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Course Title : CELL BIOLOGY AND BIOINSTRUMENTATION
Course Code : 22PGBOT104

Unit – V

SEPARATION TECHNIQUES

Topic: Chromatography

(Gel Filtration, Ion Exchange & Affinity)

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Chromatography

(Gel filtration, Ion Exchange and Affinity)

Chromatography

- The process or technique of separating molecules or components in a mixture solution (gas or liquid) according to the differential absorption and elution
- Invented in 1906 by the Russian botanist Michael Tswett
- Chromatography is the physical separation of a mixture into its individual components.
- Used in qualitative and quantitative analysis of biological and chemical substances

Types of Chromatography

- ✓Adsorption Chromatography
- ✓Partition Chromatography
- ✓Ion Exchange Chromatography
- ✓Molecular Exclusion Chromatography
- ✓Affinity Chromatography

Gel Filtration

Gel permeation chromatography/

Size exclusion chromatography/

Molecular Exclusion Chromatography

- Separation is based on size, shape and molecular weight.

- The stationary phase consists of beads containing pores that span a relatively narrow size range.
- Samples pass through the pores.
- Mixtures applied to column, smaller molecules enter through the pores and come out. By selecting the gel beads of different porosity the molecules can be separated.

1. *Dextran:* is a homopolysaccharide of glucose residues.

- it's prepared with various degrees of cross-linking to control pore size.
- It's bought as dry beads, the beads swell when water is added.
- The trade name is sephadex.
- It's mainly used for separation of small peptides and globular proteins with small to average molecular mass.

*Polyacrylamide:*these gels are prepared by cross linking acrylamide with N,N-methylene bis acrylamide.

The pore size is determined by the degree of cross-linking.

The separation properties of polyacrylamide gels are mainly the same as those of dextrans.

They are sold as bio-gel P. They are available in wide range of pore sizes.

Agarose: linear polymers of D-galactose and 3,6 anhydro-1-galactose.

It forms a gel that's held together with H bonds. It's dissolved in boiling water and forms a gel when it's cold.

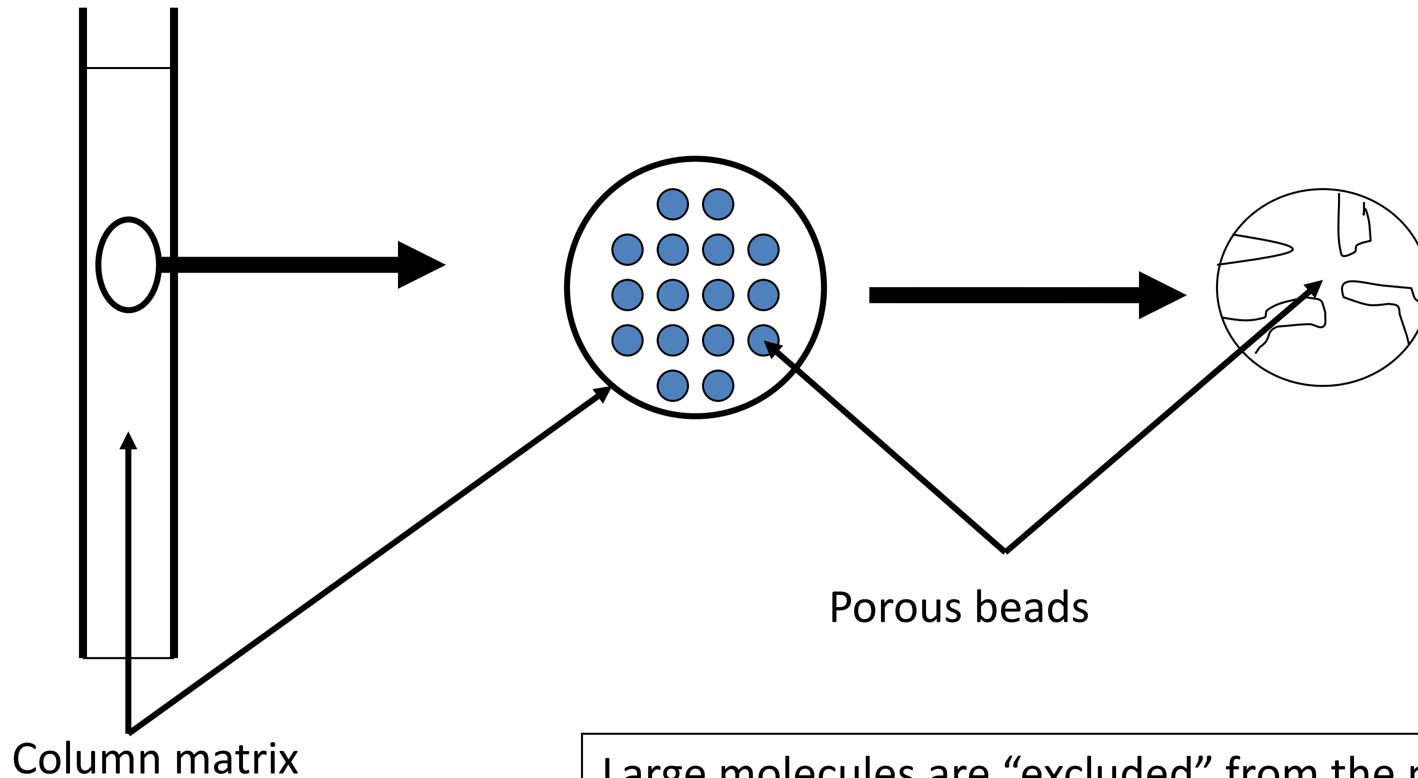
The concentration of the material in the gel determines the pore size.

