Column purification

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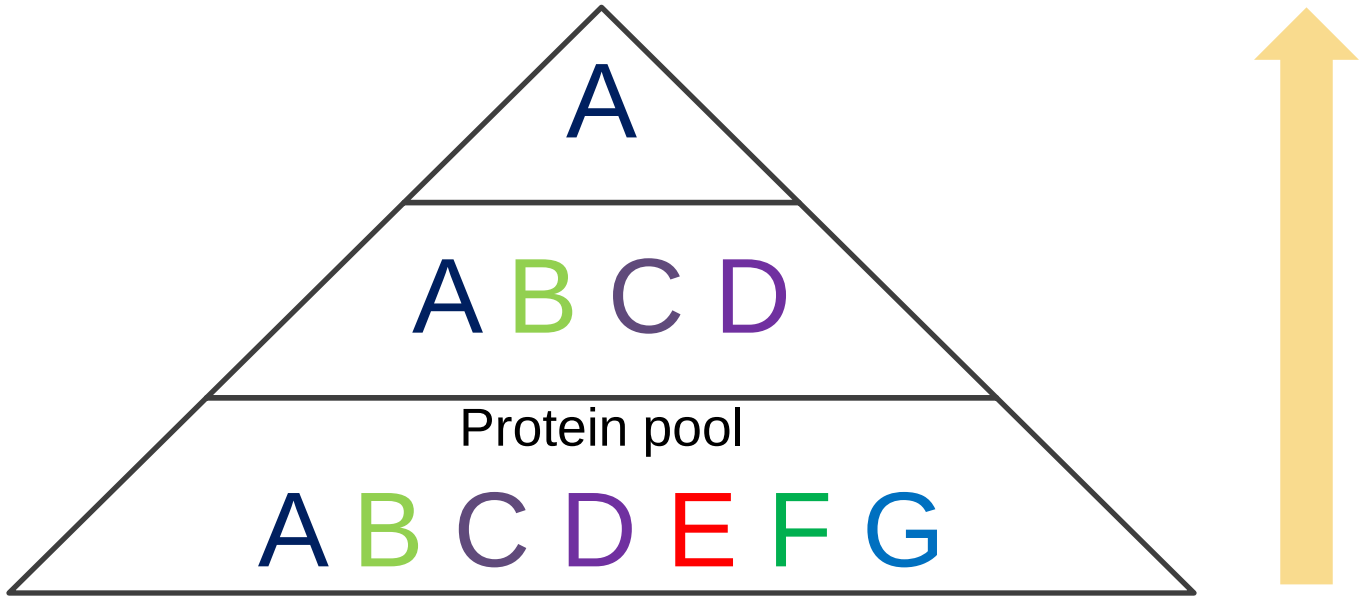
INTRODUCTION

Purpose of protein purification

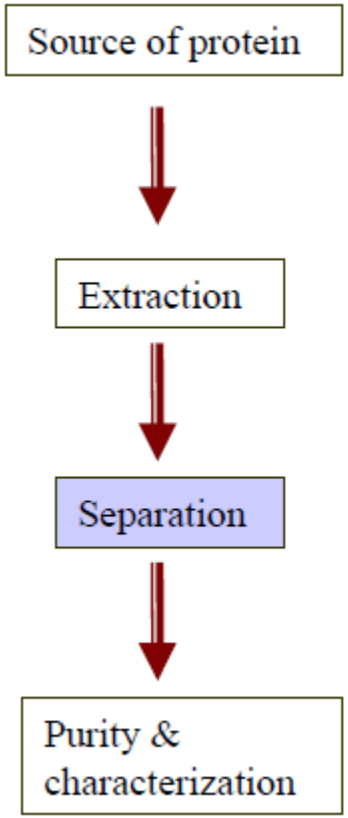
To obtain interest protein A in a short time from protein pool (A,B,C,D,E,F and G)

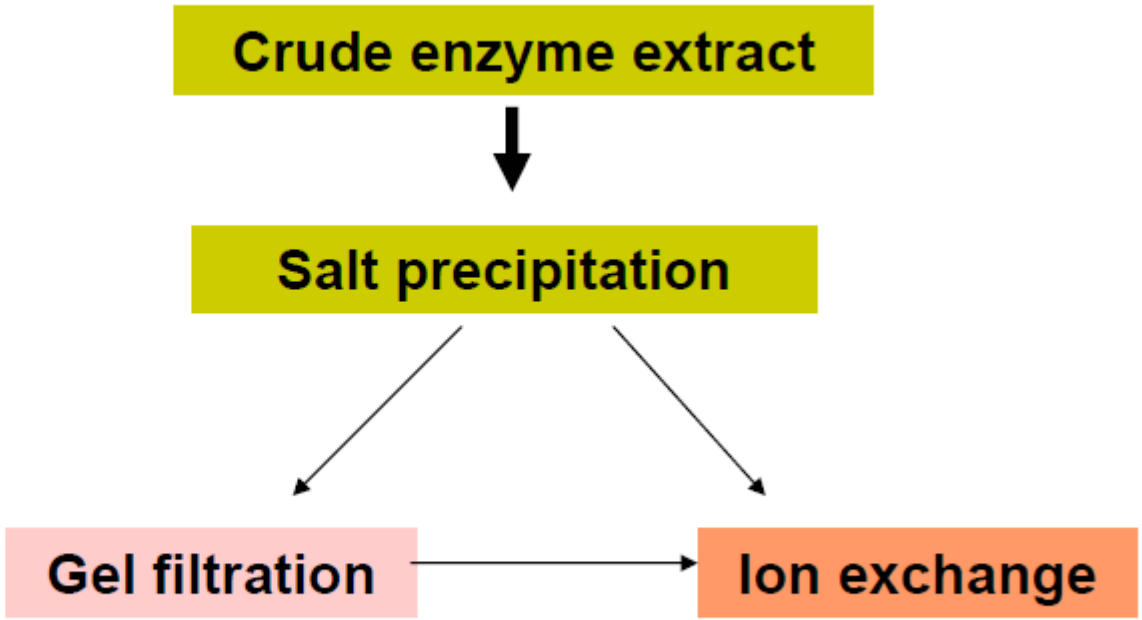


INTRODUCTION

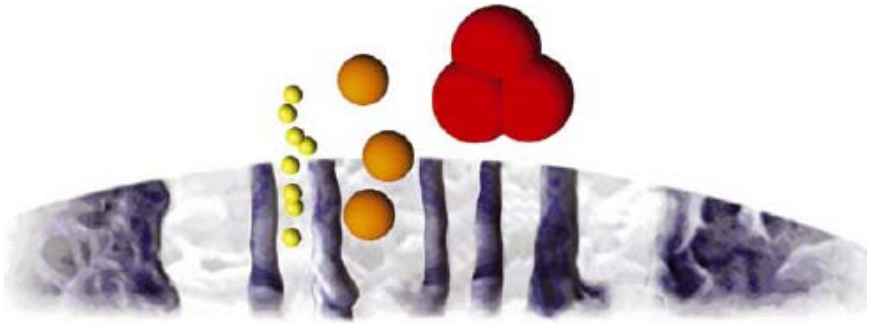
- **Gel filtration chromatography**
- **Ion-exchange chromatography**
- **Affinity chromatography**
- **Application**

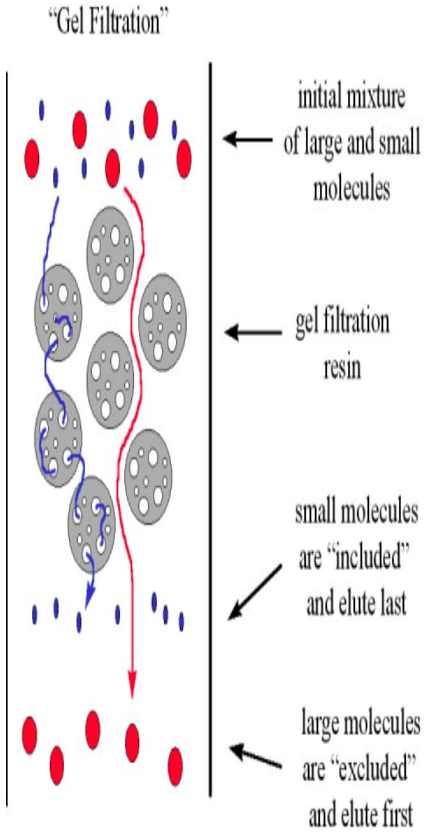
INTRODUCTION





Gel filtration chromatography

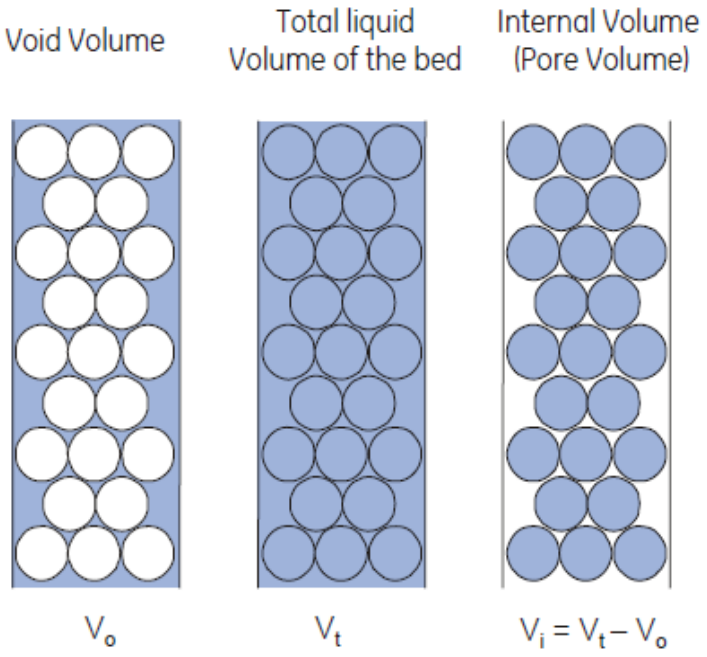




Separates molecules according to **differences in size as they pass through a gel filtration medium**(lack of reactivity or adsorptive properties) packed in a column

Well suited for **biomolecules that may be sensitive to changes in pH, concentration of metal ions or co-factors and harsh environmental conditions**

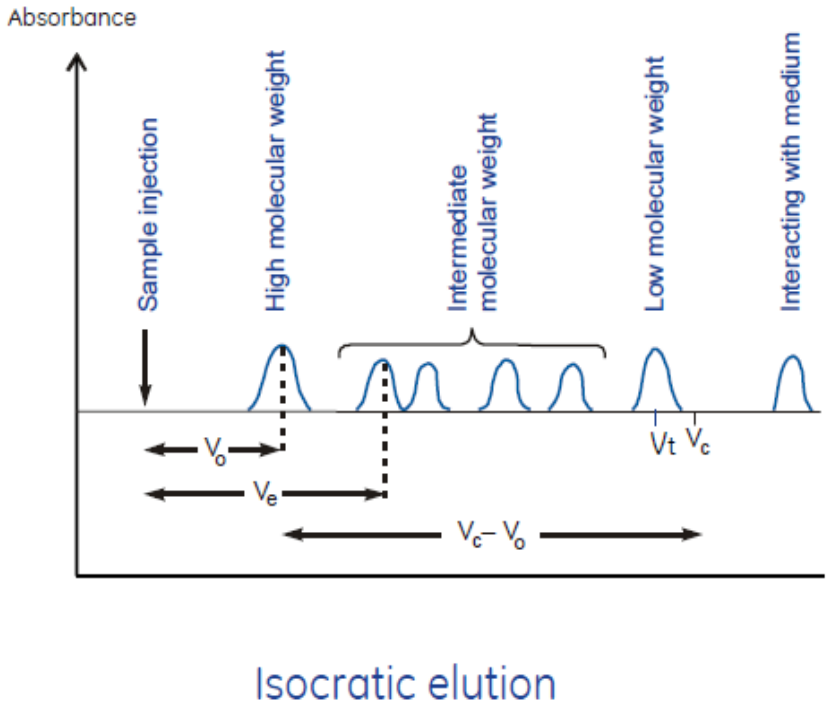
Gel filtration chromatography



Porous gel filtration matrix in a packed bed

Separation is achieved using a porous matrix to which the molecules, for steric reasons, have different degrees of access (smaller molecules have greater access and larger molecules less)

Gel filtration chromatography



Isocratic elution

The **protein zones eluted are detected by an in-line UV monitor** and **fractions are collected** for subsequent specific analysis or further preparation steps.

