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Review

Evaluation of commonly used electrophoretic methods for the analysis of proteins and peptides and their application to biotechnology

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Abstract

This account gives an overview and evaluation of the current methods of most commonly used electrophoretic techniques in the analysis of various polypeptides. A general review of some background information and pitfalls associated with the application of these methods in the characterization of the size and charge properties of proteins or peptides is presented. It is intended to provide some guidelines to those researchers first exposed to this important and powerful methodology in modern life sciences. Electrophoretic characterizations of several major classes of proteins with vastly different molecular sizes and/or charges are taken as exemplars to highlight the versatility of electrophoretic protocols and their great potential for facilitating protein purification and structural analysis of some complex and scarce biological samples when coupled to other modern analytical methods. Major electrophoretic techniques reviewed herein include:

1. Discontinuous polyacrylamide gel electrophoresis (DISC-PAGE)
2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
3. Pore gradient electrophoresis (PGE)
4. Isoelectric focusing (IEF) and
5. Two-dimensional gel electrophoresis (2D PAGE).

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1. Introduction

Electrophoresis is a process of separating charged molecules by migration in an electrical field. The method has a long and distinctive history for protein characterization since Tiselius first introduced it to the analysis of complex serum proteins in 1933 [1]. It is undoubtedly the most common technique in all fields of traditional biochemical study related to protein molecules. Recently it has become even more pre-eminent in molecular and cellular biology in light of its wide-spread use for high-resolution DNA sequencing analysis [2] using polyacrylamide as an electrophoretic medium in place of agarose. The electrophoretic analysis of proteins and peptides in

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polyacrylamide gels has also continued to grow in importance as to replace almost all other matrix media such as starch, cellulose and agarose in routine protein analysis [3,4].

It is noteworthy that the main techniques of protein electrophoresis have changed only marginally for the past three decades in spite of the advent of many new procedures and applications in the intervening years. We have made an endeavor to compare the advantages and shortcomings associated with the major electrophoretic techniques routinely used in protein analysis in order to save time and effort by ''trial and error'' approaches generally adopted by researchers first exposed to electrophoretic methods. Major electrophoretic techniques that are commonly used and reviewed herein include:

1. Discontinuous polyacrylamide gel electrophoresis (DISC-PAGE)
2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
3. Pore gradient electrophoresis (PGE)
4. Isoelectric focusing (IEF), and
5. Two-dimensional gel electrophoresis (2D PAGE).

2. Discontinuous polyacrylamide gel electrophoresis (DISC-PAGE)

Discontinuous gel electrophoresis system in contrast to continuous buffer system, which uses a uniform buffer system of constant pH in the gel and electrode buffer, makes use of discontinuous or called multiphasic buffer systems by employing different buffer ions in the gel as compared to those in the electrode reservoirs. Most commonly used discontinuous buffer systems consist of two different buffers and pHs, i.e. the system possesses discontinuities of both buffer compositions and pH. In these discontinuous buffer systems, the sample is loaded onto a large-pore ''stacking'' gel of lower pH and buffer concentration, which is polymerized on top of the small-pore resolving gel of higher pH and buffer concentration.

The discontinuous systems currently used were all derived from the basic principles and designs of Davis and Ornstein [5,6]. The major advantage of these discontinuous buffer systems over continuous buffer systems is that relatively large volumes of dilute protein samples without prior sample concentration or lyophilization can be applied to the gels but good resolution of sample components can still be obtained. The proteins are concentrated into extremely narrow zones or stacks during migration through the large-pore stacking gel before getting into the small-pore resolving gel for their *bona fide* separation during electrophoresis. The buffer used is usually referred to as Tris–HCl/glycine system. Glycine is only partially dissociated so that its effective mobility is low at the pH of stacking gel (about 6.8–7.0). Chloride ions have a much higher mobility at this pH whilst the mobilities of proteins are intermediate between that of chloride and glycine. A steady state is thus established where the products of mobility and voltage gradient for glycine and chloride are equal, these charged species then moving at similar velocities with a sharp boundary between them [3,4]. Therefore the moving boundary sweeps up the moving proteins so that they become concentrated into a very thin starting protein zone or ''stack''. Because of the high-resolution obtainable with discontinuous buffer systems as compared to the continuous one, DISC-PAGE is usually more suitable for demonstrating the purity and multiplicity of protein components than that of continuous system unless the protein(s) studied are not compatible with the buffer and pH of the stacking gel leading to the precipitation of protein components during the stacking process. Under these conditions, we can add a dissociating agent such as SDS (sodium dodecyl sulfate) to the buffers of the discontinuous system (a discontinuous buffer system with SDS added to buffers of stacking and resolving gels) and obtain the same or even better high-resolution fractionation of protein mixtures under dissociating conditions (see Section 3).

Although Tris–HCl/glycine native buffer system [5,6] is undoubtedly the buffer of choice for electrophoretic separations of most native proteins, there is no universal buffer system ideal for all proteins with vastly different charge and size properties. However, based on the theory of discontinuous (multiphasic) zone electrophoresis [7] and a knowledge of pK and ionic mobility data of buffer constituents, several thousand discontinuous buffer systems have been designed for use in the useful pH range 3–11 [8,9] and can be requested as a computer output. It is a common practice to try an Ornstein–Davis high pH system which resolves proteins at pH 8.8–9.5 before

resorting to other buffer systems for some exceptional proteins.

It is also worth emphasizing that gel electrophoresis of continuous buffer system may be needed if some native proteins aggregate easily in the stacking gel to cause the proteins to fail to enter the lower resolving gel or resulting in consistent streaking of protein bands after electrophoresis. In these special cases, in order to obtain a good resolution for the continuous buffer system:

1. the sample must be applied in as small a volume as possible to give a thin starting zone;
2. additional zone-sharpening can be obtained by loading the protein sample in a buffer which has a lower ionic strength than that of the gel and electrode buffer [3,4].

Fig. 1 shows a typical example of discontinuous polyacrylamide gel electrophoresis under native (DISC-PAGE) or dissociating conditions for the comparison of electrophoretic patterns in the analysis of one enzyme isolated from the crude venom of Taiwan cobra. These were carried out on the polyacrylamide gels using a slab-gel instead of the rod-gel system.

