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SEPERATION OF PROTEINS BY CHROMATOGRAPHIC TECHNIQUES

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ABSTRACT:

The diversity in biomolecules makes them difficult to separate in their pure form. In order to augment the biological activity of many important biomolecules it is very necessary to separate and purify them in their purest form. This review deliberates the chromatographic purification of biomolecules as one of the most emerging technology. In this the basic principle for the separation of biomolecules along with different types of chromatography, their principle and applications is discussed. This review also focused on use of combination of two or more chromatographic techniques for the separation of therapeutic proteins like monoclonal antibodies (mAbs) and enzymes to achieve their native activity. Affinity chromatography is a separation method based on specific binding interaction between an immobilised ligand and its binding partner. For example include antibody/antigen, enzyme/ substrate, and enzyme/ inhibitor interaction. Binding to the solid phase may be achieved by column chromatography whereby the solid medium is packed onto a column, the initial mixture run through the column to allow settling, a wash buffer run through the column and the elution buffer subsequently applied to the column and collected. These steps are usually done at ambient pressure. Alternatively, binding may be achieved using a batch treatment, for example, by adding the initial mixture to the solid phase in a vessel, mixing, separating the solid phase, removing the liquid phase, washing, re-centrifuging, adding the elution buffer, re-centrifuging and removing the elute. Sometimes a hybrid method is employed such that the binding is done by the batch method, but the solid phase with the target molecule bound is packed onto a column and washing and elution are done on the column.

KEY WORDS:

Chromatography introduction, column chromatography, Affinity Chromatography, Separation of proteins

INTRODUCTION:

Chromatography:

Chromatography is an important biophysical technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analysis. Proteins can be purified based on characteristics such as size and shape, total charge, hydrophobic groups present on the surface, and binding capacity with the stationary phase. Four separation techniques based on molecular characteristics and interaction type use mechanisms of ion exchange, surface adsorption, partition, and size exclusion. Other chromatography techniques are based on the stationary bed, including column, thin layer, and paper chromatography. Column chromatography is one of the most common methods of protein purification.

Based on this approach three components form the basis of the chromatography technique.

- Stationary phase: This phase is always composed of a "solid" phase or "a layer of a liquid adsorbed on the surface a solid support".
- Mobile phase: This phase is always composed of "liquid" or a "gaseous component."
- Separated molecules

The type of interaction between stationary phase, mobile phase, and substances contained in the mixture is the basic component effective on separation of molecules from each other. Chromatography methods based on partition are very effective on separation, and identification of small molecules as amino acids, carbohydrates, and fatty acids. However, affinity chromatographies (ie. ion-exchange chromatography) are more effective in the separation of macromolecules as nucleic acids, and proteins. Paper chromatography is used in the separation of proteins, and in studies related to protein synthesis; gas-liquid chromatography is utilized in the separation of alcohol, esther, lipid, and amino groups, and observation of enzymatic interactions, while molecular-sieve chromatography is employed especially for the determination of molecular weights of proteins. Agarose-gel chromatography is used for the purification of RNA, DNA particles, and viruses [4].

Stationary phase in chromatography, is a solid phase or a liquid phase coated on the surface of a solid phase. Mobile phase flowing over the stationary phase is a gaseous or liquid phase. If mobile phase is liquid it is termed as liquid chromatography (LC), and if it is gas then it is called gas chromatography (GC). Gas chromatography is applied for gases, and mixtures of volatile liquids, and solid material. Liquid chromatography is used especially for thermal unstable, and non-volatile samples .

The purpose of applying chromatography which is used as a method of quantitative analysis apart from its separation, is to achive a satisfactory separation within a suitable timeinterval. Various chromatography methods have been developed to that end. Some of them include column chromatography, thin-layer chromatography (TLC), paper chromatography, gas chromatography, ion exchange chromatography, gel permeation chromatography, high-pressure liquid chromatography, and affinity chromatography .

Types of chromatography

- Column chromatography
- Ion-exchange chromatography
- Gel-permeation (molecular sieve) chromatography
- Affinity chromatography
- Paper chromatography
- Thin-layer chromatography
- Gas chromatography
- Dye-ligand chromatography
- Hydrophobic interaction chromatography
- Pseudoaffinity chromatography
- High-pressure liquid chromatography (HPLC).

Column chromatography:

Column chromatography separates substances based on differential adsorption of compounds to the adsorbent as the compounds move through the column at different rates which allows them to get separated in fractions. This technique can be used on a small scale as well as large scale to purify materials that can be used in future experiments. This method is a type of adsorption chromatography technique.

