BIOCHEMISTRY 1

2ND CLASS

UNIVERSITY OF ANBAR-COLLOGE OF SCIENCE

BIOLOGY DEPARTMENT

2020-2021

Protein purification

Lecture five(5)

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College of Science

References:

Harper's Illustrated Biochemistry

Lippincott Biochemistry

Lehninger Principles of Biochemistry

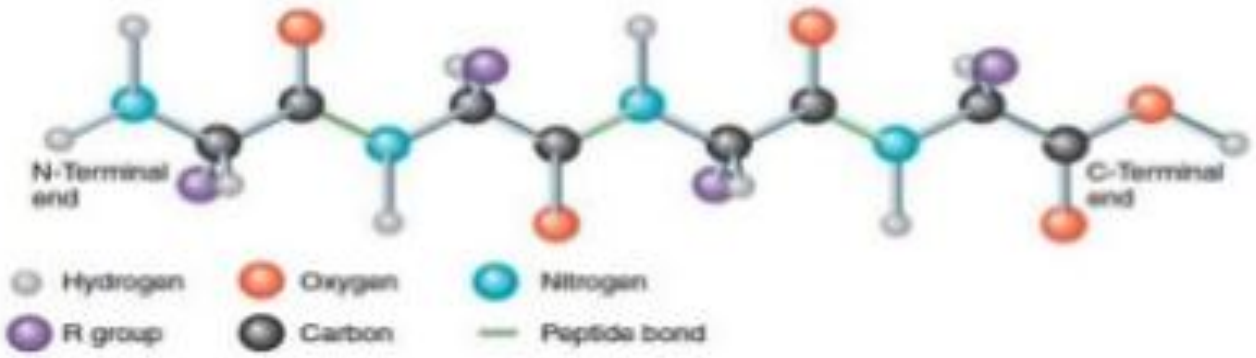
Stryer Biochemistry

Content

- Introduction
- Purification of Protein
  - Salting Out
  - Dialysis
  - Gel filtration chromatography
  - Ion-exchange chromatography
  - Gel electrophoresis
  - Isoelectric focusing
  - HPLC
  - Mass spectrometry
- Applications of purification of proteins

Introduction

- Proteins are polymers composed of hundreds or even thousands of amino acids linked by peptide bonds.

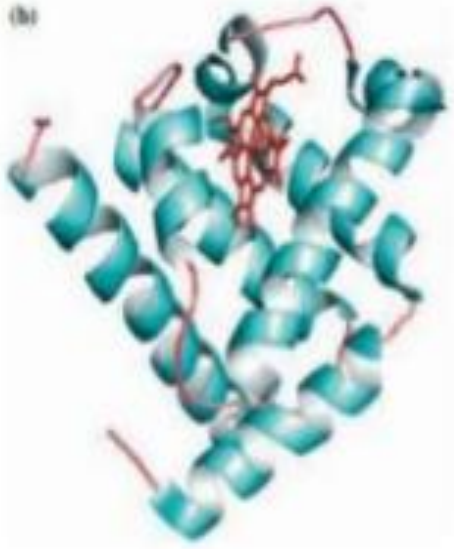


- Proteins fall into three basic classes according to shape and solubility:
  1. Fibrous Protein – Collagen
  2. Globular Protein – Myoglobin
  3. Membrane Proteins – Bacteriorhodopsin

1. Fibrous Protein

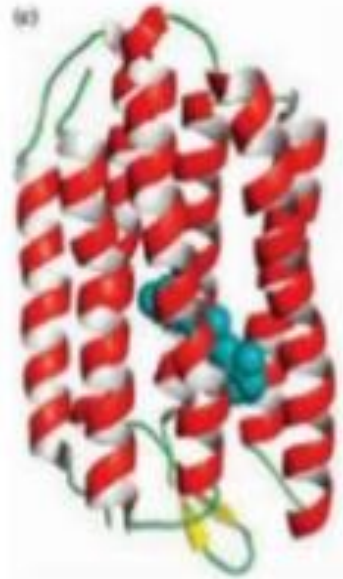
- Regular linear structures
- Play structural roles in the cell (matrix formation)
- Often water Insoluble

2. Globular Protein



- Roughly spherical in shape
- Often water soluble
- Hydrophobic amino acid side chains present in the interior of the molecule while hydrophilic side chains are outside exposed to the solution

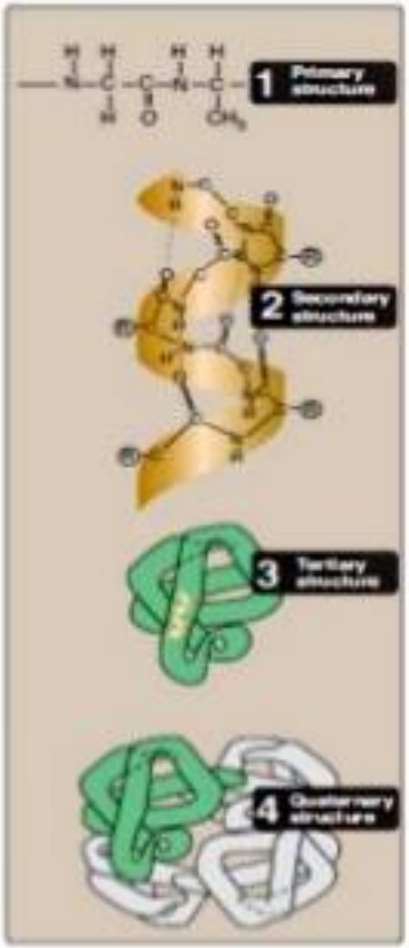
3. Membrane Protein



- Hydrophobic amino acid side chains present outside
- Play role in the cellular transport
- Often water insoluble but soluble in detergents

Organisation of Proteins

- Primary Structure
  - Stabilized by peptide bonds only. E.g.: Each chain of insulin
- Secondary Structure
  - α–helix, β-sheet, β-bends, motifs etc.
  - Stabilized by covalent (peptide, disulfide) & non-covalent interactions (H-bonding, electrostatic forces, Hydrophobic interactions and Van der wall forces).
- Tertiary Structure
  - More complex form of primary structure
  - Stabilized by covalent & non-covalent interactions
- Quaternary Structure
  - Consist of two or more polypeptides linked to each other by covalent and non-covalent interactions.



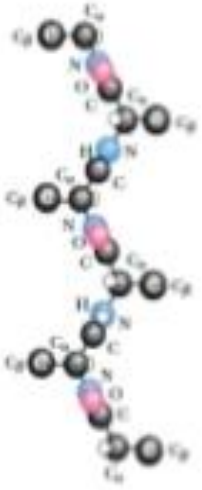
Primary Structure

E.g: Each chain of human Insulin

Secondary Structure

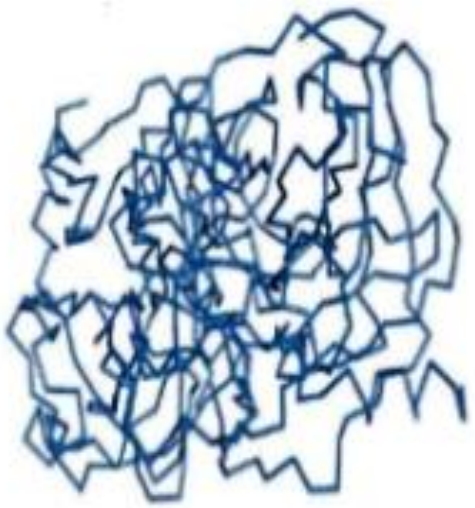
α- helix

β- helix



E.g: Myoglobin (α- helix)

Tertiary Structure



E.g: Chymotrypsin

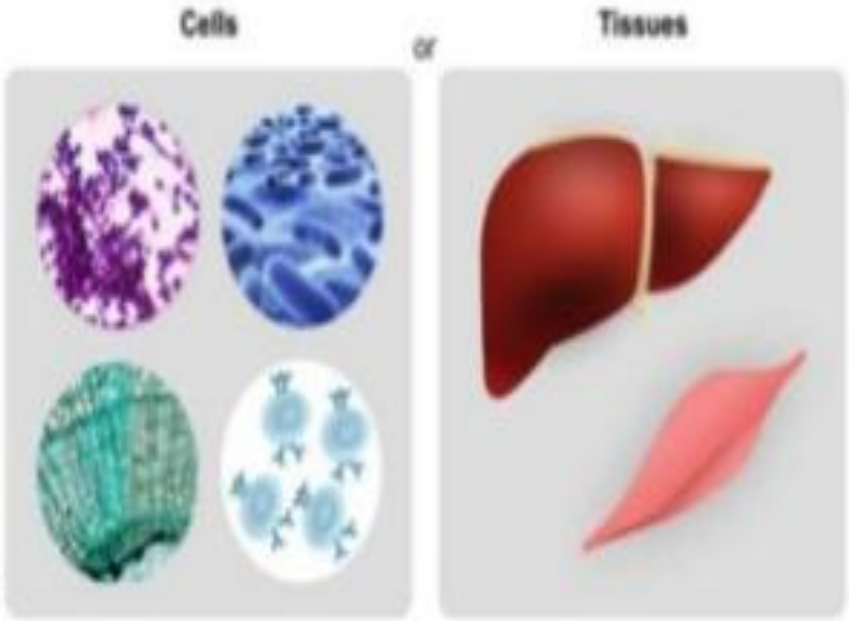
Purification of Proteins

Why we need to purify proteins?

- To identify structure and funtion of the protein of interest.
- To study protein regulation and protein-protein interactions.
- To produce antibodies
- Proteins(enzymes) are used in assays
- Perform structural analysis by X-ray crystallography

Where do we get proteins ?

- Whole organisms
- Organs or tissues
- Embryos
- Tissue culture cells
- Proteins from expression systems
  - Genes cloned into a vector, inserted into a living organism which makes the protein



Numbers of genes:

Humans	~40,000 genes
Yeast	~6000 genes
Bacteria	~3000 genes

Solubility of proteins important for purification:
60-80% soluble, 20-40% membrane

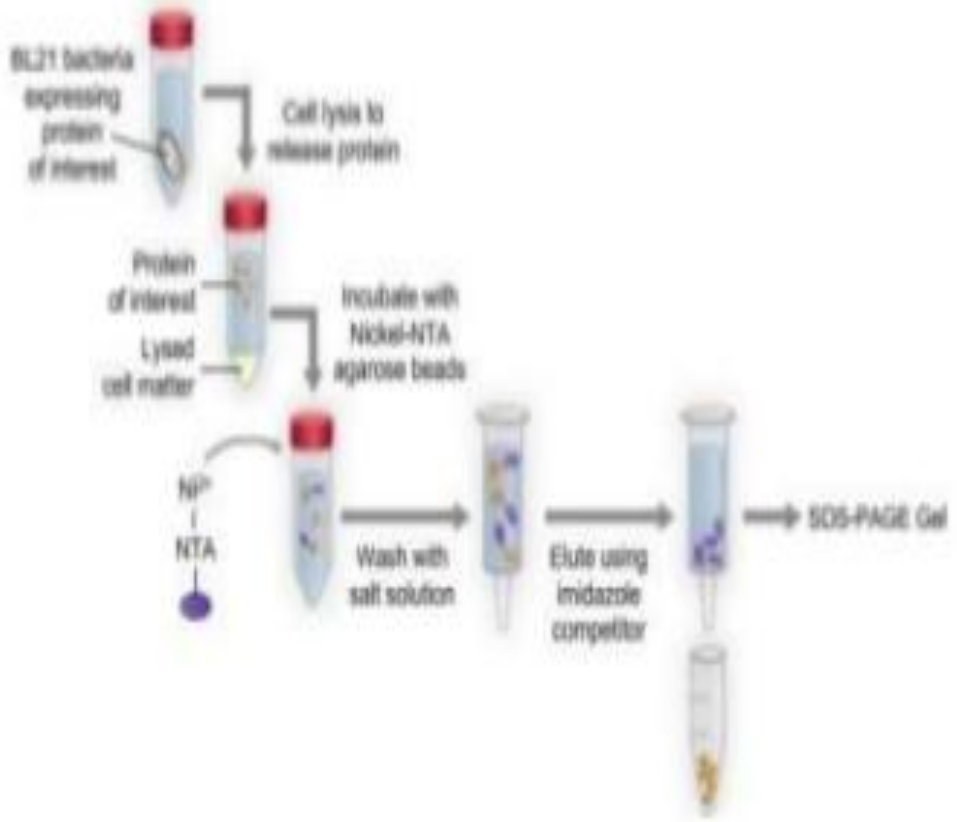
Some proteins expressed at high levels (collagen, hemoglobin)
Some proteins expressed at low levels (repressors, signaling)

Fibrous proteins - structural (collagen, elastin, keratin)
Globular proteins - structure and/or function (actin, enzymes)

Steps of purification and analysis
(1) Choose protein to purify
(2) Choose source (natural or expressed)
(3) Soluble in aqueous solution?? (problem with membrane proteins)
(4) Stability
(5) Purify
(6) Study (activity, structure, mechanism of action, etc.)

Steps of Protein Purification

- Step 1. Solubilization of protein
    - A. Homogenization
    - B. Centrifugation
    - C. Filtration
- Step 2. Stabilization of proteins
- Step 3. Isolation of proteins
- **Note**: Some biological materials themselves constitute a clear or nearly clear protein solution suitable for direct application to chromatography columns after centrifugation or filtration.



