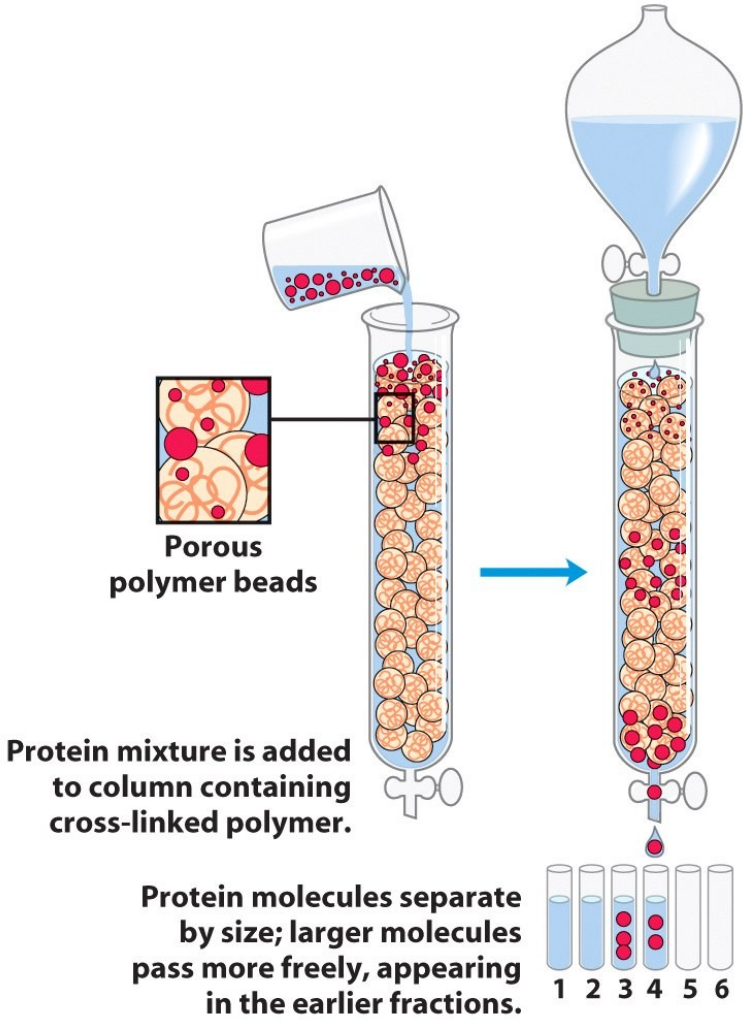
10. Separation in Solution

1. Gel Filtration

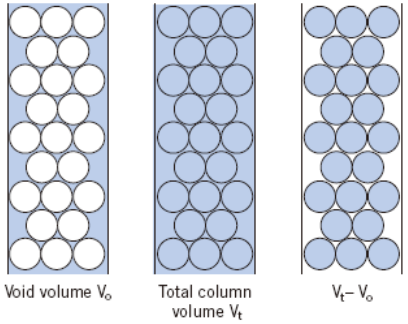
Gel Filtration

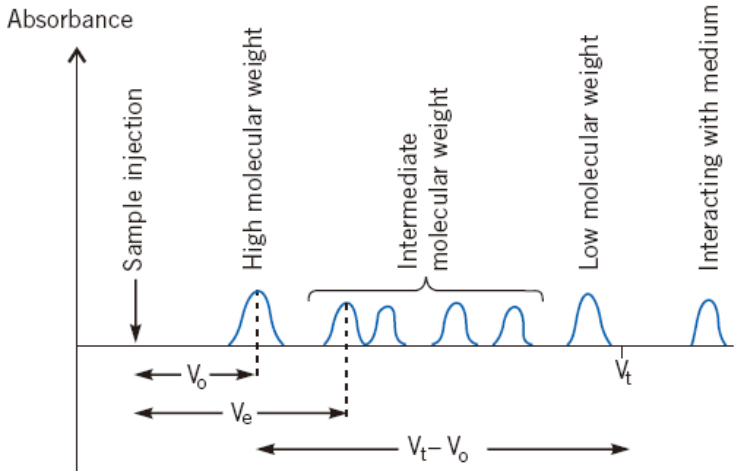
- **Other names**
  - Gel permeation
  - Gel exclusion
  - Molecular sieving
- **Principle**
  - Separation of protein according to differences in size
  - Limited accessibility of protein to cross-linked porous matrix
    - Small molecules diffuse in and out of the pores → stay longer on the column
  - No binding of protein to the matrix
    - Buffer condition does not affect resolution
- **Application**
  - Purification
  - Molecular weight determination

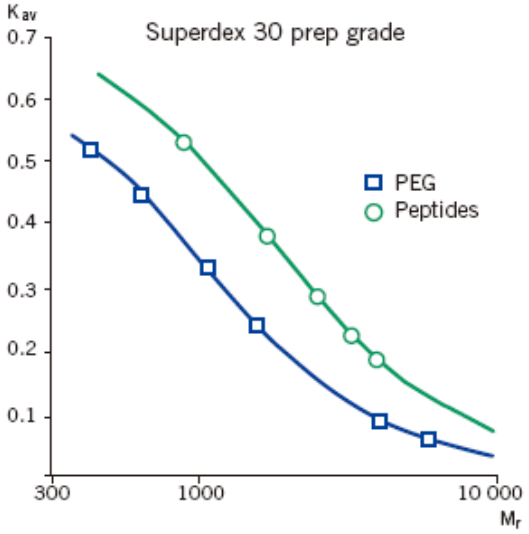


Principles of Gel Filtration

- **Partition coefficient K_{av}**
  - Proportion of pores occupied by the molecule
  - $K_{av} = (V_e-V_o)/(V_t-V_o)$
  - Depending on protein behavior and the nature of the gel beads
  - Independent of column size
- **K_{av} for gel filtration chromatography**
  - 0 (High molecular weight protein) $<K_{av}<1$ (Low molecular weight protein)
  - $K_{av}>1$: non-specific binding of the molecule
  - $K_{av}<0$: Channeling in the chromatography bed → need repacking



