Swayam Course - Analytical Techniques

Week: 8, Module 21 - SDS PAGE(Sodium Deodyl Sulphate Polyacrylamide Gel Electrophoresis)

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Learning Objectives:

1. Introduction

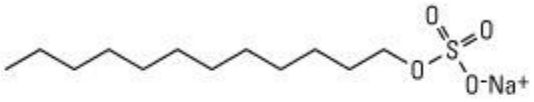
The process of moving charged molecules in solution by applying an external electric field across the conducting solution is known as electrophoresis. The molecules in an electric field move with a speed dependent on their charge, shape, and size under the influence of an electromotive force. For macromolecules like proteins, electrophoresis is carried out by applying the solution over a porous

matrix through which, under the influence of an electric field, different species of molecules in the sample move through the matrix at different rates which leads to resolution among them.The electric field consists of a potential difference generated by applying a negative charge and a positive charge at two separate ends of a matrix. The molecules having a high molecular weight/ size experience a more pronounced retardation in their migration through the matrix; this in essence is the physical basis of the resolution achieved by electrophoretic separation methods.

2. Protein fold and the role of SDS

Proteins have a globular fold in their secondary and tertiary conformation due to the presence of disulfide bonds, hydrophobic interactions, and hydrophilic interactions with their aqueous environment. Therefore, for optimal separation and for accurate analysis of peptide size to occur under an electric field, it is important to break the secondary and tertiary structure of the proteins in the sample.

Sodium dodecyl sulfate (SDS) is a anionic detergent having both a hydrophobic end (the dodecyl group) and a hydrophilic end (the sulfate group); it can also be called an amphipathic surfactant. The tertiary structure of most proteins is often a result of hydrophobic interactions at the core of the protein. These hydrophobic interactions are broken by the hydrophobic end of SDS by making interactions with the hydrophobic side chains of the amino acids. The sulfate group can disrupt hydrogen bonding in secondary protein structure by same mechanism. Disulfide bonds holding tertiary or quaternary structure together can be broken by using a reducing agent like **beta-mercaptoethanol (BME) or dithiothreitol (DTT)**. Additionally, heating the protein sample also facilitates the denaturation and unfolding process allowing chemicals like SDS and DTT to interact with the protein.



Sodium dodecyl sulfate (SDS)
MW 288

Apart from denaturing the protein, SDS also has an additional purpose. During the process, each protein gets coated with SDS molecules and the sulfate group has a negative charge, as a result each protein molecule attains a net negative charge due to which, when an external electrical field is applied to the gel in buffer, each protein molecule will flow toward the positive electrode. This allows the acrylamide matrix to separate the proteins based on size. As the SDS-covered “negatively charged” protein molecules move through the gel matrix, smaller molecular weight proteins are able

to navigate through the pores in the matrix more rapidly than those with high molecular weight. Thus, the proteins in the sample are separated by size and relative molecular weight irrespective of charge.

- **Proteins solubilised in SDS bind the detergent uniformly along their length**

to a level of 1.4 g SDS/g protein

3. The polyacrylamide matrix

Polyacrylamide makes a small-pores gel and can be used to separate most proteins, ranging in molecular weight from <5,000 to > 200,000.

3.1 Determining the pore size of the gel

The size of the pores in a polyacrylamide gel is dependent on two parameters: total solids content (%T) and the ratio of cross-linker to acrylamide monomer (%C). The %T is the ratio of the sum of the weights of the acrylamide monomer and the cross-linker in the solution, expressed as % w/v, thus at %T increases the pore size.