The pores of agarose gel are much larger than those of sephadex or bio-gel p.

It's useful for analysis or separation of large globular proteins or long linear molecules such as DNA

- The gel filtration material that will be used in the experiment below is called Sephadex G-75 and it will separate molecules with molecular weights from 3,000 to 70,000. Molecules with molecular weights larger than 70,000 will be excluded from the beads.

Theory



Large molecules are "excluded" from the pores and travel through the column fastest

Small molecules are "included" – can diffuse into the pores and elute later

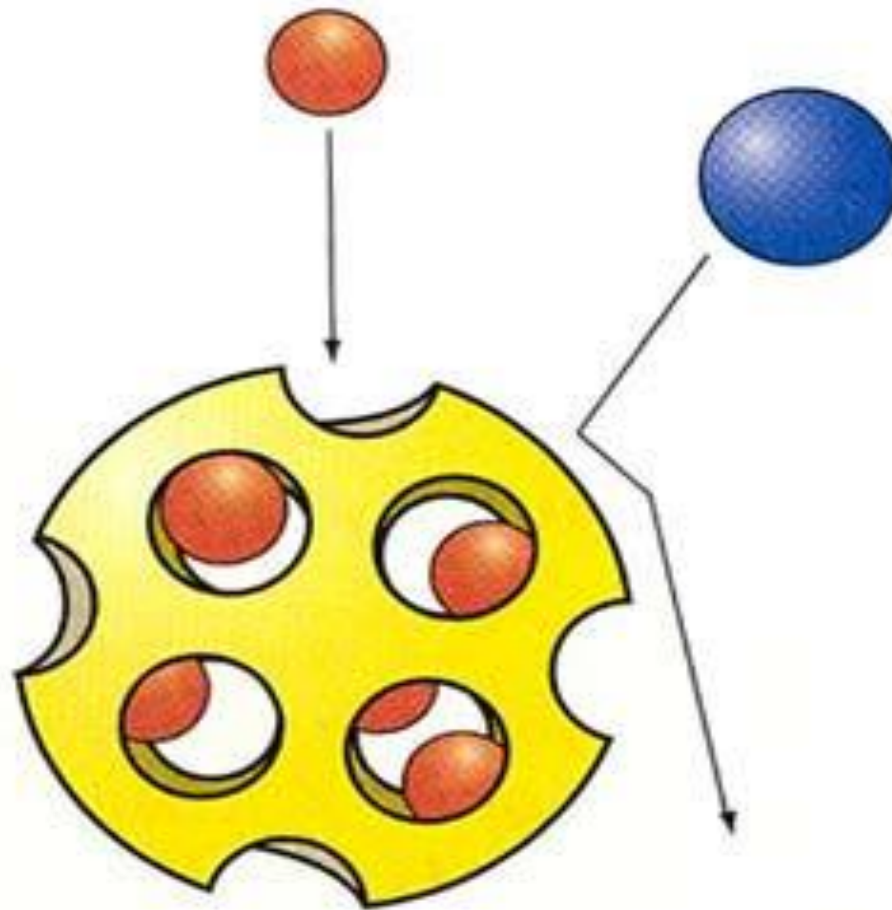
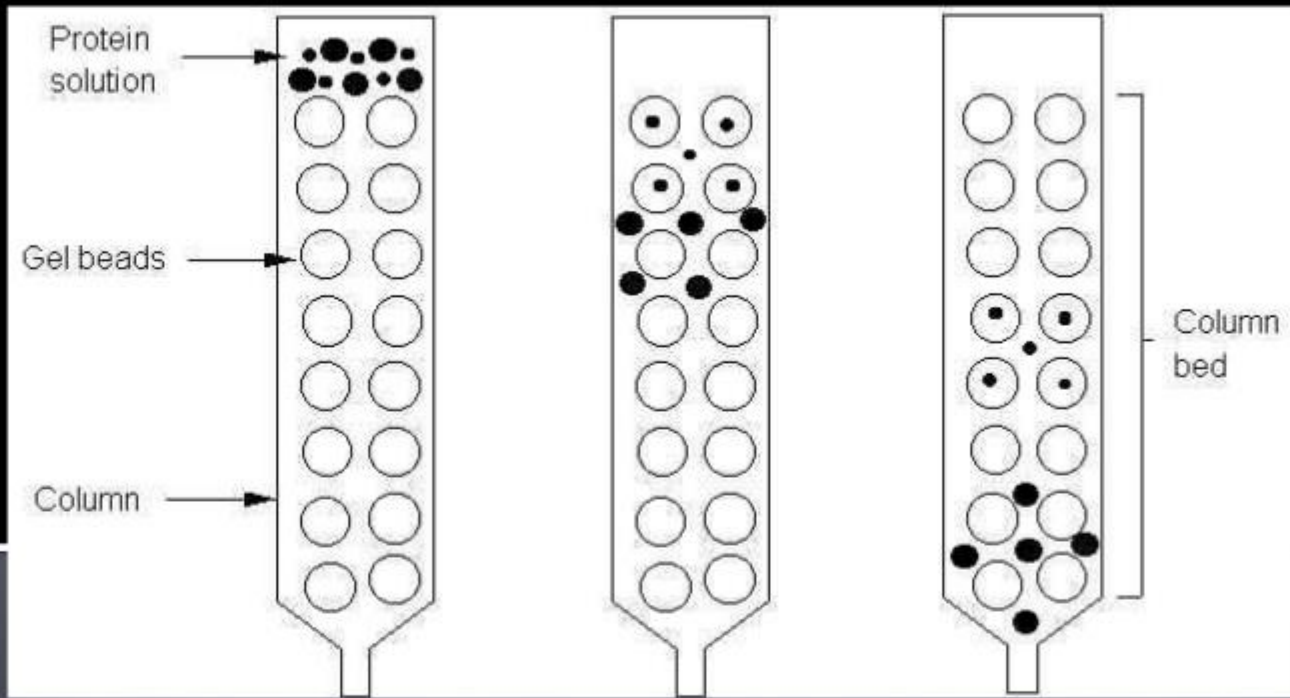


