S. S. College, Jehanabad

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Subject: Zoology

Topic: Separation techniques – SDS PAGE

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SEPARATION TECHNIQUES - SDS-PAGE

SDS-PAGE is mostly used to separate proteins accordingly by size. This is one of the most powerful techniques to separate proteins in the basis of their molecular weight. It is no applied in the separation of nucleic acids such as DNA and RNA.

Principle

This technique is discontinuous gel system, which utilizes negatively charged (anionic) detergent Sodium Dodecyl Sulfate (SDS) which dissociates proteins into their individual polypeptide subunits and gives a uniform negative charge along each denatured polypeptide. It forces polypeptides to extend their conformations to achieve similar charge : mass ratio. SDS treatment therefore eliminates the effects of differences in shape so that chain length, which reflects their molecular mass, is the sole determinant of migration rate of proteins in the process of electrophoresis.

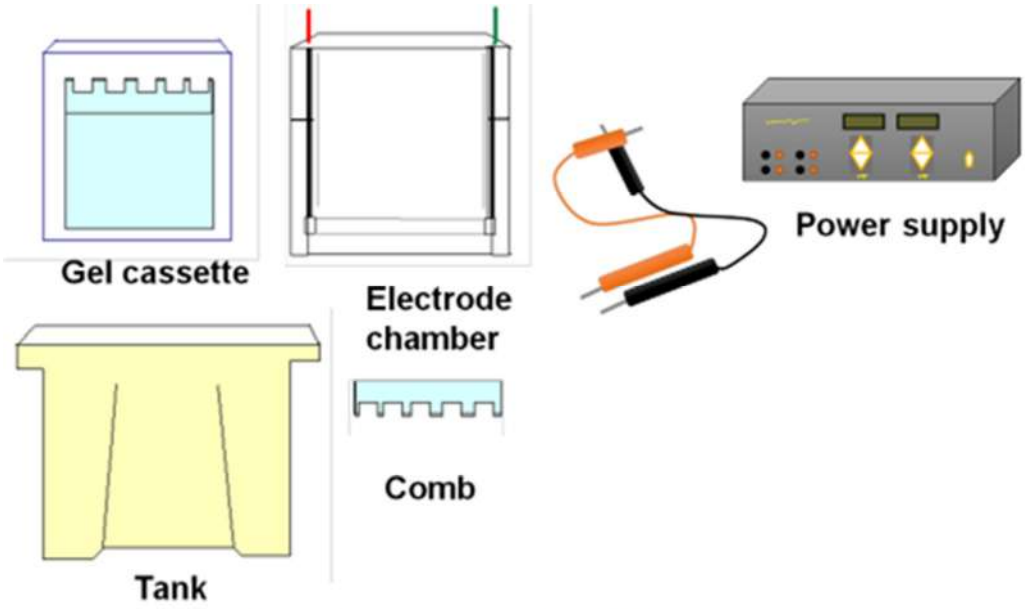
When these denatured polypeptides are loaded at the cathode end of an electrophoretic tank having polyacrylamide gel (as the supporting media) and subjected to an electric field, then we get clear bands of proteins arranged in an decreasing order of their molecular mass from the cathode to anode. The rate of movement is influenced by the gel's pore size and the strength of electric field. In SDS- PAGE, the vertical gel apparatus is mostly used. This technique uses three different buffers, namely running Buffer, stacking gel buffer of pH 6.8, and resolving gel buffer of pH 8.8. It concentrates the diluted protein sample into a narrow band. Although it is used to separate proteins on a routine basis, SDS-PAGE can also be used to separate DNA and RNA molecules.

