BIOC4004 - Industrial Biochemistry
Lecture 07 - Fri Jan 23, 04

Topics for the Day:

- Protein Purification and Analytical Techniques
  - Differential solubility
  - Chromatography
    - ion exchange
    - hydrophobic
    - reverse phase
    - affinity
    - size exclusion
  - Electrophoresis
    - SDS-PAGE
    - IEF
    - 2D
- Setting up purification schemes
- Heterologous expression revisited

Order	Group	Member 1	Member 2	Member 3	Member 4	Topic 1
7	1	Thad B.	Sandra V.	???	???	Agrifoods
3	2	Keldeagh L.	Chris J.	Pratik L.	Brian B.	Pathways
2	3	Sharon H.	Jen W.	Bethany D.	Kerry R.	Vacc or Ab eng.
1	4	Johanna L.	Jenny N.	Kathy C.	Mona L.	Diagnostics
6	5	Frances C.	Jason F.	Greg S.	Justin S.	Vacc or Ab eng.
5	6	Maria B.	Martha P.	Monique B.	Zain B.	Prot. Engineering
4	7	Jonathan G.	Robyn E.	Jason O'B.	Steve A.	Bioremediation
8	8	Alex D.	Hardeep N.	Jen E.	Brianna B.	Bioinformatic tools
The following students have just registered and do not have a group yet:						
Ali Shafea						
Daniel Kolczynski						
Thaila Riden						

Protein Purification

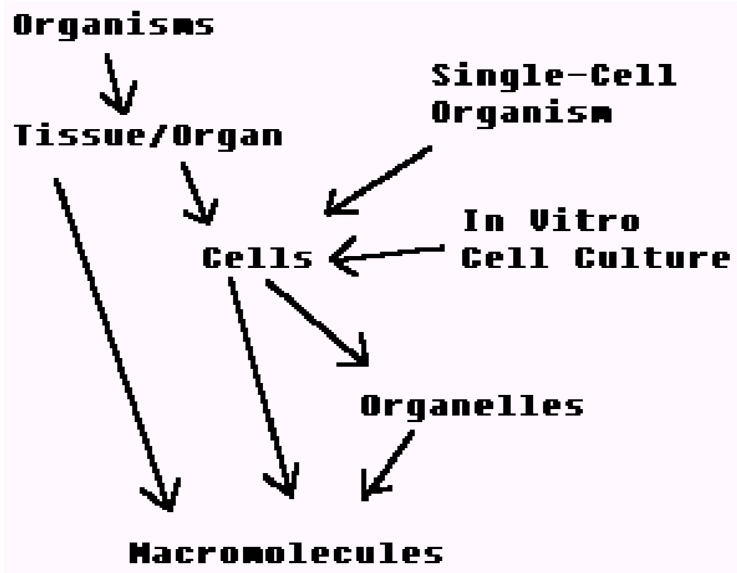
In order to work on a protein, you must isolate it and purify it

Why purify ?

- Need protein for functional or structural studies
- Need protein for industrial or pharmaceutical application
- Development of antibody for the protein
- Identification of protein sequence

How to Purify ?

- Develop method according to
    - Scale (analytical, medical, industrial)
    - Level of purity required (crude, highly purified, pyrogen-free)
    - Application (functional, structural, antibody, sequencing…)
        - native conformation vs non-native conformation
    - Source material (original, recombinant)



- For analytical purposes:
    - need lower levels of protein:
        - enzymology: 0.1-100 mg
        - crystallography: 0.1-1.0 g
- For industrial purposes:
    - therapeutic: 10g-100 kg
    - industrial: kg to tonnes

- Facilitate things by starting with “enriched” source
    - ie. recombinant source necessary for industrial uses!! (less expensive)

Protein Purification

- Need to extract protein from source material in soluble form
- Disrupt the source material (unless protein secreted outside cell)
- Separate protein of interest from others based on
    - physical characteristics
    - chemical characteristics

Cellular Disruption Methods

TECHNIQUE	COMMENTS
homogenation	Cells are placed in homogenizer or blender and disrupted by shear forces or grinding. Many different types of homogenizers are available depending on the application. Most consist of a pestle with a defined clearance and can be hand or motor driven. Homogenation can be combined with osmotic methods. Heating can be a problem.
presses	Cells are placed under high pressure in a stainless steel chamber. The chamber is opened while under pressure and the cells exit through a small orifice at a controlled rate. The rapid exposure to atmospheric pressure causes the cells to lyse. Optimal pressure for cell disruption needs to be determined empirically for each type of cell and experimental condition.
N_2-cavitation	Cells are placed under high pressure in a N_2 atmosphere. The pressure is suddenly released, causing the N_2 dissolved within cells to boil off and rupture the cells. Both the pressure and rate of pressure release can be controlled.
osmotic, hypotonic	Cells are placed in an isomolar solution of a permeable solute or a hypotonic solution. Water will enter cell which results in swelling until the membrane ruptures. The technique is gentle, but may not lyse cells with walls or rigid cytoskeletons(eg., bacteria, plants, etc.).
sonication	Ultrasonic waves are used to disrupt cells. Heating is a problem and the process tends to vesiculate membranes and subcellular compartments.
freeze-thaw	Ice-crystals formed during freezing will rupture cell membranes. It will generally take multiple cycles of freeze-thaw to disrupt all of the cells.
detergents	Detergents will disrupt the lipid bilayer allowing the contents to be released and will solubilize membrane proteins. Many detergents denature proteins and they are often difficult to remove.
chaotropic agents	Chaotropic agents solubilize proteins by disrupting the structure of water. Protein denaturation is a problem.
enzymatic	Bacteria, yeasts and plant cells are often treated with enzymes to remove the cell wall. Enzymatic treatment is often combined with other disruption methods.

