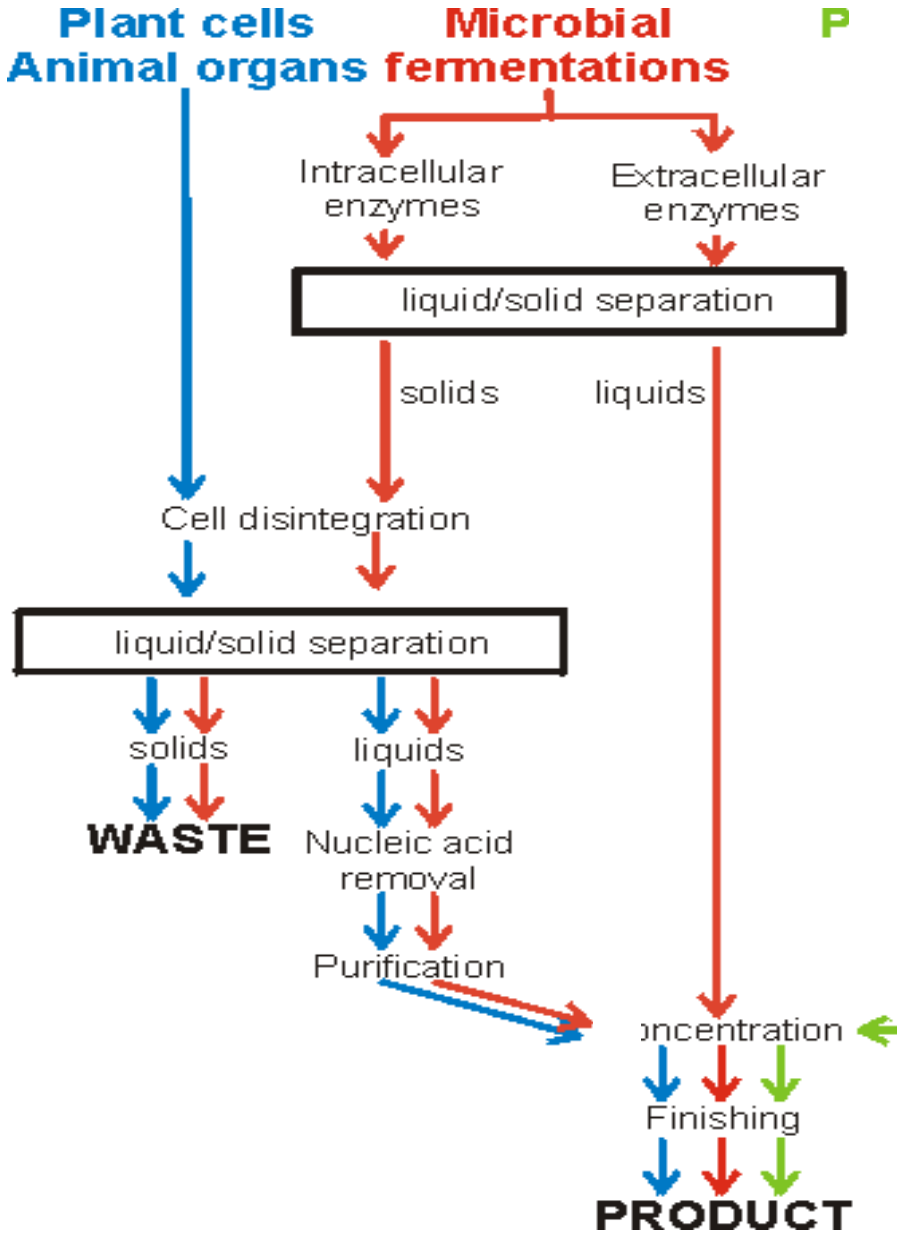