Instrumentation

The SDS-PAGE is performed in a discontinuous fashion with only gel cassette submersed within the buffer but not electrodes. The electrophoresis chamber has two platinum electrodes placed on the both sides of an end and are connected to the external power supply from a power pack which supplies a direct current or DC voltage. The tank, which is vertical, filled with the running buffer and the gel casted is submerged inside the buffer. There are additional accessories needed for casting the agarose gel such as comb (to prepare different well), spacer, gel caster etc. The system consists of three components, which are as follows;

Electrophoresis apparatus: SDS-PAGE uses vertical tank for the electrophoresis buffer. The schematic diagram of a vertical gel electrophoresis apparatus is given below. It has two buffer chambers, upper chamber and a lower chamber. Both chambers are fitted with the platinum electrodes connected to the external power supply from a power pack which supplies a direct current or DC voltage. The upper and lower tank filled with the running buffer is connected by the electrophoresis gel casted in between two glass plates (rectangular and notched). There are additional accessories needed for casting the polyacrylamide gel such as comb (to prepare different well), spacer, gel caster etc.

Power supply: A power supply of 100-200 volts is needed. This is ideal for running and transferring protein resolving gels.



Different components of SDS-PAGE apparatus

Staining box: These are trays in which the gels are stained and made up of clear plastics. These are resistant to most organic dyes, silver and other stains.

Buffer and Reagents

Buffer and reagents required for agarose gel horizontal electrophoresis and its purpose are as follows;

- ***N, N, N', N'-tetramethylethylenediamine (TEMED):*** It catalyzes the acrylamide polymerization.
- ***Ammonium persulfate (APS):*** It is an initiator for the acrylamide polymerization.
- ***Tris-HCl:*** It is the component of running and gel casting buffer.
- ***Glycine:*** It is the component of running buffer.
- ***Bromophenol blue:*** It is the tracking dye to monitor the progress of gel electrophoresis.
- ***Coomassie brilliant blue R250:*** It is used to stain the polyacrylamide gel.
- ***Sodium dodecyl sulfate:*** It is used to denature and provide negative charge to the protein.
- ***Acrylamide:*** Monomeric unit used to prepare the gel.
- ***Bis-acrylamide:*** Cross linker for polymerization of acrylamide monomer to form gel.

All these chemical reagents can be classified under four major components under reagent solution for electrophoresis, which are as follows;

Supporting media: SDS-PAGE is used as the supporting medium. It is a white crystalline powder, when acrylamide is dissolved in water; it undergoes polymerization reaction to form a net like structure called polyacrylamide gel. Polyacrylamide is a polymer ($CF_2CHCONH_2$-) formed from acrylamide subunits that can also be readily cross-linked. This type of electrophoresis has a discontinuous system of gel, i.e., we have two different systems of gels present in the electrophoretic tank physically placed one over another. These gels are as follows:

- *Resolving gel:* This is also called as separating or running gel. The separating gel constitutes about 2/3rd of the length of gel plate and is prepared by 5-10% of acrylamide. The pores in this gel (which is formed after the polyacrylamide is cross-linked) are numerous and smaller in diameter which impacts sieving property to this gel.
- *Stacking gel:* It is poured on the top of resolving gel and a gel comb is inserted which forms the well. It is the upper layer of gel that constitutes 1/3rd of the gel plate. The percentage of acrylamide is chosen depending on the size of protein that one wishes to identify or probe in the sample. The smaller is the known weight, higher is the percentage that should be used. Generally, the percentage of acrylamide in stacking gel is 2 – 5%. It is highly porous and devoid of molecular sieving action.

Buffer: There are two types of buffers that are used in SDS-PAGE. The lower reservoir, which has the running gel, has amine buffers. It is adjusted by using HCl. The upper reservoir, which has stacking gel, also has amine buffer but its pH is slightly above that of running gel buffer and is adjusted with glycine instead of HCl.

Dissociating agents: SDS is the most common dissociating agent used to denature native proteins to individual polypeptides. When a protein mixture is heated to 100°C in presence of SDS, the detergent wraps around the polypeptide back bone.

