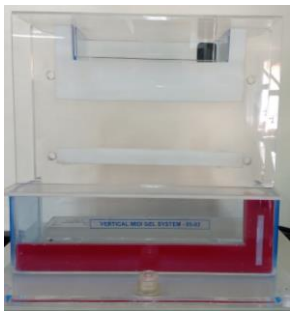

UNIT 5

ELECTROPHORESIS

Structure

5.1 INTRODUCTION

In the previous block we have studied the preliminary techniques widely used for the isolation and separation of proteins from living cells. While studying you might have come across terms like osmosis, sedimentation co-efficient, partition, adsorption, mobile and stationary phase. Similar to these techniques in this block we will be discussing major techniques for characterization and analysis of proteins that are known as E*lectrophoresis, Sequencing methods and Mass spectrometry* which are based on the native properties of proteins such as, charge, size and shape.

Current unit explores the fundamental principles, types and applications of electrophoresis technique that is used for characterization of proteins. We would also study the modifications adopted in this technique according to the nature of the proteins. Several applications have been developed to meet the demands of exploratory research work carried out in the field of biosciences and medicine with special emphasis to clinical biochemistry and diagnosis.

The journey of electrophoresis begins in the year 1861, when Quincke performed the first recorded measurements of electrophoretic phenomenon. Tiselius popularized the utility of electrophoresis in biochemistry by describing his moving boundary apparatus in 1937. The term electrophoresis denotes the migration of a charged particle under the influence of an applied electric filed. It is cleared in the previous blocks that biomolecules like amino acids, peptides, proteins and nucleic acids possess charge at any given pH and exist as electrically charged moieties (either anions or cations) in solution.

Expected Learning Outcomes

After studying this unit, you should be able to:

- define the terms like purity, molecular weight and sedimentation coefficient;
- describe the principles of various electrophoresis techniques;
- know the differences between various types of electrophoretic techniques;
- importance of different types of electrophoretic techniques; and
- grasp the versatility of electrophoresis with special emphasis to characterization of proteins.

5.2 ELECTROPHORESIS

In nature proteins available as free forms and also conjugated with other molecules like carbohydrates and nucleic acids. As we all know that purity always play a significant role in our life, as purity represents the quality of a product.

Determination of Purity:

Coming to the determination of purity of proteins, is determined by their *chemical properties* like composition of amino acids, number of polypeptide chains. Here you need to remember that purity of proteins is performed after *isolating* them from the cell and subcellular organelles.

In this unit we will be exploring the various electrophoresis techniques used to characterize the proteins based on their behavior in the applied electric field. This behavior of proteins is dependent on the shape, size and composition of amino acids. Let us discuss the working principle behind electrophoresis and the types with special reference to their applications.

Basic Principles

Electrophoresis is mainly based on the separation of charged particles or molecules in the presence of an electric field. In an applied electric field ion move towards oppositely charged electrodes i.e., anions (-ve) move towards positively charged anode and cations (+ve) move towards negatively charged cathode (Fig. 5.1). As we studied in our earlier unit-2 of block-1, most of the biomolecules bear charge. Where as carbohydrates being uncharged can be separated after undergoing *derivatization* process. This Unit illustrates how these charged molecules are being separated in an applied electric field.

