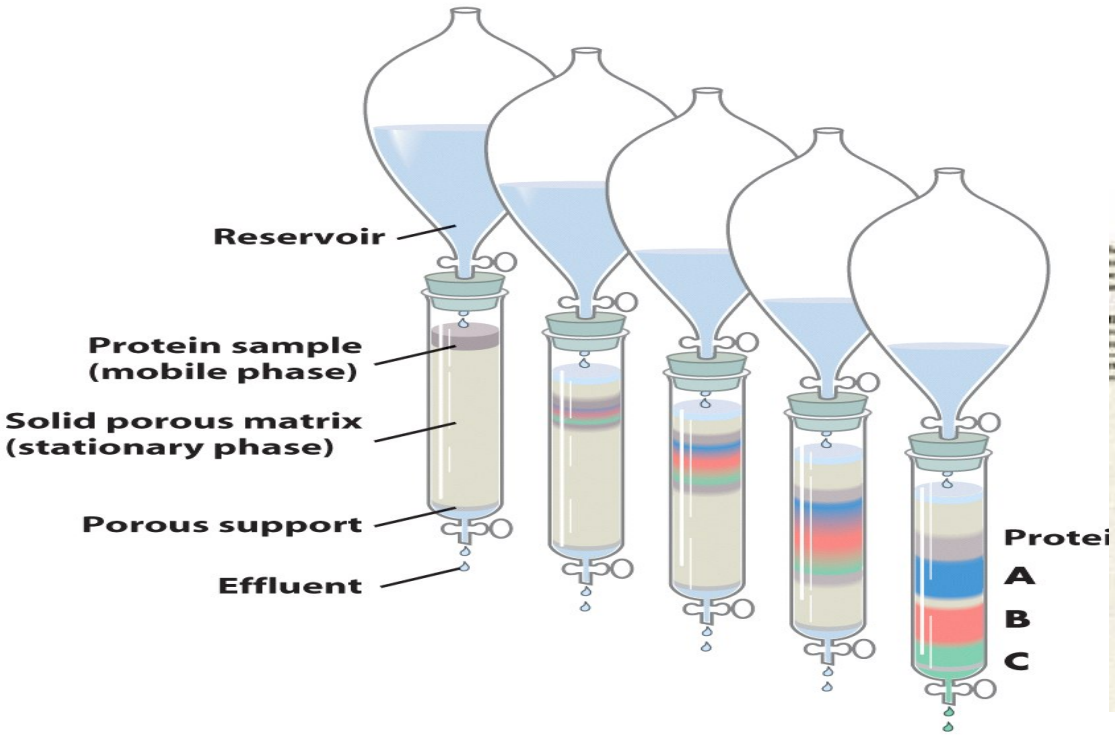
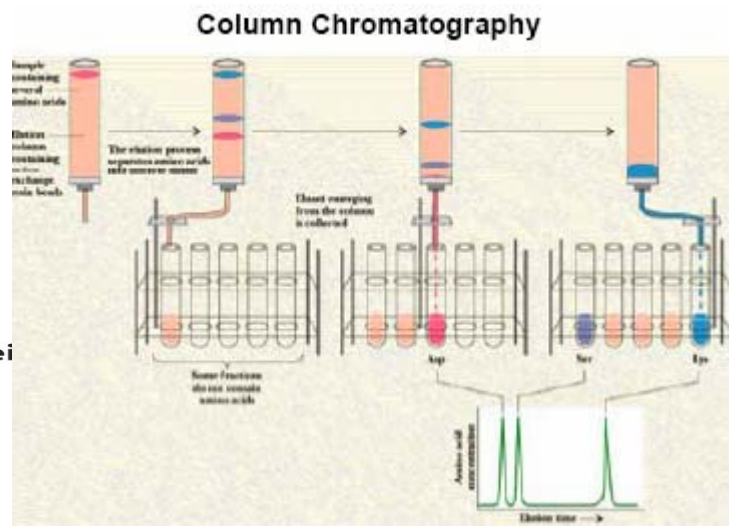
Column chromatography: General concepts





Chromatography for protein purification

Purification strategy

- Amount of protein required

  -Analytical: < 1 mg

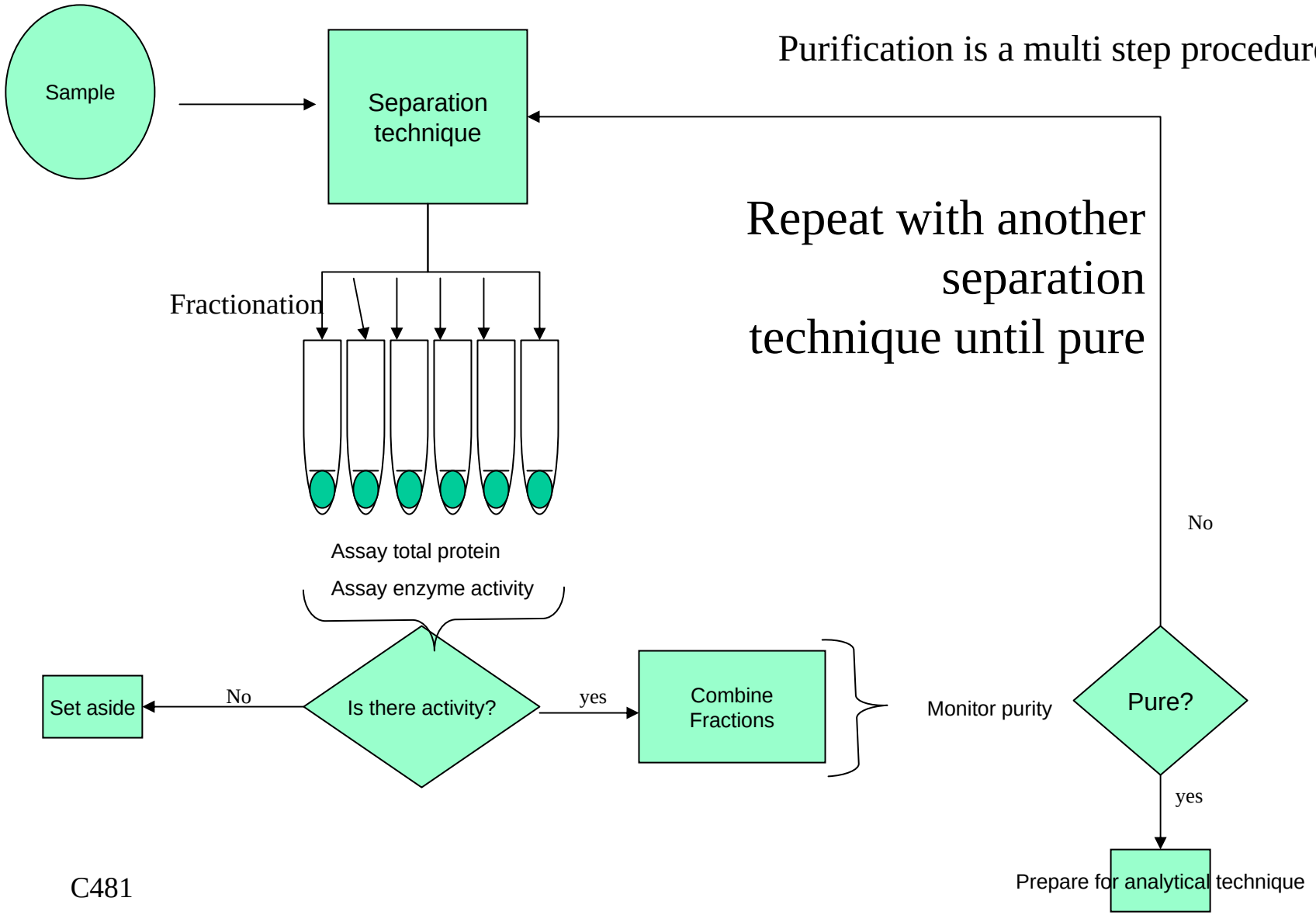
  -Preparative: < 1 g

- Source material

  -Natural sources: large quantity of resources (tissue)

  -Recombinant: *E. coli*, Insect cells, Yeast, cell lines, etc

Purification is a multi-step procedure.



Yield of purification procedure

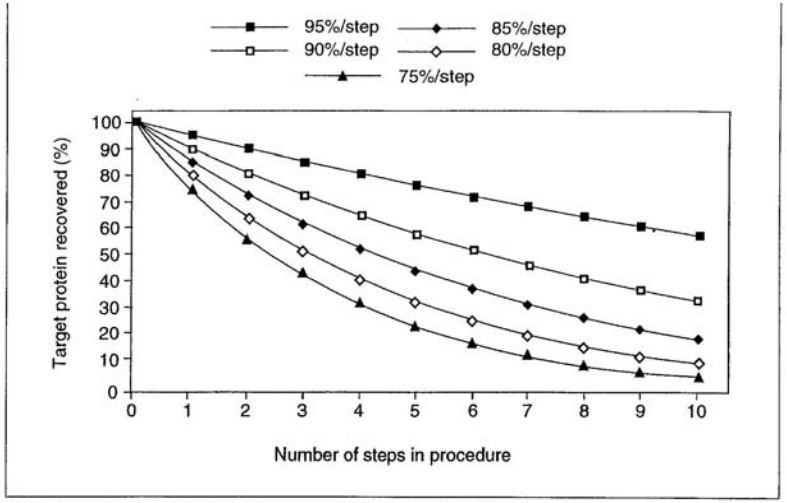


Figure 8.1.1 Theoretical yields from multistep protein purifications. Each curve represents a multistep process with a given percentage yield per step. The percentage of the original quantity of target protein remaining at the end of purification is plotted against the number of purification steps.