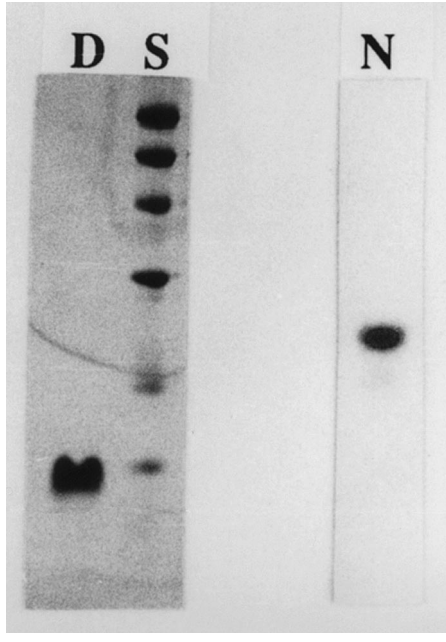

Fig. 1. Gel electrophoresis of the fractionated venom toxin under native (DISC-PAGE) and denaturing conditions (SDS-PAGE) in the presence of 5 mM dithiothreitol (5% stacking/14% resolving gels). Lane S, standard proteins used as molecular mass markers (in kDa): phosphorylase b (94), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (30), soybean trypsin inhibitor (20), and lysozyme (14). Both gel buffer systems are Tris–HCl/glycine buffer used for discontinuous PAGE with the exception that SDS-PAGE contains SDS in the buffer. The gels were stained with Coomassie blue. Lanes N and D indicate the electrophoretic positions for purified venom phospholipase A_2 (PLA_2) from Taiwan cobra under native (DISC-PAGE) and denaturing conditions, respectively. Note that the isolated PLA_2 shows one major band under native and denatured states with a molecular mass of 14 kDa.

3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

3.1. General background and principles

One of the landmark papers in the application of gel electrophoresis to protein analysis was the report by Shapiro et al. [10] who described the effect of SDS on proteins, a strong protein-dissociating detergent, in improving gel electrophoresis of proteins on polyacrylamide gel matrix. Later on it was established as a facile method of choice for the estimation of molecular masses of protein subunits by SDS-gel electrophoresis [10,11]. There are several different buffer systems for SDS-PAGE, most of them are carried out on continuous or uniform buffer system. Nevertheless, by far the most widely used SDS-PAGE is the discontinuous system originally described by Laemmli [12] based upon the original Ornstein–Davis native buffer system [5,6] in the presence of SDS. The simplicity and high-resolution separation patterns obtained from the method, plus the fact that dilute samples with microgram amounts of sample proteins can routinely be analyzed, have made the report of Laemmli's SDS-PAGE system one of the most cited methodological papers in life sciences. It is also the most widely used method for the determination of the complexity and molecular masses of constituent polypeptides in a protein sample both on 1D and 2D slab-gels (see below).

The majority of proteins bind SDS in a constant weight ratio (1.4 g of SDS per gram of polypeptide) [13], effectively masking the intrinsic charge of the polypeptide chains, so that the net charge per unit mass becomes approximately constant. Thus SDS–

polypeptide complexes have essentially identical charge densities and migrate in polyacrylamide gels of correct porosity strictly according to their effective molecular radius, which roughly approximates to molecular size. Therefore electrophoretic separation occurs solely as a result of the effect of ''molecular sieving'' through the gel matrix [3,4]. For Laemmli's SDS-PAGE, SDS is added as a 0.1% (w/v) to the gel and electrode buffer, this SDS concentration being well above the critical micelle concentration for this anionic detergent to maintain a constant charge density of SDS–protein complex. However, for optimal reaction of polypeptides with SDS it is customary to keep protein samples in 1–2% SDS (w/v) and boil for 2–5 min in the presence of reducing reagents such as 5% 2-mercaptoethanol or 20 mM dithiothreitol to cleave disulfide bonds and ensure optimal binding of SDS to denatured polypeptides.

3.2. Molecular mass estimation

With uniform concentration gels, there is a linear relationship between $\log_{10}$ (molecular mass) and relative mobility (*Rf*) or distance migrated by the SDS–polypeptide complex [10,11]. The conventional method for the estimation of molecular masses of polypeptides by the SDS–phosphate uniform buffer system of Weber and Osborn [11] possesses the advantages that it is easier to prepare the continuous uniform gel and buffer system, and that it is less susceptible than the Laemmli system [12] to experimental variations of each individual researcher, which may induce artifacts caused by incorrect multiphasic buffer concentrations and pH adjustment in gel preparation. However, the continuous buffer gel suffers the major drawback of inferior resolution of complex protein mixtures when compared with that of discontinuous SDS-PAGE. Unless one is concerned with the anomalous electrophoretic behavior experienced by a specific and purified protein on Laemmli's buffer system, the old protocol of SDS-PAGE in the uniform gel is less commonly used in the molecular mass estimation for complex protein mixtures from various cell extracts. It is also to be noted that conventional SDS-PAGE carried out in rod–tube gels has been superseded by the easier and superior slab-gels especially for the purpose of molecular mass estimation since sample and marker polypeptides can be electrophoresed on a single slab-gel and therefore under identical conditions to get more reproducible and accurate results for the comparison of multiple samples.

For any given gel concentration the linear relationship between $\log_{10}$ (molecular mass) and relative mobility is valid only over a limited range of molecular mass. For example, improved resolution of dilute protein samples in the molecular mass range of 14–95 kDa using the Laemmli SDS-discontinuous buffer system, the linear relationship holding over this range can usually be obtained with a 5% stacking and 14% *T* (percentage total monomer, i.e. grams acrylamide plus bisacrylamide per 100 ml) resolving gel (Figs. 1 and 2). However, for the fractionation of polypeptides smaller than about 12 000–15 000 molecular mass, a special buffer system needs to be adopted (Tricine buffer system, see below). Moreover, many glycoproteins also behave anomalously even when SDS and