1.A. Homogenization

- On the laboratory scale:
  - ✓ Sonication
  - ✓ French Press
  - ✓ Enzymatic or chemical cleavage of the cells
  - ✓ Freeze-thawing
- For larger scale:
  - ✓ High pressure homogenization
  - ✓ Bead milling

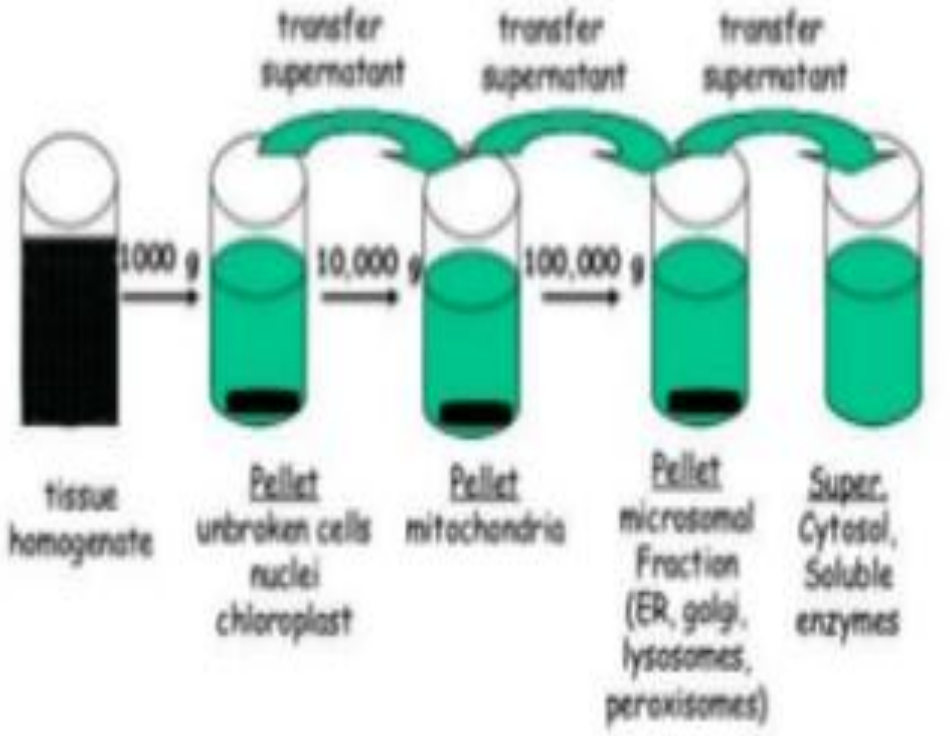
Sonicator

French Press

High Pressure Homogenizer

1.B. Centrifugation

- Centrifugation is done to isolate the proteins that are components of the membrane or subcellular assembly (For e.g: mitochondrial protein)
- Cell lysate is centrifuged at a speed that removes only the cell components denser than the desired organelle.



Step 2. Stabilization of Proteins

- Care must be taken to preserve protein structure and function after it is removed from its natural environment where it was stable.
- pH – To prevent denaturation or loss of function, proteins are placed in buffered solutions at or near their native pH.
- Temperature – Protein purification is normally carried at low temperature ~ 0°C. while some proteins are thermally stable at high temperatures.
- Inhibition of proteases
- Retardation of microbes that can destroy proteins
  - Sodium azide is often used.

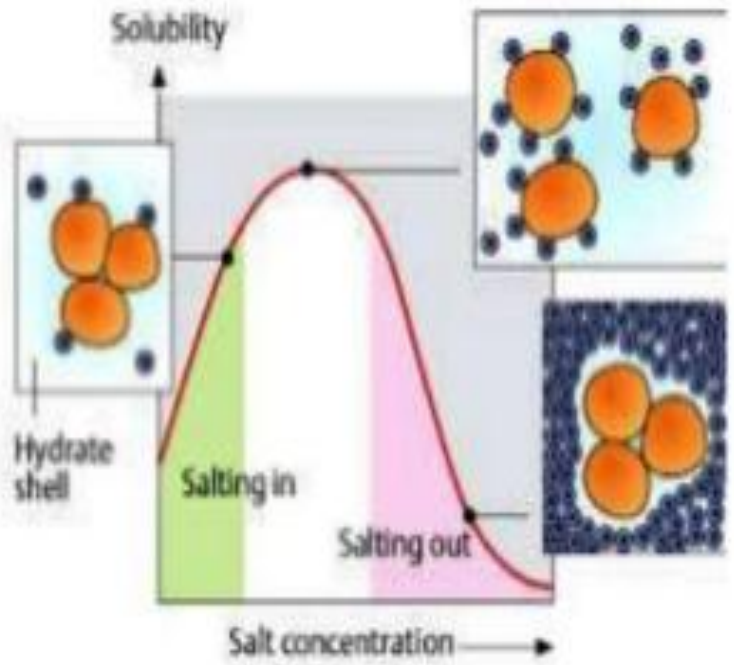
Step 3 – Isolation of Proteins

- Method of isolation of proteins differs from one protein to other.
- **Proteins can be isolated on the basis of following properties:**

Characteristic	Procedure
Solubility:	1. Salting out
Molecular size:	1. Dialysis 2. Gel filtration chromatography
Ionic charge:	1. Ion-Exchange Chromatography 2. Gel electrophoresis 3. Isoelectric focusing
Binding specificity:	1. Affinity chromatography

3.1. Solubility

- **Salting-In:** Most globular proteins tend to become increasingly soluble as the ionic strength is raised due to the addition of salt. This phenomenon is known as salting-in of proteins.
- **Salting-out:** As the salt concentration increases, this lead to diminishment of electrostatic attraction between protein molecules by the presence of abundant salt ions. This phenomenon is known as salting-out of proteins.
- The salt concentration at which protein precipitates differs from one protein to another.
- **Salting out is one of the most commonly used protein purification procedures.**
- **Ammonium sulfate** is the most commonly used reagent
  - High solubility (3.9 M in water at 0 ºC)
  - High ionic strength solution can be made (up to 23.5 in water at 0 ºC)

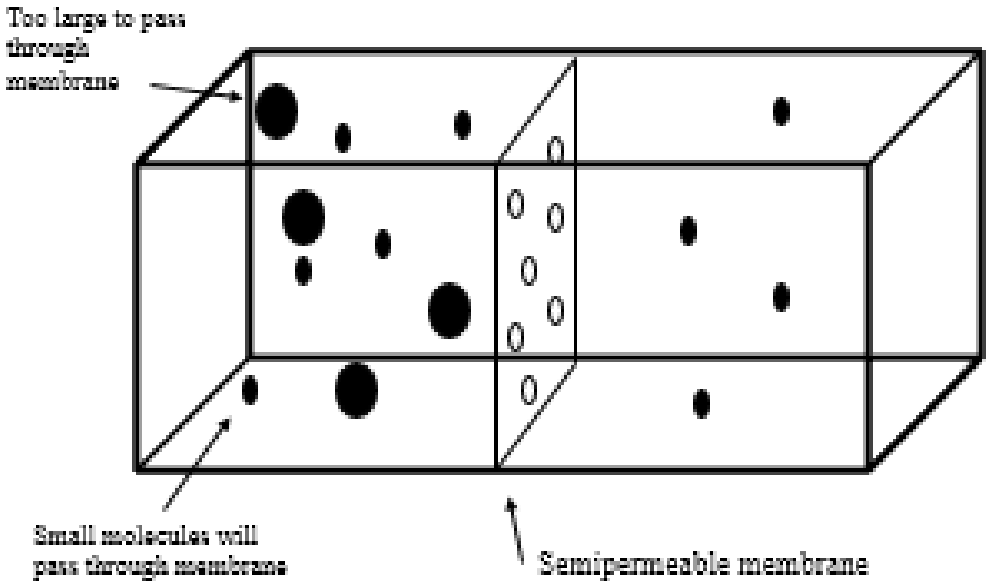


Purify

Solubility used for many years - lower solubility at high salt conc. called "salting out"

selectively precipitate proteins using $(NH4)_2SO_4$

To remove excess salt then perform:
Dialysis - separate proteins from solvents, remove $(NH4)_2SO_4$



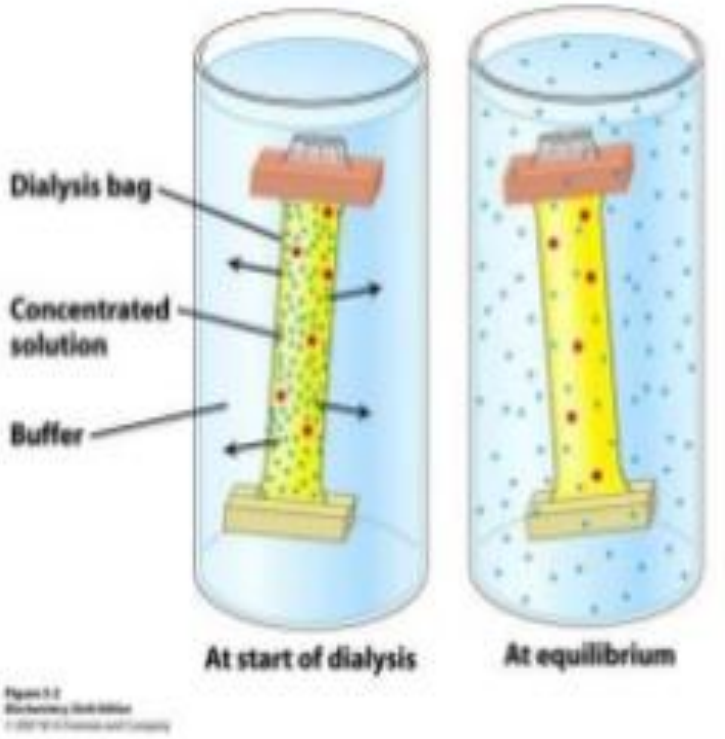
Purify

Characteristic:	Procedure:
Charge	1. Ion exchange chromatography 2. Electrophoresis 3. Isoelectric focusing
Size:	1. Dialysis and ultracentrifugation 2. Gel electrophoresis 3. Gel filtration (size exclusion) chromatography
Specificity:	1. Affinity chromatography
Polarity:	1. Adsorption chromatography 2. Paper chromatography 3. Reverse-phase chromatography 4. Hydrophobic chromatography

3.2 Molecular Size

A. Dialysis

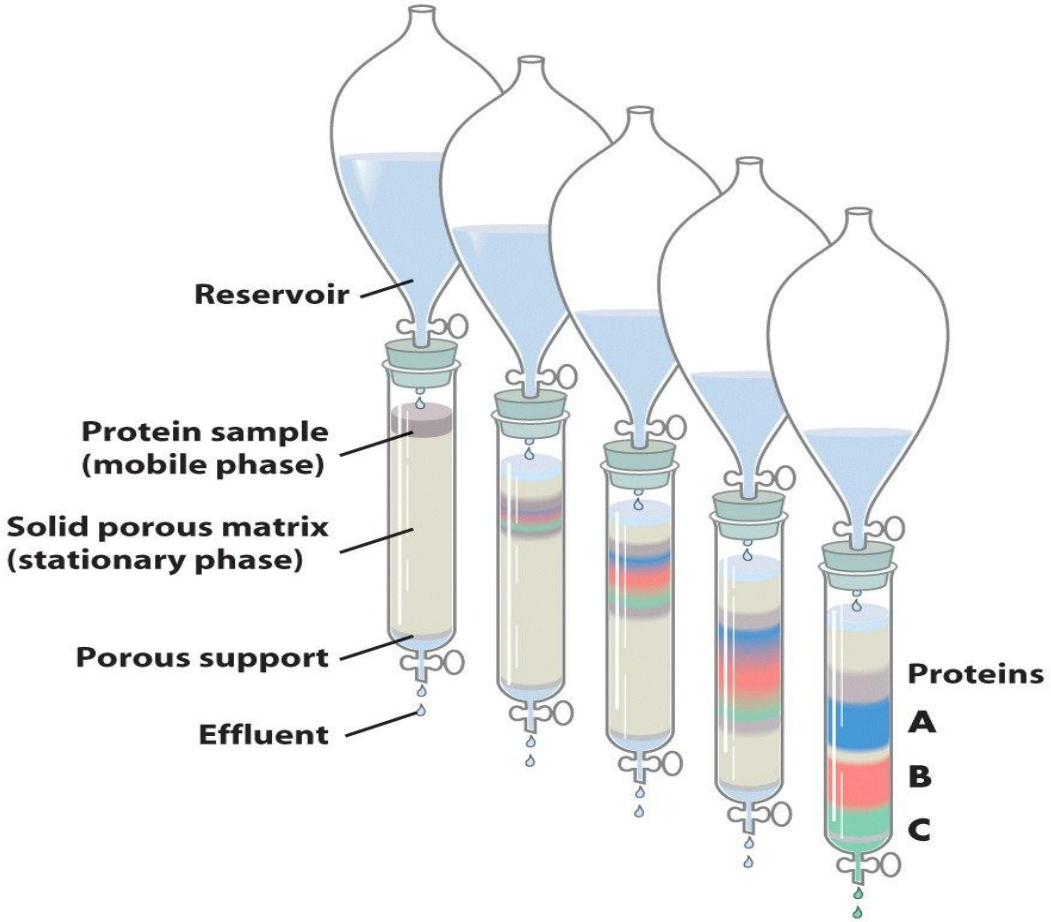
- Proteins can be separated from small molecules such as salt by *dialysis* through a semipermeable membrane such as cellulose membrane with pores.
- Protein mixture is placed inside the dialysis bag which is then submerged in buffer solution.
- Molecules having dimensions significantly greater than the pore diameter are retained inside the dialysis bag.
- Smaller molecules and ions capable of passing through the pores of the membrane diffuse down their concentration gradient and emerge in the solution outside the bag.