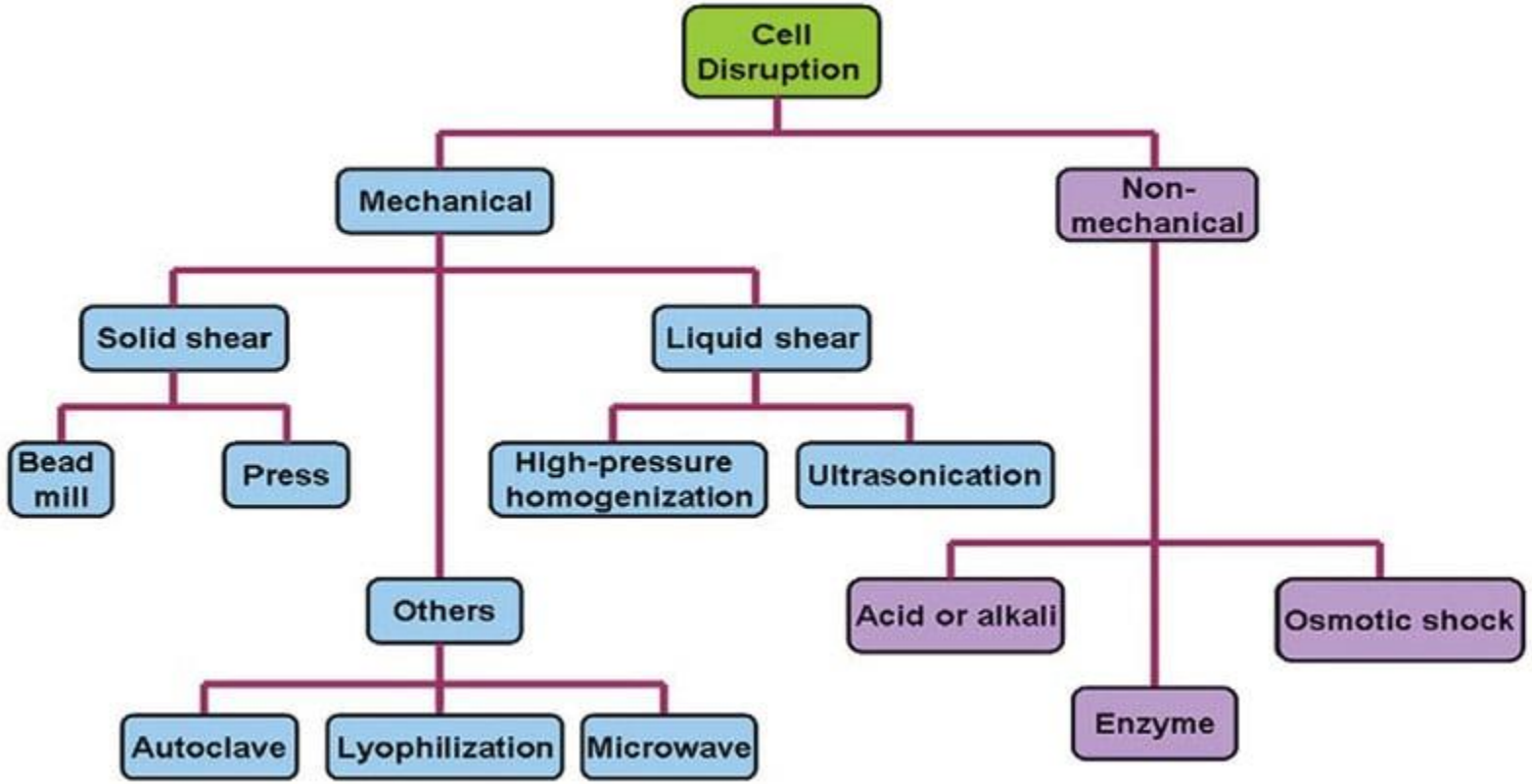
M2/L3,4: Purification of proteins

