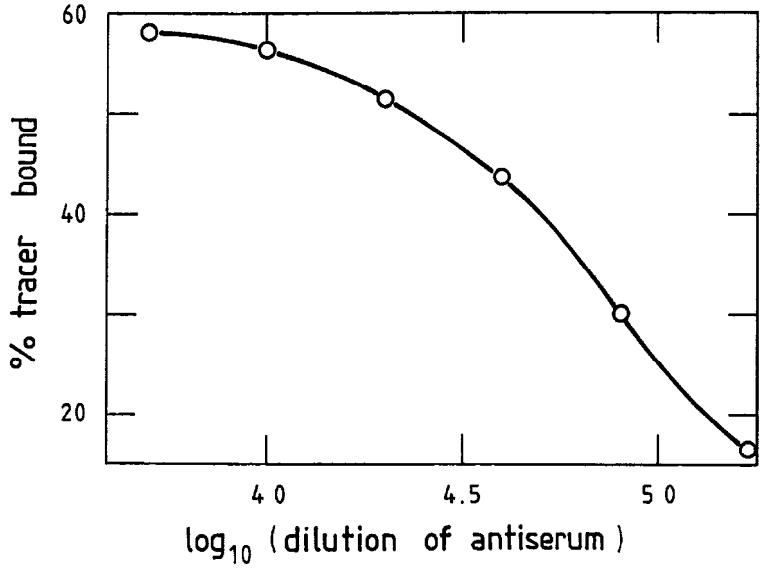

Fig. 1. Antiserum dilution curve. Radioiodinated kallikrein (100 µL; 7000 counts/min) was incubated with serial dilutions (1:5000 to 1:160,000) of antiserum (100 µL) to kallikrein from rat submandibular gland in a total vol of 400 µL for 20 h at 25°C. The bound antigen was separated from the free antigen by incubation at 37°C for 2 h with a solid-phase second antibody. The bound radioactivity was measured and was expressed as a percentage of the total radioactivity that was used.

the zero standard are added 200 µL of buffer, whereas 100 µL of buffer are added to every other tube. The standard solutions (100 µL) of antigen or unknown solutions are then added to the relevant numbered tubes in duplicate. Next, the antiserum (100 µL) at the chosen dilution is added to all tubes except for the three tubes to be used to measure total counts and the three tubes to be used to measure nonspecific binding. Finally, the radioactive tracer (100 µL) is added to every tube. The contents of each tube are thoroughly mixed with the aid of a vortex mixture. Each tube is then left at a constant temperature until equilibrium is reached (often 24–48 h at 4°C). Many variations to the described procedure are possible (*see* Notes 2–5).

7. When equilibrium has been reached, the free and bound antigen are separated.
8. Although it is possible for either or both phases to be counted, it is normal for the radioactivity associated with the antibody to be measured. There should be good agreement between the counts of each tube in a particular pair or set of three.

9. The counts associated with the three tubes representing nonspecific binding are averaged and, in turn, are subtracted from the average counts for each set of tubes.
10. The resultant, average specific counts associated with the three zero standard tubes can be expressed as a percentage of the average specific counts of the three tubes containing just tracer. If the conditions of the assay have been satisfactory, the counts of the zero standard should be 30–50% of the total count of tracer.
11. If so, the average, specific counts of each set of duplicates can then be expressed as a percentage of the average specific counts of the three zero standards.
12. A standard curve is constructed from the calculated results (*see* Note 1).

3.2. Separation of Free and Bound Antigen

3.2.1. Fractional Precipitation Using Polyethylene Glycol

1. A solution of polyethylene glycol 6000 (20%) and potassium iodide (6.25%) is prepared in 0.1*M* sodium phosphate buffer containing 0.9% sodium chloride and 0.01% sodium azide, final pH 7.4, with thorough mixing. A separate solution (1.4%) of bovine γ globulin is made in the same buffer.
2. To each of the tubes requiring separation of bound and free antigen is added at 4°C γ globulin solution (200 µL) and polyethylene glycol/potassium iodide solution (1 mL). Each tube is vortexed and is allowed to remain at 4°C for 15 min (*see* Note 6).
3. Each tube is then centrifuged at 4°C at 5000*g* for 30 min.
4. The supernatants are carefully removed by aspiration at a water pump. The precipitate at the bottom of each tube is then counted. If necessary, the precipitates can be washed at 4°C with the polyethylene glycol/potassium iodide solution (800 µL) followed by vortexing and centrifugation (*see* Note 7).

3.2.2. Double (Second) Antibody Method

1. First, the optimal dilution of the second antiserum has to be carefully assessed. Serial dilutions of the second antiserum are made in 0.05*M* sodium phosphate buffer, pH 7.4, containing 0.5% nonimmune serum and 50 m*M* EDTA.
2. Samples containing tracer (100 µL), first antiserum at optimal dilution (100 µL) and buffer (200 µL) are allowed to come to equilibrium at constant temperature (*see* Notes 8 and 9).
3. Aliquots (100 µL) of the different dilutions of the second antiserum are added. The tubes are vortexed and are kept at constant temperature

for a further 18–24 h. The tubes are then centrifuged at 5000*g* for 30 min. The supernatants are aspirated at a water pump, and the tubes are counted.

4. The highest dilution of the second antiserum that results in the precipitation of a maximum percentage of the tracer represents the minimum amount of second antiserum that should be employed in the separation.
5. Aliquots (100 µL) of the chosen dilution of the second antiserum are added to each of the sample tubes requiring separation of free and bound antigen. The tubes are then treated as in step 3 (*see* Notes 10 and 11).

4. Notes

1. The purpose of a radioimmunoassay is to measure the concentration of a particular antigen in a unknown sample by comparison with standard solutions of the antigen. From the measurements of the bound and free radioactivity in the presence of various known amounts of antigen is constructed a standard curve. The amount of antigen in the test sample is then found from that curve by sample interpolation. The standard curve can be represented in several different ways. One straightforward method is to plot the proportion of tracer bound expressed as a percentage of that in the zero standard against the corresponding concentration of standard antigen (*see* Fig. 2). However, to avoid the problems associated with the subjectivity of drawing a curve, it is better to construct a linear plot. One such linear transformation that Fig. 3 is very widely used is shown in Fig. 3, where the logit of the tracer bound (y) is plotted against log concentration.

$$\text{logit } y = \log_e \left(\frac{y}{100 - y}\right) \text{ and} \tag{2}$$

$$y = \frac{B - N}{B_O - N} \times 100 \tag{3}$$

where B = counts associated with a certain concentration of antigen, B_o = counts associated with the zero standard, and N = counts associated with nonspecific binding. Often the logit plot becomes nonlinear near its extremes, and in that case, the points in those regions are not employed in construction of the straight line.

