Biochemistry

Protein

Separation & purification of proteins

Cell contains different kinds of proteins, it is necessary to had a pure preparation of given protein before we determine its amino acid composition and sequences.

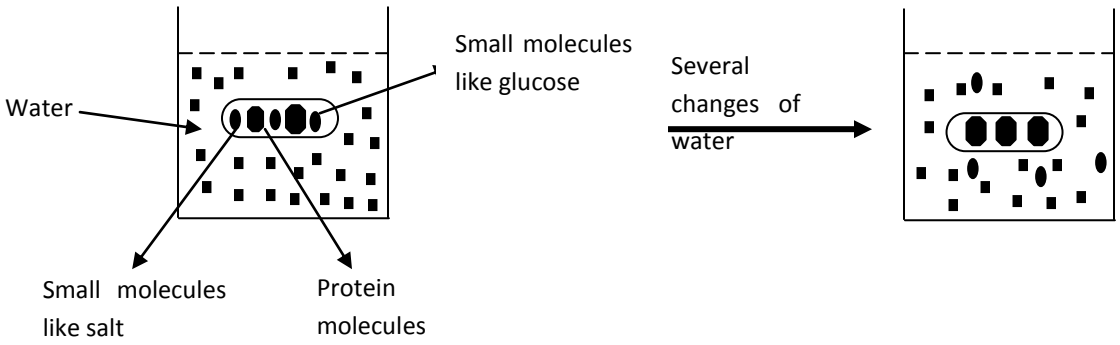
Protein can be separated from a mixture by using some of their known physical properties.

Dialysis

Non protein organic and inorganic solutes are easily removed by dialysis where large molecules such as proteins are retained inside a bay of material with ultramicroscopic pores (like cellophane or semi permeable membranes).

In order to convert the solute composition within the bad into the composition wanted, the external fluid must be repeatedly changed to maintain the required final composition.

Dialysis used routinely to remove small molecules or to change buffer condition gently, also used in concentration.

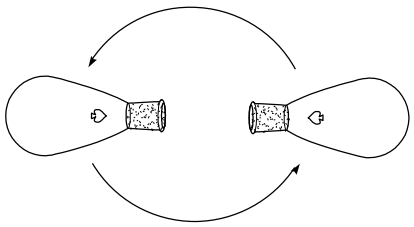


Separation according to their: -

1. *Molecular size:*

a. **Density – gradient or zonal centrifugation:** were different density – gradient of sucrose solution is used and placed in a plastic centrifuge tube were the concentration of sucrose is 20% in top layer and 60% in the bottom layer, the protein mixture is layered at the top and centrifuged in horizontal centrifuge. The individual proteins will separated into bands, the lowest being the heaviest molecule.

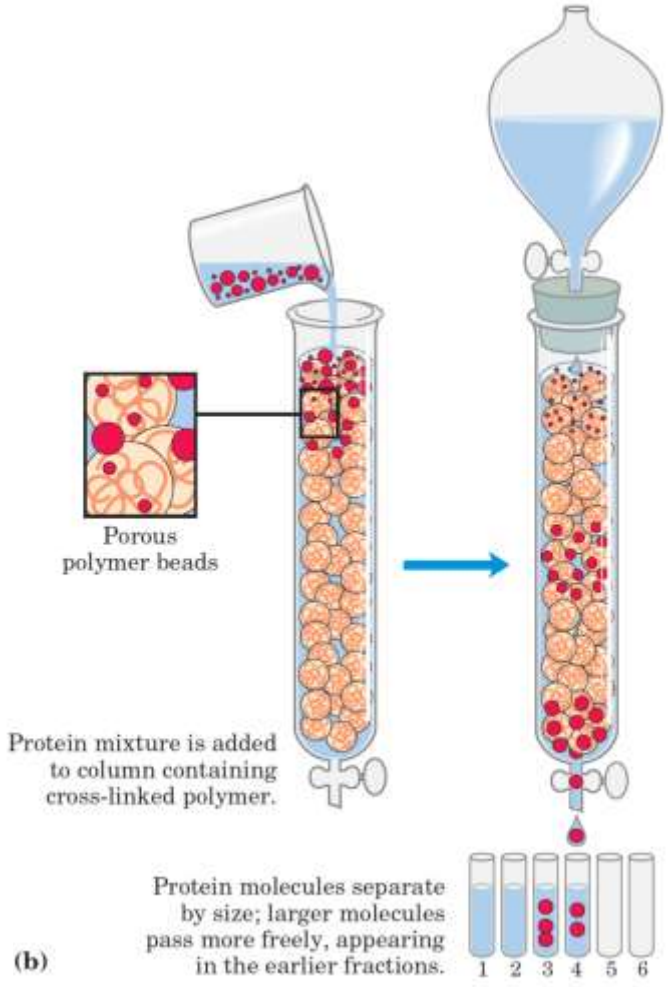
Sedimentation velocity for each protein differ from other according to different size