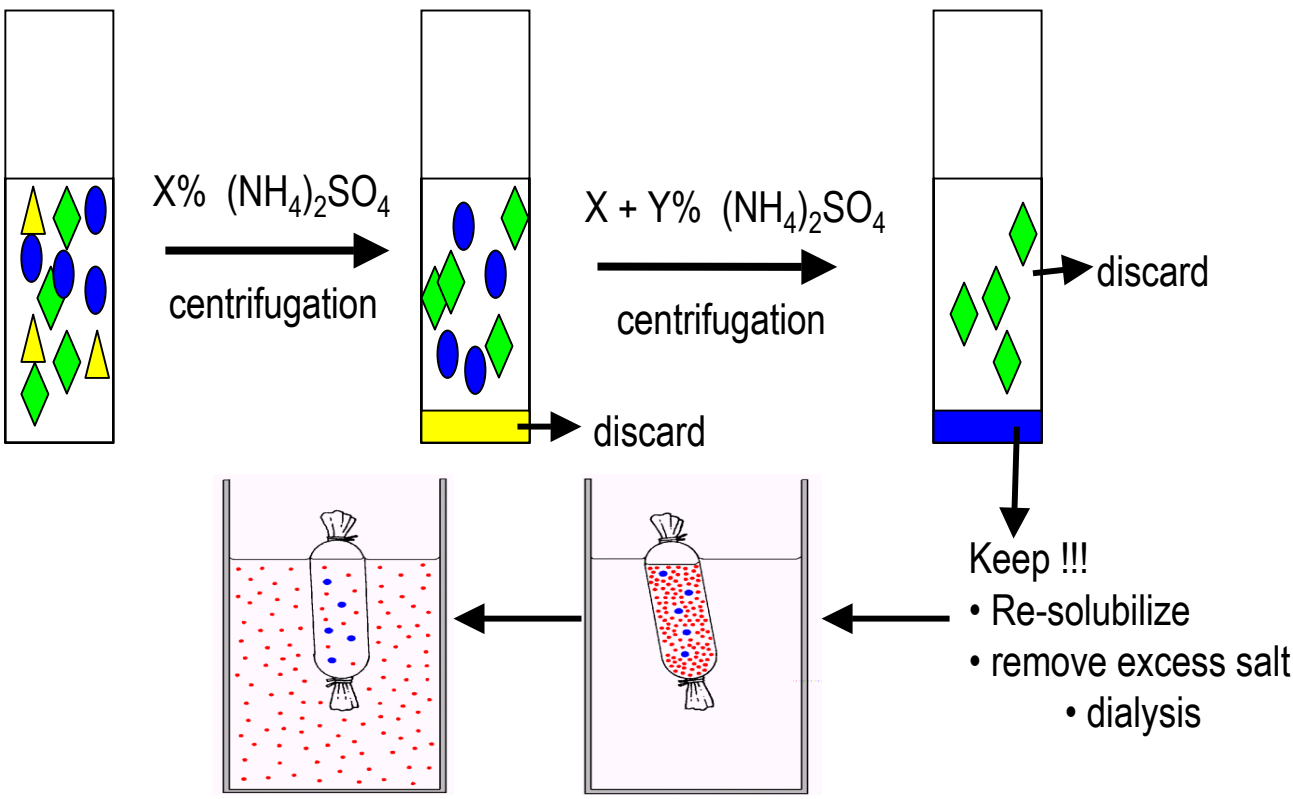
Protein Purification

Differential Protein Solubility

- individual proteins can be separated based on different physical and chemical properties
- common techniques:
  - differential solubility →
    - salt, $(NH_4)_2SO_4$
    - solvents (acetone)
    - acidic pH
    - high temperature
  - chromatography
  - electrophoresis
- salting-out effect
  - as [salt] ↑ less H_2O is available for hydration of protein
  - proteins will aggregate, or precipitate, according to their hydrophobicity