2. There are a very large number of variations to the procedure outlined for incubation of antigens and antiserum. The best set of conditions for a particular case can be worked out by trial and error. Of particular importance is the volume of the unlabeled antigen in the incubation mixture. In theory, an increase in that volume relative to the other components should lead to an increase in sensitivity of the assay, i.e.,

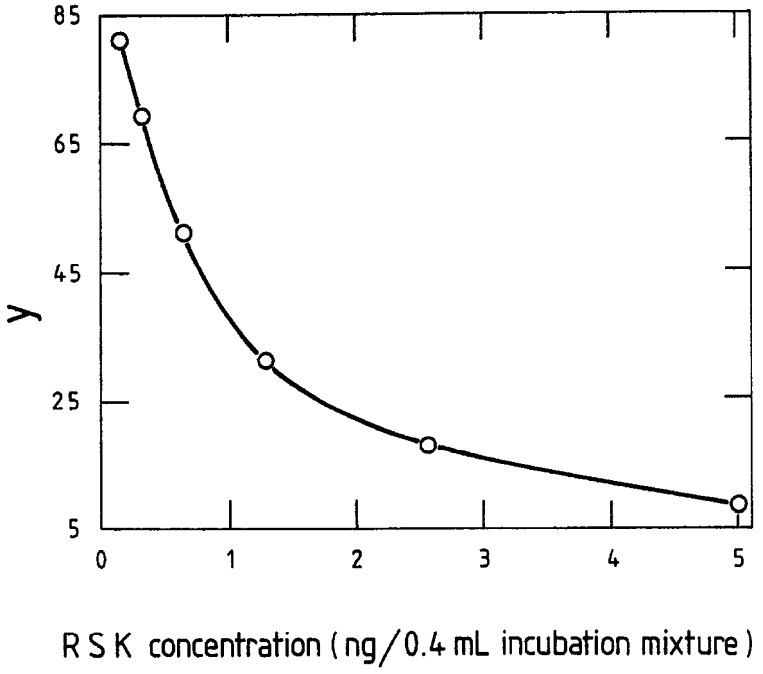

Fig. 2. Standard curve for radioimmunoassay. Radioiodinated kallikrein (100 µL; 7000 counts/min) was incubated with standard solutions of kallikrein from rat submandibular gland (200 µL containing 0.16–5.12 ng kallikrein) and antiserum to the enzyme (100 µL of 1:60,000 dilution) for 20 h at 25°C. The bound radioactivity was separated from the free radioactivity by use of a solid-phase second antibody on incubation for 2 h at 37°C and was counted.

$$y = \frac{B - N}{B_o - N} \times 100$$

B = counts associated with a certain concentration of antigen, B_o = counts associated with the zero standard, and N = counts associated with nonspecific binding.

detection of a lower amount of antigen. In practice, there may actually be a decrease in sensitivity owing to interference in the antigen–antibody binding by other substances present in the sample.

3. Other factors that often increase the sensitivity of the assay include:
   a. "Late" addition of the tracer. The labeled antigen is added a considerable time after the unlabeled species and antiserum have been allowed to interact, but before the attainment of equilibrium.

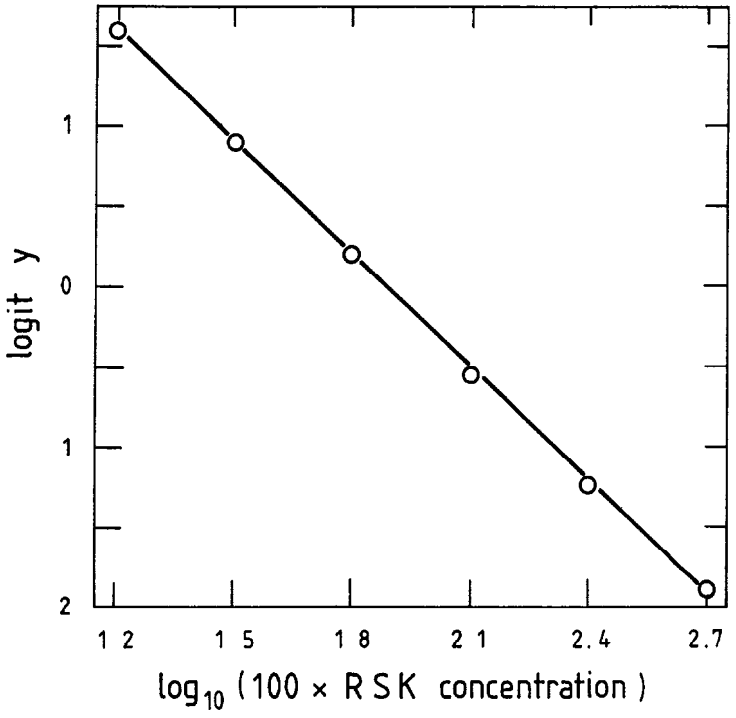

Fig. 3. Logit-log plot of the standard curve. For details, *see* legend to Fig. 2.

$$\text{logit } y = \log_e \left(\frac{y}{100 - y}\right)$$

b. Decrease in the amount of antiserum. However, a limit will be reached beyond which a further reduction in antiserum will be counterproductive because of loss of precision of the assay.
c. Decrease in the amount of tracer. Again, there is a limit beyond which a further reduction will produce no significant change in sensitivity. It should be noted, however, that very high sensitivity may not be required of a radioimmunoassay. What is far more important is that the assay should be able to measure accurately, precisely, and reproducibly antigen in the range of concentrations found in biological tissues and fluids, preferably without the need for sample dilutions. However, the following note should also be borne in mind.

4. One major problem of radioimmunoassay, particularly when applied to undiluted biological samples, is that substances in the samples interfere with antigen–antibody interaction. High concentrations of salts

and plasma proteins decrease antigen–antibody binding. Thus, it may be necessary to dilute the samples to reduce such effects or to include such substances in the standard antigen solutions.

5. Another problem may be encountered if the biological samples contain proteolytic enzymes, which will degrade the antigen. In those cases, it is necessary to include a broad-spectrum inhibitor in the assay, e.g., aprotinin.
6. Precipitation by polyethylene glycol is very sensitive to fluctuations in temperature. Hence it is essential to keep all solutions and tubes at 4°C during the whole procedure.
7. One problem that is often associated with fractional precipitation using polyethylene glycol is the occurrence of significant nonspecific binding, i.e., high blanks. The inclusion of potassium iodide in the polyethylene glycol solution has been found to reduce precipitation of free antigen *(3)*. Washing of the precipitate with polyethylene glycol/potassium iodide solution may further reduce the non specific binding.
8. When carrying out the double (second) antibody method, normal serum or nonimmune γ globulin from the species in which the first antiserum was raised can be included in the incubation mixture, rather than be added with the second antiserum. However, the presence of components of the complement system in serum samples can affect the formation of the immune precipitate. Thus, it is usual to include EDTA (0.05–0.1*M*) in the incubation and separation buffers to inactivate those components and avoid any such difficulties.
9. If the concentrations of reactants are low, it can take a considerable time for complete immunoprecipitation to occur. The use of higher amounts of γ globulin or serum will greatly speed up the process, but, of course, higher amounts of the second antiserum will also be needed.
10. Many of the difficulties associated with the double antibody method can be avoided through the use of a second antibody that is covalently linked to a suitable, inert, solid matrix. Because of the solid phase nature of such a second antibody, immune precipitation occurs relatively quickly, and a carrier nonimmune γ globulin is not required. Of course, considerable effort, second antiserum, and a suitable solid support are necessary to produce the solid phase second antiserum *(7)*. Certain preparations are commercially available but are expensive, e.g., antibodies, raised in sheep against rabbit γ globulins, coupled to cellulose or antibodies, raised in goat against rabbit γ globulins, coupled to polyacrylamide.