Ravikrishnan Elangovan,
Department of Biochemical Engg and Biotechnology
Indian Institute of Technology - Delhi

Enzyme Production



Cell lysis


Cell Disruption
Mechanical
Non-mechanical
Solid shear
Liquid shear
Bead mill
Press
High-pressure homogenization
Ultrasonication
Others
Acid or alkali
Osmotic shock
Autoclave
Lyophilization
Microwave
Enzyme

Concentration and Primary Purification

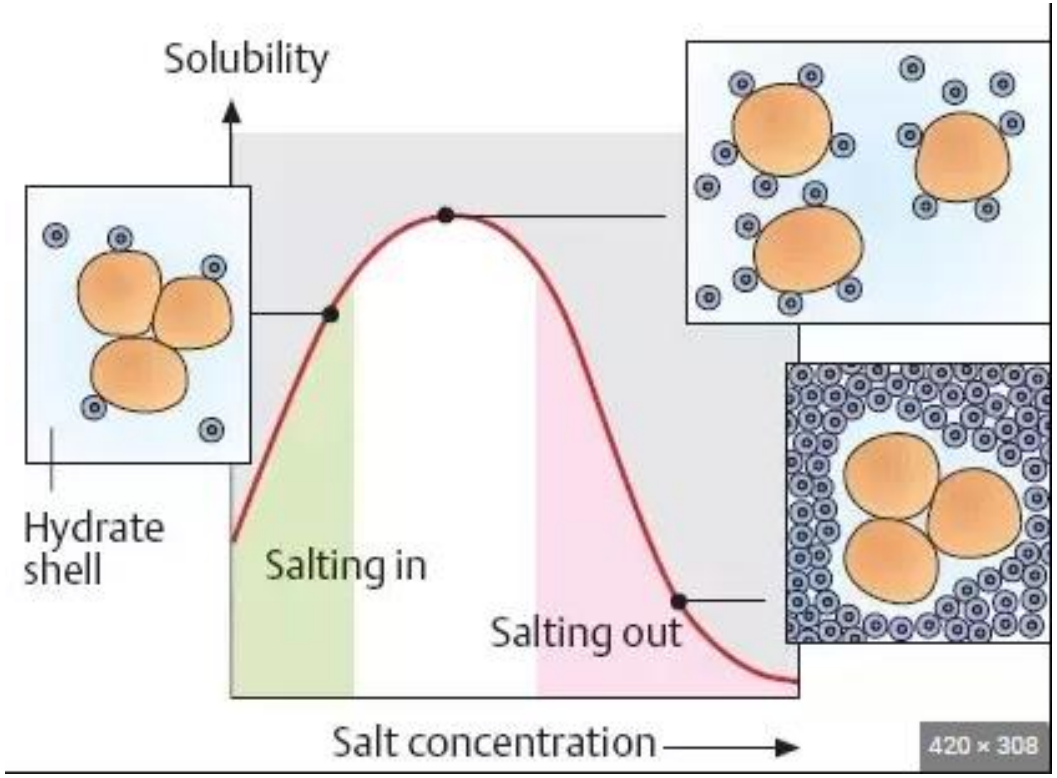
Large volumes of dilute solution ⇒ Manageable amount

In laboratory scale:

- Ultrafiltration
- Precipitation
- Ion-exchange
- Dialysis
- Freeze drying
- Addition of dry Sephadex G-25

Concentration by precipitation-1

Precipitation method	Example
Addition of neutral salts	Ammonium sulphate
Addition of organic solvents	Ethanol or acetone
Addition of organic polymers	Polyethylene glycol
Affinity precipitation	Addition of ligand, antibody
Adjustment of solution pH	Changing the pH of solution to pI value of desired protein
Selective denaturation	If desired protein is more stable than contaminating protein in denaturing conditions



Concentration by precipitation-2

One of the oldest methods

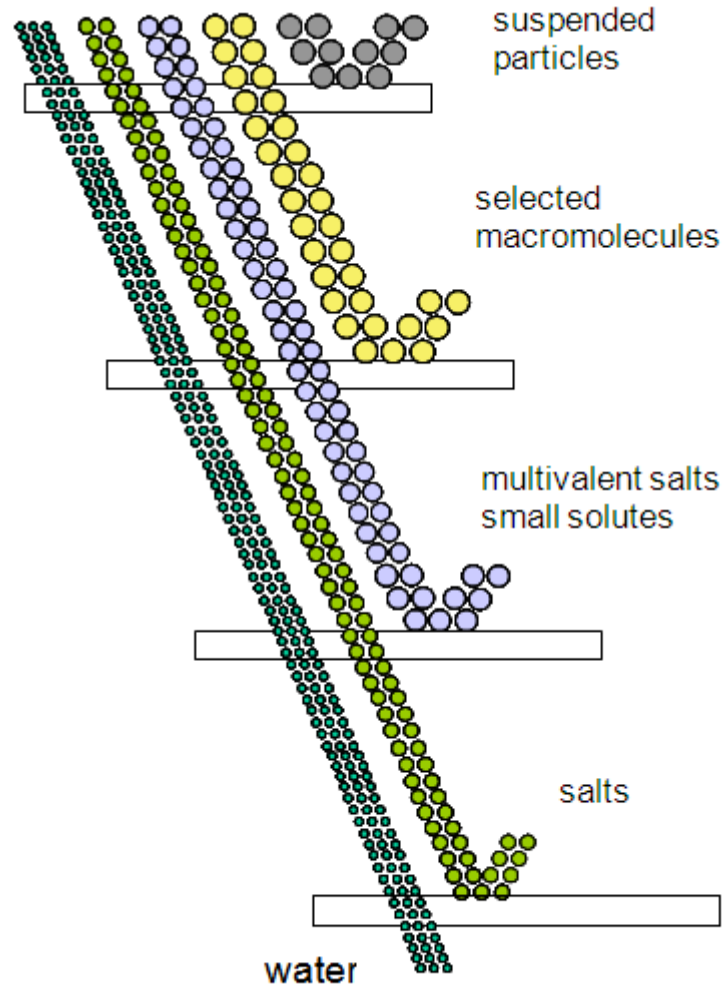
☺ Straight forward to perform
☺ Uncomplicated equipment
☺ High recovery of biological activity