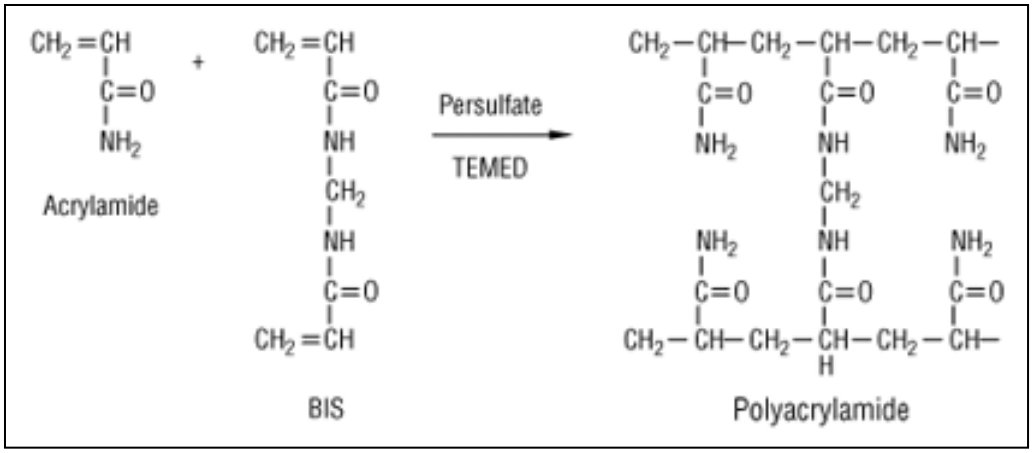
$$\%T = \frac{\text{g acrylamide} + \text{g cross-linker}}{\text{Total volume, ml}} \times 100$$

$$\%C = \frac{\text{g cross-linker}}{\text{g acrylamide} + \text{g cross-linker}} \times 100$$

3.2 Structure and polymerization of matrix

The protein samples are overlayed on top of a porous matrix made up of repeating units of acrylamide while N,N'-methylenebisacrylamide (bis) acts as a branching agent or a cross linker. When a mixed solution of acrylamide and cross-linker monomers co-polymerize into long chains that are covalently cross-linked, a polyacrylamide gel is formed. These gels are physically tougher than agarose gels, and the polymerization process is also more complex.

The polymerization process is a free- radical catalysed reaction, the most common source of free-radicals is the quaternary amine, N,N,N',N'-tetramethylethylenediamine (TEMED) as the catalyst and ammonium persulphate as the initiator peroxide . Generally APS and TEMED are used at approximately equimolar concentrations in the range of 1 to 10 mM



Polymerization of the polyacrylamide gel

Stock Solution	Stacking gel	Separation gel
Acrylamide (30%) + bis-Acrymalide (0.8%) (ml)	1.0	2.13
Tris buffer 1M pH 8.8 (ml)	-----	3.18
Tris buffer 1M pH 6.8 (ml)	1.25	----
Glycerol 87% (ml)	----	0.5
Bi-distilled water (ml)	7.1	2.625
Ammonium persulfate 10% (µl)*	50.0	37.5
TEMED (µl)**	10.0	15.0

Preparing a discontinuous polyacrylamide gel

Polymerization of acrylamide generates heat and a rapid polymerization process can generate excessive heat, causing convection inconsistencies in the gel structure. It is therefore important to adjust the concentration of the catalyst so that the polymerization occurs in a controlled manner.

It is important that the matrix be electrically neutral, the presence of fixed charged groups on the matrix can cause the flow of water toward one or the other electrode, usually the cathode. This phenomenon, called electroendosmosis markedly decreases the resolution of the separation.

4. Electrophoresis buffers

For electrophoretic protein separations based on the mobility of the different species, the pH of the solution must be kept constant to maintain the charge and, hence, the mobilities of the proteins. Tris-glycine buffer is used for protein electrophoresis, tris is the buffering system while glycine is the

conducting species. SDS-PAGE utilizes a discontinuous buffer system to concentrate, samples into a very sharp zone in the stacking gel before actual resolving takes place.

In the case of the Tris-Glycine system, mainly three ions are involved:

1. Chloride (-), from the gel buffer, serves as the leading ion as it has the highest attraction to the anode with respect to other anions in the system.
2. Glycine (-), the primary anion provided by the running buffer it serves as the trailing ion due to its partial negative charge and remains behind the more highly charged chloride ions.
3. Tris Base (+) is the common ion present in both the gel and the running buffers. The gel and buffer ions in the Tris-Glycine system form a pH of 9.5 in the resolving portion of the gel.