Chromatography

Important steps in chromatography

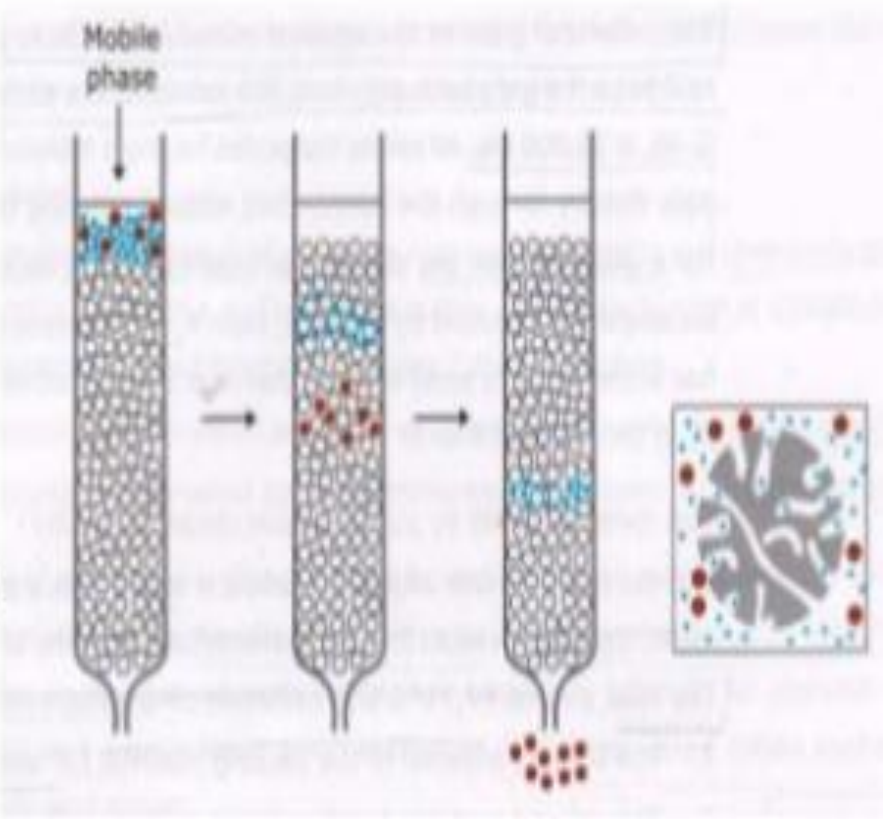
1. Pack column - Column is packed with material (resin) that can absorb molecules based on some property (charge, size, binding affinity, etc.)
2. Wash column - Molecules washed through the column with buffer
3. Collect fractions - Fractions are taken, at some point your molecule will elute



3.2 Molecular Size

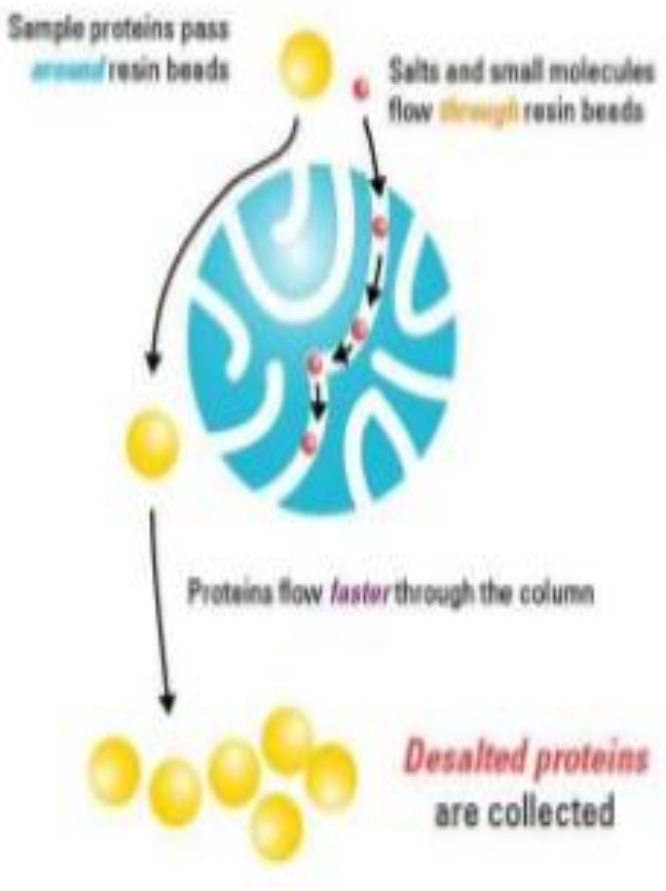
B. Gel-filtration Chromatography

- Also Known as molecular exclusion chromatography.
- Stationary Phase: Porous beads.
  Eg: Sephadex, Sepharose etc
- Mobile Phase: Buffer
  Eg: Tetrahydrofuran, Choloform, Dimethyl formamide



B. Gel-filtration Chromatography

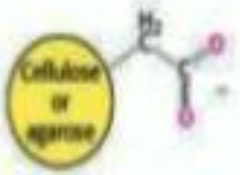
- The beads are made of an insoluble but highly hydrated polymer such as dextran or agarose or polyacrylamide.
- Sample is applied at the top of the column consisting of porous beads in the buffer.
- Small molecules of the sample can enter these beads but large ones cannot.
- Small molecules are distributed inside the beads and between them whereas large molecules are located only in the solution between the beads.
- Large molecules flow more rapidly through this column while small molecules take a longer, tortuous path, will exit last.



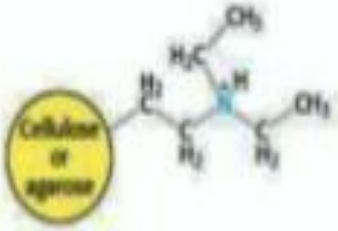
3.3 Ionic Charge

A. Ion-exchange chromatography

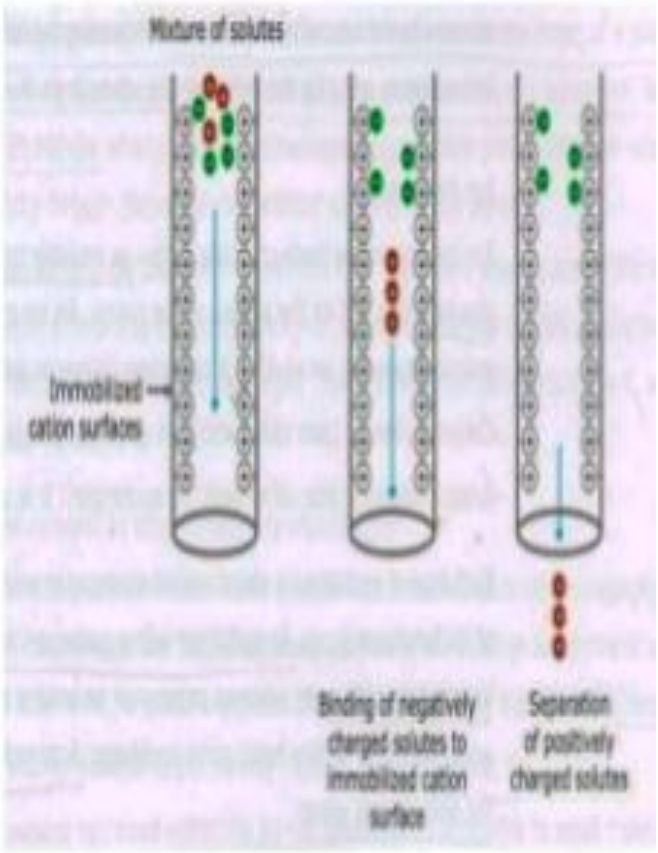
- Stationary phase: Ion-exchangers
- Mobile phase: Buffers of variable concentrations
- Principle: Ion-exchange chromatography relies on the attraction between oppositely charged ion-exchangers and analyte (protein sample).
- Ion-exchangers:



Carboxymethyl (CM) group
(cation-exchanger)



Diethylaminoethyl (DEAE) group
(anion-exchanger)

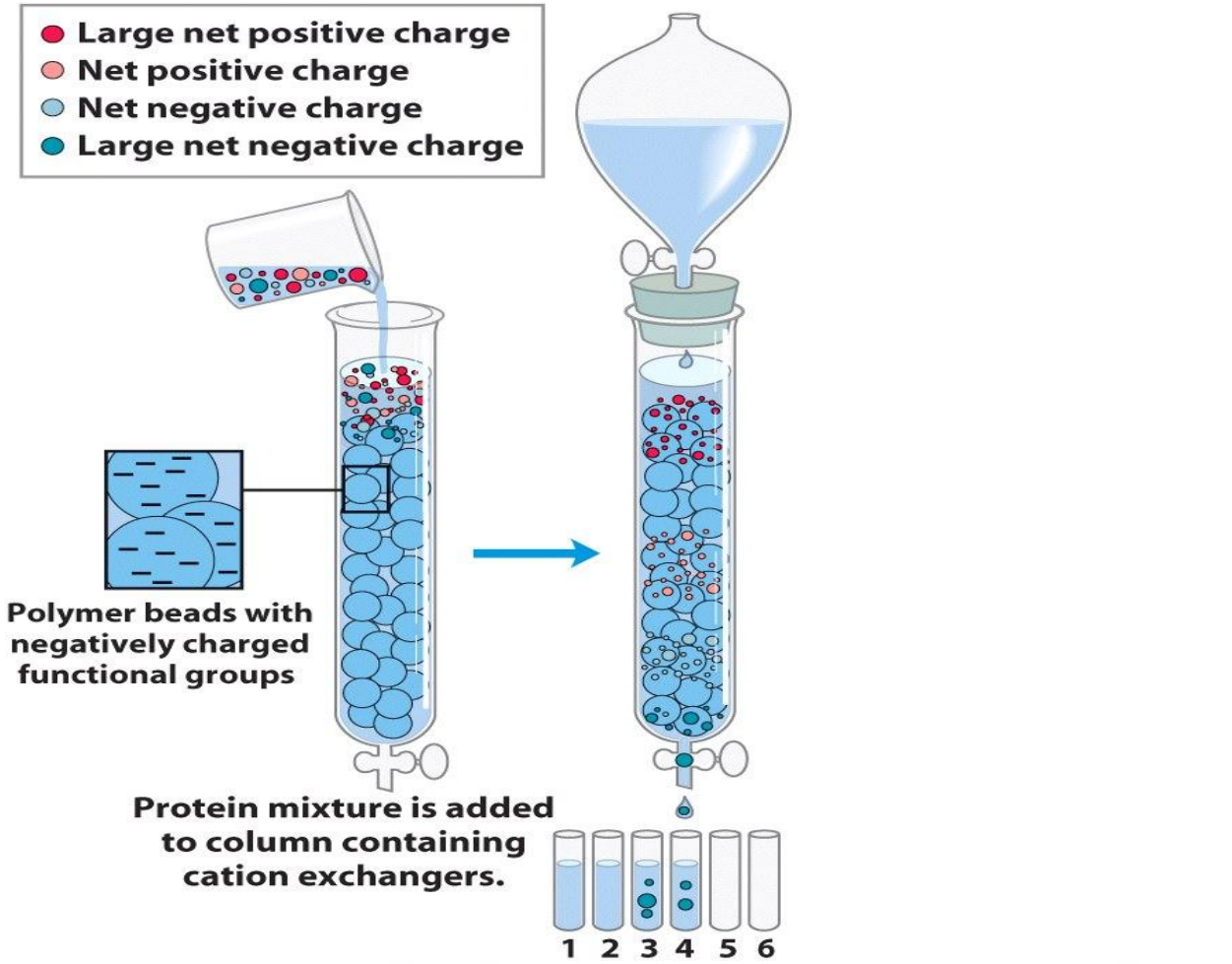


Ion exchange chromatography

Separate by charge

Elute protein

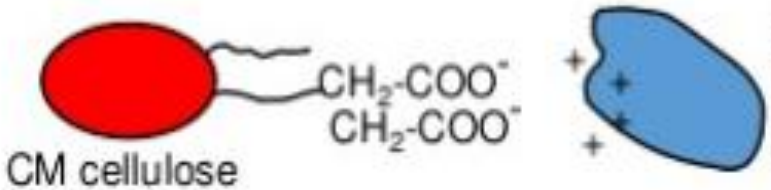
- Increase salt or pH to elute protein of interest



Proteins move through the column at rates determined by their net charge at the pH being used. With cation exchangers, proteins with a more negative net charge move faster and elute earlier.

A. Ion-exchange chromatography

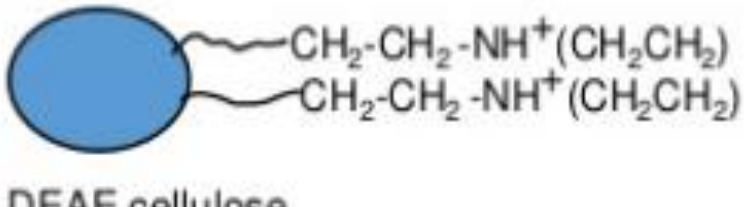
- Ion exchange resins contain charged groups.
- If these groups are *acidic* in nature they interact with positively charged proteins and are called **cation** exchangers.



CM cellulose
cation exchanger

Positively charged (basic) protein or enzyme

- If these groups are *basic* in nature, they interact with negatively charged molecules and are called **anion** exchangers.