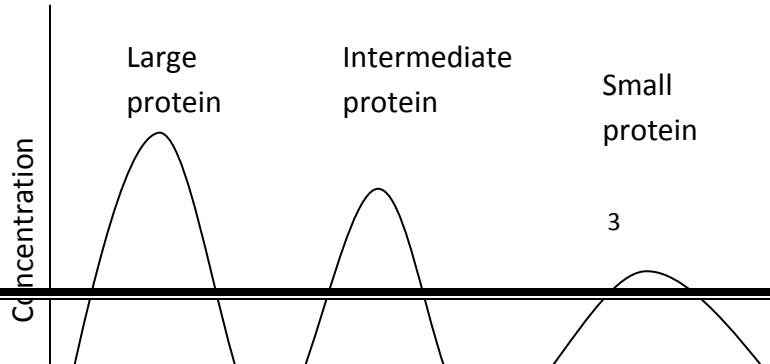


b. **Gel – filtration or molecular sieving:** this is a type of chromatography were the mixture of protein is passed down a column of very small porous beads of hydrophilic polymer (like dextran derivatives) which is widely used.

The smaller protein molecules can penetrate into pores but large one cannot penetrate into the beads and pass through down the column more rapidly.

Protein of intermediate size will pass down the column at intermediate rates depending on the degree of their penetration into the beads, such a column of gel – filtration is called molecular sieve.





Fraction number

Elution pattern

Q_1: In what order would the following proteins emerge upon gel – filtration of mixture on sephedex G – 200?

Myoglobin {M.w.} → 16,000
Cytochrome C {M.w.} → 12,000
Catalase {M.w.} → 500,000
Chymotrypsinogen {M.w.} → 26,000
Serum albumin {M.w.} → 65,000

Answer: cytochrome C is the smallest protein in the set and it will be diffused into all the space within the beads and it will not leave the column until the column has been washed with a volume equal to its total volume. Catalase is the largest protein; it would be completely excluded from the sephedex beads

∴ Therefore order of elution: -

1.Catalase 2.Serum albumin 3.Chymotrypsinogen 4.Myoglobin 5.Cytochrome C.

2. ***Solubility differences: -***

Solubility of proteins various with: -

- pH
- ionic strength
- nature of solvent
- temperature

a. pH: the solubility of most protein is lowest at its isoelectric pH so protein can be precipitated by increase or decrease the pH. Since they have different isoelectric pH, this is known as *isoelectric focusing*.

Note: a minimum in solubility occurs at the isoelectric point. Proteins typically have on their surface charged amino acid that undergoes polar interactions with the surrounding water. The total charged on the protein is the sum of the side chain charges and these depend on the pH. At pI all molecules have no charges and aggregate together, at pH values away from their pI, they would have similar charges and repletion.

