BT 510 Analytical Biotechnology Lab

SDS-PAGE of protein

THEORY/PRINCIPLE:

Electrophoresis is the process of migration of charged molecules in response to an electric field. The rate of migration depends on the net charge, size and shape of the molecule, the voltage gradient of the electric field E, and the frictional resistance of the supporting medium f, which impedes their movement. Proteins have a net charge at any pH other than their isoelectric point (pI), thus when placed in an electric field, proteins will migrate towards the electrode of the opposite charge. This principle is used to separate molecules of differing charges.

Electrophoresis in acrylamide gels is referred to as Polyacrylamide gel electrophoresis (PAGE). Polyacrylamide gels which were first used for electrophoresis by Raymond &Weintraub (1959) are chemically inert and particularly stable. By chemical copolymerization of acrylamide monomers with cross linking reagent N-N'-methylene bisacrylamide a clear transparent gel which exhibits little endosmosis is obtained.

The polymerization of acrylamide is an example of free radical catalysis and is initiated by the addition of Ammonium per sulfate and a catalyst N,N,N'N'-Tetramethylenediamine(TEMED).TEMED catalyses the decomposition of the persulfate ion to give a free radical

$$S_2O_8^{2-} + e^- \rightarrow SO_4^{2-} + SO_4^-$$

If this free radical is represented as $R^\cdot$ (where the dot represents an unpaired electron) and M as an acrylamide monomer molecule than the polymerization can be represented as follows.

$$R^\cdot + M \rightarrow RM^\cdot$$

$$RM^\cdot + M \rightarrow RMM^\cdot$$

$$RMM^\cdot + M \rightarrow RMMM^\cdot$$

In this way long chains of acrylamide are built up being crosslinked by introduction of bisacrylamide forming a mesh like structure in which the holes of the mesh represent the pores. Overall protein mobility through polyacrylamide gel is proportional to the pore size which is a function of both the acrylamide concentration (%T) and that of bisacrylamide crosslinker (%C.).In general the pore size is inversely proportional to %T.

$$\%T = \frac{\text{Acrylamide (g)} + \text{Bisacrylamide (g)} \times 100\%}{100 \text{ ml}}$$

$$\%C = \frac{\text{Bisacrylamide(g)} \times 100\%}{\text{Acrylamide (g)} + \text{Bisacrylamide (g)}}$$

%T gel	Mr range
5-12	20,000-150,000
10-15	10,000-80,000
>15	<15,000

The proteins may be run in denaturating conditions in presence of SDS or in native condition devoid of denaturants called as native- PAGE of proteins.

In native or non-denaturing gel electrophoresis SDS is not used and the proteins retain their native structure and enzymatic activity. Although the resolution is not as high as that of SDS-PAGE but the technique is useful when the enzymatic activity of a protein need to be assayed following electrophoresis. The migration of proteins in non-denaturating gel is due to both the net charge and the size of the protein.

SDS-PAGE is the most commonly used gel electrophoretic system for analyzing proteins. This method is based on the separation of proteins according to size and can also be used to determine the relative molecular mass of proteins. SDS is an anionic detergent which binds strongly to and denatures proteins to produce linear polypeptide chains. On average one SDS molecule will be present for every two aminoacids. The presence of β-mercaptoethanol assists in protein denaturation by reducing all disulfide bonds. The detergent binds to the hydrophobic region of the denatured protein in a constant ratio of about 1.4g of SDS/gm of protein. The protein-SDS complex carries net negative charges, hence move towards the anode and the separation is based on the size of the proteins. Most SDS-PAGE gels are cast with a molar ratio of Bisacrylamide:Acrylamide of 1:29 which has been shown empirically to be capable of resolving polypeptides that differ in size by little as 3%.

The Polyacrylamide gel is cast as a separating gel topped by a stacking gel. The stacking gel has properties that cause the proteins in the sample to be concentrated into a narrow band at the top of the separating gel. This is achieved by utilizing differences in ionic strength and pH between the resolving buffer and the stacking gel and involves a phenomenon known as isotachophoresis. The stacking gel is of high porosity and buffered with Tris-cl buffer at pH 6.8, whereas separating gel contains high percentage of acrylamide and is cast in Tris-cl buffer at pH 8.8. The upper (and lower) electrophoresis buffers contain Tris at pH 8.3 with glycine as counter ion.