11. One particularly useful modification of the double antibody method is to include a small amount of ammonium sulfate or polyethylene glycol with the second antiserum *(8)*. The inclusion of the chemical precipitant speeds up the immune precipitation and enables a lower amount of second antiserum to be used.

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CHAPTER 47

Enzyme-Linked Immunosorbent Assay (ELISA)

Denis J. Reen

1. Introduction

The ELISA technique *(1)* is a simple, sensitive, rapid, reliable, and versatile assay system for the quantitation of antigens and antibodies. Because of the extreme discriminating power of antibodies to recognize an almost infinite array of antigenic structures, the application of ELISA to analyte measurement is almost unlimited. ELISAs have been developed in many configurations depending on the particular application of the assay. In practice, ELISA as a solid-phase technique may be classified into two main types: (1) competitive assays using either an antigen–enzyme conjugate or an antibody–enzyme conjugate, and (2) noncompetitive assays using a double antibody "sandwich" technique where the second antibody has an indicator enzyme conjugated to it. In solid-phase ELISA, one of the immunoreactants (antibody or antigen) is immobilized onto a solid support (microtiter plate) by adsorption, through noncovalent interactions. The immobilized antibody is then incubated with test solution containing the analyte of interest. Following a period of incubation and washing, the bound antigen is detected, by the addition of an enzyme-conjugated antibody that binds to the remaining antigenic sites on the antigen. Following further incubation and washing, the amount of antigen present is visualized by the addition of a chromogenic or fluorogenic substrate, depending on the sensitivity

From: *Methods in Molecular Biology, Vol. 32: Basic Protein and Peptide Protocols*
Edited by: J. M. Walker

required. The sensitivity of any immunochemical procedure depends on the amplification system used to detect the specific interaction between antigen and antibody. ELISA relies on the production of color produced by an enzyme-labeled antibody or antigen, following hydrolysis of a chromogenic substrate. Since one molecule of an enzyme can catalyze the hydrolysis of many substrate molecules, a direct and simple, high-amplification system is achieved. The concentration of the substrate product is directly proportional to the concentration of standards used in the assay, as well as the analyte antigen. The choice of ELISA system is determined primarily by the specificity and purity of available antibodies or antigens. When two purified monoclonal antibodies with specificities for different epitopes on the antigen are available, the method of choice is the "sandwich"-type ELISA. This is also the case with having one or two purified polyclonal antibodies. In the case of having only a single antigen-specific polyclonal antibody, the antibody can be directly conjugated to an enzyme to form the top layer of the "sandwich," while using the unlabeled antibody as the coating reagent. In other conditions, a competitive assay employing either a labeled antigen or antibody is the method of choice. The ELISA method described here is the noncompetitive double antibody "sandwich" method (*see* Fig. 1) using an alkaline phosphatase detection system. Depending on the affinity of the antibodies available, the limit of detection for protein antigens can be as low as 10 pg/mL.

2. Materials (for Noncompetitive Assays)

1. 96-well flat-bottomed polystyrene microtiter plates.
2. Adjustable micropipets, 0–20, 20–200, 200–1000 µL.
3. Multichannel pipet 0–250 µL (eight channel).
4. Plate washing device: Automated plate washer; manual hand-held pipet connected to a reservoir; wash bottle or multichannel pipet.
5. Manual or automated microplate ELISA reader.
6. 0.1*M* Carbonate/bicarbonate buffer, pH 9.6: Make up 0.1*M* solutions of Na_2CO_3 (10.6 g/L) and $NaHCO_3$ (8.4 g/L), then mix 29.3 mL 0.1*M* Na_2CO_3 with 70.7 mL 0.1*M* $NaHCO_3$. Note: The bicarbonate solution is unstable and should not be kept for more than 1 wk.

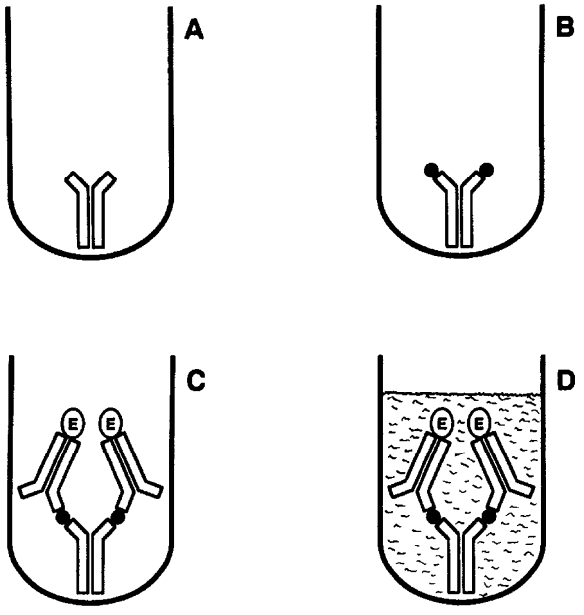

Fig. 1. Schematic representation of ELISA technique (noncompetitive assay). (A) Solid-phase adsorbed antibody on microwell plate. (B) Formation of antigen–antibody complex. (C) Detecting enzyme-labeled second antibody combining with "solid-phase" antigen "sandwich." (D) Addition of substrate results in color development.

7. PBS-Tween: Make up 0.5*M* phosphate-buffered saline (PBS), pH 7.2. Weight out 8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, and 0.2 g KH_2PO_4. Dissolve in 1 L distilled H_2O. Then add 0.5 mL Tween 20.
8. 10% Diethanolamine buffer, pH 9.8: Dilute 9.7 mL diethanolamine with 80 mL distilled H_2O and adjust to pH 9.8 with 1*M* HCl. Make up to 100 mL with distilled H_2O (stable at 4°C).
9. Coating antibody: IgG fraction or affinity-purified polyclonal or monoclonal antibody, specific for the analyte (commercial sources or laboratory prepared). (*See* Chapter 43.)
10. Enzyme-conjugated antibody: Alkaline phosphatase conjugated IgG specific for the analyte (commercial sources or laboratory prepared; *see* Chapter 44).
11. Reference reagent: A pure preparation of the analyte, for setting up a reference curve.