Gel filtration chromatography

A pplication

•Desalting or group separation
• Protein fractionation
• Characterizing the molecular dimensions of proteins

STRATEGIC PLANNING

Selecting Matrix and Column for Desalting

The exclusion limit, the smallest-sized protein molecule that will be excluded from the pores of the matrix.
The typical length of columns for laboratory use is ～10 cm.

Selecting Matrix and Column for Protein Fractionation

Selecting Matrix and Column for Molecular Size Determination

Table 8.3.1 Gel Filtration Matrices Suitable for Desalting[a]

Gel type	Exclusion limit		Supplier[d]
	M_r[b]	Radius (Å)[c]	
Sephadex G-10	700	7	PB
Bio-Gel P-2	1,800	10	BR
Sephadex G-25	5,000	14	PB
Bio-Gel P6DG	6,000	15	BR
Sephadex G-50	30,000	25	PB
Bio-Gel P-30	40,000	28	BR

[a]Data as reported by manufacturers.
[b]Molecular mass of smallest protein excluded from matrix.
[c]Apparent radius of smallest spherical protein excluded from matrix, calculated as $R = 0.808\ M_r^{1/3}$, where R is the radius and M_r the molecular mass (Hagel, 1989).
[d]Abbreviations: BR, Bio-Rad; PB, Amersham Pharmacia Biotech. Addresses and phone numbers of suppliers are provided in the *SUPPLIERS APPENDIX*.

Table 8.3.4 Gel-Filtration Matrices for Protein Fractionation or Size Determination[a]

Gel type	Fractionation range (M_r)	Particle size (μm)	Supplier[b]
Toyopearl HW 40S	100-10,000	25-40	TH
Superdex 30 prep grade	200-10,000	34	PB
Toyopearl HW 50S	500-80,000	25-40	TH
Sephacryl S-100 HR	1,000-100,000	47	PB
Toyopearl HW 55S	1,000-700,000	25-40	TH
Superdex 75 prep grade	3000-70,000	34	PB
Sephacryl S-200 HR	5,000-250,000	47	PB
Superose 6 prep grade	5,000-5,000,000	34	PB
Superdex 200 prep grade	10,000-600,000	34	PB
Sephacryl S-300 HR	10,000-1,500,000	47	PB
Sephacryl S-400 HR	20,000-8,000,000	47	PB
Sephacryl S-500 HR	20,000-30,000,000	47	PB
Toyopearl HW 65S	50,000-5,000,000	25-40	TH
Toyopearl HW 75S	500,000-50,000,000	25-40	TH

[a]Data as reported by manufacturers.
[b]Abbreviations: PB, Amersham Pharmacia Biotech; TH, Toso Haas. Addresses and phone numbers of suppliers are provided in the *SUPPLIERS APPENDIX*.

Gel filtration chromatography

Table 8.3.5 Empty Columns Suitable for Protein Fractionation or Size Determination[a]

Columns	Dimensions[b] (length × inner diameter, cm)	Maximum operating pressure (MPa)	Supplier[c]
HR series	20 × 0.5–50 × 1.6	3–5	PB
G series	25 × 1.0–100 × 4.4	3-7	AM
XK series	20 × 1.6–100 × 5	0.5	PB

[a]Data as provided by manufacturers. All columns are made of borosilicate glass.

[b]Column dimensions given here are those suitable for protein fractionation and size determination. Other column dimensions may also be available.

[c]Abbreviations: AM, Amicon; PB, Amersham Pharmacia Biotech. Addresses and phone numbers of suppliers are provided in the *SUPPLIERS APPENDIX*.

Table 8.3.6 Prepacked Columns for Protein Fractionation or Size Determination[a]

Column type[b]	Fractionation range (M_r)[c]	Particle size (μm)	Dimensions available (length × inner diameter, cm)	Supplier[d]
Superdex Peptide	100-7,000	13	30 × 1	PB
HiLoad Superdex 30 prep grade	200-10,000	34	60 × 1.6 60 × 2.6	PB
TSK SW 2000	500-60,000	10	30 × 0.75 60 × 0.75	TH
HiLoad Sephacryl S-100 HR	1,000-100,000	47	60 × 1.6 60 × 2.6	PB
TSK SW 3000	1,000-300,000	10	30 × 0.75 60 × 0.75	TH
Protein-Pak 60	2,000-30,000	10	30 × 0.78	WA
Superdex 75 HR 10/30	3,000-70,000	13	30 × 1	PB
HiLoad Superdex75 prep grade	3,000-70,000	34	60 × 1.6 60 × 2.6	PB
Bio-Sil SEC 125	5,000-100,000	10	60 × 0.75 60 × 2.15	BR
G2000SW_{XL}	5,000-150,000	5	30 × 0.75	TH
HiLoad Sephacryl S-200 HR	5,000-250,000	47	60 × 1.6 60 × 2.6	PB
TSK SW 4000	5,000-1,000,000	13	30 × 0.75 60 × 0.75	TH
Superose 6 HR 10/30	5,000-40,000,000	13	30 × 1	PB
Protein-Pak 125	10,000-80,000	10	30 × 0.78	WA
Glass GF-250	10,000-250,000	4	30 × 1	DU
Bio-Sil SEC 250	10,000-300,000	10	60 × 0.75 60 × 2.15	BR
G3000SW_{XL}	10,000-500,000	5	30 × 0.75	TH
Superdex 200 HR 10/30	10,000-600,000	13	30 × 1	PB
HiLoad Superdex 200 prep grade	10,000-600,000	34	60 × 1.6 60 × 2.6	PB
HiLoad Sephacryl S-300 HR	10,000-1,500,000	47	60 × 1.6	PB
G4000SW_{XL}	20,000-10,000,000	7	30 × 0.75	TH
Glass GF-450	25,000-900,000	6	30 × 1	DU

[a]Data as reported by the manufacturers.

[b]In general, matrices of natural polymers (e.g., Superdex, Sephacryl, or Superose) have larger pore volumes than silica-based materials (e.g., TSK SW, Protein-Pak, Bio-Sil SEC, or GF-250/450).

[c]Selectivity of materials decreases with increased width of the fractionation range and also with decreased pore volume.

[d]Abbreviations: BR, Bio-Rad; DU, DuPont; PB, Amersham Pharmacia Biotech; TH, Tosoo Haas; WA, Waters. Addresses and phone numbers of suppliers are provided in the *SUPPLIERS APPENDIX*.

Gel filtration chromatography

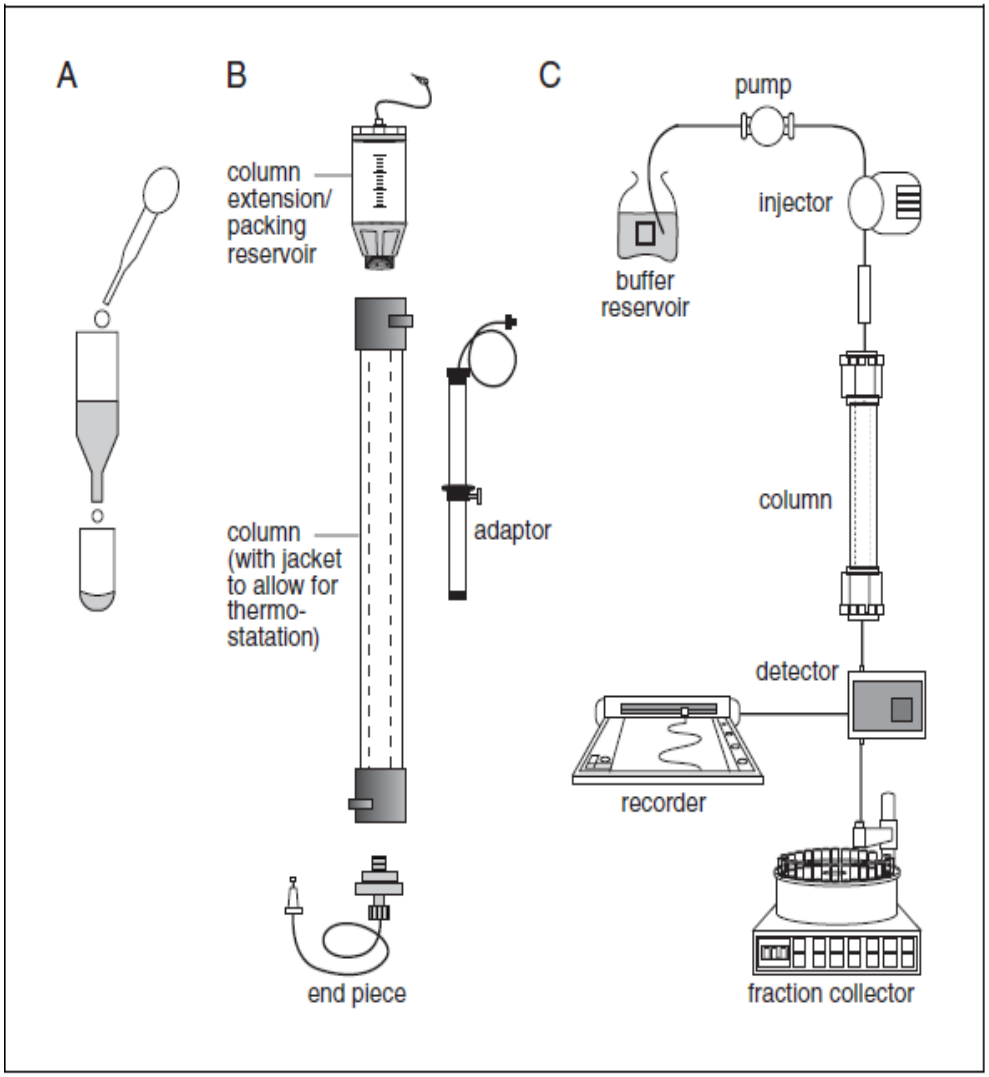


Figure 8.3.1 Equipment used for gel filtration. (**A**) Simple setup for desalting using an open-bed column made with a Pasteur pipet. (**B**) Column and attachments. (**C**) Complete automated chromatography system. Courtesy of Amersham Pharmacia Biotech.