☹ Many precipitants are highly corrosive
☹ Inefficient if initial protein concentration is low
☹ Some precipitants are highy inflammable, some are expensive
☹ Many precipitants must be disposed carefully
☹ In many cases, precipitant must be removed totally

Concentration by ultrafiltration-1

- Most widely applied method both in laboratory and industrial scale
- Ultrafiltration membranes (pore diameters: 1 – 20 nm)
- Molecular mass cut-off: 1 – 300 kDa (globular proteins)
- Traditional materials: cellulose acetate and cellulose nitrate
- Nowadays: PVC and polycarbonate
- Concentration polarization can be a problem...

MF	0.1 - 3 bar 0.1 - 5 µm
UF	2 - 10 bar 20 nm - 0.1 µm
NF	5 - 30 bar » 1 nm
RO	10 - 100 bar 0.1 - 1 nm (close)



Concentration by ultrafiltration-2

☺ Gentle

☺ High recovery rates (even > 99 %)

☺ Quick

☺ Little ancillary equipment is needed

☺ Some degree of protein purification

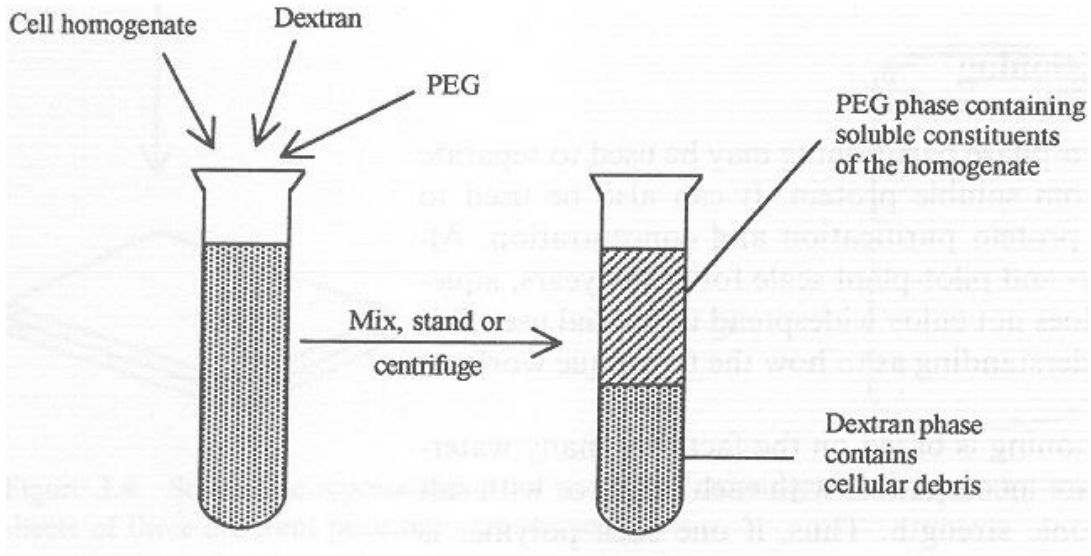
☹ Susceptibility to rapid membrane clogging

Removal of Whole Cells and Debris-2

- **Aqueous two-phase partitioning**

☺ Gentle
☺ Stabilization of proteins
☺ Yield of protein activity high
☺ Easy scale-up

☹ Empirical



Removal of Whole Cells and Debris-3

- **Removal of nucleic acids**
  - Liberation of large amounts of nucleic acids increases viscosity of cellular homogenate

    $\Rightarrow$ difficult to process
  - Nucleic acid removal is especially important in the preparation of therapeutic proteins
  - Methods: precipitation (by polyethylenimine) or treatment with nucleases

- **Removal of lipids**
  - It is a contaminant and can interfere with subsequent purification steps
  - Removal: Glass wool or a cloth of very fine mesh size

Column Chromatography

Separation of different protein types from each other according to their differential partitioning between two phases:

1. A solid stationary phase
2. A liquid mobile phase

Separation based on size and shape, overall charge, presence of surface hydrophobic groups, and ability to bind various ligands

