# Separation Techniques/Bioseparation

## Ph.D. Courses

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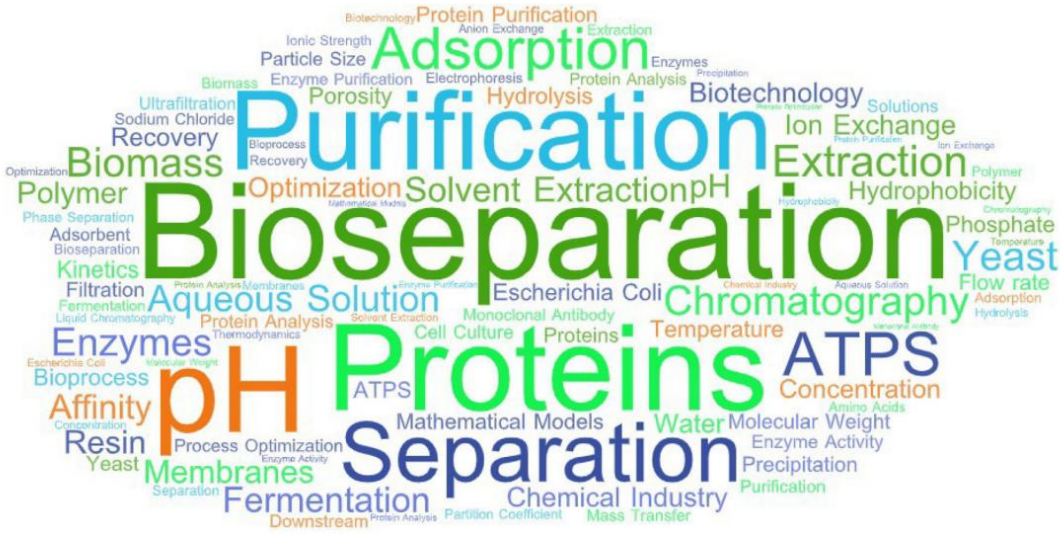
**Reference book:** **"Bioseparations: Principles and Techniques" by Sivasankar**

This course introduces the fundamental principles of separation operations for the recovery of products from biological processes, membrane filtration, centrifugation, cell disruption, extraction, and process design.



## Protein Separation and Identification

- The primary, secondary, tertiary, and quaternary structure of a protein determine its physical, chemical, biochemical, physical, chemical, and biological properties and it summarizes the differences in the properties of different proteins or changes the conditions to make them different. Using a variety of properties at the same time, in the case of both yield and purity, choose the method of protein purification.
- The overall goal of the protein purification is trying to increase the purity of products or specific activity, requires reasonable, speed, high yield, purity for purification.
- The reason why a protein can be purified from thousands of protein mixtures is that different proteins have very different physical, chemical, physicochemical and biological properties.
- These properties are caused by different sequences and number of proteins' amino acids, and connected in the main chain of the polypeptide amino acid residues is positive, burden on electricity, polar or non-polar, hydrophilic or hydrophobic.
- In addition, the polypeptide can fold into a very determined secondary structure of (alpha helix, beta, folding, and various Angle), tertiary structure, and quaternary structure. Formed unique size, shape, and distribution of residues on the protein surface and a set of reasonable fractional separation steps can be designed by taking advantage of the difference in properties between the protein to be separated and other proteins.
- The protein mixture can be separated according to the method corresponding to the different properties of the protein as shown in the following table:

## Gel Electrophoresis

Agarose is a natural linear polymer extracted from seaweed that forms a gel matrix by hydrogen bonding when heated in a buffer and allowed to cool. For most applications, only a single-component agarose is needed and no polymerization catalysts are required (Figure 3.1). Therefore, agarose gels are simple and rapid to prepare. They are the most popular medium for the separation of moderate and large-sized nucleic acids and have a wide range of separation but a relatively low resolving power since the bands formed in the gels tend to be fuzzy and spread apart. This is a result of pore size and cannot be largely controlled. These and other advantages and disadvantages of using agarose gels for electrophoresis are summarized in Table 3.1.