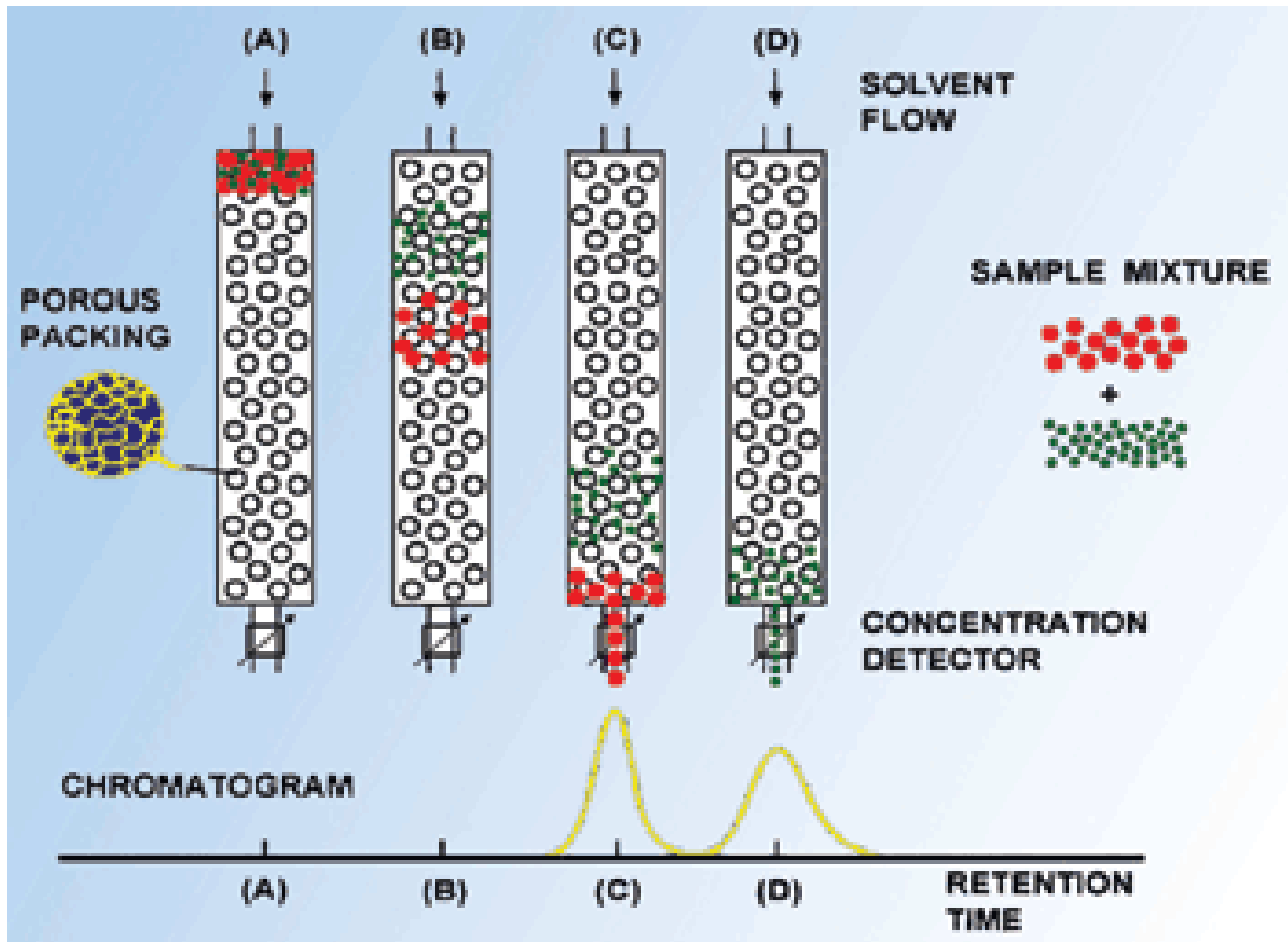
Figure **Gel filtration chromatography. (a)** Principle of the method. A resin bead is schematically represented as a "whiffle ball" (yellow). Large molecules (blue) cannot fit into the beads, so they are confined to the relatively small buffer volume outside the beads. Thus, they emerge quickly from the column. Small molecules (red), by contrast, can fit into the beads and so have a large buffer volume

