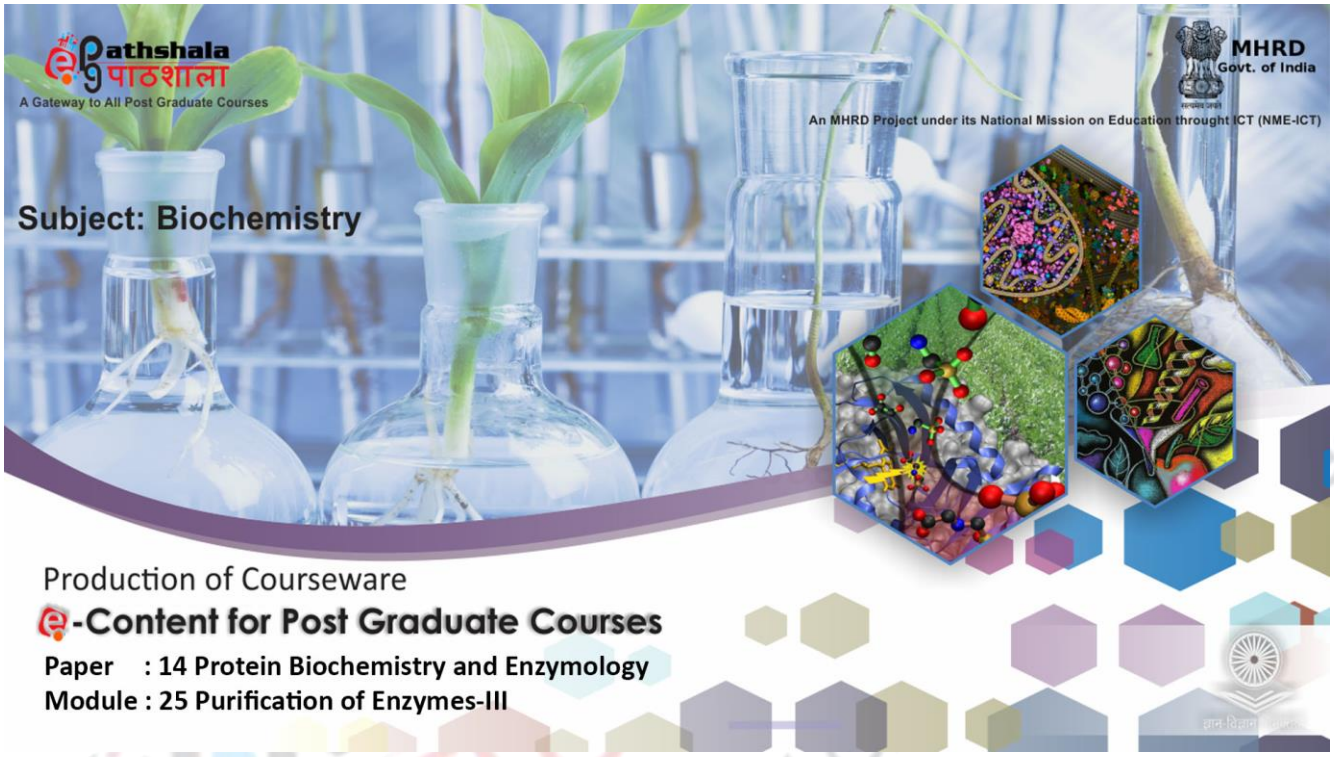

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Development Team

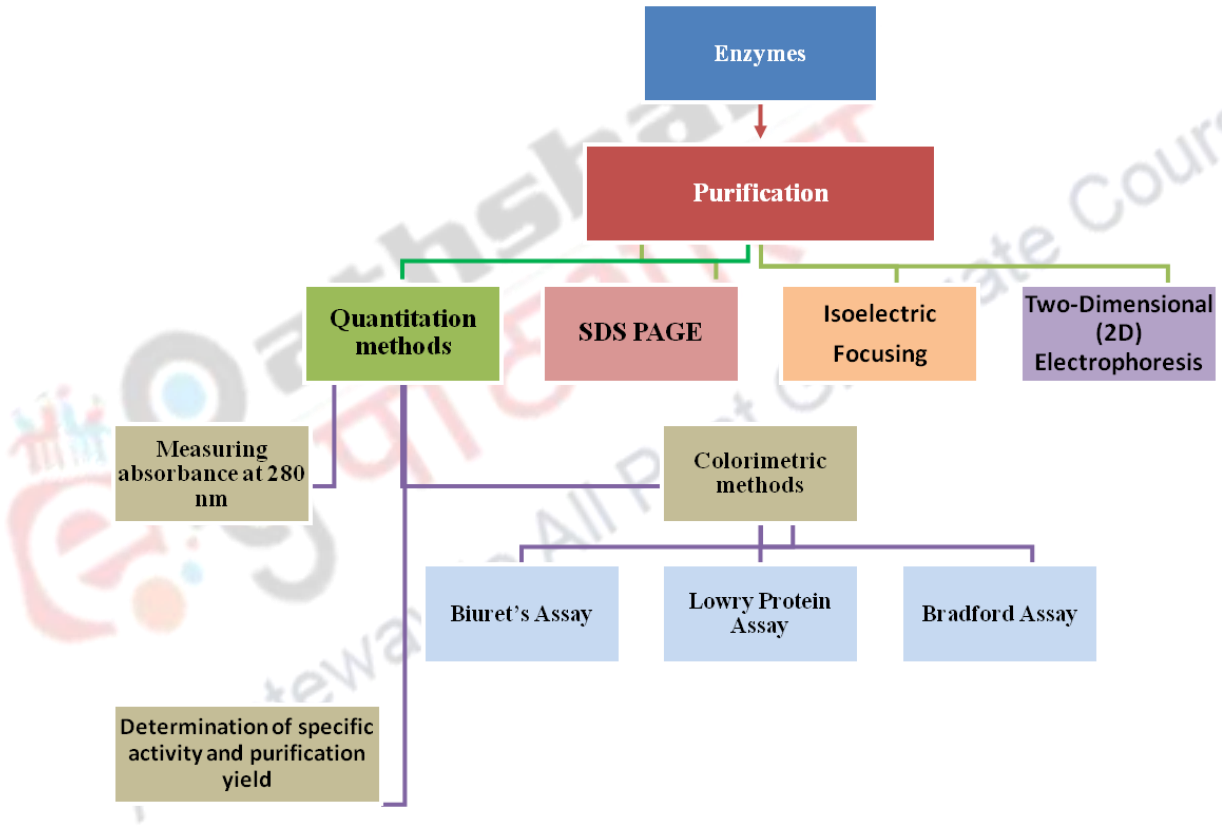
Principal Investigator	Dr. Sunil Kumar Khare, Professor, Department of Chemistry, IIT-Delhi
Content Writer:	Dr. Rajeshwari Sinha,Center for Science and Environment, Delhi
Content Reviewer:	Dr. Tanmay Dutta, Assistant Professor, Department of Chemistry, Indian Institute of Technology, Delhi
Paper Coordinator:	Dr. Archna Sachdeva, Professor, Division of Biochemistry, Indian Agricultural Research Institute (IARI), New Delhi

Description of Module	
Subject Name	Biochemistry
Paper Name	08 Protein Biochemistry and Enzymology
Module Number/Title	25 Purification of Enzymes-III

1. Objectives

1. Understanding methods of quantitative analysis of proteins
2. Determination of specific activity and purification yield
3. Explaining SDS-PAGE electrophoresis
4. Explaining iso electric focussing and 2D electrophoresis.

2. Concept Map



3. Description

3.1 Quantitation of proteins

The quantitative estimation of proteins is a very important and necessary step throughout the entire purification process. Knowledge of the amount of protein present after each step in purification is an essential criterion. Depending on the amount and purity of the protein available, different chromatographic methods can be selected for protein separation. The various methods of estimating protein quantitatively are shown in Figure 1.