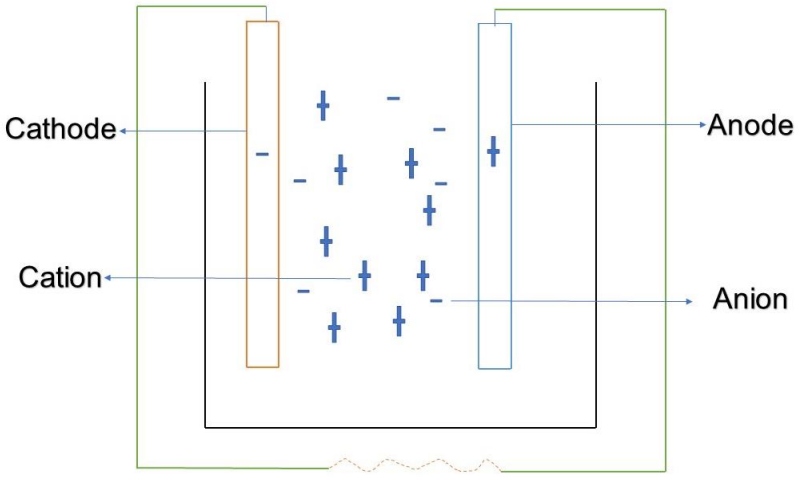

Fig. 5.1: Depicting the principle of electrophoresis.

Derivatisation is the process where uncharged molecules converted in to charge by coupling them with borates and phosphates. Even if two molecules have the similar charge, they might not migrate together because there may be a difference in their molecular weights, which will make them to have different charge/mass ratio. This difference is crucial for gel electrophoresis.

To know how exactly charged molecule separates under elctric field, consider a potential diffeence (voltage) applied across the anode and cathode, which leads to a potential gradient, “E”, which intern is the applied voltage, “V” and divided by the distance, “d”, between electrodes. When ‘E’ is applied, the force on a molecule having a charge of ‘q’ *coulombs* is ‘Eq’ newtons. This is the force that moves a charged particle towards electrode.

However, there is a *frictional resistance* that interupts the migration of charged particle. This force is an indicator of hydrodynamic size of the particle, its shape, the pore size of the gel and the viscosity of the buffer.

There fore velocity, ‘*v*’, of a charged molecule in an electric field is given by equation:

$$v = Eq / f$$

where ‘*f*’ is frictional coefficient

Electrophoretic mobility (μ), of an ion represented as: *v*/E, which is the ratio of velocity of the ion to the field strength.

Types of Electrophoresis

Eletrophoresis can be divided in to 3 types based on the supporting media used

- Agar gel electrophoresis
- Polyacrylamide gel electrophoresis
- Cellulose acetate or paper electrophoresis

Irrespective of the type of electrophoresis, support media is a mandate, which is popularly known as gel matrix. Basic components of a electrophoretic unit are buffer reservoirs, glass plate, comb plate, spacer arms and a power pack. Using appropriate buffer system is essential to maintain a constant ionisation state. Elctrophoresis can be performed as vertical or horizontal gel systems. Typical gel dimensions ranges between 12 cm x 14 cm, with a thickness of 1-2 mm.

Factors like sample charge, size and shape will affect the elerophoretic mobility of molecules along with elctricfield and the nature of buffer.

5.3 GEL MATRIX

Gels are porous materials, size of the pores with respect to that of biomolecule determines whether the molecule to be seperated will enter the pore and be retarded or will bypass it. Hence seperation not only depends on the charge but also on the size of the molecule. Agar and polymerised acrylamide are the two routinely used matrices.

5.3.1 Agarose

Agarose madeup of two galactose based polymers, such as agarose and agaropectin (Fig. 5.2). Sulphated agaropectin is the key element in the development of electroosmosis, which will be the deciding factor for eletrophoretic seperation. Boiling at 40^{o} C with aqueous buffers solubilizes agar and upon cooling forms gel at 38^{o}C. In practice less concentration of agar forms high pore size, hence widely used to separate high molecular weight molecules like proteins and nucleic acids.

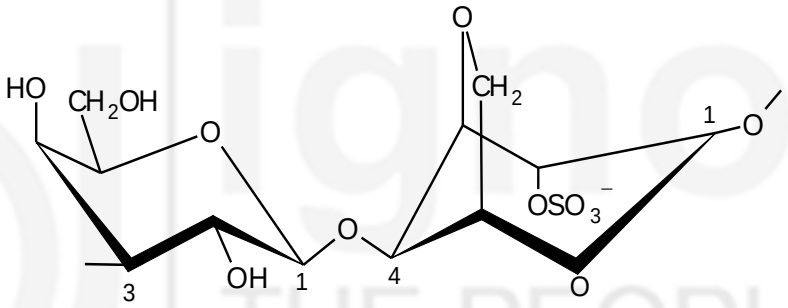

Fig. 5.2: Structure of Agarose.