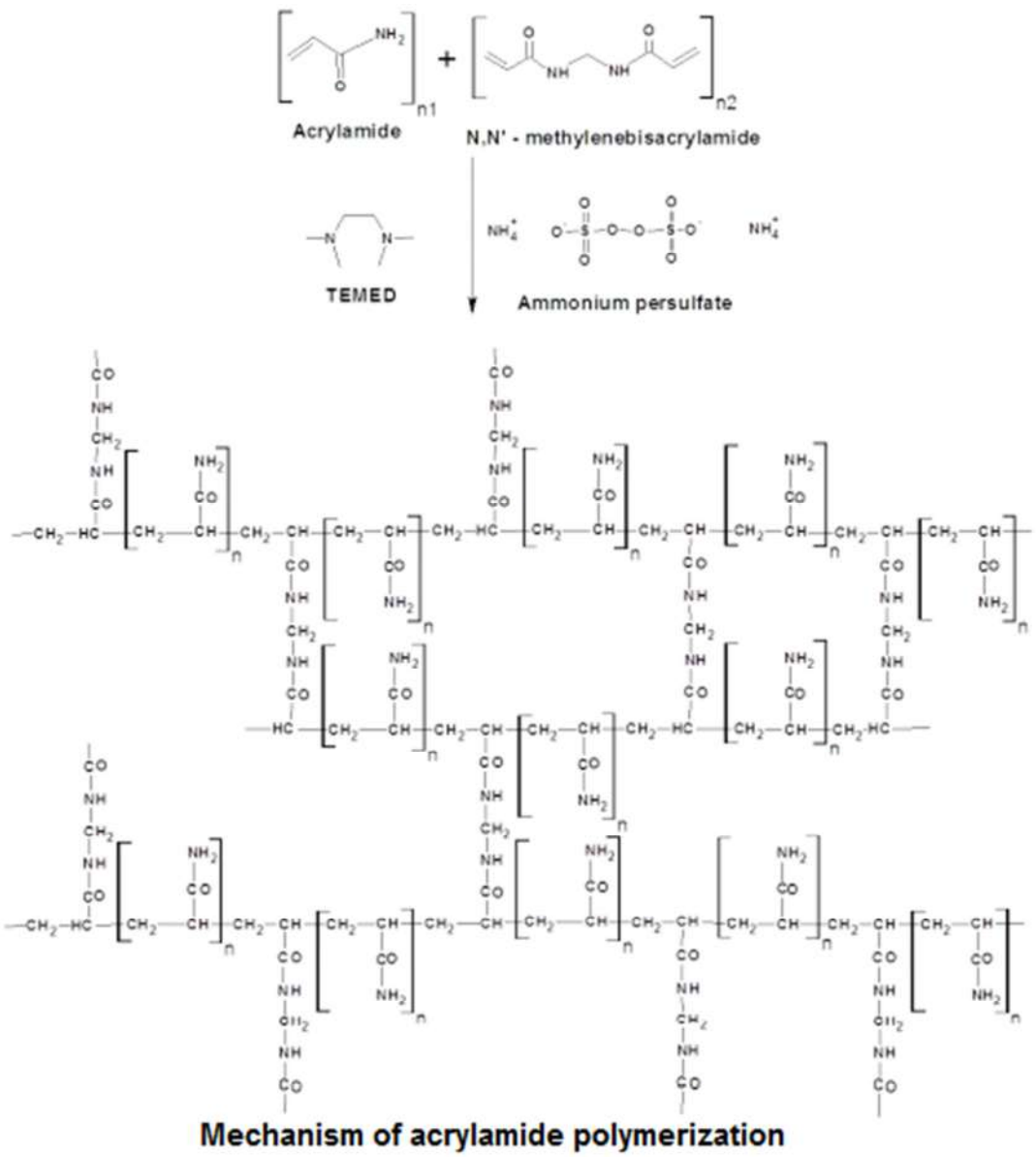
It binds to polypeptide in a constant weight ration 1.4 g/g of polypeptide. In this process, the intrinsic charges of polypeptides become negligible when compared to the negative charges contributed by SDS. Thus, polypeptides after treatment become rod-like structure possessing uniform charge density that is the same net negative charge per unit length.