- **Assessment of protein purity**
  Functional
  Structural

Steps in purification scheme

- Capture stage
  - Goals: 1) Concentrating the target protein from the source material
    2) Removing as much of the contaminants as possible
  - Use adsorption chromatography techniques (ion exchange, hydrophobic-interaction, affinity)

    Gel filtration is not an adsorption chromatography

    Higher flow rate to reduce potential product degradation
  - Large volume: batch mode

- Intermediate stage
  - Goals: high resolution and high yield
  - Exploiting differences in other physicochemical attributes

    After initial column, ***the target and contaminant proteins share similar functional or structural attribute***

    Using other type of chromatographic technique

♦ Polishing stage

– Removing trace amount of contaminants (including structural variants)
– Exploiting small differences in a different aspect of physicochemistry

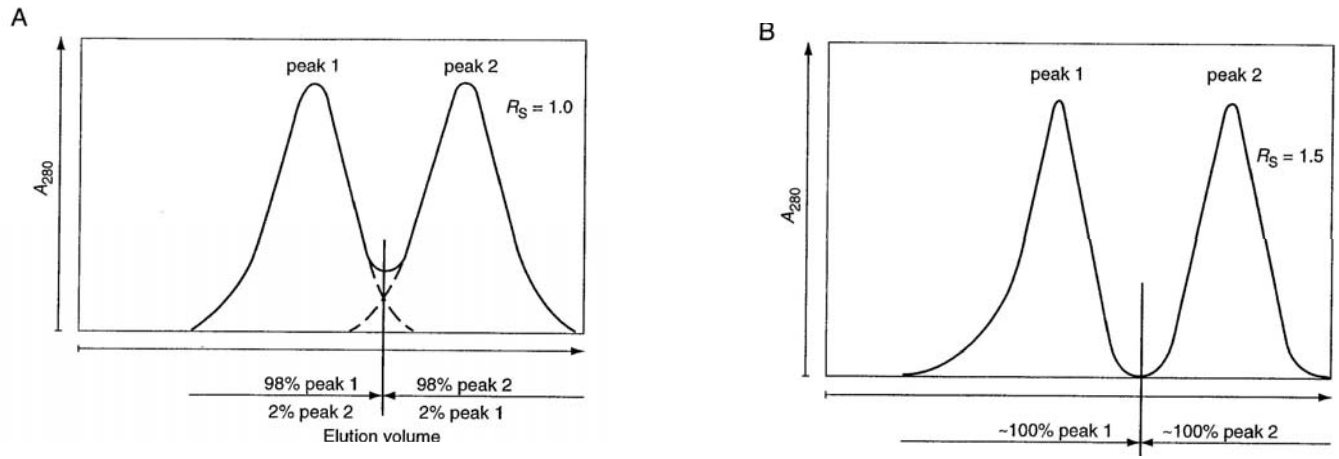
Another type of chromatographic technique

• Chromatography – differential separation of sample components between a mobile phase and a stationary phase

Stationary phase – spherical particles which are packed into a column
Mobile phase – protein mixture is introduced into the mobile phase

Parameters in chromatography

- Resolution
  - A measure of the relative separation achieved between two chromatographically distinct components



  - The aim of each purification step
  - Proportional to the capacity, efficiency and selectivity

$$R_s = \frac{1}{4}\left(\frac{k}{1+k}\right)\left(N^{1/2}\right)\left(\frac{\alpha-1}{\alpha}\right)$$

k – average **capacity factor** for the two peaks
N – **efficiency factor** for the system
α – **selectivity factor** of the medium

- ***Capacity (retention factor k)***
  - A measure of the amount of protein which can be adsorbed from solution onto a unit volume or weight of the stationary phase

$$k = \frac{V_{R2} - V_m}{V_m}$$

V_{R2}: elution volume of peak 2
V_m: volume of the mobile phase (the total bed volume)

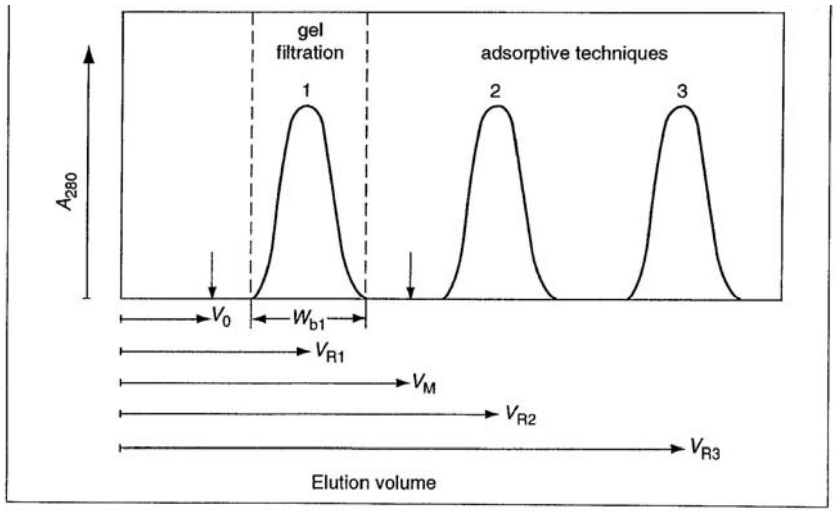


Figure 8.1.4 Hypothetical chromatogram. V_o = void volume; V_{R1} = elution volume for peak 1; V_{R2} = elution volume for peak 2; V_{R3} = elution volume for peak 3; V_M = volume of mobile phase; W_{b1} = peak width for peak 1; W_{b2} = peak width for peak 2.

  - High capacity chromatography is frequently used early on in a purification strategy
    High capacity: $V_{R2} > V_m$
    Adsorption techniques, ion exchange, hydrophobic interaction and affinity chromatography, have high capacity (gel filtration is not)

- ***Efficiency (N)***
  - A measure of zone broadening (peak width) on a column
    Cause of zone broadening – diffusion
  - Improved with uniformity in particle size and with decreasing bead size
    Uneven packing, trapped air, poor mixing during gradient formation
  - Also defined as the number of plates for the column under specific experimental conditions

$$N = 5.54\left(\frac{V_R}{W_h}\right)^2$$

V_R: the elution volume
W_h: the peak width at half the peak height

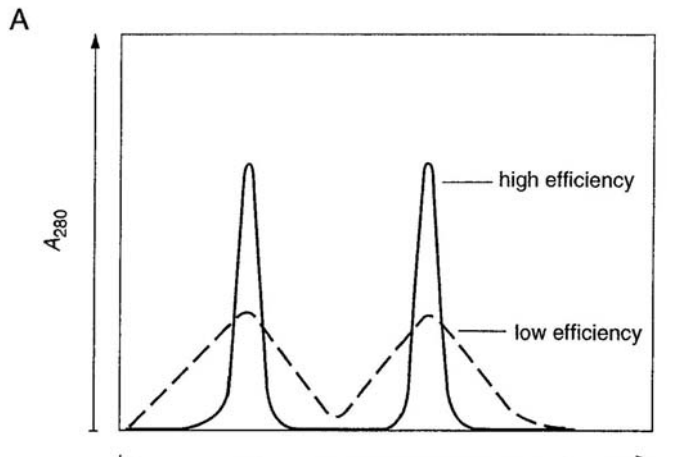
- ***Selectivity (α)***

  – More important than N in determining resolution
  High selectivity → a high degree of resolution

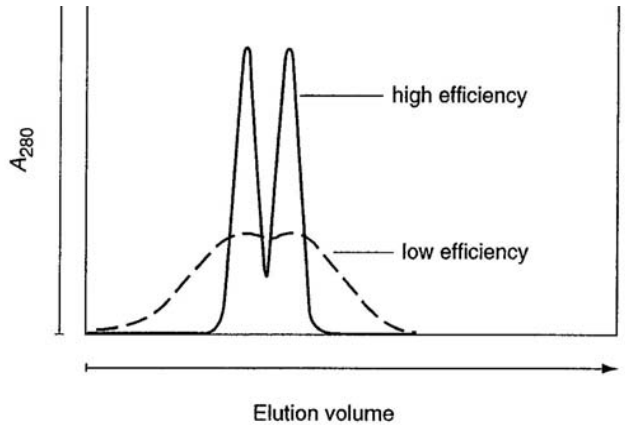
$$\alpha = \frac{V_{R2} - V_m}{V_{R1} - V_m} \approx \frac{V_{R2}}{V_{R1}}$$

V_{R1}, V_{R2}: elution volume of the first and second peaks
V_m: volume of the mobile phase

  – Depends partly on the chromatographic technique employed
  Improved by experimental conditions (pH and ionic strength of the mobile phase)
  – Factor to be exploited to improve resolution
  – Usually reserved for the last stage in a purification



N ⇒ Measure of Zone Broadening



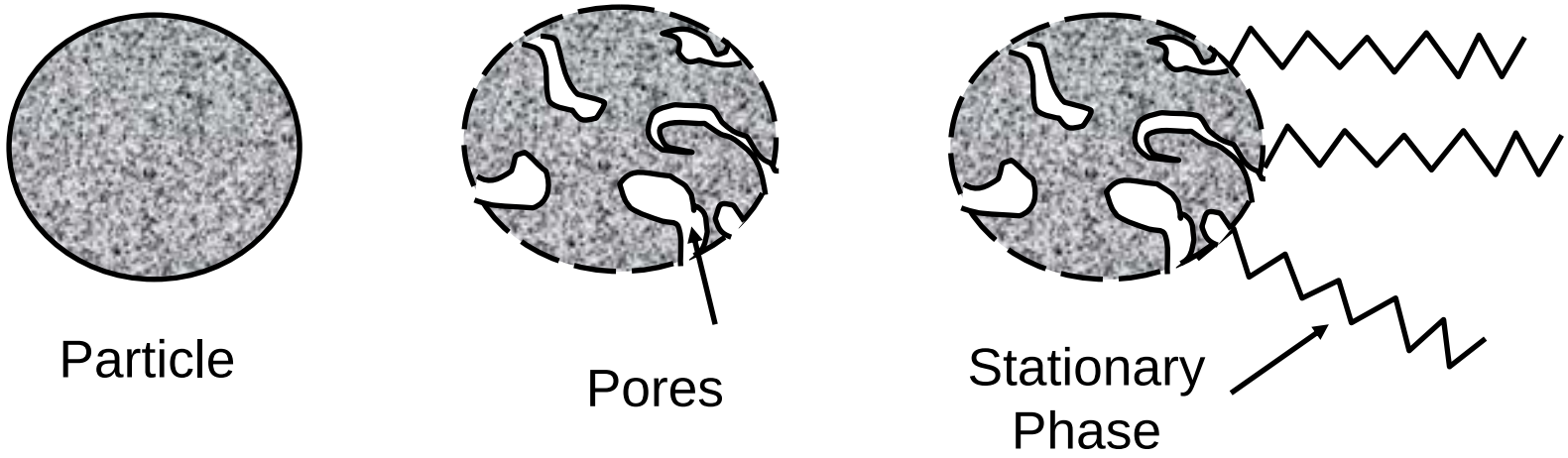
α ⇒ Measure of Resolution

- Matrix materials (Stationary phase)