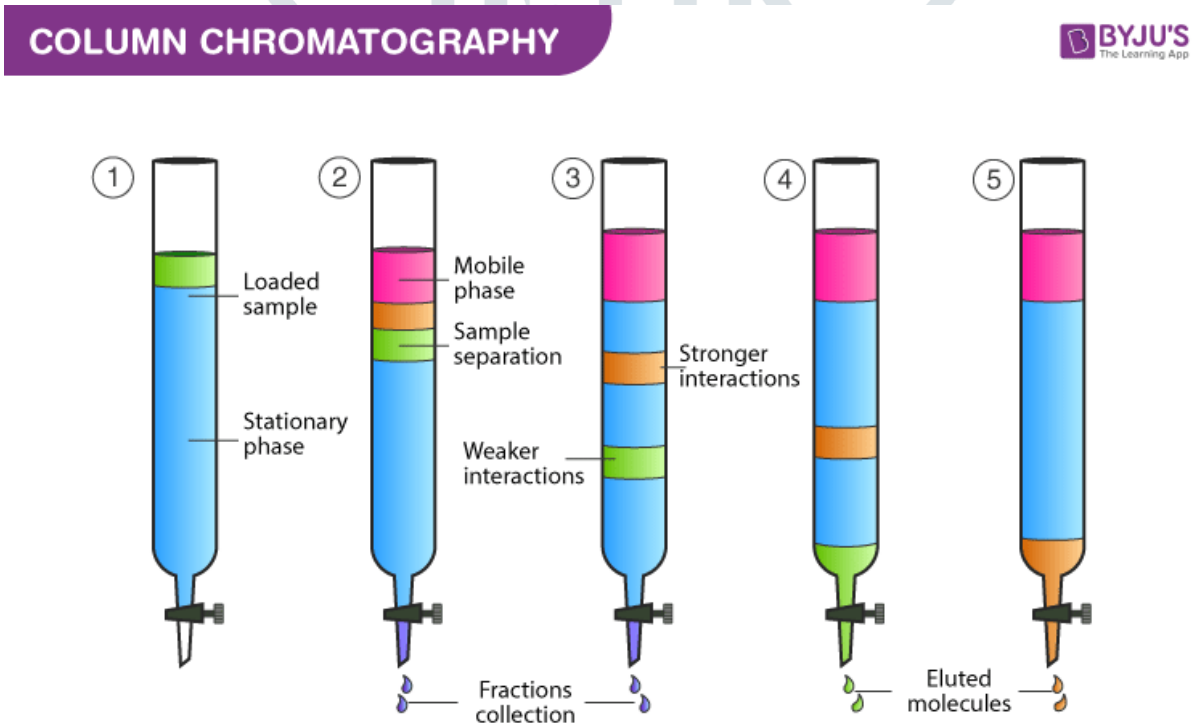
Column Chromatography Principle:

When the mobile phase along with the mixture that needs to be separated is introduced from the top of the column, the movement of the individual components of the mixture is at different rates. The components with lower adsorption and affinity to the stationary phase travel faster when compared to the greater adsorption and affinity with the stationary phase. The components that move fast are removed first whereas the components that move slowly are eluted out last.

The adsorption of solute molecules to the column occurs in a reversible manner. The rate of the movement of the components is expressed as:

R_f = the distance travelled by solute/ the distance travelled by the solvent

R_f is the retardation factor.



Elution:

Elution is a chemical process that involves removing a material's ions by ion exchange with another material. The chromatographic technique of extracting an adsorbed substance from a solid adsorbing media using a solvent. The eluent is the solvent or mobile phase that passes through the column. When the polarity of the eluent matches the polarity of the molecules in the sample, the molecules desorb from the adsorbent and dissolve in the eluent.

The fraction of the mobile phase that transports the sample components is known as eluent. The mixture of solute and solvent that exits the column is known as an eluate. The eluate is made up of the mobile phase and analytes. A substance that separates and moves constituents of a mixture through the column of a chromatograph. The eluent in liquid chromatography is a liquid solvent whereas in gas chromatography is a carrier gas.

Column Chromatography Procedure:

Before starting with the Column Chromatography Experiment let us understand the different phases involved.

Mobile phase – This phase is made up of solvents and it performs the following functions:

1. It acts as a solvent-sample mixture that can be introduced in the column.
2. It acts as a developing agent – helps in the separation of components in the sample to form bands.
3. It acts as an eluting agent – the components that are separated during the experiment are removed from the column
4. Some examples of solvents used as mobile phases based on their polarity are – ethanol, acetone, water, acetic acid, pyridine, etc.