Table 3.1. Advantages and Disadvantages of Agarose Gel Electrophoresis.

| Advantages | Disadvantages |
|---|---|
| Nontoxic gel medium | High cost of agarose |
| Gels are quick and easy to cast | Fuzzy bands |
| Good for separating large DNA molecules | Poor separation of low molecular weight samples |
| Can recover samples by melting the gel, digesting with enzyme agarose or treating with chaotropic salts | |

Polyacrylamide gels are chemically cross-linked gels formed by the polymerization of acrylamide with a cross-linking agent, usually N, N'-methylenebisacrylamide (Figure 3.1). The reaction is a free radical polymerization, usually carried out with ammonium persulfate as the initiator and N,N, N', N'-tetramethylethylendiamine (TEMED) as the catalyst. Although the gels are generally more difficult to prepare and handle, involving a longer time for preparation than agarose gels, they have major advantages over agarose gels. They have a greater resolving power, can accommodate larger quantities of sample without significant loss in resolution and the purity of the sample recovered from polyacrylamide gels is extremely high. Moreover, the pore size of the polyacrylamide gels can be altered in an easy and controllable fashion by changing the concentrations of the two monomers. Thus, it is commonly used to separate proteins and smaller fragments of DNA. It should be noted that polyacrylamide is a neurotoxin (when unpolymerized), but with proper laboratory care it is no more dangerous than various commonly used chemicals. Some advantages and disadvantages of using polyacrylamide gels for electrophoresis are depicted in Table 3.2.