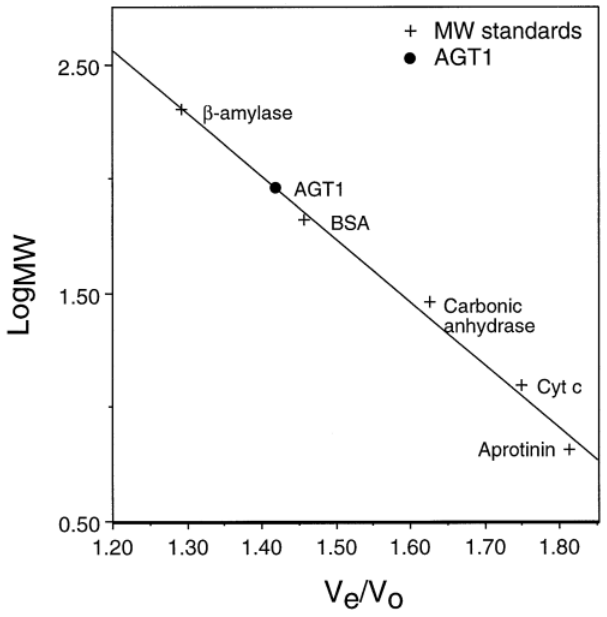




Applications

- **Determination of protein molecular weight**
  - Using calibrations of elution volumes of known proteins
  - Elution volume is related to stokes radius
    - A sphere with a hydrodynamic behavior equivalent to that of a particular irregular shaped particle
  - For proteins with similar shape, calibration curve between logMW and K_{av} can be used for to determine molecular weight of an unknown protein

- **Purification of proteins**
  - High resolution than the exact position of elution is important
  - High resolution: 1/3~2/3 of separation range

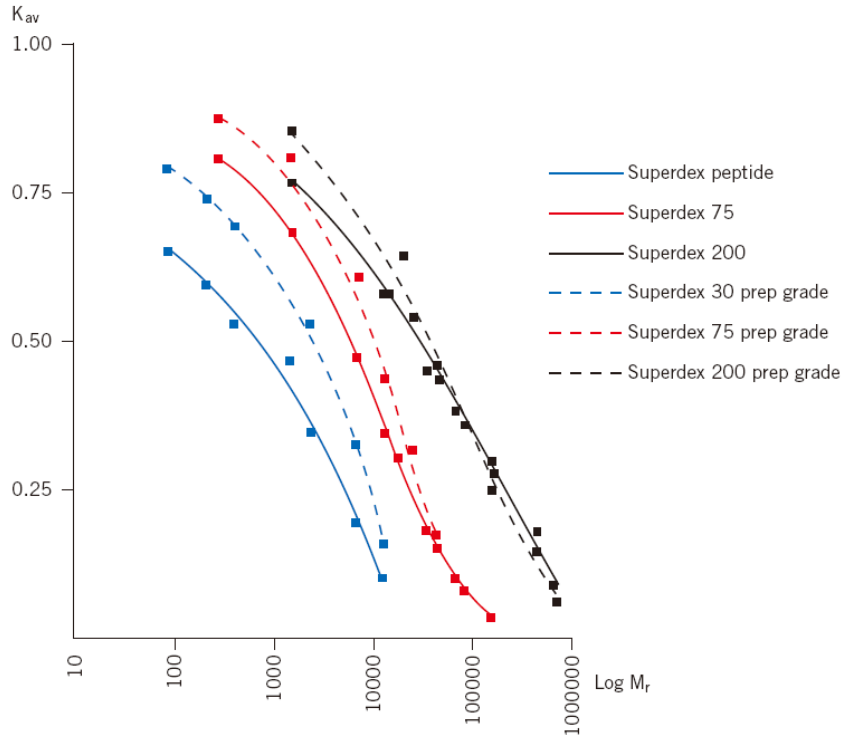


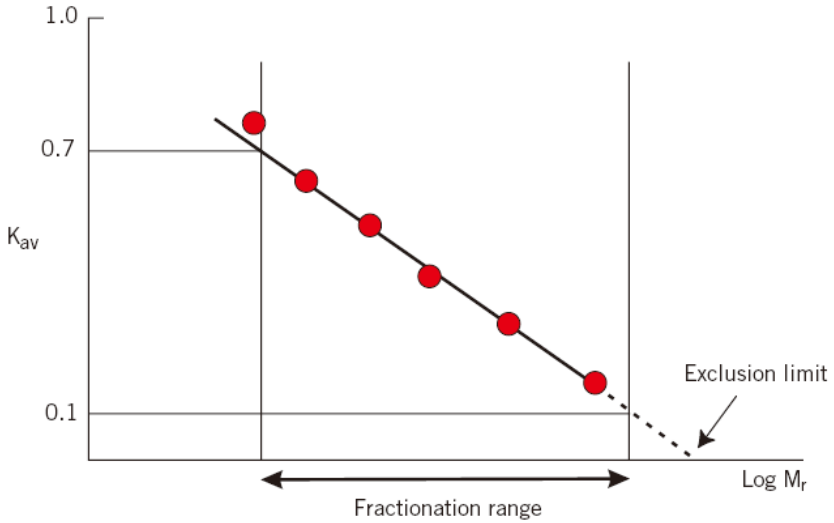
β-amylase: 200 kDa
BSA: 66 kDa
Carbonic anhydrase : 29 kDa
Cytochrome c :12.4 kDa
Aprotinin: 6.5 kDa

Liepman A. H., et al., 2001, The Plant Journal

Selection of Media

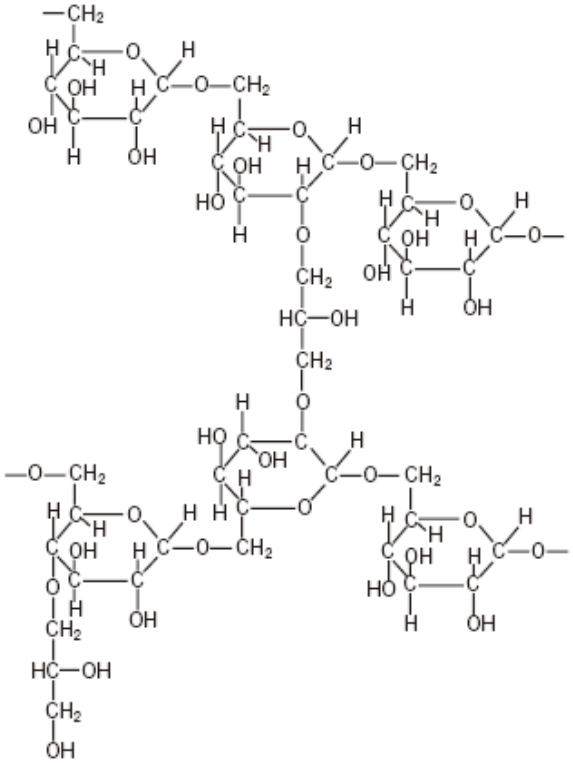
- **Depending on purpose**
  - High resolution fractionation
  - Group separation
- **Depending on the size of the target protein**
  - The target protein should be within the fractionation range (K_{av} 0.1 to 0.7)
  - Mw 100 ~ 8×10^8