12. Substrate: *p*-nitrophenyl phosphate (1 mg/mL) in 10% diethanolamine buffer, pH 9.8.

3. Method

1. Dilute the coating antibody to 10 µg/mL protein in 0.1*M* carbonate/bicarbonate buffer, pH 9.6 (*see* Note 1).
2. Add 100 mL of diluted antibody to each well of the microtiter plate. Cover the plate with plate sealer, and incubate overnight at 4°C.
3. Aspirate each well of the plate, and wash with three to five quick changes of PBS-Tween (fill each well). After the last wash, blot the plate against clean paper toweling to remove any remaining wash buffer (*see* Note 2).
4. Add 100 mL of each dilution of standard antigen reagent, in triplicate, to the plate. Doubling dilutions of standard reagent are made up freshly, usually in the range 10–1000 pg/mL (*see* Note 3). Add 100 µL of appropriate dilutions of test antigen in triplicate, diluted in PBS-Tween, to the plate. PBS-Tween without added antigen can be used as a negative control. Cover the plate, and incubate at room temperature (approx 20°C) for 2 h to allow antigen binding to the antibody-coated plate to occur (*see* Note 8).
5. Empty and wash the plate as in step 3 above. Add 100 µL of optimally diluted enzyme-conjugated antibody (approx 1/1000 dilution) to each well (*see* Note 1). Cover the plate and incubate at room temperature for 2 h (*see* Note 6).
6. Empty and wash plate, as described in step 3. Add 100 µL *p*-nitrophenyl phosphate solution. Cover the plate and incubate at room temperature for 30 min.
7. If required, the reaction can be stopped by adding 100 µL 3*M* NaOH to each well.
8. Read the absorbance at 405 nm (*see* Note 10.)
9. A standard curve of absorbance vs $\log_{10}$ antigen concentration is constructed, and the concentration of the antigen in the test sample is read off the standard curve (*see* Note 4).

4. Notes

1. The optimum concentration of coating antibody and enzyme-conjugated antibody should be determined in advance, by a series of reagent combination experiments. This is best carried out by a checkerboard-type titration experiment. Doubling dilutions of both coating antibody and detecting antibodies are set up according to the experimental conditions of the test system. An "ideal" antigen concentration (e.g., 100

ng/mL) which would be expected to be within the sensitivity range of the assay, is included in the test. The optimal concentration for each antibody is the combination for each antibody giving maximum signal. Where a double antibody system is used to detect the bound antigen to the antibody-coated plate, a titration experiment should be carried out to determine the optimum concentration of each of the two antibodies in the detection system.

2. Antibody-coated plates can be stored, after thorough washing and drying, for 7–14 d at 4°C, provided the plate is well sealed.
3. Tween 20 is added to PBS in the washing solution and the reagent diluent to minimize nonspecific protein binding to the solid surface. It is sometimes necessary to add 1% protein (bovine serum albumin [BSA]) to the PBS-Tween buffer to reduce nonspecific binding of the enzyme–antibody conjugate or sample further to acceptable levels. The coating antibody can be either a monoclonal or polyclonal antibody.
4. Where the "background signal" remains unacceptably high after the addition of BSA to the diluent and washing buffers, a "blocking" step may be introduced after Section 3., step 3 as follows: Add 100 µL PBS-Tween–1%BSA to each well. Cover the plate and incubate at room temperature for 1 h. Empty and wash the plate as described in Section 3. Continue on to Section 3., step 4.
5. If a suitable quality antibody is available, it is advisable to use a $F(ab)_2$ fragment of both the coating and the enzyme-labeled antibody, in order to minimize crossreactivity of rheumatoid factors in biological fluids, with the Fc portion of IgG.
6. Where only a nonenzyme-conjugated detecting antibody is available, an extra step can be introduced to detect antigen. Following incubation with the nonconjugated antibody, the plate is emptied and washed as in Section 3., step 3. One hundred microliters of optimally diluted (*see* Note 1) alkaline phosphatase conjugated antispecies antibody (specific for the second antibody, which must be different from the species of the coating antibody) are added to each well and incubated at room temperature for 2 h.
7. When monoclonal antibodies are used, the epitope specificity of the coating antibody must be different from that of the antigen conjugated antibody detection system. Although this is also preferable when using polyclonal antibodies, it is possible to obtain perfectly acceptable data using the same polyclonal antibody on both sides of the "sandwich."
8. Duration of incubation times with test solutions, antibody–enzyme conjugate, and substrate may be reduced or extended to achieve maximum sensitivity, depending of the quality of both antibodies.

9. Because of the extreme sensitivity of the technique, particular care should be taken with pipeting techniques, dilution steps, preparation of standard curve reagents, and so forth. Make sure all pipets are precisely calibrated and that the quality of tips is sufficiently high.
10. If a dual-wavelength ELISA plate reader with wavelength correction is available, set it to 540 nm. This will correct for optical interference owing to imperfections in the microtiter plate. Readings made directly at 405 nm can be used, but will be higher than those using the wavelength correction factor.

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CHAPTER 48

Free Zone Capillary Electrophoresis

David J. Begley

1. Introduction

Electrophoresis within silica capillaries is a relatively new technique, with the first publications appearing in the 1970s followed by a rapid expansion and establishment of the technique in the 1980s. Commercial machines for the application of capillary electrophoresis appeared in the late 1980s and now a number of machines are manufactured.

All of the commercially available machines function in essentially the same way, although there is of course individual variation. This account of capillary electrophoresis is tailored not to any particular machine, but is written in a manner that describes the basic theory and method in a way that can be easily varied to suit a particular application. This chapter contains a detailed description of a capillary electrophoresis apparatus and some theory of the operation, together with protocols and notes for performing separations.

High-performance capillary electrophoresis (HPCE) can be used to separate a wide variety of solutes, both charged and uncharged, and is particularly suited to the separation of small peptides and proteins. Separations can be performed under a variety of conditions with free zone capillary electrophoresis (FZCE), where the solutes being separated are in simple solution in buffer. A variant of the technique is capillary isoelectric focusing (CIF), where the ampholyte and sample are premixed and loaded into the capillary before application of a voltage. A pH gradient is formed, and separation of samples according to their isoelectric point (pI) takes place within the capillary.

From: *Methods in Molecular Biology, Vol. 32. Basic Protein and Peptide Protocols*
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This chapter will be essentially confined to FZCE separations, since these have found the greatest application in the biological sciences *(1–6)*.

Capillary electrophoresis is normally carried out in fused silica capillaries with internal diameters between 10 and 100 μm. Capillary length may be varied with a longer capillary producing a greater spatial separation between two solutes of similar mobility. A popular capillary for a variety of separations would be one of 25-μm internal diameter and 20 cm in length. A potential difference is normally applied across the two ends of the capillary with a power source. Electrophoresis is usually conducted under constant voltage conditions with the current finding its own level. Typical operating parameters under differing conditions are shown in Table 1. Separation under normal conditions produces significant amounts of heat, but this is not usually a problem that affects the quality of a separation or leads to denaturation of protein, since the large surface area of a capillary in relation to its volume efficiently dissipates the heat produced. Power sources for use in capillary electrophoresis normally supply voltages up to 30 kV or more.

1.1. Apparatus and Theory of Operation

A typical layout for a capillary electrophoresis apparatus is shown in Fig. 1. The apparatus consists of the silica capillary, which is usually coiled to make it more manageable. The ends of the capillary project into two buffer reservoirs that contain silver electrodes. A facility for changing the polarity of these two electrodes usually exists, although for the majority of applications, the left-hand electrode on the figure will be the anode and the right-hand the cathode.