Gel filtration chromatography-An Overview



Larger particles come out first, while smaller particles come in later fractions

Theory

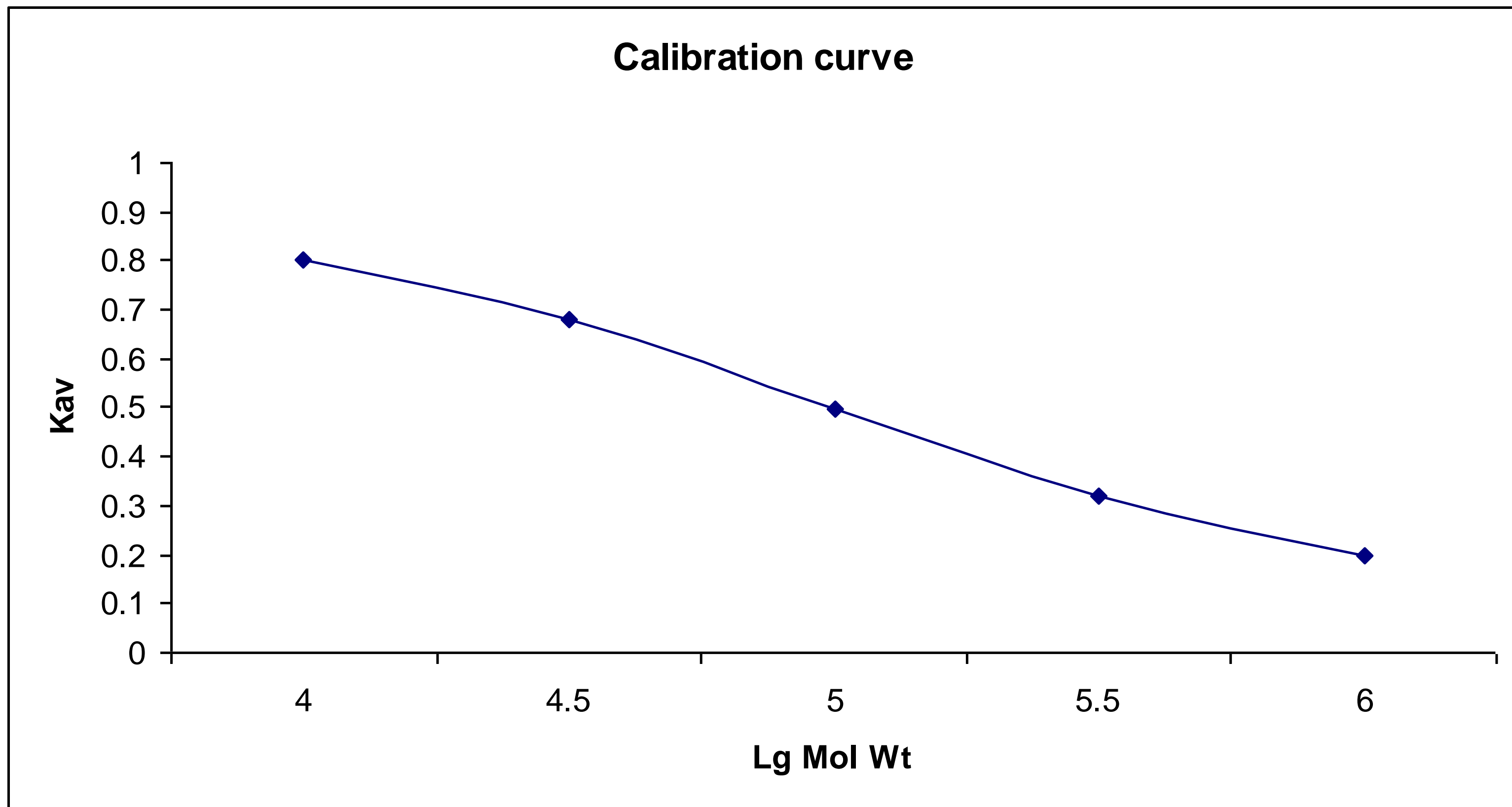


Estimation of molecular weights

- To do this, several proteins with known molecular weights are run on the column and their elution volumes determined. If the elution volumes are then plotted against the log molecular weight of the corresponding proteins, a straight line is obtained for the separation range of the gel being used. If the elution volume of a protein of unknown molecular weight is then found, it can be compared to the calibration curve and the molecular weight determined.

Determination of Molecular Weight

- Calibrate column with known standards
- Plot Kav against lg Mol Wt



Advantages

- It's the best method for separation of molecules differing in molecular weight because:

1. It doesn't depend on temperature, pH, ionic strength and buffer composition. So separation can be carried out under any conditions.
2. There is very little adsorption
3. There is less zonal spreading than in other techniques.
4. The elution volume is related to the molecular weight