Gel filtration chromatography

•**Sample preparation** : too high sample viscosity causes instability of the separation and an irregular flow pattern. This leads to very broad and skewed peaks and back pressure can increase.

•**Buffer preparation:** select a buffer and pH that are compatible with protein stability and activity and in which the product of interest should be collected.

•**Elution and flow rates**: use flow rates that allow time for molecules to diffuse in and out of the matrix in order to achieve a separation

•**Resolution** : the degree of separation between peaks of a gel filtration separation

Factors that influence the final resolution

Sample volume, the ration of sample volume to column volume, column dimensions, particle size, particle size distribution, packing density, pore size of the particles, flow rate, and viscosity of the sample and buffer

Gel filtration chromatography

Experimental tips

• Always use filtered buffers and samples to reduce the need for additional column maintenance

• Always use well degassed buffers to avoid the formation of air bubbles in the packed column during a run

• Buffers, pre-packed columns and samples should be kept at the same temperature to prevent air bubbles forming in the column

• Filter cleaning solutions before use and always re-equilibrate the column with 2-3column volumes of buffer before the next separation

Gel filtration chromatography

Column packing

1. If the gel matrix is a dry powder, allow the medium to swell in an appropriate buffer. (Avoid using magnetic stirrers, spatulas or glass rods since they may damage the matrix.)
2. we the bottom filter by injecting distilled water through the effluent tubing. Close the end piece outlet. Mount filter and bottom end piece onto the column
3. if the slurry volume is greater than the volume of the column, attach a packing reservoir to the column
4. mount column and packing reservoir vertically on a laboratory stand.
5. fill the column with distilled water or buffer to a height of approximately 2cm above the column end piece. Avoid air bubbles.
6. pour the well-mixed and well-degassed suspension in a single operation down the inside wall using a glass rod. Avoid introducing air bubbles.
7. Connect the pump outlet to the inlet of the packing reservoir. Open the column outlet and start the flow of buffer. Pass 2-3 column volumes of buffer through the column in order to stabilize the bed and equilibrate completely.

Gel filtration chromatography

Column packing

8. Maintain the packing flow rate for at least 3 column volumes after a constant bed height is obtained
9. Mark the bed height on the column and close the column outlet. Remove the packing reservoir
10. Add buffer carefully to fill the column and form an upward meniscus
11. Connect all tubings. Slacken the adaptor tightening mechanism and insert the adaptor at a n angle into the column so that no air is trapped under the net. Slide the adaptor slowly down the column until the mark is reached. Note that the outlet of the adaptor should be open and the column outlet should be closed.
12. Adjust the tightening mechanism to give a sliding seal between the column wall and o-ring. Screw the adaptor onto the column
13. Continue packing the column for approximately 10 minutes. Stop the pump, close the column outlet and move the top adaptor down onto the surface of the medium push the adaptor further 3mm into the medium the column is now ready for equilibration

Gel filtration chromatography

Procedure

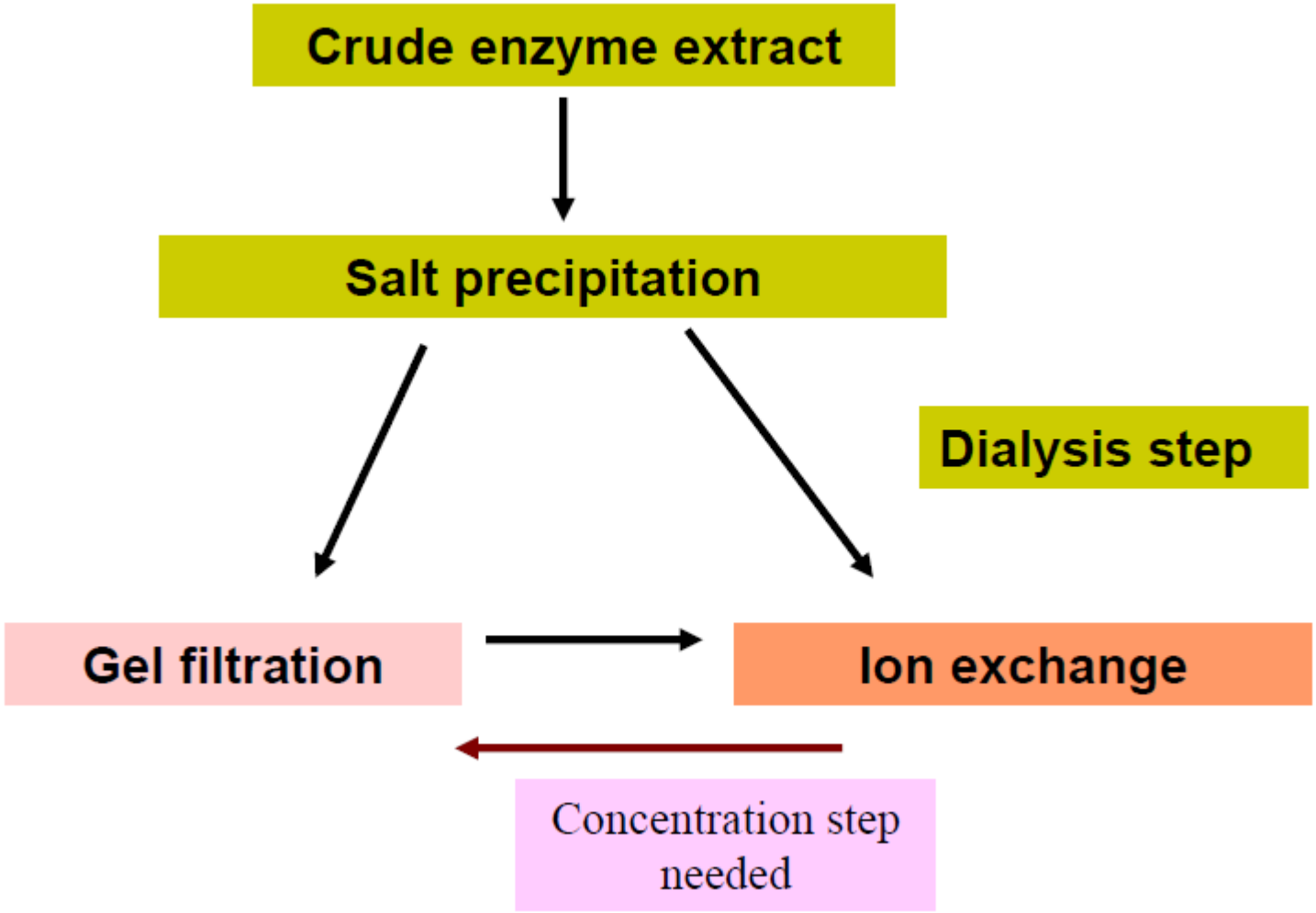
Separation

1. For first time use, or after long term storage, equilibrate the column with 1 column volume of buffer, but containing 0.05M NaCl at 30cm/h
2. Equilibrate with 2 column volumes of buffer containing 0.15M NaCl at 50 cm/h
3. Reduce linear flow to 3cm/h. Apply a sample volume equivalent to 0.5-4% of the column volume.
4. Elute with 1column volume of buffer
5. Before applying a new sample, re-equilibrate column with 1 column volume of buffer at 50cm/h and until the baseline monitored at A280 is stable

Cleaning

1. Wash with 1 column volume of 0.5M NaOH at a flow of 25cm/h to remove most non-specifically adsorbed proteins.
2. Wash with 1 column volume of distilled water at 25cm/h
3. Re-equilibrate with 2 column volumes of buffer at a flow of 50cm/h or until the baseline monitored at A280 and the pH of the eluent are stable.

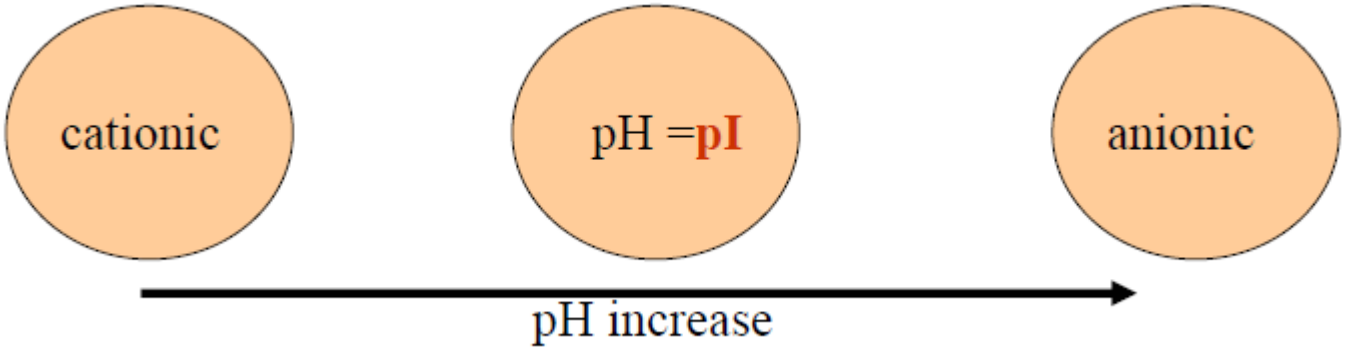
Ion-Exchange chromatography



Ion-Exchange chromatography

Principle of ion exchange chromatography

Proteins are charged molecules. At specific pH, it can exist in **anionic (-), cationic (+) or zwitterion (no net**charge) stage.