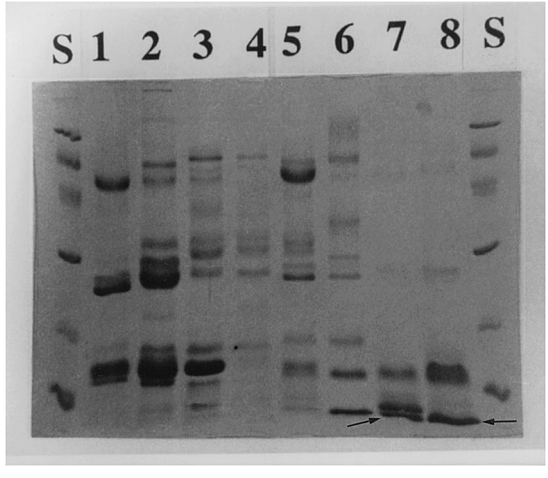

Fig. 2. Gel electrophoresis of the crude venoms of various snake species under denaturing conditions (SDS-PAGE, 5% stacking/14% resolving gels) in the presence of 5 mM dithiothreitol. Lane S, standard proteins used as molecular mass markers (in kDa): phosphorylase b (94), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (30), soybean trypsin inhibitor (20), and lysozyme (14). The gel was stained with Coomassie blue. Lanes 1–8 correspond to eight different snake species of *Agkistrodon acutus, Trimeresurus mucrosquamatus, Agkistrodon piscivorus leukostoma, Agkistrodon piscivorus piscivorus, Crotalus atrox, Crotalus adamanteus, Naja naja siamensis* and *Naja naja atra*, respectively. The arrows indicate positions of the dye front which co-migrates with protein molecules, possessing molecular masses of less than 12 kDa.

thiol reagent are in excess, yielding artefactually high molecular mass estimates [3 and the references therein]. To circumvent this problem, SDS-PAGE should be carried out at increasing polyacrylamide gel concentrations, at which molecular sieving predominates over the charge effect and the apparent molecular masses of glycoproteins decrease and may approach their real molecular masses. Other protein samples which do not follow the linear relationship of molecular size and relative mobility are some polypeptides after chemical modification, collagenous polypeptides with high proline content, and some extremely basic or acidic proteins. They all fail to bind SDS at a ratio approaching that of 1.4 grams per gram protein generally found for most proteins and give rise to abnormally high molecular masses as estimated by SDS-PAGE. The anomalous behavior of these proteins in SDS-PAGE, similar to that of glycoproteins, is shown to be alleviated by increasing acrylamide concentrations.

3.3. Estimation of molecular masses of small proteins or peptides

If the polypeptides to be fractionated fall into the size range of smaller than 12 kDa, SDS-PAGE even at % T=15% cannot adequately resolve these small protein components, all of them moving together with the standard dye front and showing up as a dense and thick band in the front end of gel after staining (see Fig. 2). When one does SDS-PAGE at 10% acrylamide gel, protein components below 18–20 kDa all migrate with the buffer or dye front and fail to be resolved by molecular sieving effect under most SDS-PAGE systems. This problem can be rectified by including high concentrations of urea (4–8 M) both in the sample buffer and gel solution to improve the molecular sieving of SDS–oligopeptides complexes in the gel matrix [14]. The system of discontinuous SDS-PAGE with urea is shown to be capable of separating small polypeptides in the range 1–100 kDa using 8–15% acrylamide gel and a high bisacrylamide crosslinker (5–20% C).

3.4. SDS-PAGE in Tricine gels

As described above, oligopeptides with molecular masses below about 15 kDa are not well resolved by SDS-PAGE using the Laemmli buffer system even in 15% polyacrylamide gels. This can sometimes be improved by using 12.5% polyacrylamide gels prepared with a high ratio of bisacrylamide crosslinker to total monomer (% C=5–10% instead of the normal 2.5–3.0%) or the inclusion of 8 M urea in a continuous or discontinuous system. The other alternative protocol is to use Tricine as the tracking ion in an SDS-discontinuous buffer system.

Schagger and von Jagow [15] first introduced this approach which does not rely upon the use of urea for the analysis of small proteins and oligopeptides. They lowered the pH of the gel buffer and replaced glycine, the tracking ion in the Laemmli buffer system with Tricine in the running buffer. At the pH values during the process of electrophoresis, Tricine migrates much faster than glycine in the stacking gel and proteins are not stacked as strongly as in the Laemmli system with the benefit that small SDS–polypeptide complexes separate more easily from stacked SDS-micelles in lower percentage gels resulting in the cleaner separation and sharper bands. The 10% T/3% C resolving gel is recommended as an ideal choice in this system for initial examination of a complex sample which may contain small polypeptides since it spans the molecular mass range up to 100 kDa yet allows researchers to detect polypeptides down to a molecular mass of 1 kDa. The omission of glycine and urea in this system may also favor the subsequent protein microsequencing of eluted polypeptides from this gel system. Moreover, the buffer system tolerates the application of high amounts of protein. Fig. 3 shows a SDS-PAGE electrophoretic pattern using Tricine gel for the protein analysis and molecular mass estimation of small toxin peptides from the cobra venom.

4. Pore gradient electrophoresis (PGE)

4.1. General background and principles

It has been long recognized that high resolution of proteins in polyacrylamide gel is mainly due to the gel matrix acting as a molecular sieve that differentially retards protein molecules according to their molecular size as compared to that of gel pores of the matrix [6,16]. This effect can be exploited to advantage by using a gel with a continuous concentration gradient

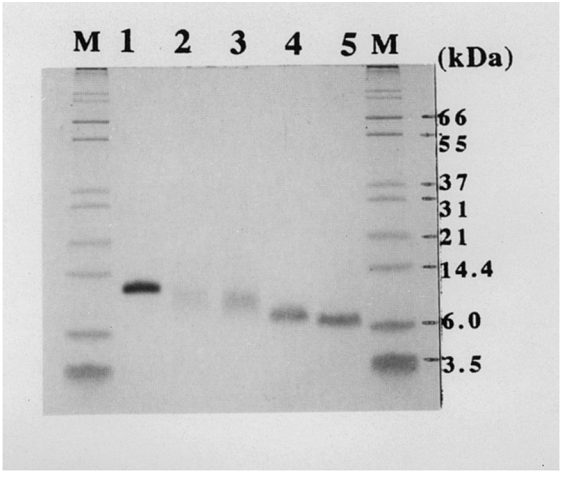

Fig. 3. Gel electrophoresis of purified toxin fractions from Taiwan cobra under denaturing conditions (Tricine/SDS-PAGE, 10% resolving gel) in the presence of 5 mM dithiothreitol. Lanes M, standard proteins used as molecular mass markers (in kDa): bovine serum albumin (66), glutamic dehydrogenase (55), lactate dehydrogenase (37), carbonic anhydrase (31), soybean trypsin inhibitor (21), lysozyme (14.4), aprotinin (6.0) and insulin B chain (3.5). Lanes 1–5 correspond to the purified toxin proteins of crude venom from reverse-phase HPLC for: (1) phospholipase A_2, (2) cobrotoxin, (3) NTX 5–1, (4) NTX 12–1 and (5) cardiotoxin, respectively. The gel was stained with Coomassie blue. Note that Tricine gel system can separate small toxins of less than 10 kDa even at 10% gel concentration.