  - Roles of matrix
    Solid substrate
    Modified with various functional groups

  - Physicochemical properties of the matrices
    Mechanical stability: flow rate
    Chemical stability: sterilization and maintenance
    Capacity: minimize the bed volume
    Pore size
    Pore shape
    Inert surface of the matrix
    Matrix density

Column – Packing Material



- Packing material - spherical
  - Particle size: 2.5, 3.5, 5 micron (μm)
  - Pores: Holes to provide more surface area for analyte interaction
  - Stationary phase: Groups bonded onto the particle
    - Reverse phase: C18, C8
    - --$(CH_2)_{17}CH_3$ hydrophobic group (dislikes water) or lipophilic (oil-like)

- Types of matrix
  - Cellulose
    Linear polysaccharide
    Very hydrophilic
    Inert to proteins but unstable to mineral acids, alkalis and oxidants
    Unstable at high flow rates and may collapse
    In the early purification steps
  - Agarose
    Polysaccharide from agar
    Very porous hydrophilic gel structure
    Minimal non-specific adsorption
    Stable over pH3-14 range
    Pore size controlled by the level of cross-linking and the amount of agarose
    Stable at fast flow

– Dextran

An extracellular polysaccharide

Very hydrophilic matrix

Chemically inert and easily derivatized

Stable over pH 2-12

Pore size controlled by the amount of cross-linker, relatively homogenous

Soft, not recommended for fast flow

– Silica

Acidification of sodium silicate

Incompressible, commonly used in HPLC

Hydrophilic and easily derivatized

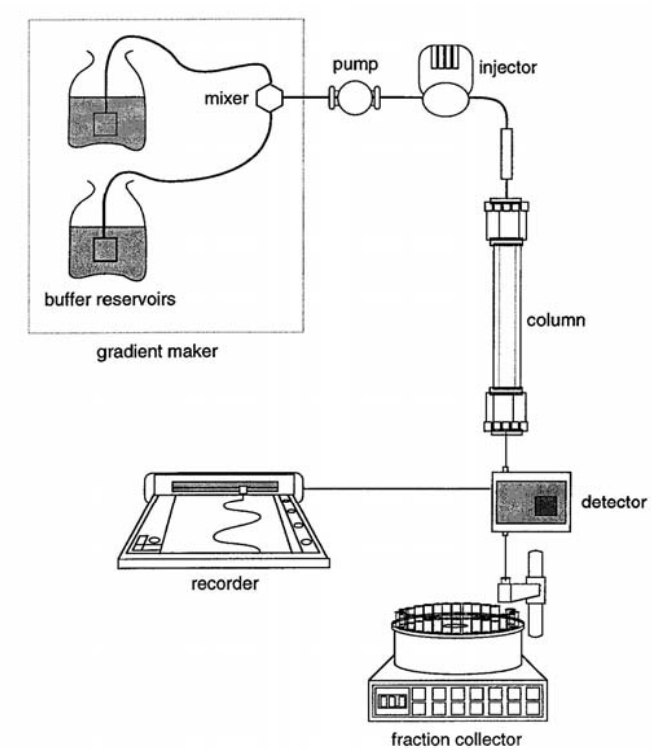
Unaffected by organic solvents and mechanically stable

Dissolve above pH 8, working under pH < 7

- Mobile phase
  - ♦ Components
    - – Buffer, to maintain certain pH
    - – Salt, to provide ionic strength

- Chromatographic experiment
  - ♦ Components

    Pump: deliver an even flow of liquid
    Column: where the protein separation occurs
    Detector: provide a continual measurement of a physical parameter of the eluent
    Recorder: give a continuous visual read-out of the detector output
    Fraction collector: separate the column eluent into samples

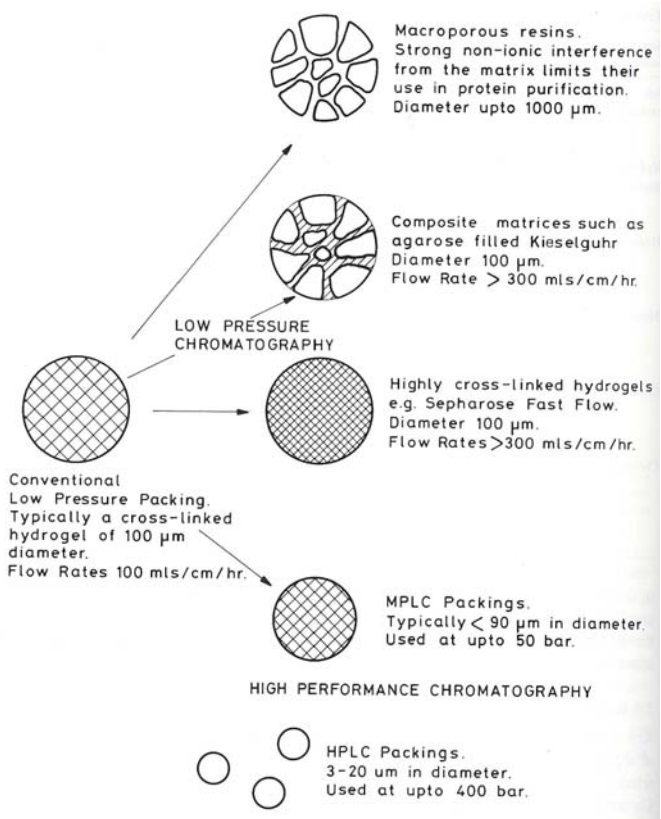


♦ Operation ranges

LPLC: < 5 bar(45 psi)

MPLC: 6-50 bar (90 – 750 psi)

HPLC: > 50 bar (750 psi)



♦ Types of chromatography

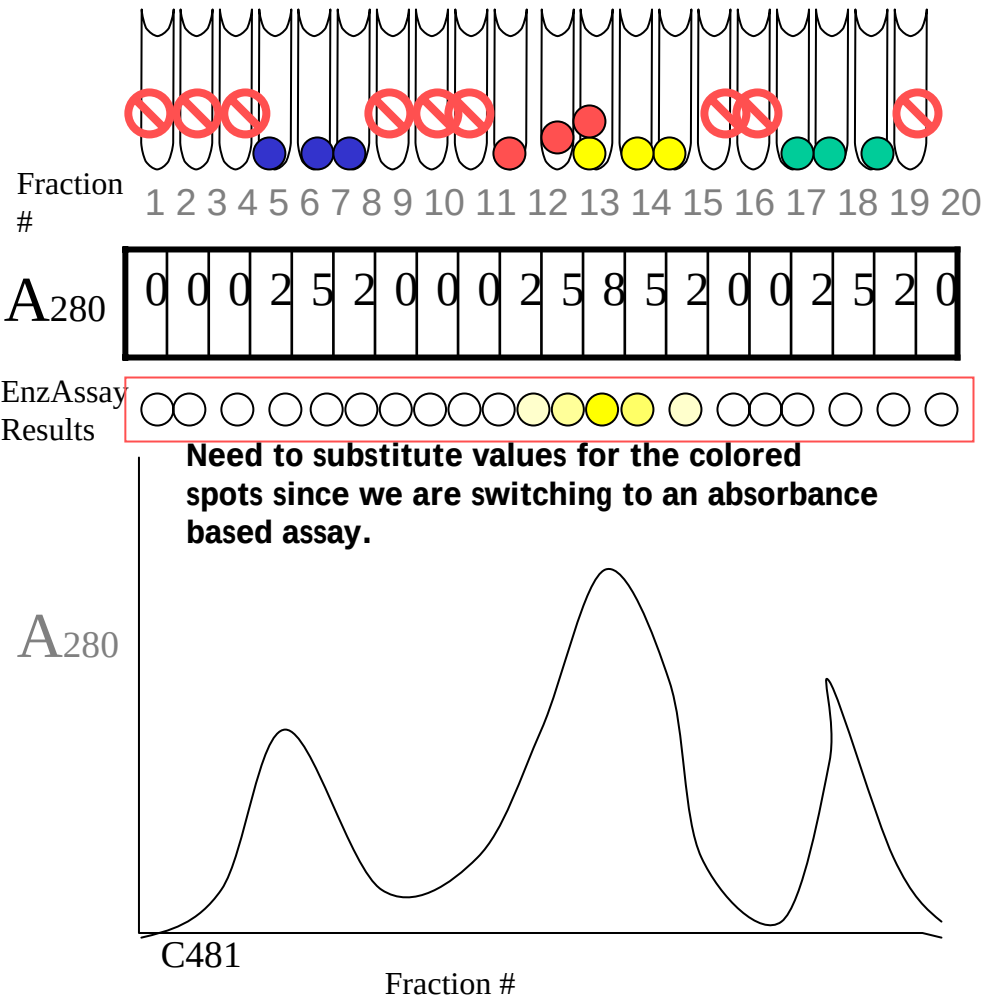
Based on charge (ion exchange)

