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Proteins – structure, properties and their separation by SDS-polyacrylamide gel electrophoresis

Vikas Kumar Roy[1], N. Senthil Kumar[2] and G. Gurusubramanian[1*]

[1] *Department of Zoology;* [2] *Department of and Biotechnology, Mizoram University, Aizawl 796 004, India*

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Abstract

This paper deals with three basic aspects: 1) basics of protein structure in relation to physico-chemical characterization, protein stability, importance of disulfide bond and their structure; 2) basic principles involved in polyacrylamide gel electrophoresis (PAGE) and SDS-PAGE and the rationale behind the use of disc electrophoresis; and 3) detailed protocol involved in SDS-PAGE in terms of reagent preparation, sample preparation and loading, staining and destaining, calculation of relative mobility and pairwise comparisons between species.

Key words: Amino acid; electrophoresis; PAGE; protein; structure .

Introduction

Proteins typically make up more than half the dry weight of cells. They contribute to the structure of a cell and are responsible for cellular functions such as catalysis and molecular recognition. Phenotype is determined by the proteins and not by the genes (i.e. genotype). Efforts are now being directed towards the characterization of the proteome, or the complete set of proteins found in a cell, tissue or organism.[1] The proteome is a dynamic entity in that genes are expressed at specific times and places and environmental conditions will also influence gene expression. Furthermore, many proteins are found in specific subcellular compartments or modified post-translationally (e.g. phosphorylation, glycosylation, acylation, proteolysis, etc.). The subcellular location of a protein as well as the post-translational modifications will have an impact on cellular phenotype. Proteomics deals with techniques for a complete understanding of protein function and their applications (Fig. 1).

Corresponding author: Gurusubramanian
Phone: +91-9862399411 Fax: 0389 - 2330834/2330644
E-mail: gurus64@yahoo.com

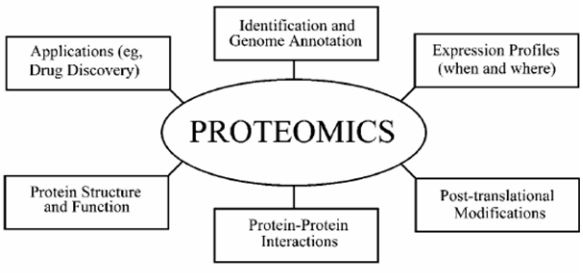

Figure 1. Entities of proteomics.

Protein function is also dependent on protein -protein interactions and resolving these networks of protein-protein interactions is also central to understanding protein function and cellular phenotype. Knowing the structure of proteins also contributes to understanding protein function. Protein structure can be determined by X-ray crystallography and biophysical techniques. Some elements of protein structure can also be inferred from the protein sequence and comparing these sequences to related proteins in which the structures have been determined. The information about a protein's structure and function can then be used in applications such as drug development[2].

PROTEIN STRUCTURE

Proteins are polymers of L-α-amino acids. The α refers to a carbon with a primary amine, a carboxylic acid, a hydrogen and a variable side-chain group, usually designated as 'R' (Fig. 2). Carbon atoms with four different groups are asymmetric and can exhibit two different arrangements in space due to the tetrahedral nature of the bonds. The L refers to one of these two possible configurations the four different groups on the α-carbon can exhibit. Amino acids of the D-configuration are not found in proteins and do not participate in biological reactions.[3]

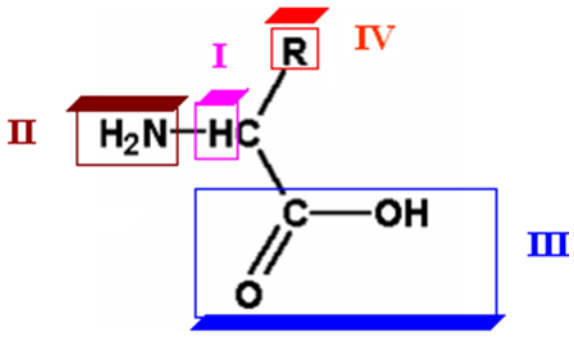

Figure 2. Arrangement of four groups with a carbon atom in an amino acid.

Polar and non-polar amino acids and hydrophobicity

Twenty different amino acids, distinguished by their side-chain groups, are found in proteins (Table 1). The side-chain groups vary in terms of their chemical properties such as polarity, charge and size. These various side-chain groups influence the chemical properties of proteins as well as determine the overall structure of the protein. For example, the polar amino acids tend to be on the outside of the protein where they interact with water and the non-polar amino acids are on the inside forming a hydrophobic core. Hydrophobicity is a chemical prop-

Table 1. Categories of L-α-amino acids.