Applications

- Purification of enzymes and other proteins.
- Estimation of molecular weight mainly for globular proteins:

ION EXCHANGE CHROMATOGRAPHY

Principle……

Reversible exchange of ions between ions present in the mobile phase and ion exchange resin.

Ion Exchange Chromatography

Ion exchange chromatography -- is a separation based on charge

Used for almost any kind of charged molecules --- large proteins, small nucleotides and amino acids

Ion-exchange chromatography preserves analyte molecules on the column based on ionic interactions

Mobile phase – buffer, pH and salt concentration---opposite charged solute ions attracted to the stationary phage by electrostatic force

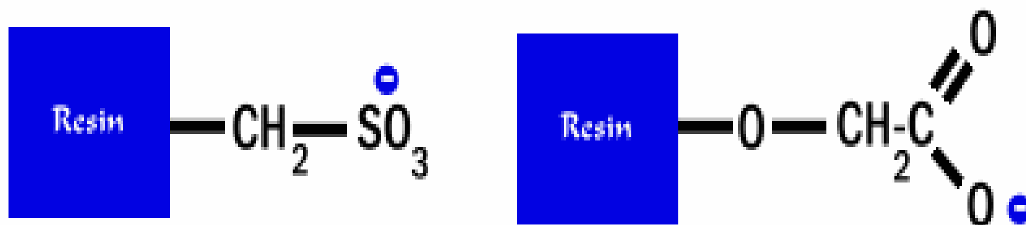
Stationary phase– resin is used to covalently attach anions or cations onto it

Types of IEC….

- anion exchangers
- cation exchangers

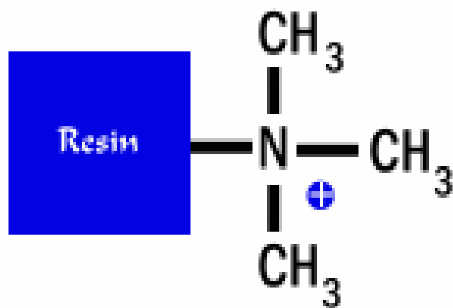
Cation exchange chromatography

---positively charged molecules are attracted to a negatively charged solid support. Commonly used cation exchange resins are S-resin, sulfate derivatives; and CM resins, carboxylate derived ions