along the gel so that the proteins are forced to migrate through progressively smaller pores, the sizes of which are regulated by the gel concentration [16,17]. In theory there is a gel concentration which is optimal for the resolution of a given pair of proteins. However, it is difficult to find a single gel concentration which will give a maximum resolution of the components of an unknown and complex protein mixture. Thus in practice it is often better to use gels containing a linear or non-linear concentration gradient of polyacrylamide gel. The average pore radius of these gels decreases with increasing gel concentration, so that there is an effective band-sharpening effect during electrophoresis and proteins with a wider range of molecular masses can be separated. The use of this gradient gel is often referred to as ''pore gradient electrophoresis'' (PGE), by which proteins are driven through pores of successively decreasing sizes until they are brought to their pore limits in the gel according to their sizes.

4.2. Estimation of molecular masses by PGE

With uniform concentration gels, there is a linear relationship between $\log_{10}$ molecular mass and *Rf* value or distance migrated by the SDS–polypeptide complex. However, with linear concentration gradient gels, the linear relationship is better related by $\log_{10}$ molecular mass and $\log_{10}$ polyacrylamide concentration (*% T*) [18–20]. Experimentally the calculation of *% T* is better facilitated by employing linear than other non-linear gradients. The dependence of electrophoretic mobility of protein species on gel concentration, *% T* (percentage total monomer acrylamide), was first described and exploited by Ferguson [21] to estimate native molecular masses of proteins in gel electrophoresis (Ferguson plot). It has since been used to estimate molecular masses of native and subunits of denatured proteins for all uniform and gradient gels and remains to be one of the most fundamental and semi-quantitative bases for the estimation of molecular masses of proteins in gel electrophoresis.

4.3. Molecular mass estimation by Ferguson plots

Most proteins under native conditions, i.e. in the absence of dissociating agents such as SDS, move in the electric field according to their charges and sizes. By measuring the mobility of each protein in a series of gels of different acrylamide concentration, one can construct the Ferguson plot, a plot of $\log_{10}$ relative mobility (*Rf*) versus gel concentration (*% T*). In the construction of these Ferguson plots, the charge aspect of proteins is eliminated and the slope of each plot, K_R (retardation coefficient), is only a measure of molecular size [22]. Ferguson plot analysis has been most useful and quite accurate for determining the molecular size of globular native proteins. Fig. 4 exemplifies the molecular mass estimation for four major charge isomers of γ-crystallin isolated from bovine eye lenses by Ferguson plot under native-gel PAGE conditions [23]. However, the problem of molecular mass determination with native proteins is that it is only valid if the standard proteins used to generate calibration curves have the same shape with similar degrees of hydration and partial specific volume. SDS virtually eliminates conformational and charge density differences among different proteins and reduces the effect of variability in partial specific volume and

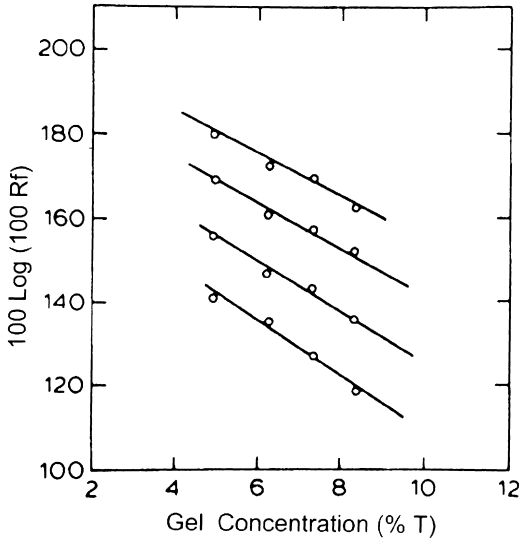

Fig. 4. Ferguson plot of bovine γ-crystallin subfractions [23]. The gel buffer system was that of Tris/glycine buffer according to Davis's system [5]. Four different gel concentrations (5.0%, 6.25%, 7.30% and 8.30%) were used in the determination of the slope for each subfraction, which is proportional to native molecular masses of the studied proteins.

hydration. Therefore it is in general to be expected that molecular mass estimation is more accurate with SDS-PAGE instead of DISC-PAGE under native conditions for any unknown proteins using Ferguson plots [24].

4.4. Electrophoretic behavior of glycoproteins and small peptides in PGE

Unlike their electrophoretic behaviors in uniform and homogeneous concentration gels, there is a good indication that glycoproteins do not behave anomalously during SDS-PAGE using gradient gels because the molecular sieving effect seems to predominate over the anomalous surface charge effect caused by reduced binding of SDS to most glycoproteins [19].

It was shown that the molecular masses of proteins separated in linear SDS-PAGE gradient gels can be estimated from plots of $\log_{10}$ molecular mass (M) versus the square root of the migration distance ($D^{1/2}$) [25]. This linear relationship can be applied to reduced SDS–protein complexes, non-reduced SDS–protein complexes, glycoproteins and non-glycoproteins. It is noteworthy that this relationship is time-independent so that electrophoresis can be terminated at the appropriate time in order to gain optimal resolution of the protein bands of interest rather than waiting until the dye indicator to reach the end of the gel. In light of these advantages gradient gels may therefore become the gel medium of choice to circumvent the anomalous behavior for glycoproteins usually encountered by SDS-PAGE in uniform gels.

