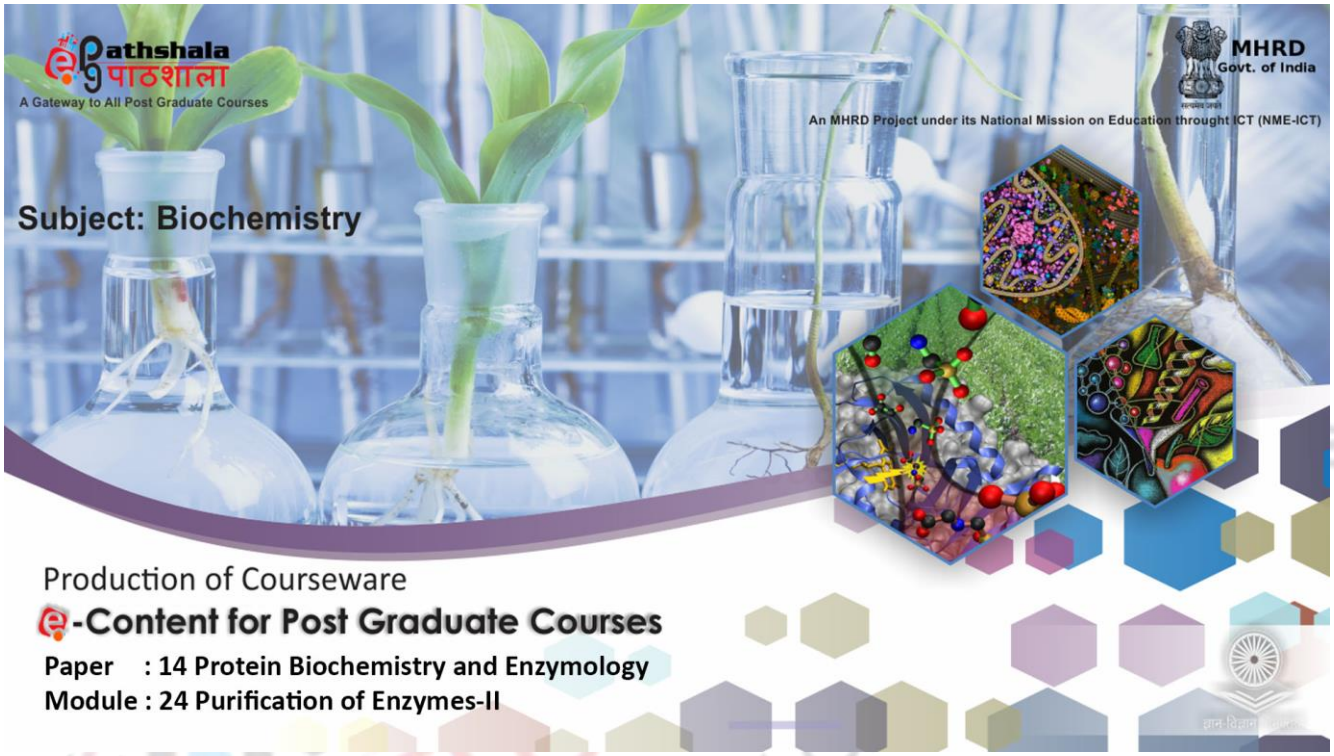


Production of Courseware

e-Content for Post Graduate Courses

Paper : 14 Protein Biochemistry and Enzymology

Module : 24 Purification of Enzymes-II

athshala पाठशाला

MHRD Govt. of India

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

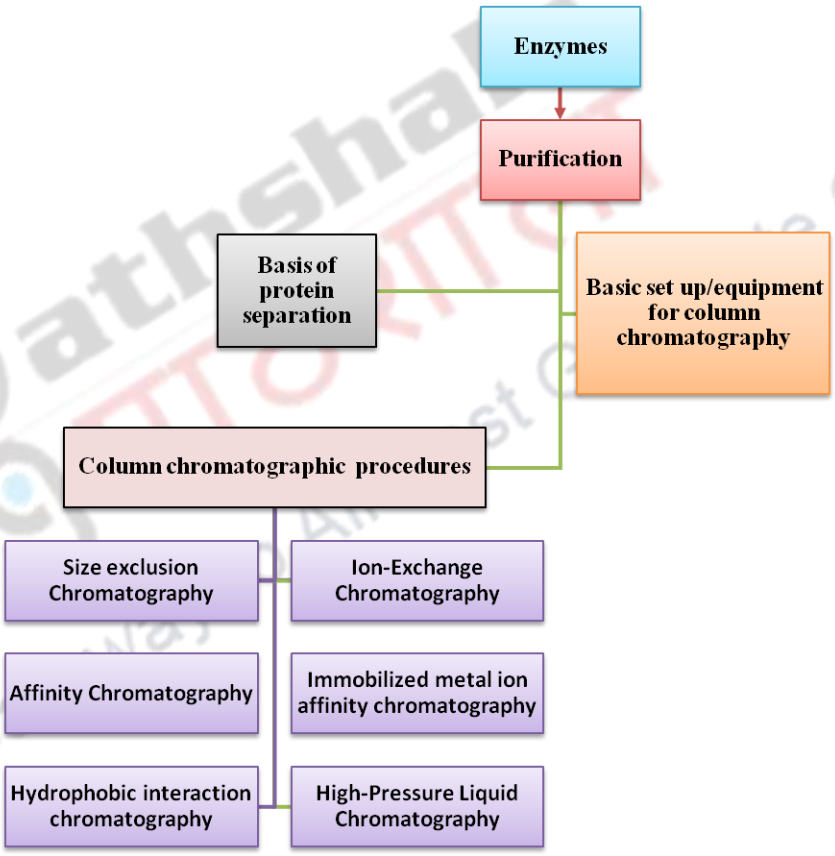
Description of Module	
Subject Name	Biochemistry
Paper Name	08 Protein Biochemistry and Enzymology
Module Number/Title	24 Purification of Enzymes-II

A Gateway to All Post Graduate Courses

1. Objectives

1. Understanding the basis of protein separation
2. Explaining the basic column chromatographic equipment and set up
3. Explaining various column chromatographic procedures in protein purification
4. Understanding principles, specifics of each procedure and their advantages.

2. Concept Map



3. Description

3.1 Basis of protein separation

As we have studied earlier, proteins can be obtained from natural sources like plants, animals or microbes or by their over-expression in a host organism. The next step involves purifying the protein to remove associated contaminants so as to obtain the desired protein in functional form and in substantial amounts. The purification scheme must be optimized in such a way that maximum yield of pure enzyme is achieved in less time and in fewer number of steps.