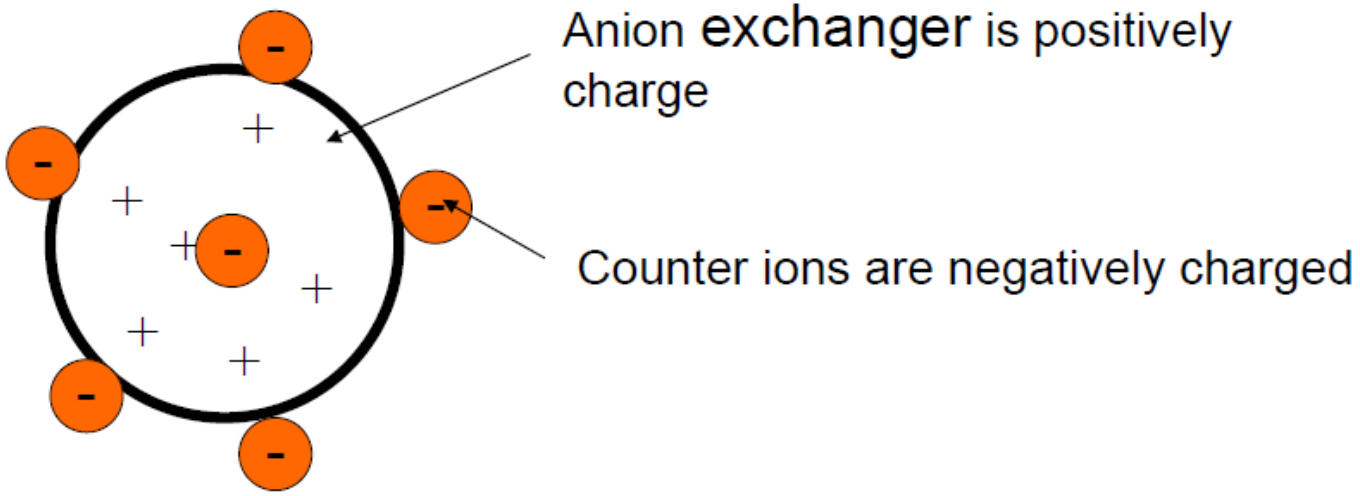


In Ion exchange chromatography separation is based on the charges carried by the protein molecules

Ion-Exchange chromatography

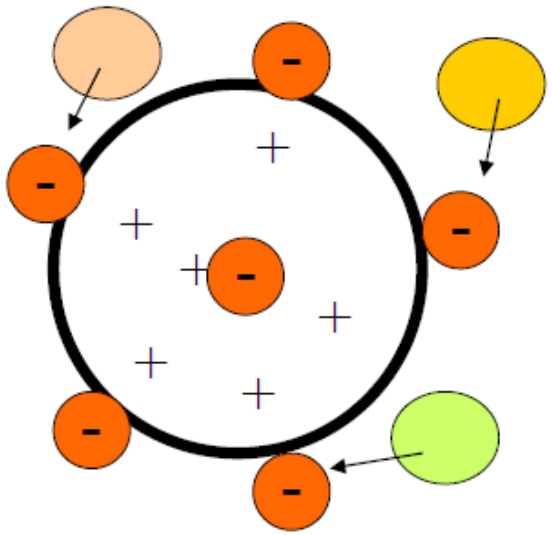
Anion exchange chromatography

Positively charged functional groups are bound to the insoluble matrix



Ion-Exchange chromatography

Anion exchange chromatography



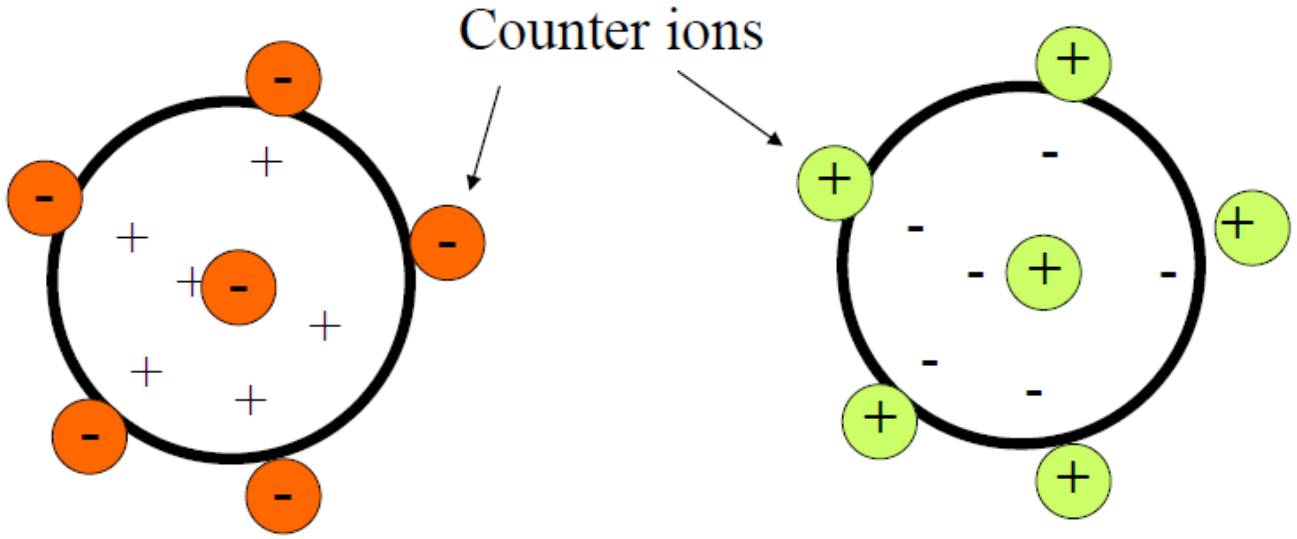
Negatively charged proteins can replace the counter ions and be bound to the ion exchanger.

Counter ions in the eluting buffer can then exchange for the protein species, thus releasing the proteins from the ion exchanger

Separation is based on the degree of of binding strength of the proteins to the ion exchanger

Ion-Exchange chromatography

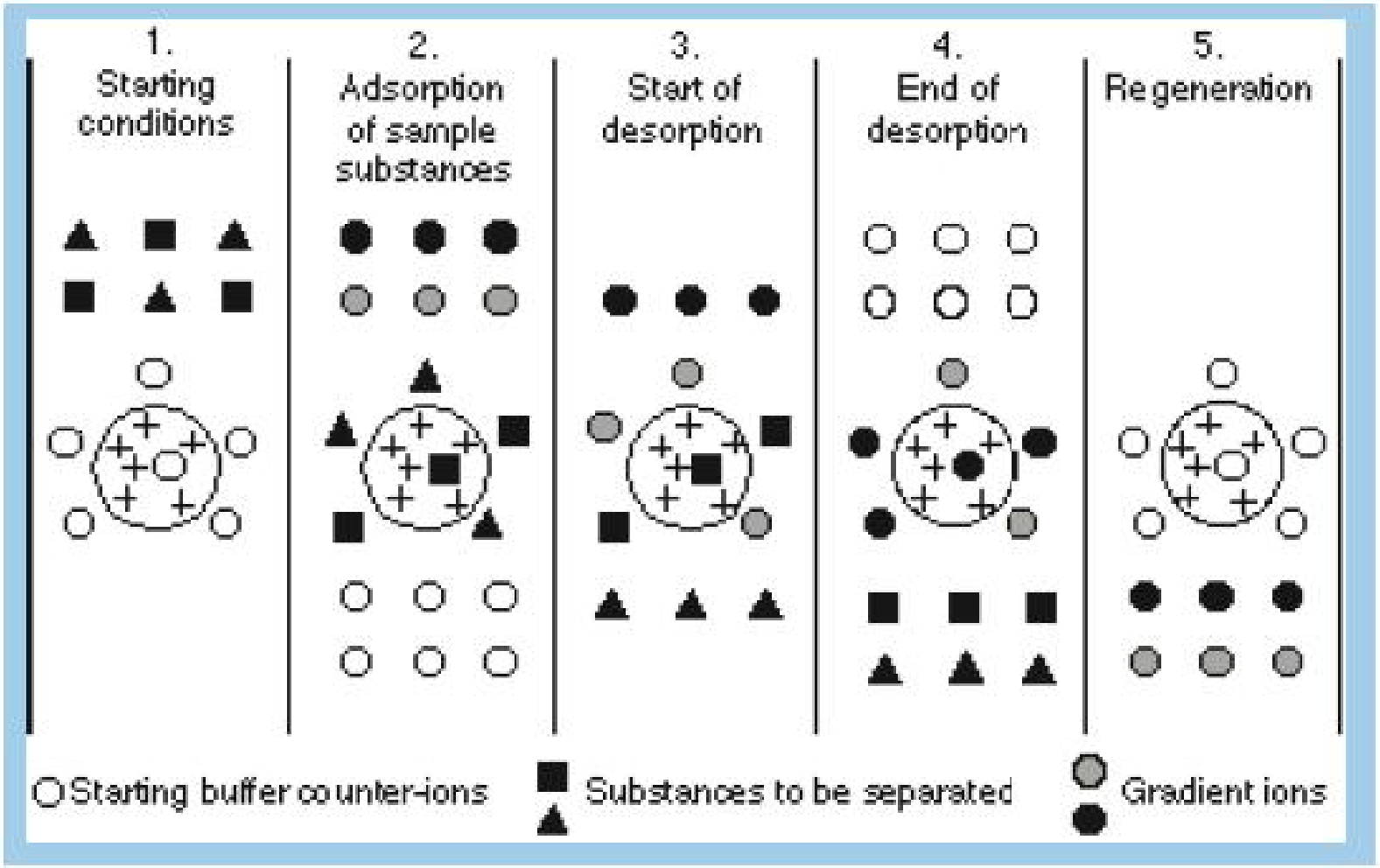
Ion exchangers – Functional groups



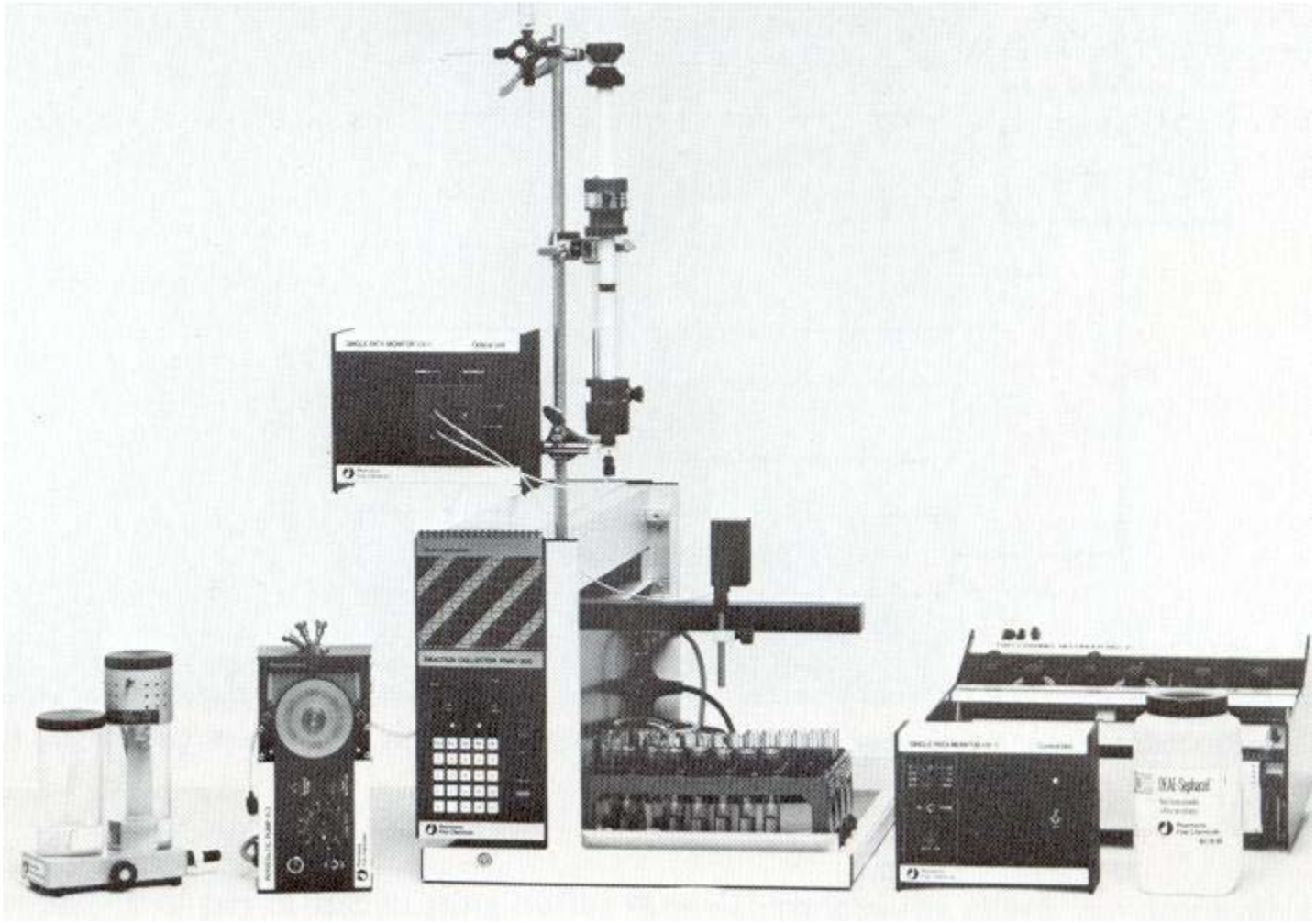
Anion exchanger
Aminoethyl (AE-)
Diethylaminoethyl (DEAE-)
Quaternary aminoethyl (QAE-)