When a potential difference is applied across the ends of the capillary and because the walls of the capillary have a standing charge (the ζ potential), an electroendo-osmotic flow of water is produced from anode to cathode. This is shown diagrammatically in Fig. 2. Thus, migration of a positively charged solute from anode to cathode along the capillary is partially produced by the voltage gradient applied and partly the result of electroendo-osmotic flow. This electroendo-osmotic flow makes it possible to achieve the separation of uncharged solutes, such as steroids, in a silica capillary. With uncharged solutes, the electroendo-osmotic flow of water provides the

Table 1
Operating Parameters for Capillary Electrophoresis under Differing Conditions[a]

Buffer	pH	Voltage, kV	Current, μA	Resistance, Meg Ω	Power, watts
Phosphate 100 m*M*	2.5	8.0	12.6	634.9	0.101
Phosphate 100 m*M*	4.4	8.0	25.25	316.8	0.202

[a]Note that the resistance approximately halves with the change in pH, and the current and the power dissipation (heat production) double.

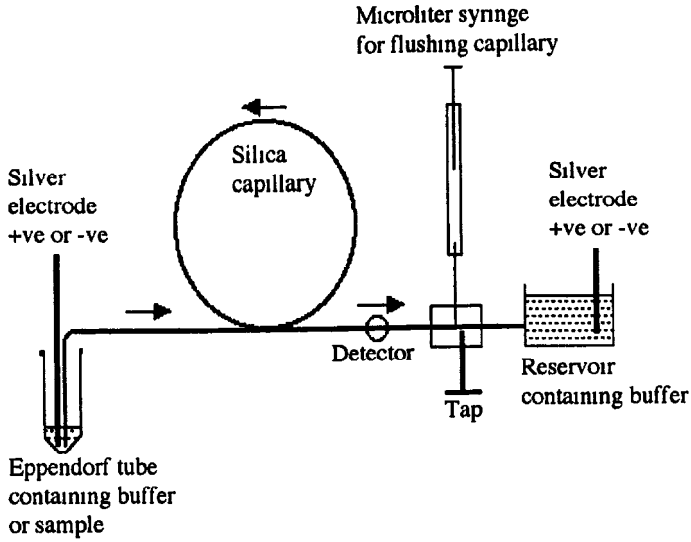

Fig. 1. A typical layout for a high performance capillary electrophoresis apparatus, (HPCE). For electrophoretic loading, an Eppendorf tube containing the sample is offered up to the left-hand electrode, and the capillary and a loading voltage applied for a set time. With suitable Eppendorf tubes, the sample contained in loading buffer may be as little as 10 μL. The tube containing the sample is then substituted with one containing the running buffer and the run conducted. After the run, the tap can be closed and the capillary back-flushed with running buffer between runs. At pH 2.5 with positively charged analyses, the left-hand electrode will normally be the anode and the right-hand electrode the cathode.

propulsive force, and solutes of similar molecular weight are separated by a combination of differences in their mass and also partly by a chromatographic interaction with the capillary wall. For a charged species, the apparent rate of migration (U_{app}) is the product of the

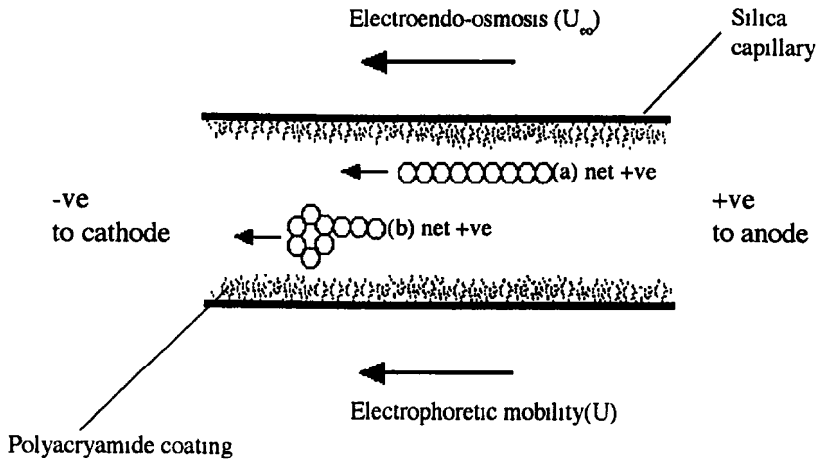

Fig. 2. Migration of solute in the silica capillary The ζ potential on the wall of the capillary produces an electroendo-osmotic flow (U_{eo}) of water from the anode to cathode. Coating the capillary internally with polyacrylamide reduces this standing charge and reduces the magnitude of U_{eo}. The electrophoretic mobility of a charged solute (U) is dependent on the mass/charge ratio and the applied potential difference. Thus the actual rate of migration (U_{app}) is equal to $U + U_{eo}$ With coated capillaries, the degree of electroendo-osmosis (U_{eo}) is minimized For two solutes of equal charge and mass, such as (a) and (b) The configuration of the molecule can also influence the rate of migration (U) with (b) exhibiting a greater molecular sieving and/or chromatographic effect than (a) (*See also* Fig 4)

electrophoretic mobility (U), plus the rate at which the solute is propelled by the electroendo-osmotic flow (U_{eo}), and is related to the mass/charge ratio of the solute (Fig. 2).

Electroendo-osmosis can be greatly reduced by coating the inner surface of the capillary with a thin layer of polyacrylamide. This layer of polyacrylamide abolishes the ζ potential and reduces the electroendo-osmotic flow to virtually zero. Under these conditions U_{app} and U are virtually identical, and the mobility of solutes in the electrical gradient is a product of the mass charge ratio. Highly charged solutes with a small mass exhibit the greatest mobility. The separation of charged solutes is improved in coated capillaries, because when there is fluid flow in the capillary, the solute front is elliptical in shape as a result of laminar flow in the capillary, whereas when the propulsive force is purely the electrical gradient the solute is migrating

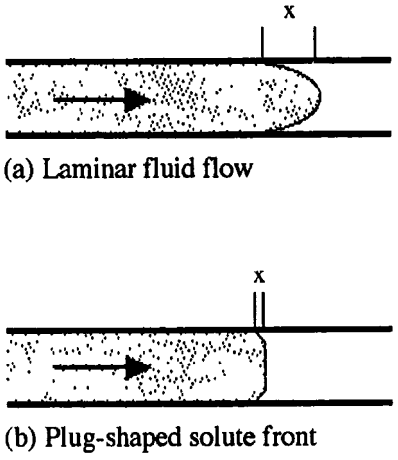

Fig. 3. Factors influencing the profile of the solute front. (a) When there is solvent movement through the capillary as a result of the electroendo-osmotic flow of water, a laminar flow profile is set up where the solvent near the wall of the capillary moves more slowly as a result of solvent drag. (b) In coated capillaries where the solutes are migrating through the solvent down an electrical gradient, this effect is minimized and the solute front is plug-shaped, and thus produces a sharp well-defined zone (x)

through the solvent and the solute front is "plug" shaped (Fig. 3). This means that solute spread is minimized and peaks produced by the detector are sharper. Coating of the capillaries also reduces any nonspecific adsorption of proteins onto the capillary wall.

The detector usually comprises a beam from a deuterium lamp (190–380 nm; UV) or tungsten lamp (380–800 nm) that is focused through the capillary. The wavelength can be selected via a holographic monochromator to give a bandwidth of approx 5 nm. A typical wavelength setting for the detection of peptides and proteins would be 200 nm. The transmitted light is then detected, and the signal amplified to give typical full-scale deflections of 1.0–0.005 AUFS on a chart recorder. Fluorescence, conductivity, and electrochemical methods of detection are all possible with capillary electrophoresis, but UV absorption is most commonly used.