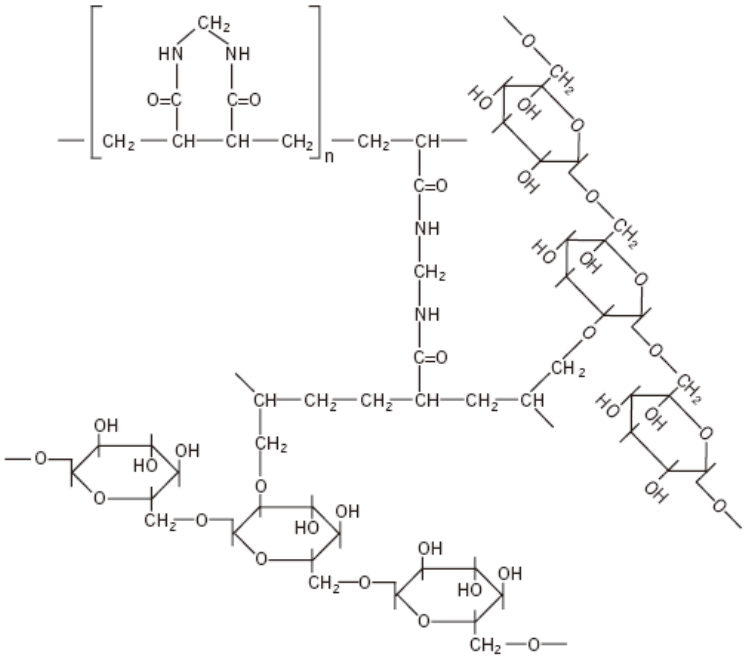
Gel Filtration Media

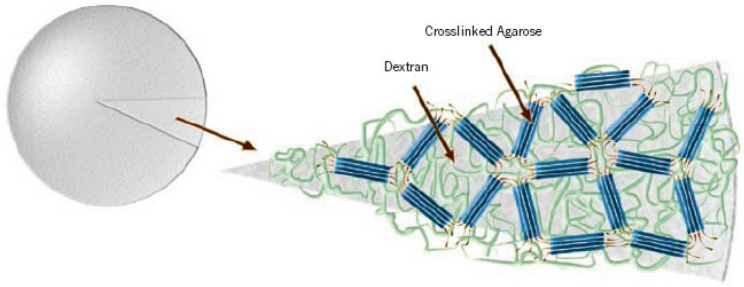
- **Cross-linked dextran**
  - Sephadex
    - Controlling the degree of cross-linking and particle size
    - For rapid group separation of high and low molecular weight substances
      - Desalting, buffer exchange and sample clean up
      - e.g Sephadex G-25
        - » Excellent for removing salt and contaminant from protein of > 5 kDa
- **Cross-linked agarose : Higher porosity and strength**
  - Superose
    - Broad fractionation range, not suitable for industrial scale