Stains: The stains are used to see the bands of separated proteins after the process of electrophoresis. Coomassie Brilliant Blue R-250 (CBB) is the most popular protein stain. It is an anionic dye, which binds with proteins non-specifically.

Procedure

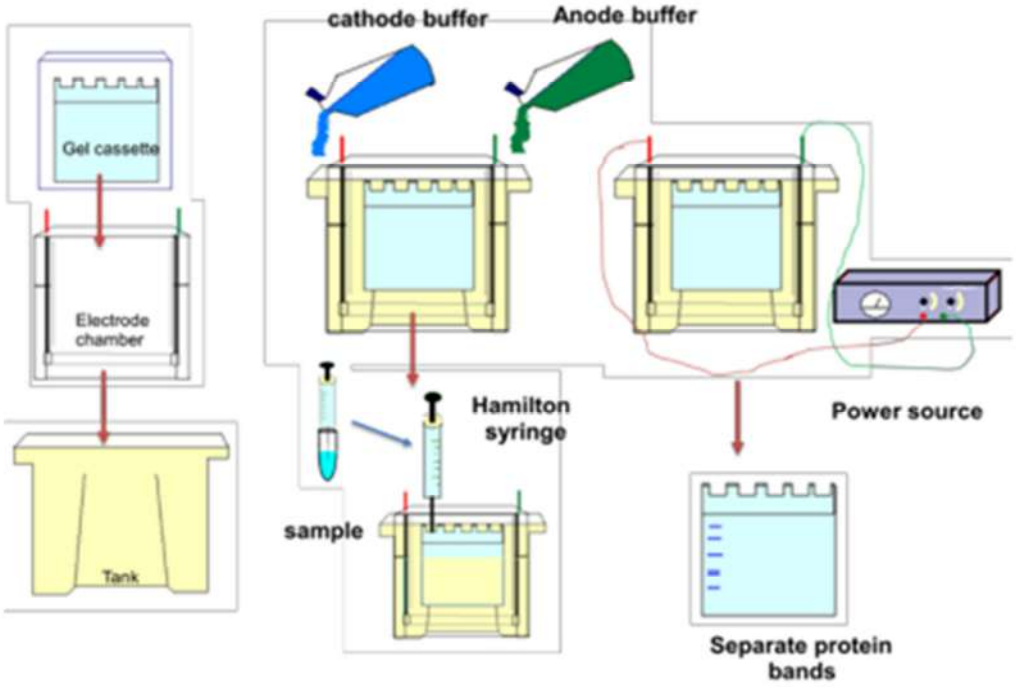
It starts with casting of polyacrylamide gel, loading of samples in the gel and running the samples in vertical electrophoresis system. The different step of the Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) is as follows;

Casting of the gel: The acrylamide solution (a mixture of monomeric acrylamide and a bifunctional crosslinker bisacrylamide) is mixed with the TEMED and APS and poured in between the glass plate fitted into the gel caster. In this method, persulfate in the presence of TEMED forms oxygen free radicals and induces the polymerization of acrylamide monomer to form a linear polymer. These linear monomers are interconnected by the cross linking with bis-acrylamide monomer to form a 3-D mesh with pores. The size of pore is controlled by the concentration of acrylamide and amount of bis-acrylamide in the gel. In a vertical gel electrophoresis system, we cast two types of gels, stacking gel and resolving gel.



Mechanism of acrylamide polymerization

First the resolving gel solution is prepared and poured into the gel cassette for polymerization. A thin layer of organic solvent (such as butanol or isoproponal) is layered to stop the entry of oxygen (oxygen neutralizes the free radical and slow down the polymerization) and make the top layer smooth. After polymerization of the resolving gel, a stacking gel is poured and comb is fitted into the gel for construction of different lanes for the samples.