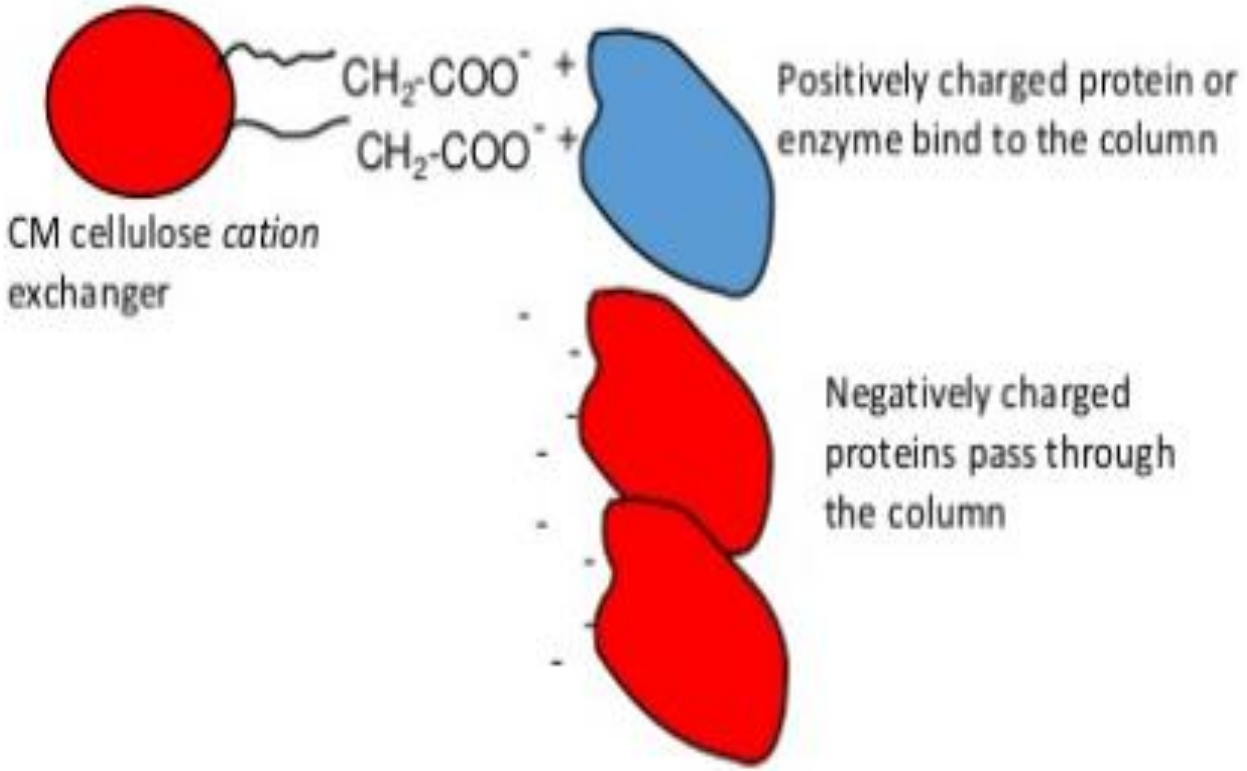


DEAE cellulose
anion exchanger

Negatively charged (acidic) protein or enzyme

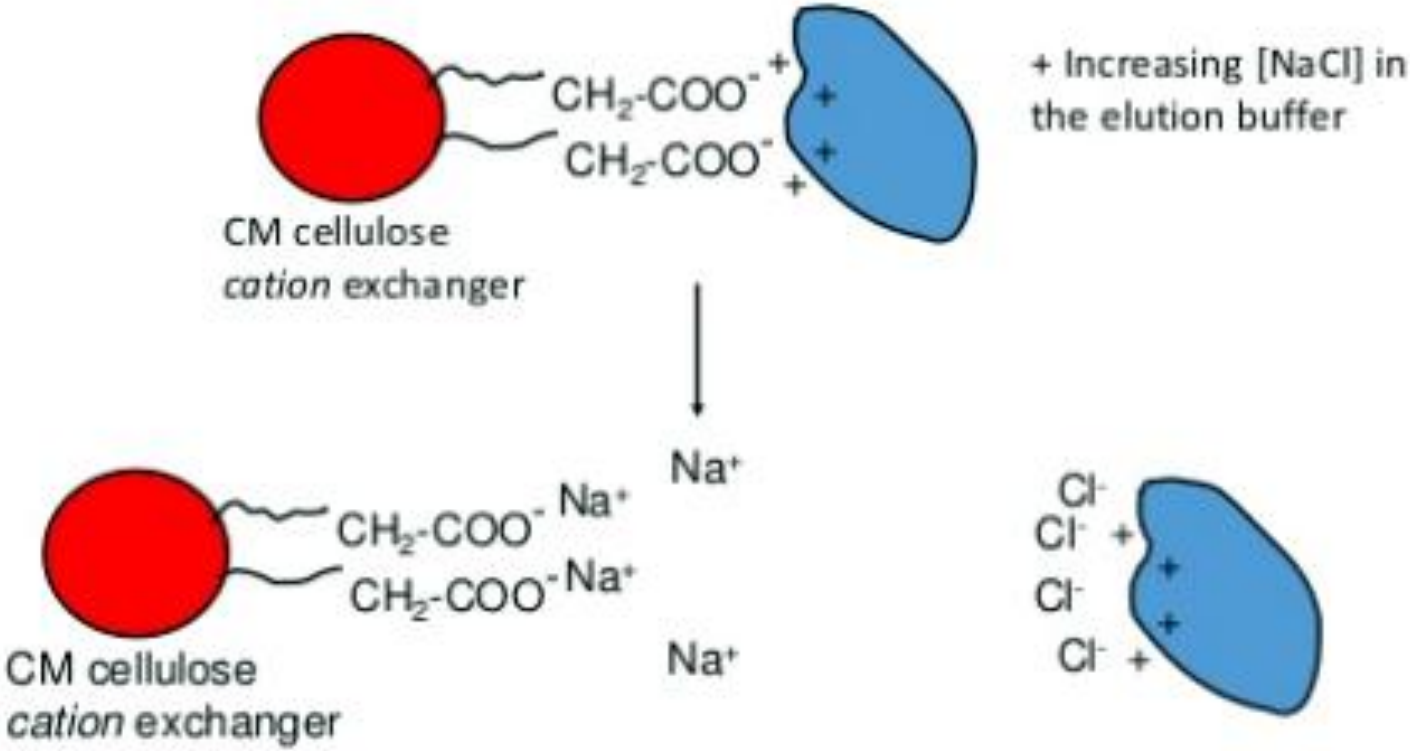
A. Ion exchange chromatography

If a protein has a net positive charge at pH 7, it will usually bind to a column of beads containing carboxylate groups (cation-exchangers).



A. Ion exchange chromatography

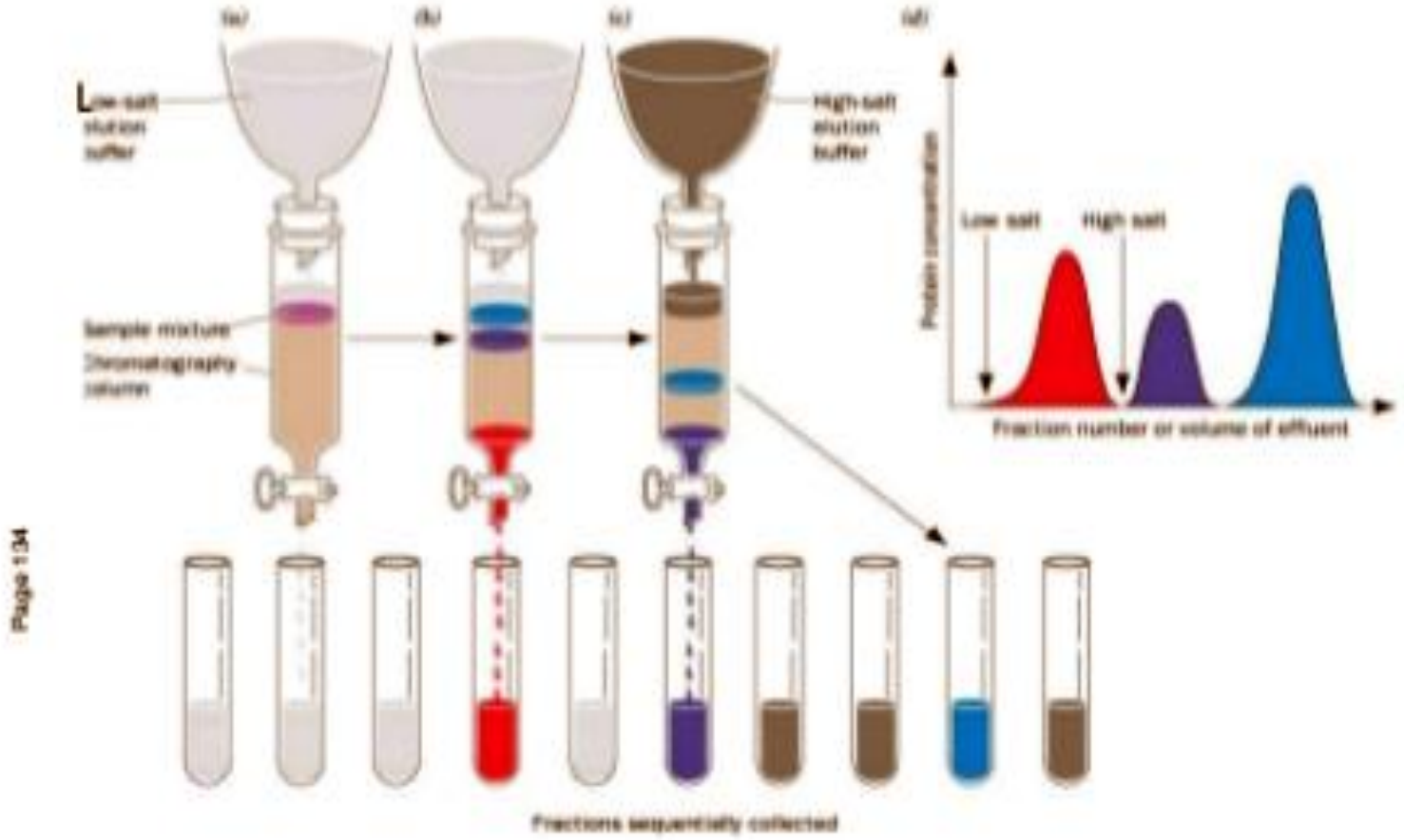
To elute our protein of interest, add buffer of higher salt concentartion. Na^+ will interact with the cation resin and Cl^- will interact with our positively charged protein to elute off the column.

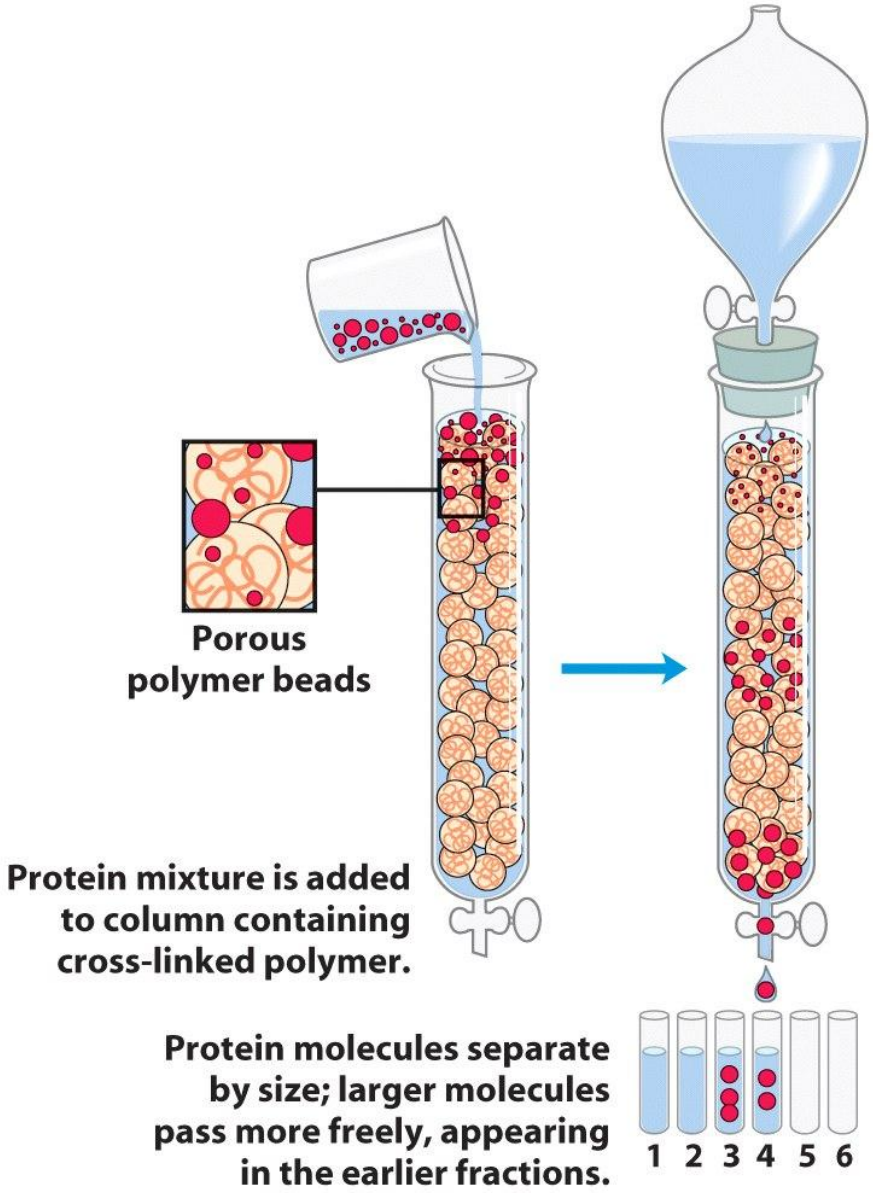


A. Ion exchange chromatography

- Proteins will bind to an ion-exchanger with different affinities.
- As the column is washed with buffer, proteins which have relatively low affinities for the ion-exchange resin will move faster through the column than the proteins that bind tightly to the column to the ion-exchangers.
- The greater the binding affinity of a protein for the ion exchange column, the more it will be slowed in eluting off the column.
- Proteins can be eluted by changing the elution buffer to one with a higher salt concentration and/or a different pH (stepwise elution or gradient elution).

Figure: Ion exchange chromatography using stepwise elution.





Size exclusion (gel filtration) chromatography

Separate by size

As wash with buffer:

Small molecules enter the beads

Large molecules move between the beads

A. Ion-exchange chromatography

There are four steps of ion-exchange chromatography:

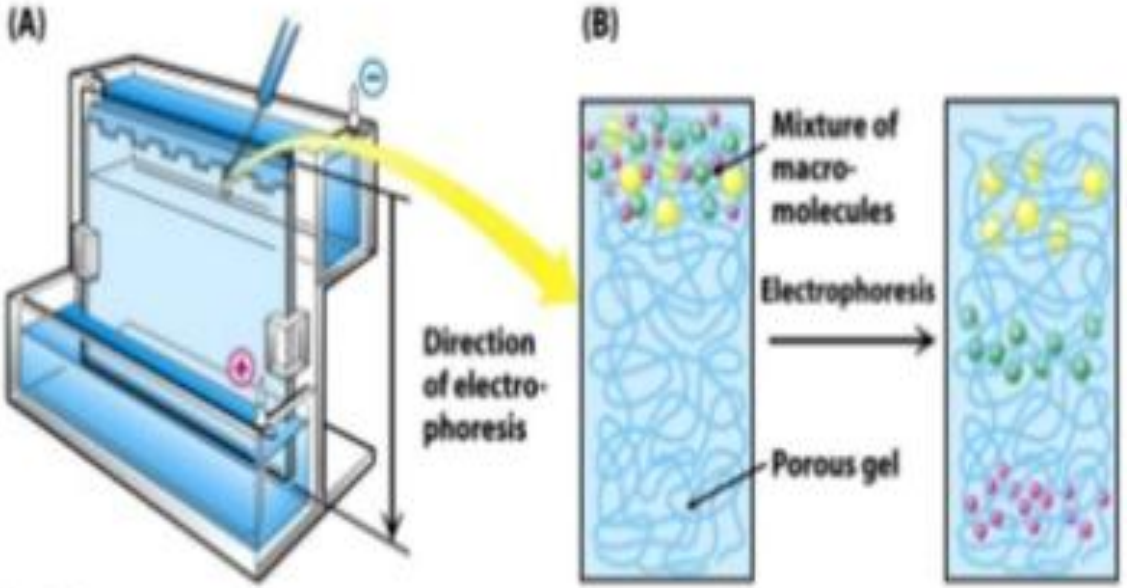
1. **Equilibration** – stabilization of the ion-exchangers with oppositely charged ions in the buffer. E.g: Na^+Cl^-
2. **Sample application and wash** (Protein bound to the ion-exchangers remain attached while other gets removed during wash)
3. **Elution** – Removal of bound protein from the ion exchangers with the help of increased concentration of elution buffer.
4. **Regeneration** – Preparing the ion exchangers for the next round of protein purification.