4.1 Choosing the right buffer

1. **pK_a value**

A buffer with a pK_a that is very close to the desired pH should be chosen, buffers with a pKa in the range of 7-9 are best suited for most electrophoretic applications.

2. **Formal charge**

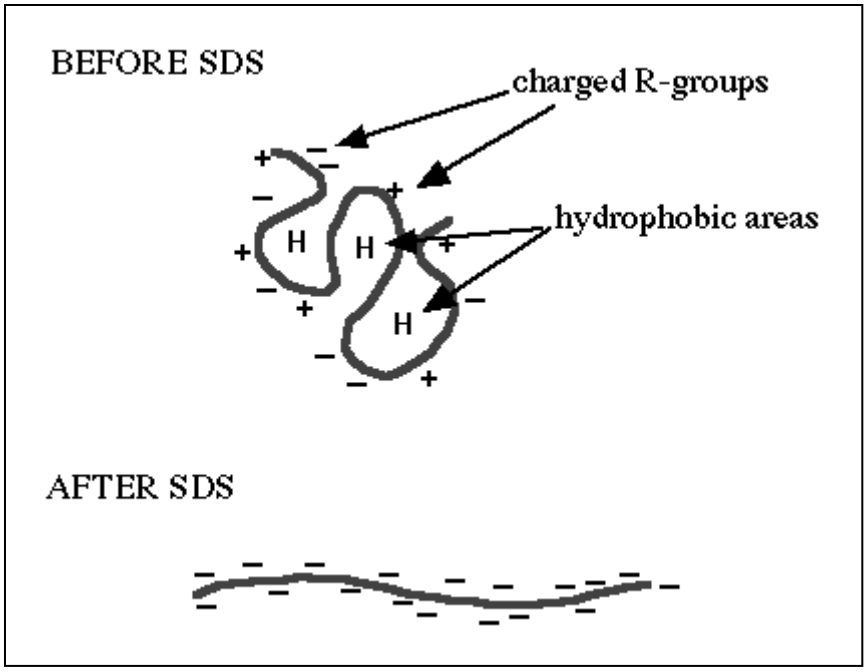
Buffers generating ions with high charge should be avoided as it can increase current and deplete the ions in the buffer very quickly. Tris base and borate are preferred buffer species as they are uncharged for part of the time at the desired pH and thus have a reduced electrophoretic mobility.

3. **Molecular size**

Buffer species having high molecular size move slowly in electrophoresis. Tris is preferred as it moves slower than chloride or phosphate because of its low charge/mass ratio which significantly inhibits buffer depletion.

5. Basis of separation of proteins by SDS PAGE

The pre-requisite for size based separation is that the ratio of charge to mass must be constant. The solubilisation of proteins in SDS confers on them a uniformly negative charge; the proteins also attain a fully extended conformation in its presence.



The effect of SDS on protein conformation

The end result of SDS "coating" is that the protein molecules in the sample achieve a fixed charge to mass ratio and are uniformly negatively charged. Under the influence of an applied electric field, the proteins now have a tendency to move towards the positive electrode. The migration velocities towards the positive electrode depend on how easily the protein molecules are able to traverse through the porous matrix. In this aspect, molecular size becomes the determining factor. Larger sized proteins experience a more pronounced retardation in their movement through the polyacrylamide matrix as compared to smaller ones. This differential migration rate leads to a size based resolving effect among the proteins present in the sample.