5.3.2 Polyacrylamide

Components used to synthesize the matrix are acrylamide monomer, N,N'-methylenebisacrylamide (bis), ammonium persulphate (APS) and tetramethylenediamine (TEMED). Where APS activates acrylamide monomers by its freeradical generating ability and activated monomers polymerizes to form polymers and they get cross linked in the presence of bis, in the whole process TEMED acts as a catalyst (Fig. 5.3). The pore size of the gel can be controlled by the amount of acrylamide and bis. Most of the components used in the preparation are neurotoxins and thus care should be taken while preparing.

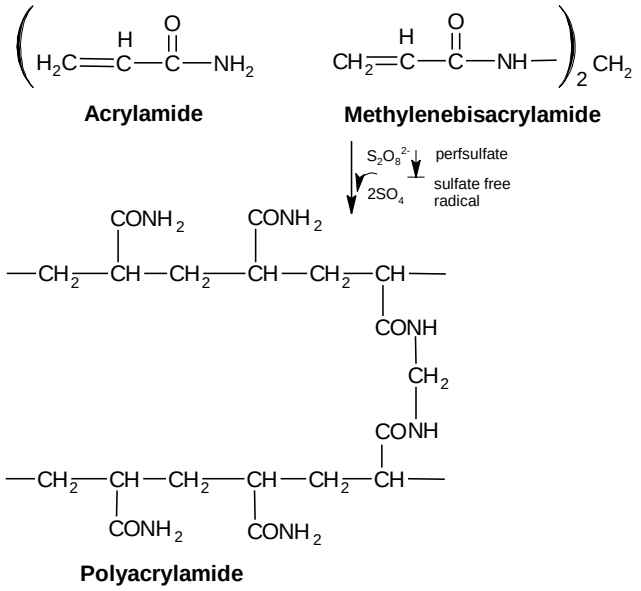

Fig. 5.3: Synthesis of polyacrylamide.

SAQ 1

1. Define the principle of electrophoresis
2. Give the composition of Agar and Polyacrylamide

5.4 ELECTROPHORESIS OF PROTEINS

In this section we will be studying about polyacrylamide electrophoresis, isoelectro focussing and 2-D gel electrophoresis. In this context, let us begin with understanding the essential terminology used to describe the characteristic features of a protein.

Molecular weight of protein: It is obtained by multiplying the average weight of total of amino acids present in a particular protein. Example: A protein with 300 amino acids possesses 33 kDa i.e., average weight of an amino acid is 110 Da, therefore 300 x 110 = 33000 Da or 33kDa.

Molecular extinction coefficient: This is a unit to measure the light absorbance ability of a chemical substance at specific wavelength. However, the value of extinction coefficient is used to measure molar concentration of a solution at a particular wavelength. The units used to express are: $(mg/ml)^{-1}cm^{-1}$ i.e., absorbance divided by the concentration and the path length. However you will know more about these units while studying BBCS-183 course.

5.4.1 SDS-PAGE

Seperation of proteins mainly depends on the nature of proteins to be seperated. Here we are going to study about few electrophoresis mehtods which are in wide usage. Addition of SDS [CH_3-$(CH_2)_{10}$-$CH_2OSO^-_3Na^+$] to perofrm PAGE referred as SDS-PAGE. As we studied in our previous blocks about proteins, the structure of proteins stabilized by hydrophobic interactions, disulphide bridges and hydrogen bonds. To study the individual polypeptides

of a protein we need to break the above bonds. Solubilising agents like Urea, SDS and β-mercaptoethanol are used to separate proteins in to their individual peptides; Where urea breaks hydrogen bonds, SDS and β-mercaptoethanol breaks hyrophobic and disulphide bridges respectively.