One of the main advantages of gradient gel electrophoresis is that the migrating proteins are continually entering areas of gel with decreasing pore sizes such that the advancing edge of the migrating protein zone is retarded more than the trailing edge, resulting in a marked sharpening of the protein bands. In addition, the gradient of different pore sizes in one slab-gel increases the range of molecular masses which can be fractionated simultaneously. However, it is important to note that even a gradient gel in the 3–30% T range cannot resolve polypeptides smaller than about 12 kDa using the standard Laemmli SDS-discontinuous buffer conditions.

As in the continuous or uniform gel buffer of SDS-PAGE, small polypeptides down to 1 kDa can be separated using 10–18% gradient gels if 6–8 M urea (final concentration) is included in the gel and sample buffers to decrease the mobilities of protein molecules [26]. It is also noteworthy that even gradient gels can generally achieve a higher resolution for a protein mixture than uniform gels, they cannot match the resolution of any particular pair of protein components possessing similar size and/or charge obtainable with gels of a uniform, optimized polyacrylamide concentration. If all the proteins of interest fall into a narrow molecular mass range in gradient gel, SDS-PAGE should then be carried out with several different uniform concentration gels to obtain optimal resolution of the components.

5. Isoelectric focusing (IEF)

5.1. Isoelectric focusing using carrier ampholytes

IEF is a high-resolution electrophoretic method in which amphoteric protein molecules are separated in the presence of a continuous pH gradient [27]. Under these conditions proteins migrate according to their charges until they reach the pH values at which they

have no net charge (i.e. their isoelectric points, p*I*). The proteins will attain a steady state of zero migration and will be concentrated or focused into very narrow zones [28]. In great contrast to zone electrophoresis, where the constant (buffered) pH of the separation medium establishes a constant charge density at the surface of the polypeptide and causes it to migrate with constant mobility (in the absence of molecular sieving), the surface charge of an amphoteric compound in IEF keeps changing, and decreases as it moves along a pH gradient until it reaches its equilibrium position, i.e., the region where the pH equals to its p*I*. The pH gradient is created and maintained by the passage of an electric current through a solution mixture of amphoteric small compounds (less than 1 kDa molecular mass) which have been prepared within closely spaced p*I*s, encompassing a given pH range. Their electrophoretic transport causes the carrier ampholytes to stack according to their p*I*s, and a pH gradient, increasing from anode to cathode, is established [27,28]. Two counteracting effects of diffusion versus electrophoresis are the primary causes of the residual current observed under isoelectric steady-state conditions. Finally as time progresses, the sample protein molecules reach their equilibrium isoelectric point. Therefore p*I* is an intrinsic physico-chemical parameter of any amphoteric compound in the solution under electric field.

The practical limit of carrier ampholyte-IEF is in the pH 3.5–10 interval. Most protein p*I*s cluster between pH 6 and 8. An upper molecular weight limit of about 750 000 can be separated in IEF using low-concentration acrylamide gel. It should be noted that IEF is an equilibrium technique in contrast to most other electrophoretic techniques which are essentially out of equilibrium (steady-state conditions). Therefore IEF allows excellent resolution of proteins at their equilibrium conditions resulting in picking up the difference in p*I* by only 0.01 pH units (with immobilized pH gradients, up to ~0.001 pH units, see below); the protein bands may be the sharpest among all electrophoretic techniques due to the focusing effect.

In IEF it is essential to use conditions that minimize molecular sieving effects, which necessitates the use of gels of low acrylamide concentration (3–5% *T*). Although in 2D PAGE (see below) it is customary to run IEF in rod gels, it is usually easier to handle the low-concentration IEF gels in horizontal slab-gels of ultrathin (0.02–0.5 mm) thickness, which can be cast on thin plastic supports to improve heat dissipation and ease of handling. It is generally known that it is not a trivial matter for preparing the IEF gels due to the inherent pH gradient instability which always results in shift in pH gradient inside the gels (cathodic drift). Especially troublesome is the quality of the ampholytes used to prepare the gradient, which usually changes from batch to batch from different suppliers. Therefore it is advisable for beginners to use the precast ready-made IEF gels which are more consistent in gel compositions and easier to handle by an inexperienced user. A range of ready-made wet IEF gels bound to plastic supports is commercially available, covering both broad (pH 3.5–9.5) and narrow (such as pH 4–5 or 6–8) pH intervals. They are simple to use and give more reproducible results with different users although they are too expensive to be used routinely for protein separation purpose.

IEF can be performed under denaturing conditions in the presence of high levels of urea or non-ionic detergent to help to prevent the precipitation of proteins during IEF. However, it should be cautioned that the use of any charged detergents such as SDS is incompatible with IEF. Salt concentration of samples should therefore be minimized by gel filtration, ultrafiltration or dialysis, if band distortion, extended focusing times and excessive heating effects are to be avoided during IEF. Users should also bear in mind that some minor bands showing up in the gel may not be genuine, being the artefactual result of heterogeneity formed by the interaction between some charged species such as dye indicators and ampholytes which are small peptides by nature. A duplicate run using another IEF gel and different sample buffer should be carried out to confirm or refute the anomalous results. Fig. 5 shows an IEF run in the presence of 6 M urea for the analysis of lens protein fractions isolated from ostrich eye lenses [29].

5.2. IEF using immobilized pH gradient

An important recent innovation in IEF, which was first described in 1982, has been the development of Immobiline reagents (Pharmacia-LKB) for the preparation of polyacrylamide gels containing immobilized pH gradients (IPGs) [30]. IPGs are based on the