% Acrylamide	Molecular weight range (kDa)
7	50-500
10	20-300
12	10-200
15	3-100

1.1 The importance of buffer components in protein resolution

Mostly in SDS PAGE, discontinuous Laemmli buffer system is used, where the buffer in the gel and tank are different. Typically, the system consists of **a pH 6.8 stacking gel, a pH 8.8 resolving gel and a pH 8.3 electrode buffer.** The stacking gel has larger pores owing to the low concentration of acrylamide. Glycine can either be positive, negative or neutral depending on the ambient pH, when electric field is applied the negatively charged glycine ions in the pH 8.3 electrode buffer are forced to enter the stacking gel where the pH is 6.8. In this condition, glycine exists in its zwitterionic (neutral form) due to which its flow is retarded, here the chloride ions form the gel front with glycine at the back, the protein molecules are concentrated between these two zones. At the interface of resolving gel, the pH is 8.8 at which the glycine molecules are negative and migrate much faster than proteins. As a result the proteins are rendered in a very narrow band at the interface of the two gel portions, and since the resolving gel has a lower pore size, the size based resolution can now begin.

6. Protocol

6.1 Gel casting

1. Assemble vertical slab gel apparatus using 1mm spacer, make sure the plate is thoroughly cleaned and dried before use.
2. Seal the glass plates on 3 sides with 1% agarose.
3. Pour the separating gel/ resolving gel mixture to a level of approximately 2.5 cm below the glass plates, gently layer 250µl of TDW over the gel surface.
4. Allow to polymerize. Make sure to prepare the solution freshly each time it is required. As soon as ammonium persulphate is added, the gel should be poured quickly before the acrylamide polymerizes.
5. After polymerization remove water from the top.
6. Pour the prepared 5% stacking gel over the resolving gel/ separating gel.
7. Immediately insert a comb and allow polymerizing the gel.

6.2 Sample loading

1. Prepare the required volume of sample + equal volume of sample buffer.
2. Heat the sample in boiling water bath for 5 min to denature the protein. Immediately keep them on ice to retain the denature stage.
3. Remove the comb from the mould; wash the well with distilled water.
4. Mount the gel on electrophoretic apparatus.

5. Add electrophoresis buffer to the top and bottom reservoir of the electrophoretic apparatus. Load the sample along with marker protein into the wells (20µl).

6.3 Electrophoresis

1. Attach the apparatus to the power supply unit and apply 8 V/Cm for stocking gel (70V) and 15 V/Cm for resolving gel (150-200V).
2. Electrophoresis is continued until bromophenol blue reaches the bottom of the gel.
3. Dismantles apparatus and remove gel from between the plates and place in a tray containing distilled water cut a small corner of the gel to indicate the direction of loading.

7. Visualization of the gel

7.1 Coomassie Brilliant Blue R-250

It is an anionic dye, which binds non-specifically to proteins. The structure of this dye is mainly non-polar, and it is used in a methanol solution acidified with acetic acid. Proteins in the gel are fixed by acetic acid and simultaneously stained. The excess dye incorporated into the gel can be removed by using a destaining solution. The proteins are detected as blue bands on a clear background. As SDS is also anionic, it may interfere with staining process. Therefore, large volume of staining solution at least ten times the volume of the gel, are recommended. It can detect 50ng of protein band.

7.2 Silver staining.

Silver staining is used when more sensitivity for detection is needed, as it increases the sensitivity typically 50 times. The exact chemical mechanism by which this happens is still largely unknown. Silver nitrate is reduced in presence of proteins to give a brownish colored spot.

8. Applications

1. To establish the size of different proteins in a sample having a variety of proteins as in cell culture or tissue lysate.
2. Protein identification based on size can be done using protein molecular weight markers.
3. The purity of a sample can be established by checking if unwanted protein molecules are present in it or not.
4. Identification of the subunit characteristics of proteins can be done, if the protein is multi-subunit then the denaturing conditions should break the disulphide linkages and render the two subunits separate which can be visualized as distinct bands on the gel.