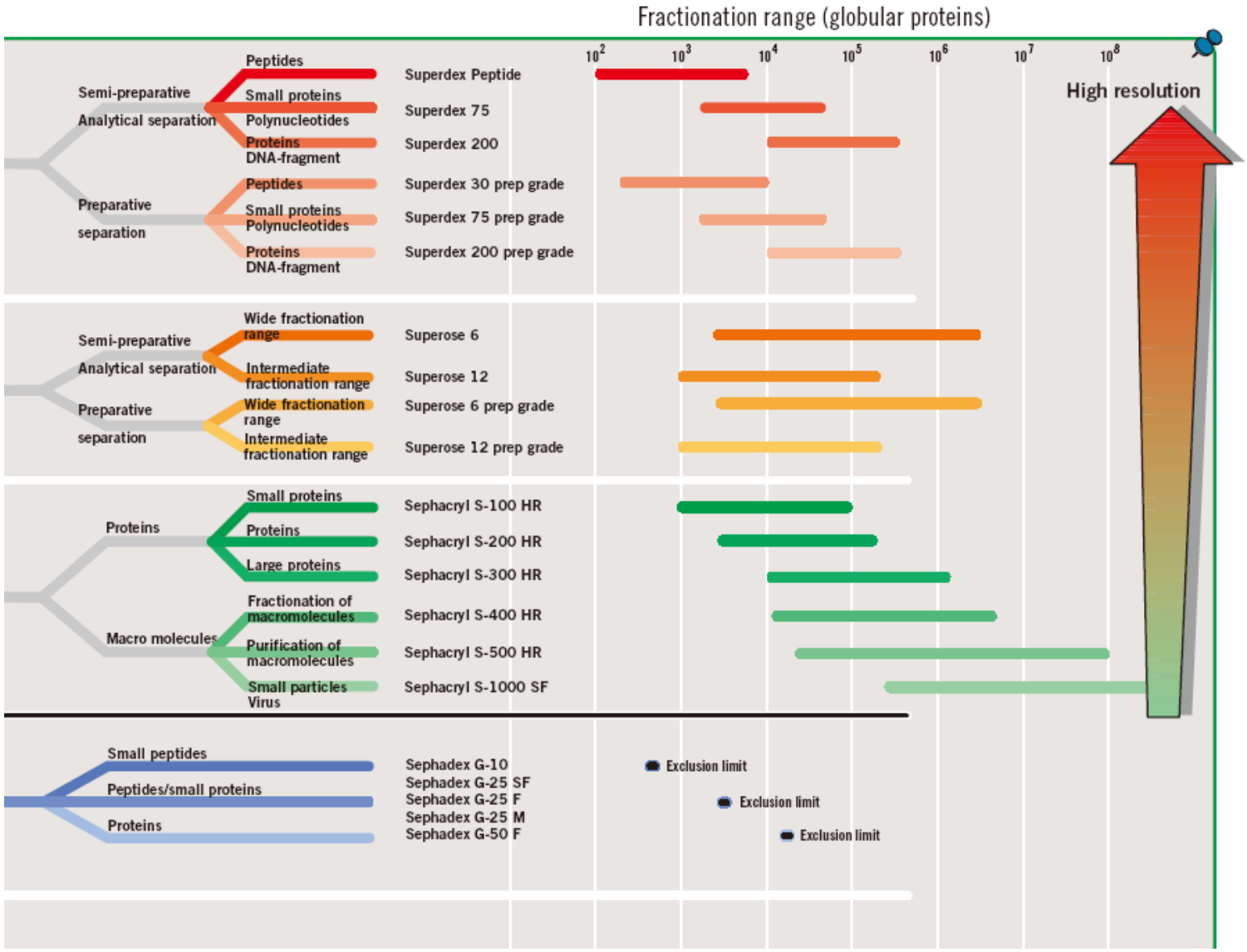
Gel Filtration Media

- **Composite**
  - Sephacryl
    - Cross-linked allyl dextran with N,N'-methylene bisacrylamide
    - Fast, high recovery at laboratory and industrial scale
  - Superdex
    - Highly cross-linked agarose + covalent attachment of dextran chain
    - Very high stability
    - Fast, high resolution and recovery



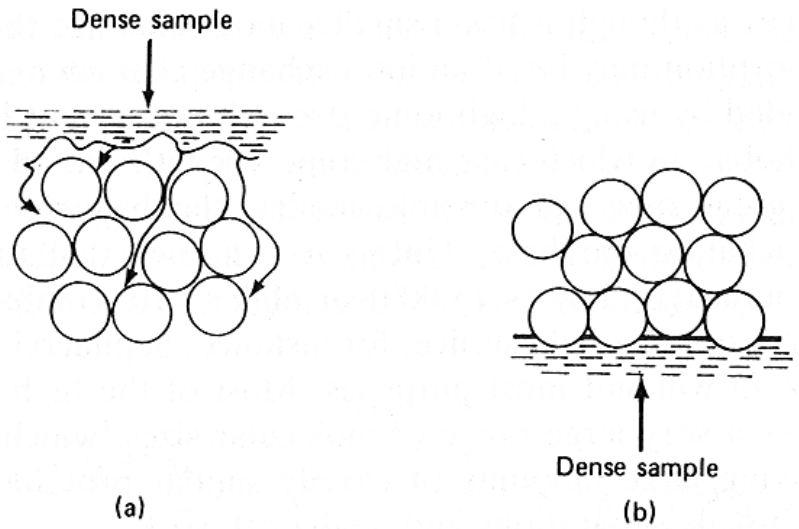


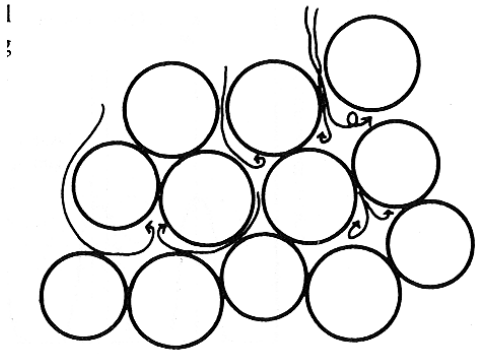
Selection of Media



Factors Affecting Resolution

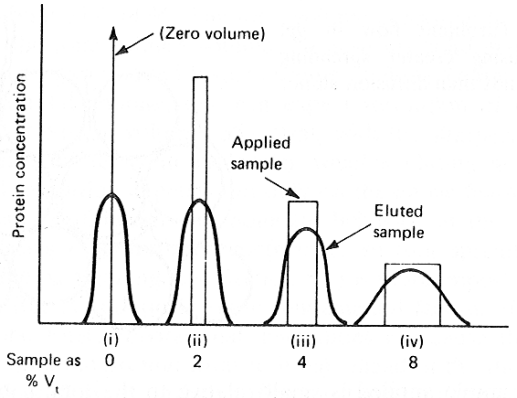
- **Sample**
  - The ratio of sample volume to column volume
  - Viscosity of the mobile phase
- **Flow rate**
- **Column**
  - Column dimension or shape
    - Squat column : poor resolution by minor deviation of flow
    - Length ~ 20-40x of diameter
  - Particle size
    - The smaller the better, but need higher pressure
  - Particle size distribution
  - Packing density
  - Porosity of the particle
- **Gravitational instability**
  - Dense solution moves faster than expected
- **Turbulent flow between particles**

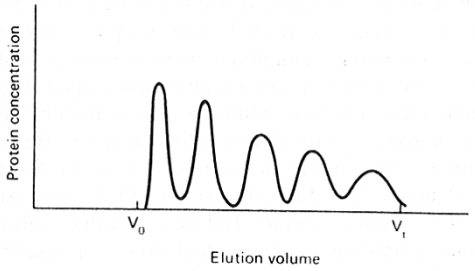


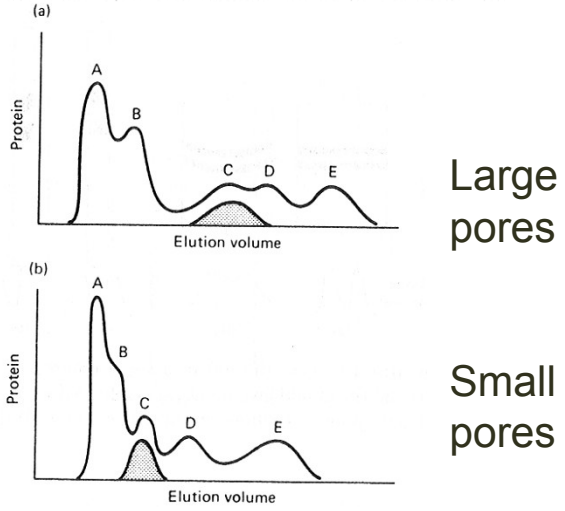


Factors Affecting Elution Pattern

- **Sample volume**
  - 1~3% of column volume
  - Smaller volume
    - Similar level of broadening because diffusion, natural chromatographic spreading, and nonideal flow
  - Higher volume
    - Band broadening
- **Protein size**
  - Smaller proteins
    - High diffusion coefficient and longer stay in the column
    - More diffusion and band broadening
- **Pore size**