Prior to start the experiment, protein sample to be seperated is boiled for 5minutes in a sample buffer containing bromophenol blue, SDS and β-mercaptoethanol, which completely denatures protein and imparts net negative charge to the polypeptide chains. Bromophenol blue functions as tracking dye to monitor the electrophoretic run.

SDS-PAGE consists of two parts of gel, viz seperating gel (10-15% acrylamide,10 cm) and a shorter stacking gel (2-4% acrylamide, 1 cm). A plstic comb is inserted in between glass plates and gel solution will be poured and comb is removed after polymerisation. This provides loading wells for sample application. Seperating gel is first poured in between glass plates and allowed to polymerize, followed by 1 ml of stacking gel (Fig. 5.4 a & b). Although the gel is formed between glass plates that are clamped together and held apart by spacer arms. Preboiled protein sample loaded in to the wells of the stacking gel and was allowed to separate in the presence applied electric field. Proteins start migrating towards anode, where smaller proteins move fastly and larger one bit slowly due to their molecular seive properties. Current is turned off as the blue dye reaches the bottom of the gel. Then the gel removed from the glass plates and stainied for 2-3 h with the specific stainer (Table 5.1). Then washed overnight with destainer solution (7% acetic acid). This removes unbound background dye from the the gel leaving stained clearly visible blue protein bands (Fig.5.5).

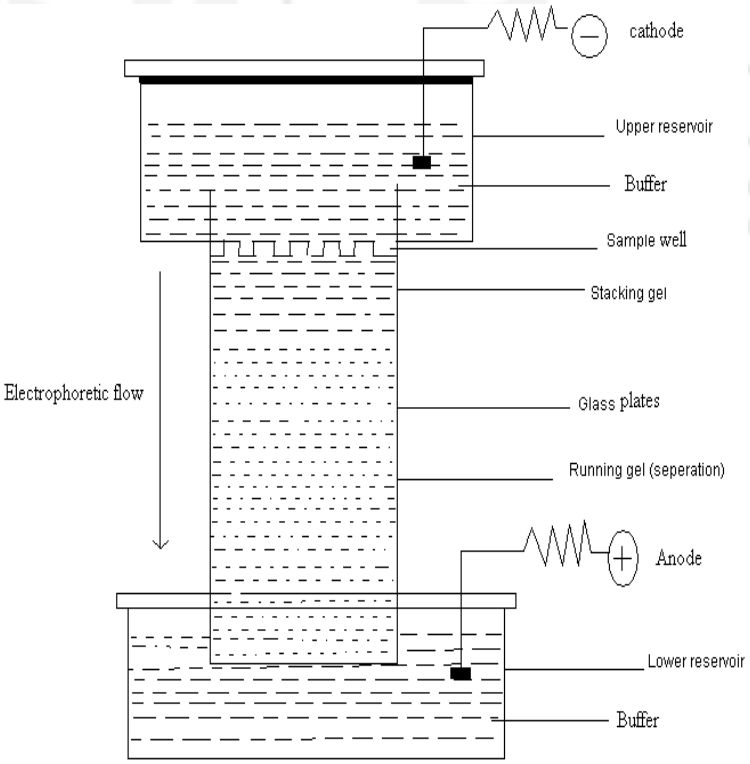

Fig. 5.4 a): Schematic representation of PAGE.

Fig. 5.4 b): Image showing Power pack, electrophoretic unit and accessories used in PAGE.