Different steps in performace of vertical gel electrophoresis to resolve sample

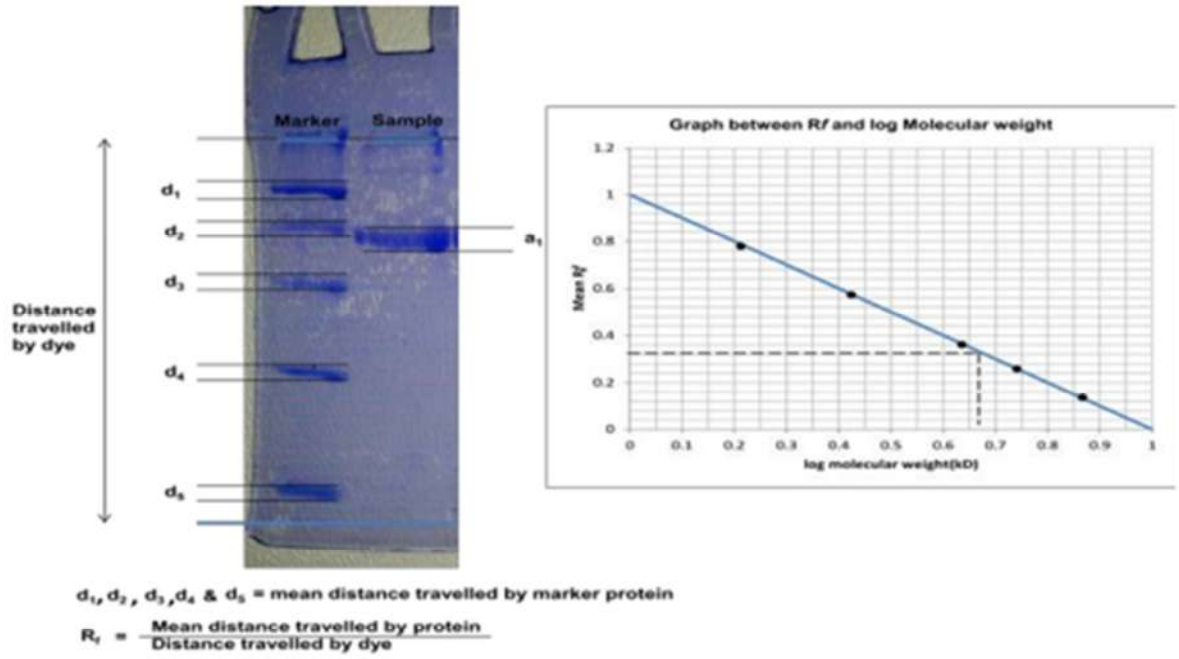
Running and Staining: The detail step by step procedure is as follows;

First of all the solution of proteins to be analysed is mixed with SDS, an anionic detergent which denatures secondary structure. Besides addition of SDS, proteins may optionally be boiled in the presence of a reducing agent, such as Di-Thio-Threitol (DTT) or 2-mercaptoethanol, which further denatures the proteins by reducing disulfide linkages, thus overcoming some forms of tertiary protein folding, and breaking up quaternary protein structure (Oligomeric subunits). This is known as reducing SDS-PAGE, and is most commonly used. Non-reducing SDS-PAGE (no boiling and no reducing agent) may be used when native structure is important in further analysis (e.g., enzyme activity, shown by the use of zymograms). The denatured proteins are subsequently loaded into the wells of stacking gel flooded with stacking buffer.

This end is connected with the cathode of power supply. Then an electric current is applied across the gel, causing negatively charged proteins to migrate across the gel towards anode. After crossing the stacking gel, denatured proteins enter the running gel which has its own buffer system (running buffer).

Depending on their size, each protein move differently through the gel matrix; short proteins move more easily fit through the pores in the gel, while larger ones move with more difficulty.