Example: $(Ala – Asp – Gly)_5$ at pH 6.0

$(Asp – Ser – His)_3$ at pH 9.0

b. Ionic concentration: proteins show a variation in solubility that depends on the concentration of salts in the solution. These effects may involve in specific interaction between charged side chains and solution ions. Most protein require small amount of neutral salts in aqueous solution to dissolve them. Increase the solubility by the effect of salt is referred as ***salting in***. High salt concentration will precipitate most of protein, saturating ammonium sulfate produce a greatly

decreased solubility of the protein, this result is called ***salting out***. Were the molecules tending to associate with each because at high salt concentration, protein – protein interactions become ***more favor*** than protein – solvent interactions. Different portions can be precipitate by using different concentration of salt like: $(NH_4)_2SO_4$, NaCl, $MgCl_2$.

c. **Solvent fractionation:** solvents like *alcohol* and *acetone* decrease the solubility of most proteins in water and cause their precipitation. The protein will lose much of their electric charges so the repulsive forces between protein molecules decreased and leading to precipitation.

   For example:

   1) TCA (Trichloro acetic acid) is a potent precipitate agent for protein.
   2) Used different concentration of *ethanol* at low temperature to separate the plasma proteins.

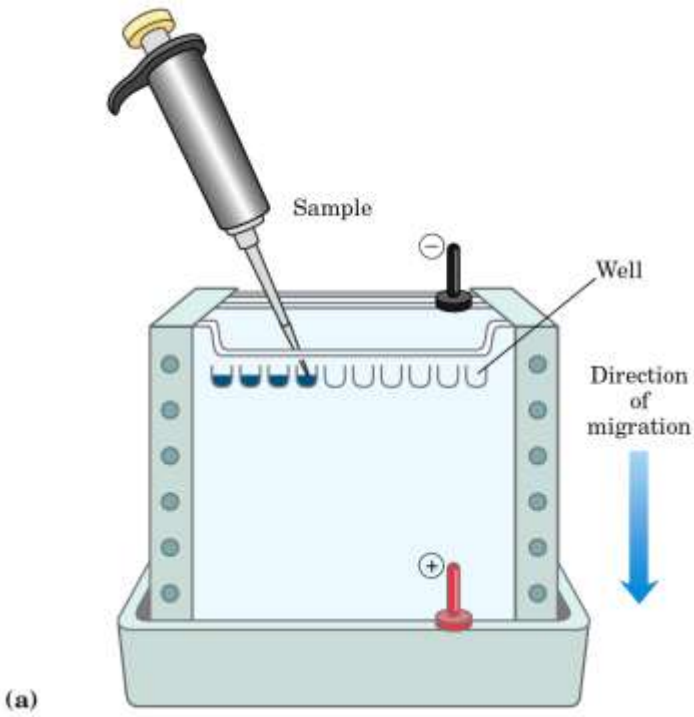
Note: {example of salting out}

The protein of blood serum can be separated into albumin & globulin by precipitation with sodium sulfate (salting out). The protein fraction precipitate is globulin & fraction remaining in solution is albumin.

d. **Temperature:** the solubility of globular protein increased from 0°C to 4°C above this temperature the molecules tend to denaturation & precipitation. But this cannot use in separation of protein from a mixture in the native state.

3. ***Electric charges:***

Different proteins carry different electric charges on their molecules depending on the acidic and basic group in R side chain; also they have different isoelectric pH (electrophoresis).