Cation exchanger
Carboxymethyl (CM-)
Phospho
Sulphopropyl (SP-)

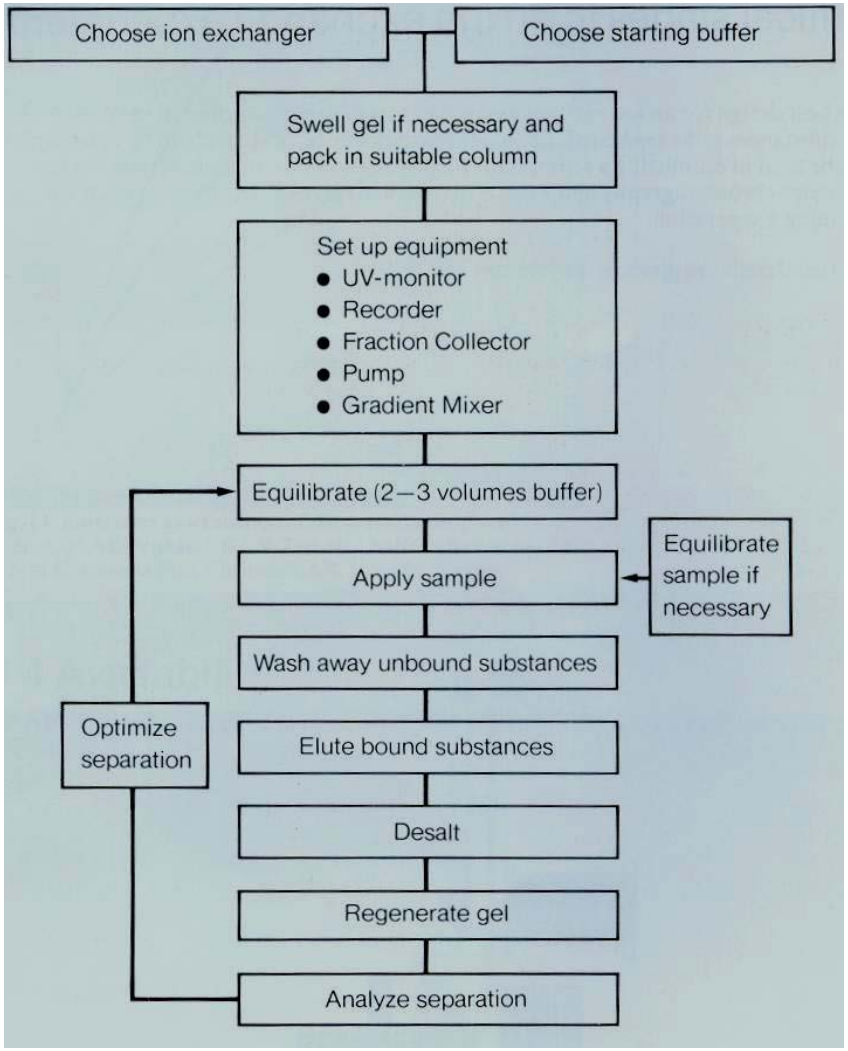
Ion-Exchange chromatography



Ion-Exchange chromatography



Ion-Exchange chromatography



Ion-Exchange chromatography

Steps

1. Sample application and adsorption and equilibration

2. Elution of column with specific buffers to achieve protein separation -salt gradient ionic strength gradient

3. Regeneration of ion-exchangers

Remarks

- Sample diffuses into ion exchanger surface and matrix
- Unbound proteins will be removed
- Weakly bound proteins will be eluted first, followed by those which are tightly bound
- Ion-exchanger can be reused

Ion-Exchange chromatography

Choosing your ion-exchanger: know your proteins

•Stability of proteins
stable below pI value, use **cation-exchanger**
stable above pI value, use **anion-exchanger**

•Molecular size of proteins
<10,000 mw, use matrix of small pore size
10,000-100,000 mw, use Sepharose equivalent grade

•Other specific requirements
Inactivation of specific buffer types, then limit choice of ionexchanger

Ion-Exchange chromatography

Strength and Capacity of Ion-exchangers

Strength

•determined by functional group
•strong or weak ion-exchangers -reference to extent of ionisation with pH
•strong - complete ionisation over a wide pH range

Example:
DEAE = weak ion-exchanger
SP = strong ion-exchanger

Capacity

•quantitative measure of ionexchange ability to bind ions
•depends on number of available functional group
•Information usually provided by manufacturer

Ion-Exchange chromatography

Choice of buffers. Use the correct buffers

Consideration	
Buffer types	
Cationic buffers with anion-exchangers →	DEAE: Tris, Imidazole, pyridine
Anionic buffers with cation-exchangers →	CM: acetate, citrate, phosphate
pH	
1 unit ± pI value of protein →	anion exchanger: 1 unit > pI cation exchanger: 1 unit< pI
facilitate binding	
ionic-strength →	determined experimentally
for separation	Salts used: NaCl, KCl

Ion-Exchange chromatography

Ionic strength of buffer & elution types

To achieve good separation, choice of buffer ionic strength is as important as choice of ion-exchangers

Initial strength: LOW ionic strength

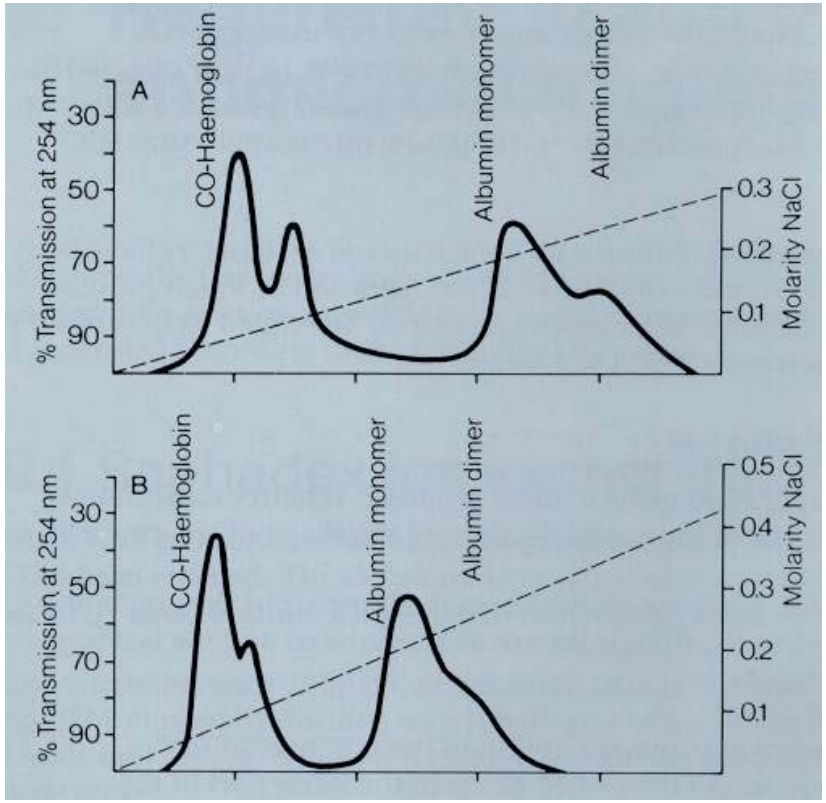
Different approaches taken for different purposes
isocratic elution
- ionic strength manipulated to elute or retain protein

gradient elution
- increasing ionic strength (low to high)
- continuous gradient
- step gradient

Ion-Exchange chromatography

Elution gradient and flow rates are important factors in ion-exchange chromatography

