Gel Electrophoresis of Proteins

A Practical Approach

SECOND EDITION

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Preface

SINCE the first edition of this book, the electrophoretic analysis of proteins in polyacrylamide gels has continued to grow in importance as an essential research technique in the life sciences. Whilst some techniques have changed only marginally, many new procedures and applications have arisen in the intervening years. This second edition seeks to reflect these changes.

Without doubt, one-dimensional polyacrylamide gel electrophoresis is currently the most widely used form of the technique in all areas of the life sciences and so a greatly extended first chapter is devoted to this topic. This chapter also covers many of the recently developed methods for analysing gels, especially the use of different staining and blotting protocols. The subsequent chapters describe in great practical detail the other major gel electrophoretic techniques that are now in common use, including isoelectric focusing, with both conventional and immobilized pH gradients, two-dimensional gel electrophoresis, peptide mapping, and immuno-electrophoresis.

Our hope is that readers of this methods manual will find it to be as instructive and valuable a laboratory companion as many colleagues were kind enough to say they found the first edition.

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Abbreviations

ACM	*N*-acroyloyl-morpholine
AMPS	2-acrylamide-2-methyl propane sulphonic acid
ANS	1-aniline-8-naphthalene sulphonate
AP	alkaline phosphatase
BAC	*N,N'*-bisacrylylcystamine
BCIP	5-bromo-4-chloro-3 indolyl phosphate
Bis-ANS	bis(8-*p*-toluidino-1-naphthalene sulphonate)
CA	carrier ampholyte
CIE	crossed immunoelectrophoresis
CPCL	cetylpyridinium chloride
CTAB	cetyltrimethylammonium bromide
DAB	3,3' diaminobenzidine
DATD	*N,N'*-diallyltartardiamide
DBM	diazo-benzyloxymethyl
DDA	dodecyl alcohol
DDE	didodecyl ether
DDS	didodecyl sulphate
DHEBA	*N,N'*-(1,2 dihydroxyethylene) bisacrylamide
DMAPN	3-dimethylamino-propionitrile
DMSO	dimethylsulphoxide
DNP	dinitrophenol
DPT	diazophenylthioether
DTT	dithiothreitol
EDIA	ethylene diacrylate
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
FIA	fluorescent immunoassay
FITC	fluorescein isothiocyanate
HbF	fetal haemoglobin
HRP	horseradish peroxidase
IEA	immunoelectrophoretic analysis
IEF	isoelectric focusing
IPG	immobilized pH gradient
LGT	low gelling temperature (agarose)
MDPF	2-methoxy-2,4-diphenyl-3(2H)-furanone
MTT	methyl thiazolyl tetrazolium
NBT	nitroblue tetrazolium
NCS	Nuclear Chicago solubilizer
NEPHGE	non-equilibrium pH gradient electrophoresis
OPA	*o*-phthaldialdehyde
PAS	periodic acid – Schiff
PCMB	*p*-chloromercuribenzoic acid
PITC	phenylisothiocyanate
PMS	phenazine methosulphate

PMSF	phenylmethylsulphonyl fluoride
POPOP	1,3-bis-2-(5-phenyloxazole)
PPO	2,5 diphenyloxazole
PVDF	polyvinyl-difluoride
QAE	quaternary amino ethyl
RIA	radioimmunoassay
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSA	sulphosalicylic acid
TCA	trichloroacetic acid
TEMED	*N,N,N',N'*-tetramethylethylenediamine

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One-dimensional polyacrylamide gel electrophoresis

B. DAVID HAMES

1. Introduction

Many years after its first use, polyacrylamide gel electrophoresis continues to play a major role in the experimental analysis of proteins and protein mixtures. Although two-dimensional gel separations of proteins have the highest resolving power, one-dimensional polyacrylamide gel electrophoresis is still the most widespread form of the technique since it offers sufficient resolution for most situations coupled with ease of use and the ability to process many samples simultaneously for comparative purposes. The basic protocols for preparing and running one-dimensional polyacrylamide gels have changed relatively little in recent years but there have been considerable advances in the analysis of proteins separated by polyacrylamide gel electrophoresis; for example, silver staining and a whole range of blotting methodology.

This chapter describes in detail the practicalities of one-dimensional polyacrylamide gel electrophoresis of proteins, together with theoretical considerations where appropriate. Not surprisingly, many of the methods and approaches described here are also applicable to two-dimensional separations which are described later in this book (Chapter 3).