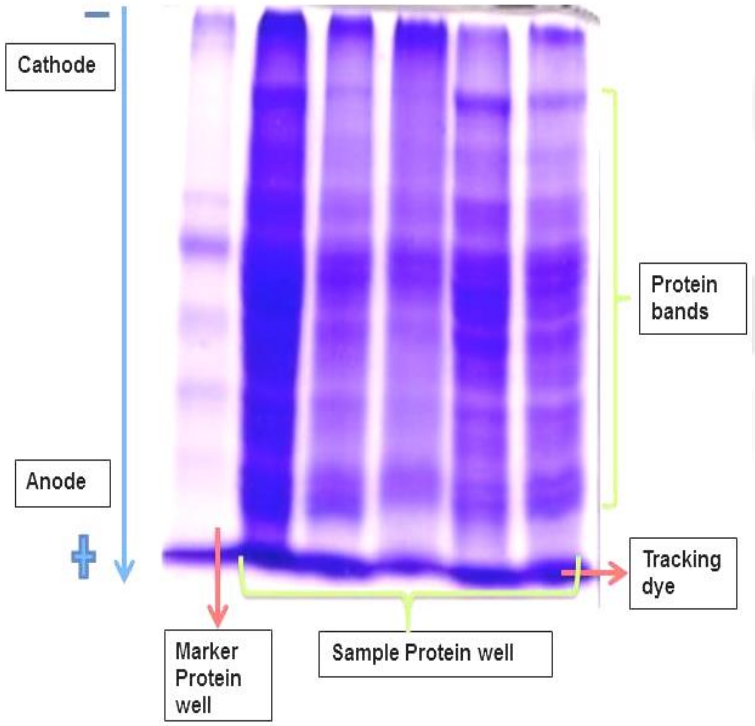

Fig. 5.5: Gel with separated protein bands after SDS-PAGE.

Table 5.1: Different types of stains used in electrophoresis

Compund	Stain	Remarks
Proteins	Bromophenol blue Coomassie Brilliant Blue R-250 Dansyl chloride Aqueous anilinonapthalene sulfonate Lissamine green	Visual Visual Fluorescent Fluorescent Visual Visual
Nucleic acids	Nigrosine Etidium bromide Methylene blue Methyl green-pyronine Lanthanum acetate +	Fluorescent Visual, RNA DNA-blue, RNA-red DNA, RNA-orange red Visual
Lipoproteins	acridine	Visual
Polysaccharides	Toluidine blue Sudan black in 60% ethanol Iodine	DNA-bluish purple, RNA-blue, Proteins-red

5.4.2 Native gel

Polyacrylamide gel prepared without SDS is known as native gel, it is widely used to study the enzymes. As enzymes get denatured by SDS treatment and loose their catalytic property. In native gel electrophoresis all proteins bear their original charge and will get seperated based on their electrophoretic mobility and seiving effect of the gel. Where as all other steps involved in this are similar to that of SDS-PAGE. Native gel is incubated in a solution containing substrate specific to enzyme, which will produce a coloured spot on the gel at the site of enzyme.

5.4.3 Gradient gel

Polyacrylamide is used to preapre this gel, where pore size continue to decrease towards the bottom of the gel and it is achieved by increasing the concentration of acrylamide. When macromolecules tend to separate in an applied electric field, initially the macromolecules move according their electrophoretic mobility, as they reach down the gel they are retardted by small pore size. Hence, in this gel migration is more dependent on size rather than electrophoretic mobility. This method is extensively used to separate crude protein samples.

5.4.4 Isoelectric focusing

It is advised to refer “properties of amino acids” from unit-3 of BBCCT-101 before reading this topic.

This technique was invented by H. Svenson a Swidish scientist, which has got high resolution power. In this technique seperation of proteins achieved based on their zwitter ion state i.e., isoelectric point , hence called as IEF. To perfrom

this ready-made IEF strips are available with various ranges of p^H and length ranging from 7cm to 11cm.

In conventional electrophoresis the p^H between anode and cathode is constant and movement of molecules will be towards oppositely charged electrodes (Fig.5.1). Where as in IEF, a stable p^H gradient is existing between both electrodes, which gradually increases from anode to cathode. Proteins will have net positive charge in acidic p^H as most amino gropus are protonated and as p^H increases towards base, carboxylic groups carrying a negetive charge increases. Due to which most protiens get net negetive charge in this p^H. After reaching isoionic point, the net charge of the protein molecule is zero and they become immovable (Fig. 5.6). Imagine if a aprotein introduced at low p^H i.e., at anode bears a net positive charge. This happens, due to the freely available protons, which may bind to amino (NH_2) group and converts it in to ammonia (NH_3^+). This protein when reaches isoelectric point becomes electrically neautral (zwitterion) and become immovable. IEF has been extensively used for seperation and identification of plama or serum proteins.