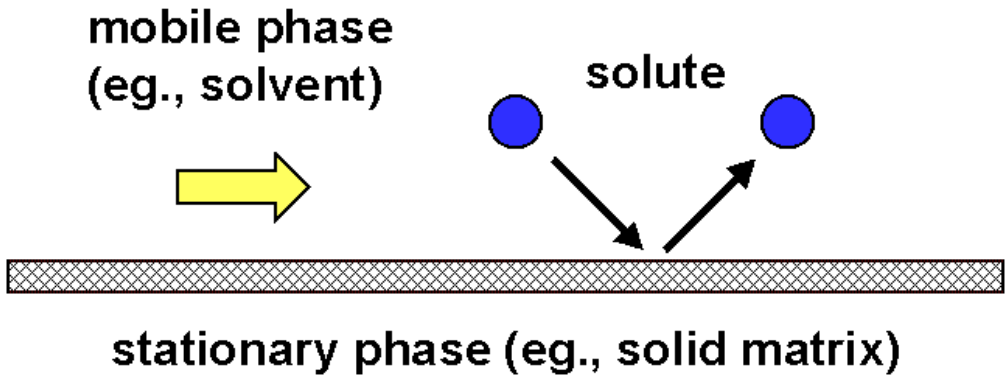
Ammonium Sulfate Precipitation

- different proteins will precipitate at different $(NH_4)_2SO_4$ concentrations

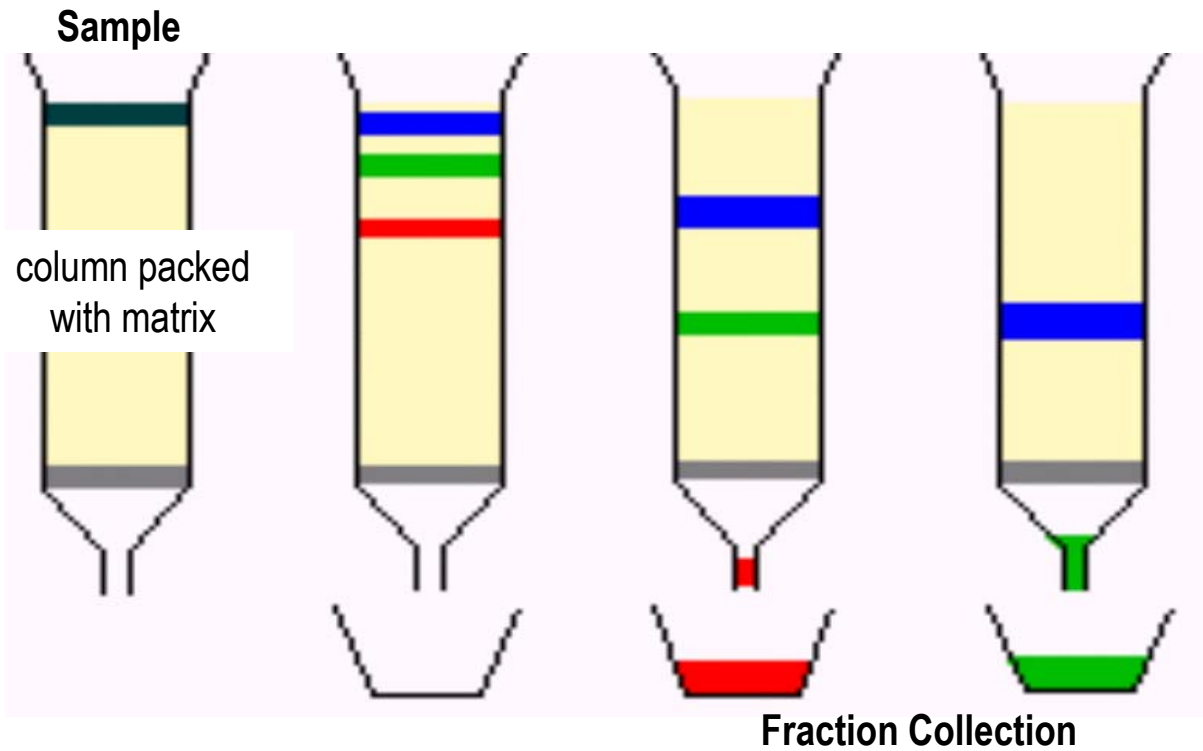


Protein Purification

Chromatography



• separate according to differential interactions between solute and solid phase



Generic Protocol

1. Prepare Column (±)
2. Apply Sample
3. Wash
4. Elute
5. Analyze Fractions

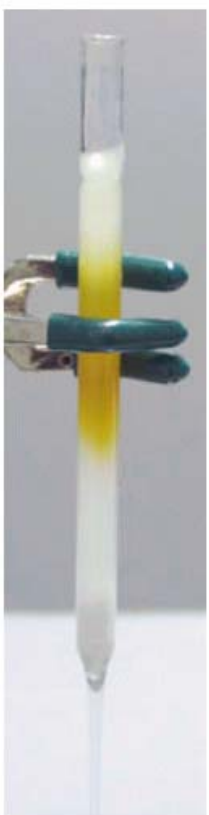
Types

- ion exchange
- hydrophobic
- gel filtration
- affinity

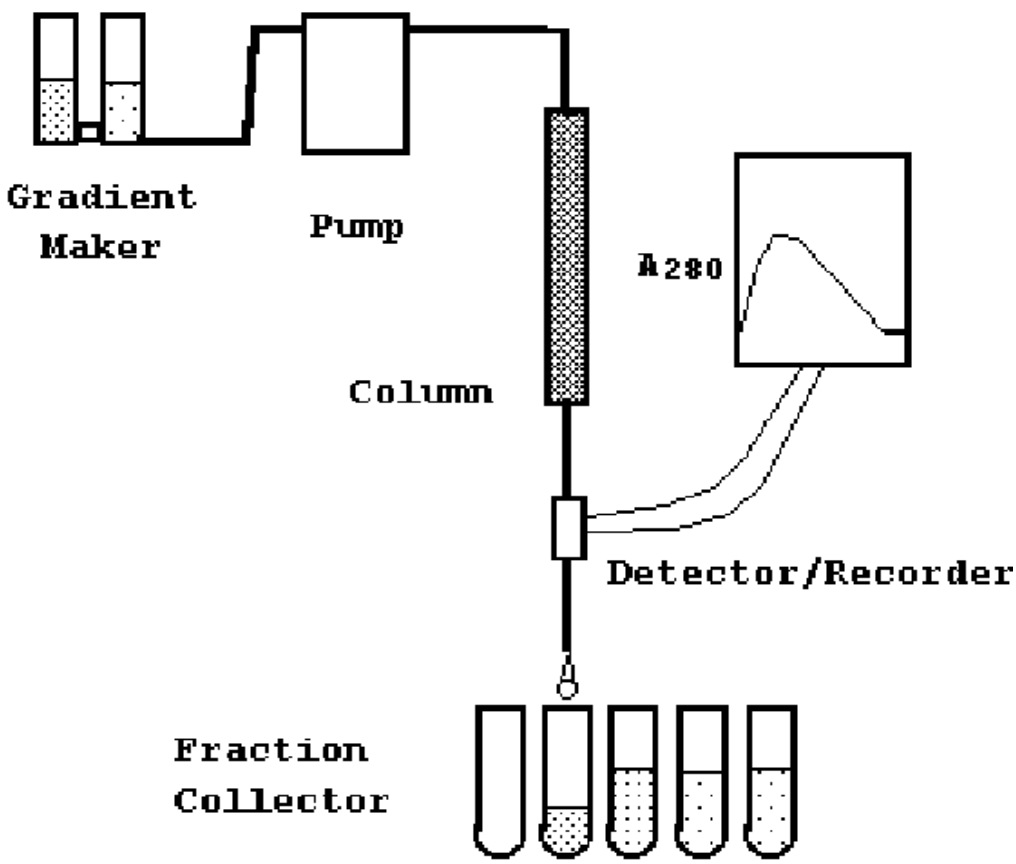
Advances in Chromatography: HPLC and FPLC

- HPLC: high performance (pressure) liquid chromatography
- FPLC: fast protein liquid chromatography

    - conventional chromatography:
        - limitation on matrix particle size(affects flow rate)
        - limitation on packing of the matrix
        - slow flow rate (low volume pumps or gravity)
            - diffusion and loss of resolution
    - HPLC:
        - pumps with faster flow rates(increased pressure)
        - decreased particle size (increased surface area)
        - increased strength (tolerate higher pressure)



gravity

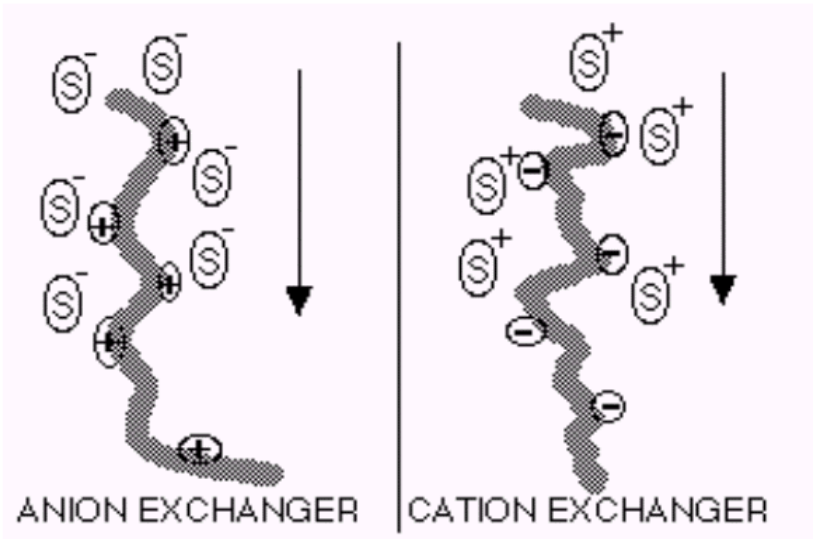


Different Types of Chromatography

Media Type	Discrimination
Ion Exchange	Charge
Gel Filtration	Size and Shape
Hydrophobic	Surface Hydrophobicity
Reverse Phase	Total Hydrophobicity
Affinity	Specific Amino Acids