L-α-AMINO ACIDS	
Nonpolar	**Polar**
Alanine (A[#])	Arginine (R[*])
Glycine (G) - Conformational flexibility	Asparagine (N) - Do not ionize
Isoleucine (I[#])	Aspartic acid (D[@])
Leucine (L[#])	Cysteine (C) Participate in redox reactions and can form disulfide links
Methionine (M) Hydrophobic, but the thioether group is a potent nucleophile	Glutamic acid (E[@])
Phenylalanine (F[§])	Glutamine (Q)- Do not ionize
Proline (P) – Rigid, affects on protein conformation	Histidine (H) Active site of many enzymes, as well as binding metal ions.
Tryptophan (W[§])	Lysine (K[*])
Valine (V[#])	Serine (S[#])
	Threonine (T[#])
	Tyrosine (Y[§])

[#] Hydrophobic interactions and provide for a variety of surfaces and shapes.
[§] Hydrophobic interactions and interacting with other flat molecules.
[@] Negatively charged at neutral pH (pKa = 4.3-4.7).
[*] Positively charged at neutal pH (pKa >10).
[#] Weakly ionizable (pKa~13) and participate as active groups in some enyzmes.

erty which promotes the aggregation of nonpolar compounds with each other in an aqueous environment. These hydrophobic interactions are not an attractive force per se, but are forced upon non-polar compounds in a polar environment.

Peptide bond and shape of a protein

The covalent linkage between two amino acids is known as a peptide bond. A peptide bond is formed when the amino group of one amino acid condenses with the carboxyl group of another amino acid to form an amide (Fig. 3). This arrangement gives the polypeptide chain a polarity in that one end will have a free amino group, called the N-terminus, and the other end will have a free carboxyl group, called the C-terminus.

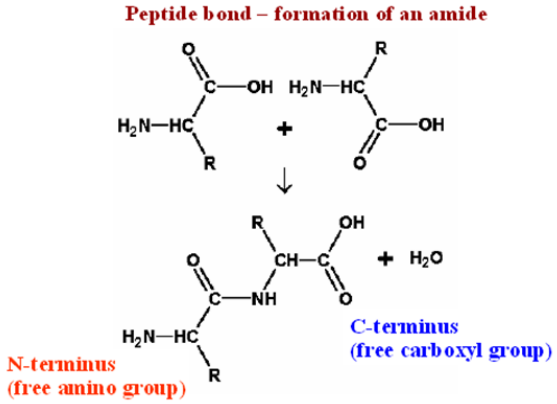

Figure 3. Formation of a peptide bond.

Peptide bonds tend to be planar which gives the polypeptide backbone some rigidity. However, rotation can occur around both of the α-carbon bonds resulting in a polypeptide backbone with different potential conformations in regards to the relative positions of the R-groups. Interactions between the R-groups will limit the number of potential conformations and proteins tend to only fold into a single functional conformation. In other words, the conformation or shape of the protein is due to the interactions of the side-chain groups with one another and with the polypeptide backbone. The interactions can be between amino acids that are close together in a polypeptide or between amino acids that are far apart or even on different polypeptides. These different types of interactions are often discussed in terms of primary, secondary, tertiary and quaternary protein structure (Table 2).

Table 2. Levels of protein structure.

Interactions between amino acids within and different polypeptides	
Primary	Refers to the amino acid sequence and the location of disulfide bonds between cysteine residues (i.e. covalent bonds).
Secondary	Refers to interactions between amino acids that are close together (e.g. α-helix, β-sheet, β-turn, random coil).
Tertiary	Refers to interactions between amino acids that are far apart (e.g. motifs, domains).
Quaternary	Refers to interactions between two or more polypeptide chains (i.e. protein subunits).

Levels of Protein Structure

Primary and secondary structure

The primary amino acid sequence (Fig. 4) and positions of disulfide bonds strongly influence the overall structure of protein. In regards to the primary amino acid sequence, certain side chains will permit, or promote, hydrogen-bonding between neighboring amino acids of the polypeptide backbone resulting in secondary structures such as β-sheets or α-helices (Fig. 5). Alternatively, certain R-groups may interfere with each other and prevent certain conformations.

In the α-helix conformation the peptide backbone takes on a 'spiral staircase' shape which is stabilized by H-bonds between carbonyl and amide groups of every fourth amino acid residue (Fig. 5). This restricts the rotation of the bonds in the peptide backbone resulting in a rigid struc-

EAVDLVENKKYEEALEKYNKIISFGNPSAMIYTKRA-SILLNLKRPKACIRDCTEALNLNV

Figure 4. Primary structure of a protein.