Stacking principle: Glycine at pH 6.8 of the stacking gel remain in neutral zwitterionic form with only a fraction 1% in the negative glycinate form. This prevents glycine to be an effective carrier of current. The Cl^- ions remain effective current carriers at pH 6.8 and migrate rapidly towards the anode. The SDS-coated protein molecules and dye which have charge to mass ratio >glycine but less than that of Cl^- must now migrate to carry the electrophoresis current behind the Cl^- and ahead of the glycine. There is only a small quantity of protein-SDS complexes so they concentrate in a thin band sandwiched between the cl- ions and the glycine molecules at the interface between stacking and separating gels.

The higher pH of the separating gel favours ionization of glycine, carrying a higher charge to mass ratio than that of the proteins. Now the newly formed glycinate ions move faster than the proteins with mobility approaching that of the cl^- ions. The negatively charged protein-SDS move according to their relative mobilities and are separated by the sieving effect of the separating gel according to size. The high mobility of the tracking dye assures that it will migrate faster than the proteins.

Protein resolved in the gel can be stained with either Coomaassie brilliant blue or with silver stain. Silver staining is the most widely used high sensitivity staining method which is reported to be 100 times more sensitve than Coomassie blue with a detection limit about 0.1-1ng of protein. Coomassie blue are electrostatically attracted to charged groups on the protein, forming strong dye:protein complexes that are further augmented by vanderwaals forces, hydrogen bonding and hydrophobic bonding. On the other hand selective reduction of silver ions to metallic silver at gel sites occupied by proteins is the principle of silver staining. It depends on the differences in the oxidation-reduction potentials in the sites occupied by the proteins in comparison with adjacent sites in the gel that do not contain proteins.

METHODOLOGY:

a) Materials Required:

i) Equipments:

1. Electrophoresis apparatus for vertical slab gels with a size of 0.75mm X 10cm X 12cm.
2. Power supply.
3. Micropipette for loading samples

ii) Chemicals/Reagents/Buffers:

1. Stock acrylamide solution: 30g acrylamide, 0.8g bisacrylamide. Make up to 100ml in distilled water and filter through whatman No1 filter and store in amber bottle at 4°c.

(***CARE: Acrylamide monomer is a neurotoxin. Take care in handling acrylamide (wear gloves)and avoid breathing).***

2. Buffers:

a) Separating gel buffer: 1.875M Tris-cl, pH 8.8

b) Stacking gel buffer : 0.6M Tris-Cl. pH 6.8

3. 10% w/v Ammonium persulfate. Make fresh. Store at 4°c. (***Care: Always use in Fume hood)***

4.10% w/v Sodium dodecyl sulfate (SDS)

5.N,N,N’,N’-tetramethylethylenediamine(TEMED)

6. Sample buffer

0.6M Tris-HCl,pH 6.8	5.0ml
10% SDS	0.5g
Sucrose	5.0g
β-mercaptoethanol	0.25ml
Bromophenol blue (0.5% stock)	5.0ml

Make up to 50ml with distilled water

7. Electrophoresis buffer: Tris (12g), glycine (57.6g), and SDS (2.0g). Make up to 2l with water. No pH adjustment is necessary
8. Protein Stain: 0.1% Coomassie brilliant blue R250 in 50% methanol, 10%glacial acetic acid. Dissolve the dye in the methanol and water component first, and then add the acetic acid. Filter the solution through whatmann filter paper. (***Note:Coomassie brilliant blue is harmful by inhalation or ingestion. Wear appropriate gloves & safety glasses while handling)***
9. Destaining solution: 10% methanol, 7% glacial acetic acid
10. Protein sample
11. Standard Protein molecular weight markers.

iii) Glasswares and others:

Conical flask
Beaker
Graduated cylinder

b) Method:

1. Clean the internal surfaces of the gel plates with methylated spirits, dry, and then join the gel plates together to form the cassette, clamp it in a vertical position.
2. In an Erlenmeyer flask or disposable plastic tube, prepare the separating gel by mixing the following: ***(NOTE1*)**

	For15% gels	For 10%gels
1.875M tris-HCl, pH8.8	8.0ml	8.0ml
Water	11.4ml	18.1ml
Stock acrylamide	20.0ml	13.3ml
10%SDS	0.4ml	0.4ml
Ammonium persulfate (10%)	0.2ml	0.2ml

3. Degas this solution under vacuum for about 30sec**. *(NOTE2)***
4. Add 14µl of TEMED and gently swirl the flask to ensure even mixing.
5. Using a Pasteur pipette transfer this separating gel mixture to the gel cassette carefully down one edge. Continue adding this solution until it reaches a position 1cm from the bottom of the comb that will form the loading wells.