3.3 Ionic Charge

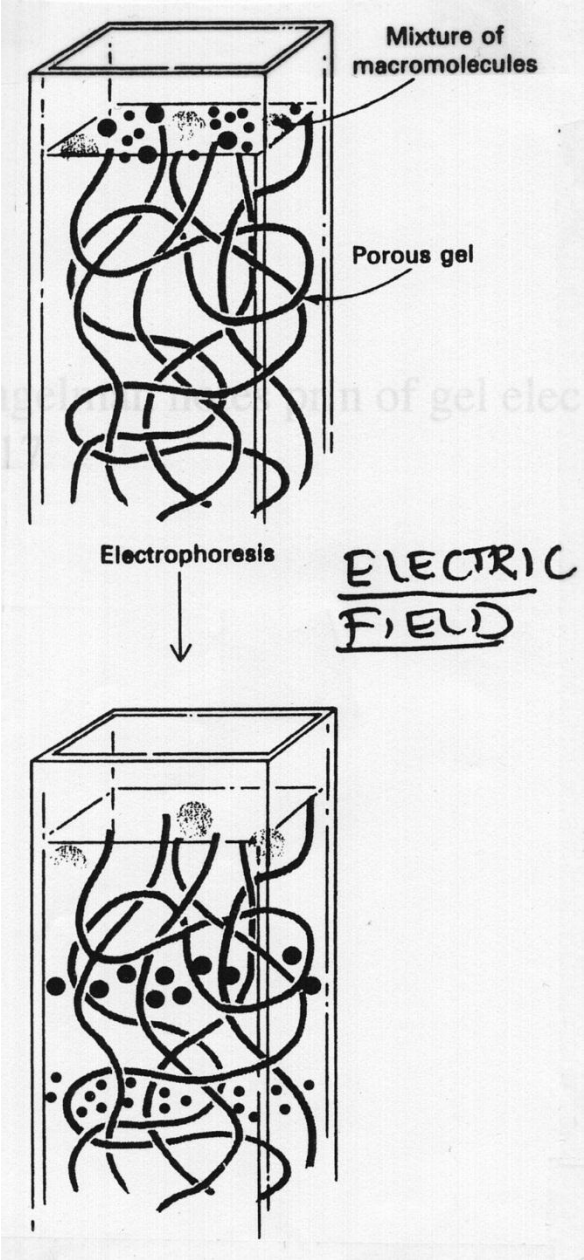
B. Gel Elecrophoresis

Apparatus:

1. Buffer tank
2. Buffer
3. Electrodes
4. Power supply
5. Support media
6. Tracking dye



Gel Electrophoresis



B. Gel Electrophoresis

- **Principle:** Any charged ion or molecule migrates when placed in an electric field, the rate of migration depend upon its net charge, size, shape and the applied electric current.
- Can be represented by following equation:

$$V = \frac{E * q}{f}$$

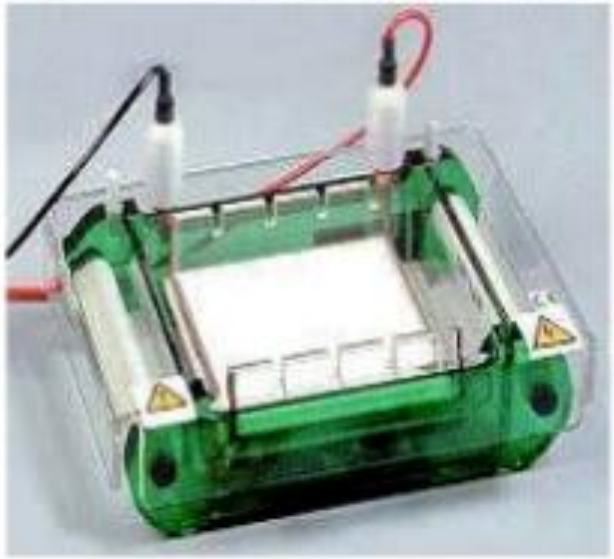
- V = velocity of migration of molecule
- E = electric field in volts per cm
- q = net electric charge on the molecule
- f = frictional coefficient

B. Gel Electrophoresis

- Electrophoresis is the movement of charged particles through an electrolyte when subjected to an electric field.
- Cations move towards cathode.
- Anions move towards anode.
- Commonly used in biological analysis, particularly in the separation of proteins, peptides and nucleic acids.
- Polyacrylamide is used as supporting media for the separation of proteins.

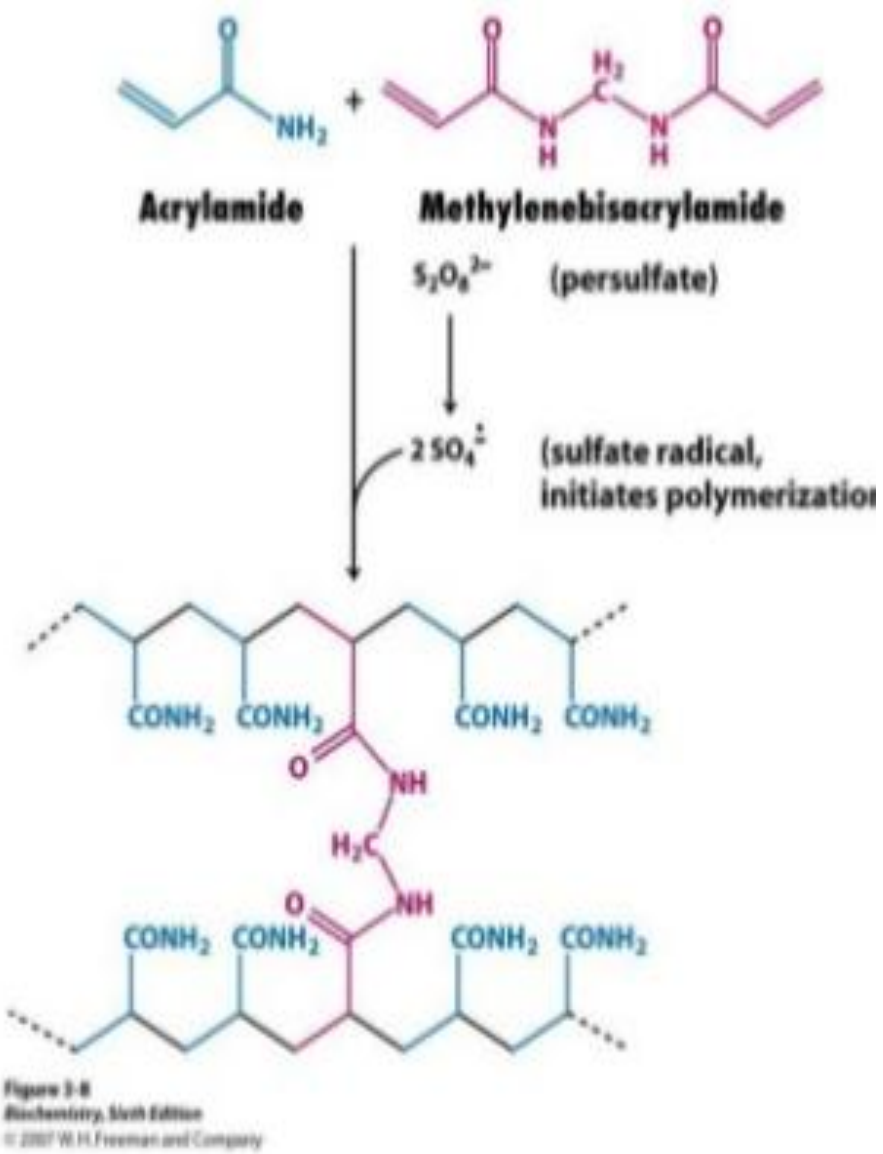
B. Gel Electrophoresis

- Based on gel casting technique, classied into:
  1. Horizontal
  2. Vertical



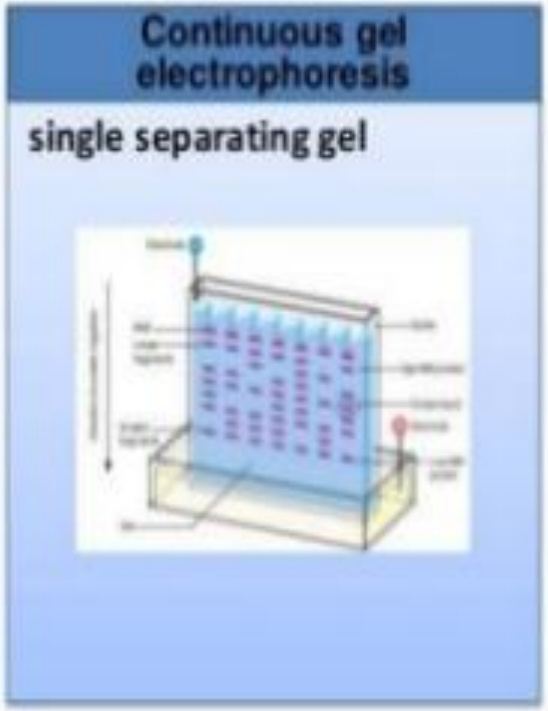
B. PAGE

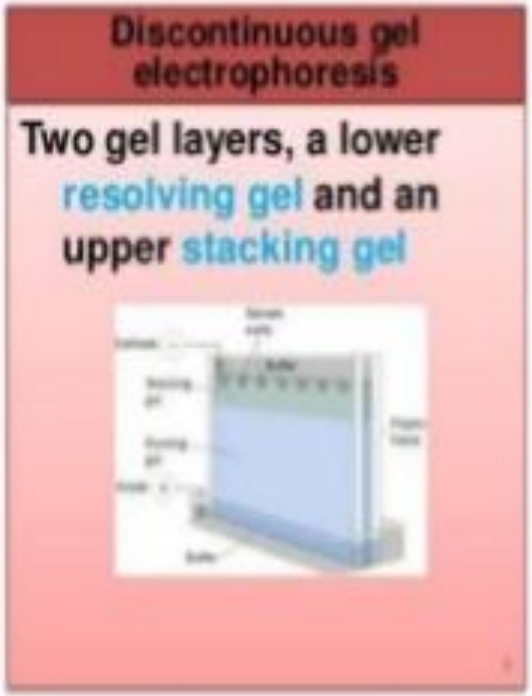
- PAGE is always vertical because in **HGE** gels are cast in a tray which are exposed to atmospheric oxygen.
- Oxygen inhibits the polymerization of acrylamide so it interferes with the creation of the gel.



B. PAGE

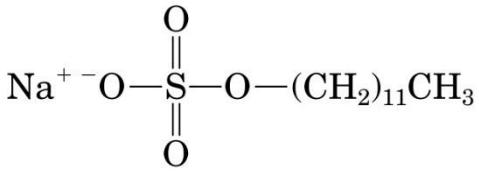
- Vertical PAGE can be of two types:
  1. Continous Gel Electrophoreis
  2. Discontinous Gel Electrophoresis





SDS Gel Electrophoresis

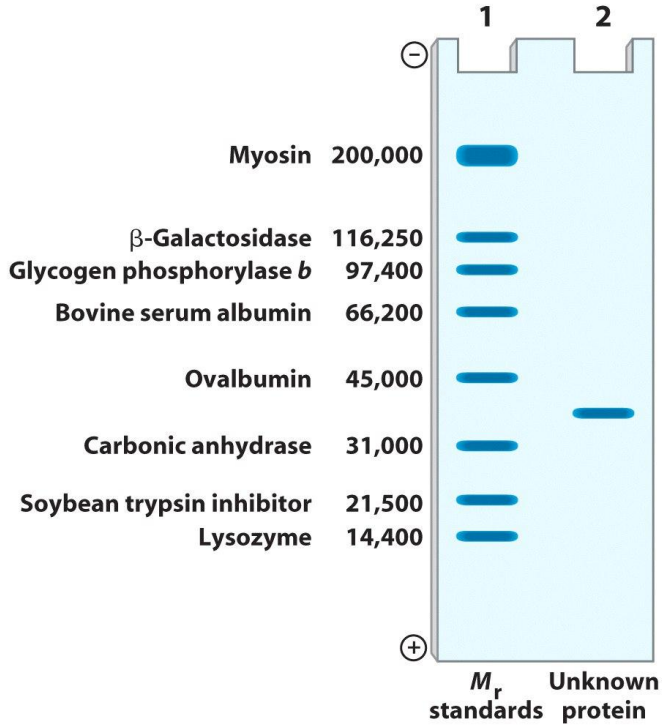
Used to estimate purity and molecular weight, separate proteins by size
Denature protein by adding SDS (then separate by size only)

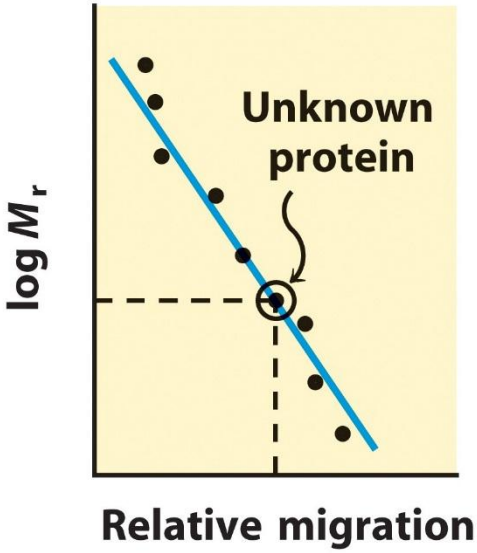


Sodium dodecyl sulfate
(SDS)