Stationary phase – It is a solid material which should have good adsorption properties and meet the conditions given below:

1. Shape and size of particle: Particles should have a uniform shape and size in the range of 60 – 200µ in diameter.
2. Stability and inertness of particles: high mechanical stability and chemically inert. Also, no reaction with acids or bases or any other solvents was used during the experiment.
3. It should be colourless, inexpensive and readily available.
4. Should allow free flow of mobile phase
5. It should be suitable for the separation of mixtures of various compounds.

Proteins:

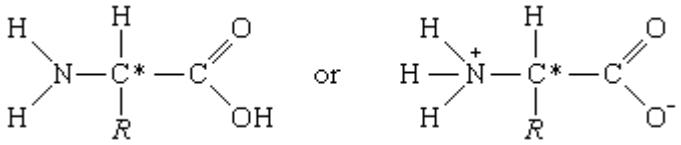
Proteins are large, complex molecules that play many critical roles in the body. They do most of the work in cells and are required for the structure, function, and regulation of the body's tissues and organs.

Proteins are made up of hundreds or thousands of smaller units called amino acids, which are attached to one another in long chains. There are 20 different types of amino acids that can be combined to make a protein. The sequence of amino acids determines each protein's unique 3-dimensional structure and its specific function. Amino acids are coded by combinations of three DNA building blocks (nucleotides), determined by the sequence of genes.

General structure and properties of proteins:

The amino acid composition of proteins:

The common property of all proteins is that they consist of long chains of α-amino (alpha amino) acids. The general structure of α-amino acids is shown in . The α-amino acids are so called because the α-carbon atom in the molecule carries an amino group (—NH_2); the α-carbon atom also carries a carboxyl group (—COOH).



In acidic solutions, when the pH is less than 4, the —COO groups combine with hydrogen ions (H^+) and are thus converted into the uncharged form (—COOH). In alkaline solutions, at pH above 9, the ammonium groups (—NH^+_3) lose a hydrogen ion and are converted into amino groups (—NH_2). In the pH range between 4 and 8, amino acids carry both a positive and a negative charge and therefore do not migrate in an electrical field. Such structures have been designated as dipolar ions, or zwitterions (i.e., hybrid ions).

Although more than 100 amino acids occur in nature, particularly in plants, only 20 types are commonly found in most proteins. In protein molecules the α-amino acids are linked to each other by peptide bonds between the amino group of one amino acid and the carboxyl group of its neighbour.

Functions of Proteins

Positive negative attractions between different atoms in the long amino acid strand cause it to coil on itself again and again to form its highly complex shape. Folded proteins may combine with other folded proteins to form even larger more complicated shapes.

The folded shape of a protein molecule determines its role in body chemistry. Structural proteins are shaped in ways that allow them to form essential structures of the body. Collagen, a protein with a fibre shape, holds most of the body tissues together. Keratin, another structural protein forms a network of waterproof fibres in the outer layer of the skin.

Functional proteins have shapes that enable them to participate in chemical processes of the body. Functional proteins include some of hormones, growth factors, cell membrane receptors and enzymes.

Classification of Proteins

Protein molecules are large, complex molecules formed by one or more twisted and folded strands of amino acids. Each amino acid is connected to the next amino acid by covalent bonds.

1. Primary (first level) – Protein structure is a sequence of amino acids in a chain.
2. Secondary (secondary level) – Protein structure is formed by folding and twisting of the amino acid chain.
3. Tertiary (third level) – Protein structure is formed when the twists and folds of the secondary structure fold again to form a larger three dimensional structure.
4. Quaternary (fourth level) – Protein structure is a protein consisting of more than one folded amino acid chain.

Proteins can bond with other organic compounds and form “mixed” molecules. For example, glycoproteins embedded in cell membranes are proteins with sugars attached. Lipoproteins are lipid-protein combinations.
Nucleic Acids

The two forms of nucleic acid are deoxyribonucleic acid and ribonucleic acid. The basic building blocks of nucleic acids are called nucleotides. Each nucleotide consists of a phosphate unit, a sugar and a nitrogen base. DNA nucleotide bases include adenine, thymine, guanine and cytosine. RNA uses the same set of bases, except for the substitution of unit cells for thymine.

Nucleotides bind to one another to form strands or other structures. In the DNA molecule, nucleotides are arranged and twisted, and a double strand called a double helix. The sequence of different nucleotides along the DNA double helix is the “master code” for assembling proteins and other nucleic acids.