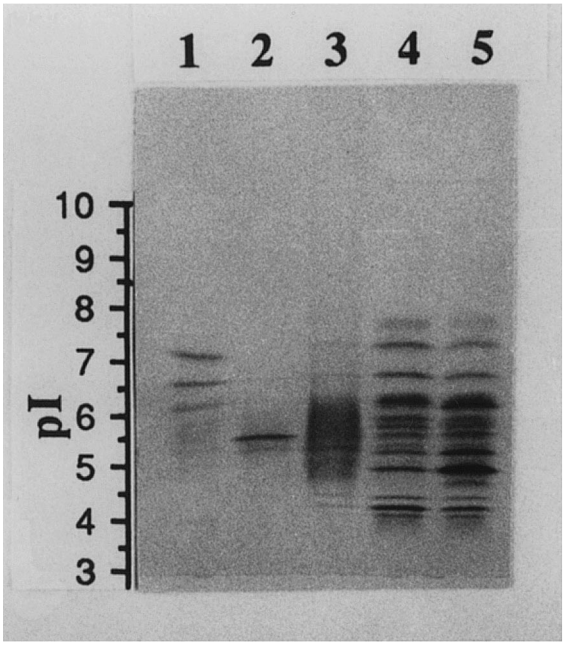

Fig. 5. Isoelectric focusing (IEF) of ostrich lens crystallins under denaturing conditions [29]. Lanes 1–5 correspond to the five peak fractions of anion-exchange chromatography. The slab-gel contained 6 M urea and 0.1% β-mercaptoethanol, and the electrophoresis was run at an initial voltage of 200 V for 6 h until a final current of less than 0.5 mA was reached. The approximate p*I*s of various crystallin polypeptides were estimated from a commercial p*I* calibration kit (range 4.7–10.6). The gel was stained with Coomassie blue.

principle that the pH gradient, which exists prior to the IEF run itself, is copolymerized and thus insolubilized within the fibers of a polyacrylamide matrix (i.e. immobilization of the buffer on the gel matrix). The buffers of IPG are no longer amphoteric, as in conventional IEF, but are bifunctional [31].

The Immobiline reagents are a series of seven acrylamide derivatives containing either a carboxyl (acidic Immobilines) or a tertiary amino (basic Immobilines) group, forming a series of buffers with different p*K* values. The main advantage of the IPG system is that during polymerization the buffering groups forming the pH gradient are covalently attached and immobilized via vinyl bonds to the polyacrylamide backbone. This results in pH gradients which are effectively and infinitely stable, thereby eliminating the deleterious effects of pH gradient drift (cathodic drift) commonly encountered in conventional IEF using ampholytes [32]. However, the electroendosmosis, which can still cause problems in the extreme pH ranges (i.e. below pH 5 and above pH 9).

IEF using immobilized pH gradient IPG methodology is in principle better and more controllable than conventional IEF using ampholytes especially regarding stability in pH gradient setup in the gel matrix. Recently the customized pre-cast IPGs are prepared in rehydratable gels, which simplify the use of IPG technology and are compatible with additives such as urea and detergents since they can be reswollen in a solution containing the desired additives. However, these ready-made IPGs usually span in a narrow range of pH 4–7 which exclude their application in the alkaline ranges (>pH 7). With the improvements in the coming years we may expect IPG to be established as a alternative to conventional IEF.

6. Two-dimensional gel electrophoresis (2D PAGE)

6.1. General background and principles

The limitation of 1D electrophoretic separations for the analysis of complex protein mixtures is that closely spaced protein bands or peaks tend to overlap, so that any 1D separation method such as SDS-PAGE can only resolve only a relatively small number of proteins (generally fewer than 50). A new method, so called 2D PAGE, was developed in 1975 by O'Farrell [33] to overcome the shortcomings of 1D PAGE or IEF, which usually arise as a result of the co-migration of closely related polypeptides.

Combining the features of two different separation procedures, 2D PAGE can resolve more than 1000 different proteins in the form of a 2D polypeptide map [33]. In the first step, the sample is dissolved in a small volume of a solution containing a non-ionic detergent (e.g. 0.5% (w/v) NP-40), the denaturing reagent urea, and reducing agents such as dithiothreitol or mercaptoethanol. This sample buffer solution can dissolve and dissociate most of the proteins from any tissues without changing their intrinsic charges. Then the polypeptide mixture is separated by IEF as described above. In the second step, the narrow gel containing the separated proteins is soaked in SDS and the proteins are further fractionated according to the size

by SDS-PAGE on a slab-gel. Each polypeptide chain now migrates as a discrete spot on the gel according to its molecular weight. The only proteins left unresolved will be those that have both an identical size and an identical isoelectric point, a relatively rare situation.

There has been an increasing emphasis on the separation and characterization of polypeptides by 2D PAGE in recent years [34], which has been claimed to be the most sensitive and high-resolution method for the analysis of protein composition in any cell extract or protein mixture. The second dimension of SDS-PAGE can usually be run in gradient gels to even improve the resolution. For higher sensitivity, cell extract or protein mixture can be labeled with a radioisotope before running 2D PAGE. Up to 2000 individual polypeptide chains can be resolved on a single 2D gel, enough to account for most of the proteins in a bacterium.

One reason for the popularity of this method is that, because the two dimensions separate proteins on the basis of two independent parameters (their isoelectric points in one dimension and mobility in an SDS-PAGE in the other), one usually obtains an excellent resolution of even very complex protein mixtures. Although the principle of 2D PAGE is relatively simple and straightforward, methodology of 2D PAGE is generally a technically demanding, time-consuming plus skill-intensive procedure in practice. The art of running good 2D gels can only be learned from experience gained in the laboratory. It is absolutely essential to remove any particulate material by centrifuging samples at least 10 000–15 000 g for 5 min in a microcentrifuge before processing for 2D electrophoresis. Fig. 6 shows a typical 2D PAGE separation pattern for the resolution of total lens proteins from the caiman lens [35].

Some of other variations on the original method of O'Farrell exist, but for the most part they are not widely used, with perhaps just two exceptions: the use of Immobilines for the IEF dimension and the trend towards capillary gels for the first dimension [36].

