VIVEKANANDA COLLEGE
THAKURPUKUR
KOLKATA-700063

NAAC ACCREDITED 'A' GRADE

Topic: Chromatography,SDS-PAGE and IEF

Course Title: Proteins and enzymes

Paper:GE-2 (CC-2)

Unit:I

Semester:2

Name of the Teacher:Nineesha Sen Banerjee

Name of the Department:Biochemistry

Chromatography

Chromatography is commonly used in biotechnology for purifying biological molecules, like proteins, for medicine or other uses. Chromatography allows the separation of individual components from complex mixtures. It consists of a mobile phase, which consists of the solvent and product mixture, and a stationary phase through which the mobile phase travels. Some examples of the stationary phase include paper, and glass beads, commonly known as resins.

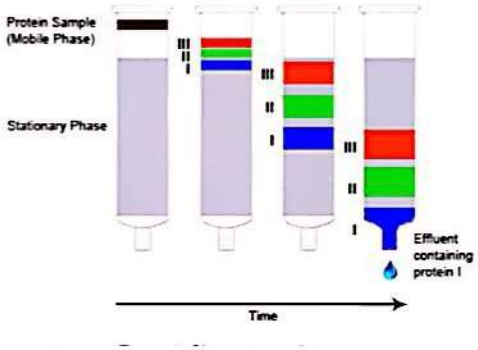


Figure 1: Chromatography setup

Figure 1 above shows the typical setup of a lab chromatography column. The mobile phase is added to the stationary column and gravity pulls the mobile phase downwards, causing the proteins present in the mixture to separate and elude from the columns in separate and distinct portions. Molecules travel through the stationary phase at different rates because of their unique properties, such as weight, size or electronic charges. Different chromatography setups utilizes the differences in one of these properties to separate mixtures of different compounds. Some examples include: Ion Exchange Chromatography (IEC), which separates compounds with different charges using charged resins as the stationary phase.

Gel filtration chromatography, commonly referred to as size exclusion chromatography (SEC), which separates compounds with different molecular sizes. In SEC, the stationary phase consists of resins which contain tiny holes, which allows some smaller molecules to pass through these pores. Affinity chromatography is a method of separating biochemical mixtures based on a highly specific interaction between antigen and antibody, enzyme and substrate, or receptor and ligand. It is able to target a specific compound for separation, and the stationary phase would contain the complementary molecule. This separation technique has the highest molecular specificity.

In this article, we will be focusing on IEC and SEC.

3. Mechanism

Ion Exchange Chromatography (IEC)

Ion Exchange Chromatography (IEC) utilizes the principle of the electrostatic forces of attraction to separate a mixture of charged proteins. Charged proteins are classified into positively and negatively charged proteins based on the presence of different charged functional groups. At a pH of 7, amino acid groups such as Histidine, Arginine and Lysine are positively charged while Aspartic acid and Glutamic acid are negatively charged.

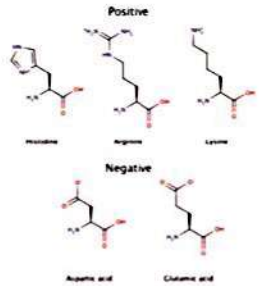
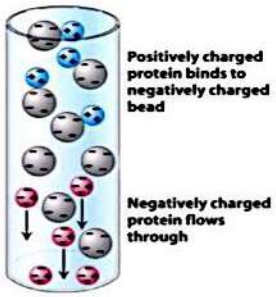


Figure 2: Charged amino acid groups

The apparatus involved in Ion Exchange Chromatography (IEC) consists of a chromatography column filled with beads of a particular charge (stationary phase), opposite to that of the protein of interest. When the protein mixture (mobile phase) is poured through the chromatography column, proteins with the same charge as the charged polymer beads are repelled and eluded from the column rapidly. These proteins are then collected in a flask at the bottom of the column as the effluent. On the other hand, proteins with a complementary charge are attracted to the charged polymer beads and remain in the column as the residue.

Figure 3: Mechanism of Ion Exchange Chromatography (IEC)

Figure 3 shows the mechanism for a cation exchange system, with the anion exchange using



positively charged resins instead to bind to the negatively charged proteins.

After removal of the effluent, a salt buffer solution such as NaCl solution is utilized as an eluding agent. When poured down the chromatography column, the charged ions within the buffer solution would compete with the protein molecules for binding to the polymer beads. As a result, the residue will then be eluded and collected. When separating proteins with differing magnitude of charges, buffer solutions of increasing concentrations can be used to separate these proteins.

Size-Exclusion Chromatography (SEC)

Size-Exchange Chromatography (SEC) utilizes the principle of varying protein molecular sizes for separation. The apparatus required involves a porous polymeric material with numerous microscopic holes being placed within the chromatography column. The sizes of these holes are determined by the sizes of the proteins, as it must allow some molecules to pass through while preventing others from doing so. The imperfect packing of the resin means that there are gaps between the resin particles, and also with the column walls where the larger molecules will move through.