Ion Exchange Chromatography (IEC)

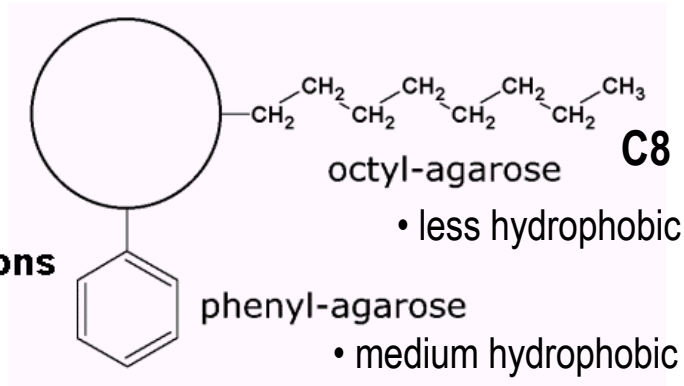
- based on charge-charge interactions between solid matrix and solute



- Interactions depend on:
  - pH: affects protein charge
  - Salt concentration: affects strength of interaction
- Weak exchangers used for protein separation
  - better resolution of small charge differences
- Load in conditions that promote binding to column
- Elute with salt / pH gradient

Hydrophobic Interaction Chromatoraphy (HIC)

- **separates proteins based on differences in hydrophobicity**
  - **absorb proteins to hydrophobic matrix**
  - **high salt promotes hydrophobic interactions**
    - **eg, 1 M $(NH_4)_2SO_4$**



← increasing salting out effect

anions: PO_4, SO_4, Cl, Br, NO_3, ClO_4, I, SCN

cations: NH_4, Rb, K, Na, Li, Mg, Ca, Ba

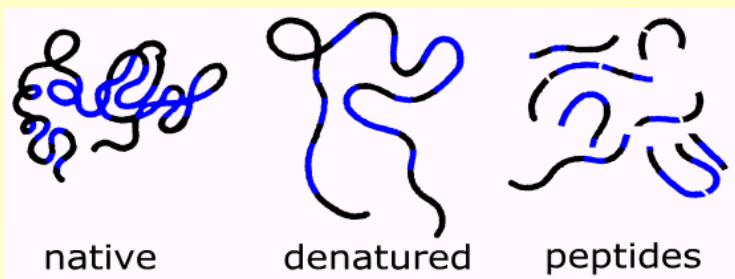
increasing chaotropic effect →

- Interactions depend on:
  - Salt Concentration: affects strength of interaction; increases hydrophobic int.
  - Solvent Polarity: affects propensity for hydrophobic interactions
  - Chaotropic agents: reduces polarity of water; decrease hydrophobic int.
- Elute by reducing the hydrophobic interactions (usually salt conc.)

Reverse Phase Chromatography (RPC)

	HIC	vs	RPC
Mobile Phase	Polar Solvent		Nonpolar Solvent → eg. hexane
Conditions	Native		Denatured
Solute Discrimination	Surface Residues		Total Residues

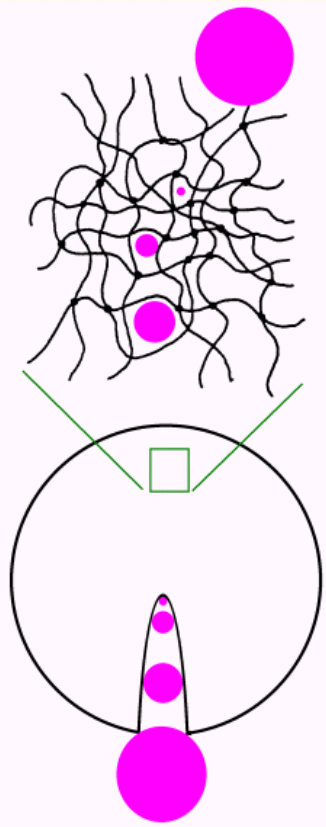
- **separation based on total hydrophobicity**
- **generally used to separate small peptides**

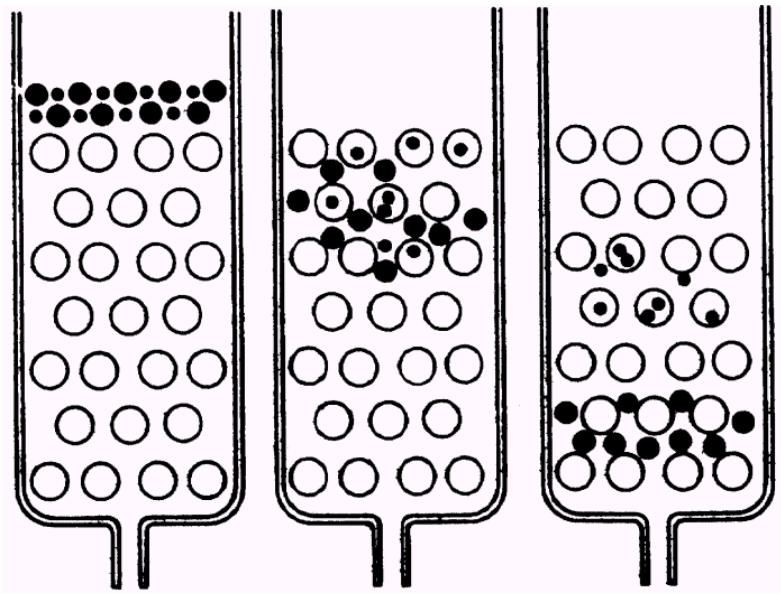


Gel Filtration Chromatography

- separation based on size, aka
  - molecular sieve chromatography
  - size exclusion chromatography
- media composed of cross-linked polymers
- 'pore' size of matrix determines degree of interaction
  - larger molecules are excluded and migrate faster
  - smaller molecules are included and are retained longer