The various properties on the basis of which proteins can be separated include solubility, size, charge, hydrophobicity as well as binding affinity. Chromatographic separation of protein mixtures, based on these aforementioned properties, are among of the most widely and commonly used means of protein purification. A variety of chromatographic purification techniques are available. Some of the most commonly used methods are shown in Table 1.

Table 1. Commonly used chromatographic procedures for protein purification

Property	Purification method
Solubility	Precipitation with ammonium sulphate (salting out)
Size/shape	Size exclusion (gel filtration) chromatography
Charge	Ion-exchange chromatography
Binding affinity	Affinity chromatography
Hydrophobicity	Hydrophobic interaction chromatography

3.2 Column chromatographic procedures for protein purification

Column chromatography is based on the principle of separation of a one/more proteins from a protein mixture by passing the mixture through a column containing an appropriate matrix, to which the protein of interest may bind and can be later eluted out from the column using an eluant. The basic equipment required for chromatography is discussed further, although specialized and expensive equipments are also available.

3.2.1 Basic set up for Column Chromatography

A schematic representation of protein purification set up is shown in Figure 1.

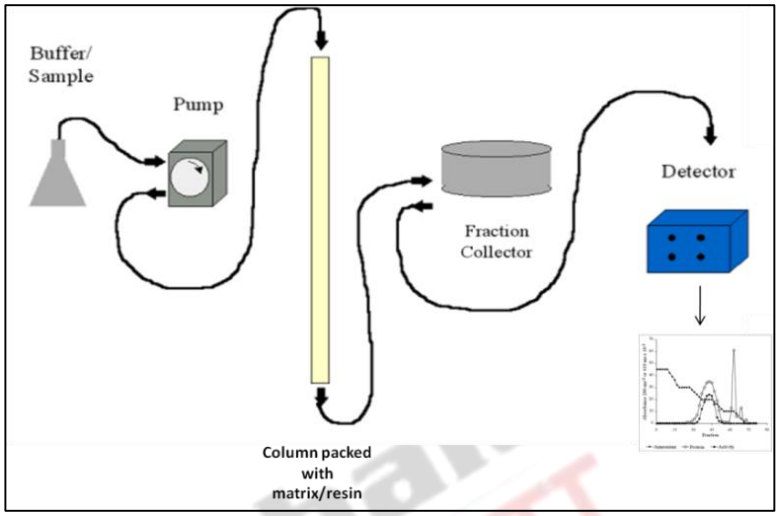


Figure 1. Schematic protein purification set up

The basic components required for column chromatography are as follows:

1. **Column**- A columnar glass reservoir (of various lengths and diameters) is cleaned and pre-packed with a matrix/resin (stationary phase) and attached to chromatographic set up.
2. **Stationary phase**- The stationary phase usually consists of an inert matrix or a resin which can be directly packed into the column or pre-swelled/pre-equilibriated before column packing.
3. **Mobile phase/solvents**-The mobile phase is constituted by various buffers which are used for column washing, equilibration, and elution of bound protein from the stationary phase. Different chromatographic methods require different mobile phases.
4. **Protein Sample-** The protein sample to be purified is an essential component which should be present in sufficient concentration and loaded onto the chromatographic column.
5. **A pump**- The chromatographic set up is usually connected to a peristaltic pump with variable and adjustable flow rate to push buffer through the column and prevent low pressure conditions or creation of air bubbles which may disrupt the column packing and affect purification process.
6. **Fraction collector**- This is necessary for collecting the fractions as and when they are eluted out of the column. The fraction collector is sometimes automated to collect a specific volume of fraction based on number of drops or on time, after which, the fraction shifts to the next empty collecting tube placed in the collector.
7. **A detector**-The fraction collector is usually connected to a detector which records protein absorbance at 280 nm. The absorbance of these individual fractions can either be read on a UV spectrophotometer or on a chart recorder, which is often directly connected to the detector.

8. **Standard assay to measure protein content and activity**: After the proteins are detected in relevant fractions eluted from the column, there should be a standard assay to determine the specific quantity of a specific protein eluted out. An understanding of the total protein that was loaded and eluted out through the column is also needed. The fractions containing the protein of interest can be assayed for enzyme/protein activity after each step before proceeding to the next step of purification.

   A laboratory set up for protein purification is shown in Figure 2.

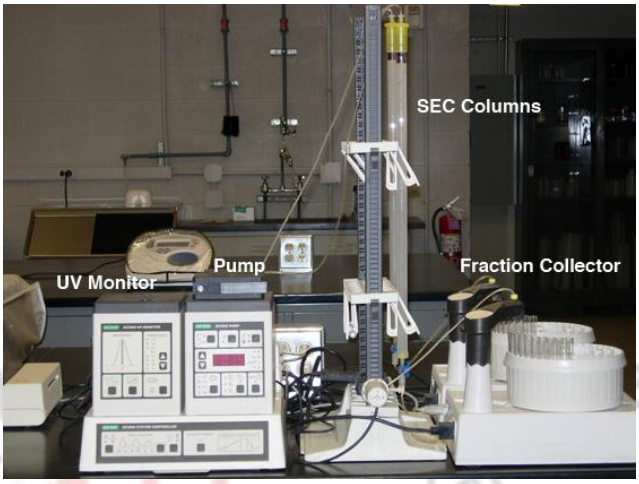


Figure 2. Laboratory set up for protein purification

3.2.2 Systems for Column Chromatography-Column chromatography can be performed with automated systems (wherein a pump is used to pass solvent over a packed column as described above), or can be run by gravity flow (in which the solvent runs down the column due to force of gravity). There are advantages and disadvantages to each system are shown in Table 2.

Table 2. Advantages and disadvantages of automated and gravity controlled column chromatography

	Automated Column chromatography	Gravity Flow Column chromatography
Advantages	• Self operated • Often coupled to an absorbance detector • Programmable • Easy to set up an elution gradient • Reproducible results produced	• Less expensive • The user has more control

Disadvantages	• Requires costly equipment • Possibility of back flow on column • Maximum flow rate dependent on pressure limit of column	• More labor intensive and time consuming • Reproducibility is difficult at times • Flow rate depends on gravity

3.2.3 Preparation of resins

The steps for preparing resin are shown in Figure 3. Resins for protein purification are available in dry or pre-swollen form. In case dry, the resin needs to be hydrated before it is used for column packing. This is done by mixing the dry resin with buffer and leaving it for overnight incubation at room temperature (25°C). After the hydrated resin has settled, very fine particles, which remain suspended at the top are carefully decanted and discarded. The next step is the equilibration of the hydrated resin in the purification buffer. Finally, the slurry is degassed (to minimize the formation of air bubbles) prior to packing the column.