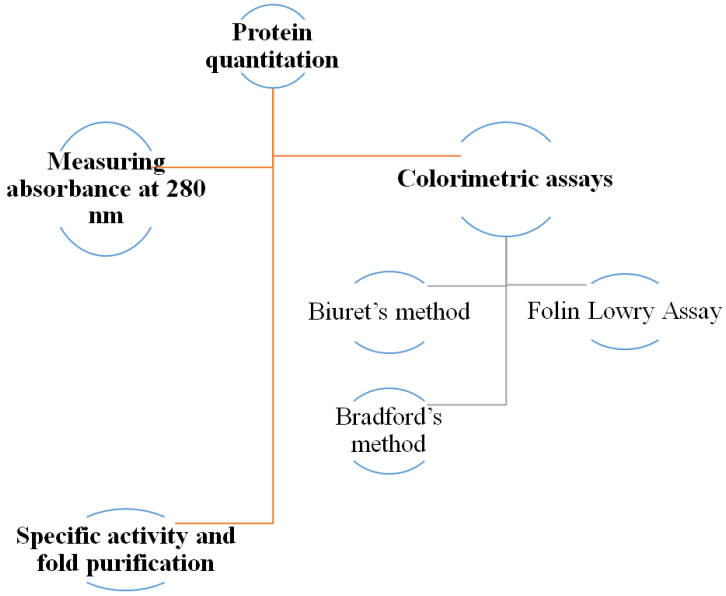


Figure 1. Methods of quantitative protein estimation

3.1.1 Measuring absorbance at 280 nm

Measurement of the protein absorbance at 280nm (UV range) is one of the most simplest and direct assay method for proteins present in solution. Amino acids containing aromatic side chains like those of tyrosine, tryptophan and phenylalanine exhibit a strong UV-light absorption 280 nm (Figure 2). Thus, proteins and peptides will also absorb UV-light in proportion to their aromatic amino acid content and concentration.

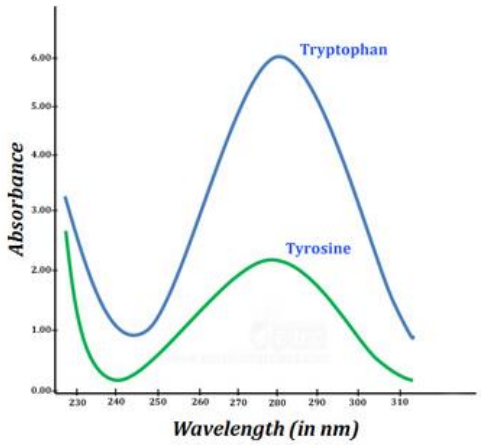


Figure 2 Absorption peak of amino acids at 280 nm

3.1.2 Colorimetric methods

Several colorimetric protein assay techniques are available, which are used routinely for determining protein concentration. The principle of these colorimetric approaches is that when protein is added to the coloring reagent, a complex is formed leading to a color change which can be read colorimetrically and which is proportional to the concentration of protein added. A standard curve consisting of known concentrations of a purified reference protein is plotted previously to assist in the determination of protein concentration.

The various colorimeric methods used for protein estimation are (i) Biuret's method (ii) Folin Lowry method (iii) Bradford's method.

- **Biuret's method**

The Biuret's reagent comprises of hydrated copper (II) sulfate, together with potassium sodium tartrate, in an alkaline medium of NaOH. When mixed with proteins, the Copper (II) ion (Cu^{2+}) complexes with peptide nitrogens in proteins to generate a pink or violet blue colour complex (Figure 3).

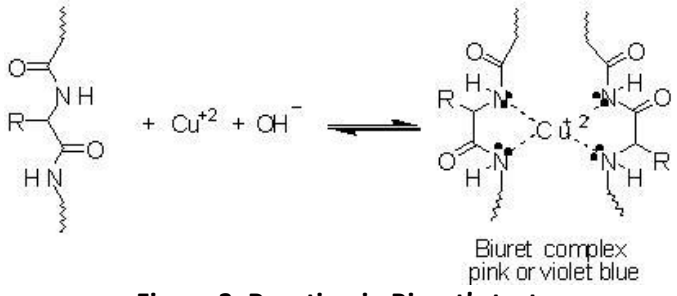


Figure 3. Reaction in Biuret's test

The colouration of the complex is proportional to the concentration of the protein and can be read spectrophotometrically at 550 nm.

Properties of Biuret's method:

a) It is not affected by any contaminating amino acids since complex formation depends on atleast a dipeptide structure.
b) It is not interfered by the presence of nucleic acids.
c) The test can be interfered by ammonia or amines. Thus, false positive results can be obtained if ammonium sulfate salts, or TRIS (trishydroxymethyl amino ethane) is present in the sample. This should be avoided
d) The method requires a high concentration of protein in th range of 5–160 mg/mL.

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- **Lowry Protein assay**

The Lowry method is a modification of the Biuret method. After treatment with Cu^{2+} (as in Biuret's method), the protein is treated with **Folin-Ciocalteu reagent** (a mixture of phosphotungstic acid and phosphomolybdic acid) under alkaline conditions. The product of this reaction is **heteropolymolybdenum blue**, which absorbs strongly at 750nm (Figure 4).