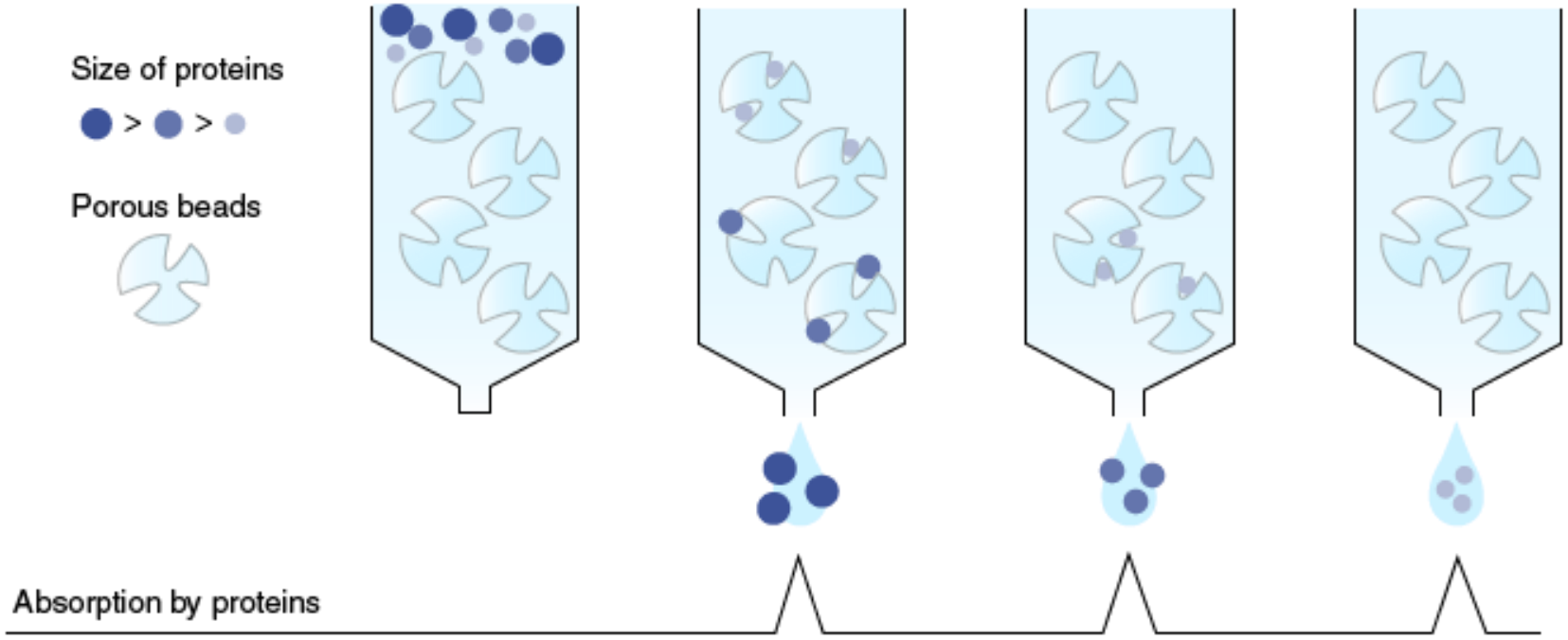
Different Chromatographic Techniques

Technique	Basis of separation
Ion exchange chromatography	Differences in protein surface charge at a given pH
Gel filtration chromatography	Differences in mass or hydrodynamic radius of different proteins
Affinity chromatography	Specific interaction between desired protein and appropriate ligand
Hydrophobic interaction chromatography	Differences in surface hydrophobicity of proteins
Chromatofocusing	Separates proteins on the basis of their isoelectric points

Gel Filtration Chromatography-1

- Also named as Size Exclusion Chromatography
- Separation based on size and shape
- Porous gel matrix in bead form is used:
  e.g. xlinked dextran, agarose, acrylamide
- Large proteins come first....

Gel Filtration Chromatography-2



Gel Filtration Chromatography-3

EXAMPLES

- **Sephadex:** dextran based, G-25 to G-200
- **Sephacryl:** allyl dextran based, more rigid and physically stable so suitable for large scale
- **Sepharose:** agarose based, lack of physical stability
- **Bio-Gel P:** acrylamide based

  A: agarose based
- **Fractogel:** A copolymer, very high degree of mechanical stability

Gel Filtration Chromatography-4

- Long chromatographic columns are needed (length/width = 25-40)
- Rarely employed during the initial stages