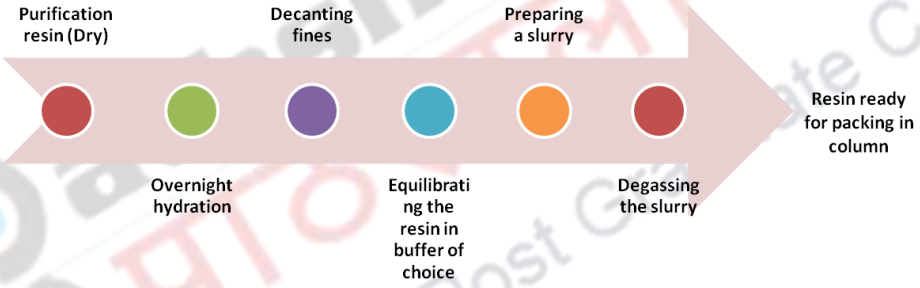


Figure 3. Preparation of resin for purification

3.3 Types of chromatography

There are a number of chromatographic methods that are in use for purification purposes. The major types of column chromatography include size exclusion chromatography (SEC), ion exchange chromatography (IEX), affinity chromatography, immobilized metal ion affinity chromatography, hydrophobic interaction chromatography (HIC) and high pressure liquid chromatography. Usually a protocol for protein purification may contain one or more chromatographic steps. It is essential to choose the most appropriate chromatographic method(s) for optimized protein purification.

3.2.1 Size exclusion Chromatography

Size exclusion or gel-filtration chromatography separates proteins on the basis of size of protein molecule (Figure 4). The stationary phase comprises of porous beads made of polymer such as dextran or agarose or Polyacrylamide which are both insoluble and highly hydrated. Examples of commonly used beads which are used commercially are Sephadex, Sepharose, and Bio-gel. Extent of cross-linking can be controlled to determine pore size.

When protein mixture sample is applied to such a column, small molecules are able to diffuse into the internal volume of these beads and are thus distributed in the aqueous solution both inside the beads. On the contrary, large protein molecules are unable to enter the beads and are located only in the solution between the beads (Figure 4).

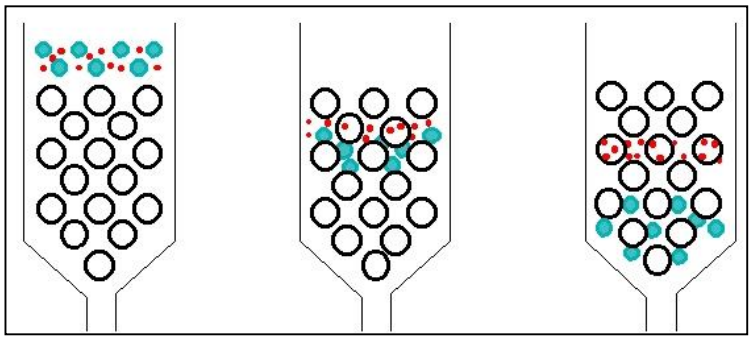


Figure 4. Basis of Size exclusion chromatography

When the mobile phase (buffer) is passed through a column, the large molecules flow more rapidly through this column and emerge first from the column. Intermediately sized molecules, which occasionally enter and exit from the beads, will flow out from the column at an intermediate position. The last to exit from the column are smaller sized protein molecules, which take a longer path (Figure 5).

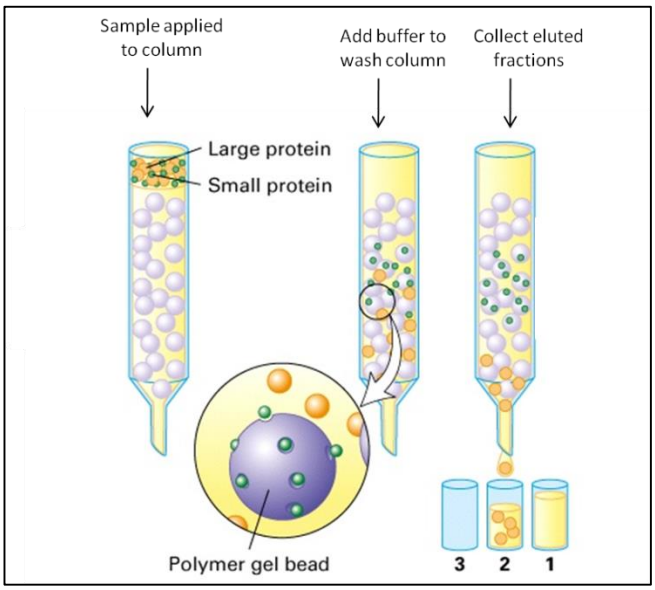


Figure 5. Gel Filtration Chromatography.