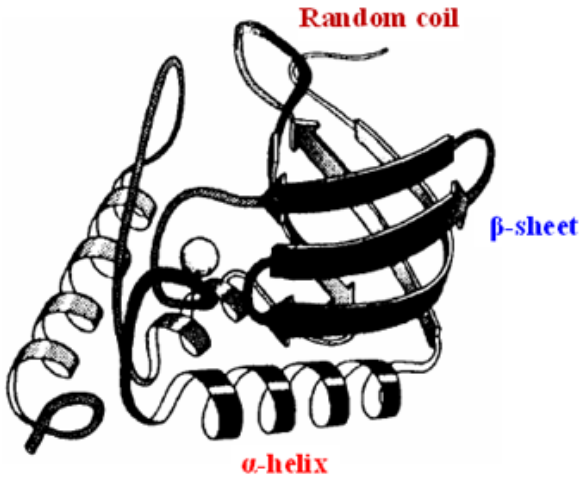

Figure 5. Secondary and tertiary structure of a protein.

ture. β-sheets are also rigid structures in which the polypeptide chain is nearly fully extended with the R-groups alternating between pointing up and down. β-sheets interact either in parallel (both with same orientation in regards to N- and C-termini) or anti-parallel fashion (Fig. 5). Certain amino acids promote the formation of either α-helices or β-sheets due to the nature of the side-chain groups. Some side-chain groups may prevent the formation of secondary structures and result in a more flexible polypeptide backbone, which is often called random coil conformation (Fig. 5).

Disulfide bond

The other aspect of primary protein structure is the position of disulfide bonds. The amino acid cysteine has a free thiol group that can be oxidized to form a covalent bond with another cysteine (Fig. 6). These disulfide bonds can form between cysteine residues that are relatively close or far apart within a single polypeptide chain, or even between separate polypeptide subunits with a protein. In this regard, disulfide bonds can contribute to secondary, tertiary and quaternary aspects of protein structure. Proteins containing disulfide bonds will be sensitive to reducing agents (such as β-mercaptoethanol) which can break the disulfide bond.

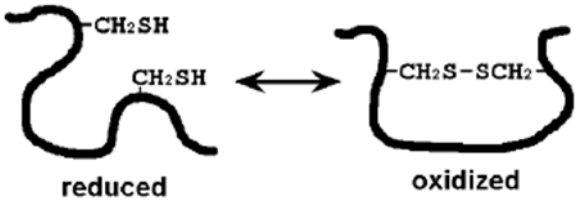

Figure 6. Formation of disulfide bond.

Tertiary and quaternary structure

The various secondary structures can interact with other secondary structures within the same polypeptide to form motifs or domains (i.e. tertiary structure). A motif is a common combination of secondary structures and a domain is a portion of a protein that folds independently. The tertiary structure will represent the overall three dimensional shape of a polypeptide (Fig. 5).

A typical protein structure is a compact entity composed of the various secondary structural elements with protruding loops of flexible (i.e., random coil) sequence. This is often depicted in a ribbon diagram (Fig. 5) in which β-sheets are drawn as flat arrows with the arrowhead representing the N-terminal side and α-helices are drawn as flat spirals. The flexible loops are represented by the strings connecting the secondary structural elements (random coil). Many proteins are composed of multiple subunits,or distinct polypeptide chain that interact with one another. This is referred to as quaternary structure.

Protein Stability

Proteins are often fragile molecules that need

Figure 7. Protein denaturation.

to be protected during purification and characterization. Protein denaturation refers the loss of protein structure due to unfolding (Fig. 7). Maintaining biological activity is often important and protein denaturation should be avoided in those situations. Elevated temperatures, extremes in pH, and changes in chemical or physical environment can all lead to protein denaturation (Table 3). In general, things that destabilize H bonding and other forces that contribute to secondary and tertiary protein structure will promote protein denaturation. Different proteins exhibit different degrees of sensitivity to denaturing agents and some proteins can be re-folded to their correct conformations following denaturation.

The optimal conditions for maintaining the stability of each individual protein need to be determined empirically. In general, though, protein solutions should be kept cold (< 4°C) except during assays and other procedures requiring specific temperatures. Many proteins are especially labile and need to be stored at -20°C or -80°C. However, repeated freezing and thawing of protein solutions is often deleterious. Adding 50% glycerol to storage buffers will lower the freezing point and allow storage at -20°C. Solutions for working with proteins will often contain heavy-metal chelators and/or antioxidants as protectants. In addition, proteases may be released during cell disruption and it may therefore be necessary to include protease inhibitors (Table 3).

Table 3. Factors affecting protein stability.