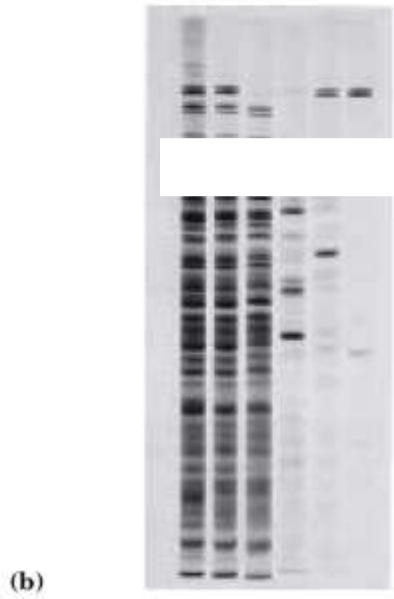


FIGURE 3–19 Electrophoresis. (a) Different samples are loaded in wells or depressions at the top of the polyacrylamide gel. The proteins move into the gel when an electric field is applied. The gel minimizes convection currents caused by small temperature gradients, as well as protein movements other than those induced by the electric field. **(b)** Proteins can be visualized after electrophoresis by treating the gel with a stain such as Coomassie blue, which binds to the proteins but not to the gel itself. Each band on the gel represents a different protein (or protein subunit); smaller proteins move through the gel more rapidly than larger proteins and therefore are found nearer the bottom of the gel. This gel illustrates the purification of the enzyme RNA polymerase from *E. coli*. The first lane shows the proteins present in the crude cellular extract. Successive lanes (left to right) show the proteins present after each purification step. The purified protein contains four subunits, as seen in the last lane on the right.

Electrophoresis carried out on a support media which may be a strip of paper or cellulose acetate or in a hydrophilic gel in order to keep protein molecules from diffusing in aqueous phase, the gel used in electrophoresis is starch, polyacrylamide agarose.

<u>Paper electrophoresis:</u> this divided to:

1. Low voltage paper electrophoresis for <u>small</u> molecules.
2. High voltage paper electrophoresis for <u>large</u> molecules.

<u>Example:</u>

1. At fixed pH → 6.0, the amino acid has positive charge like Lys, Arg, His that move to the cathode. The amino acid has negative charge like Asp, Glu move toward the anode. And other amino acid will remain at or near the origin since they have no ionizing group other than α – NH_2 & α – COOH. These have about the same isoelectric point.
   After electrophoresis the location of amino acid is detected by dried and sprayed with ninhydrin and heated, a blue or purple spots will appear.
2. For each of the amino acid and peptides below, determine the direction of migration at the indicated pH.
   a. Glu at pH 1
   b. Asp – His at pH 10

   <u>Ans.:-</u>

   a. Glu migrate toward the cathode
   b. Asp – His migrated to anode
3. What are the relative electrophoretic motilities of Gly, Leu, Asp, and Glu (at pH → 4.7)?

   <u>Ans.: -</u>

   Gly - 0.0169
   Leu - 0.0098
   → Gly move toward negative pole faster than Leu

   Glu + 0.01
   Asp + 0.0129
   → Asp moves faster than Glu toward positive pole

   <u>Note:</u>

Electrophoretic mobility of a compound depends on the charge/mass ratio.

$$mobility \propto \frac{pH - pI}{Mw}$$

Mw = 120

Positive mobility → movement toward positive pole

Negative mobility → movement toward negative pole

Factors affect "the rate of movement in electrophoresis"

1. Support media.
2. Composition and concentration of buffer.
3. Voltage & current & heating.

Q_2: In what direction the following proteins move in an electric field?

Egg albumin pI → 3.5 at pH → 5.0
β – lactoglobulin pI → 5.4 at pH → 5.0 & pH → 7.0
Ans.: -