Based on size (gel filtration)

Based on hydrophobic interaction (including reverse phase chromatography)

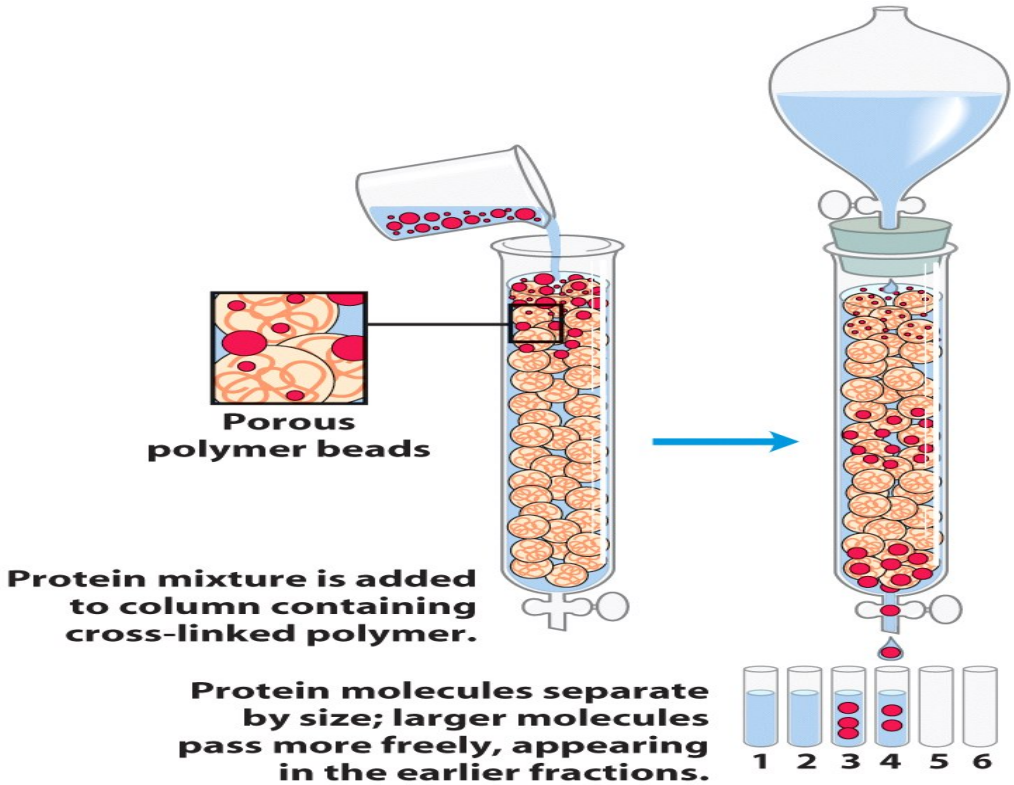
Based on specific affinity (Affinity chromatography)

Check fractions from the column: measure A280



- Fraction absorption at 280nm A280 was monitored.
- Enzyme activity can be determined by performing an enzyme assay on each fraction that contains protein.

Chromatography based on SIZE: Size Exclusion/Gel Filtration

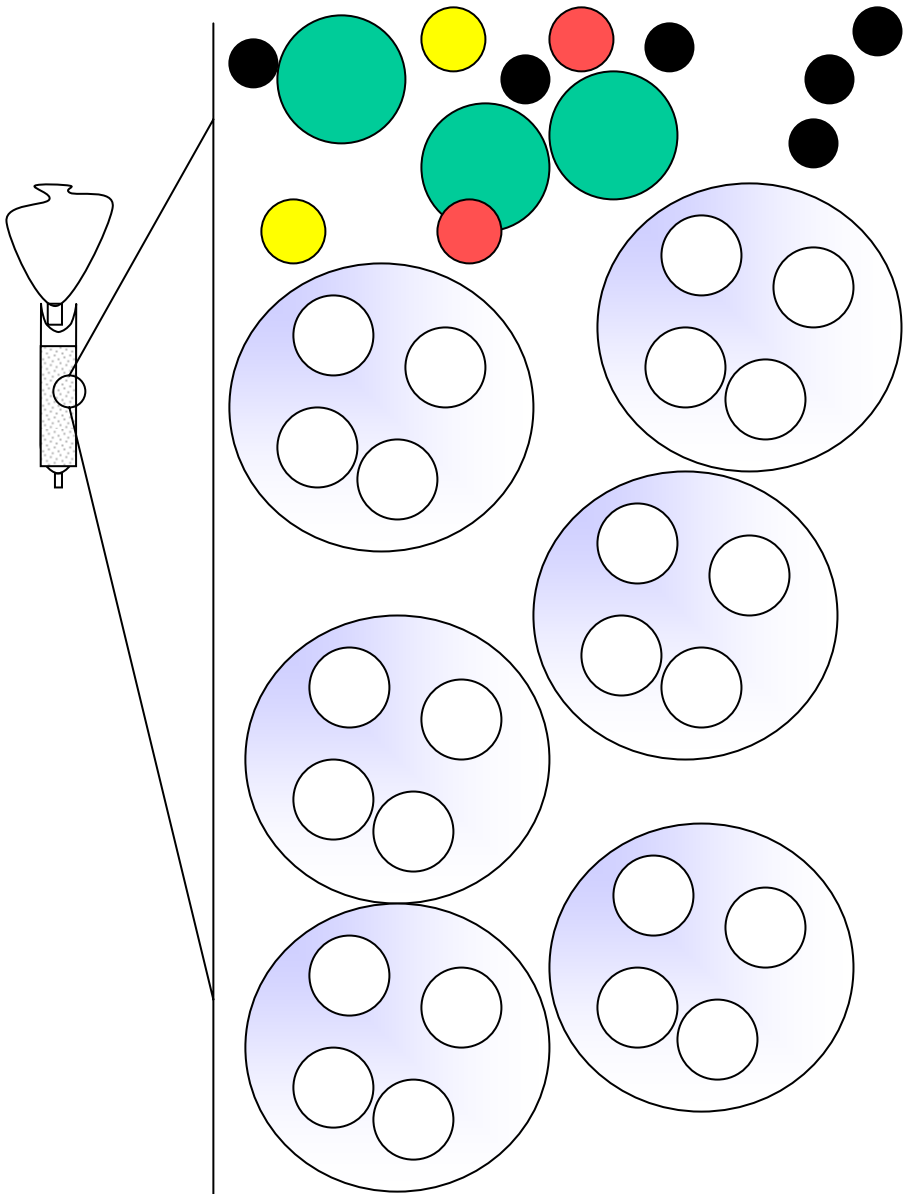


- Gel filtration
  - ♦ Separates biomolecules on the basis of size (diffusion)
  - ♦ Frequently used in the polishing stage of purification
    - – To remove the minute contaminate
    - – To remove salts from solution (desalting)
  - ♦ Fractionation range

    the ***exclusion limit***: the smallest-sized protein molecule that will be excluded from the pores of the matrix

Table 3. Properties of Superdex

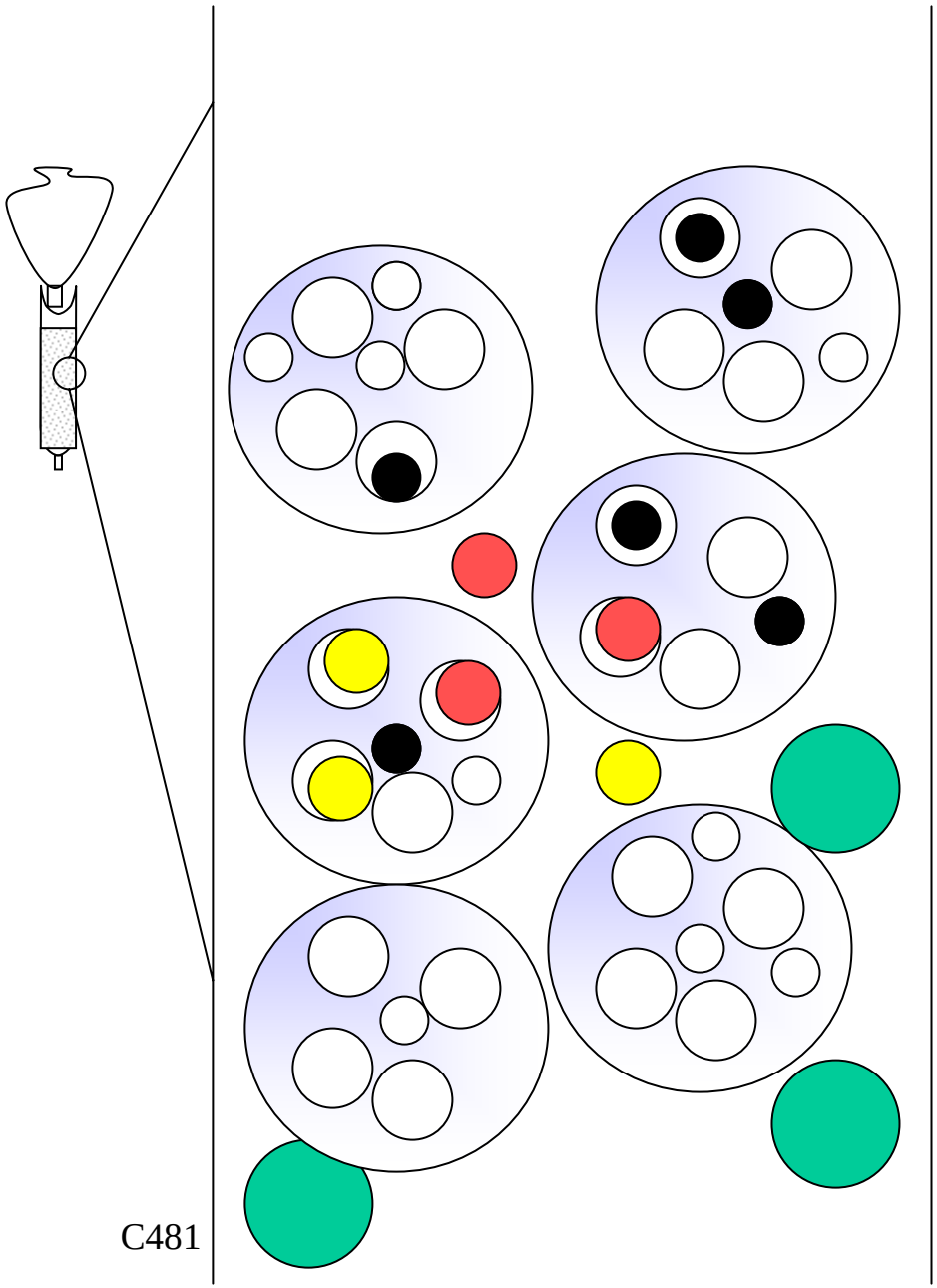
Gel type	Bead size µm	Fractionation range Globular proteins	Fractionation range Dextrans
Superdex 30 prep grade	24 – 44	– 10 000	–
Superdex 75 prep grade	24 – 44	3 000 – 70 000	500 – 30 000
Superdex 75	11 – 15	3 000 – 70 000	500 – 30 000
Superdex 200 prep grade	24 – 44	10 000 – 600 000	1 000 – 100 000
Superdex 200	11 – 15	10 000 – 600 000	1 000 – 100 000