- Dextran (=Sephadex®)
- Agarose (=Sepharose®)
- Polyacrylamide



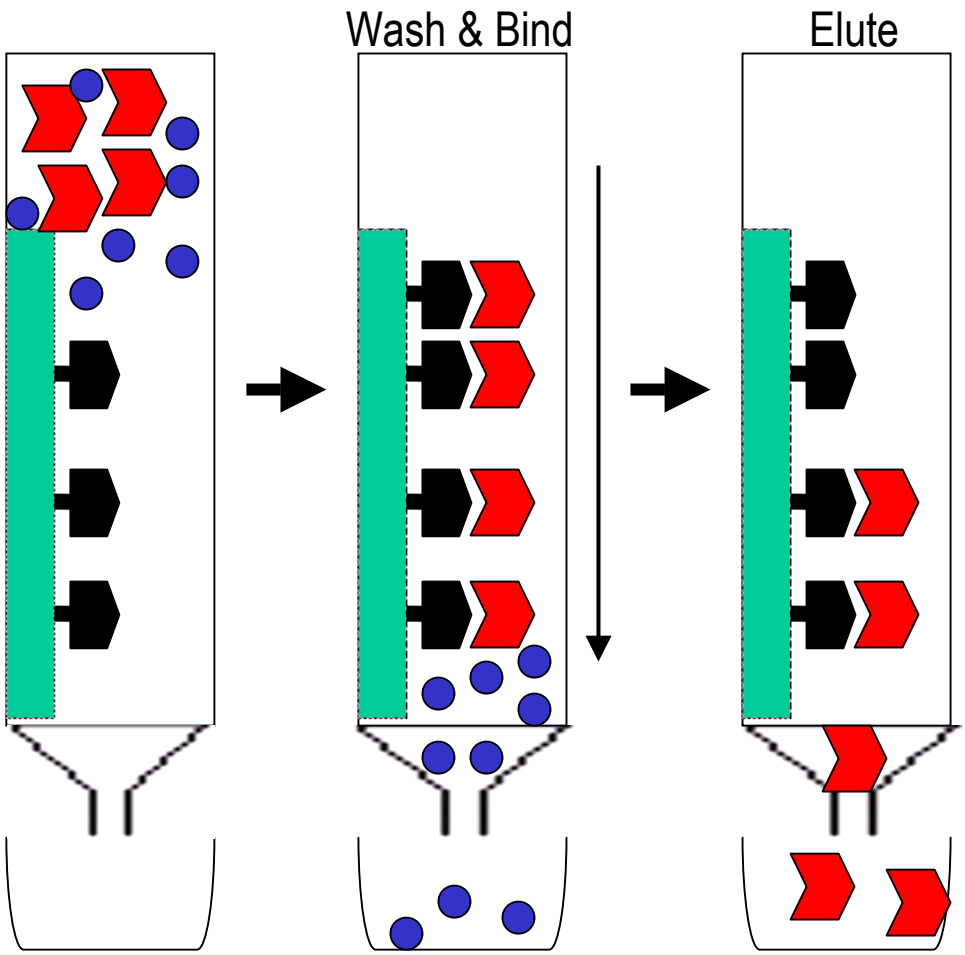


Sephadex	
Code	Range (kDa)
G-25	1-5
G-50	2-30
G-100	4-150
G-150	5-300
G-200	5-600

- Interactions depend on:
  - pore size(retention of small molecules)
  - there is no binding, only filtration
- good for desalting or exchanging buffer (fast!)
- not good for purification

Affinity Chromatography

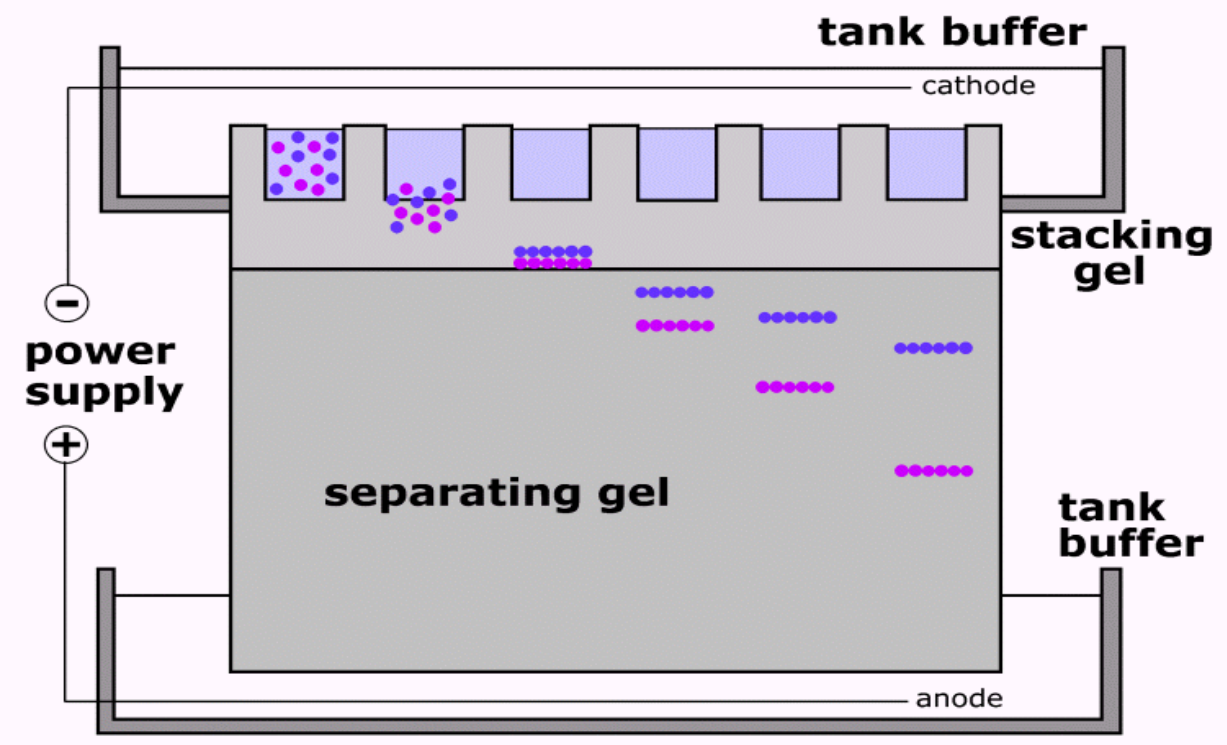
- based on specific binding of protein to "ligand"
- ligand attached to matrix (eg, CNBr-activated sepharose)
  - linker arms
  - matrix should not absorb contaminants
  - covalent attachment of ligand should not alter binding properties
  - binding should be specific, but affinity not so high as to prevent elution
- elution: destabilize binding
  - compete with free ligand
  - change pH, ionic strength
  - chaotropic or denaturing agents