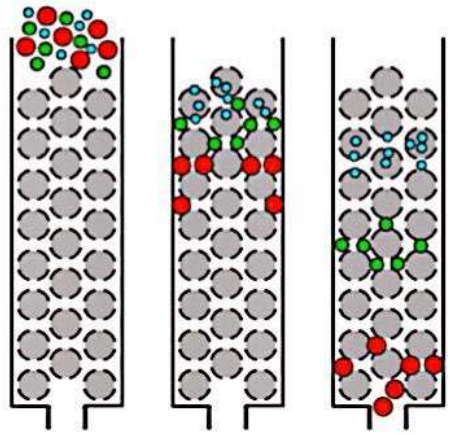
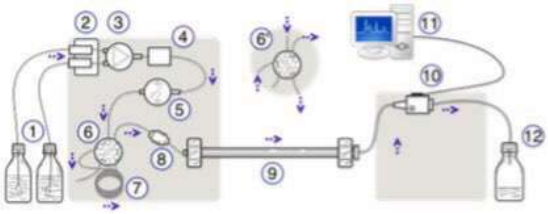


Figure 4: Mechanism of Size-Exclusion Chromatography (SEC)
The protein mixture is first poured into the column and protein molecules larger than the pores of the polymer beads (shown in red) are unable to fit through. Instead, they flow

downwards rapidly and are collected as eluent. For proteins with a molecular size smaller than the polymeric pores (as shown in blue and green in Figure 4), they flow through the polymer at a slower rate and are only collected after all the bigger protein molecules have been eluded.
The eluded protein mixture would then be poured into another chromatography column consisting of polymeric beads with a smaller pore size than the first column. The protein mixture that flows through the column rapidly can be concluded to have a molecular size ranging from the pore size of the first to the second polymeric material. As explained above, the smaller protein molecules would be entrapped within the pores of the polymeric beads and spend a longer time within the column before being eluded. Numerous repetitions involving chromatography columns of decreasing polymeric pore sizes would be required in order to obtain an efficient separation of the original protein mixture.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY
High-performance liquid chromatography (HPLC; formerly referred to as high-pressure liquid chromatography) is a technique in analytical chemistry used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column.



Schematic representation of an HPLC unit. (1) Solvent reservoirs, (2) Solvent degasser, (3) Gradient valve, (4) Mixing vessel for delivery of the mobile phase, (5) High-pressure pump, (6) Switching valve in "inject position", (6') Switching valve in "load position", (7) Sample injection loop, (8) Pre-column (guard column), (9) Analytical column, (10) Detector (i.e., IR, UV), (11) Data acquisition, (12) Waste or fraction collector.

Chromatography can be described as a mass transfer process involving adsorption. HPLC relies on pumps to pass a pressurized liquid and a sample mixture through a column filled with adsorbent, leading to the separation of the sample components. The active component of the column, the adsorbent, is typically a granular material made of solid particles (e.g., silica, polymers, etc.), 2–50 µm in size. The components of the sample mixture are separated from each other due to their different degrees of interaction with the adsorbent particles. The pressurized liquid is typically a mixture of solvents (e.g., water, acetonitrile and/or methanol) and is referred to as a "mobile phase". Its composition and temperature play a major role in the separation process by influencing the interactions taking place between sample components and adsorbent. These interactions are physical in nature, such as hydrophobic (dispersive), dipole–dipole and ionic, most often a combination.

Application:

HPLC has been used for manufacturing (e.g., during the production process of pharmaceutical and biological products), legal (e.g., detecting performance enhancement drugs in urine), research (e.g., separating the components of a complex biological sample, or of similar synthetic chemicals from each other), and medical (e.g., detecting vitamin D levels in blood serum) purposes.

SDS PAGE

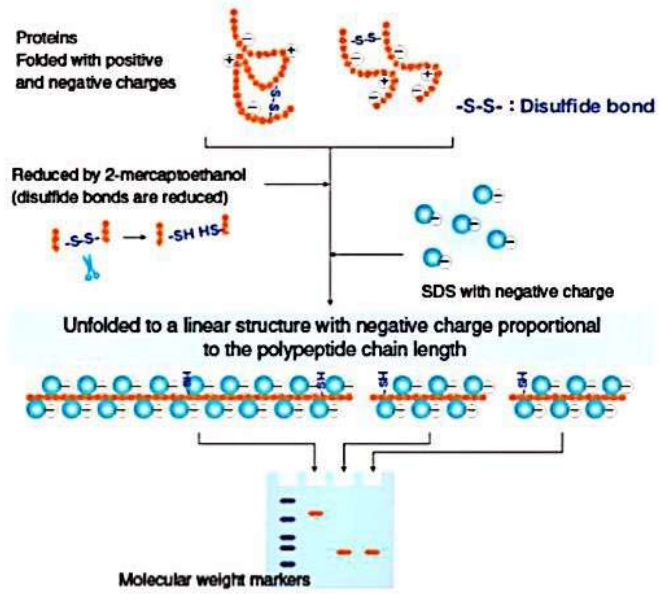
SDS-PAGE is an analytical technique to separate proteins based on their molecular weight.