Gel filtration 2 - close up of beads

- The matrix of a size-exclusion chromatography column is porous beads.

Run column

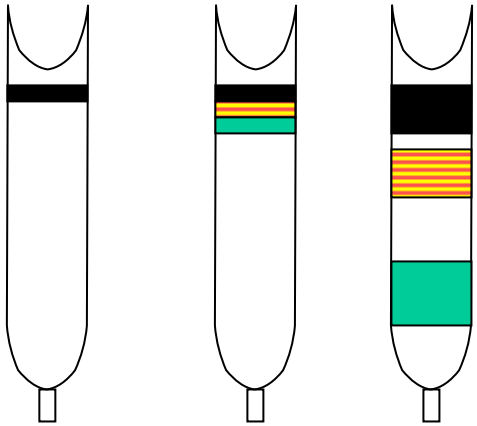


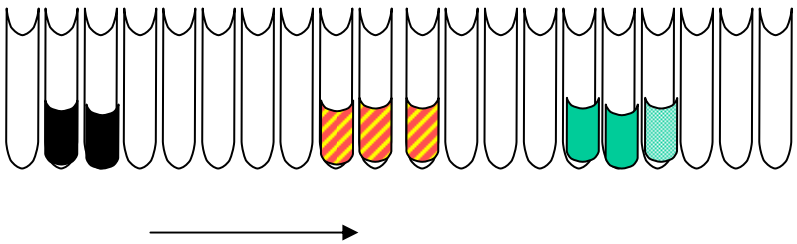
C481

Gel filtration 3 - run close up of column

- The matrix of a gel filtration column are beads with pores.
- The large green proteins can’t fit in pores so flows faster.
- The red/yellow medium sized proteins get trapped in the pores.
- The black small proteins stay trapped in pores longer.

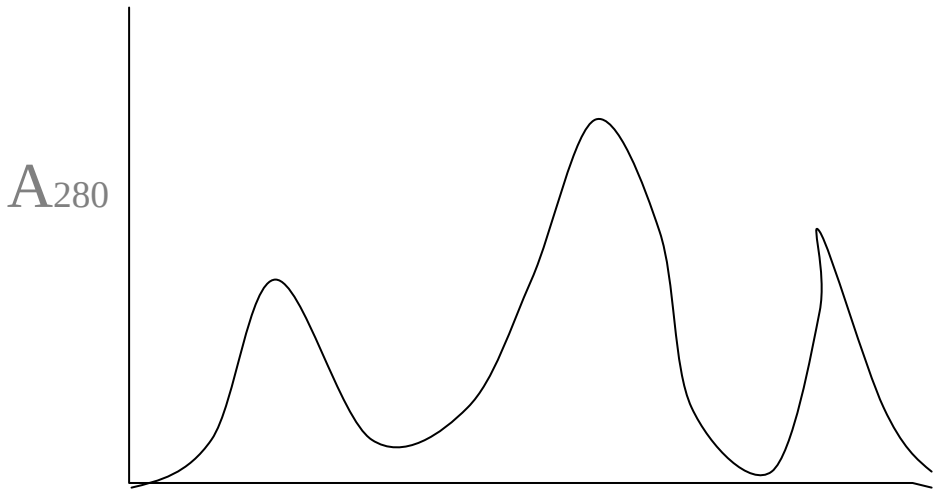
Gel filtration 4 - zoom out



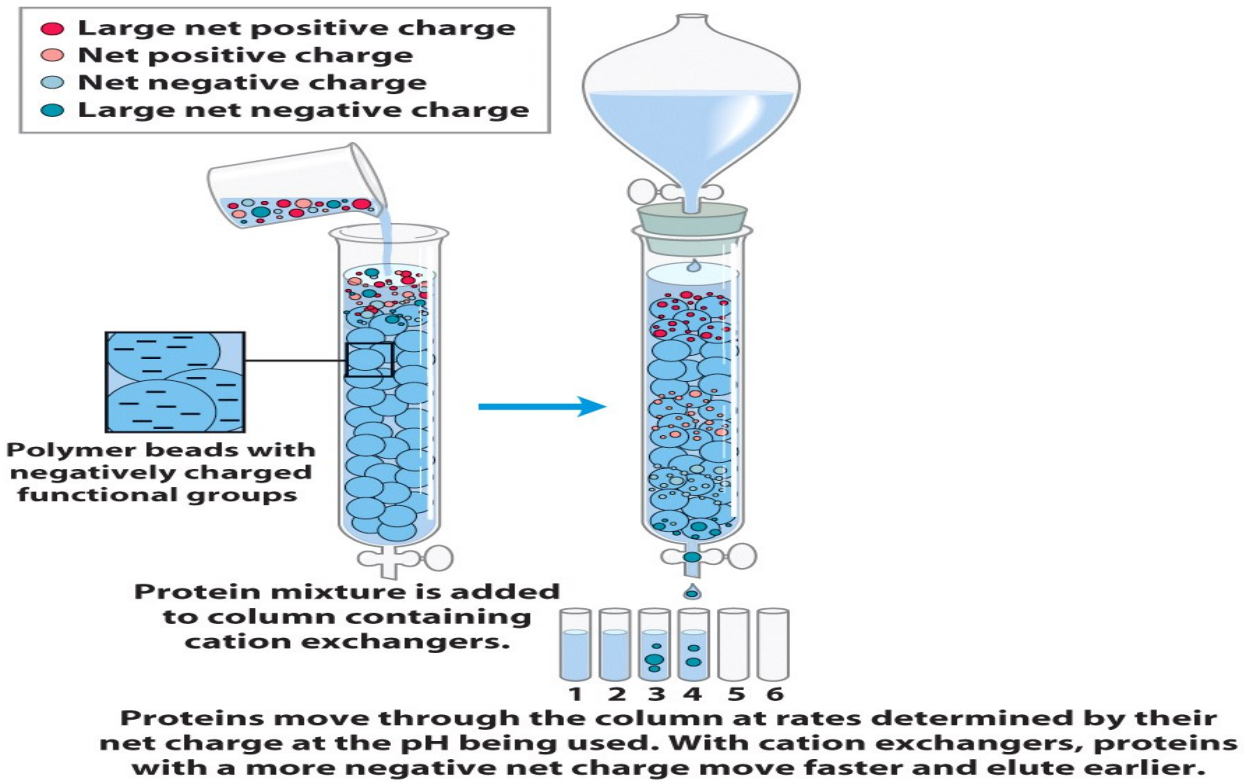


Tubes march in from left

Click on the peak that represents the protein of the largest molecular weight?

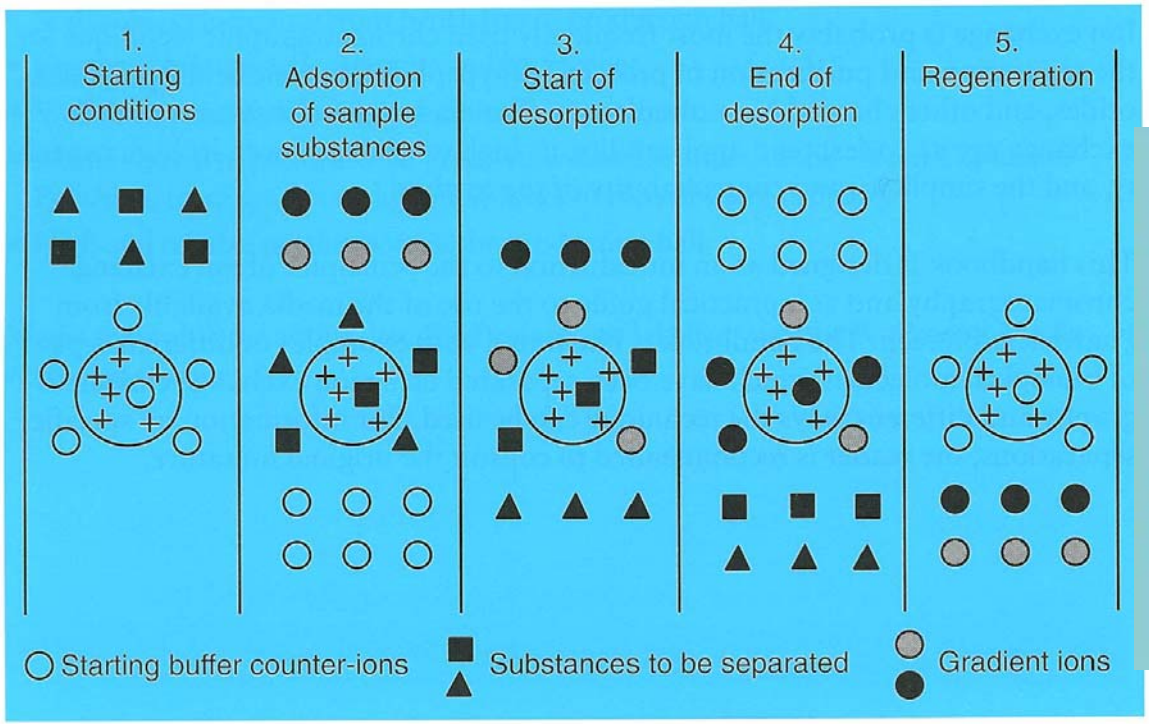


Chromatography based on CHARGE: Ion/Cation Exchange



Ion exchange chromatography

- ***Separates biomolecules on the basis of charge characteristics***
- ***Adsorption and desorption mechanism***