Ligand:
- substrate
- co-factor
- inhibitor
- interacting protein

Gel Electrophoresis

- Proteins can be separated with extremely high resolution by electrophoresis
- PAGE (polyacrylamide gel electrophoresis)
    - Native PAGE: separate proteins according to their overall charge
        - charge depends on pH
        - native means intact 3-D structure: affects migration
    - SDS-PAGE: Protein can be denatured in SDS / beta-mercaptoethanol
        - constant negative charge
        - constant mass/charge ratio
        - "fully" denatured: no 3-D structure, migration according to size
        - if activity needed, avoid full denaturation (no boiling, less SDS)

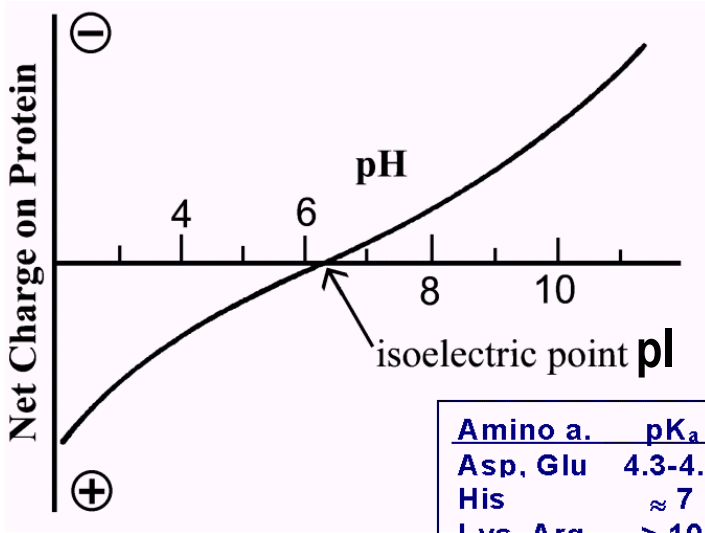


	Stacking Gel	Separating Gel
Acrylamide	3-4.5%	6-20%
pH	6.8	8.8
Ionic Strength	0.125 M Tris	0.375 M Tris

- Stacking increases resolution
- proteins compressed at the interphase

Isoelectric Focusing

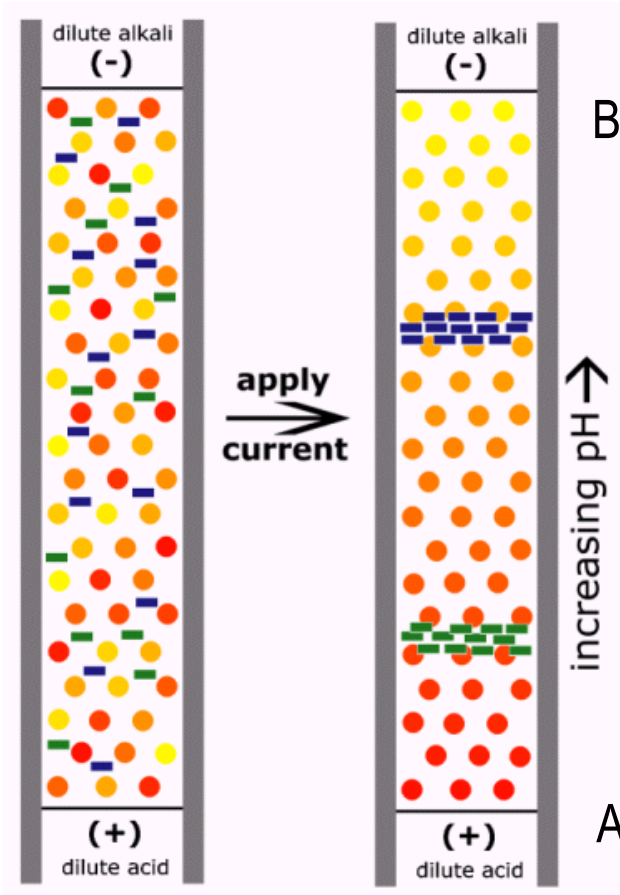
- Proteins are separated according to isoelectric point
- Size does not contribute to separation
    - native
    - denaturing
        - urea
        - non-ionic detergents



Amino a.	pK_a
Asp, Glu	4.3-4.7
His	≈ 7
Lys, Arg	> 10

Carrier Ampholytes

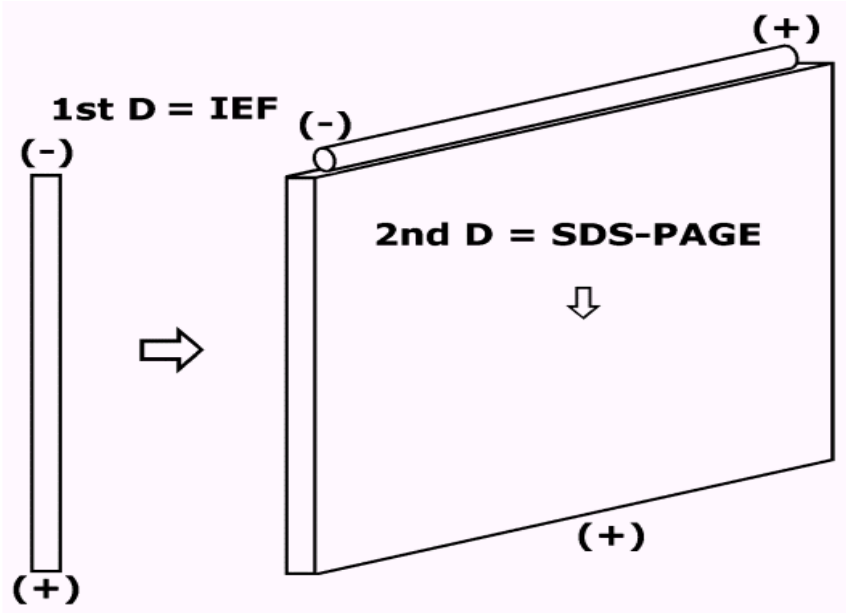
- used to establishpH gradient
- mixture of aliphatic amines
    - with carboxylic groups
    - with sulfonic acid groups
- gradient range depends on the mixture of ampholytes
    - wide range (eg. 7 pH units)
    - narrow range (eg. 3 pH units)
- anode: dilute acid (H_3PO_4)
- cathode: dilute base (NaOH)