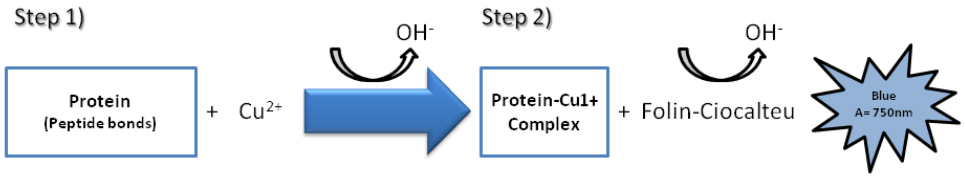


Figure 4 Reaction in Lowry Protein Assay

Properties of Folin-Lowry method:

a) It is more sensitive and can detect lower concentrations of protein (0.01–1.0 mg/mL).
b) The assay as well as standard curve must be performed at lower protein concentrations since the linear dependence is achieved only at low protein concentrations.
c) It is sensitive to presence of contaminants.
d) The assay needs to be performed with precision.

- **Bradford Assay**

The basic principle of this assay is that a dye, Coomassie Brilliant Blue binds to proteins under acidic conditions to form a blue coloured complex that absorbs strongly at 595nm (Figure 5).

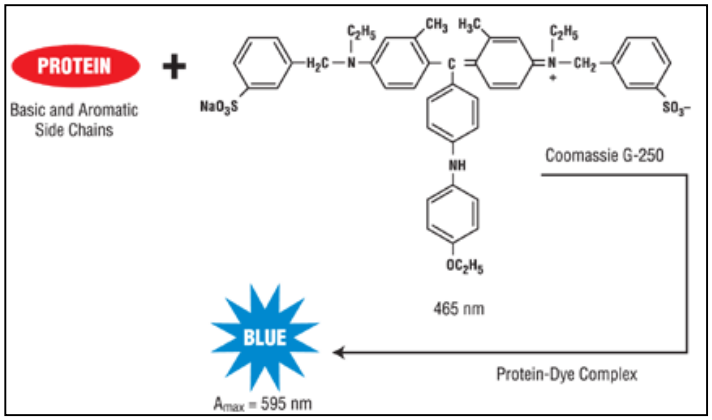


Figure 5 Reaction in Bradford's assay

Properties of Bradford's method:

- The assay is independent of the amino acid composition
- A standard curve and dilution of the sample is always essential for this method because the assay is linear for a very short range of protein concentration (0 µg/mL to 2000 µg/mL).
- The assay is interfered by the presence of detergents like SDS.
- The dye stains not only proteins but also cuvettes, test tubes, floors etc. This must be taken care of. Cuvette and test tube stains can be removed by washing with alcohol (ethanol).

3.1.3 Determination of specific activity and purification yield

The success of a protein purification scheme is also evaluated by determining specific activity and purification yield after each purification step. At each step, the parameters which are measured are discussed briefly below:

- *Total protein*-The total amount of protein present in a fraction is obtained by determining the protein concentration present in each fraction. This is obtained using one of the protein quantitation methods stated above. Bradford's method is most widely used. Its unit is usually in mg or µg.
- *Total activity*- The activity present in any fraction is obtained by measuring the enzyme activity using assay specific to the enzyme. The total activity can be obtained by adding the activity in each fraction. Its unit is usually expressed as U.
- *Specific activity*- Specific activity is a parameter which gives us an idea of the purity of the enzyme/protein in the mixture. It is the activity of the enzyme per unit mass of the protein obtained by dividing the total activity by total protein. Its unit is U/mg. The purer the protein, the higher is the specific activity.

- *Yield*-It is a measure of how much activity is retained after each step in purification. The enzyme activity retained after each purification step is divided by the total initial activity of the crude extract and is expressed as a percentage (%).
- *Purification fold*- It is an index of how much the specific activity of the target protein has increase in comparison o the initial specific activity when the target protein was present in the crude extract. It is obtained by dividing the specific activity (after any particular purification step), by the specific activity of the initial crude extract. It has no units. Higher the purification fold, greater is the extent of protein purification that has been achieved.

An exemplary purification table is shown in Table 1.

Table 1. An exemplary purification table for enzyme purification

purification step	total protein (µg)	total phytate-degrading activity (U)	Specific activity (U mg^{-1})	purification (fold)	recovery (%)
Crude extract	54000	138	2.6	-	-
0-90% $(NH_4)_2SO_4$ precipitation	26539	117	4.4	1.7	84.8
DEAE Sepharose CL6B	5693	110	19.3	7.4	79.7
CM Sepharose CL 6B	1156	62	53.6	20.6	44.9
Sephacryl S-200 HR	287	36	125.4	48.2	26.1
Mono S HR 5/5	211	28	132.7	51.0	20.3

An efficient and good purification scheme should take into consideration both the purification fold as well as the yield.