Almost any buffer that does not have an excessive absorption of light at the chosen wavelength for detection can be used. For a buffer of a given pH, if the solute has a net positive charge, migration will

be from anode to cathode, the rate of migration for a given molecular mass being proportional to the charge. Thus, if the buffer pH is close to the isoelectric point (pI) of the solute in question, the rate of migration through the capillary will be slower. This effect can be used to advantage to separate similar molecules with similar isoelectric points, for example, isoforms of a protein or enzyme. A buffer with a pH close to the pI of a solute, in this manner, accentuates the relative differences in mobility of solutes with a similar pI with respect to their elution time. The time taken for the solute to move through the capillary to the detector is called the elution time (T_e), and depends on the mass/charge ratio under the conditions of separation and the length of the capillary and the voltage gradient, (i.e., the driving force through the capillary). Figure 4 illustrates the powerful resolution that can be obtained with FZCE. Typical elution times for some proteins in relation to their physical characteristics are shown in Table 2.

The sample can be introduced into the capillary by:

1. Displacement loading (sometimes called vacuum or pressure injection);
2. By electrokinetic loading (sometimes called electroendo-osmotic loading); and
3. Electrophoretic loading.

With displacement loading, the sample is injected directly into the capillary via a suitable manifold or drawn into the capillary by applying a negative pressure at the far end. Both of these methods suffer from problems with reproducibility. Electrokinetic loading is achieved with uncoated capillaries, and the sample is moved into the capillary by applying a potential difference that induces an electrophoretic movement of the solutes and an electroendo-osmotic flow. It is a combination of these two effects that moves sample into the capillary. Only with coated capillaries is pure electrophoretic loading achieved, where the electrophoretic mobility alone moves the solute into the capillary. It is important to appreciate that the amount of solute that moves into the capillary in a given time and, with a given potential difference, is proportional to the electrophoretic mobility of that solute under the loading conditions. Thus, with a mixture of solutes of differing electrophoretic mobilities more of the more mobile solute is loaded relative to the solutes of lesser mobility. With electrokinetic

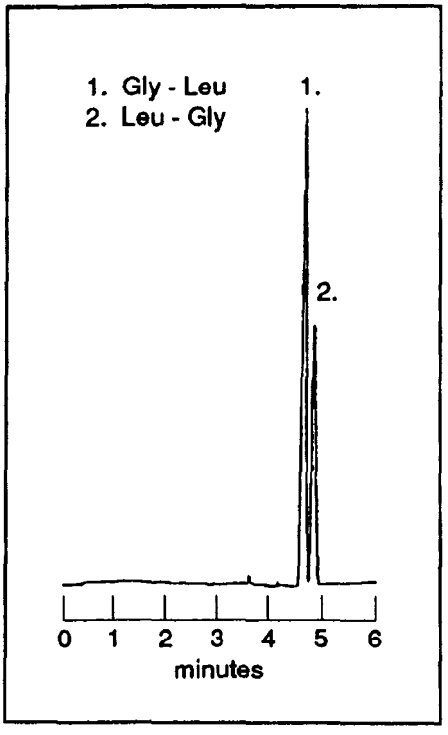

Fig. 4. Separation of two dipeptides glycyl-L-leucine and leucyl-glygine. These two peptides can be separated with free zone capillary electrophoresis. Their slightly different configurations allow separation to be achieved by a combination of molecular sieving and a chromatographic interaction with the walls of the capillary. Sample: Mixture of glycyl-L-leucine and leucyl-glycine, 25 μg/mL Buffer: Phosphate/HCl, pH 2.5. Load· 10 m*M* buffer, 4 kV, 4 s, 2.2–2.6 μA. Run: 100 m*M* buffer, 8 kV, 13.8 μA. Detection: 200 nm.

Table 2
Elution Time, T_e, for Some Proteins at pH 4 4

Protein	T_e	Mol wt, Daltons	pI	Mean radius, nm
IgG	8.4	150,000	6.4–8.9	5.34
Transthyretin (prealbumin)	10.2	61,000	4.7	3.25
Albumin	15	69,000	4.8–4.9	3.58

loading, this effect is reduced and, with displacement loading, does not occur. The protocols that follow assume electrophoretic loading with coated capillaries.

2. Materials

1. Buffer: For the separation of peptides and proteins, a typical buffer would be 100 m*M* phosphate/HCl buffer of pH 2.5. At this pH, all proteins and peptides will have a net positive charge and will migrate from anode to cathode. The buffer should contain 0.1% hydroxypropylmethylcellulose (HPMC). This has the effect of protecting the polyacrylamide coating of the capillary and also increases the viscosity of the buffer, which slightly retards the movement of solute through the capillary and accentuates separation in relation to mass and shape. To make this buffer, first dissolve sufficient HPMC in HPLC-grade water in a volumetric flask. This is a slow process. Do not warm the solution excessively, since this degrades the HPMC. Then add sufficient sodium di-hydrogen phosphate for a 100 m*M* solution, adjust the pH to 2.5 with dilute HCl, and bring to volume. This buffer will store in the refrigerator at 4°C for a week or so, but check the pH and look out for the growth of bugs!
2. Sample: Dissolve the protein or peptide or a mixture of proteins or peptides to be separated in a sample of 10 m*M* phosphate buffer, pH 2.5 (i.e., one-tenth of the concentration of the separating buffer). A sample concentration of 50 µg/mL is a good starting point. This is assuming that the sample that you wish to separate is available in crystalline form in a bottle. Biological samples present a little more difficulty and often need special attention (*see also* Note 1).

3. Methods

3.1. Loading the Sample

To load the sample electrophoretically, an Eppendorf tube containing the sample, which may be as little as 10 µL in a suitably sized tube, is offered up to the cathode and capillary tip. A potential difference, for example, 8 kV, is applied for 8 s, and the sample is loaded. In a mixture, the amount of each solute loaded into the capillary is proportional to the electrophoretic mobility, the loading time, and the voltage applied, and is thus proportionally different for each solute. This is shown graphically for bovine serum albumin (BSA) in Fig. 5. Solutes will have a greater mobility in a buffer of lower ionic concentration than the running buffer. Hence, the loading buffer should