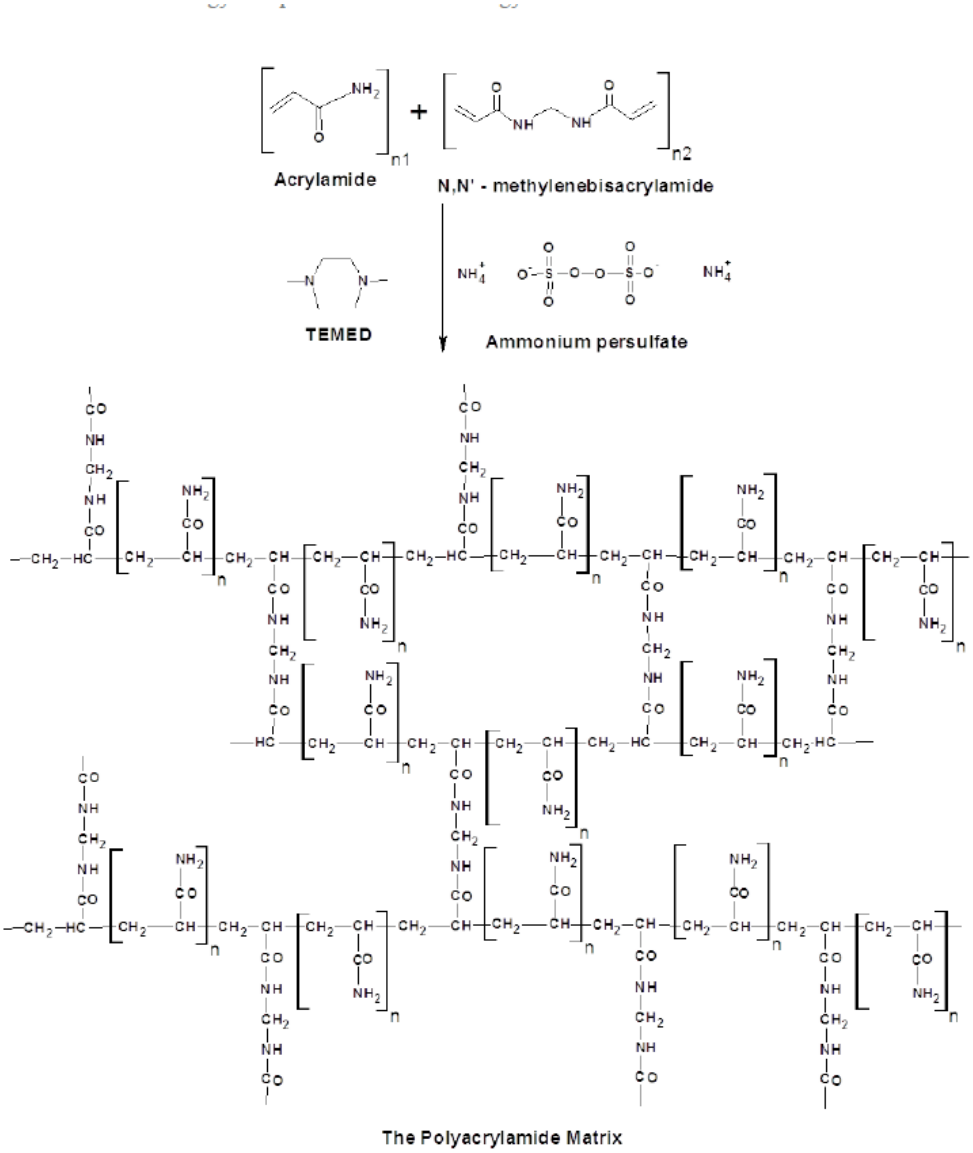



**Figure 3.1: Gels Commonly Used in Electrophoresis.**

**(A) Agarose is composed of agarobiose,**

**(B) The polymerization of acrylamide and bisacrylamide to form polyacrylamide gel. The polymerization reaction is initiated by persulfate radicals and catalyzed by TEMED.**

Table 3.2. Advantages and Disadvantages of Polyacrylamide Gel Electrophoresis.

| Advantages | Disadvantages |
|---|---|
| Stable chemically cross-linked gel | Toxic monomers |
| Sharp bands | Gels are tedious to prepare and often leak |
| Good for separation of low molecular weight fragments | Need new gel for each experiment |
| Stable chemically cross-linked gel | |

**SDS PAGE: Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis (PAGE).** of proteins with a polyacrylamide matrix, commonly called polyacrylamide gel electrophoresis (PAGE) is one of the most widely used techniques to characterize complex protein mixtures. It is a convenient, fast and inexpensive method because it requires only the order of micrograms quantities of protein. They are usually run in a vertical format and the gel rigs contain an upper and lower buffer reservoir (Figure 3.2). The samples are loaded in wells that contact the upper buffer reservoir which will house the negative cathode. The proteins migrate towards the positive anode when the electric current is applied.

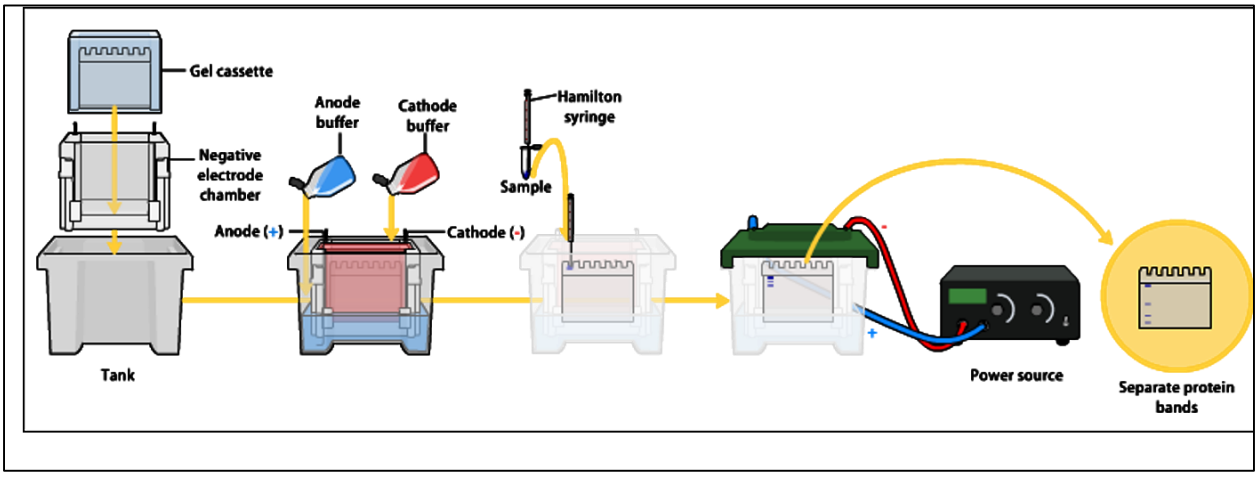



**Figure 3.2 Polyacrylamide Gel Electrophoresis (PAGE).** a typical set up for PAGE. A vertical gel is placed into a rig with an upper and lower buffer reservoir. The upper reservoir contains the gel wells where the protein is loaded and will house the cathode (negative charge). The proteins will run towards the anode (positive charge) when an electric field is placed on the system, in relation to the protein size, shape and charge.