3.2 SDS Page Electrophoresis

SDS-PAGE or Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (PAGE) is an important approach to analyze how much effective the purification strategy has been through visualization of the proteins present after each step of purification. The technique is based upon the principle that SDS-denatured and negatively charged protein molecules migrate towards a positive electrode in the presence of an electric field. In the presence of an electric field, the proteins in the gel migrate with different migration rates based on the mass, simultaneously getting separated.

3.2.1 The electrophoresis gel matrix and set up

Electrophoretic separations are mostly performed in gels which serve as a molecular sieve. A typical set up for SDS PAGE electrophoresis is shown in Figure 6.

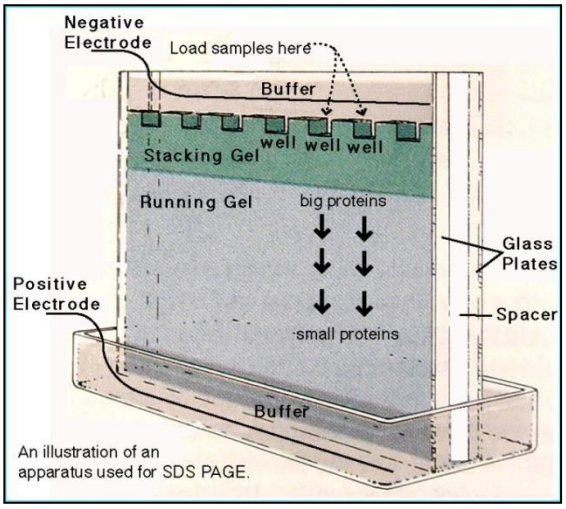


Figure 6. Polyacrylamide Gel Electrophoresis Set up

The electrophoresis is performed in a thin, vertical slab of polyacrylamide gels, formed by the polymerization of acrylamide and cross-linked by N, N'-methylenebisacrylamide (Figure 7).

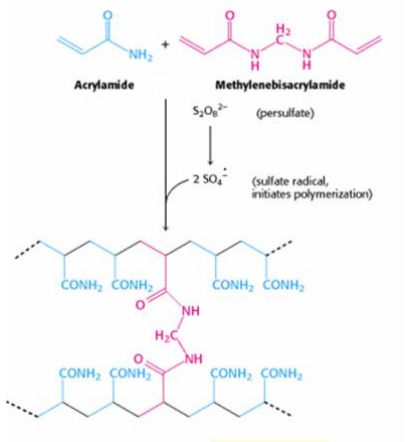


Figure 7. Formation of a Polyacrylamide Gel

Protein molecules that are smaller in size as compared to the pore sizes in the gel, can readily move through the gel, whereas molecules with sizes larger in size than the pores travel comparatively slowly. Intermediate-size molecules move through the gel at intermediate rates. The direction of movement of the proteins in the gel is from top(cathode) to bottom (anode).

3.2.2 Role of SDS

When proteins are dissolved in a solution of sodium dodecyl sulfate (SDS)(Figure 8), and further boiled, the secondary and tertiary structure of native proteins are completely disrupted. Mercaptoethanol or dithiothreitol is also added further to reduce disulfide bonds. The denatured protein is now unfolded into a linear structure. Anions of SDS bind to these linear chains at a ratio of about one SDS anion for every two amino acid residues. This SDS-protein complex now has an overall large net negative charge that is proportional to the mass of the protein. This negative charge conferred by the SDS molecules is usually much greater than the charge on the native protein, thus rendering the native charge on the protein as insignificant (Figure 9).