SDS forms micelles and binds to proteins

Determination of unknown protein molecular weight





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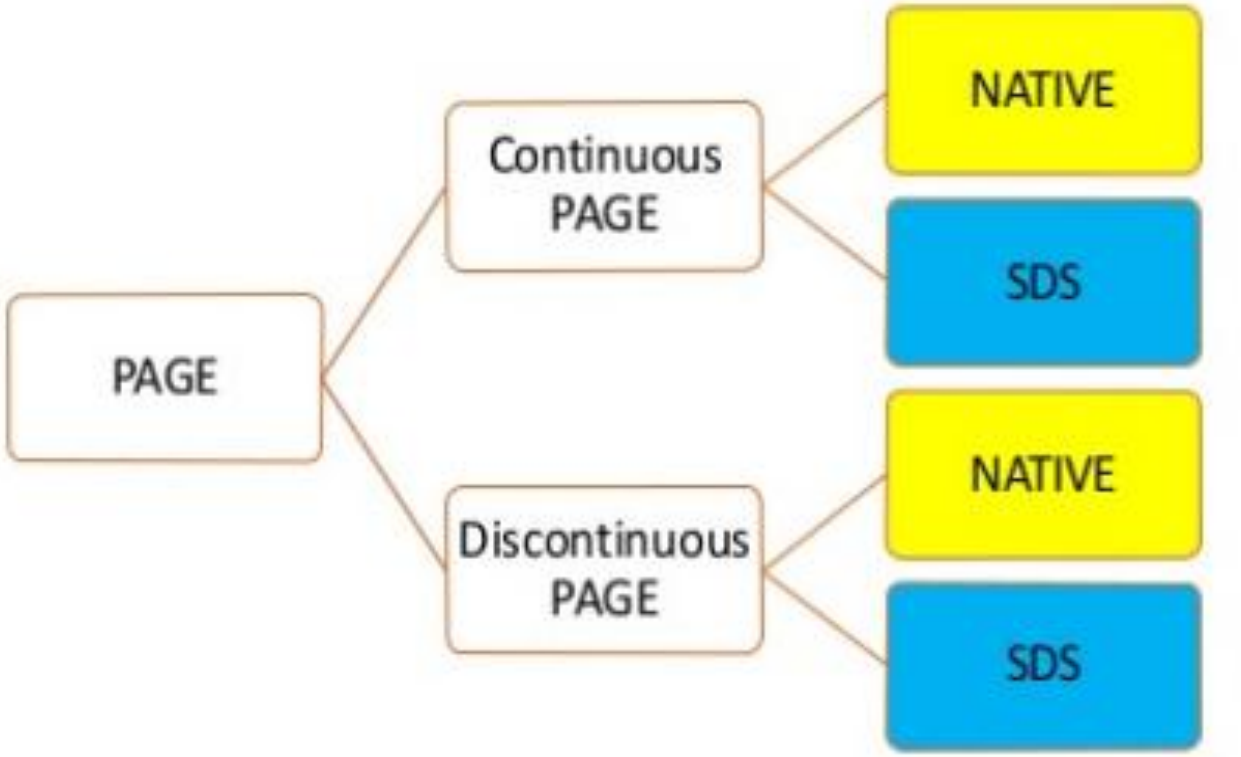
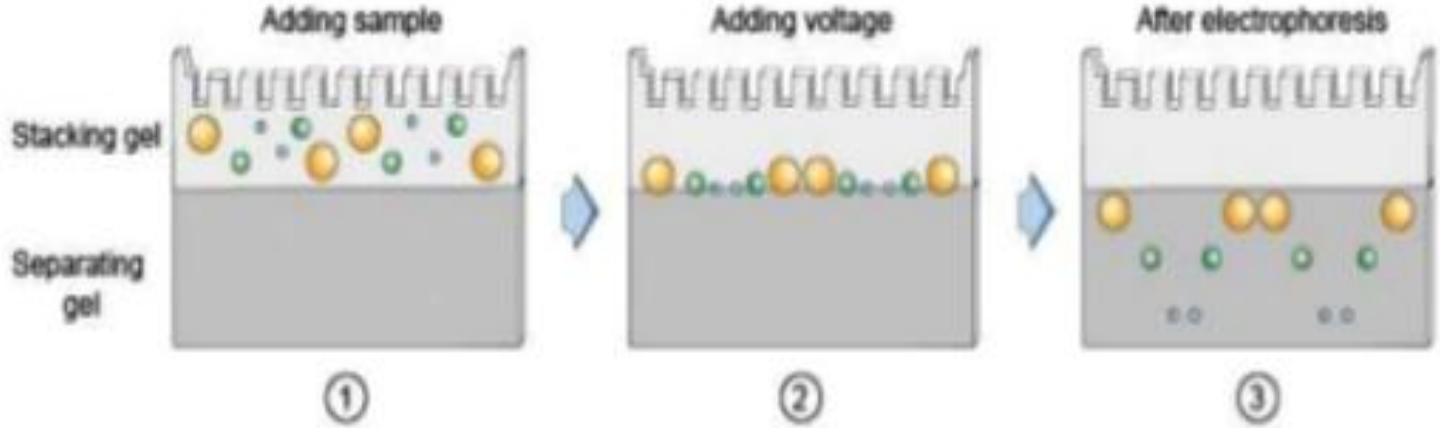
Continuous Electrophoresis	Discontinuous Electrophoresis
Consist of single gel	Consist of two or more different gels (Sample gel, stacking gel and separating gel)
pH is uniform throughout the gel.	pH differ from one gel to another.
Pore size is uniform throughout the gel.	Pore size varies from one gel to another.

B. PAGE


PAGE
Continuous PAGE
Discontinuous PAGE
NATIVE
SDS
NATIVE
SDS

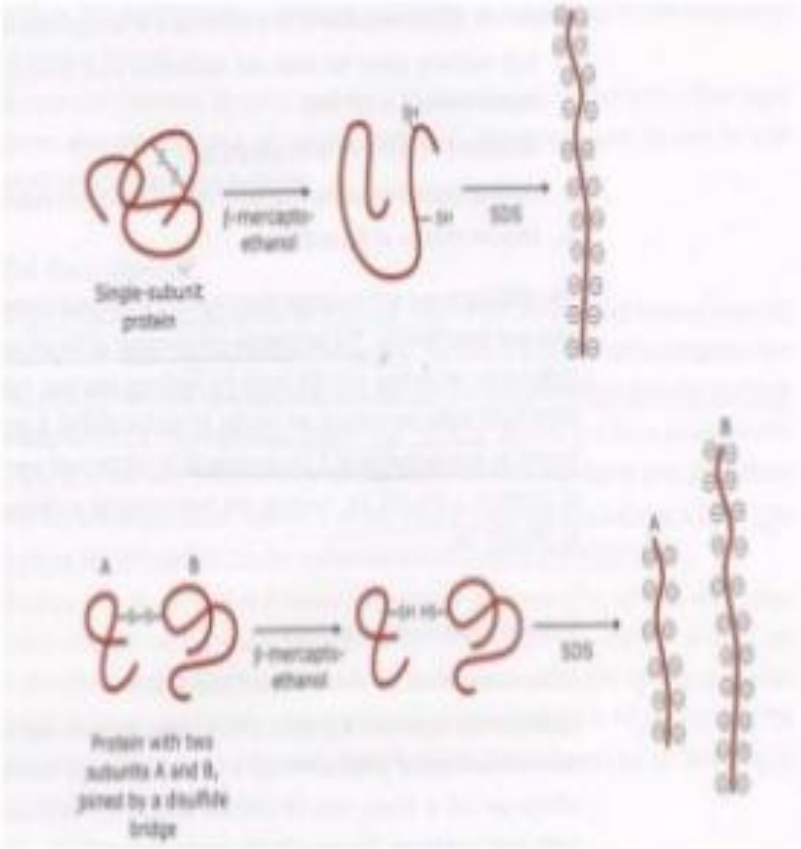
B. PAGE

- Purpose of using two layers of gel
  - **Stacking gel (5%)** needed to concentrate all the proteins in one band , so that they will start migrating in running gel together at the same time.
  - **Separating gel (12%)** or running gel allows to separate the proteins based in their molecular weight.



B. PAGE

- **Native – PAGE:** Proteins may be run in their native state (i.e., tertiary or quaternary structure)
- **SDS – PAGE:** SDS stands for *Sodium dodecyl sulphate.* SDS is an anionic buffer which contain β-mercaptoethanol and SDS. β-mercaptoethanol reduces disulphide bridges that stabilize protein tertiary structure and denatures the protein. Contributes uniform negative charge to the polypeptides.



B. PAGE

- **TRIS – Glycine buffer:** The sample is usually dissolved in glycine-chloride buffer, pH 8 to 9, before loading on the gel. Glycine exist in two forms at this pH, a zwitterion and an anion.
- Stacking gel has a pH of 6.7. At this pH, the concentation of glycine zwitterion increases and hence no electrophoretic mobility. The relative mobilities in the stacking gel are:

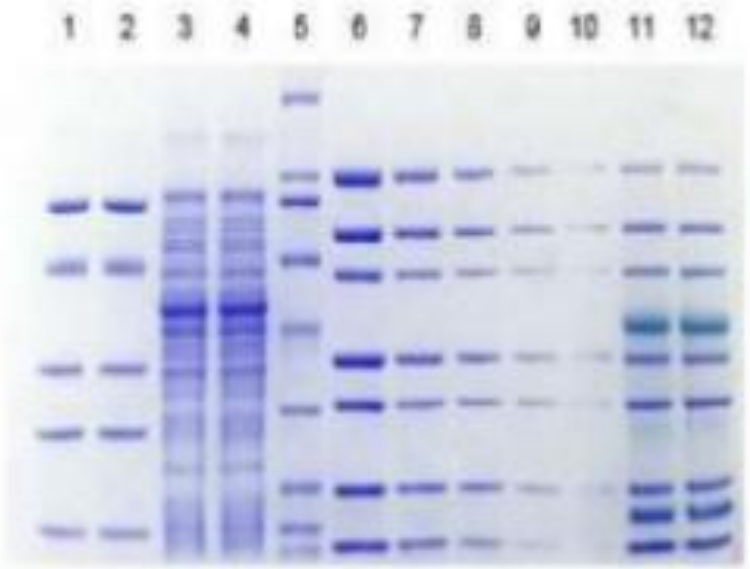
  Chloride > Protein sample > Glycine

- Separating gel has a pH of 8.9. At this pH, the glycinate conc. Increases due to glycine move faster than the protein molecules. The relative mobilities in the separating gel are:

  Chloride > Glycinate > Protein sample

B. PAGE

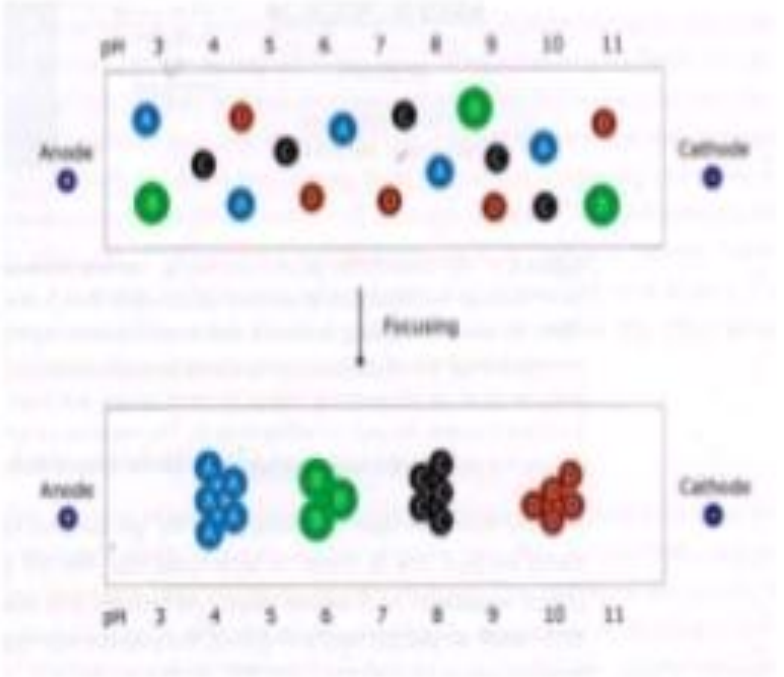
- **Tracking dye:** As proteins and nucleic acids are mostly colourless. Ionic dyes are used to detect the mobility of the protein. Very commonly used tracking dye is ***bromophenol blue*.**



3.3 Ionic Charge

C. Isoelectric Focusing

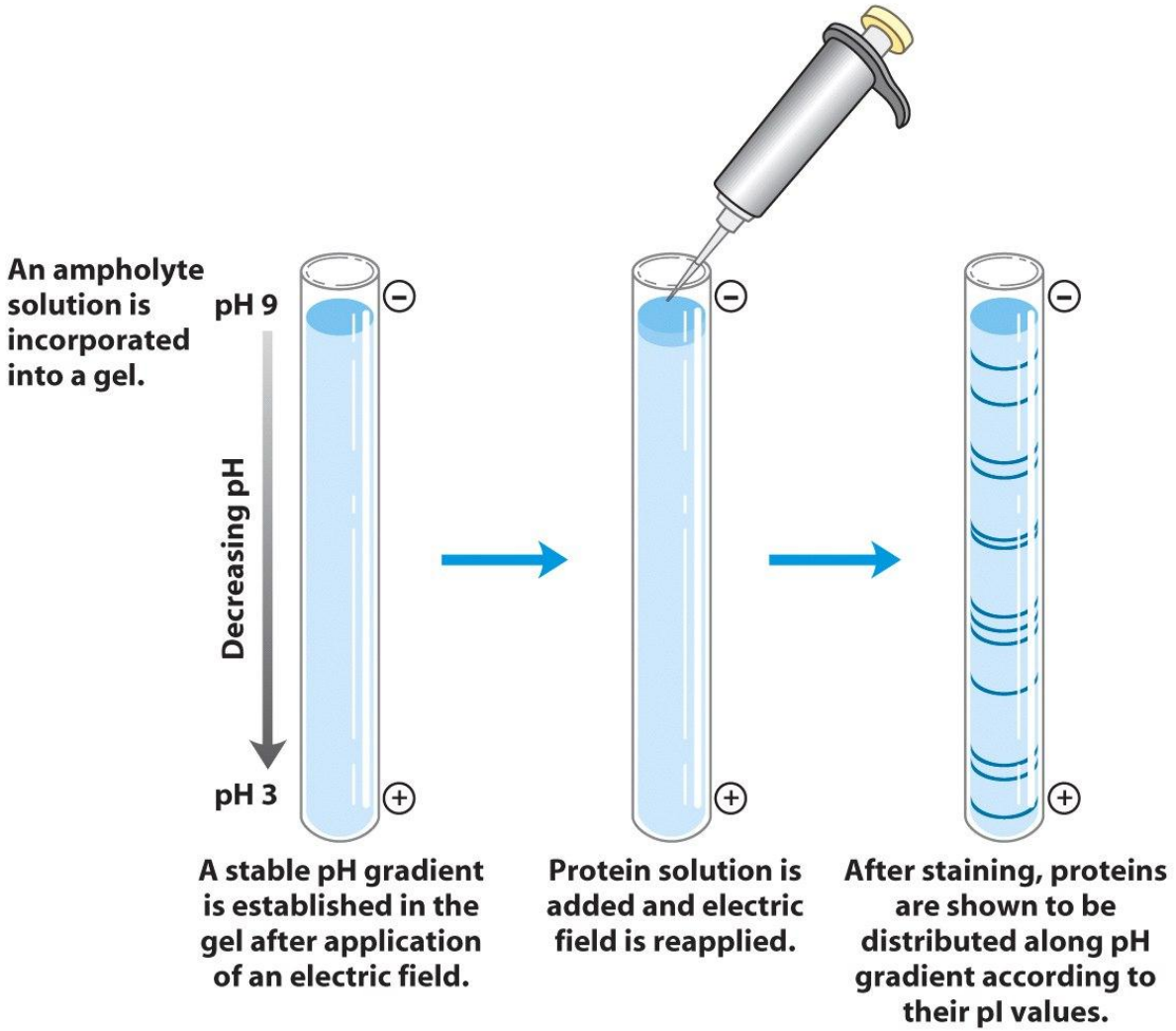
- Isoelectric focusing is an electrophoretic method in which proteins are separated on the basis of their isoelectric point (pI).
- It makes the use of the property of proteins that their net charge are determined by their local environment.
- **Principle:** At the isoelectric point, the net charge of the protein become zero. Therefore, mobility of proteins becomes zero in an electric field.