☹ Protein solution is significantly diluted

☹ Column flowrates are often considerably lower

Ion-Exchange Chromatography-1

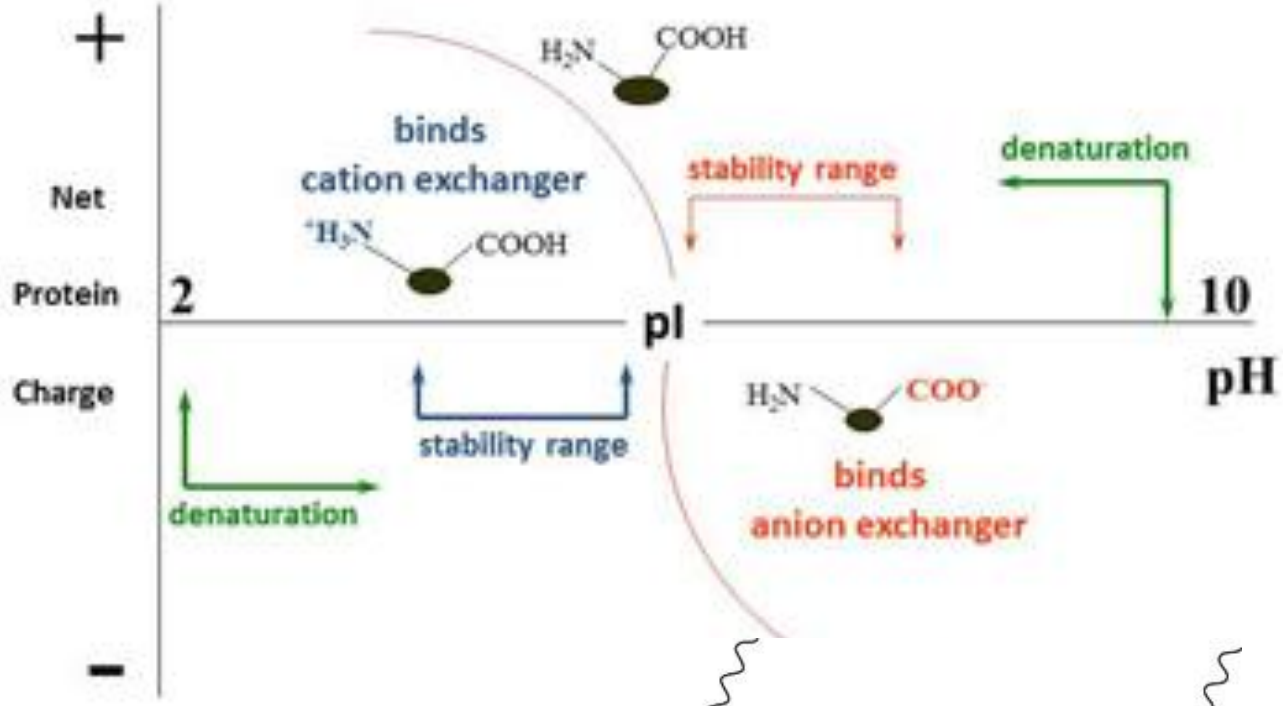
FACTS

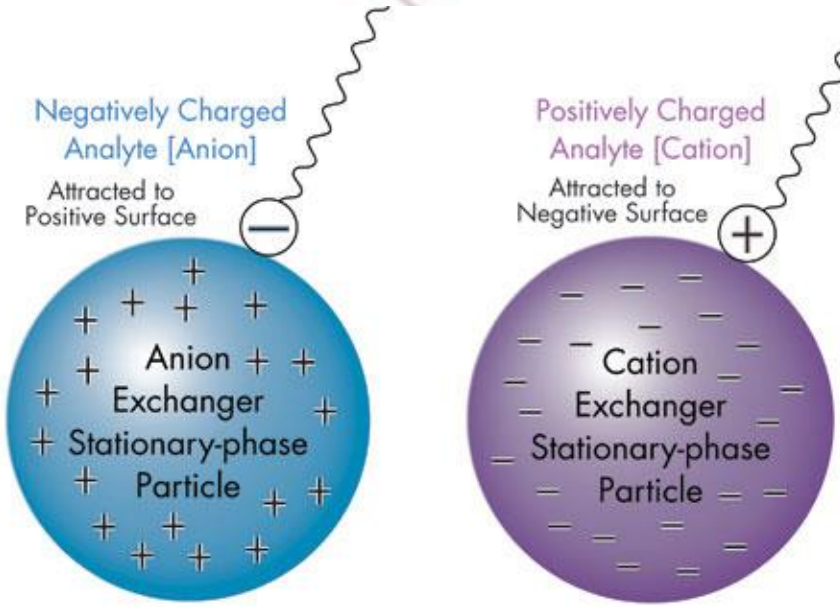
- Proteins possess both (+) and (-) charges
- At pH=7:
  - Aspartic and glutamic acid have negatively charged side groups
  - Lysine, arginine, histidine have positively charged side groups
- pH of medium vs. pI of protein

Ion-Exchange Chromatography-2

PRINCIPLE

- Reversible electrostatic attraction of a charged molecule to a solid matrix possessing opposite charge
- Elution is done by increasing salt concentration or changing pH





Ion-Exchange Chromatography-3

- Single most popular chromatographic technique...