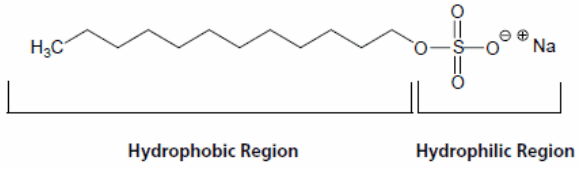


Figure 8. The SDS molecule

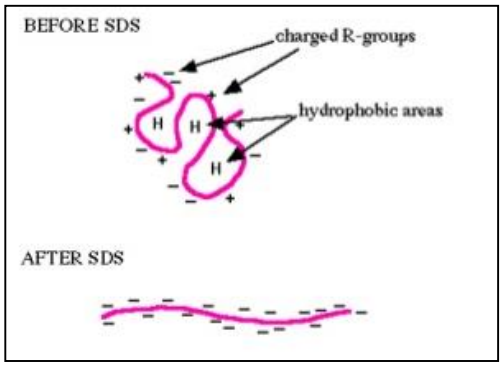


Figure 9. Linearization and charge-masking of proteins with SDS

3.2.3 The discontinuous buffer system

The SDS Page electrophoresis functions through the use of a discontinuous Laemmli buffer system, which implies that different buffers are used in the gel and tank. The stacking gel is buffered by Tris-HCl to pH 6.8, the running gel buffered by Tris-HCl to pH 8.8 and an tank buffer is buffered at pH 8.3 (Figure 10).

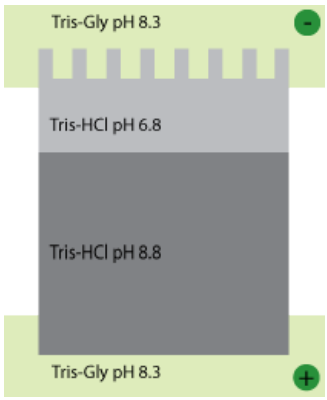


Figure 10. Discontinuous buffer system in SDS PAGE

3.2.4 Migration of ions and proteins during electrophoresis

Figure 11 outlines the different charge states of glycine (positive, neutral or negative) under different pH conditions.

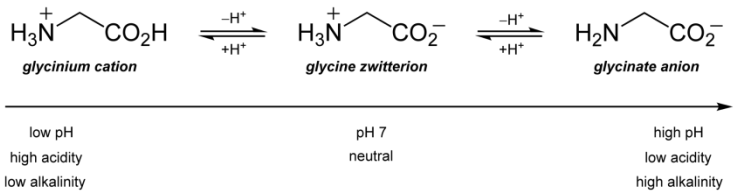


Figure 11. Different charge states of glycine

The migration of ions and proteins during SDS PAGE electrophoresis is explained below (Figure 12).

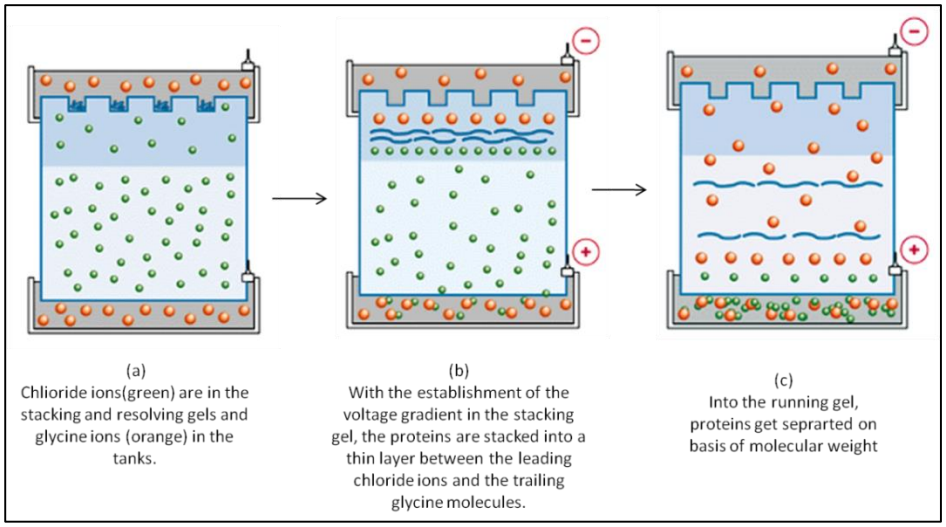


Figure 12. Migration of ions and proteins during electrophoresis

At the start of SDS-PAGE protein electrophoresis, the chloride ions are in the stacking and resolving gels and negatively-charged glycine is present in the tanks (Figure 12 a).

When the electric field is set up, the negatively-charged glycine ions enter the stacking gel from the electrode/tank buffer. In this environment (pH 6.8), the glycine molecules become zwitterionic in nature. With no net charge on them, the glycine molecules move very slowly in the electric field. On the other hand, the Cl- ions (from Tris-HCl), move much rapidly in the electric field resulting in the formation of an ion front that migrates ahead of the glycine.