Factor	Possible Remedies
Temperature	Avoid high temperatures. Keep solutions on ice.
Freeze-thaw	Determine effects of freezing. Include glycerol in buffers. Store in aliquots.
Physical denaturation	Do not shake, vortex or stir vigorously (Protein solutions should not foam.)
Solution effects	Mimic cellular environment: neutral pH, ionic composition, etc.
Dilution effects	Maintain protein concentrations > 1 mg/ml as much as possible.
Oxidation	Include 0.1-1 mM DTT (or β-ME) in buffers.
Heavy metals	Include 1-10 mM EDTA in buffers.
Microbial growth	Use sterile solutions, include anti-microbials, and/or freeze.
Proteases	Include protease inhibitors. Keep on ice.

ELECTROPHORESIS

Electrophoresis is an analytical tool to examine the movement of charged molecules in an electric field. It is capable of analyzing and purifying different types of bio molecules especially proteins. In this technique, a polymerized gel like matrix can be used as a support medium. The migration of molecules in influenced by three parameters, namely 1) applied electric field, 2) gel matrix and 3) the size, shape, charge and chemical composition of the molecules to be separated.

Theory of electrophoresis

The charged particle moves at a velocity that depends directly on the electrical field (E) and charge (q) but, inversely on the friction of the molecules.

$$v = Eq/f$$

where, E= electric field (volts/cm); q= net charge on the molecules; f= frictional coefficient of the mass and shape of the molecule and v= velocity/mobility of the molecule.

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Gels are formed from long polymers in a cross-linked lattice (Fig. 8). The space between the polymers is the pores. Higher concentrations of the polymer will result in smaller average pore sizes. Polyacrylamide gels are formed by covalently cross-linking acrylamide monomers with bis-acrylamide with a free radical like persulfate ($SO_4^{\cdot}$). The cross-linking of the acrylamide polymers results in 'pores' of a defined size. The total acrylamide concentration and the ratio of bis-acrylamide to acrylamide will determine the average pore size. Polyacryalamide gels are prepared by polymerization of acrylamide and cross linking agents N,N'-methylene-bis-acrylamide. The polymerization reaction is controlled by a catalysts ammonium persulphate (APS) and N, N, N', N'-tetramethylethylenediamine (TEMED). The resolving power and molecular size range of a gel depends on the concentration of acrylamide and bis-acrylamide. Lower concentration of acrylamide and bis-acrylamide give gels with larger pores allowing higher molecular weight proteins. In contrast, higher concentration gives gel with smaller pores allowing analysis of low molecular weight proteins. Polymerization of gel is influenced by concentration of APS and TEMED.

Since the gel is solid with respect to the mold, all molecules are forced through the gel. Smaller molecules will be able to pass through this lattice more easily resulting in larger molecules having a lower mobility than smaller molecules. In other words, the gel acts like a molecular sieve and retains the larger molecules while letting the smaller ones pass through. Therefore, the frictional coefficient is related to how easily a protein passes through the pores of the gel and size will be the major determinant of the mobility of molecules in a gel matrix. Protein shape and other factors will still affect mobility, but to a lesser extent. Substituting size for the frictional coefficient results in:

mobility = (voltage)(charge)/(size)

In other words, the mobility of a protein during gel electrophoresis is primarily a function of its charge/mass ratio.

Discontinuous or disc electrophoresis

Discontinuous gels consist of two distinct gel regions referred to as stacking gel (upper layer - 1/3 portion) and separating/resolving gel (bottom layer - 2/3 portion) (Table 4 and Fig. 9)

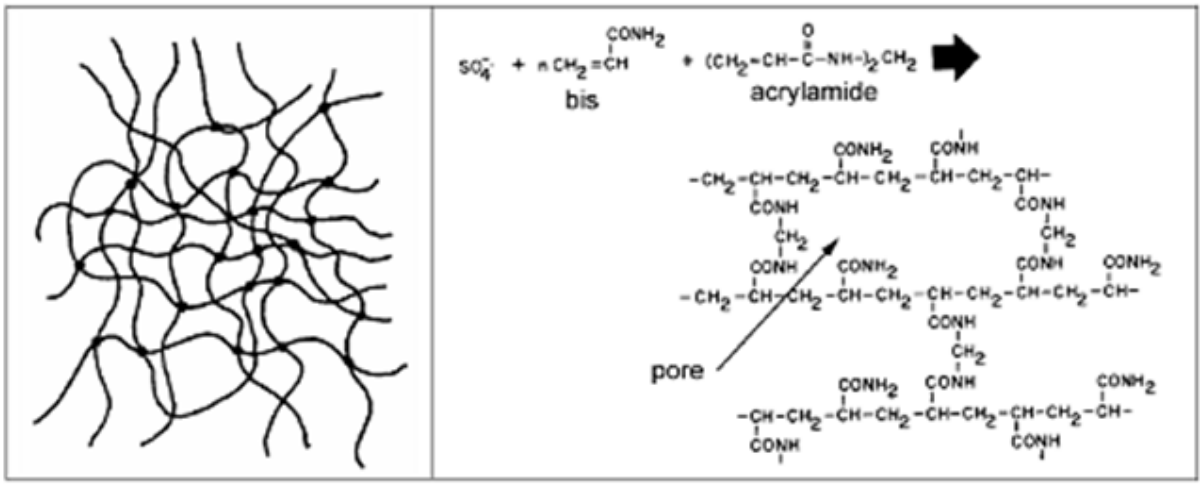

Figure 8. Diagrammatic representation of gels. Gels are formed by cross-linking polymers into a 3-dimensional meshwork or lattice (left). This results in a porous semisolid material that solutions can pass through.