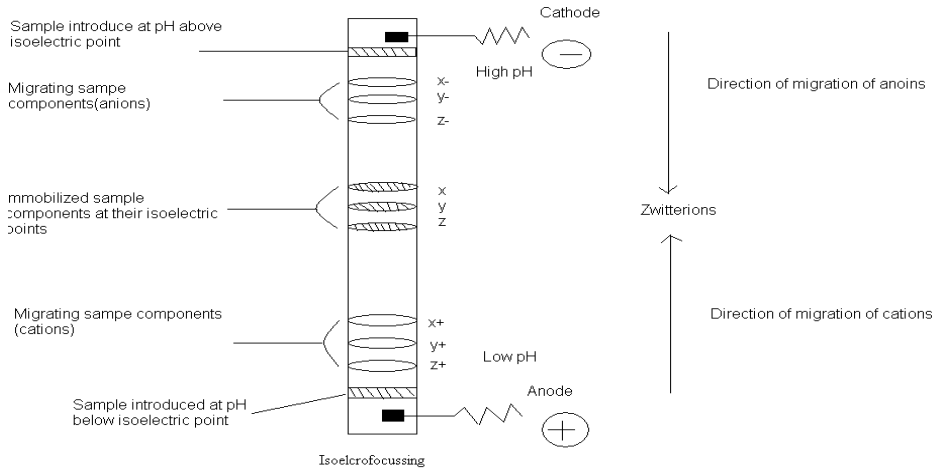

Fig. 5.6: Schematic diagram showing separation of proteins by IEF.

Note: *The resolution power of IEF is much higher than conventional electrophoresis. For example, by performing IEF plasma can be resolved in to 40 individual bands, whereas it is limited to 6 bands in regular paper electrophoresis.*

5.4.5 Two-dimensional Gel Electrophoresis

This technique has evolved by combining the resolving power of IEF and SDS-PAGE. In this protein sample first subjected to IEF, and then the IEF gel is transferred on to the top of the pre-casted SDS gel to perform PAGE, hence it is named as 2-D (Fig. 5.7). IEF and SDS-PAGE separate proteins based on their *isoelectric point* and *molecular weight*, both these properties are unique for any protein. Thus 2-D has got an added advantage over any other electrophoretic technique with regard to separation of proteins. The resolving power of 2-D is so high that a mixture containing 5000 proteins can be separated in to individual proteins spots. 2-D is widely used to analyze crude protein samples. In simple words, 2-D can be assumed as performing IEF in horizontal direction and SDS-PAGE in vertical direction for the same protein sample.

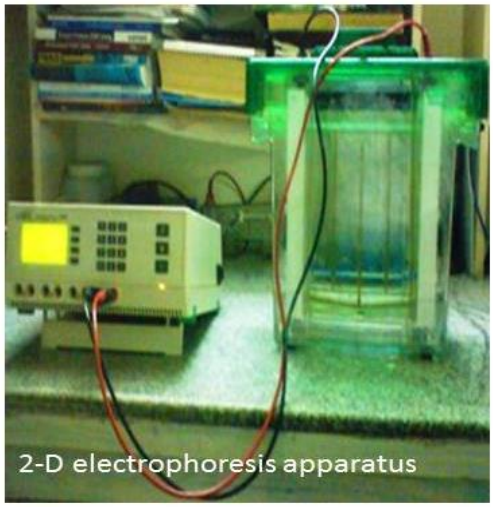

Fig. 5.7: 2-D electrophoresis apparatus and Gel.