2. Why polyacrylamide gel?

Any charged ion or group will migrate when placed in an electric field. Since proteins carry a net charge at any pH other than their isoelectric point, they too will migrate and their rate of migration will depend upon the charge density (the ratio of charge to mass) of the proteins concerned; the higher the ratio of charge to mass the faster the molecule will migrate. The application of an electric field to a protein mixture in solution will therefore result in different proteins migrating at different rates towards one of the electrodes. However, since all proteins were originally present throughout the whole solution, the separation achieved is minimal. Zone electrophoresis is a modification of this procedure whereby the mixture of molecules to be separated is placed as a narrow zone or band at a suitable distance from the

electrodes such that, during electrophoresis, proteins of different mobilities travel as discrete zones which gradually separate from each other as electrophoresis proceeds. In theory, separation of different proteins as discrete zones is therefore readily achieved provided their relative mobilities are sufficiently different and the distance allowed for migration is sufficiently large. However, in practice there are disadvantages to zone electrophoresis in free solution. First, any heating effects caused by electrophoresis can result in convective disturbance of the liquid column and disruption of the separating protein zones. Second, the effect of diffusion is constantly to broaden the protein zones and this continues after electrophoresis has been terminated. To minimize these effects, zone electrophoresis of proteins is rarely carried out in free solution but instead is performed in a solution stabilized within a supporting medium. As well as reducing the deleterious effects of convection and diffusion during electrophoresis, the supporting medium allows the investigator to fix the separated proteins at their final positions immediately after electrophoresis and thus avoid the loss of resolution which results from post-electrophoretic diffusion. The fixation process employed varies with the supporting medium chosen.

Many supporting media are in current use, the most popular being sheets of paper or cellulose acetate, materials such as silica gel, alumina, or cellulose which are spread as a thin layer on glass or plastic plates, and gels of agarose, starch, or polyacrylamide. These media fall into two main classes. Paper, cellulose acetate, and thin-layer materials are relatively inert and serve mainly for support and to minimize convection. Hence separation of proteins using these materials is based largely upon the charge density of the proteins at the pH selected, as with electrophoresis in free solution. In contrast, the various gels not only prevent convection and minimize diffusion but in some cases they also actively participate in the separation process by interacting with the migrating particles. These gels can be considered as porous media in which the pore size is the same order as the size of the protein molecules such that a molecular sieving effect occurs and the separation is dependent on both charge density and size. Thus two proteins of different sizes but identical charge densities would probably not be well separated by paper electrophoresis, whereas, provided the size difference is large enough, they could be separated by polyacrylamide gel electrophoresis since the molecular sieving effect would slow down the migration rate of the larger protein relative to that of the smaller protein.

The extent of molecular sieving depends on how close the gel pore size approximates the size of the migrating particle. The pore size of agarose gels is sufficiently large that molecular sieving of most protein molecules is minimal and separation is based mainly on charge density. In contrast, starch and polyacrylamide gels have pores of the same order of size as protein molecules and so these do contribute a molecular sieving effect. However, the success of starch gel electrophoresis is highly dependent on the quality of the starch gel itself, which, being prepared from a biological product, is not reproducibly good and may contain contaminants which can adversely affect the quality of the results obtained. On the other hand, polyacrylamide gel, as a synthetic polymer of acrylamide monomer,

can always be prepared from highly purified reagents in a reproducible manner provided that the polymerization conditions are standardized. The basic components for the polymerization reaction are commercially available at reasonable cost and high purity although for some purposes extra purification may be required. In addition, polyacrylamide gel has the advantages of being chemically inert, stable over a wide range of pH, temperature, and ionic strength, and is transparent. Finally, polyacrylamide is better suited to a size fractionation of proteins since gels with a wide range of pore sizes can be readily made whereas the range of pore sizes obtainable with starch gels is strictly limited. For these and other reasons, polyacrylamide gels have become the medium of choice for zone electrophoresis of most proteins although starch gels have been widely used for the analysis of isoenzymes. Starch gel electrophoresis has been reviewed by Gordon (1), Smith (2), and Andrews (3). Agarose gels are used for the fractionation of molecules or complexes larger than can be handled by polyacrylamide gels, especially certain nucleic acids and nucleoproteins. In addition, agarose is widely used in immunoelectrophoresis where zone electrophoresis of proteins is coupled to immunological detection and quantitation (Chapter 4).

This chapter is concerned with analytical zone electrophoresis of proteins in polyacrylamide gels plus modifications which allow small-scale preparations of proteins of interest. Detailed quantitative approaches to analytical zone electrophoresis and special techniques for large-scale preparation of proteins by zone electrophoresis are not described.

3. Properties of polyacrylamide gel

3.1 Chemical structure

Polyacrylamide gel results from the polymerization of acrylamide monomer into long chains and the crosslinking of these by bifunctional compounds such as *N,N'*-methylene bisacrylamide (usually abbreviated to bisacrylamide) reacting with free functional groups at chain termini. Other crosslinking reagents have also been used to impart particular solubilization characteristics to the gel for special purposes (Section 7.5.2). The structure of the monomers and the final gel structure are shown in *Figure 1*.

3.2 Polymerization catalysts

Polymerization of acrylamide is initiated by the addition of either ammonium persulphate or riboflavin. In addition, *N,N,N',N'*-tetramethylethylenediamine (TEMED) or, less commonly, 3-dimethylamino-propionitrile (DMAPN) are added as accelerators of the polymerization process.