Note that proteins have a net electrical charge if they are in a medium having a pH different from their isoelectric point and therefore have the ability to move when subjected to an electric field. The migration velocity is proportional to the ratio between the charges of the protein and its mass. The higher charge per unit of mass the faster the migration.

Proteins do not have a predictable structure as nucleic acids, and thus their rates of migration are not similar to each other. Furthermore, they will not migrate when applying an electromotive force, when the pH of the system is the same as isoelectric point. PAGE gels that are run in this fashion are called Native PAGE, as the proteins are still folded in their native state found in vivo. In this situation, proteins migrate according to their charge, size and shape. Alternatively, proteins may be denatured prior to electrophoresis. The most common way to denature the proteins is by adding a detergent such as sodium dodecyl sulfate (SDS) (Figure 3.3).

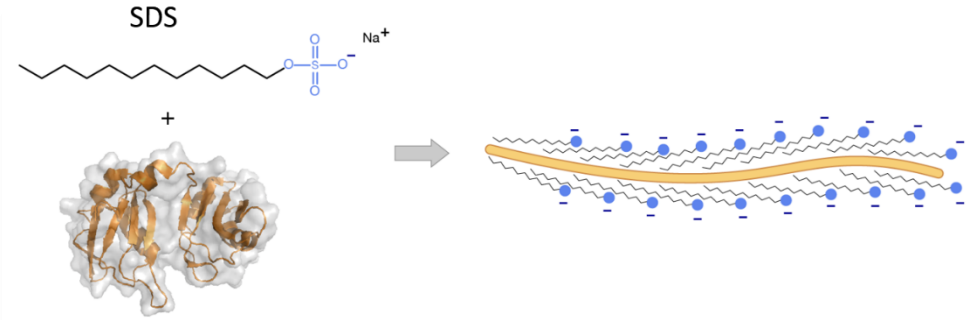



**Figure 3.3** Polyacrylamide Gel Electrophoresis (PAGE). Sodium dodecyl sulfate (SDS) is often used to denature proteins prior to PAGE analysis, causing proteins to migrate based on size only.

This not only denatures the proteins, but it also coats the protein with a negative charge, such that all of the proteins will run towards the positive lead when placed into an electric field. This type of electrophoresis is referred to as SDS-PAGE and separates proteins exclusively according to molecular weight. A reducing agent that breaks disulfide bonds, such as β merceptoethanol is often added to the loading buffer as well, causing proteins to fully denature and dissociate into the monomer subunits. This ensures that the proteins migrate through the gel in direct relation to their size, rather than by charge or shape.

Beta-mercaptoethanol is a reducing agent in the loading buffer that cleaves disulfide bonds, which are unaffected by SDS. Together with SDS, it ensures the unfolding of the protein, making the structure of the protein primary. The primary structure is necessary for the separation of proteins to be based on their molecular weight rather than their shape (Figure 3.4).