6.2. Analysis and quantitation of two-dimensional gel patterns

Analysis of 2D gels in its simplest form can be carried out by superimposing one photographic image over another. However, better and more accurate

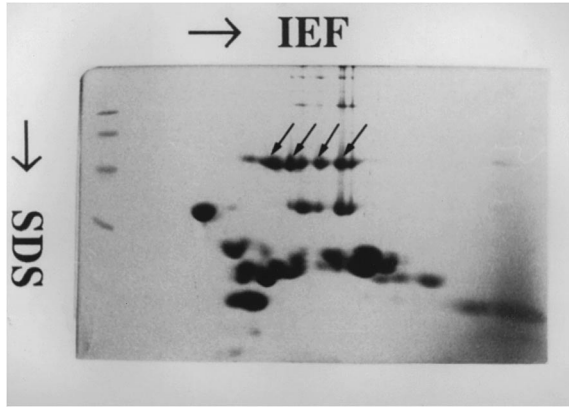

Fig. 6. 2D gel analysis of the caiman lens homogenate. About 50–100 μg of crude homogenate was layered on a 5% polyacrylamide gel for isoelectric focusing (IEF) using carrier ampholytes of pH 3.5–10 in the first dimension (basic end is to the left), followed by discontinuous SDS-gel electrophoresis in the second dimension (higher molecular weight is at the top). A concurrent run of four standard marker proteins [phosphorylase b (94), bovine serum albumin (66), ovalbumin (45) and carbonic anhydrase (30) in kDa] in the second dimension was shown on the left lane. The sample buffer was in 8 M urea and 0.1% β-mercaptoethanol. Arrows indicate the positions of δ-crystallin subunits with approximate p*I*s between 5.9 and 6.8 determined from a p*I* calibration kit (range 4.7–10.6).

results can usually be obtained by computer analysis of the gels [37]. Reproducible gels from which data can be collected on several hundred and perhaps over a thousand proteins from a single gel of one sample can now be analyzed automatically by scanning (gel scanner) and computer analysis with various versions of computer algorithms for data collection and analysis. The system must be able to compare and match up patterns from several gels to allow accurate identification of spots for quantitative analysis. With the recent advent of powerful personal computers and the associated analysis softwares, it has become increasingly obvious that 2D protein gels can now be satisfactorily and reproducibly analyzed if they are digitized and the features of protein data displayed on the gels abstracted using an automated system.

7. Conclusion and perspectives

7.1. General consideration

Traditionally protein purification and characterization occupied a central position in many areas of

biochemical research. With the recent advances in gene cloning and expression it becomes even more obvious that the need for a facile method of analyzing various proteins from different sources is always a prerequisite for structure-function studies of any proteins of interest. By the current "state-of-the-art" electrophoretic protocols, most protein components of many sources regardless of their relative abundance in crude extracts could be analyzed by various protocols of electrophoresis with ease and accuracy. Therefore in the initial stage of embarking structural characterization of biological macromolecules it is generally important to be familiar with the available electrophoretic techniques in order to find a suitable protocol for analyzing proteins of interest. In this regard the review is intended to provide a practical guide to the readers for the methods which are most commonly used in terms of the quality of results by protein researchers first exposed to this important methodology.

7.2. *Factors affecting separation: problems and precautions*

It should be emphasized that electrophoresis is mainly a qualitative technique in which only changes in electrophoretic mobility are generally interpretable. Many years after its first use, polyacrylamide gel electrophoresis (PAGE) continues to play a major role and remain the method of choice in the experimental analysis of proteins and protein mixtures.

SDS-PAGE is more powerful than any previous method of protein analysis principally because it can be used to separate any proteins regardless of their inherent solubility in aqueous solution. Membrane proteins, protein components of the cytoskeleton and proteins that are part of large macromolecular aggregates can all be resolved as separate species. Since SDS-PAGE separates proteins strictly according to their sizes, it provides information about the molecular weight and the subunit composition of any protein complex. While other physical methods can also give rise to the same information afforded by SDS-PAGE, they lack the simplicity in the procedure and short time required for SDS-PAGE. Most noteworthy is that SDS-PAGE is a relatively simple and reproducible technique which requires only micrograms of samples, yet is capable of very high resolution as it can separate proteins differing in mobility by as little as 1% (a difference in molecular mass of 1 kDa for a 100 kDa protein). It should be kept in mind that the molecular mass estimation by SDS-PAGE is a relative method which requires that molecular mass marker proteins be included with each gel run and a standard curve drawn for that particular gel under the specified conditions.

It is noteworthy that alterations of even a single amino acid may sometimes change the mobility of proteins in SDS-PAGE of Laemmli's discontinuous buffer system up to ±10% [38–40]. Therefore it is possible for proteins with identical molecular size to appear as multiple bands, which may arise from inherent amino acid differences in their primary sequences due to the high-resolution resolving power of discontinuous SDS-PAGE. The anomalous electro-

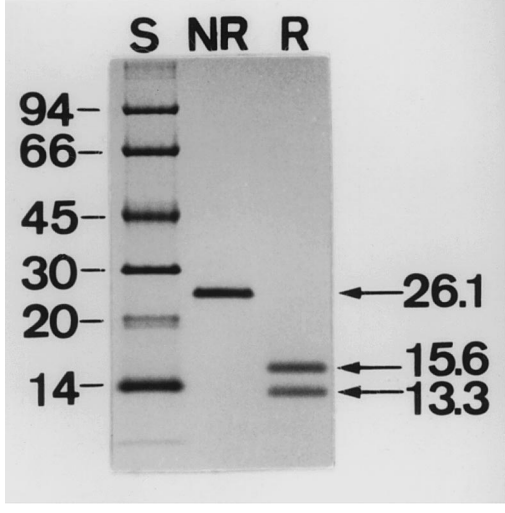

Fig. 7. SDS-gel electrophoresis (SDS-PAGE) of the purified two-chain venom factor [54] under denaturing conditions in the absence (NR lane) and presence (R lane) of 1% dithiothreitol. Lane S, relative electrophoretic mobilities of standard proteins used as molecular mass markers (in kDa): phosphorylase b (94), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (30), soybean trypsin inhibitor (20), and lysozyme (14). The gel was stained with Coomassie blue. Note that the purified factor shows a pure single band with an estimated molecular mass of 26.1 kDa, consisting of two subunits of 15.6 and 13.3 kDa upon reduction. These two stained subunit bands were electroblotted to polyvinylidene difluoride (PVDF) membrane according to the published protocol [48]. The stained protein bands were cut out and transferred to a microsequencing sequencer for N-terminal sequence determination.