Table 4. Composition of gels and separation of protein

	Stacking Gel	Separating Gel
Acrylamide	3-4.5%	6-20%
pH	6.8	8.8
Ionic Strength	0.125 M Tris	0.375 M Tris
Acrylamide Concen. (%)	Range of separation of protein (kD)	
15	12-13	
10	16-68	
7.5	36-94	
5.0	57-212	

and a Tris-glycine tank buffer. The buffers used to prepare the two gel layers are of different ionic strengths and pH. The stacking gel has a lower acrylamide concentration (larger pore size -3-4.5%), a lower pH (6.9) and a lower ionic strength than the separating gel whereas the resolving gel has a higher pH (8-9) and a higher acrylamide concentration (smaller pore size-6-20%).

RATIONALE BEHIND THE USE OF RESOLVING AND STACKING LAYERS IN A SLAB GEL

The sample is usually dissolved in glycine chloride buffer (pH-8-9) before loading on the gel. The glycine exists primarily in two forms, i.e. zwitterions at low pH (6.9-stacking gel) and anion at high pH (8-9-resolving gel).

Stacking gel

The sample pH is greater than the pH of stacking gel. After loading the sample on to the well of gel, the protein molecules present in the sample are in dispersed state and in anionic form. When electric field is applied on the gel, the glycine-chloride buffer ions and sample move in to the stacking gel which has pH of 6.9. In this pH, the glycine ion is in the form of zwitterion with net charge zero and no electrophoretic mobility. But the chloride ion and sample are in anionic form at pH 6.9 and act as mobile ions. The sample will tend to accumulate and

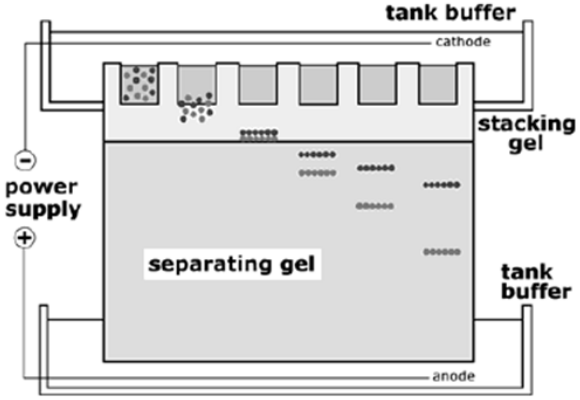

Figure 9. Discontinuous gel electrophoresis.

form a thin, concentrated band sandwiched between chloride and glycinate. The chloride ion and protein carry the most of the current.

Resolving gel

The ionic front of concentrated band reaches the resolving gel with pH 8-9, the zwitterionic glycine is changed in to anionic form. In resolving gel anionic glycine and chloride carry most of the current. The proteins present in the sample encounter with high pH and smaller pore size. The increase in pH would tend to increase electrophoretic mobility but, smaller pores decrease mobility. Hence, the relative rate of movement of protein in lesser than chloride and glycine. This process lead to better separation of proteins based on charge/mass ratio and a discrete size and shape.

Separation of proteins

The lower ionic strength of the stacking gel results in a greater local electric field strength than in the resolving gel. The field strength difference combined with the lower acrylamide concentration results in proteins having a higher mobility in the stacking gel than in the resolving gel. In addition, the glycine in the tank buffer has a higher mobility in the resolving gel than in the stacking gel because of the pH differences (Table 5). Therefore, proteins will migrate faster

Table 5. Separation of proteins disc electrophoresis.

Stacking gel	Resolving gel
Low ionic strength, low pH, large pore size- low acrylamide concentration	High ionic strength, high pH, small pore size – high acrylamide concentration
Increase in local electric field strength	Decrease in local electric field strength
Increase the protein mobility	Decrease the protein mobility
Compression of protein between glycinate and chloride	Charge/mass ratio high- more charge and low mass - High mobility of protein
	Charge/mass ratio low- less charge and high mass - low mobility of protein
	Increase in resolution

than the glycine in the stacking gel. When proteins reach the resolving gel their mobility is decreased because of the increased acrylamide concentration and decreased field strength, whereas the increase in pH results in glycine having a higher mobility. All of these factors result in the proteins becoming compressed at the interface between the two gels and thus increasing resolution (Fig. 9 and Table 5). Resolution in non-discontinuous electrophoresis depends partially on the volume of the sample. However, stacking also occurs at the interface of the sample and gel, especially if a high voltage is applied.