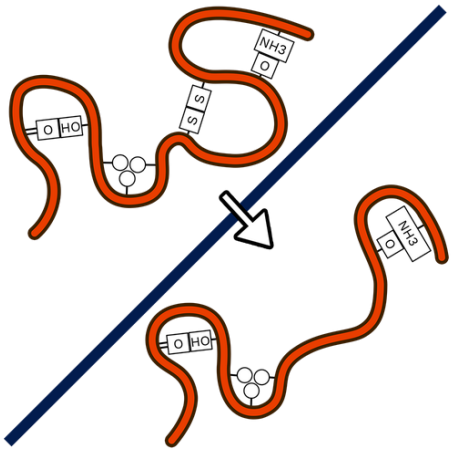



Figure 3.4: Role of Beta-mercaptoethanol in SDS-PAGE

## What is the mechanism of stacking protein sample in the discontinuous gel system?

This technique uses 3 different buffers (1) Running Buffer (2) Stacking gel buffer of pH 6.8 (3) Resolving gel Buffer of pH 8.8. The discontinuous gel system concentrates the diluted protein sample into a narrow band, allowing application of diluted protein sampleStacking gel buffer is composed of TrisHCl pH 6.8, SDS as the resolving gel buffer contains Tris pH 8.8, SDS and the pore size is large compared to the resolving gel. The mobility of the chloride ion present in the buffer is more than the protein in the sample. The glycine moves slower than the protein sample and as a result protein sample get sandwiched between fast-moving chloride ion and slow-moving glycine. Due to high electrophoretic mobility, the protein molecules run fast and stack between the leading and trailing ions. Once the protein sample enters into the resolving gel (resolving buffer pH 8.8), glycine ions don't stop the migration of protein and protein molecules separate as per their size or molecular weight.

**Vertical Gel Instrument**- The schematic diagram of a vertical gel electrophoresis apparatus is given in (Figure 3.5). It has two buffer chambers, an 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.

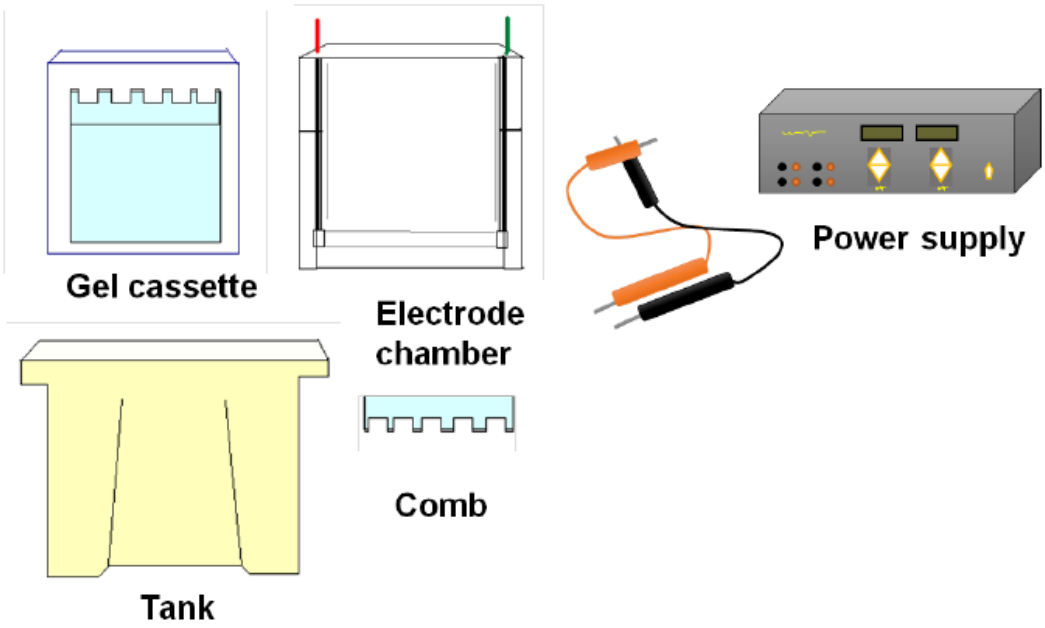



**Figure 3.5: Different components of vertical gel electrophoresis apparatus.**

Buffer and reagent for electrophoresis- The different buffer and reagents with their purpose for vertical gel electrophoresis is as follows-

1. N, N, N', N'-tetramethylethylenediamine (TEMED)-it catalyzes the acrylamide polymerization.

2. Ammonium persulfate (APS)-it is an initiator for acrylamide polymerization.

3. Tris-HCl- it is the component of running and gel casting buffers.

4. Glycine- it is the component of the running buffer.

5. Bromophenol blue- it is the tracking dye to monitor the progress of gel electrophoresis.

6. Coomassie brilliant blue -it is used to stain the polyacrylamide gel.

7. Sodium dodecyl sulfate is used to denature and provide a negative charge to the protein.

8. Acrylamide- monomeric unit used to prepare the gel.

9. Bis-acrylamide- cross linker for polymerization of acrylamide monomer to form gel.

**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. **What is the mechanism of acrylamide polymerization?** Ammoinum persulfate in the presence of TEMED forms oxygen free radicals and induces the polymerization of acryalide 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. First the resolving gel solution is prepared and poured into the gel cassette for polymerization. A thin layer of organisc 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.