phoretic mobilities of proteins were also reported in the literature, which were caused by slight variation of buffer composition such as pH or the amounts of reducing agents present in the stacking and resolving gels. Sometimes even the applied voltages during electrophoresis can affect the mobility and resolution of some proteins [41]. For example the difference of mobility difference between αA and αB subunits of α-crystallin with similar molecular mass of 20 kDa (shown as 20 and 22 kDa subunit bands in SDS-PAGE of Laemmli's system) can be eliminated by either adding urea [38] to the gel or lower the applied voltage from 150–200 to 50–100 V during electrophoresis [41]. It appears that the disappearance of mobility difference for homologous polypeptides may be due to the equalization of frictional velocities of SDS–protein complexes in the gel matrix resulting in less molecular sieving effect for proteins in the presence

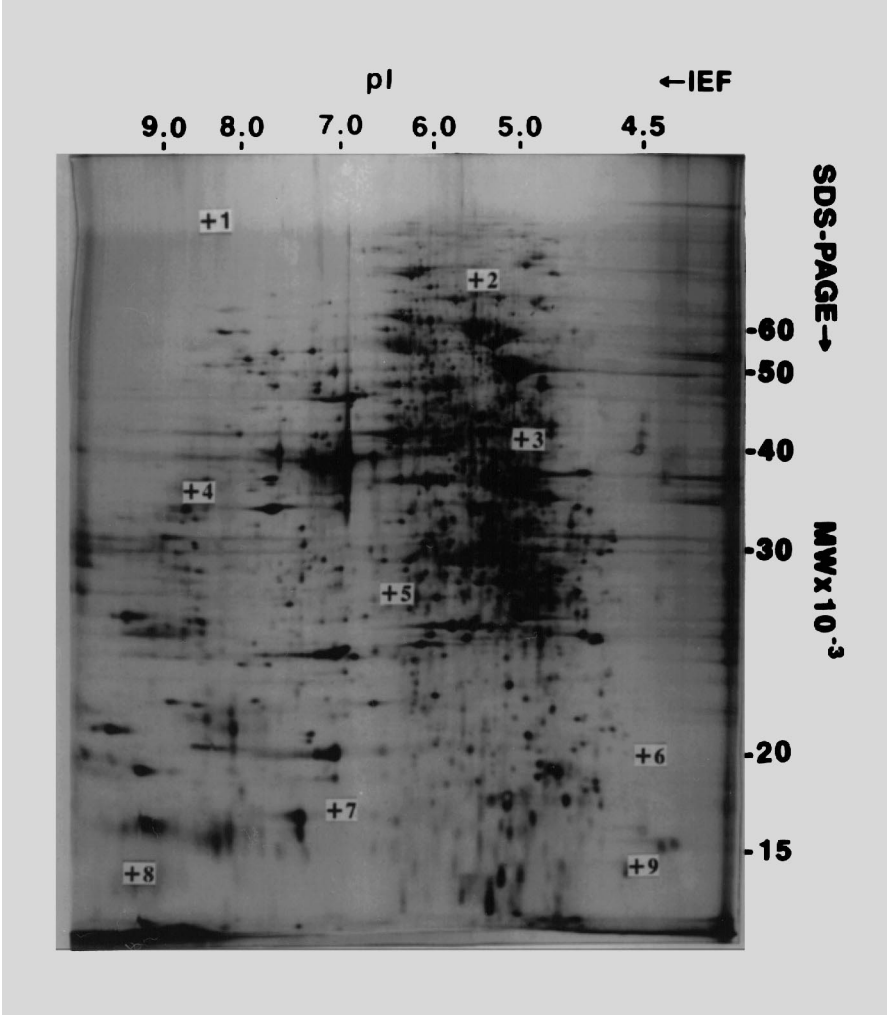

Fig. 8. 2D gel analysis of the proteins from *Fusarium sporotrichioides*, a fungus strain of plant pathogens. An immobiline dry strip consisting of a linear pH gradient (range 3.0–10.5) was used for isoelectric focusing (IEF) in the first dimension. SDS-gel electrophoresis in the second dimension was carried out without the stacking gel. The gel was subjected to silver staining. The labeled numbers indicate nine known proteins used as internal standards for calibration of 2D coordinates of protein spots using laser scanner and computer data analysis and storage. From this gel 1244 protein spots were well resolved and 103 spots were electroblotted to PVDF membrane for N-terminal sequencing analysis [55].

of urea or lower voltages. Therefore despite the advantage of high-resolution power of SDS-PAGE using Laemmli's system caution is warranted in the interpretation of the number and molecular masses of protein subunits. The most serious disadvantage of SDS-PAGE in general is that after electrophoresis it is difficult to characterize the separated proteins in terms of their enzymatic or biological activities since it may be difficult or impossible to remove SDS after PAGE.

Although the electrophoretic methods described earlier will separate the vast majority of cellular proteins, particular classes of proteins tend to be insoluble under the usual buffer conditions and so need special electrophoretic systems for their fractionation. Histone proteins are usually separated by an acetic acid–urea buffer system to detect charge variants [42]. Nuclear non-histone proteins need SDS and urea for complete dissolution [43]. Other proteins which require the combined use of SDS and urea include ribosomal proteins and some integral membrane proteins [3 and references therein].

7.3. Current status and future perspectives

The five major electrophoretic techniques described have changed relatively little in practice for the past two decades but there have been considerable advances in the detection and analysis of proteins separated by polyacrylamide gel electrophoresis, for example, sensitive silver [44,45] and immuno-staining protocols [36,46].

One of the major recent advances in the analysis of proteins after polyacrylamide gel electrophoresis has been the development of techniques for the electrophoretic transfer of the separated proteins from the gel to a thin support matrix (so called electroblotting or Western blotting), most commonly a nitrocellulose membrane, to which they bind and are immobilized. Under ideal conditions, over 90% of the protein can be transferred. The most important advantage of electroblotting is that it transfers the separated proteins from a gel matrix (which hinders protein analysis) to the surface of a filter sheet where the protein molecules are readily accessible. Indeed some analyses can be carried out easily only by using Western blots rather than gels. Currently a whole range of membrane blotting methodology [47,48] is developed which can be applied to 1D (Fig. 7, including SDS-PAGE, IEF and native gels) and 2D PAGE (Fig. 8) for the electroblotting of minor protein bands or spots directly for sequence analysis when coupled with the sensitive microsequencing and mass spectrometry [49–53]. These later developments in 20 page have laid a firm methodological basis in the quantitative analysis and protein mapping of genome-encoded proteins, i.e., the emerging field of Proteomics.

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