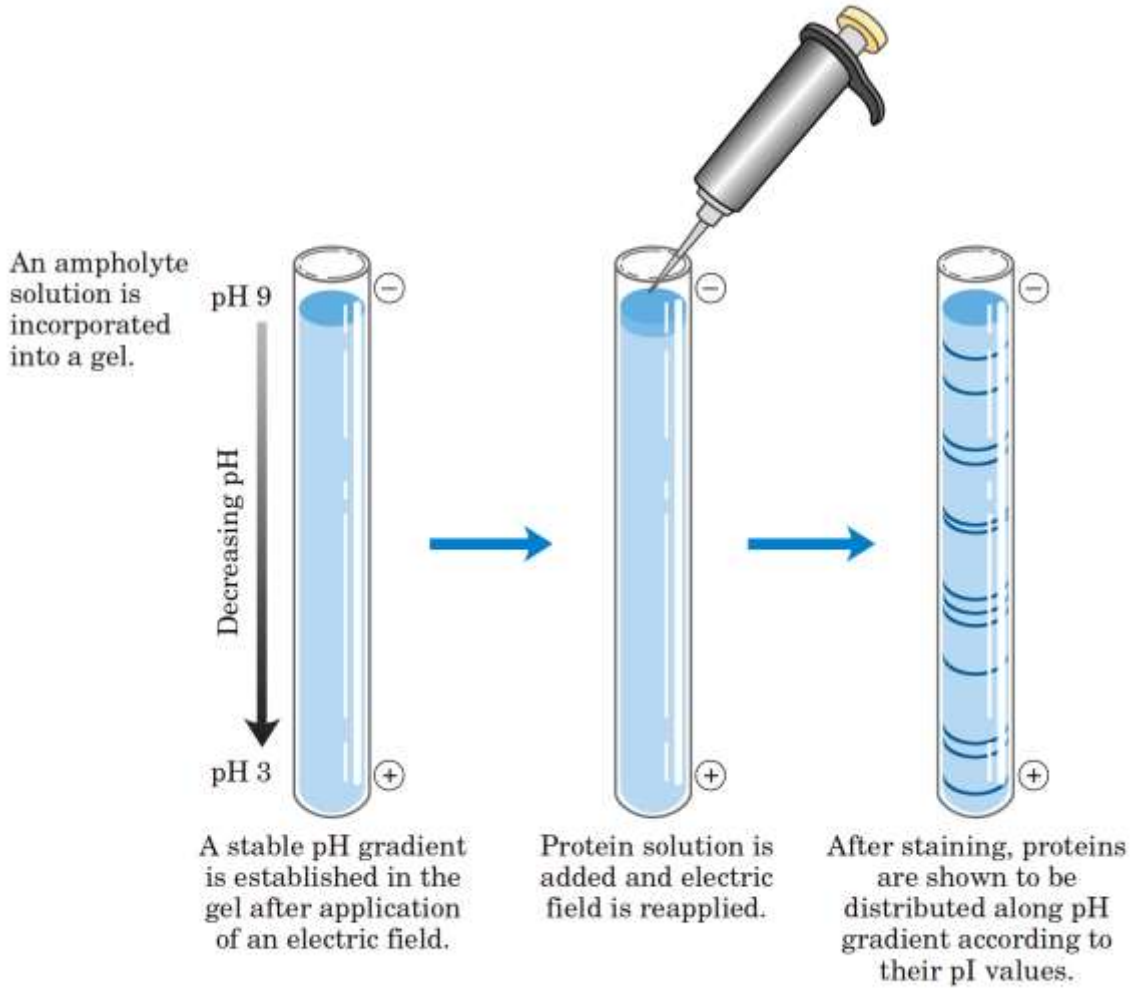
Egg albumin at pH 5.0 is above isoelectric point and the protein carry ***negative*** charge so it will move toward the anode.

β – lactoglobulin at pH 5.0 is below its pI at this pH the protein will move toward cathode because they carry ***positive*** charge, at pH 7.0 the protein will be negative and will move toward the anode.

Isoelectric focusing

This technique is wieldy used now, it combine the effects of an electric field (at pH gradient) on the protein in this technique a suitable amount of protein is introduced in such a system covering the pH range of their isoelectric point.

The molecule move toward anode or cathode until they reach the point at which the pH is that of their pI



Chromatography: -

It is separation technique used for separation of molecules by continues redistribution between two phases, one is stationary phase and the other is mobile phase.

A variety of attractive forces between the stationary phase and substance to be separated let to different rate of migration of substance molecule by the moving phase.