Gel filtration chromatography is also known as molecular sieve chromatography, gel permeation chromatography. The advantages of gel filtration chromatography / size exclusion chromatography are:

- Separation process is carried out under very mild conditions.
- High resolution can be achieved.
- Compatibility with many solvents. Thus wide range of buffer systems may be used
- Gel filtration can be used for buffer exchange, desalting or to obtain an estimate of molecular size

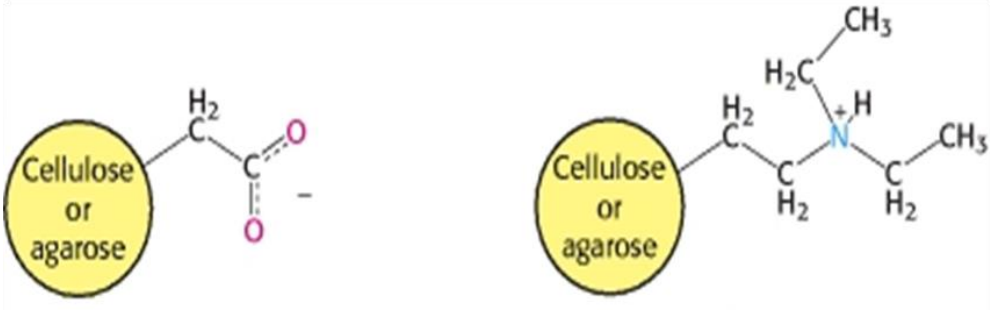
Disadvantages of gel filtration chromatography:

- Chromatography is very sensitive to column packing. Column packing should be done ensuring there are no cracks or air bubbles trapped in between.
- Non-specific interactions between protein and resin can often lead to decrease in resolution.

3.2.2 Ion-Exchange Chromatography

Ion-exchange chromatography (IEC) is the most frequently used chromatographic technique used for separation of proteins which are charged in nature. The basis for separation of proteins in IEC is the net charge on the proteins and their ionic interactions with the matrix that comprises the stationary phase in chromatography.

The stationary phase comprises of a column of beads containing carboxylate groups (cation exchanger). Positively charged proteins (cationic proteins) will readily bind onto a cation exchange column (for eg, carboxymethyl-cellulose (CM-cellulose) columns) due to electrostatic attraction. Similarly, negatively charged proteins can be separated on positively charged diethylaminoethyl-cellulose (DEAE-cellulose) columns (anion exchanger) (Figure 6 a and b).



(a) (b)

Figure 6. (a) Carboxymethyl (CM) group (ionized form) (b) Diethylaminoethyl (DEAE) group (protonated form)

Separation of positively and negatively charged proteins on an anion exchange matrix is shown in Figure 7.

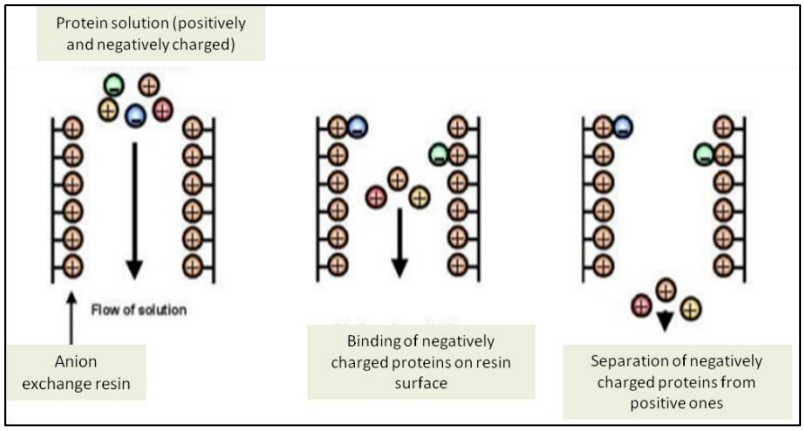


Figure7 . Separation of positively and negatively charged proteins on an anion exchange matrix

A common strategy for elution of such a bound protein is to use an increasing salt gradient, that is to increase the concentration of a salt (for e.g. NaCl) in the eluting buffer. The sodium ions will compete with positively charged groups on the protein for binding to the resin. Weakly charged proteins get eluted at lower salt concentrations, while proteins with a higher charge density get eluted at high salt concentrations (Figure 8). Gradient elution also leads to increased resolution.