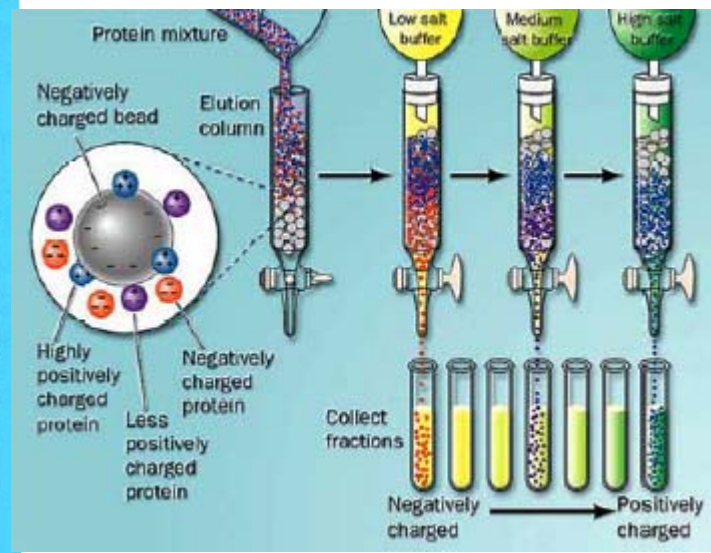


Fig. 1. The principle of ion exchange chromatography (salt gradient elution).

♦ Matrix attached with charge groups

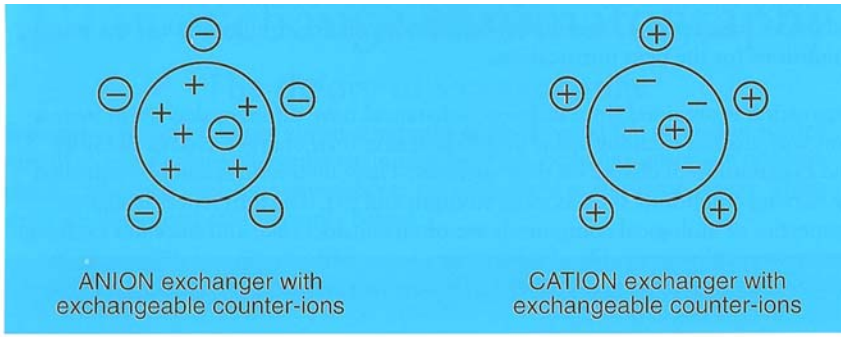


Fig. 2. Ion exchanger types.

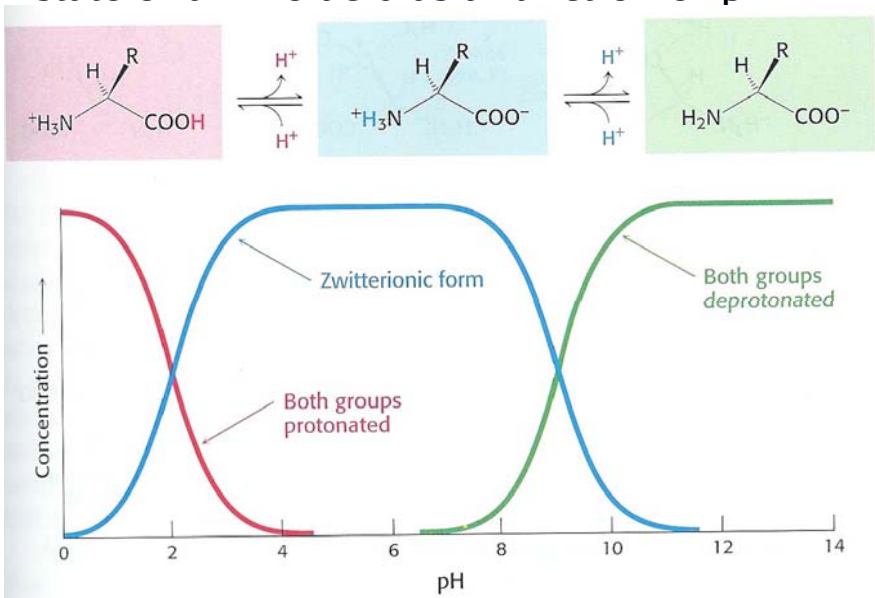
– ***Anion exchanger***
Functional group is *positively* charged

– ***Cation exchanger***
Functional group is *negatively* charged

Table 1. Functional groups used on ion exchangers.

Anion exchangers	**Functional group**
Diethylaminoethyl (DEAE)	$-O-CH_2-CH_2-N^+H(CH_2CH_3)_2$
Quaternary aminoethyl (QAE)	$-O-CH_2-CH_2-N^+(C_2H_5)_2-CH_2-CHOH-CH_3$
Quaternary ammonium (Q)	$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-N^+(CH_3)_3$
Cation exchangers	**Functional group**
Carboxymethyl (CM)	$-O-CH_2-COO^-$
Sulphopropyl (SP)	$-O-CH_2-CHOH-CH_2-O-CH_2-CH_2-CH_2SO_3^-$
Methyl sulphonate (S)	$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2SO_3^-$

- Charges of a protein and pH of the buffer
  - Ionization state of amino acid as a function of pH



The net charge of amino acid in zwitterion form is zero

  - Isoelectric point (pI) of protein

pH at which the net charge of a protein is zero

Estimate pI: http://www.expasy.org → primary sequence analysis

pH below pI, the net charge of protein is positive

pH above pI, the net charge of protein is negative

– Other ionizable groups

Table 1.2 *Intrinsic pK_a Values of Ionizable Groups Found in Proteins*

Group	Observed pK_a[a]
α-Amino	6.8 – 8.0
α-Carboxyl	3.5 – 4.3
β-Carboxyl (Asp)	3.9 – 4.0
γ-Carboxyl (Glu)	4.3 – 4.5
δ-Guanido (Arg)	12.0
ϵ-Amino (Lys)	10.4 – 11.1
Imidazole (His)	6.0 – 7.0
Thiol (Cys)	9.0 – 9.5
Phenolic hydroxyl (Tyr)	10.0 – 10.3

[a] The ranges of values are given by different model compounds used to represent an isolated amino acid residue. The values for the terminal α-amino and α-carboxyl groups especially depend on the identity of the terminal residue. Values from C. Tanford, *Adv. Protein Chem.* 17:69 – 165 (1962); A. Bundi and K. Wüthrich, *Biopolymers* 18:285 – 297 (1979); J. B. Matthew et al., *CRC Crit. Rev. Biochem.* 18:91 – 197 (1985).

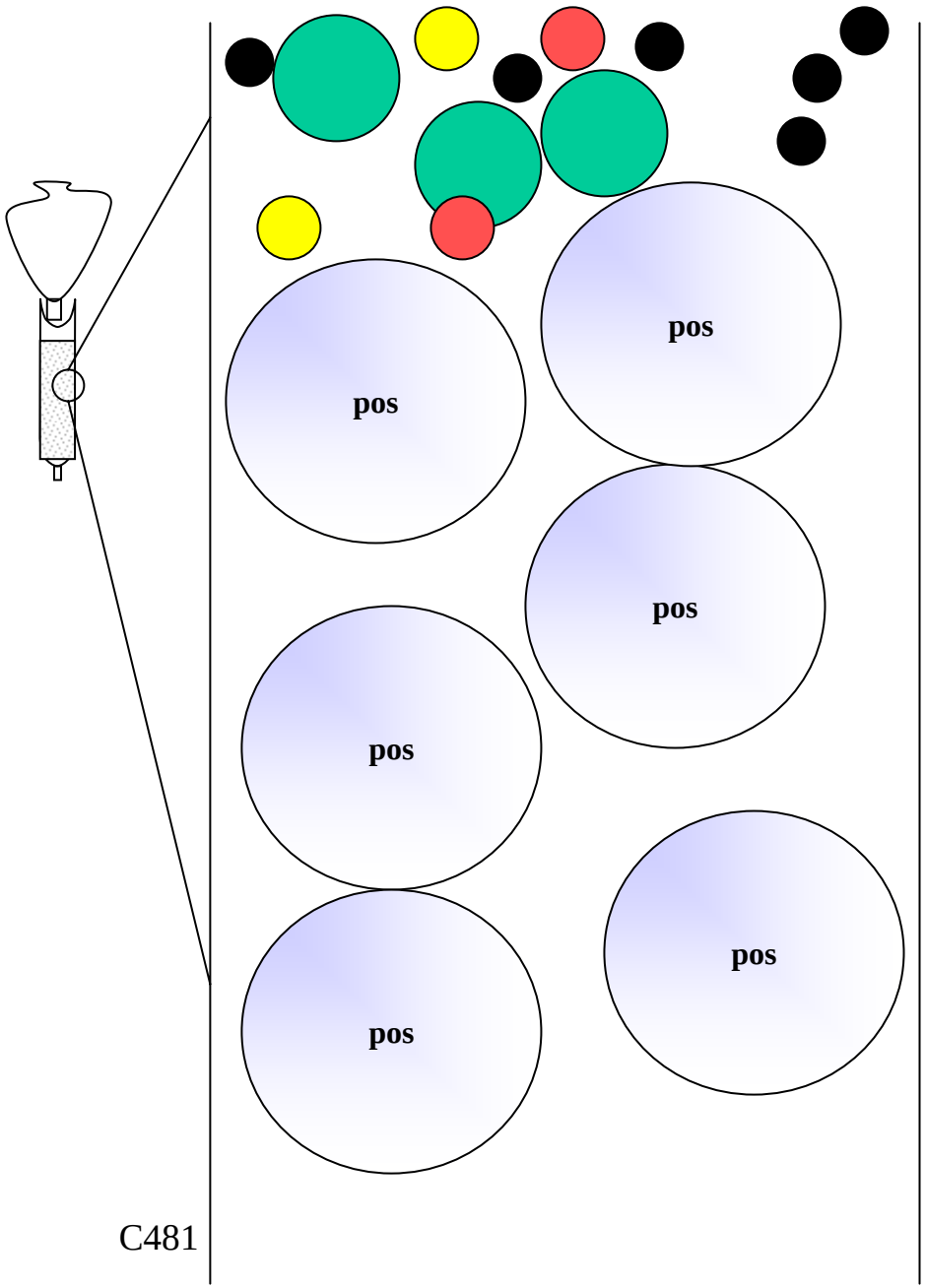
♦ Experimental conditions
  – matrix medium
  – buffer (pH, salt conc.)