**Running of the gel:** The sample is prepared in the loading dye containing SDS, β-mercaptoethanol in glycerol to denature the sample and presence of glycerol facilitates the loading of sample in the well. As the samples are filled vertically there is a distance drift between the molecules at the top Vs at the bottom in a lane. This problem is taken care once the sample run through the stacking gel. The pH of the stacking gel is 6.8 and at this pH, glycine is moving slowly in the front where as Tris-HCl is moving fast. As a result, the sample gets sandwiched between glycine-Tris and get stacked in the form of thin band. As the sample enters into the resolving gel with a pH 8.8, the glycine is now charged, it moves fast and now sample runs as per their molecular weight (due to SDS they have equal negative charge). After tracking dye reaches to the bottom of the gel, gel is taken out from the glass plate with the help of a spatula. Gel is stained with coomassie brilliant blue dye. The dye stains protein present on the gel.

## Detection of Proteins in Gels

Proteins separated on a polyacrylamide gel can be detected by various methods, for instance, dyes and silver staining (Figure 3.6).

### Dyes

The Coomassie blue staining allows the detection of up to 0.2 to 0.6 µg of protein and is quantitative (linear) up to 15 to 20 µg. It is often used in methanol-acetic acid solutions and is discolored in isopropanol-acetic acid solutions.

## Materials:

1. Polyacrylamide gel containing protein bands.

2. Coomassie Brilliant Blue R250 staining solution:

3. Destaining Solution: 15% (v/v) Methanol and Acetic Acid (10% v/v) in Triple Distilled water.

4. plastic or glass container with lid.

5. platform shaker

## Procedure:

1. Remove polyacrylamide gel from the electrophoresis unit and place in plastic container with ~10 volumes Coomassie Brilliant Blue staining solution.

2. Agitate slowly on a platform shaker for 30-60mins.

3. Discard staining solution and wash the gel with triple distilled water.

4. Add 5 to 10 gel volumes of destaining solution.

5. Agitate slowly on a platform shaker for 30-60mins.

6. If the color of the destaining solution is intense blue, replace it with the new destaining solution.

**Results:** A typical gel picture during coomassie brilliant blue staining and destaining is given in the (Figure 3.6). In the beginning, whole gel will appear blue/black but

the dye will be removed from the background and give discrete appearance of protein bands.

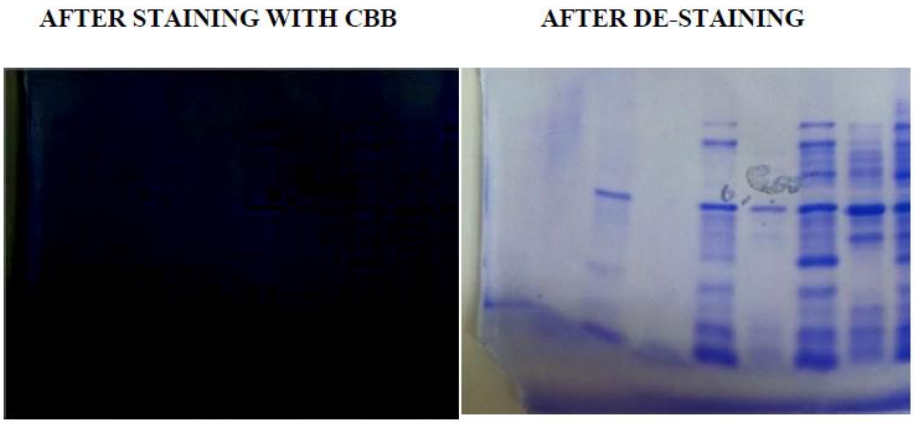



**Figure 3.6: Coomassie Brilliant Blue Staining of Polyacrylamide gel.**

**Two-Dimensional Gel Electrophoresis**: Two-dimensional gel electrophoresis (2-DE) is based on separating a mixture of proteins according to two molecular properties, one in each dimension. The most used is based on a first dimension separation by isoelectric focusing and second dimension according to molecular weight by SDS-PAGE (Figure 3.5).