Isoelectric focusing gel electrophoresis

determine the isoelectric point (pI) of a protein

separates proteins until they reach the pH that matches their pI (net charge is zero)

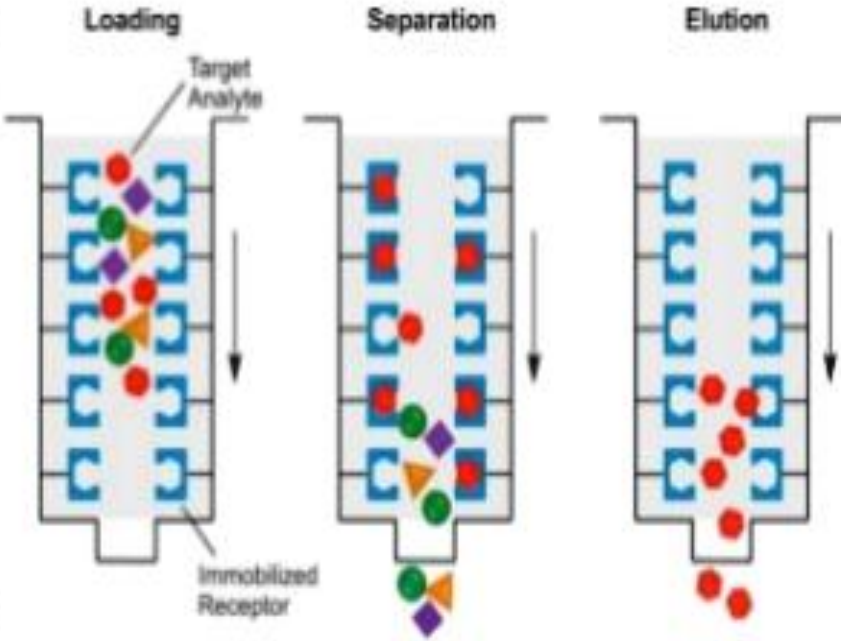


3.3 Ionic Charge

- Procedure of isoelectric focusing:
  - A cell extract is fully denatured by high concentration of urea (8M).
  - Denatured cell extract is poured on the polyacrylamide gel layered with ampholytes.
  - When placed in an electric field, the ampholytes will separate and form a continuous pH gradient based on their net charge. Charged proteins migrate through the gradient until they reach their isoelectric point.
  - At pI, its net electrical charge becomes neutral and stops migrating.

3.4 Binding Affinity

- Affinity chromatography:
  - — Principle: Many proteins can bind specific molecules very tightly but non covalently. The substance to be purified is specifically and reversibly adsorbed to a ligand (binding substance), immobilized by a covalent bond to a chromatographic bed material.
  - — Steps:
    1. Incubate crude sample with the immobilized ligand.
    2. Wash away non bound sample components from solid support .
    3. Elution of the molecules of interest in a purified form.

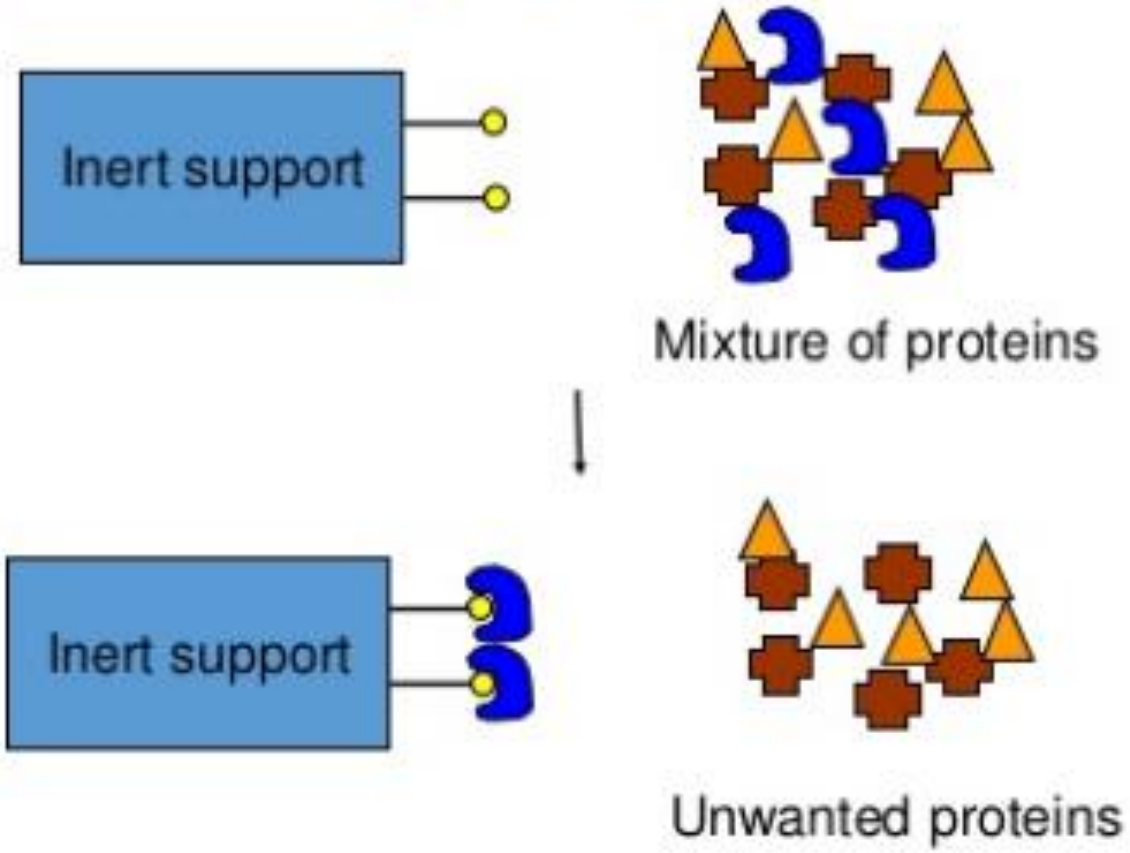


Affinity Chromatography

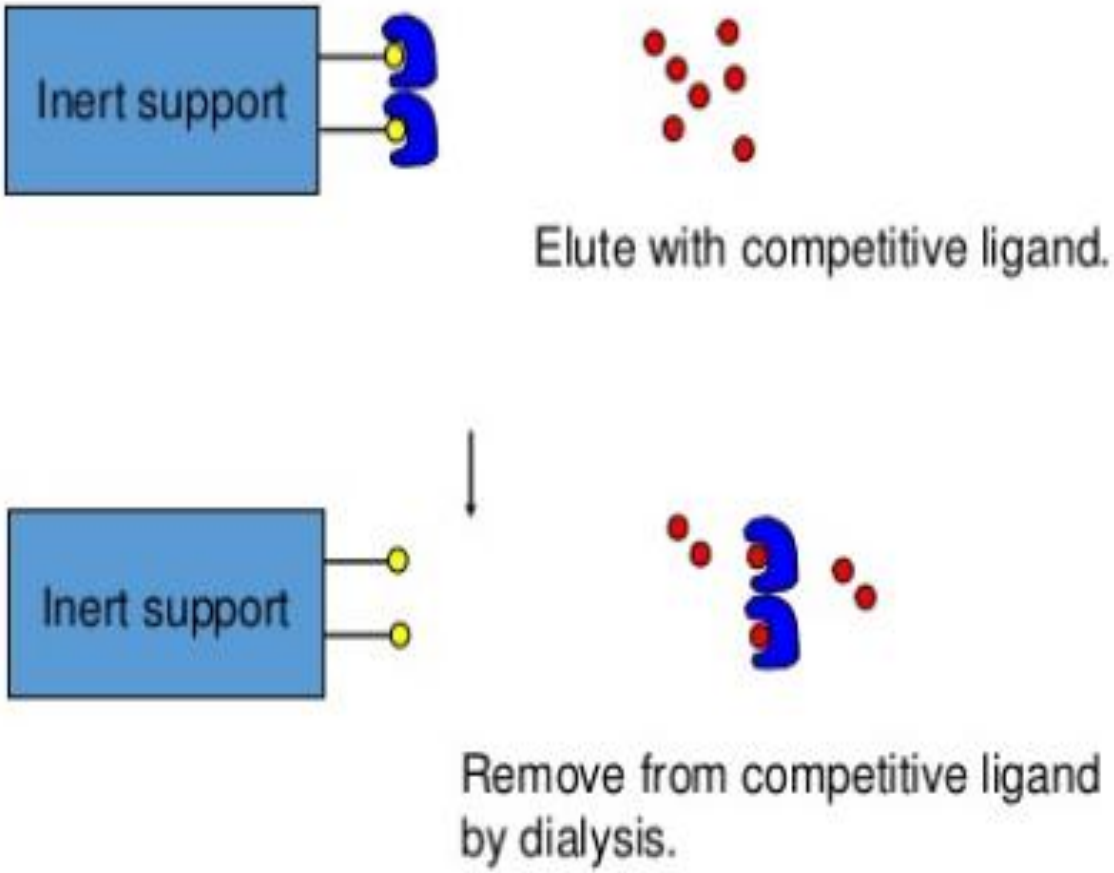
Table 1.3 Typical biological interactions used in affinity chromatography

Types of ligand	*Target molecules or molecules of interest*
Enzyme	Substrate analogue, inhibitor, cofactor
Antibody	Antigen
Lectin	Polysaccharide, glycoprotein, cell surface receptor, cell
Nucleic acid	Complementary base sequence, nucleic acid binding protein
Hormone	Receptor
Avidin	Biotin
Calmodulin	Calmodulin-binding molecule
Poly(A)	RNA containing poly(U) sequences
Glutathione	Glutathione-S-transferase or GST fusion proteins
Proteins A and G	Immunoglobulins
Metal ions	Poly (His) fusion proteins, native proteins with histidine

Affinity chromatography



Affinity chromatography

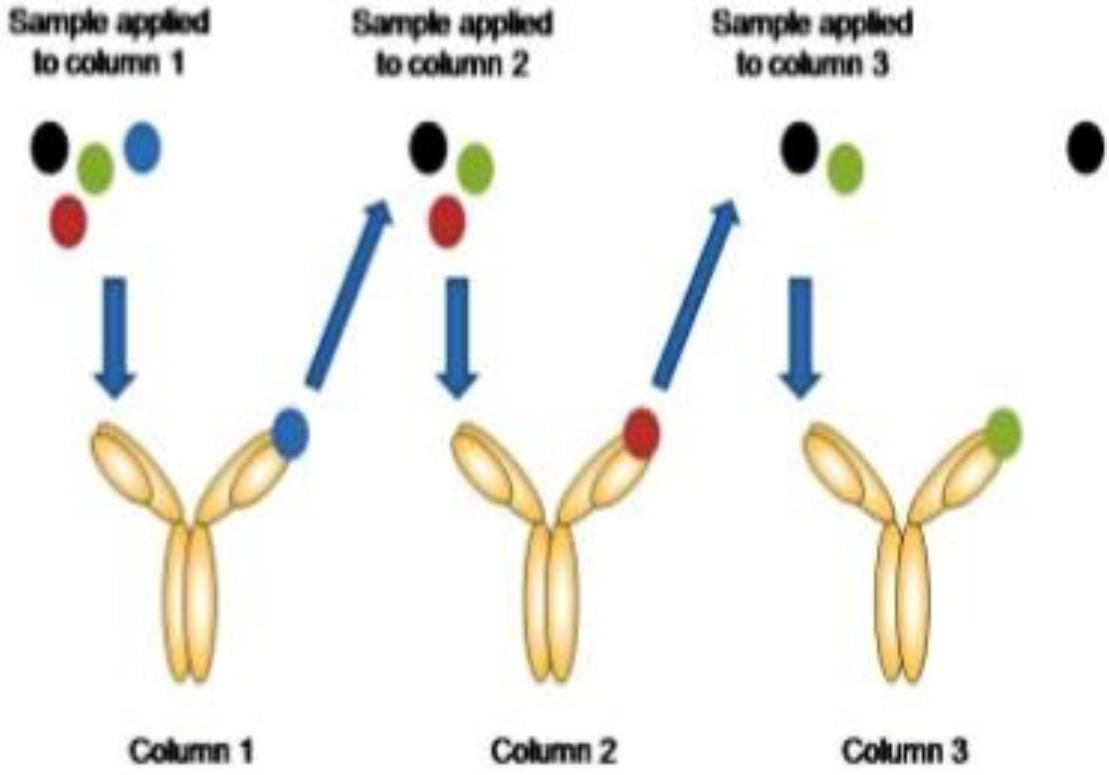


Affinity chromatography

- To remove the protein of interest from the column, you can elute with a solution of a compound with higher affinity than the ligand (competitive)
- You can change the pH, ionic strength and/or temperature so that the protein-ligand complex is no longer stable.