A steep voltage gradient is created, resulting in two narrowly separated fronts of migrating ions; the highly mobile Cl- front, followed by the slower, zwitterionic glycine molecule front with proteins in the stacking gel having an intermediate mobility between these two. The proteins are thus stacked into a thin layer between the leading chloride ions and the trailing glycine (Figure 12 b).

At the running gel (pH 8.8), the glycine molecules become negatively charged and can now migrate much faster and thus, they accelerate past the proteins. The proteins remain stacked in a narrow band at the interface of the stacking and running gels. Further, since the running gel has an increased acrylamide concentration as compared to the stacking gel, the pore sizes in the gel are sill smaller and the movement of the proteins is further slowed down. Higher molecular weight proteins move more slowly through the porous acrylamide gel compared to lower molecular weight proteins. Thus separation of the proteins begins according to their size (Figure 12 c).

3.2.5 The staining

- **Coomassie staining**

The electrophoresed SDS gel is visualized by staining with a dye such as Coomassie blue R-250. The principle of Coomaasie staining is the same as that of the Bradford's protein assay wherein Coomaasie dye reacts with proteins to generate a blue-coloured complex. Figure 13 shows a Coomassie stained SDS gel after electrophoresis.

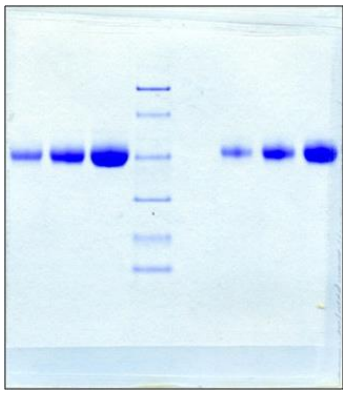


Figure 13. Coomassie staining of proteins after electrophoresis

- **Silver staining**

Another method for staining electrophoresed gels is the silver staining method. Silver staining is used in cases when the protein samples loaded onto the gel have lower concentrations and a higher sensitivity is required. This method of staining however uses simple and cheap reagents and equipments. The various steps involved in silver staining of an SDS PAGE electrophoresed gel containing proteins are fixation, sensitization, silver impregnation and development. However, silver staining requires a lot of carefully handled steps. A silver stained SDS-PAGE gel is shown in Figure 14.

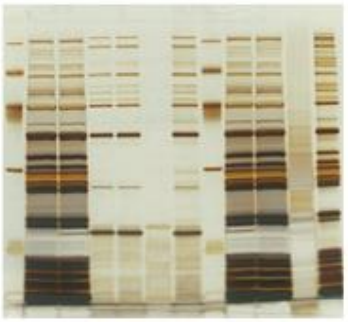


Figure 14. A silver stained SDS PAGE gel after electrophoresis

3.2.6 Sensitivity

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) method for protein visualization is a rapid and sensitive method, which also provides high degree of resolution to the protein sample being separated. While, in case of Coomassie staining, proteins with concentration in the microgram range (as little as 0.1 µg) gives a distinct and

visible band, the sensitivity is even higher in case of silver staining where protein samples in the concentration of nanogram range can be detected. Also, proteins that differ in mass by about 2% can also be separated and distinguished on an SDS gel.

3.2.7 Applications:

SDS PAGE electrophoresis can be used for determination of purification efficiency and protein molecular mass.

- **Determination of mass**

SDS PAGE electrophoresis can be used for the determination of the mass/molecular weight of the protein. The mobility of most protein molecules under SDS-PAGE conditions is linearly and inversely proportional to the logarithm of their mass. Thus mass of the protein can be determined by an extrapolation of the graph plotted between logarithm of the mass (y-axis) against the relative mobility or migration distance on the SDS gel (x-axis).

- **Efficacy of purification**

SDS-PAGE can also be used to assess the efficiency of any particular purification scheme by analyzing each fraction (containing the target protein to be purified). While the initial fractions will display a number of contaminating proteins along with the target proteins, the number of bands will diminish as the purification progresses, and only the band pertaining to the purified target protein will predominate which will refer to the fraction containing the single purified protein, free from any contaminating protein.

3.3 Isoelectric Focusing

Isoelectric focusing refers to the separation of proteins electrophoretically on the basis of their pI that is on the basis of their relative acidic and basic residues. The *isoelectric point* (pl) of a protein is the pH at which its net charge remains zero. Therefore, at this pH (isoelectric point), the electrophoretic mobility of the protein will also be zero since the proteins will not be able to migrate in any electric field. When a mixture of proteins is subjected to gel electrophoresis across a pH gradient, each protein will tend to move in the gel until it reaches a position where the pH becomes equal to the pI of the protein. At this position, the migration of the protein will stop. Figure 15 outlines the method of isoelectric focusing for separation of proteins.