☺ High level of resolution
☺ Easy scale-up
☺ Ease of usage
☺ Easy column regeneration
☺ One of the least expensive

Ion-Exchange Chromatography-4

Inert, rigid and porous matrix materials are desirable

EXAMPLES

- Cellulose-based
- Improved cellulose-based, e.g. diethylaminoethyl (DEAE) Sephacel
- Sephadex; charged groups attached to Sephadex G-25 or G-50
- Based on polymers: Agarose and Sepharose
- Alternative one: tentacle type

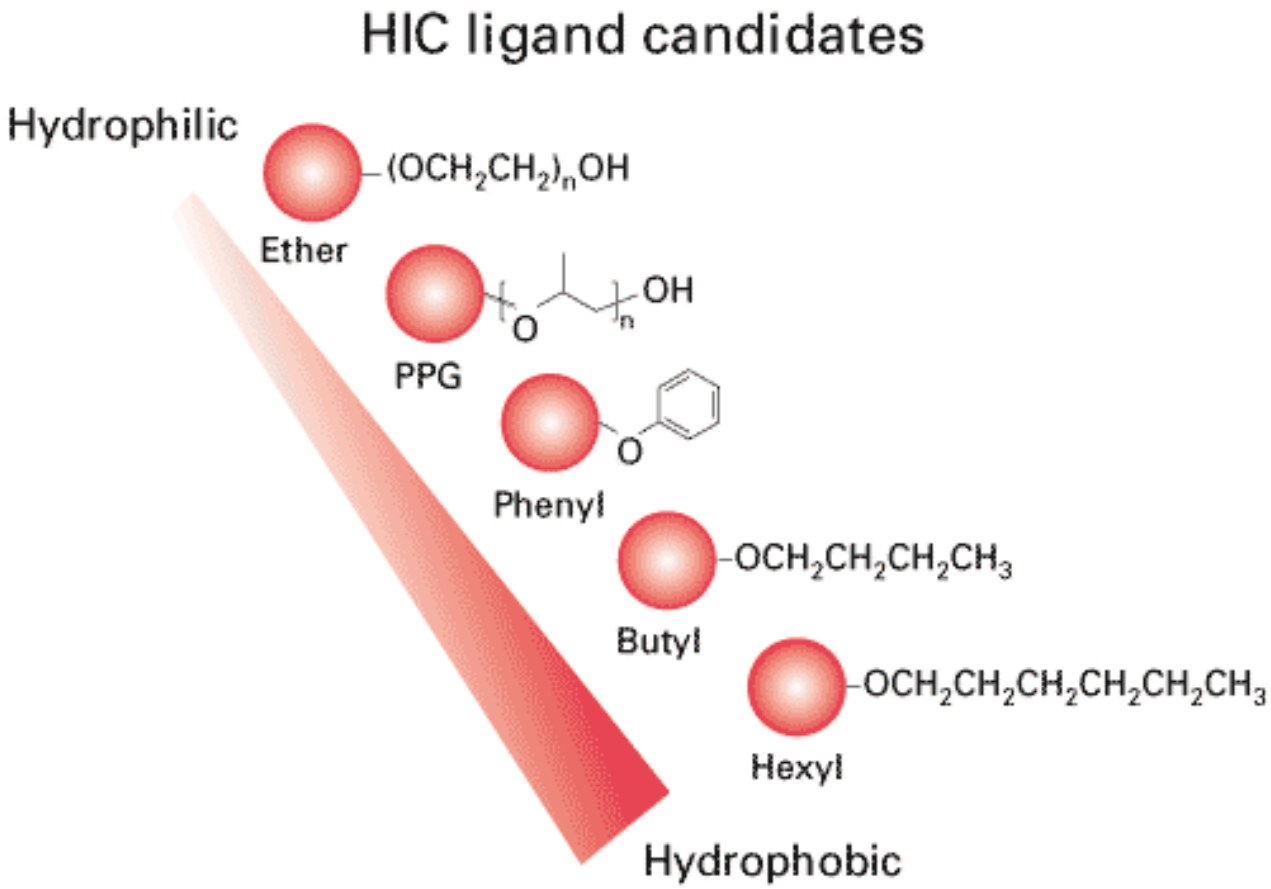
Hydrophobic Interaction Chromatography-1