SODIUM DODECYL SULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Principle

SDS [$C_{12}H_{25}NaO_4$] completely disrupts protein-protein interactions and denatures almost all proteins resulting in a complete unfolding of proteins. When protein molecules are treated with SDS, the detergent disrupts the secondary, tertiary and quaternary structure to produce linear polypeptide chains coated with negatively charged SDS molecules. The SDS binds to hydrophobic regions of denatured protein chain in constant ratio of about 1.4 g of SDS per gram of protein. The bound SDS molecules carrying negative charges mask the native charge of the protein. In addition, β-mercaptoethanol is often used to break disulfide bonds. The SDS binds to the unfolded proteins giving all proteins a similar shape (i.e. random coil or extend conformation) and a uniform charge-to-mass ratio (Fig. 10). In other words, coating proteins with a negatively charged detergent minimizes the effects of a protein's net charge. The polypeptide chains of a constant charge/mass ratio and uniform shape are produced after the treatment of SDS and mercaptoethanol. The electrophoretic mobility of the SDS-protein complexes is influenced primarily by molecular size: the larger molecules are retarded by molecular sieving effect of the gel and the smaller molecules have the greater mobility (Fig. 10). Therefore, during electrophoresis in the presence of SDS the mobility of a protein now depends primarily upon its size (i.e. mobility is inversely proportional to protein mass).

Steps involved in SDS-PAGE

1) sample preparation, 2) pouring of separating gel, 3) pouring of stacking gel, 4) loading of samples, 5) Applying electric field, 6) staining of gel, 7) destaining of gel and 8) calculation of molecular mass of proteins and relative mobility (R_f value).

Proteins to be analyzed by SDS-PAGE are solubilized in a sample buffer that typically contains 2% SDS and 5% β-mercaptoethanol and then boiled. The reducing agent is omitted in

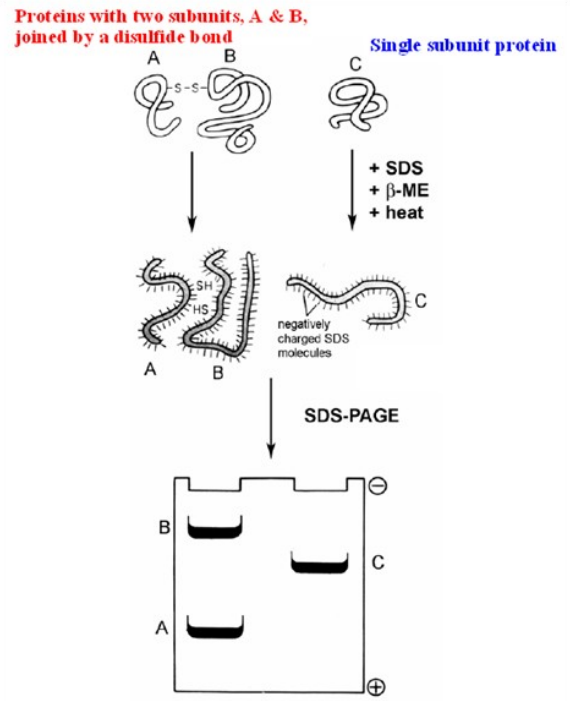

Figure 10. Separation of protein by SDS-PAGE.

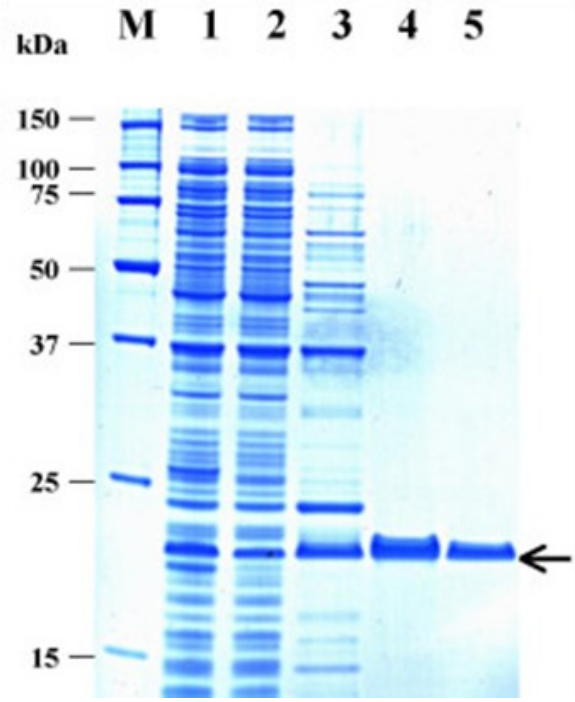

Figure 11. Detection using Coommassie blue staining.

situations where disulfide bonds need to be preserved. In situations where an enzyme activity will be measure following electrophoresis a lower SDS concentration is used and the sample is not boiled.