Affinity Chromatography (AC):

The term affinity chromatography was first introduced by Cuatrecasas et al. in 1968. Firstly, affinity chromatography was being practiced in the form of traditional chromatography. Combining the synergistic effect and selective characteristics of biomolecules with that of the efficiency, speed and other features of HPLC, AC is nowadays routinely adapted to HPLC system. Considering high performance of this system it has rapidly developed into highly selective, fast method for separating the wide variety of viruses, cells, as well as some complex biomolecules. In view of large-scale purification of biomolecules, affinity chromatography is often considered as single-step method than multi step process that incorporates SEC, IEC, and HIC.

The supports used in affinity column should be highly hydrophilic, the most commonly used supports are polyacrylamide, methacrylate, silica, agarose, porous glass, and cellulose.

Taking into consideration the applications of preparative chromatography, supports having fibrous nature were developed . Typically, in affinity chromatography, a bio specific ligand is covalently attached to the stationary phase provided either by carboxyl, amino or hydroxyl functional group which is called as spacer arm. Using standard immobilization techniques, affinity ligands such as enzyme inhibitors, hormones, lectins, antigens, antibodies, hormones or biomimetic ligands may be attached on commercially available stationary supports .

Though high specific binding is the major principle behind affinity chromatography, the binding between the product and affinity ligand should not be toostrong or else for desorption and column regeneration may require harsh conditions which results in the denaturation of the adsorbed product the isolation of IgG.

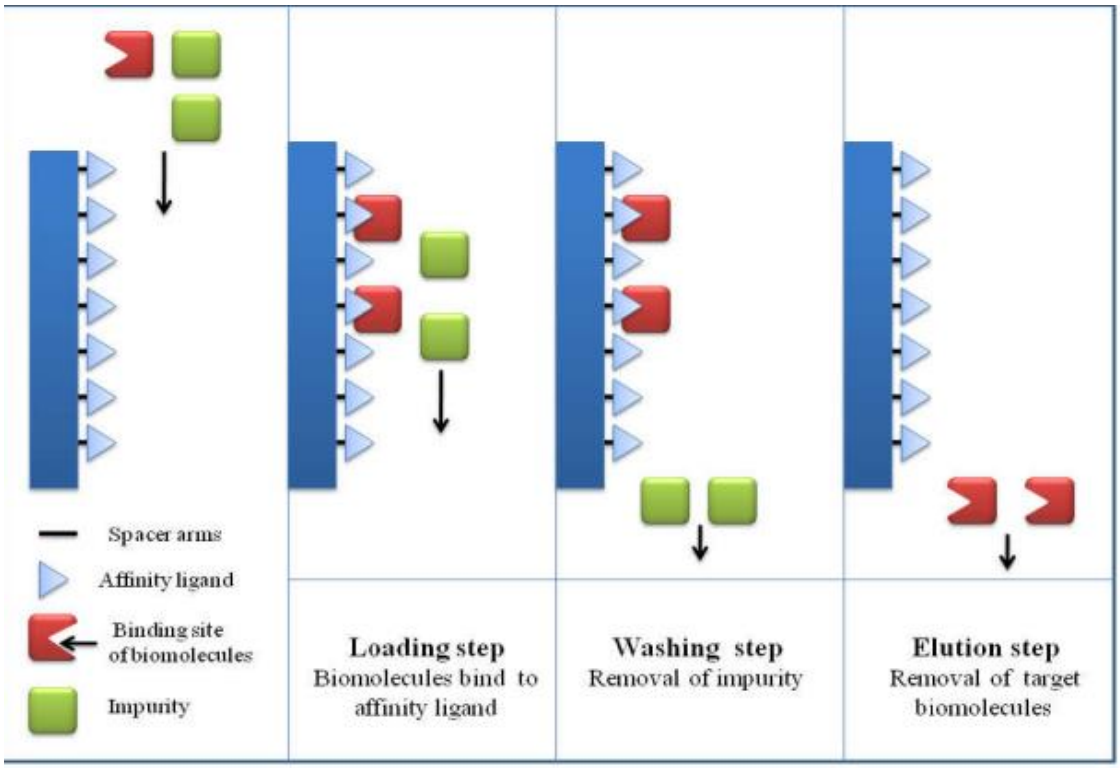


Figure 1. Separation of biomolecules using Affinity Chromatography (AC)

Size Exclusion Chromatography (SEC) or Gel Filtration Chromatography: Size Exclusion Chromatography (SEC) is used for the separation of biomolecules based on their molecular weight. This technique most widely used in polishing step of the purification of biomolecules like proteins, enzymes, and antibodies. After the purification of biomolecules by ion exchange, hydrophobic interaction, affinity chromatography, most of the remaining impurities are removed from the products using SEC.