- 8 out 20 commonly found amino acids in proteins are classified as hydrophobic
- In most proteins the majority of hydrophobic residues are buried inside the protein
- Different proteins have different degree of hydrophobic surface

Hydrophobic Interaction Chromatography-2

EXAMPLES

- Most popular resins are hydrophobic group attached xlinked agarose gels
  - e.g. octyl- and phenyl-Sepharose gels



Affinity Chromatography-1

- Described as the most powerful highly selective method
- It relies on the ability of most proteins to bind specifically and reversibly to their ligands
- Generally used in late purification steps

Affinity Chromatography-2

- Biospecific affinity chromatography
  - **General ligand approach:** Cofactors (NAD^+) or lectins
  - **Specific ligand approach:** enzyme substrates, substrate analogues or inhibitors, antibodies
- Pseudoaffinity chromatography: e.g. Dye affinity chromatography

Affinity Chromatography-3

Biospecific Affinity Chromatography

- Choice of affinity ligand
  Specificity, reversible binding, stability

- Choice of support matrix
  Stability, rigidity, inertness, porosity, derivatizable, inexpensive, reusable
  e.g. agarose, cellulose, silica and various organic polymers

- Choice of chemical coupling technology
  nonhazardous, inexpensive, rapid. Spacer arm?

Affinity Chromatography-4

☺ Increase in purity of over 1000-fold, with almost 100 % yields are reported (at least in lab scale)

☺ Drastically reduce number of subsequent steps

☹ Ligands are extremely expensive and often exhibit poor stability

☹ Ligand coupling techniques are chemically complex, hazardous, time-consuming and costly

☹ Leaching of ligand causes:

- The reduction of system effectiveness
- The presence of undesirable contaminant in product

Purification of recombinant proteins

- Same techniques but generally more straight forward because of high expression of recombinant protein
- Specific peptide or protein tags can be incorporated for rapid purification
  - Polyarginine or polylysine tag: cation exchange chromatography
  - Polyhistidine tag: metal chelate chromatography
  - Flag (a synthetic peptide) tag: immunoaffinity chromatography
- Removal of the tag is generally desirable afterwards

Protein Inactivation and Stabilization

Inactivating influence	Example	Comments
Chemical Influences	Detergents, Organic solvents, chaotrophic agents, oxidising agents, heavy metals	Most induce inactivation by interfering with non-covalent bonds which stabilizes protein structure. Some oxidizing agents can induce loss of activity by covalently modifying an amino acid residue essential to the protein's biological activity.
Biological Influences	Proteolytic enzymes, carbohydrates (glycoproteins), Phosphatases, Microbial contaminants	All induce enzyme inactivation by hydrolysis of covalent bonds. Microbial contaminants can secrete proteases to hydrolyse the protein into amino acids for their metabolism.
Physical Influences	Extremes of temperature or pH, Freeze thawing, vigorous agitation	Most induce inactivation by causing denaturation.

Approaches to protein stabilization

- Buffered solution
- Temperature control
- Minimization of processing time
- Avoid vigorous agitation or addition of denaturing chemicals
- Add substances inactivating known inactivators
- Include stabilizing agents
  - Glycerol, sugars and PEG: they decrease free water activity
  - BSA: as “bulking” protein

Storage

Optimization of storage conditions is a trial and error process...

- Optimum Temp. and pH for maximum stability
- In liquid format: add stabilizing agents, filter-sterilization is advised
- In frozen format: quickly freeze the solution, preferably in liquid nitrogen, then store in -70^{O}C
- In dry format: protein may be more stable

Lyophilization-1

- Lyophilization involves the drying of protein directly from frozen state
  - Freeze the sample
  - Apply vacuum
  - Increase the temperature $\Rightarrow$ sublimation

- Many commercial proteins (e.g. vaccines, hormones, antibodies) are marketed in freeze-dried form

Lyophilization-2

☺ One of the least harsh method for protein drying
☺ Lightweight product $\Rightarrow$ distribution easier
☺ Can be rapidly rehydrated
☺ Accepted by regulatory authorities

☹ Equipment is extremely expensive
☹ Running cost high
☹ Long processing times
☹ Some proteins exhibit an irreversible decrease in biological activity