The amount of protein that can be loaded onto a gel is limited. Overloading the gels results in the pores becoming plugged and has an adverse effect on the electrophoresis. After loading the samples into the wells of the gel an electric field is applied across the gel. Electrophoresis is usually carried out under constant voltage or constant power to minimize the resulting increase in heating that occurs during electrophoresis. A tracking dye (bromophenol blue) is included in the sample. When this dye reaches the bottom of the gel or some predetermined time afterwards the power is turned off and the proteins detected. A common way to detect proteins after electrophoresis is to stain the gel with Coomassie blue, a dye that binds proteins (Fig. 11). Gels are usually 'fixed' before staining with an acetic acid and methanol solution which precipitates proteins into the acrylamide matrix.

Calculation of relative mobility and percent commonality

Mobility in SDS gel electrophoresis is expressed as a relative mobility (Rf) (Fig. 12). The distance the protein migrated is compared to the length of the gel, or:

Rf = distance protein migrated ÷ gel length

Calculating percent commonality- pairwise comparisons between species:

% of proteins in common ÷ Total % of unique proteins x 100

PROTOCOL FOR SDS PAGE

Reagents preparation

Acrylamide and bisacrylamide – 30% (electrophoresis grade, free from metal ions)

- 29 g Acrylamide and 1 g bisacrylamide dissolve in 100 ml of distilled water.

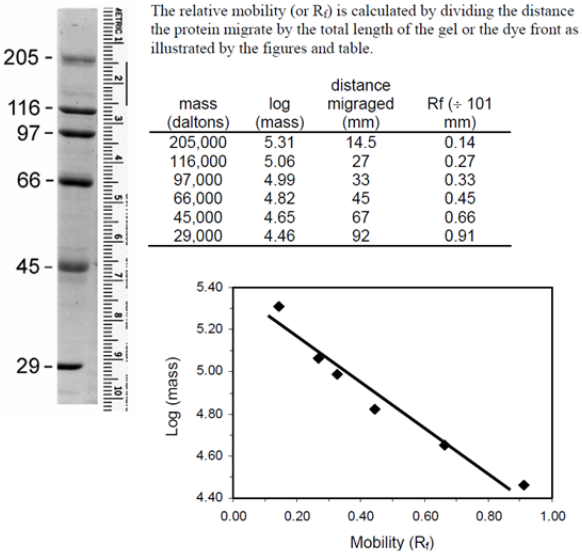

Figure 12. Calculation of relative mobility.

- [Store in dark bottle at 4°C; prepare fresh and use the solution within a month (on storage acrylamide and bisacrylamide convert into acrylic acid and bisacrylic acid); acrylamide and bisacrylamide are potent neurotoxins; pH of the solution should be 7 or <7].

SDS – 10% (electrophoresis grade)

- Dissolve 10 g of SDS in 100 ml of distilled water. Store at room temperature.

Tris buffers (use only TRIS base and no TRIS-CHLORIDE/TRIZMA)

- 1.5 M Tris (pH 8.8) – 100 ml – Resolving gel;
- 1.0 M Tris (pH 6.8) – 100 ml – Stacking gel;
- 1.0 M Tris (pH 7.6) – 500 ml – sample preparation.

TEMED – accelerates polymerization.

Ammonium per sulfate (APS) – 10% - provides free radicals for polymerization.

- Dissolve 0.1 g of APS in 1 ml of distilled water. [Store at 4°C and prepare fresh solution].

Phenylmethylsulfonyl fluoride (PMSF)-100mM

- Dissolve 17.42 mg in 1 ml of isopropanol [Prepare fresh, Store at -20°C]

NaCl - 1.0 M – 100 ml

- Dissolve 5.844 g in 100 ml of distilled water.

PBS (pH 7.4) - 0.2 M

Solution A : 0.2 M solution of monobasic sodium phosphate

- Dissolve 13.799 g of $NaH_2PO_4.H2O$ in 500 ml of distilled water.

Solution B: 0.2 M solution of dibasic sodium phosphate

- Dissolve 14.196 g of Na_2HPO_4 in 500 ml of distilled water.
- [Add 81 ml of solution B + 19 ml of solution A + 30 ml of 1.0 M NaCl and adjust volume to 200 ml].