**The general workflow in a 2-DE experiment would be:**

## Sample Preparation

The method of sample preparation depends on the aim of the research and is crucial to the success of the experiment. Factors such as the solubility, size, charge, and isoelectric point (pI) of the proteins of interest enter into sample preparation. Sample preparation is also important in reducing the complexity of a protein mixture. The protein fraction to be loaded on a 2-DE gel must be in a low ionic strength denaturing buffer that maintains the native charges of proteins and keeps them soluble.

## First-Dimension Separation

This part is performed by IEF. Using this technique, proteins are separated on the basis of their pI, the pH at which a protein carries no net charge and will not migrate in an electrical field.

## Equilibration

A conditioning step is applied to proteins separated by IEF prior to the second-dimension run. This process reduces disulfide bonds and alkylates the resultant sulfhydryl groups of the cysteine residues. Concurrently, proteins are coated with SDS for separation on the basis of molecular weight.

## Second-Dimension Separation

This part is performed by SDS-PAGE. The choice for the gel depends on the protein molecular weight range to be separated. The ability to run many gels at the same time and under the same conditions is important for the purpose of gel-to-gel comparison.

## Staining

In order to visualize proteins in gels, they must be stained in some manner. The selection of staining method is determined by several factors, including desired sensitivity, linear range, ease of use, expense, and the type of imaging equipment available. At present there is no ideal universal stain. Sometimes proteins are detected after transference to a membrane support by western blotting, which is described in more detail below.

## Image Analysis

The ability to collect data in digital form is one of the major factors that enable 2-DE gels to be a practical means of collecting proteome information. It allows unprejudiced comparison of gels and cataloging of immense amounts of data. Many types of imaging devices interface with software designed specifically to collect, interpret, and compare proteomics data. One of the biggest problems in 2-DE is the analysis and comparison of complex mixtures of proteins. Currently there are databases capable of comparing two-dimensional gel patterns. These systems allow automatic comparison of spots for the precise identification of those needed in the quantitative analysis.

## Protein Identification

Once interesting proteins are selected by differential analysis or other criteria, the proteins can be excised from gels, distained and digested to prepare their

identification by mass spectrometry. This technique is known as peptide mass fingerprinting.

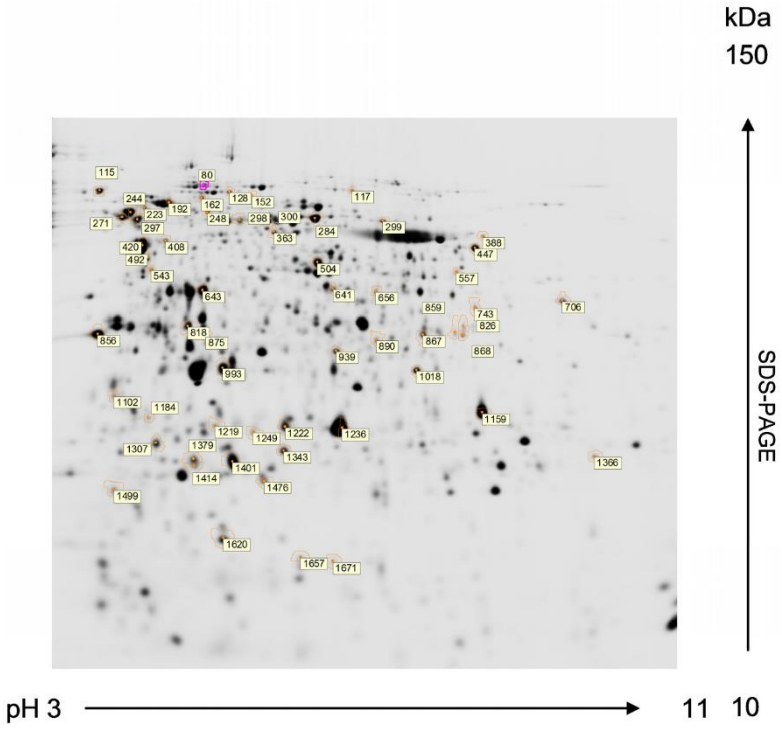



**Figure 3.7:** Two-Dimentional Gel Electrophoresis..