Gradient steepness can affect resolution

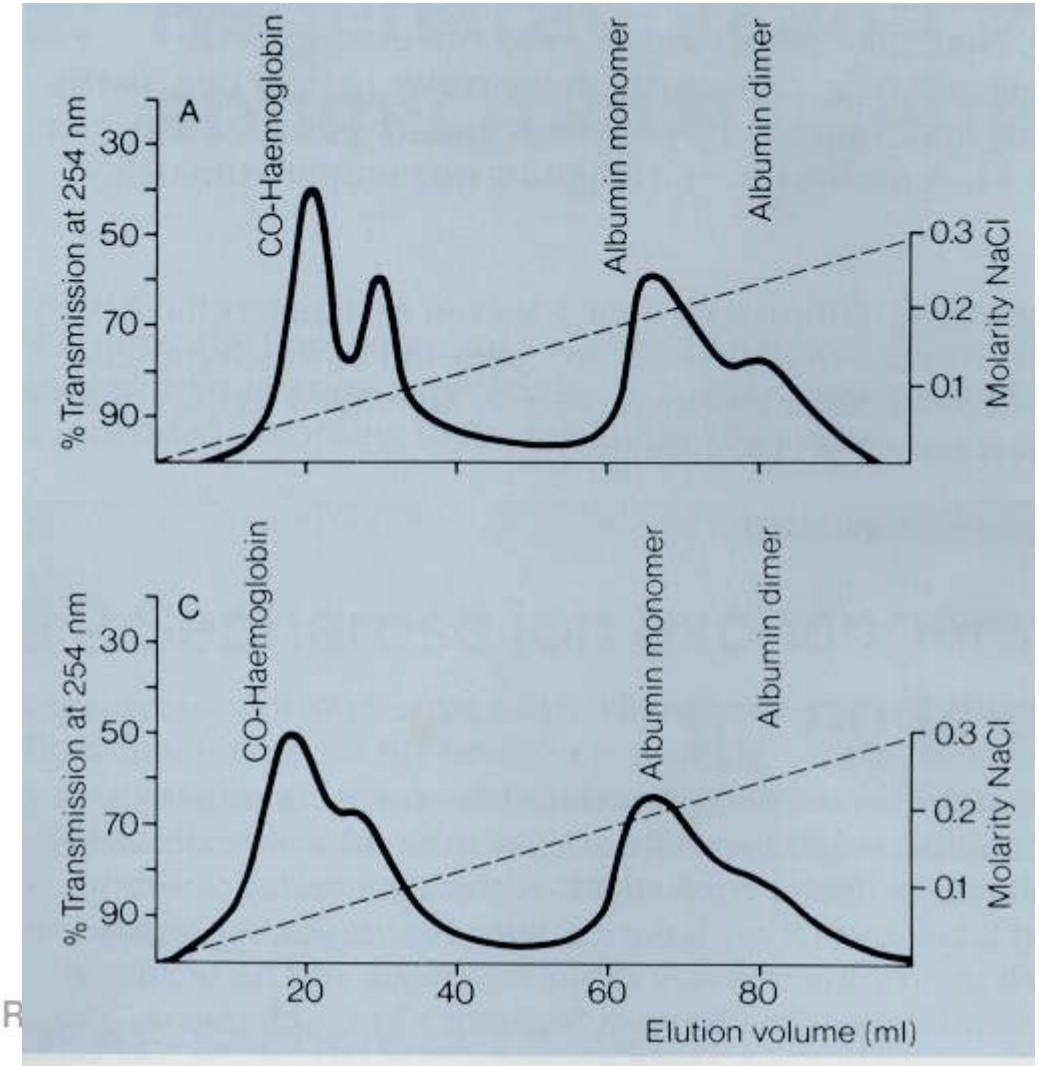


Flow rate 8ml/h
Gradient 0 to 0.3M NaCl

Flow rate 8ml/h
Gradient 0 to 0.4M NaCl

Ion-Exchange chromatography

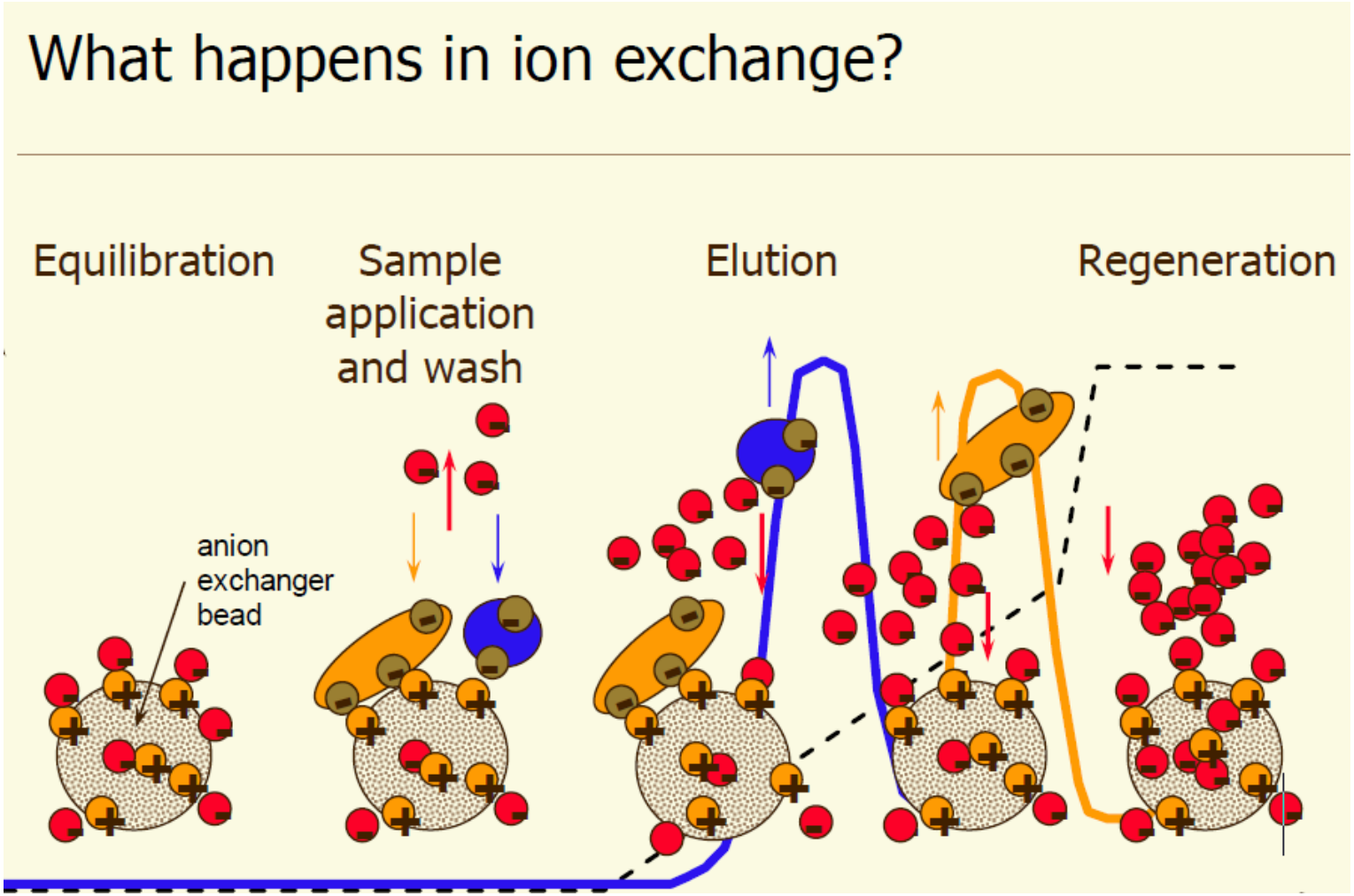
Same elution gradient but different flow rate



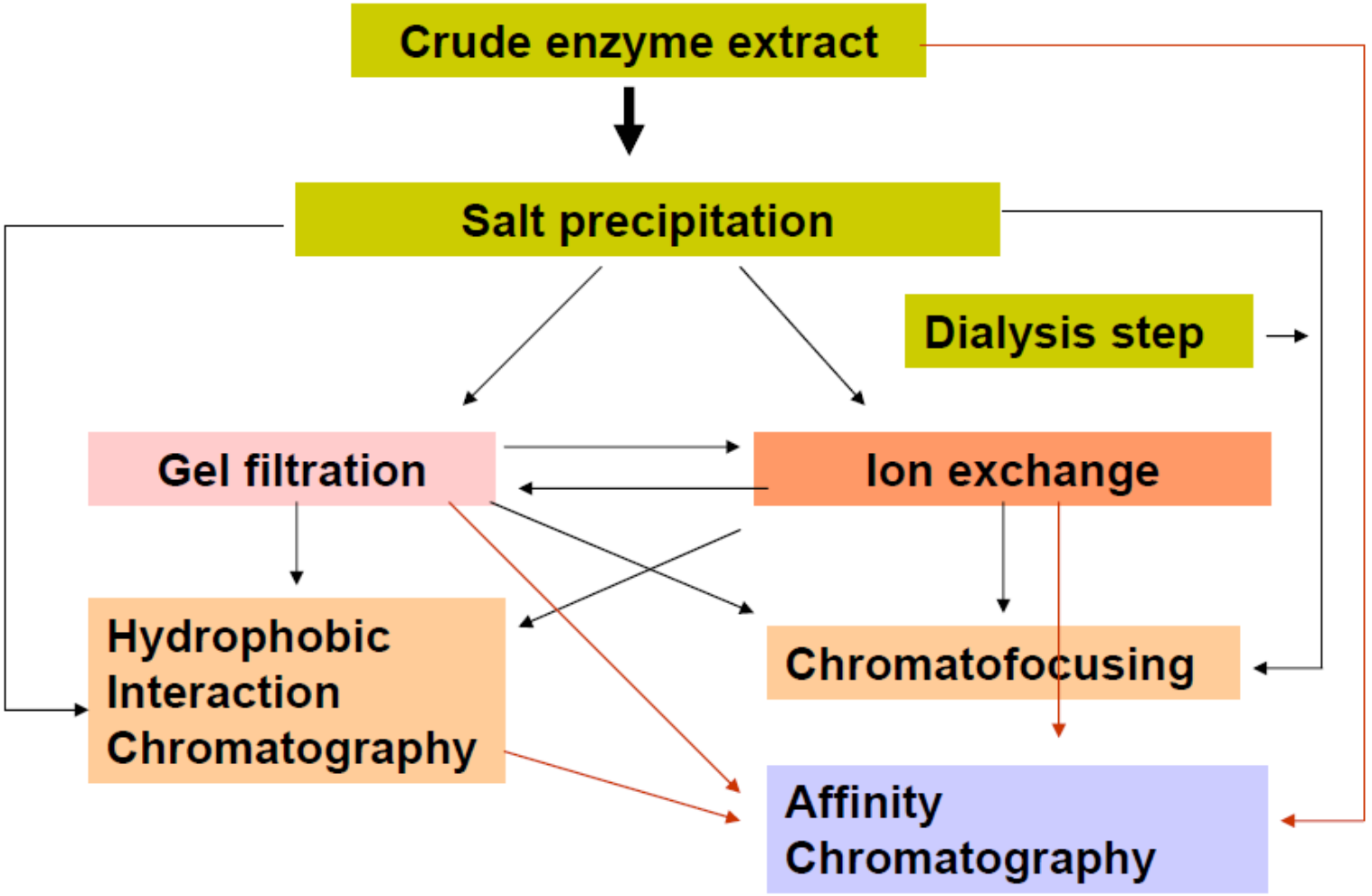
Flowrate 8 ml/h

Flowrate 20 ml/h

Ion-Exchange chromatography



Affinity chromatography



Affinity chromatography

- Separates proteins on the basis of a reversible interaction between a protein (or group of proteins) and a specific ligand coupled to a chromatography matrix

- Biological interactions between ligand and target molecule can be a result of electrostatic or hydrophobic interactions, van der Waals' forces and/or hydrogen bonding. To elute the target molecule from the affinity medium the interaction can be reversed, either specifically using a competitive ligand, or non-specifically, by changing the pH, ionic strength or polarity.