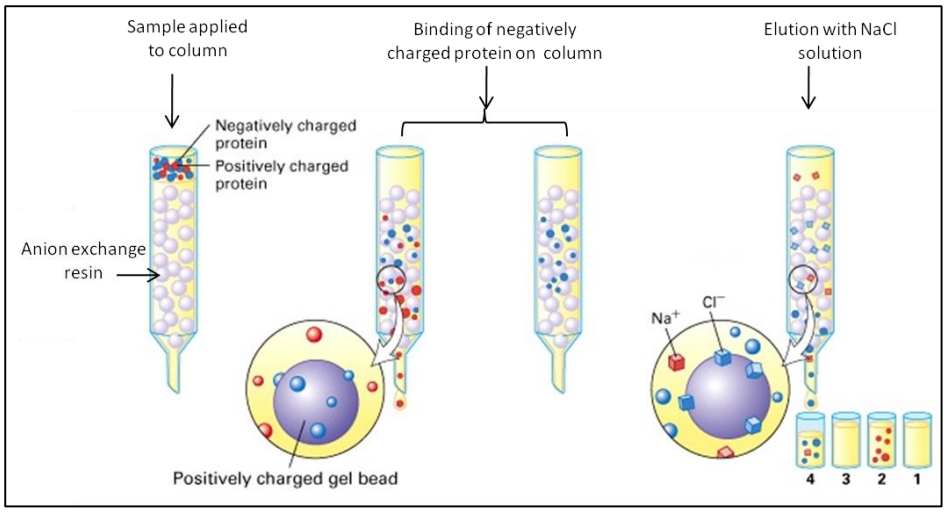


Figure 8 . Ion-exchange chromatography (anion exchange chromatography)

Advantage of IEC:

- Elution takes place under milder conditions, thus enabling the protein to retain native conformation throughout the process.
- High protein binding capacity.
- The method is easily controllable.
- Ion-exchanger resins can be reused for a number of cycles with appropriate column washing and sanitization.

3.2.3 Affinity Chromatography

Affinity chromatography exploits the high affinity of many proteins for their respective ligands. Figure 9 outlines the basic principle of an affinity purification.

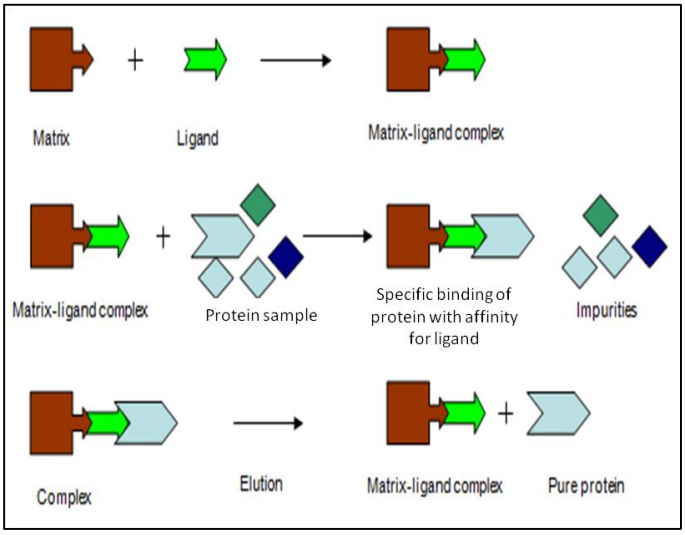


Figure 9. Schematic understanding of an affinity purification scheme

As a protein mixture is passed through a column in which a ligand is attached to a matrix, proteins which have specific affinity for the ligand bind to the column while other proteins pass out as unbound in the flow through. The column is then washed with buffer to remove all unbound protein. Elution of the bound protein is achieved by passing a high concentration of the ligand (in its soluble form) through the column or by altering chromatographic conditions such that the binding affinity is decreased.

For example, a well known example of affinity chromatography is the Lectin affinity chromatography. In this case, lectin protein concanavalin A (which has specific affinity to glucose) is purified by passing the crude extract through a column containing covalently attached glucose residues. Concanavalin A will bind to this column owing to its strong affinity to glucose. The bound concanavalin A can then be eluted out of the column by passing a concentrated solution of glucose through the column.