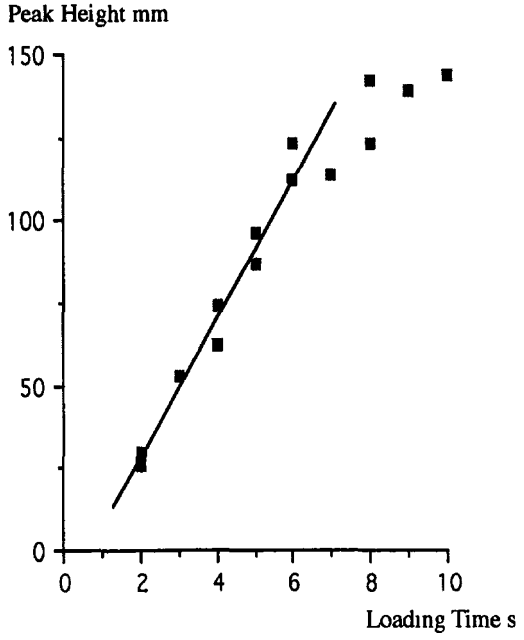

Fig. 5. Effect of time on electrophoretic loading and peak height for bovine serum albumin. Sample: Bovine serum albumin, 50 μg/mL (Sigma, A7888) Buffer: Phosphate/HCl, pH 2.5. Load: 10 m*M* buffer, 8.0 kV, 11.8–12.6 μA. Run: 100 m*M* buffer, 7.0 kV, 8.8–11.2 μA. Detection wavelength: 200 nm. Note that the amount of BSA loaded declines with longer loading times.

be 10 m*M*, and the running buffer 100 m*M*. Thus, a greater difference between the ionic concentration of the running and loading buffer will produce a greater loading of the sample. With electrophoretic loading, this phenomenon also has the useful effect of also causing the solutes to slow down once they enter the more concentrated buffer at the start of the capillary. This results in a "stacking" of the solutes at the beginning of the capillary (*see* Fig. 6) and means that all of the solutes are starting their migration essentially from the start of the capillary. The end result is a better resolution of the individual peaks in a sample, since the zone concentrations may be increased by a factor of ten. This does not occur with electrokinetic or displacement loading, where a substantial length of the capillary will contain the sample, prior to the run. For a particular sample, the experimenter must vary the loading current and loading time until an optimal load-

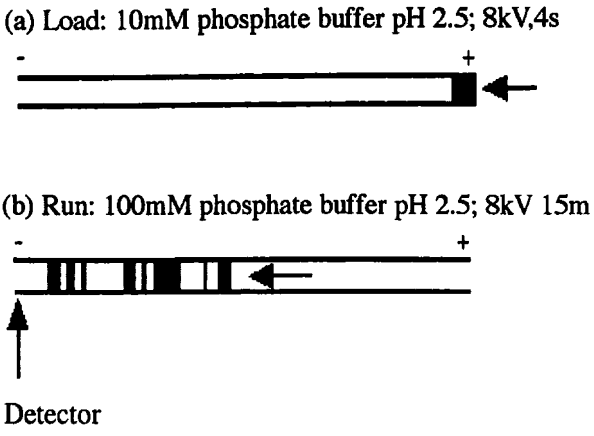

Fig. 6 Loading and running the separation. (a) When the sample is loaded from 10 m*M* buffer into a capillary containing 100 m*M* buffer, the effect is to stack the sample at the start of the capillary. (b) When the separating voltage is applied, the positively charged solutes in the mixture migrate toward the cathode at a rate predominantly determined by their mass-charge ratio to form discrete zones that are detected as absorbance peaks at the detector.

ing and separation of the solutes is achieved. For electrophoretic loading a good starting point for a peptide or protein is 8 kv for 8 s and to then vary first loading time and then voltage until loading is optimized. A voltage of 8 kV with a 25 μm × 20 cm capillary with pH 2.5 phosphate buffer will give a loading current of approx 11–14 μA. The current should be repeatable from sample to sample, when loaded under the same conditions.

3.2. Running the Separation

The run can be initially carried out at the same potential difference as the loading voltage. The run should be carried out with a constant voltage set on the power supply and, at 8 kV, will give approximately the same current as the loading conditions (*see* Table 1). Elution times for different solutes of course vary widely according to mass and charge. However, few peaks yielding useful information will elute after 25 min or so, as a result of zone spreading and distortion of the peaks. The voltage can be increased to speed the migration of slow peaks, or reduced, to slow down fast-moving solutes and improve the resolution between peaks with a similar T_e. Small peptides with mol

wt of 1 kDa or less usually elute within 15 min, whereas a complex biological sample may still be producing peaks after 20 min or so. Careful experimentation with the separating voltage must be conducted to optimize the separation. In reality, this means a careful balance between the loading conditions and the separating conditions to produce the best separation of the solutes of interest. In a complex mixture, as with the technique of high-performance liquid chromatography (HPLC), these conditions are often a compromise, and it proves impossible to separate all of the contents of a complex mixture with equal resolution.

3.3. Quantifying the Results

The best method for determining the quantity of material represented by a peak on the chart recorder is to measure the peak height. Peak area with HPCE techniques is not reliable as a measure of the quantity of solute, since a rapidly migrating solute will pass the detector quickly and produce a narrow peak, and a slowly moving solute will produce a wider peak. Thus, peak area is related to speed of migration as well as the quantity of material present. This effect is obviously most pronounced with coated capillaries, where electroendo-osmosis is reduced to a minimum. A graph relating peak height to the quantity of albumin loaded is shown in Fig. 7. The apparatus must be calibrated with known quantities of the solute being determined to give the relationship between peak height and quantity for that solute.

4. Notes

1. Sample preparation: Biological samples are often complex mixtures of solutes, and a careful pretreatment of the sample is usually required prior to any analysis *(7)*.
   a. Desalting. Samples of biological fluids will contain the ionic constituents of extracellular fluid at a total concentration of 290 mosM. This will be more concentrated than the running buffer, and thus for the reasons stated earlier, electrokinetic and electrophoretic loading under these conditions will be poor. When samples containing a high concentration of protein and peptide are available in reasonable volume, for example saliva, plasma, and seminal fluid, simply diluting the sample with HPLC-grade water will suffice to lower the salt concentration to a level where loading becomes efficient. If the

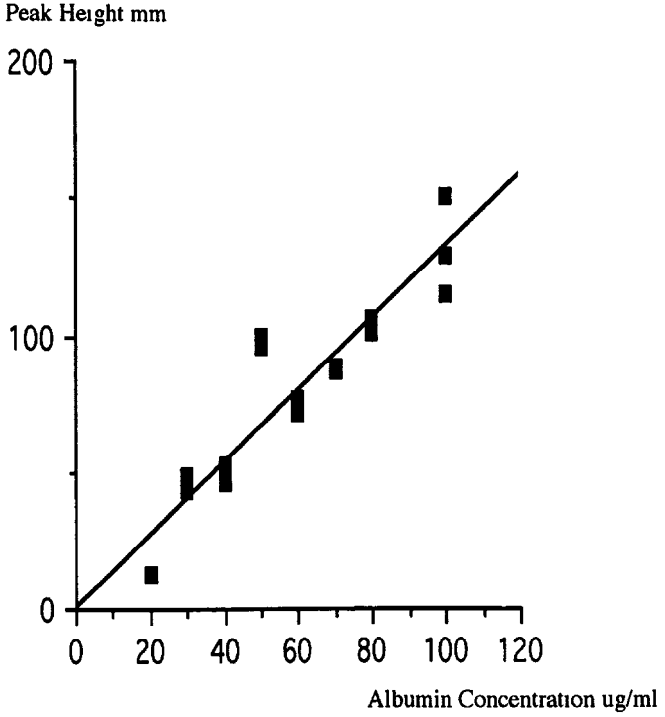

Fig 7. Relationship between concentration of bovine serum albumin in sample and peak height. Sample: Bovine serum albumin, 20–100 µg/mL (Sigma, A7888). Buffer: Phosphate/HCl, pH 2.5. Load: 10 m*M* buffer, 8.0 kV, 8 s, 11.4–12.6 µA. Run: 100 m*M* buffer, 7 kV, 10.6–12.0 µA. Detection: 200 nm $T_e = 4.31 \pm 0.03$ min ($n = 16$).

sample has a low concentration of peptide or protein, for example, cerebrospinal fluid or aqueous humor, or when only a few microliters of sample are available, dilution will be ineffective and a different strategy must be employed.