After the separation is over, the gel is gently taken out and transferred to the staining box and treated with the staining dye, e.g. Coomassie Brilliant Blue R-250 (CBB R-250). Excess of stains are removed by de-staining using acetic acid solution. The bands appear to be blue stained which are then analyzed according to the need of the experiment.



d_1, d_2, d_3, d_4 & d_5 = mean distance travelled by marker protein

$R_f = \frac{\text{Mean distance travelled by protein}}{\text{Distance travelled by dye}}$

Determination of molecular weight using SDS-PAGE. (A) SDS-PAGE (B) Determinaion of Rf

Calculation of molecular weight of the unknown protein sample:

- First of all, resolve the protein sample on the SDS-PAGE along with the molecular weight markers.
- Calculate the relative mobility '**Rf**' using the following formula:

$$\boldsymbol{Rf = \frac{Migration\ of\ protein\ from\ the\ lane}{Migration\ of\ tracking\ dye}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (\boldsymbol{iv})$$

- Plot log molecular mass (Y-axis) versus relative mobility (X-axis) of the standards.
- Perform a linear regression using a calculator or using regression software such as Microsoft Excel.
- Use the linear regression equation $(y = mx + c)$ to estimate the mass of the unknown protein.
- Finally, calculate molecular weight by following formula;

$$\boldsymbol{Log\ Molecular\ Weight} = (\boldsymbol{Slope}).(\boldsymbol{Mobility\ of\ the\ unknown}) + \boldsymbol{Y\ intercept}$$

Warning:

- *Smiling:* Uneven heating of the gel causes differential migration of proteins, with outer lanes moving slower than the middle lanes. Rapid heat transfer eliminates this defect and be achived by filling the lower tank with the buffer until sample height.
- *Diffuse Protein Bands:* Diffused pattern of the protein band appears on the PAGE. Diffused protein bands pattern can be corrected by increasing running current by 25-50%, higher concentration of acrylamide.

- *Vertical Streaking:* Vertical streaking of the protein bands appers due to overloading of the protein sample. It can be corrected by either reducing amount of the protein sample or running the gel at lower current.
- *Protein Runs faster than expected:* In few cases migration of protein is not proportional to the molecular weight, it is either more or less on gel than the expected place. It is due to the unusual very high proportion of basic or acidic amino acids.
- *Double Bands:* Appearance of double bands is due to partial oxidation or degradation of the protein sample. Addition of more amount of reducing reagent or preparing a fresh sample will reduce these artifacts.
- *Distorted Protein Bands:* Appearance of distorted or uneven protein band is due to stacking gel polymerization. It can be corrected by increasing amount of ammonium persulfate and TEMED or deaerating the stacking gel.
- *Lateral Spreading:* if a protein band appears laterally spreading, it can be avoided by reducing the time between loading of the sample and running.

Application of SDS-PAGE

SDS PAGE has several advantages over other electrophoresis techniques. It has high mobility and rapid separation of protein molecules. Since, all the proteins are negatively charged, therefore all migrate towards anode. Further, SDS is known to solubilize all proteins including very hydrophobic and even denatured proteins. Therefore, it has wide application also, which are enlisted as follows;

1. Establishing protein size
2. Protein identification
3. Determining sample purity
4. Identifying disulfide bonds
5. Quantifying proteins
6. Blotting applications

Reference

1. Lee P. et. al. Agarose Gel Electrophoresis for the separation of DNA fragments. J. Visulized Exp. 2012; 20 (10): .3791-3923.
2. https://nptel.ac.in/content/storage2/courses/102103047/PDF/mod3.pdf
3. https://people.wou.edu/~courtna/ch462/Gel%20Electrophoresis.pdf
4. https://msu.edu/course/css/451/Lecture/PT-electrophoresis%20(2009).pdf
5. https://www.edvotek.com/site/pdf/Electrophoresis_Guide.pdf
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8. https://www.iitg.ac.in/biotech/MTechLabProtocols/SDS%20PAGE.pdf
9. https://www.researchgate.net/publication/321727799_SDS-PAGE

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