Advantages of affinity chromatography

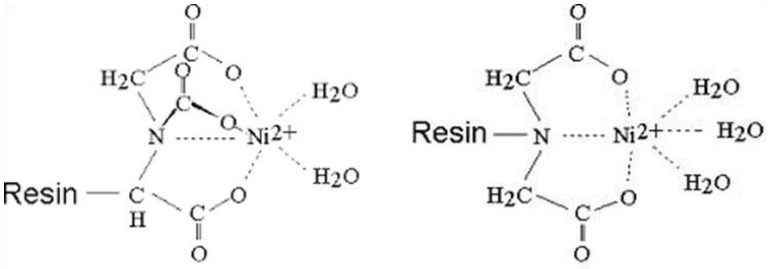
- The purification technique can be used for purifying proteins present in low concentration in samples.
- Rapid separation is achieved while avoiding contamination.

3.2.4 Immobilized metal ion affinity chromatography (IMAC)

IMAC is a specific kind of affinity chromatography which is based on the covalent binding between proteins (specifically the histidine residue in proteins) and metal ions. In this process, the mixture of proteins can be separated based on their differential ability to interact with the metal ions.

The technique is mainly used to purify recombinant proteins since naturally occurring proteins do not usually have an affinity for metal ions. Proteins are engineered to contain a poly-histidine tail such that histidine can act as a ligand for divalent metal cations. The stationary phase is simultaneously immobilized with divalent metal cations. As the His-tagged protein is passed though an IMAC column, those proteins containing a higher number of histidine residues would be able to bind to the column more tightly than those with fewer histidine residues.

The most widely preferred method for IMAC is to use an immobilized nickel column (for example, Ni-NTA (Nitrilo-triacetic acid) resin or Ni-IDA (imino-diactic acid) resin) (Figure 10).



(a) (b)

Figure 10. Composition of (a) Ni-NTA resin (b) Ni-IDA resin

The histidine residues of recombinant protein of interest bind onto this column (Figure 11).

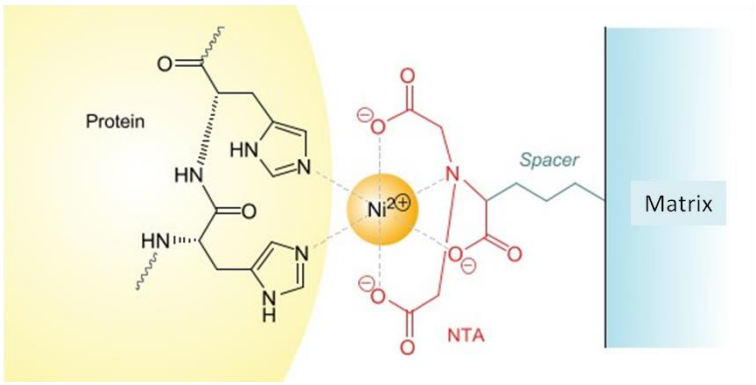


Figure 11. Protein-nickel-chelate complex

The elution of bounded protein can be achieved by three different elution strategies namely competitive elution, stripping elution and pH adjustment

3.2.5 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) is another powerful method of protein purification in which the proteins get separated on the basis of their relative hydrophobicity.

HIC media are comprised of porous and crosslinked cellulose particles, to which a hydrophobic group covalently bonded via a short spacer. For example, the hydrophobic groups commonly used are butyl, phenyl or octyl groups (Figure 12).

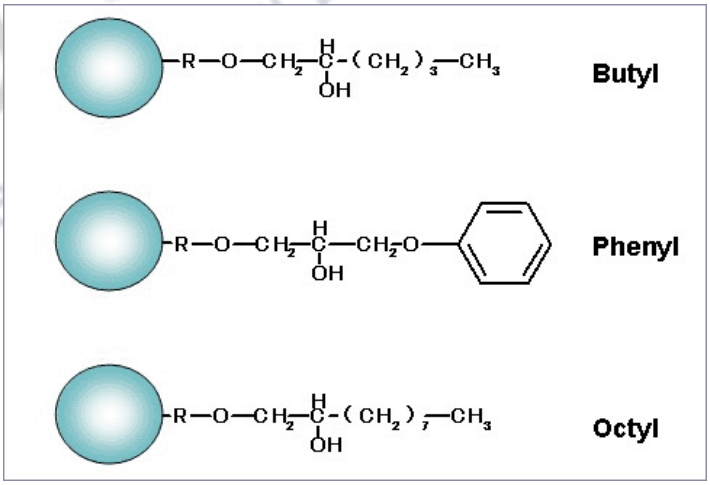


Figure 12. Composition of HIC media

Most proteins have hydrophobic patches on their surface. Protein sample to be purified is loaded onto an HIC column under high salt buffer. The high salt reduces solvation of proteins. As solvation decreases, the hydrophobic patches on the protein surface are exposed which get adsorbed onto the hydrophobic areas on the solid support (Figure 13). This is favored thermodynamically since it reduces the number and volume of exposed hydrophobic patches on protein surface.