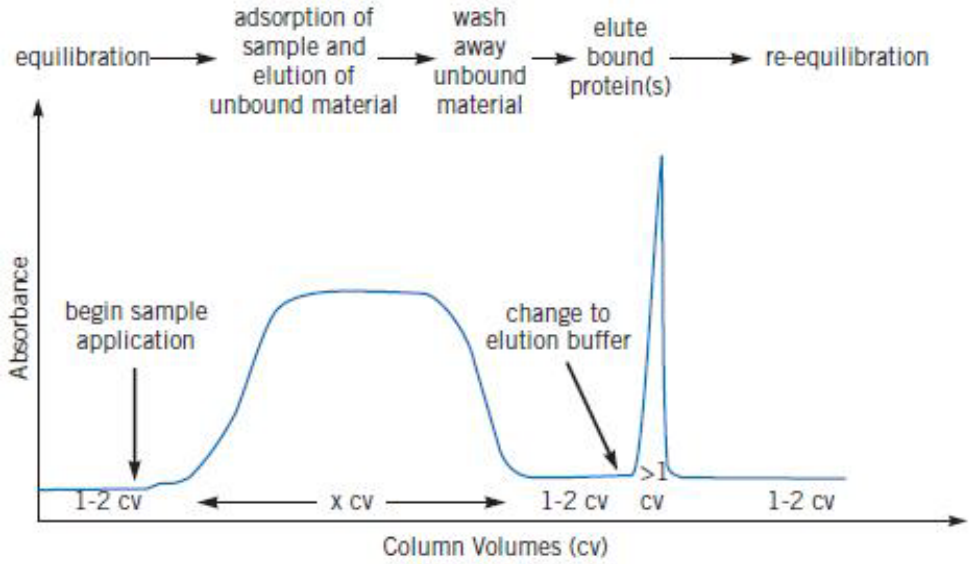
Affinity chromatography

1.

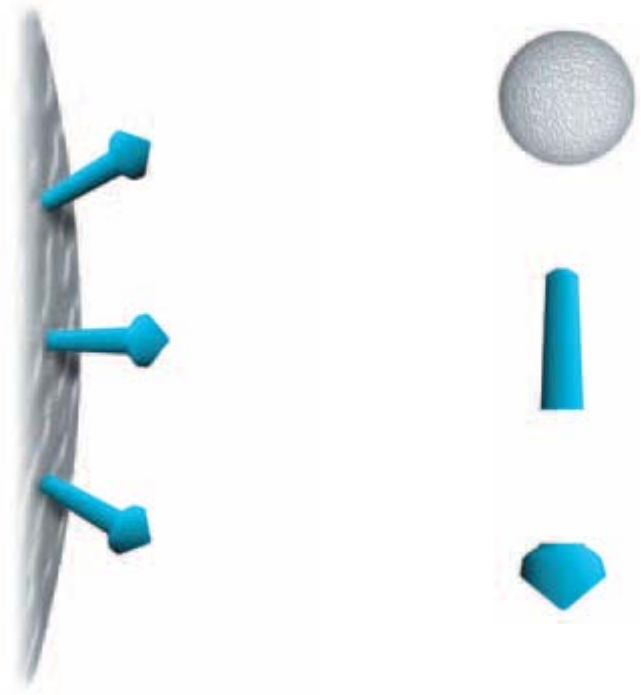
2.

3.

4.



Affinity chromatography



Matrix: for ligand attachment. Matrix should be chemically and physically inert.

Spacer arm: used to improve binding between ligand and target molecule by overcoming any effects of steric hindrance.

Ligand: molecule that binds reversibly to a specific target molecule or group of target molecules.

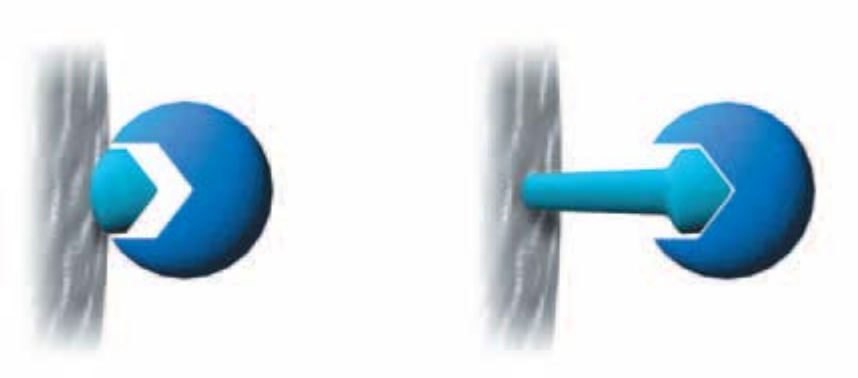
Ligand coupling: covalent attachment of a ligand to a suitable pre-activated matrix to create an affinity medium.

Pre-activated matrices: matrices which have been chemically modified to facilitate the coupling of specific types of ligand.

Affinity chromatography

Spacer arms

The binding site of a target protein is often located deep within the molecule and an affinity medium prepared by coupling small ligands, such as enzyme cofactors, directly to the matrix may exhibit low binding capacity



The ligand is unable to access the binding site of the target molecule

‘Spacer arm’ facilitates effective binding

Affinity chromatography

Elution methods

- Non-Selective
  - pH elution
  - Ionic strength elution

- Selective
  - Competitive elution

= Procedure for immuno-affinity chromatography

Affinity chromatography

Mouse Monoclonal IgG

Column: HiTrap Protein G HP, 1 ml or 5 ml

Recommended flow rates: 1 ml/min (1 ml column) or 5 ml/min (5 ml column)

Binding buffer: 0.02 M sodium phosphate, pH 7.0

Elution buffer: 0.1 M glycine-HCl, pH 2.7

Neutralization buffer: 1 M Tris-HCl, pH 9.0

1. Equilibrate column with 5 column volumes of binding buffer
2. Apply sample
3. Wash with 5-10 column volumes of the binding buffer to remove impurities and unbound material. Continue until no protein is detected in the eluent (determined by UV absorbance at 280nm)
4. Elute with 5 column volumes of elution buffer
5. Immediately re-equilibrate with 5-10 column volumes of binding buffer

Since elution conditions are quite harsh, it is recommended to collect fractions into neutralization buffer (60 µl – 200 µl 1 M Tris-HCl, pH 9.0 per ml fraction), so that the final pH of the fractions will be approximately neutral.

= Procedure for metal-chelating affinity chromatography

Poly(his) fusion protein

Affinity chromatography

Nickel solution: 0.1M NiSO4

Binding buffer: 20mM sodium phosphate, 0.5M NaCl, 10mM imidazole, pH 7.4

Elution buffer: 20mM sodium phosphate, 0.5M NaCl, 500mM imidazole, pH 7.4

1. Wash the column with 5 column volumes of distilled water
2. Load 0.5 column volumes of the 0.1M nickel solution onto the column
3. Wash with 5 column volumes of distilled water
4. Equilibrate the column with 10 column volumes of binding buffer
5. Apply sample at a flow rate 1-4ml/min or 5ml/min. collect the flow-through fraction
6. Wash with 10 column volumes of binding buffers. Collect wash fraction
7. Elute with 5 column volumes of elution buffer. Collect eluted fractions in small fractions such as 1ml to avoid dilution of the eluate
8. Wash with 10 column volumes of binding buffer. The column is now ready for a new purification and there is rarely a need to reload with metal if the same (His)6 fusion protein is to be purified

Application of column chromatography in protein purification

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ARTICLE

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Purification and Biochemical Characterization of Brazil Nut (*Bertholletia excelsa* L.) Seed Storage Proteins

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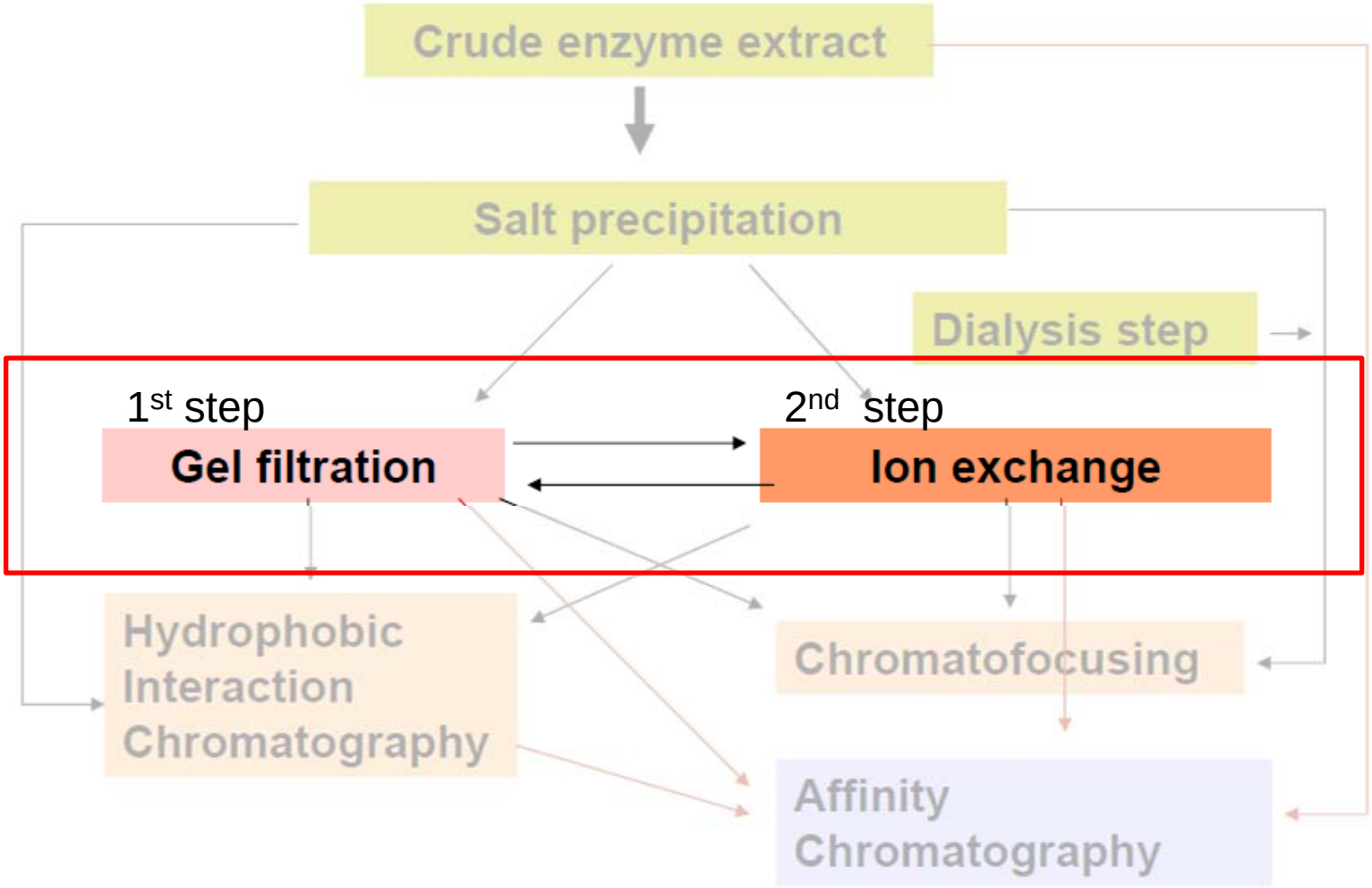
Application of column chromatography in protein purification



Application of column chromatography in protein purification

Purification of BN Seed Storage Proteins.

Defatted Brazil nut flour (2 g) was dispersed in 20 mL of 0.035M phosphate buffer containing 1M NaCl, pH7.5, by continuous mixing for 1 h at room temperature (25 ℃) to **solubilize flour proteins**.