Large pores

Small pores

2. Electrophoretic Methods

Electrophoresis for Protein Purification

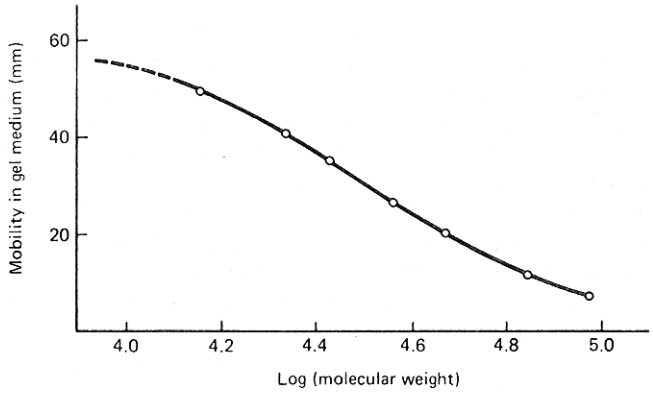
- **Electrophoresis**
  - Elctrophoresis under constant pH current
  - Isoelectric focusing
    - Separation in pH gradient
  - Isotachophoresis
    - Variation of electric field according to the conductivities of components being separated
- **Application of electrophoresis for protein purification**
  - The last choice for protein purification

Electrophoresis Principles

- **Protein mobility in an electric field**
  - $Ez = 6\pi\eta rv$ in a steady state
    - E : electric field (volts/m)
    - *z*: net number of charge
    - η : viscosity
    - *r*: particle radius (Stokes radius)
    - *v*: velocity
  - Specific mobility $u = v/E = z/6\pi\eta r$
- **Free boundary electrophoresis**
  - Difficult to separate molecules
    - Diffusion
    - Overlapping of components
    - Protein-protein interactions
      - High salt to minimize interaction → heat generation
  - Electrophoretic buffer
    - Tris-borate at pH 8-9
      - Low conductivity
      - Most proteins move to anode

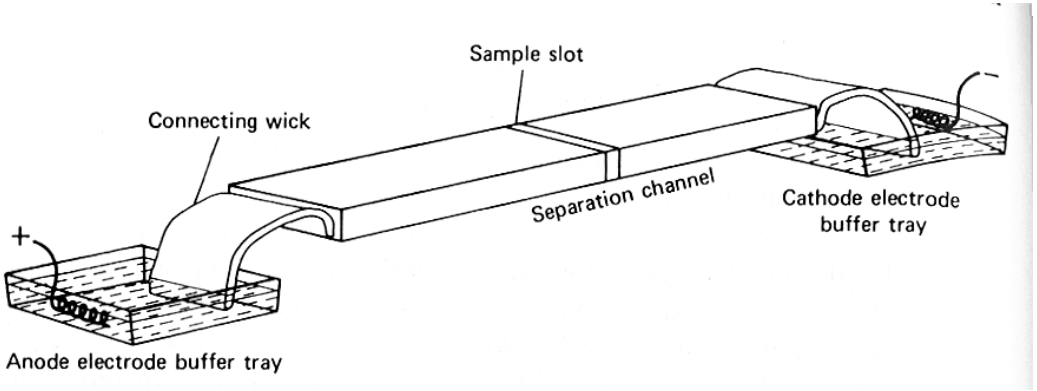
Electrophoresis Principles

- **Gel electophoresis (polyacrylamide gel)**
  - Separation by size and charge
    - Good for analysis but technically difficult for preparative work
  - Molecular sieving effect
    - Mobility is inversely proportional to logMW for similarly shaped molecules having same charge density
  - SDS-PAGE
    - $u=u_o(A-\log MW)/A$
      - A: logMW of a molecule that would not move in the gel
      - u_o : the mobility of a small molecule unaffected by the gel = $z/6\pi\eta r$
    - $u= z/6\pi\eta r \cdot (A-\log MW)/A$



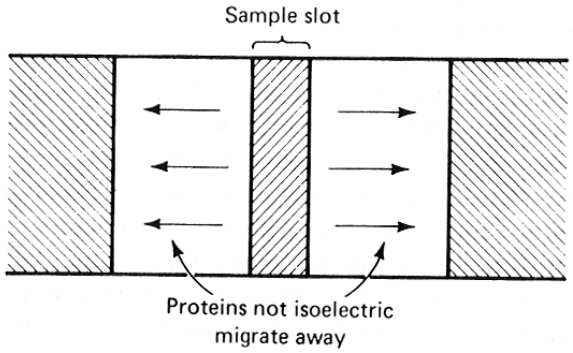
Preparative Electrophoresis: Horizontal Slabs

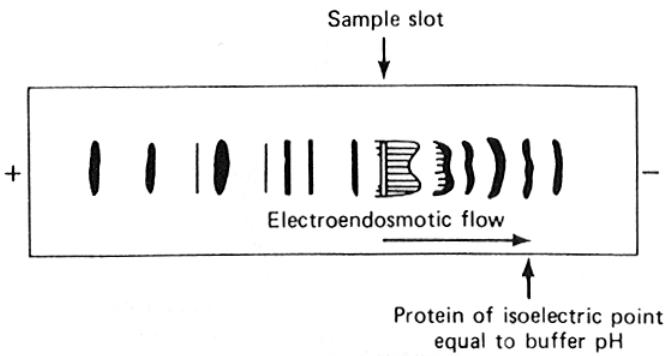
- **Components of electrophoresis**
  - Separation channel
  - Connection of channel to electrodes via buffer reservoir
- **Horizontal slab**
  - Thick slurry (buffer mixed with starch powder, Sephadex G25 beads etc.) or gel (1~2 % agarose) connected to the buffer reservoir via connecting wick
  - Slice the block containing the target protein
  - Low resolution but high capacity