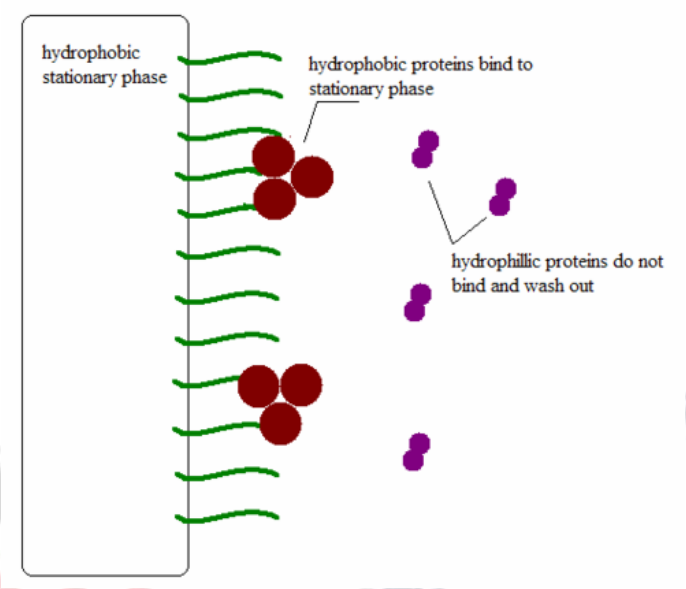


Figure 13. Principle of HIC

Proteins bound onto HIC column are eluted under low salt concentrations. Decreasing the concentrations of salts reduces hydrophobic interactions and results in proteins getting desorbed from the solid support.

Advantages of HIC

- Fast rapid chromatography due to high flow rates.
- Can be easily operated on a large scale.
- The column does not shrink at high salt concentrations
- A wide range of solvent systems can be used.

3.2.6 High-Pressure Liquid Chromatography

An advanced and improved version of the column chromatographic techniques discussed so far above is the *high-pressure liquid chromatography (HPLC).* Use of this method substantially improves the resolving power because the column matrix materials are much more finely divided enabling more interaction sites and increased separation among proteins. Since the column is made of finely divided material, pressure is required

to be applied on the column for obtaining adequate flow rates. The schematic overview of various steps in HPLC are shown in Figure 14.

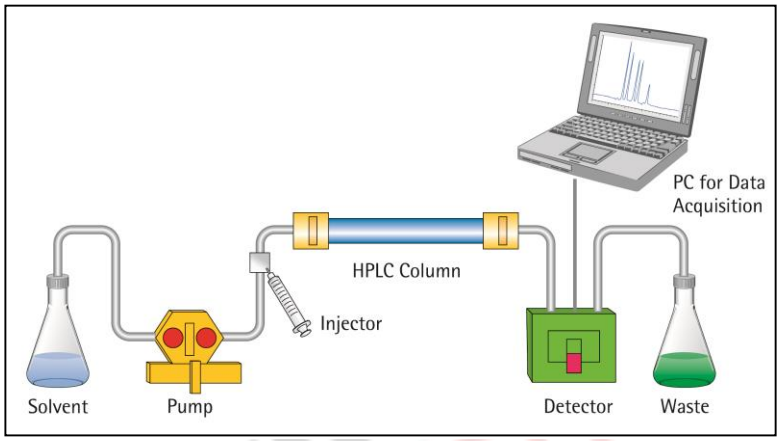


Figure 14. Scheme of HPLC

4. Summary

1. Proteins can be separated on the basis of solubility, size, charge, hydrophobicity and binding affinity.
2. The basic components of a column chromatographic set up include a chromatography column, a pump, a fraction collector and detector along with resin/protein sample and buffer systems.
3. A number of chromatographic methods that are in use for purification purposes: SEC, IEX, Affinity chromatography, IMAC, HIC, HPLC.