It is possible to desalt the sample by passing it through a desalting resin, such as AG11-A8 (Bio-Rad), or a similar ion-retarding or ion-exchange resin. If the sample volume is just a few microliters it can be passed through a resin by centrifugation. A small amount of resin may be loaded into a disposable filter holder with luer fittings containing a membrane suitable for aqueous solvents, such as an ACRO LC3A. The sample can then be loaded onto the resin, and the whole assembly put into an Eppendorf tube and centrifuged. The sample can then be retrieved from the Eppendorf tube with virtually 100% recovery.

Some luer filter assemblies are fitted with a membrane that contains a reversed-phase sorbent similar to those used in HPLC columns (SM-2 membrane, Bio-Rad). A sample of peptides in aqueous solution can be passed through the filter, and the peptides will bind strongly to the hydrophobic membrane. Remaining salts can be washed from the membrane with HPLC-grade water, and the peptides eluted with a water/acetonitrile or water/methanol mixture. The eluted peptides can then be recovered by evaporating the eluting solvent (freeze-drying) and redissolving in loading buffer.

b. Removing some components of a complex mixture: With some biological samples, the number of absorbing components in the UV range is so great that individual peaks often cannot be resolved adequately, because of the high background absorbance masking the peaks of interest. The removal of most of the material that is not of interest from the sample is the strategy to apply here. The same approach as is used in Note 1a above can be applied using various absorbents layered into the ACRO-type filter. For example, hydroxyapatite, which binds proteins *(8)*, can be put into the filter assembly. This will selectively remove proteins from a complex mixture. If the proteins are the solute of interest, they can be subsequently recovered from the hydroxyapatite. Obviously, a large number of variations can be used with this technique using various immobilized ligands on beads, such as antibodies or lectins and a wide variety of liquid chromatography column packings.

c. Extraction of peptides from tissue: Peptides in tissue samples can be extracted by homogenizing the tissue in 1*N* HCl and centrifuging. The supernatant containing the peptides can be further purified by passing the sample through a SEP-PAK (Waters Associates) sample preparation cartridge *(9)*. The peptides can then be eluted with a water/acetonitrile mixture and freeze-dried before redissolving in loading buffer.

2. Care of the capillary: The silica capillaries are remarkably robust, and it is in fact quite difficult to damage them. A capillary should perform several hundred separations before it needs replacing. The greatest danger to the capillary is blockage either as the result of particulate material in the sample being drawn into the capillary or because solute has crystallized in the capillary lumen. To guard against the former, all samples should be centrifuged before any attempt is made to load them, and if very fine suspended material is present, the sample should be passed through a 0.45-μ filter. Crystallization of a solute in the capillary is most likely to occur when the solute passes from buffer of one

composition to another, i.e., from the loading buffer into the running buffer. This effect should be borne in mind when dealing with solutes of limited solubility. If the capillary blocks, some attempts to unblock it may be successful. Back-flush the capillary with buffer under pressure. This will not damage the capillary and may shift the blockage. Some authorities have been known to use an HPLC pump to do this producing pressures in excess of 3000 psi to shift the blockage! Remember that the blockage is most likely to be at the end of the capillary where the run is started, so it is important to back-flush. Blockage as the result of crystallization can sometimes clear with time, so do not be too efficient in throwing blocked capillaries away. With time, the crystals can slowly dissolve and the blockage disappear. This process can sometimes be helped by gently heating the capillary in a laboratory oven. Remember you have nothing to lose but your capillary, and a blocked capillary is useless.

Capillary performance and life can be enhanced by regular flushing with a 0.5% SDS (sodium dodecylsulfate/laurylsulfate) solution after use. Capillaries should be stored between use in the refrigerator at 4°C and filled with distilled water. These treatments will greatly minimize the chance of a capillary blocking while being stored.

It is often a good idea to do a blank run for about 20 min each day when the apparatus is first set up. The voltage and current can then be checked for stability. This procedure also seems to "run in" the capillary for the day's work.

3. Optimizing the separation:
   a. Running conditions: Suggestions as to the initial loading and running conditions to select have been given above. However, careful experimentation with the settings on the power source can often improve a separation enormously. The best practice is to optimize the loading first by altering the voltage and the time. Try increasing and decreasing the voltage initially, and then altering the time. In general, with most solutes, shorter loading times give the best results. Several runs should be performed at various potential differences until the best separation for the solute(s) of interest are obtained.
   b. Detection wavelength: As the solutes are migrating in an electrical field, if the current is switched off, they will stop moving. Thus, when a solute of interest is in the UV light path, the power can be switched off and the solute is immobilized. The wavelength setting on the monochromator can then be adjusted until the absorbance is optimized. In this way, an absorbance spectrum for an unknown

solute separated from a mixture can be obtained. Remember that the silica capillary will also have an absorbance spectrum, so the best approach is to obtain this spectrum first with the capillary filled with buffer and to then subtract this spectrum from that of the capillary containing the solute of interest.

4. Special techniques: A particular technique, which may be useful if a sample contains a mixture of both charged and noncharged solutes to be separated, is that of micellar electrokinetic capillary Chromatography (MECC) *(10–12)*, where separation is achieved by a combination of both electrophoretic migration and micellar partitioning. Micelles are formed by adding SDS 0.05*M* (approx 1.5%) to the buffer. The SDS will solubilize hydrophobic species and form ion pairs with those of opposite charge. The separation is usually performed at pH 7.0 and with uncoated capillaries so as to induce electroendo-osmotic flow. Components of the sample have differing degrees of interaction with the micelles formed by the detergent action of the SDS. The micelles formed by SDS are anionic in nature and will therefore migrate in the opposite direction to the induced electroendo-osmotic flow, in a countercurrent manner, thus setting up the partitioning effect. The migration of the various solutes in a sample therefore ranges among the rates of electroendo-osmotic flow, electrophoretic migration, and the opposing rate of micelle migration. MECC has the advantage of allowing both charged and noncharged solutes to be separated in a single procedure, and has a far better resolution than an un-coated capillary alone. However, because the electroendo-osmotic flow and micelle movement are in opposite directions, not all of the components of a mixture may be drawn past the detector. To optimize the separation, it may be necessary to modify the composition of the buffer to increase or decrease the rate of electroendo-osmosis. Other buffer additives *(13)* are worth experimenting with, for example, detergents such as Triton X-100 and disaggregating agents, such as urea.

Acknowledgments

The author would like to thank Bio-Rad Laboratories UK Ltd., for the grant of an HPE 100 capillary electrophoresis apparatus and for the donation of research materials to the laboratory.

Further Reading

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