Preparative Electrophoresis: Horizontal Slabs

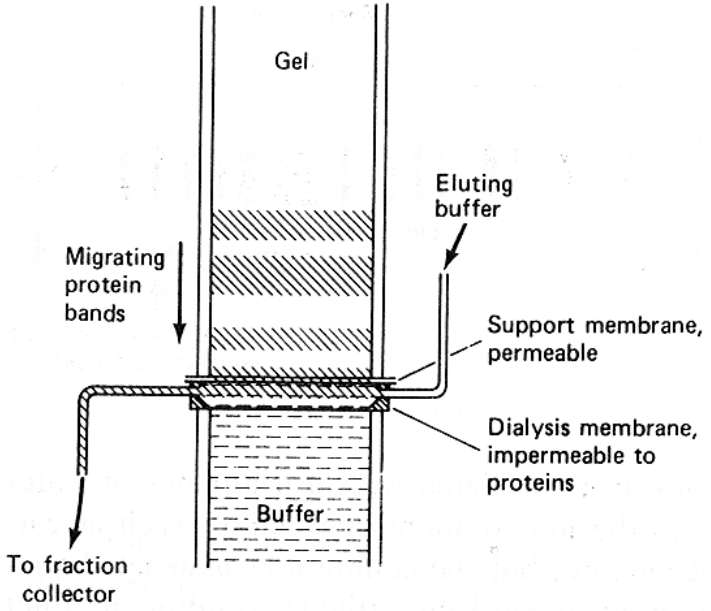
- **Isoelectric point electrophoresis**
  - Run electrophoresis under the conditions of no movement of the target protein
  - The pH might be different from isoelectric point determined by isoelectric focusing
    - Depending on the buffer
      - Binding of polyanions → lower the apparent pI
    - Electroendosmotic flow
      - The flow of bulk liquid solvents under the influence of electric field due to immobilized negative charge groups on the walls of electrophoresis channel or gels
      - Movement of associated cation to cathode → movement of water

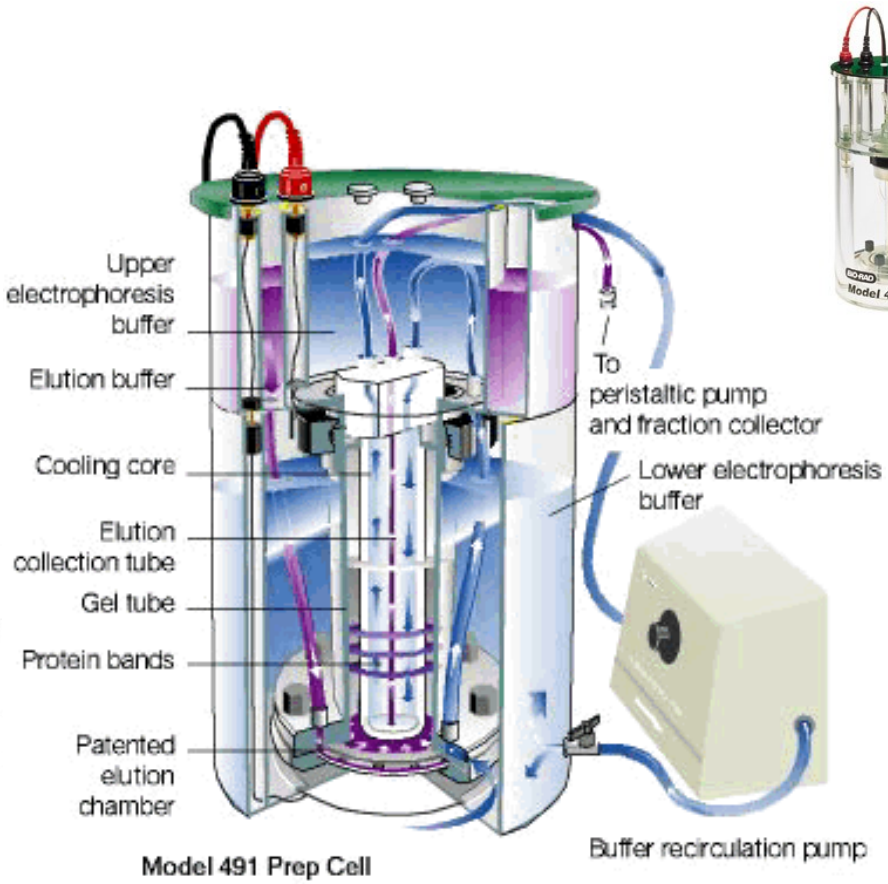




Preparative Electrophoresis: Vertical System

- Collection of emerging protein at the bottom of the gel during electrophoresis

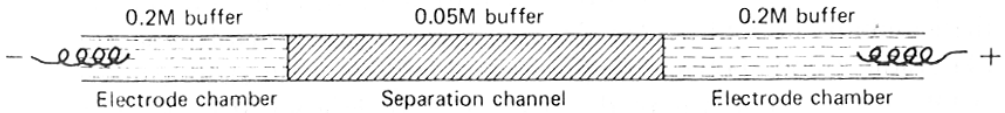




Model 491 Prep Cell

Buffer Systems for Electrophoresis

- **Buffer ions**
  - Avoid very mobile ions : generate heat
    - Metal ions : Na^+, K^+, Mg^{2+}
    - Simple anions F^-, Cl^-, Br^-. SO_4^{2-}, HPO_4^{2-}
  - Bulky organic ions with n= +1 or -1
    - Slower mobility
  - Simple cations
    - Effective in preventing protein-protein interaction
- **Buffer systems**
  - Continuous
    - Same buffer
      - 0.05-0.15 ionic strength, Tris-borate (pH8~8.5) + EDTA
    - Higher concentration in the electrode chambers to lessen voltage losses across the connections