There are different types of stationary phase either (liquid, solid or gas). The mobile phase is liquid or gas. There are many types of chromatography: -

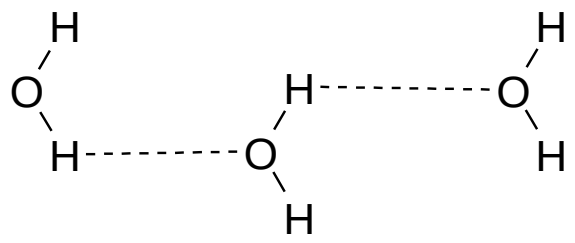
A. <u>Ion exchange chromatography.</u>

B. <u>Adsorption chromatography:</u>

In this type of chromatography active adsorptions of substance on the solid stationary phase which may be *alumina* or *silica gel*.

Competition for the adsorption site is occurred between the molecules of the mixture to be separated and the molecules of the mobile phase, where the mobile phase is either liquid or gas.

The forces involved in the adsorption chromatography is *weak forces*, include Vander Waals forces which is a short range forces of attraction between molecules and hydrogen bonds which arise between hydrogen atom and electronegative atom like O_2, N_2 and unshared pair of electron of another electronegative atom.



In liquid – solid chromatography (adsorption chromatography) the attractive forces are either Vander Waals or hydrogen bonding.

For column adsorption chromatography burette is practically used.

The mixture of substance to be separated must be applied in a solvent of low polarity, so the solvent can pass through the component without competition with the sample mixture on the adsorptive sites.

C. *T.L.C. (thin layer chromatography):*

In this technique the adsorbent is supported by a plate

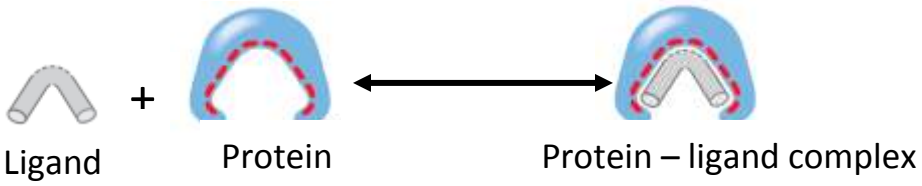
D. *Gel – chromatography*

E. *DEAE cellulose*

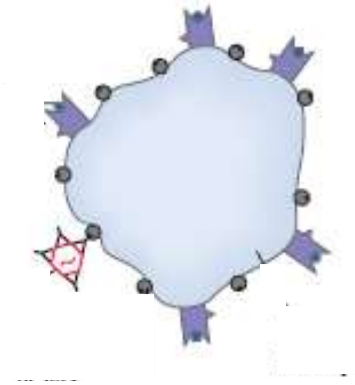
F. ***Affinity chromatography:***

This is highly specific and efficient method of isolation specific proteins because other methods are for organic molecules like distillation and solvent extraction were not suitable.

This technique used for the isolation and identification of certain enzyme, immunoglobulins and receptor hormones the bases of this technique that the protein in its normal biological action will reversibly bind another specific molecule called a ***ligand,*** very tightly to form a non covalent protein – ligand complex.