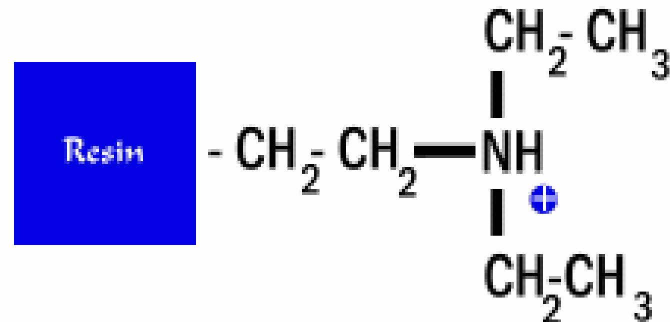


Anion exchange chromatography

---negatively charged molecules is attracted to a positively charged solid support. Commonly used anion exchange resins are Q-resin, a Quaternary amine; and DEAE resin, DiEthylAminoEthane

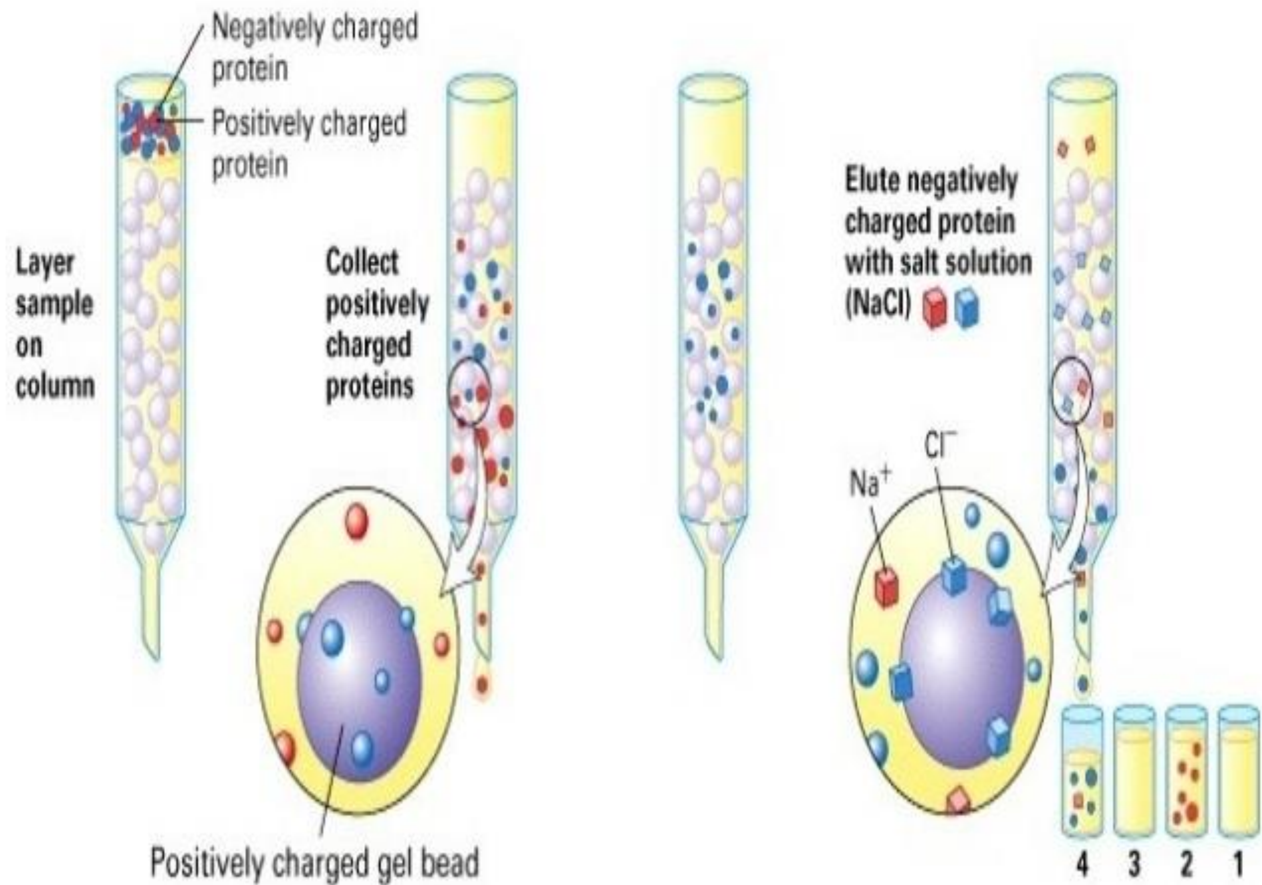


Q-anion exchanger



DEAE-anion exchanger

- ✓ Column washed with buffer to equilibrate
- ✓ Sample loading
- ✓ Flow through collection
- ✓ Elute protein