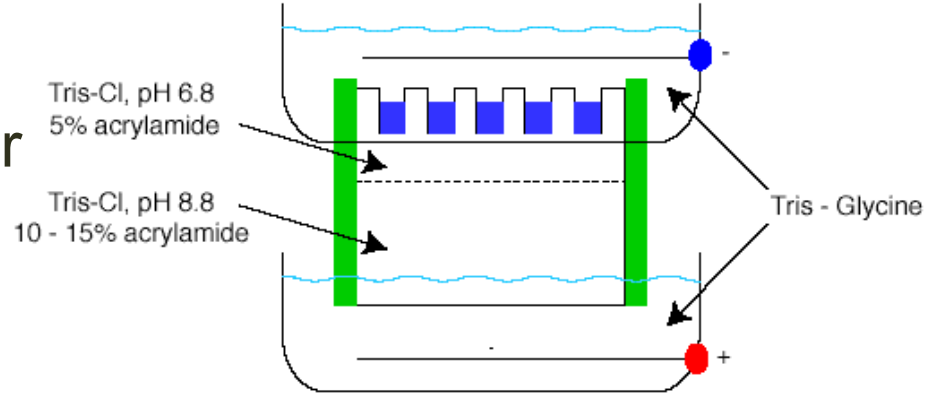


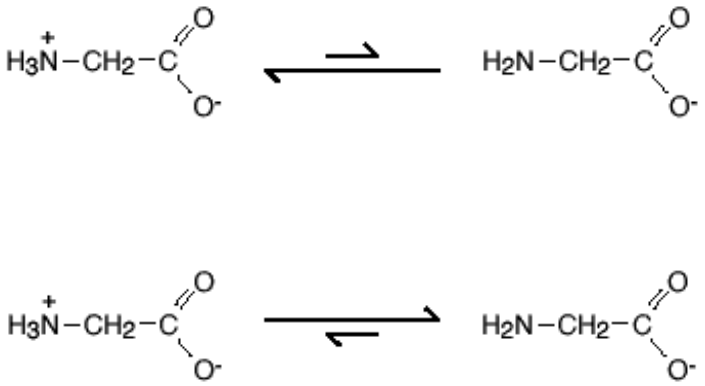
(a) Continuous

  - Discontinuous

Discontinuous Buffer Systems

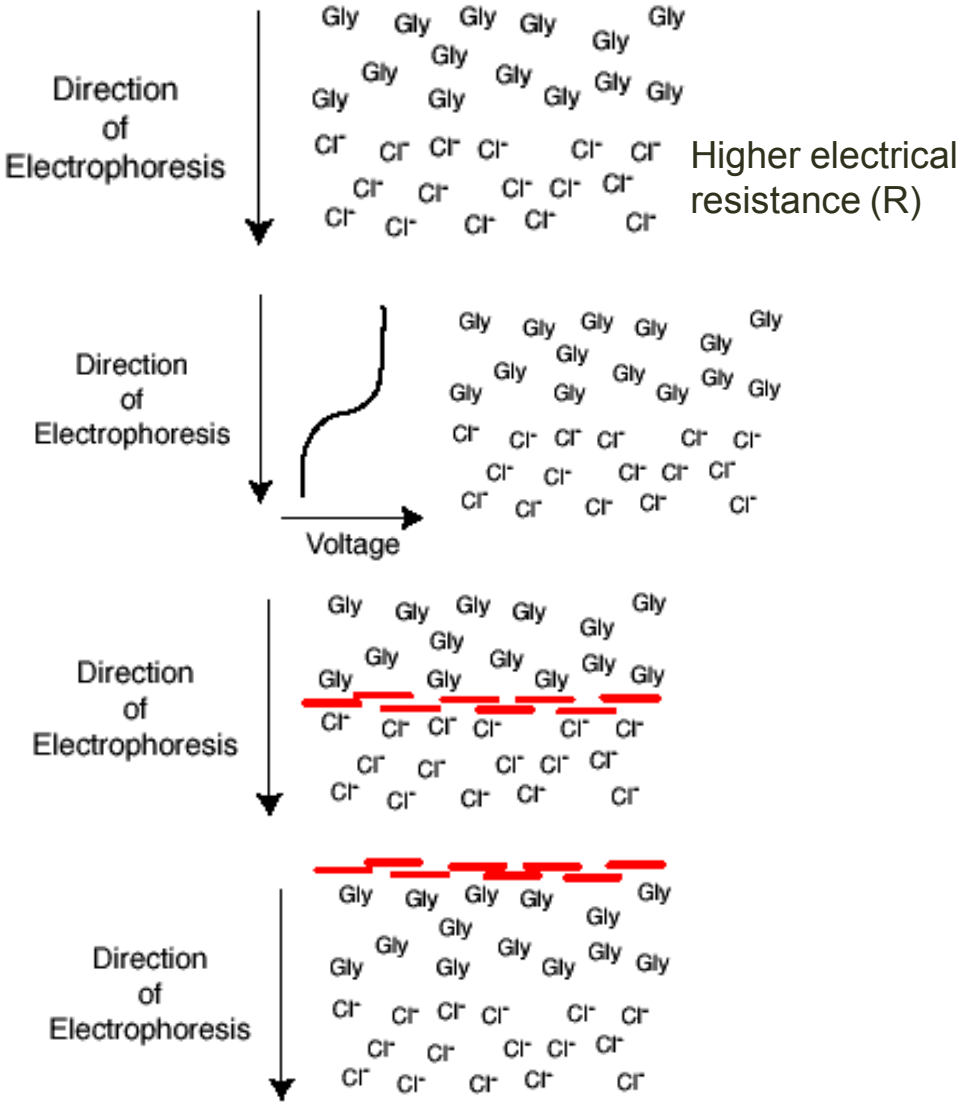
- **Principle**
  - Kohlrausch discontinuity between the starting buffer ions and the following ions with lower mobility
  - Band sharpening effect
- **Example**
  - Running buffer
    - Tris-glycine
  - Stacking gel
    - Tris-Cl, pH 6.8
    - Low motility of glycine
  - Resolving gel
    - Tris-Cl, pH 8.8
    - High motility of glycin





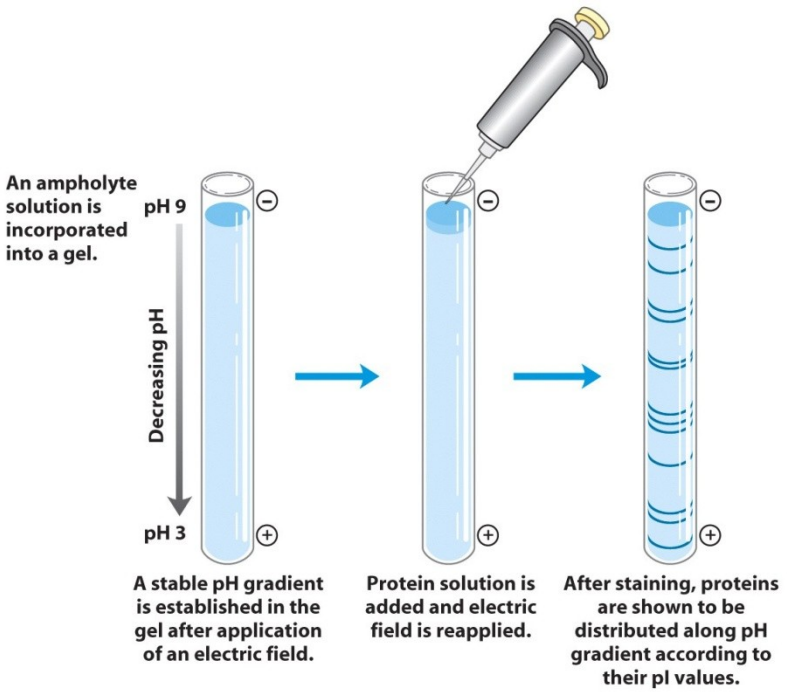
Discontinuous Buffer Systems

- **In stacking gel**
  - Formation of boundary between glycine and Cl^- with higher mobility
  - The voltage gradient sharpens the ion boundary
  - Proteins are trapped between Gly and Cl^-
- **In resolving gel**
  - Gly moves faster than proteins
  - Separation of proteins depending of charge/mass ratio, size, and shape