In the ammonium persulphate–TEMED system, TEMED catalyses the formation of free radicals from persulphate and these in turn initiate polymerization. Since the free base of TEMED is required, polymerization may be delayed or even

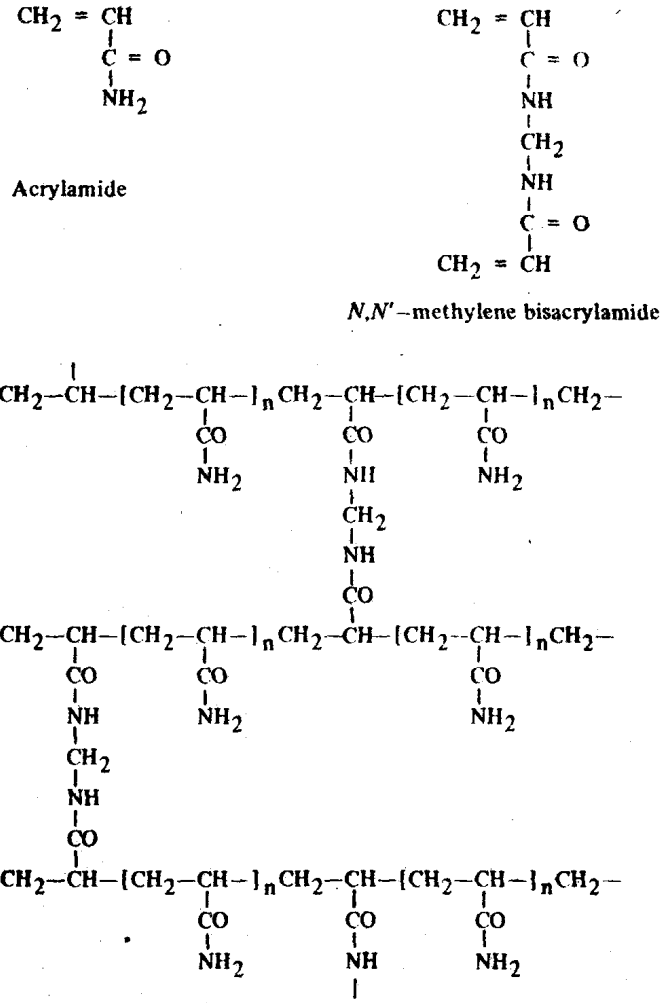

Figure 1. The chemical structure of acrylamide, *N,N'*-methylene bisacrylamide, and polyacrylamide gel.

prevented at low pH. Increases in either the TEMED or ammonium persulphate concentration increase the rate of polymerization.

In contrast to chemical polymerization with persulphate, the use of the riboflavin TEMED system requires light to initiate polymerization. This causes photo-

decomposition of riboflavin and production of the necessary free radicals. Although gelation occurs when solutions containing only acrylamide and riboflavin are irradiated, TEMED is usually also included since under certain conditions polymerization occurs more reliably in its presence.

Oxygen inhibits polymerization and so gel mixtures are usually degassed prior to use.

3.3 Effective pore size

The effective pore size of polyacrylamide gels is greatly influenced by the total acrylamide concentration in the polymerization mixture, effective pore size decreasing as acrylamide concentration increases. Gels with concentrations of acrylamide less than about 2.5%, which are necessary for the molecular sieving of molecules above a molecular mass of 10^6, are almost fluid but this can be remedied by the inclusion of 0.5% agarose (Section 9.7). At the other extreme, polyacrylamide gels will form at over 30% acrylamide at which concentration polypeptides with a molecular mass as low as 2000 experience considerable molecular sieving. As one might expect, the choice of acrylamide concentration is critical for optimal separation of protein components by zone electrophoresis and will be considered in more detail later (Section 4.6).

The composition of any given polyacrylamide gel is now usually described by two parameters, $\%T$ and $\%C$. The $\%T$ value represents the total concentration of monomer used to produce the gel (acrylamide plus bisacrylamide) in grams per 100 ml (i.e. w/v), and $\%C$ is the percentage (by weight) of the total monomer which is the crosslinking agent. For any given total monomer concentration, the effective pore size, stiffness, brittleness, light scattering, and swelling properties of the polyacrylamide gel vary with the proportion of crosslinker used. Polymerization in the absence of crosslinker leads to the formation of random polymer chains resulting only in a viscous solution. When bisacrylamide is included in the polymerization mixture, gelation occurs with random polymer chains crosslinked at intervals to form a covalent meshwork. As the proportion of crosslinker is increased, the pore size decreases. Initial studies (4) suggested that pore size reaches a minimum when the bisacrylamide represents about 5% of the total monomer concentration (i.e. $C_{Bis} = 5\%$) irrespective of the absolute value of $\%T$. However, more recent work has shown that, above about $T = 15\%$, the proportion of crosslinker required for minimum pore size increases with the value of $\%T$ (5).

As the proportion of crosslinker is increased above the value required for minimum pore size, the acrylamide polymer chains become crosslinked to form increasingly large bundles with large spaces between them so that the effective pore size increases again. The variation in pore size is substantial. Thus for a 5% polyacrylamide gel ($T = 5\%$, $C_{Bis} = 5\%$) the pore size is approximately 20 nm but at very high proportions of bisacrylamide crosslinker ($C = 30-50\%$) the pore size can reach 500–600 nm (6).