The slurry was centrifuged at 27000g for 20 min at 4 ℃, and the **supernatant was loaded on a Sephacryl S200 column (2.6x72 cm)** previously equilibrated with 0.035M phosphate buffer, pH7.5, containing 1M NaCl.

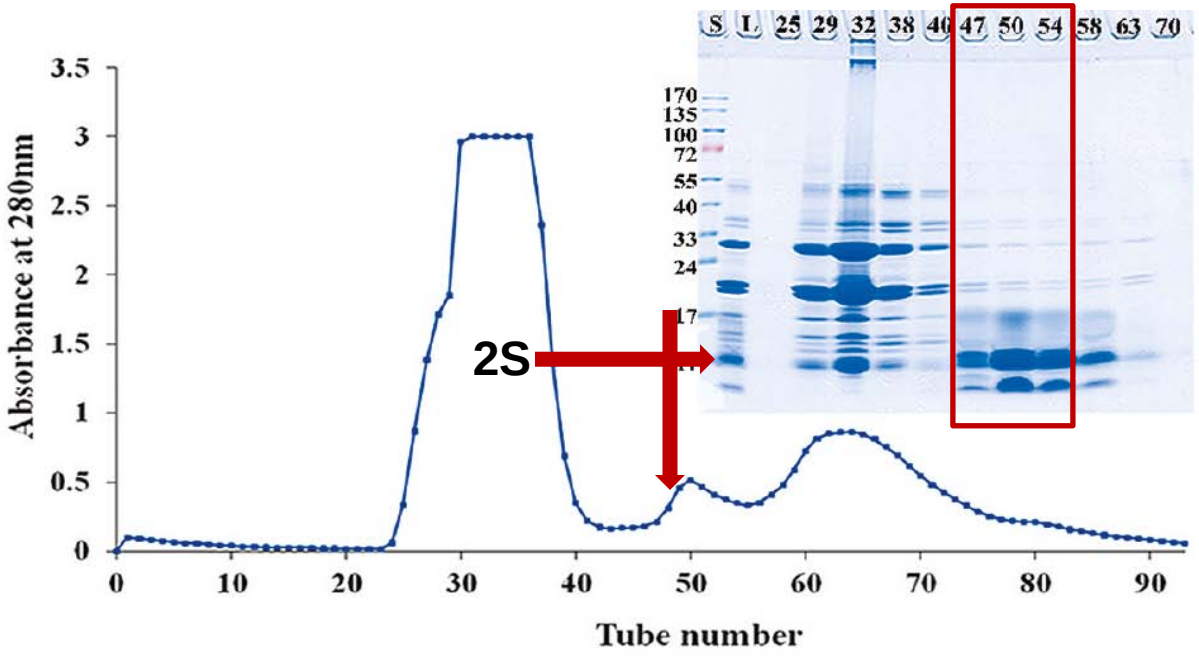
The flow rate of the column was maintained at 24 mL/h, and fractions were collected every 15 min.

All protein purification steps were done at 4 ℃, and proteins eluted from the columns were monitored by measuring absorbance at 280 nm and electrophoresis of aliquots from the select column fractions.

Application of column chromatography in protein purification

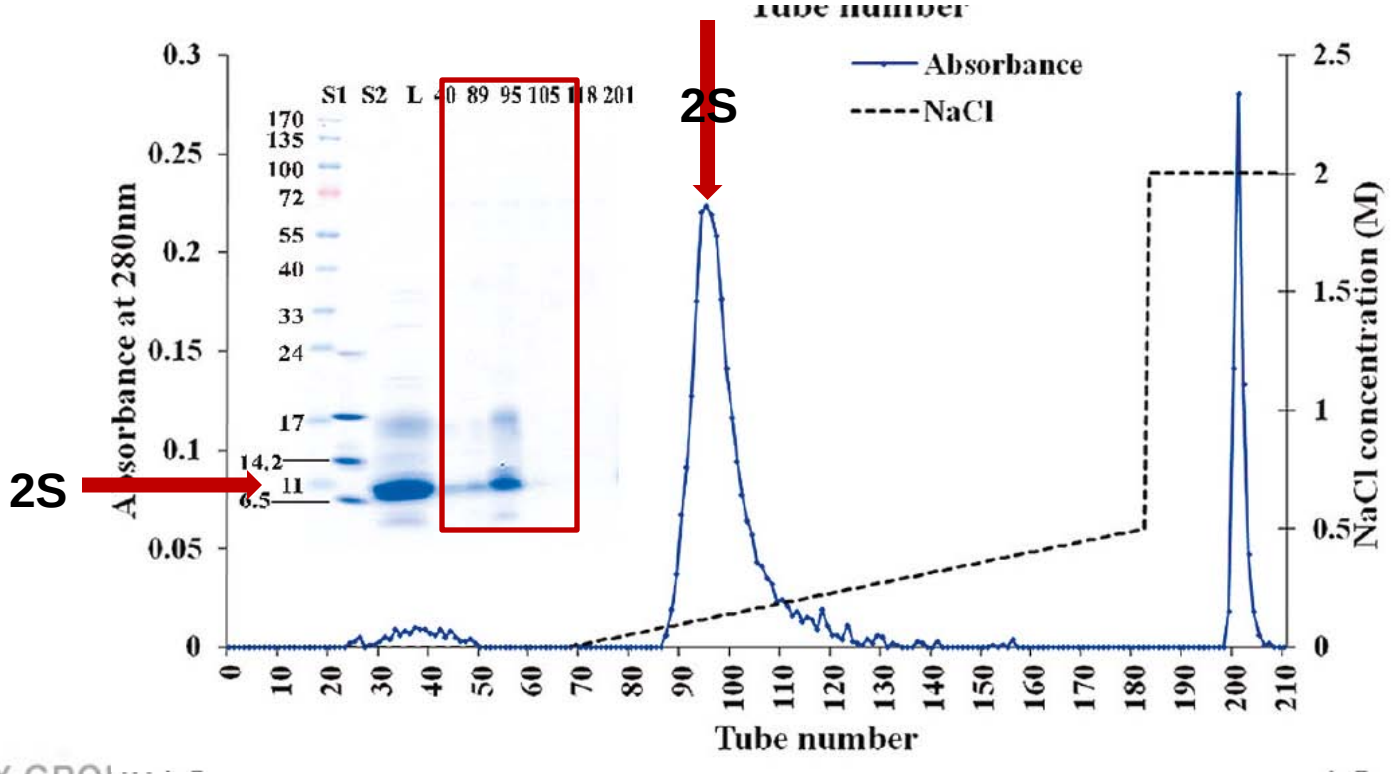
2S Albumin

The gel filtration peak rich in **2S albumin** (**tubes 47-55) was pooled and dialyzed against 0.02M Tris-HCl, pH 8.1, for 48 h with six buffer changes (3 L per change); the dialysate was loaded onto a DEAE DE-53 column (2.6x23 cm) equilibrated with 0.02M Tris-HCl, pH 8.1.**



Application of column chromatography in protein purification

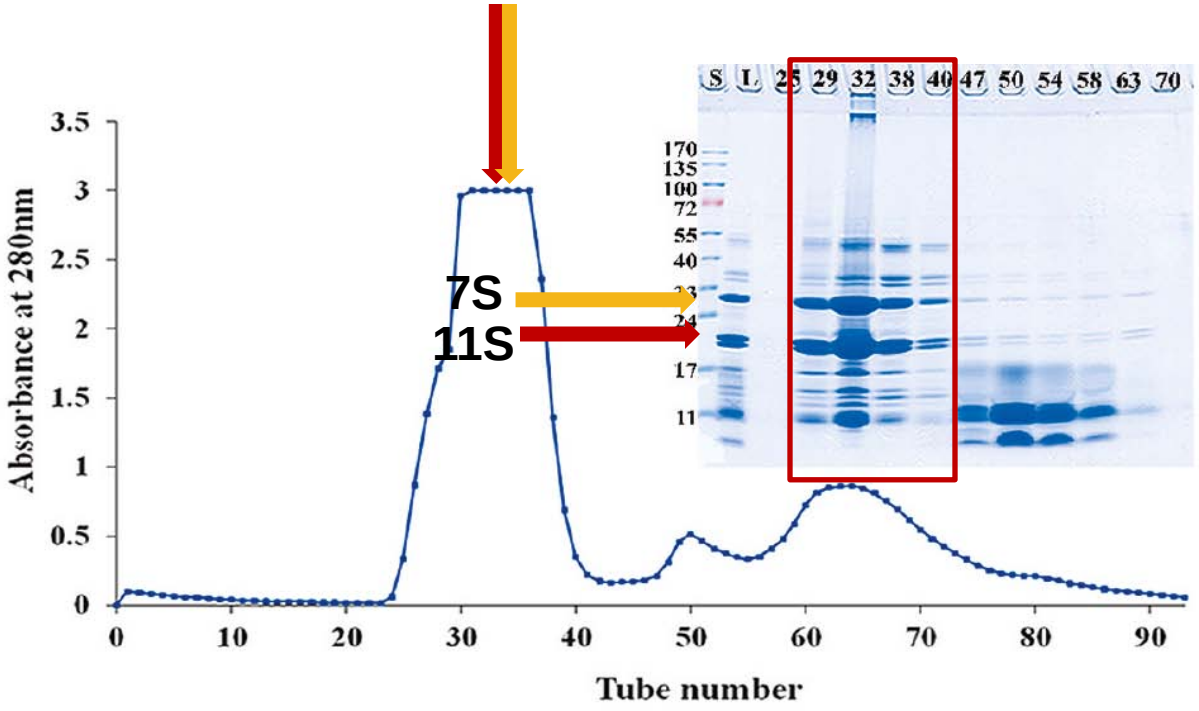
The column was flushed with the equilibration buffer until the absorbance at 280 nm returned to baseline. Adsorbed proteins were eluted with a 0-0.5 M NaCl gradient in the equilibration buffer (400 mL each).
The fractions containing **2S were pooled (tubes 89-105), dialyzed against distilled (DI) water for 48 h with six water changes (5 L per change), and lyophilized**.



Application of column chromatography in protein purification

7S Vicilin and 11S Legumin

The gel filtration peak rich in **7S and 11S globulins (tubes 26-40) was pooled, dialyzed against DI water for 48 h with six water changes, and lyophilized.** The lyophilized globulin fraction was resuspended in 0.02MTris-HCl, pH 8.1, containing 0.1 M NaCl for 1 h and centrifuged at 27000g for 20 min to remove insoluble aggregates. **The supernatant was loaded onto a DEAE DE-53 column (2.6 X19 cm) equilibrated with 0.02 M Tris-HCl, pH 8.1, containing 0.1 M NaCl**.



Application of column chromatography in protein purification

The column was flushed with the equilibration buffer until the absorbance at 280 nm of the effluent returned to the baseline. The column was subsequently eluted with a 0.1-0.4 M NaCl gradient in the equilibration buffer (250 mL each). The column flow rate was 24 mL/h, and fractions were collected every 15 min.

The peaks corresponding to 7S (tubes 12-23, Figure 1C) and 11S (tubes 52-66) were pooled separately, dialyzed againstDI water