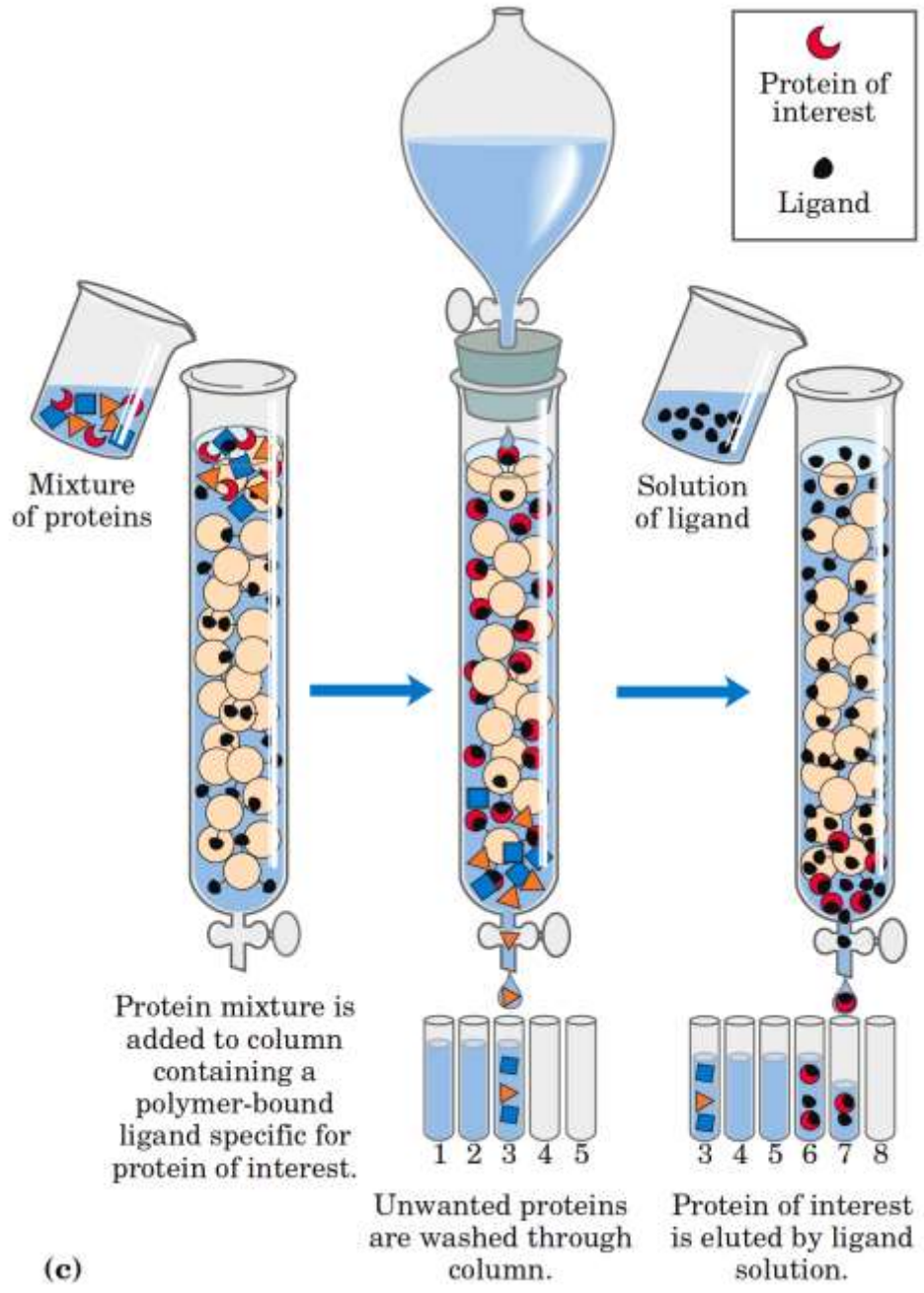


The specific ligand for the protein is attached to insoluble polymer beds.



Note: cell extract containing hundred of different protein, only one protein can be isolated and purified by one step.

Elution of protein bound to ligand is done by using another compound with high affinity to the ligand than the protein itself, or changing the condition to which binding is not occurring.



Q_4: A solution containing egg albumin pI = 4.6, β – lactoglobulin pI = 5.2, Chymotrypsinogen pI = 9.5 was layered at a column of DEAE – cellulose at pH → 5.4, the column was then eluted with pH = 5.4 buffer with increase gradient of salt concentration, what is the elution pattern?

Ans.: -

1. Chymotrypsinogen → carrying positive charge (eluting first).
2. β – lactoglobulin → carrying very small negative charge.
3. Egg albumin → carrying negative charge.