6. To ensure that the gel sets with a smooth surface very carefully run distilled water down one edge into the cassette using a Pasteur pipette.
7. While the separating gel is setting prepare the 4% stacking gel solution. Mix the following in a 100ml Erlenmeyer flask or disposable plastic tube.

0.6M Tris-Hcl, pH6.8	1.0ml
Stock acrylamide	1.35ml
Water	7.5ml
10%SDS	0.1ml
Ammonium persulfate (10%)	0.05ml

Degas this solution under vacuum for about 30 sec

8. When the separating gel has set, pour off the overlaying water. Add 14 µl of TEMED to the stacking gel. Pour the stacking gel solution directly onto the surface of the polymerized resolving gel. Immediately insert a clean Teflon comb into the stacking gel solution, being careful to avoid trapping of air bubbles. Place the gel in a vertical position at room temperature and allow to set for 20min.

Preparation of samples and running the gel:

9. About 10µl of protein sample and 5µl of sample buffer are mixed by vortexing. The sample is than heated for 5min at 95-100°C to denature the proteins. The sample is than kept in ice ***(Note3)***
10. After polymerization is complete, remove the Teflon comb. Rinse out any unpolymerised acrylamide solution from the wells using electrophoresis buffer and assemble the cassette in the electrophoresis tank. Add Tris-glycine electrophoresis buffer to the top and bottom reservoirs. (*Note: Donot prerun the gel before loading the samples, since this procedure will destroy the discontinuity of buffer system.)*

11. Load up to 5-10µl of each of the samples (unknown and standard) in a predetermined order into the wells.

12. Connect the electrophoresis apparatus to the power pack (the positive electrode should be connected to the bottom buffer reservoir), and pass a current of 30mA through the gel (constant current) for large format gels, or 200V (constant voltage) for minigels (Biorad). The gel is run until the bromophenol blue reaches the bottom of the resolving gel. This will take 2.5-3.0h for large format gels (16µm x 16µm) and about 40min for minigels (10µm x7µm) *(Safety care: Always turnoff & disconnect the power supply before removing the lid)*

13. Dismantle the gel apparatus, pry open the gel plates; remove the gel, discard the stacking gel, and place the separating gel in stain solution.

14. Staining should be carried out with shaking, for a minimum of 2h at room temperature. Destain the gel by soaking it in the methanol:acetic acid solution on a slowly rocking platform for 4-8 hrs.
15. After destaining, store the gels in H_2O containing 20% glycerol
16. The gel can now be used for immunoblotting to determine the protein sample

NOTE:

1. **Typically15% polyacrylamide gels are used for separating proteins of molecular mass in the range of 100,000-10,000 kd.However,a protein of 150,000for example would be unable to enter a 15% gel.In this case, a large pored gel(eg a 10% or 7.5% gel) would be used.**
2. **Degassing helps prevent oxygen in the solution "mopping up" free radicals and inhibiting polymerization.**
3. **β-mercaptoethanol is essential for disrupting disulphide bridges in proteins. However exposure to air decreases the reducing power of β-mercaptoethanol. Thus it should be prepared fresh.**
4. **Destain solution needs to be replaced at regular intervals since a simple equilibrium is quickly set up between the concentration of stain in the gel and destain solution after which no further destaining takes place.**
5. **It is generally accepted that a very faint protein band detected by Coomassie brilliant blue, is equivalent to about 0.1µg(100ng) of protein**
6. **Data Analysis** Label each lane on the photograph of your gel**:** The molecular weight of the unknown protein can be determined by running calibration proteins of known molecular weight on the same gel run as the unknown protein.

   A standard curve is constructed that plots relative mobility (Rf) versus Log mol weight

   R_f =(distance migrated by protein/distance migrated by tracking dye)

   The R_f of the standard protein is calculated and plotted on the graph. The R_f value of the unknown protein is calculated by measuring the distance each protein band migrated (Measure from the bottom of the well to the middle of each band) and the distance the tracking dye migrated in each lane. Using the standard curve, the molecular weight of the unknown protein is determined.

.