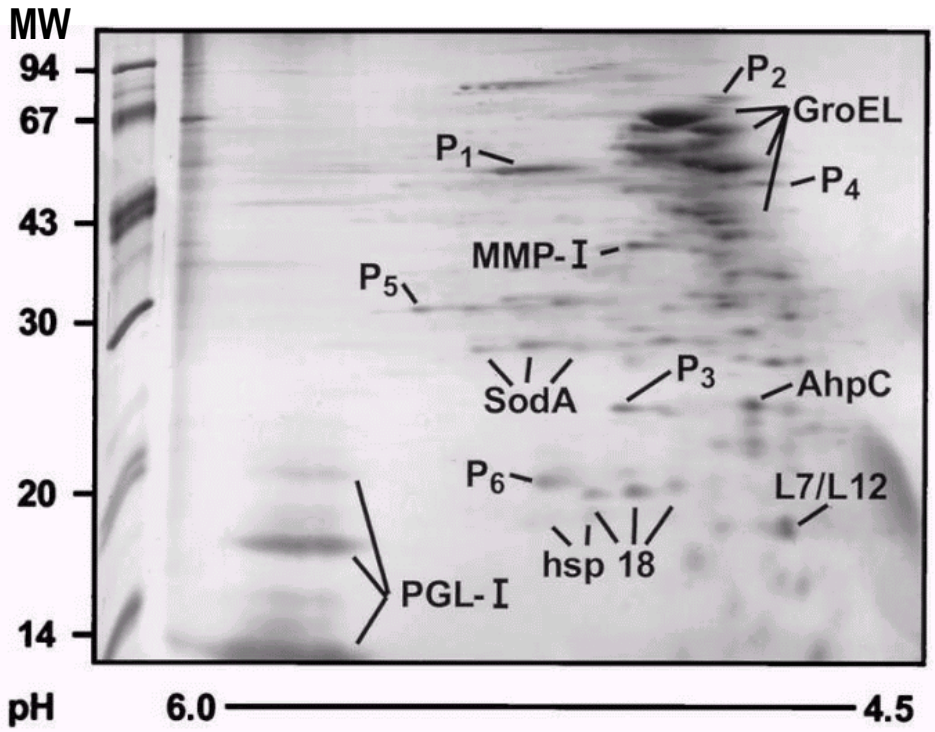


- the gel is run until equilibrium is reached
    - ie. proteins have reached their pI

2D-Gel Electrophoresis

- 1st dimension: separation according to pI (use IEF)
- 2nd dimension: separation according to molecular weight (SDS-PAGE)
- excellent resolution because two separation criteria are used

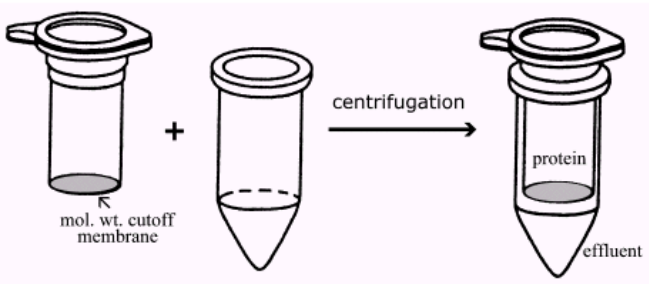


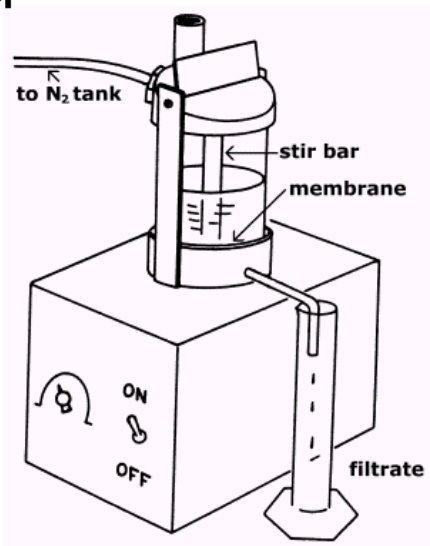


Concentrating Protein

Purification usually leads to dilution of the protein

- precipitation (eg, $(NH_4)_2SO_4$)
- lyophilization (freeze drying)
- dialysis against 50% glycerol
- 'dialysis' against solid PEG
- ultrafiltration



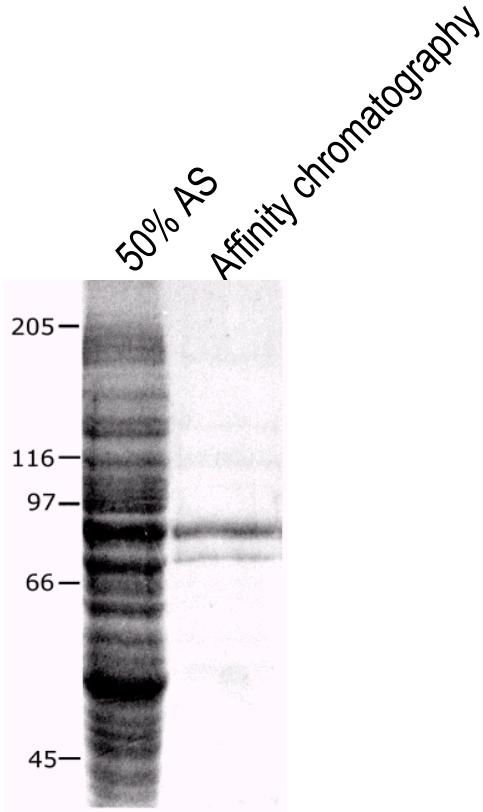


How Pure is your Protein ?

- qualitative (gel electrophoresis)
- quantitative
  - recovery (% yield)
  - fold-purification