The principle

When proteins are separated by electrophoresis through a gel matrix, smaller proteins migrate faster due to less resistance from the gel matrix. Other influences on the rate of migration through the gel matrix include the structure and charge of the proteins.

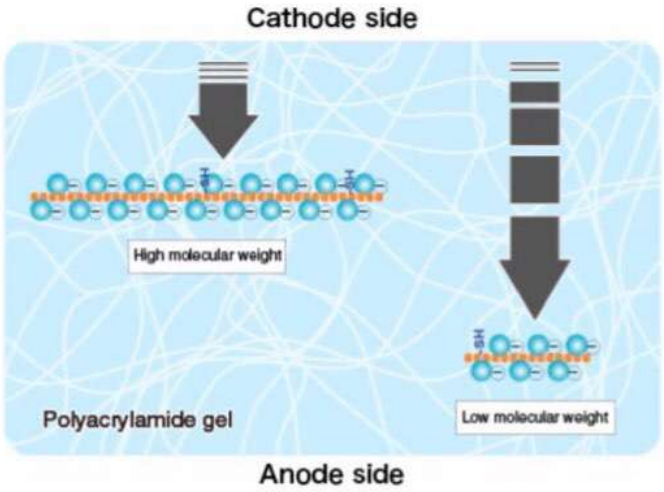
In SDS-PAGE, the use of sodium dodecyl sulfate (SDS, also known as sodium lauryl sulfate) and polyacrylamide gel largely eliminates the influence of the structure and charge, and proteins are separated solely based on polypeptide chain length.

SDS is a detergent with a strong protein-denaturing effect and binds to the protein backbone at a constant molar ratio. In the presence of SDS and a reducing agent that cleaves disulfide bonds critical for proper folding, proteins unfold into linear chains with negative charge proportional to the polypeptide chain length. Unfolded to a linear structure with negative charge proportional to the polypeptide chain length



Polymerized acrylamide (polyacrylamide) forms a mesh-like matrix suitable for the separation of proteins of typical size. The strength of the gel allows easy handling. Polyacrylamide gel electrophoresis of SDS-treated proteins allows researchers to separate proteins based on their length in an easy, inexpensive, and relatively accurate manner.

Proteins are separated based on their polypeptide chain length by electrophoresis in a polyacrylamide gel with an appropriate mesh size.



Principle of SDS-PAGE

After Separation

ISOELECTRIC FOCUSSING

Isoelectric Focusing or IEF is a method of separating proteins according to their Isoelectric points in a pH gradient. Isoelectric point denoted as pI is defined as the pH at which protein carry no net charge, or pH at which protein become immobile in an electric field.

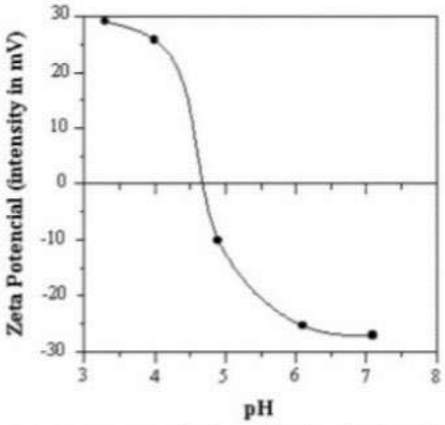
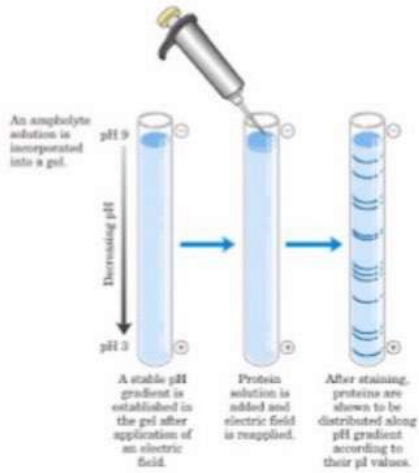


Figure 4: Determination of the isoelectric point.

Isoelectric Point determination

One of the best method for characterizing proteins, because protein separation /purification can be easily done if Isoelectric point of a protein is known.



Isoelectric Focussing Principle

The resolution of Isoelectric Focussing is very high. In normal electrophoretic methods pH between anode and cathode remains constant but in Isoelectric focussing , a pH gradient is arranged.when pH of protein is below its pI proteins become positively charged and it will migrate towards cathode. because of the pH gradient charge of the protein molecule changes while moving forward, there will be a point at which net charge of the protein becomes zero this point is called Isoelectric point. when a protein mixture or a single protein is run under an electric field at specific pH the protein stops moving.