5. In cloning and gene expression, SDS PAGE is used to establish whether the induction of the gene has occurred and the corresponding protein is being overexpressed.
6. Peptide mapping

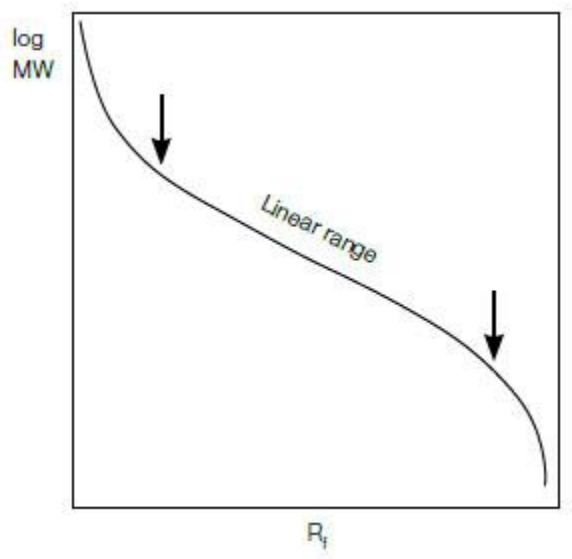
   In the process of peptide mapping, controlled cleavage of a pure protein with small amounts of a pure protease to generate peptides of characteristic, reproducible sizes is done. These peptides are then separated on PAGE to produce a "fingerprint" characteristic of the protein. Peptide mapping can be used to map cleavage sites in an unknown protein, or for identification of an unknown protein based upon its fingerprint identity with a previously tested sample.
7. Densitometric analysis

   SDS PAGE can be used to analyze the relative amount of differential proteins in multiple samples. For the purpose of measuring protein levels, intensities of specific bands, corresponding to the proteins of interest are measured using commercially available software.

9. Quantification of molecular weight using SDS PAGE

The mobility (R_f) of a molecule in gel electrophoresis is determined by its free solution mobility along with the sieving action of the gel matrix. In SDS protein electrophoresis, SDS buffer uniformly coats the proteins with negative charges, equalizing the charge to mass ratio for all proteins, thus it makes the Y_0 basically same for all the components. Ultimately, relative mobilities are determined only by the pore size of the gel whose sieving action is proportional to the molecular weight (MW) of the protein. The R_f (distance migrated from the wells) for a set of standard protein markers varies linearly with MW. After this standard plot is made, the molecular weight of the test proteins in the sample can be interpolated from the graph using their respective R_f values. The accuracy can be confidently expected in the range of 5-10 %.

$$Rf = \frac{\text{Migration distance of the protein}}{\text{Migration distance of the dye front}}$$



- If most proteins in the sample are fully denatured and the gel percentage is appropriate for the molecular weight range of the sample a linear graph is obtained. Getting a sigmoidal curve means that the sieving effect of the matrix is either too large that it restricts the path of the molecules into the gel or is nearly negligible that it allows protein molecules to migrate approximately at their free mobility.
- Glycoproteins and lipoproteins are however an exception to this rule as they are not uniformly coated with SDS and may show anomalous behavior.

Summary:

1. SDS PAGE is an electrophoretic technique used to separate proteins based on their molecular size, the matrix used for separation under an external electric field is made up of polyacrylamide and bis-acrylamide as the cross linking agent.
2. The process utilizes a discontinuous gel system (Laemmli setup) where the two gel portions, stacking gel and resolving gel having pH of 6.8 and 8.8 respectively.
3. The stacking gel has a higher pore size as compared to the resolving gel and this helps in concentrating the protein bands into a narrow zone just before they enter the resolving gel, this helps in improving the resolution of the system.
4. The running buffer is made up of glycine which is a crucial component determining the migration of the proteins as its movement is dependent on the pH of the environment in the gel.
5. Since the protein conformation is highly variable and the pre-requisite for electrophoretic separation is uniform charge/mass ratio, SDS is employed for the same. It coats the proteins providing them a uniform negative charge.
6. Proteins solubilised in SDS bind the detergent uniformly along their length to a level of 1.4 g SDS/g protein

BME or DTT are used as reducing agents to break disulfide linkages in the proteins and along with SDS they help in changing the protein conformation to log extended form which is uniformly coated with negative charge.