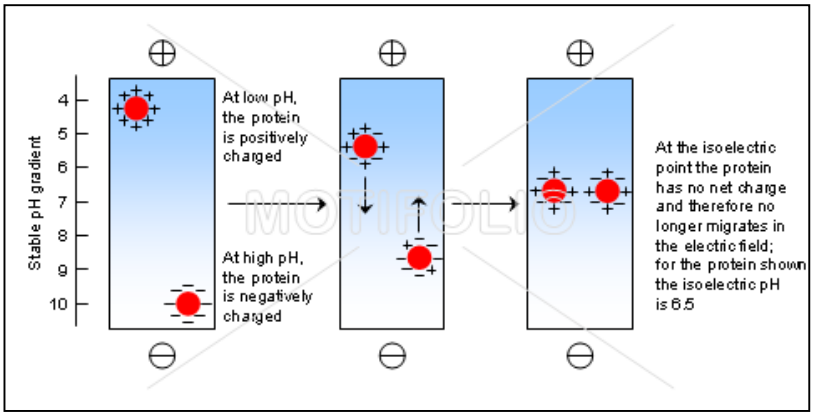
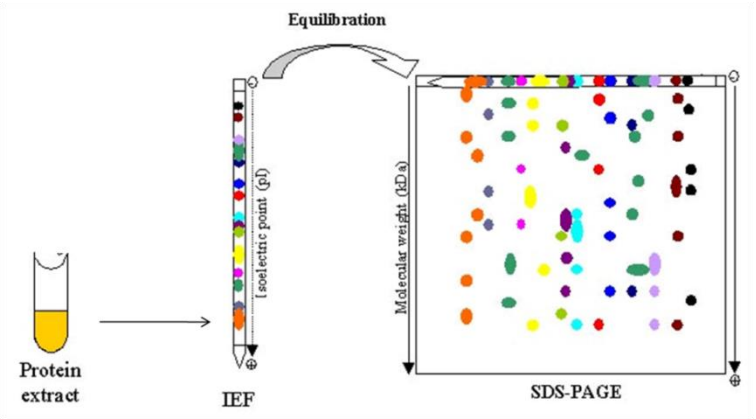


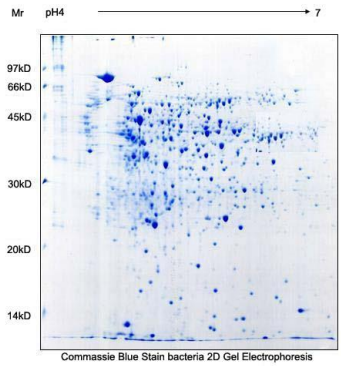
Figure 15. Separation of protein molecules by isoelectric focussing

3.4 Two-Dimensional (2D) Electrophoresis

Two-dimensional electrophoresis is an advanced method of protein separation where the separation of proteins on a gel is conducted in two dimensions. It involves the combination of isoelectric focusing with SDS-PAGE to obtain protein separations with a very high resolution.

The sample to be purified is first subjected to isoelectric focusing as described in the previous section (1 dimension). For the second dimension, the gel is then subjected to SDS PAGE electrophoresis. The proteins now move through polyacrylamide gel vertically based on how far they migrated during isoelectric focusing (Figure 16 a).Post the electrophoresis, the gel is further stained by Coomassie or silver staining to yield visible two dimensional pattern of spots (Figure 16 b). Thus, in 2D gel electrophoresis, the proteins are separated horizontally on the basis of isoelectric point and vertically on the basis of their mass (Figure 16).





Commassie Blue Stain bacteria 2D Gel Electrophoresis

(a) (b)

Figure 16 (a) Scheme of Two-Dimensional Gel Electrophoresis (b) A 2D electrophoresed gel

The proteins separated by 2D electrophoresis can further be identified by coupling with mass spectrometric techniques.

4. Summary

1. Protein quantitation is an essential step at each purification step;
2. Specific activity and purification yield are important parameters to be considered at each purification step;
3. SDS-Polyacrylamide Gel Electrophoresis (PAGE) is an important technique to analyze the effectivity of the protein purification and visualization of the proteins present at each purification step;
4. Isoelectric focusing is used for protein separation on the basis of their pI. In 2D gel electrophoresis, the proteins are separated horizontally on the basis of isoelectric point and vertically on the basis of their mass,