SAQ 2

1. Differentiate between native and SDS- PAGE
2. Match the following

i). Zwitter ion	1. IEF+ SDS-PAGE	( )
ii). Size dependent	2. Gradient gel	( )
iii). 2-D electrophoresis	3. IEF	( )

Polyacrylamide gels are also employed to separate DNA: In routine practice, 3.5% polyacrylamide gels are used to separate DNA in the range of 80 – 1000 nt and 12 % gels for fragments between 20 – 100 nt. Gradient gels ranging from 3.5 % - 7.5 % were used for wide range of sizes. This reflects that, lesser will be the pore size for smaller DNA fragments.

Note: After performing electrophoresis DNA can be recovered by i) electro elution ii) Macerating the gel piece in buffer followed by centrifugation and collecting the supernatant, however the final recovery of the DNA is done by subjecting the supernatant to precipitation with ethanol.

5.5 APPLICATIONS OF ELECTROPHORESIS

Electrophoresis has got wide range of applications in Medicine, Clinical and Pharmaceutical research. Some of the important applications are given here

- Paper elcetropphoreis is an essential technique in clinical laboratory for the seperation of serum proteins, lipoproteins, isoenzymes and haemoglobin

  PAGE is a novel elctrophoretic technique for the analysis and seperation of proteins and small fragments of nucleic acids (RNA and DNA)

- SDS-PAGE is used to resolve proteins in to its individual polypeptide chains (sub-units) based on their molecular size and weight
- Determination of *molecular weight of preoteins* by SDS-PAGE is a cutting edge application of electrophoresis. There is a linear relationship exists between electrophoretic mobility of a SDS-protein complex and logarithm of the molecular weight of the protein (Fig. 5.8)

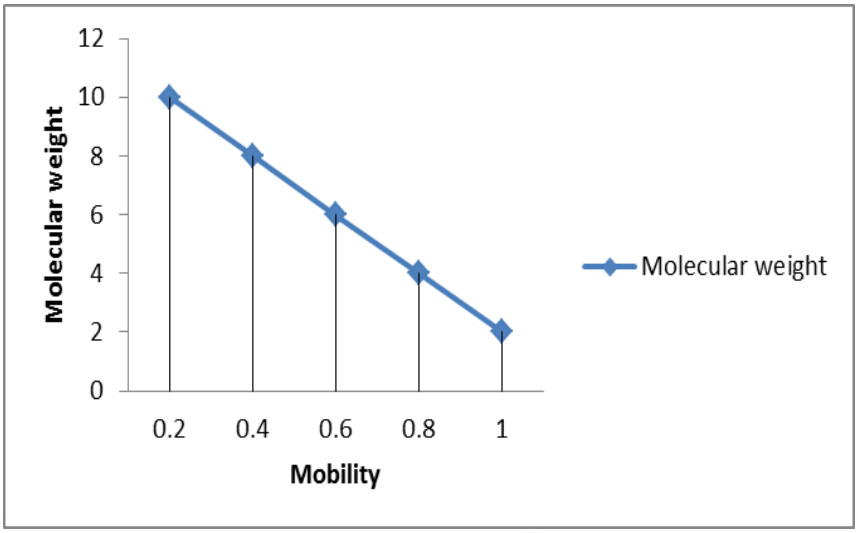

Fig.5.8: Linear plot between molecular weight of SDS-Protein against to electrophoretic mobility. Plot clearly shows that, higher the molecular weight lesser will be the mobility.

- Another way of determining molecular weight of a protein is, by running protein standards along with the unknown sample. By comparing the electrophoretic mobility of these bands one can easily identify the molecular weight of the unknown protein.
- IEF is the most versatile technique which can separate mixture proteins in to individual bands based on their isoelectric points 2-D electrophoresis can separate proteins based on their isoelectric point as well as molecular size. By using 2-D purity of a protein can be assessed, screening variations in the protein expression can be studied.