ION –EXCHANGE 1

60 Kd	20 Kd	20 Kd	5 Kd
Low pI (6)	Low pI (7)	Medium pI (7)	Hi pI (8)

- Ion-exchange column chromotography separates proteins on the basis of charge.

- We will start with 4 proteins.
- pH 7.2
- pos charged column

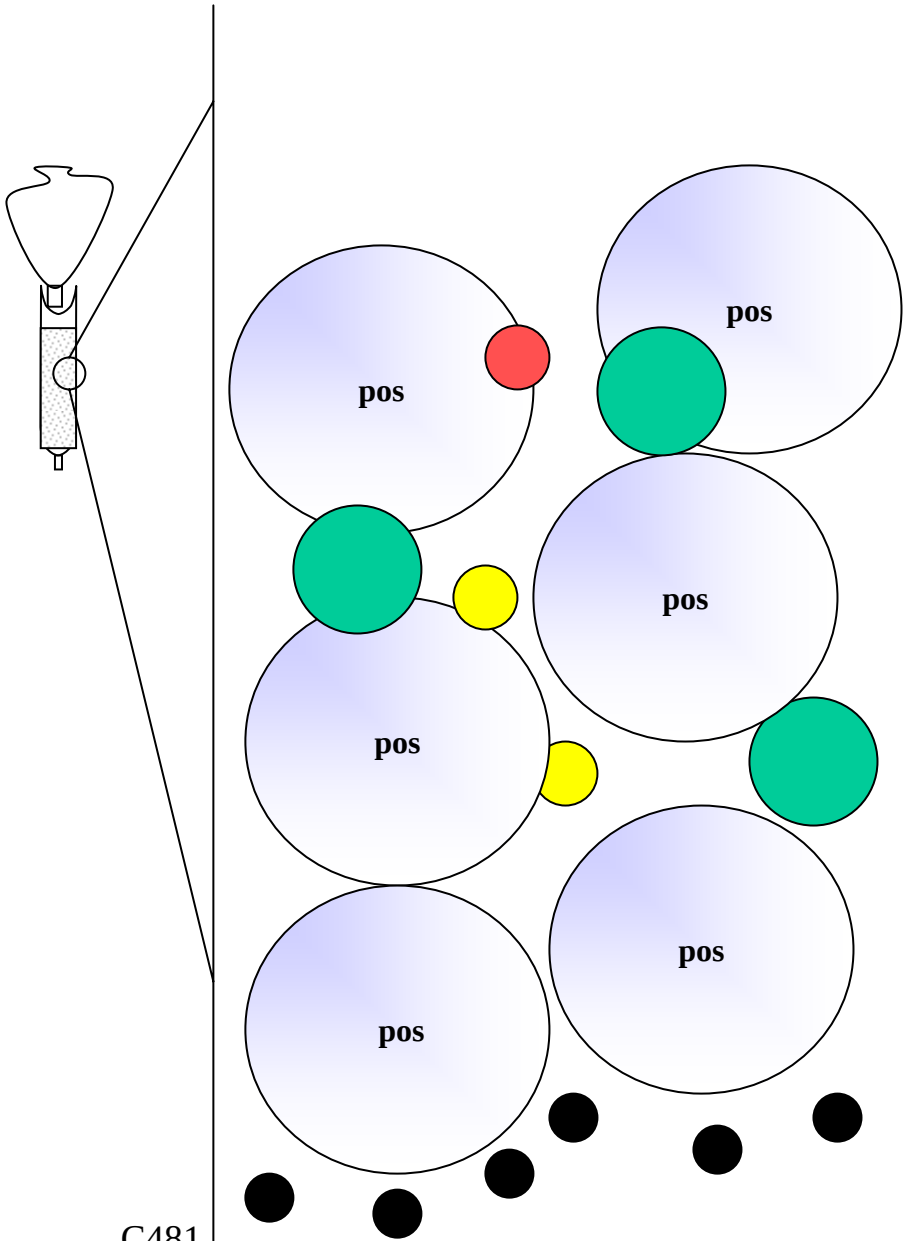


Ion Exchange 2 – loaded proteins

- The matrix of an ion exchange is positively charged.
- What do you think will happen?

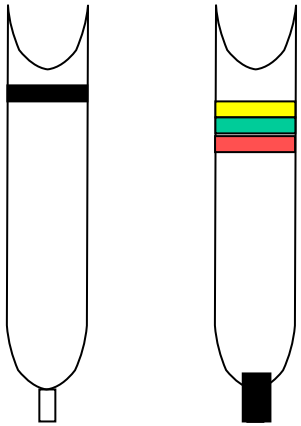
Run column

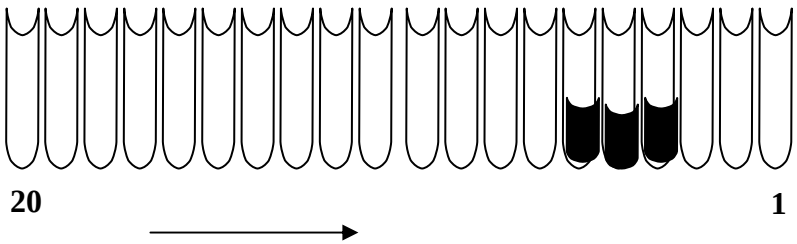
Ion Exchange 3 –column run



- The matrix of an ion exchange is positively charged.
- Only the pos charged proteins run through the pos charged column. The others “stick” to the column.