Negatively charged protein
Positively charged protein
Layer sample on column
Collect positively charged proteins
Positively charged gel bead
Elute negatively charged protein with salt solution (NaCl)
Na+
Cl−
4
3
2
1

Buffers Used In IEC

✓Buffer system 1 : Buffer A = 20 mM Tris, pH=8. Buffer B = 20 mM Tris, 1 M NaCl, pH=8.0

✓Buffer system 2: (Common CEC buffer system): Buffer A = 30 mM sodium acetate, pH=4.5. Buffer B = 30 mM sodium acetate, 1 M NaCl, pH=4

✓Buffer system 3: (AEC for proteins which are very insoluble or have a very high pI)

Buffer A = 30 mM Ethanolamine, 8M urea, pH=10.0

Buffer B = 30 mM Ethanolamine, 8M urea, 1 M NaCl, pH=10.0

Recommended Buffers for Polypeptide Ion-Exchange Chromatography

A wide range of buffers are available for use with ion-exchange chromatography. Recommended buffers for various ranges of pH are listed below.

Anion-Exchange Chromatography Buffers

Buffers for anion exchange are generally basic amines.

Buffer	Concentration	Anion	pKa	Buffering Region
L-histidine	20 mM	Cl-	6.15	5.5 - 6.8
bis-Tris	20 mM	Cl-	6.50	5.8 - 7.0
bis-Tris propane	20 mM	Cl-	6.80	6.4 - 7.3
Triethanolamine	20 mM	Cl-	7.77	7.3 - 8.2
Tris	20 mM	Cl-	8.16	7.5 - 8.8
diethanolamine	20 mM	Cl-	8.88	8.4 - 9.4

Cation Exchange Chromatography Buffers

Buffers for cation-exchange chromatography are acids.

Buffer	Concentration	Cation	pKa	Buffering Region
formate	20 mM	Na+	3.75	3.3 - 4.3
acetate	20 mM	Na+	4.76	4.2 - 5.2
MES	20 mM	Na+	6.15	5.5 - 6.7
phosphate	20 mM	Na+	2.1/7.2	2.0 - 7.6
HEPES	20 mM	Na+	7.55	7.6 - 8.2

Advantages

- ✓It is a non-denaturing technique. It can be used at all stages and scales of purification
- ✓An IEX separation can be controlled by changing pH, salt concentration and/or the ion exchange media
- ✓It can serve as a concentrating step. A large volume of dilute sample can be applied to a media, and the adsorbed protein subsequently eluted in a smaller volume
- ✓It offers high selectivity; it can resolve molecules with small differences in charge.

Disadvantages

- ✓costly equipment and more expensive chemicals
- ✓turbidity should be below 10ppm.

Conclusion

Ion exchange chromatography is more efficient than other chromatography. It could be widely used for commercial purposes.

Affinity chromatography

- based on a highly specific biologic interaction such as that between antigen and antibody, enzyme and substrate, or receptor and ligand. Any of these substances, covalently linked to an insoluble support or immobilized in a gel, may

  serve as the sorbent allowing the interacting substance to be isolated from relatively impure samples; often a 1000-fold purification can be achieved in one step.

- This form of chromatography is growing in use. Lectins are glycoproteins have an affinity for carbohydrate residues. For example, they can separate polysaccharides, glycopeptides, and oligosaccharides and cells that contain particular carbohydrate structures.

THANKS