Ethylene diamine tetra acetic acid (EDTA)-100 mM (pH 8.0) - 50 ml

- Dissolve 2.92 g in 50 ml of water, stir vigorously and adjust pH with NaOH.
- [The disodium salt of EDTA will not dissolve until the pH is adjusted to pH nearly 8.0]

NaOH- 1.0 M - 100 ml

- Dissolve 4 g of NaOH in 100 ml of water

Sample preparation buffer

Stock	Qtity taken for 1 ml	Final concentration
1.0 M NaCl	100 µl	0.1 M NaCl
1.0 M Tris Cl (pH 7.6)	10 µl	0.01 M Tris Cl (pH 7.6)
0.1 M EDTA (pH 8.0)	10 µl	0.001 M EDTA (pH 8.0)
100 mM PMSF	10 µl	1 mM PMSF
1 µg/ml Aprotinin	10 µl	0.01 µg/ml Aprotinin
Adjust the volume to 1 ml with distilled water		

- [PMSF extremely toxic, should be added from the stock solution just before the sample preparation buffer (Half life is 35 minutes at pH 8.0)]

SDS gel loading buffer - 2X

Stock	Qtity taken for 1 ml	Final concentration
1 M Tris Cl (pH 6.8)	100 µl	100 mM TRIS Cl (pH 6.8)
2-mercaptoethanol	20 µl	2% v/v - 2-mercaptoethanol
10% SDS	400 µl	4% SDS
Bromophenol blue	0.2 mg/ml	0.02% Bromophenol blue
Glycerol	200 µl	20% Glycerol
Adjust the volume to 1 ml with distilled water		

- [SDS gel loading buffer lacking mercaptoethanol can be stored at room temperature].

Tris-Glycine electrophoresis buffer/running buffer

- 5X Tris glycine buffer containing 25 mM Tris base, 250 mM glycine and 0.1% SDS.
- [Dissolve 15.1 g of Tris base and 94 g of glycine in 900 ml of distilled water. Finally, add 50 ml of 10% SDS and adjust the volume to 1000 ml with distilled water].

Stacking gel (5%) - For 5 ml

Distilled water	3400 µl
30% acrylamide-bisacrylamide mix	830 µl
1.5 M Tris (pH 6.8)	630 µl
10% SDS	50 µl
10% APS	50 µl
TEMED	5 µl

Resolving gel (10%) - For 10 ml

Distilled water	4000 µl
30% acrylamide-bisacrylamide mix	3300 µl
1.5 M Tris (pH 6.8)	2500 µl
10% SDS	100 µl
10% APS	100 µl
TEMED	4 µl

Coommassie Brilliant Blue staining solution (0.2%)

CBB R250	0.2 g
Methanol	45 ml
Glacial acetic acid	10 ml
+ Distilled water to make 100 ml	

Destaining solution

Methanol	10 ml
Glacial acetic acid	20 ml
Distilled water	70 ml

Sample preparation

- Wash the tissue fragments or cells through in PBS (ice cold).
- Centrifuge at 3000 g for 5 min at 4°C.
- Remove the supernatant and disperse the tissue fragments/cells in 5 volume of ice cold sample preparation buffer.
- Add equal volume of 2X SDS gel loading buffer.
- Boil the sample in water bath for 10 min.
- Centrifuge the sample at 10,000 g for 10 min. at room temperature.
- Transfer the supernatants to a fresh tube and discard the pellet.

Sample loading

- Mount the gel in the electrophoresis apparatus.
- Add Tris glycine electrophoresis buffer to the top and bottom reservoirs.
- Remove any bubbles that become trapped at the bottom of the gel between the glass plates.
- Do not pre-run the gel before loading.
- Load 15-25 µl of the sample into the bottom of the wells.
- Load an equal volume of 1X SDS gel loading buffer into any wells that are unused.
- Connect the electrophoresis apparatus to an electric power supply (positive red electrode should be connected to the bottom reservoir and negative black electrode to top one).
- Apply a voltage of 100V to the gel (8V/cm).
- After the bromophenol dye front reaches into the resolving gel increase the voltage

to 15V/cm.
- Run the gel until the bromophenol blue reaches the bottom of the resolving gel.

Staining and destaining of the gel

- Stain the gels for 10 minutes with 0.2% Coomassie-blue G-250. Gels thicker than 0.75 mm may need to be stained longer.
- Destain the gel with several changes of the destain solution over the next 10-15 minutes. (Works best if the first 1-2 washes carried out in the first 5-10 minutes).

Calculation of molecular mass from SDS gels.

- Molecular masses of proteins can be estimated by comparing the migration of proteins of interest to standards of known size.
- The relative mobilities of the standards are plotted against the log of their molecular masses.
- The sizes of unknown proteins are then extrapolated from the standard curve.

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