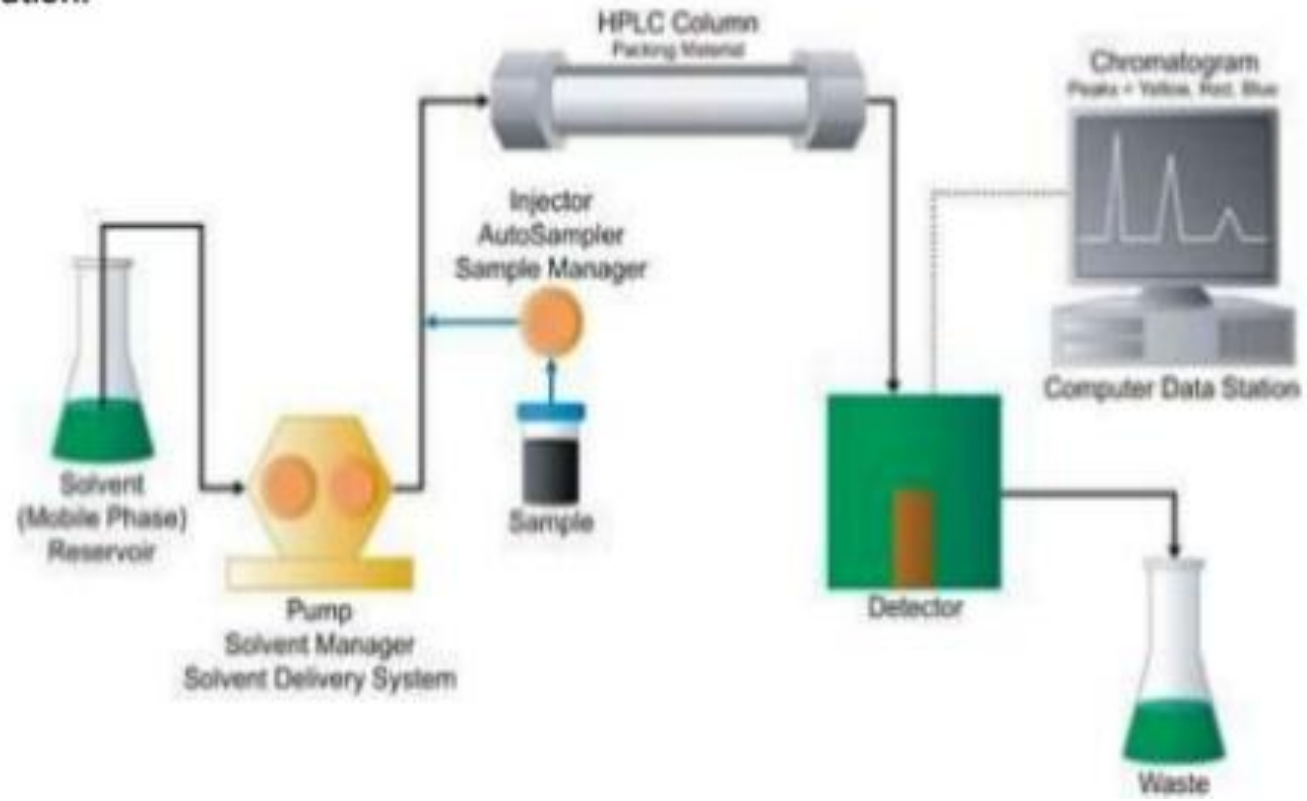
3.5 Immunoaffinity chromatography

- Monoclonal antibodies can be attached to the column material.
- The column only binds the protein against which the antibody has been raised.
- 10,000-fold purification in a single step!
- Disadvantages:
  - Difficult to produce monoclonal antibodies
  - Cost-effective



4. HPLC

- Type of liquid chromatography that use high pressure & small particle size to push a mobile phase solution through a column of stationary phase allowing separation of complex mixtures with high resolution.



4. HPLC

- Principle: Separation of compounds occur on the basis of polarity and solubility. The interaction between stationary and mobile phase allow the separation of compounds in the analyte from each other.
  - Like dissolve like i.e., polar molecules binds with polar stationary phase while non-polar molecules binds with the non-polar stationary phase.
- Stationary Phase: small diameter particles
- Mobile Phase: Solvent
- On the basis of polarity of stationary phase & mobile phase, HPLC can be of two types:

	Normal Phase HPLC	Reverse Phase HPLC
Stationary Phase	Polar	Non-polar
Mobile Phase	Non-polar	Polar

5. Mass Spectrometry

- Principle: Mass spectrometry determines the molecular mass by measuring the mass-to-charge ratio of ions in the gas phase.

The operator of the mass spectrometer involves the following steps:

1. Production of the sample in an ionized form in the gas phase;
2. Acceleration of the ions in an electric field, each ion emerging with a velocity proportional to its mass-to-charge ratio (m/z):
3. Passage of ions into a field-free region;
4. Detection of the times of arrival of the ions , the time-of-flight indicating the mass-to-charge ratio of the ions.

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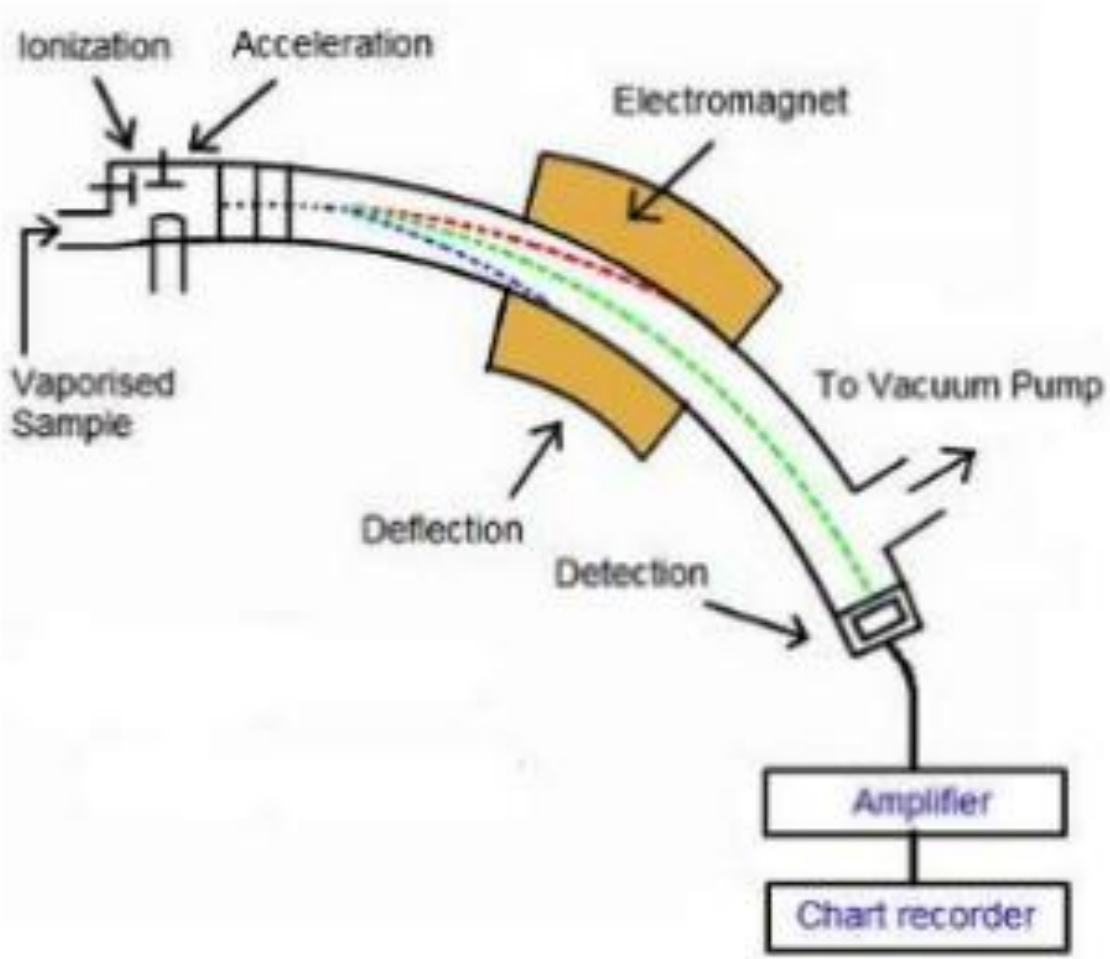

Ionization
Acceleration
Electromagnet
Vaporised
Sample
To Vacuum Pump
Deflection
Detection
Amplifier
Chart recorder

6. Control of purity

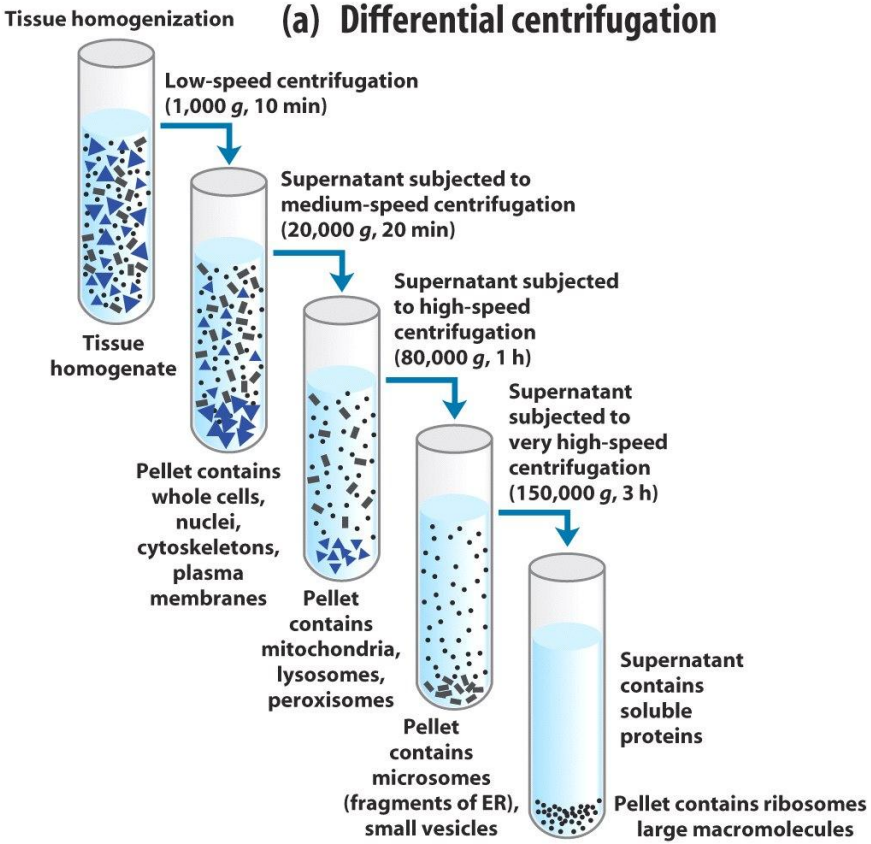
- Previously, complete removal of protein impurities was confirmed by crystallization of the proteins.
- Today, electrophoretic methods of high separation efficiency or HPLC are used.
  - ✓ The behaviour of the protein during chromatographic separation is an additional proof of purity.
  - ✓A purified enzyme is characterized by an elution peak in which the positions of protein absorbance and enzyme activity coincide and the specific activity remains unchanged during repeated elutions.

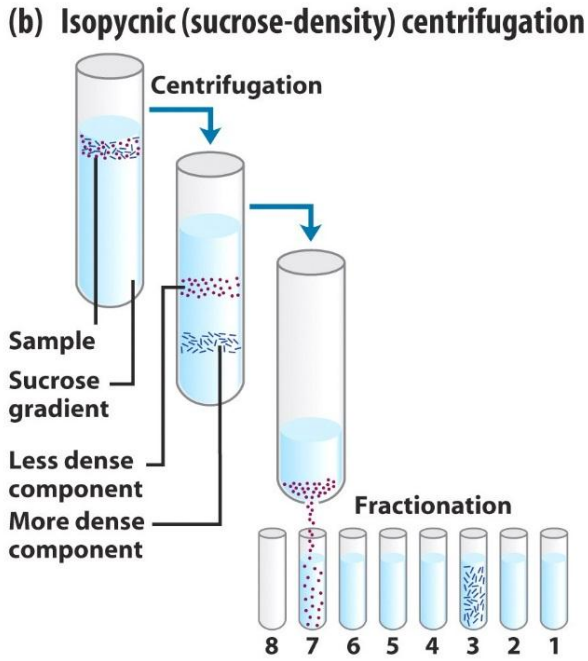
7. Applications of Purified Proteins

- Hormones – Insulin, Erythropoietin, Clotting factors
- Diagnostics – LFT, KFT, TFT
- Cellular markers - Cancer
- In recombinant DNA technology to locate gene of interest
- Vaccines
- Supplements

Centrifugation

Separate proteins by size or density
Differential centrifugation - separates large from small particles
Isopycnic (sucrose-density) centrifugation - separates particles of different densities





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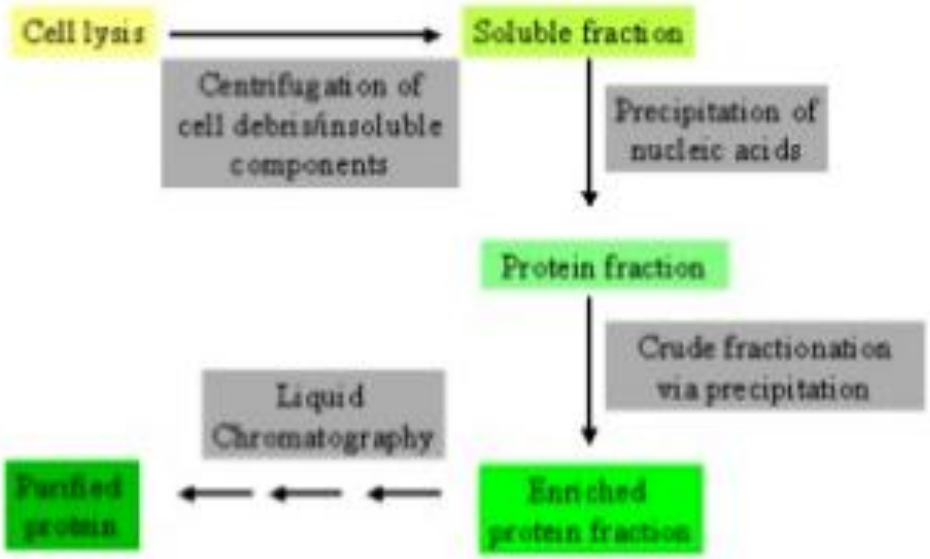
Summary


Cell lysis
Soluble fraction
Centrifugation of cell debris/insoluble components
Precipitation of nucleic acids
Protein fraction
Crude fractionation via precipitation
Liquid Chromatography
Purified protein
Enriched protein fraction

References:

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Lippincott Biochemistry

Lehninger Principles of Biochemistry

Stryer Biochemistry