SAQ 3

1. What is role SDS in SDS-PAGE?
2. At _____ point proteins stop moving in IEF.
3. Electrophoretic mobility of a protein is directly proportinal to _____ (Refer figure 5.8).
4. In 2-D electrophoresis proteins are seperted based on their _____ and _____.

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Applications of electrophoresis in characterisation of Nucleic acids:

- Seperation of large chromosomal genome can be achieved by Pulsed Field Gel Electrophoresis.
- Agarose gel electrophoresis (AGE) is extensively used in sequencing DNA, screening of mutations in DNA or RNA, determine the molecular weight of the proteins and DNA.
- AGE is widely used in Immunoelectrophoresis to investigate the *purity of specific antisera* (antibodies raised against particular antigen). Also to check the cross reactivity and antigen concentration in blood bank.
- Electrophoresis is widely used in *DNA fingerprinting* and developing *DNA restriction maps*. These two applications are well recognised for the *genetic analysis* either with in or in between different species.
- Blotting techniques (hybridization tehcniques) like Southern, Northern and Western used for identification of DNA, RNA and protein are totally dependent on the electrophoresis.
- An advanced application of electrophoresis is EMSA (electrophoretic mobility shift assay), to study the *nucleic acid-protein interaction*. This is very essential technique in studies involving cell growth and behaviour. This technique also known as *gel retardation* or *band shift assay*.

5.6 SUMMARY

Let us summarise what all has been studied and discussed in this unit:

- Seperation of charged particles in the presence of an applied electric field is the principle
- To perform electrophoresis, the basic requirements are support media in the form of agarose or polyacrylamide. Proteins seperated using native PAGE and SDS-PAGE.
- The unique nature of SDS-PAGE is to separate proteins into its individual polypetide chains.
- IEF has been extensively used for seperating proteins based on their isoelecric points.
- 2-D electrophoresis is known for its high resolving power.

S

5.7 TERMINAL QUESTIONS

1. What is protein purity? Explain the basic principle behind electrophoresis?
2. Describe SDS-PAGE and its significance in characterization of proteins?
3. Explain how 2-D electrophoresis is advantageous over PAGE?
4. Write a short note on supporting mediums (matrix) used in electrophoresis?
5. Enlist the applications of electrophoresis.

5.8 ANSWERS

SAQ 1

1. The principle operating electrophoresis is based on “the separation of charged particles or molecules in the presence of an electric field”. In an applied electric field ion move towards oppositely charged electrodes.
2. Agar is made up of two galactose based polymers, such as agarose and agaropectin. Poly acrylamide is composed of acrylamide monomer, N,N$^{'}$-methylenebisacrylamide (bis).

SAQ 2

1. Addition of sodium dodecyl sulphate is the major difference between SDS-PAGE and PAGE.
2. 3,2,1.

SAQ 3

1. Sodium Dodecyl Sulfate (SDS) is responsible to break the hydrophobic interactions of a protein and also develops net negetive charge on the protein. These two roles are very essential in the seperation of proteins through SDS-PAGE (refer section 5.4.1).
2. Isoelectri point
3. Size
4. Size and charge

Terminal Questions

1. Purity defined as the quality of the proteins isolated. Refer section 5.2 for complete details.
2. SDS-PAGE kanown as Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. It is derived from basic PAGE, where SDS being added to break the hydrophobic interactios and to impart net negetive charge over the protein. SDS-PAGE is is used to study the protein stoichiometry and determination of molecular weight of unknow protein. PAGE can separate protein based on their molecular size only, where as in 2-D sepeartion is achieved by both isoelectric point and molecular size of

protein. Both these properties are unique for any proetin. Hence it has got an edge over PAGE. Refer to topic 5.4.1 in this unit for more details.

3. 2-D electrophoresis can separate proteins based on their isoelectric point as well as molecular size. Refer section 5.4.5.

4. Agarose and polyacrylamide are the most commonly used gel materials (Refer section 5.3).

5. Electrophoresis is widely used technique to characterise the proteins. (Refer section 5.5).

5.9 FURTHER READINGS

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