Isoelectric Focusing

- **Procedures**
  - Establishment of pH gradient
    - Gel containing a mixture of low molecular weight organic acids and bases (ampholytes)
    - Application of electric field
  - Each protein migrates until it reaches the pH corresponding to its pI
- **Advantage**
  - High resolution
  - High electric field can be applied with little heating
- **Requirement**
  - The protein must be stable at its pI
  - The protein must be soluble around its pI even at zero ionic strength



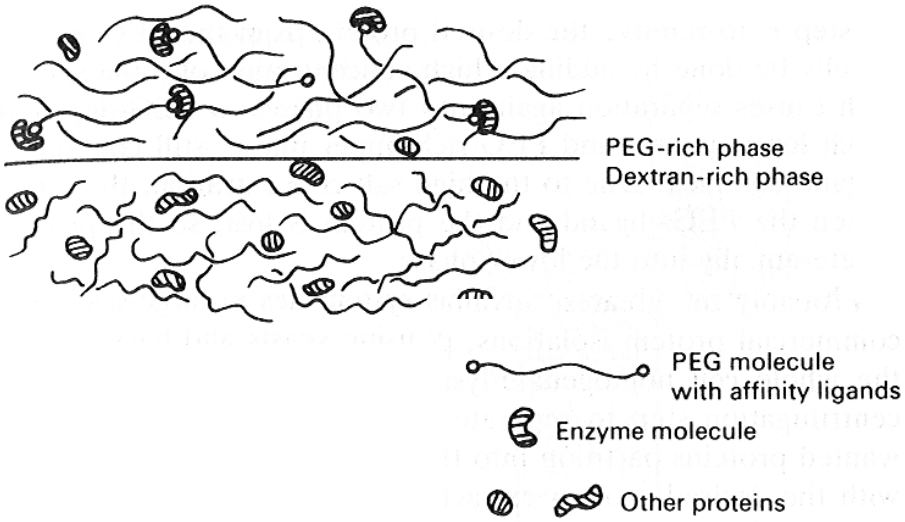
3. Liquid Phase Partitioning

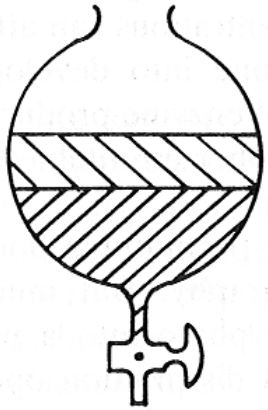
Separation of Proteins by Liquid Phase Partitioning

- **Requirement**
  - Creation of two immiscible liquid phases
  - Each liquid phase can dissolve proteins
  - Proteins should remain bioactive after dissolving
- **Phase separation**
  - Addition of certain pairs of hydrophilic polymers to aqueous solutions
    - Generation of several phases
  - Dextran and PEG
    - Phase separation
      - Dextran-rich (bottom) : Partition of most proteins
      - PEG-rich (top)
    - Useful for large scale production
      - Whole cell lysate can be applied without removing cell debris
      - High capacity : 100mg/ml

Affinity Partitioning

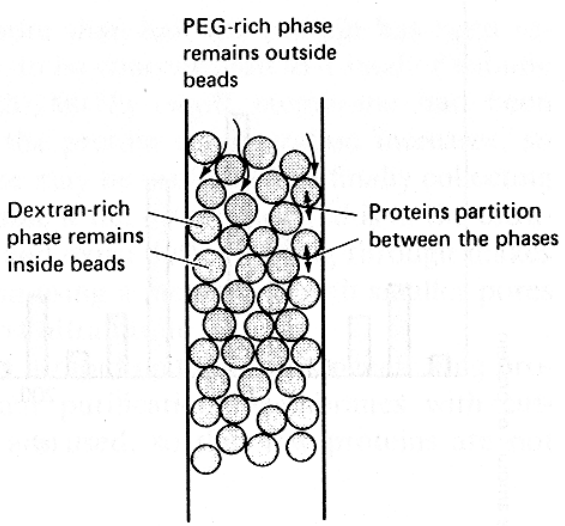
- **Dextran and PEG + PEG-ligand**
  - PEG-ligand
    - Activation and attachment of ligand to –OH of PEG
    - Mix with the bulk PEG
  - Partitioning of the target protein in the upper phase
  - Elution of the protein from the PEG phase
    - Addition of high concentration of phosphate buffer
    - Phase separation
    - Protein partition into phosphate-rich lower phase





Liquid-Liquid Chromatography

- **Immobilization of one liquid phase**
  - Equilibrate agarose bead with one of the phases
    - E.g dextran-rich phase
  - Use the other phase as moving buffer in column
  - Surface tension prevents mixing of two phases
- **Problems of phase partitioning methods**
  - Handling problems with viscous solutions
  - Cost : dextran is expensive
    - Alternative: starch derivatives
    - Temperature-dependent phase separation
      - Triton X-114
      - Copolymers of ethylene an propylene oxide
- **Advantage**
  - Gentler than adsorption chromatography
    - Suitable for labile enzymes
  - Rapid
  - High capacity
  - Requires little equipment



4. Ultrafiltration

Ultrafiltration

- **Principle**
  - Forcing liquid through semi-permeable membrane using hydrostatic pressure
    - Molecules smaller than 'cutoff' pore size of membrane → pass
    - Bigger molecules → retain
  - Less discriminating than gel filtration
- **Applications**
  - Concentration of protein
    - Cutoff 20 of 10 kDa
  - Also can have some purification effect depending on the choice of membrane cutoff size and the molecular weight of the target protein