Ion Exchange 4- zoom out

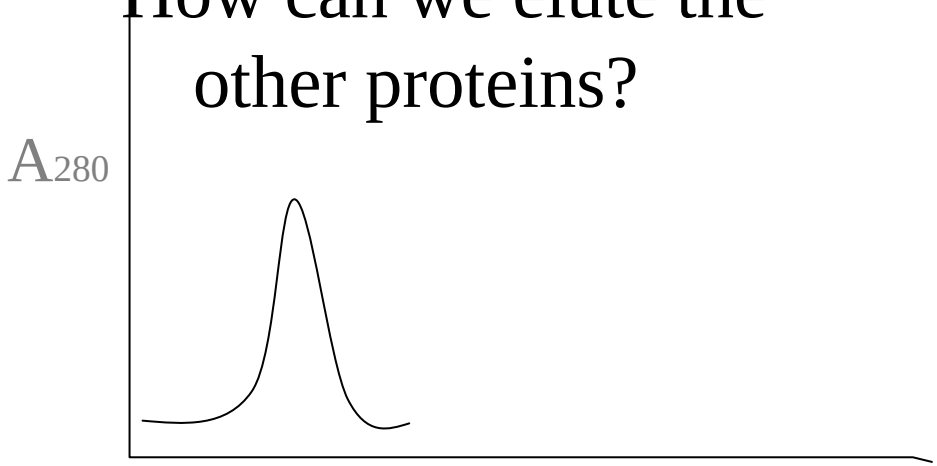




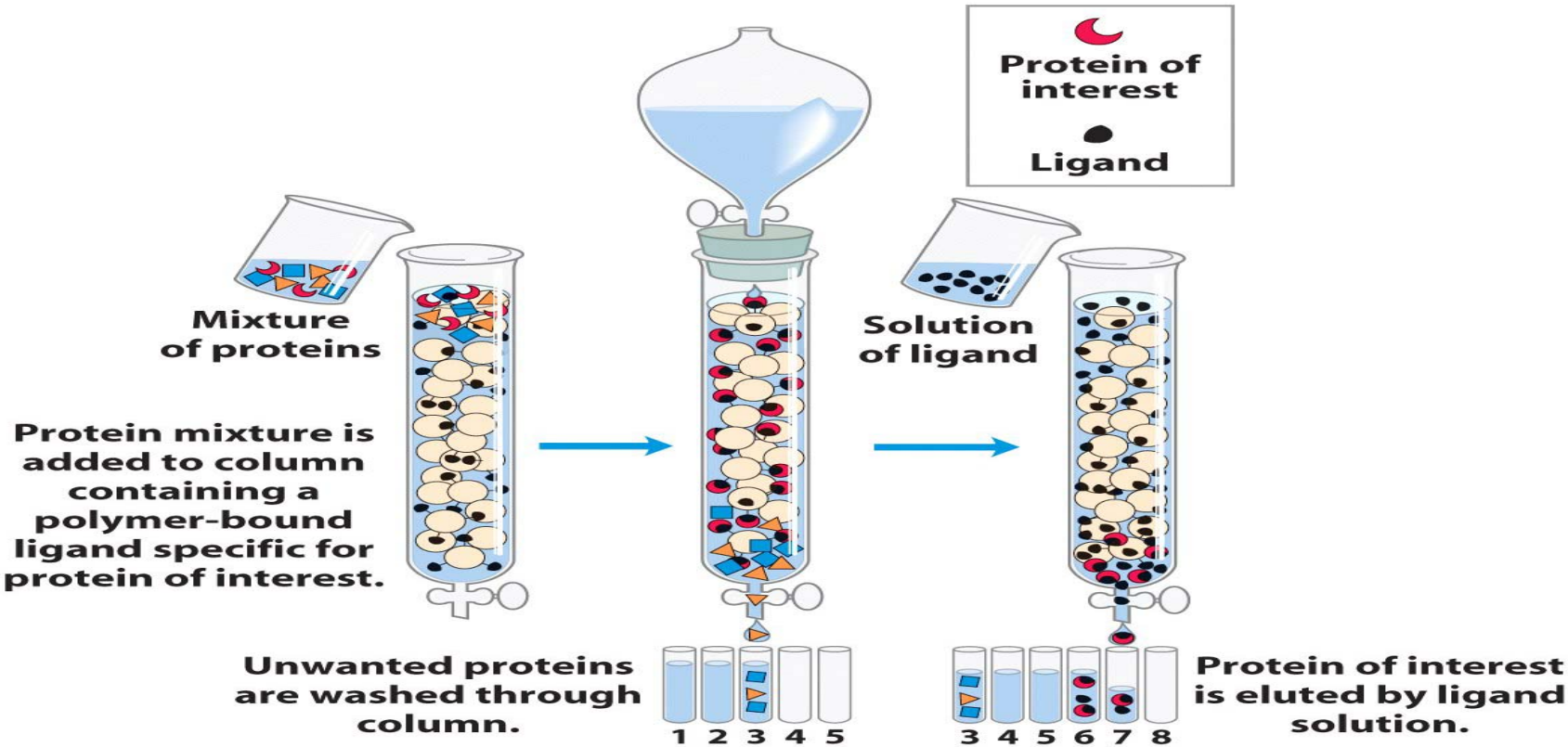
Tubes march in from left

Only the POS charged proteins run through the column.

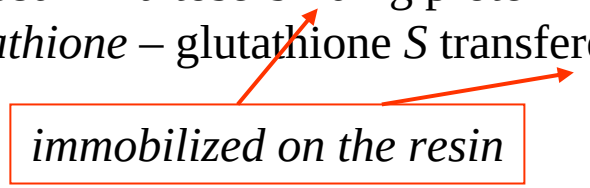
How can we elute the other proteins?



Chromatography based on Specific BINDING: Affinity Column

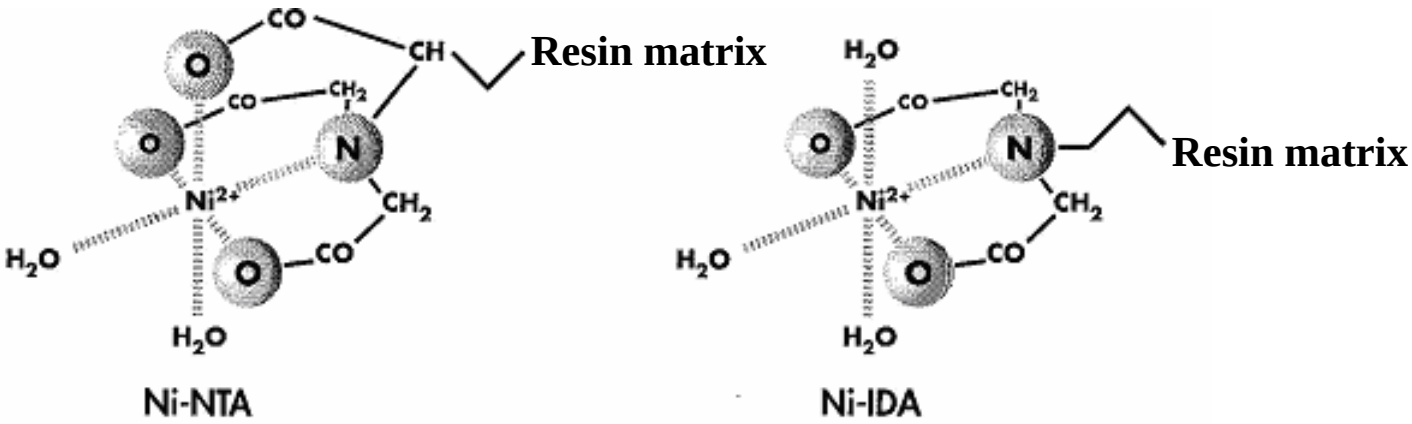


- Affinity Chromatography
  - ♦ Principle – based on specific interaction
    Examples: *Antibody* – antigen
    Hormone – receptor
    Ligand – protein: *biotin* – avidin (strepavidin)
    Maltose – maltose-binding protein
    Glutathione – glutathione *S* transferease (GST)

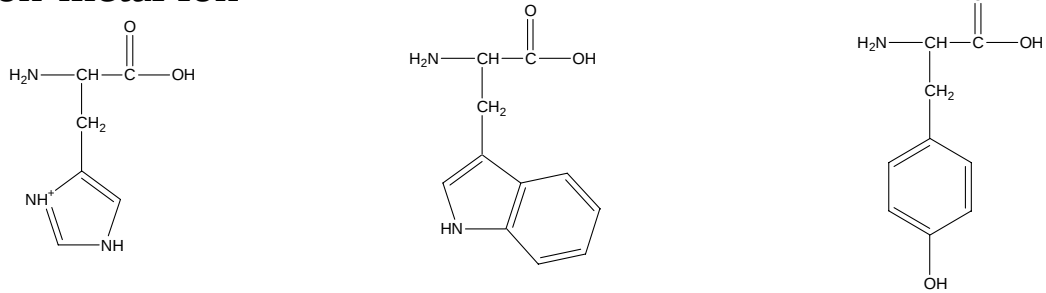


  - ♦ Steps of affinity chromatography
    - – Choice of appropriate ligand
      Specificity (or selectivity) is the key
    - – Immobilization of the ligand onto a support matrix
      CNBr (cyanogen bromide)-activated Sepharose 4B from Pharmacia
    - – Loading of the protein onto the matrix
    - – Washing away the nonspecifically bound proteins
    - – Elution of bound protein in a purified form

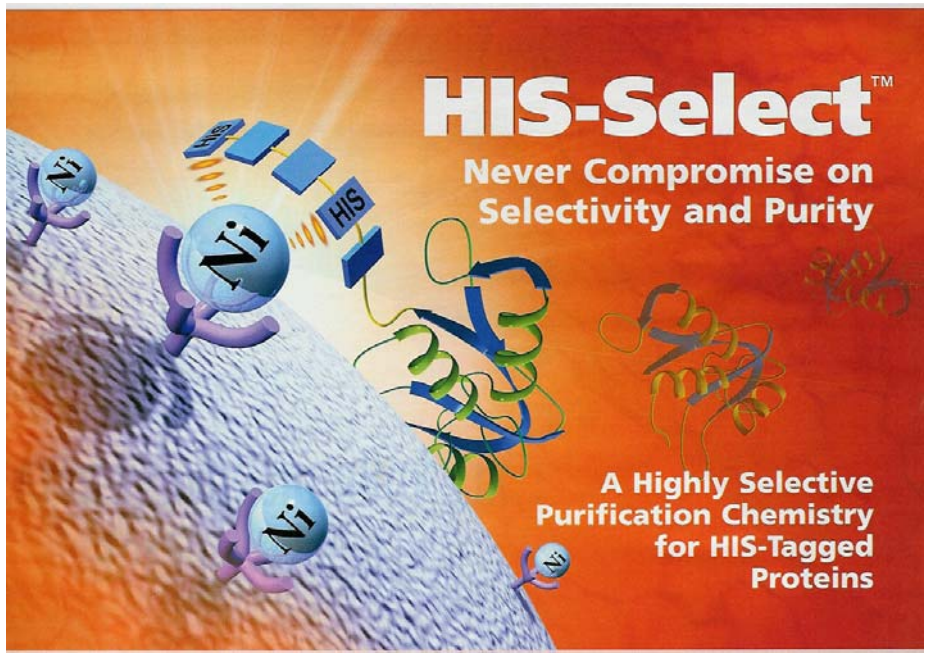
- Metal-chelate affinity chromatography
  - Matrix attached with chelating group that immobilizes transition metal ions (Ni^{2+}, Co^{2+})
    Chelating group: iminodiacetic acid (IDA)
    nitrilotriacetic acid (NTA) (more firmly attached to the matrix)



  - AA (H, W, T) as electron donors on the surface of proteins bind reversibly to transition-metal ion



– Engineering a 6 histidine tandem at the N- or C-termini of protein to facilitate binding



Experimental conditions

pH: ~ 8.0 to ensure binding

Salt: to minimize nonspecific interaction with the matrix

Elution: imidazole to compete for binding to Ni^{2+}, or lower pH

- Experimental conditions
  - Column dimension
    The longer traveling length, the better resolving power
  - Sample volume
  - Buffer (salt concentration)
    To avoid solute-matrix (nonspecific ionic and hydrophobic) interactions

High Performance Liquid Chromatography (HPLC)

Fast Performance Liquid Chromatography (FPLC)