The solution now contains different molecular weight compounds that have to be passed through the column. The smaller molecules that get diffused in the pore matrix will take longer time hence will show require more retention time. While larger size molecules which are unable to diffuse in the pores of the matrix adsorbent hence, require show retention time in the column hence elutes first. Dextran is the most widely used gel media for the separation of protein molecules over many years.

Pharmacia commercialized this material under trade name Sephadex and they have standardized this media for size-based separation of protein. Other polymeric resins are also used such as agarose, polyacrylamide, polyvinylpyrrolidone, polyvinyl ethyl carbitol and polyacrylamide. Polyacrylamide commercialized by Bio Red under tradename Bio Gel.

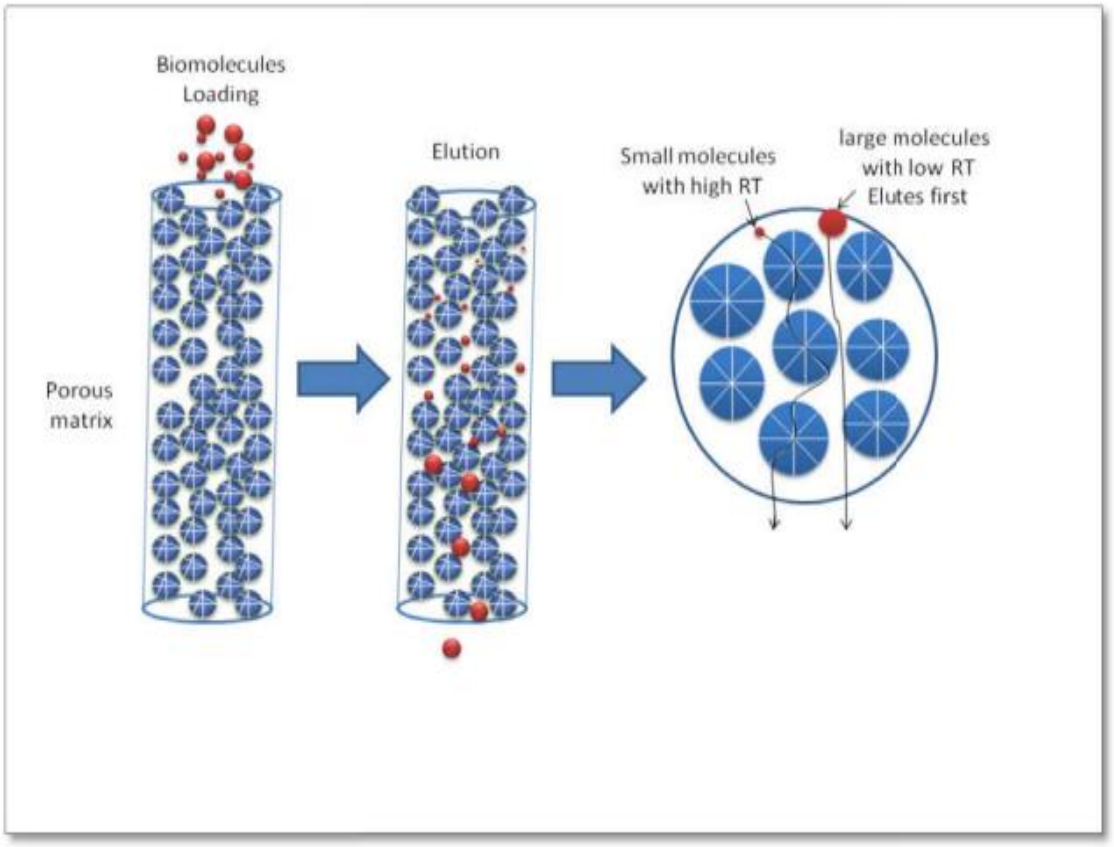


Figure 4. Principle of Size exclusion chromatography for biomolecules separation

Conclusion:

Increase in the advances in biotechnology sector augments the production of variety of variable bioproducts. These valuable bioproducts have to be efficiently separated and purified in order to retain their biological activity. Hence, the combination of novel technologies minimizing the purification steps plays a crucial role for getting maximum purification of the biomolecules. In the view of the production scale chromatography has emerged as one of the most efficient purifying technologies purifying the molecules achieving high purity. Depending upon the type of biomolecules to be separated, different types of chromatographic techniques can be used. Molecules on the basis of their molecular weight, charge, specificity, and hydrophobicity can be separated, different types of chromatographic techniques include size exclusion, ion exchange, and hydrophobic interaction chromatography. Molecules such as therapeutic proteins and enzyme, and other important proteins require combination of chromatography so as to purify them in purest form.

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