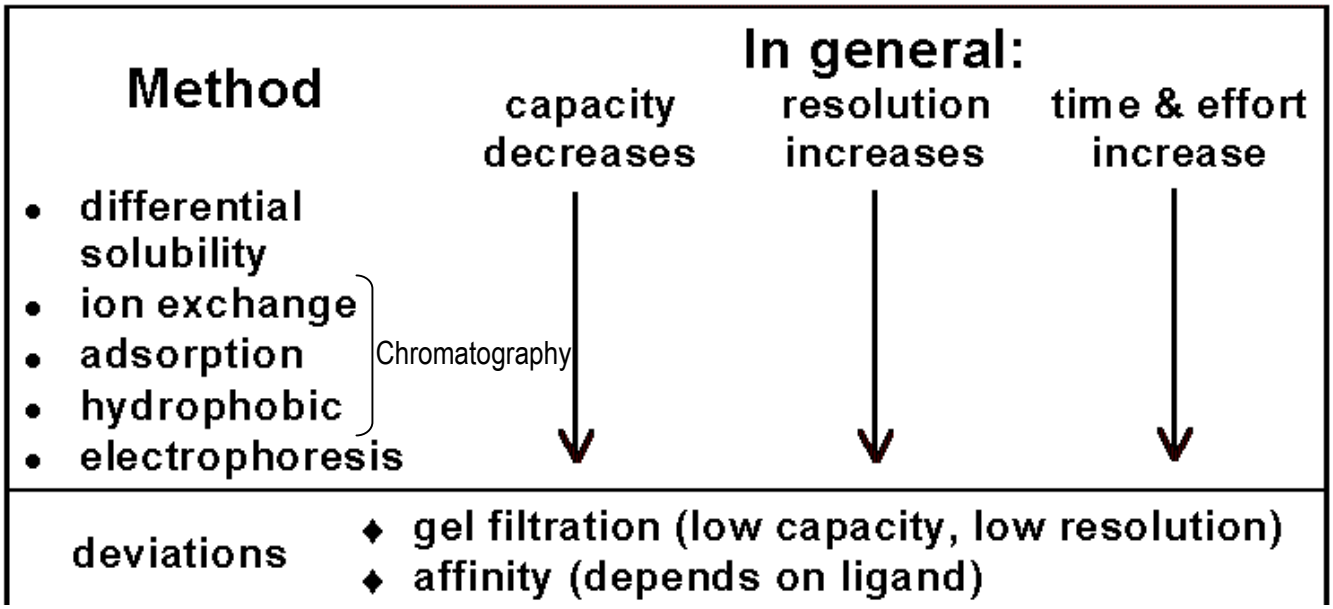
$$\% \text{ yield} = \frac{\text{total act. recovered}}{\text{total starting act.}}$$

$$\text{fold-purification} = \frac{\text{sp. act. recovered}}{\text{starting sp. act.}}$$



Protein Purification

Capacity vs. Resolution

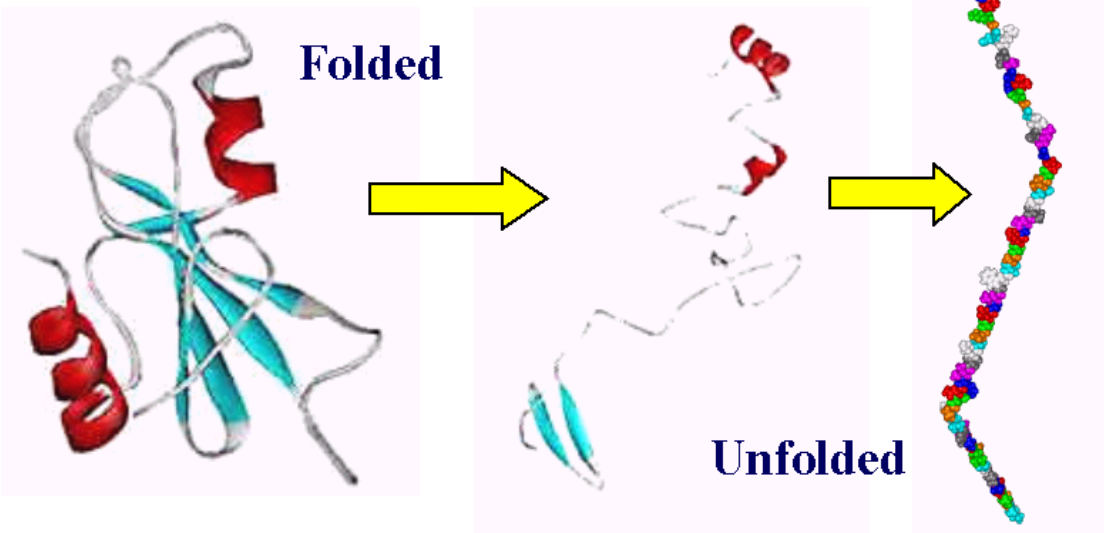


Development of Protein Purification Schemes:

- Pilot experiment (small scale) to test various techniques
  - followed by scale-up
- Start with rapid and high-capacity techniques (crude purification)
  - progress to higher resolution (low-capacity) techniques as required
- Minimize time and number of manipulations necessary
  - eg. use of compatible buffers
- Exploit any unique features that may help purify protein (chromatography)
  - eg. Yeast SNF1 and poly-His tag, hydrophobicity, etc...

Protein Denaturation

- **Denaturation** is caused by disruptive forces that overcome the bonding that keeps a protein folded properly
- unfolding leads to loss of 3-D structure
- loss of 3-D structure leads to loss of function



Factors Affecting Protein Stability

Factor	Possible Remedies
temperature	Avoid high temperatures. Keep solutions on ice.
freeze-thaw	Determine effects of freezing. Include glycerol in buffers. Store in aliquots.
physical denaturation	Do not shake, vortex or stir vigorously. (Protein solutions should not foam.)
solution effects	Mimic cellular environment: neutral pH, ionic composition, etc.
dilution effects	Maintain protein concentrations > 1 mg/ml as much as possible.
oxidation	Include 0.1-1 mM DTT (or β-ME) in buffers.
heavy metals	Include 1-10 mM EDTA in buffers.
microbial growth	Use sterile solutions, include anti-microbials, and/or freeze.
proteases	Include protease inhibitors. Keep on ice.

- You can **try** and refold a protein; more often than not this will not work
- Avoid denaturation in the first place !!!!

Why Heterologous Expression of Recombinant Proteins ?

In order to work on a protein, you must isolate it and purify it

Before recombinant DNA

- isolation and purification from original sources
- find a source tissue that is enriched for the protein of interest !!!!

- **Advantages:**
  - working with the real deal !!!!
- **Disadvantages:**
  - need high amounts of starting material
  - low yield in most cases
  - purification of individual protein from a myriad of other proteins!!!

After recombinant DNA:

- cloning of the gene, expression of the gene in heterologous system

- **Advantages:**
  - don't need to have continuous source of original source !!!!
  - higher "starting purity"
    - over-expression: higher proportion of total protein content
    - protein secretion
  - use protein engineering to facilitate purification
    - purification tags
    - protein secretion
- **Disadvantages:**
  - protein may have incorrect structure
    - folding
    - post-translational modifications

Protein chemistry is tough enough; use rDNA tech to solve some problems!!!

Components of a Heterologous Expression System

The Host ie. who's gonna express your gene ?

- bacterial cells
- unicellular eukaryotes
- insect cells
- mammalian cells
- plants
- animals

The Vector ie. how are you gonna ensure gene expression in the host?

- plasmids
- bacteriophages
- viruses
    - strong promoter
    - good transcriptional regulatory sequences
    - all of the signals to ensure good translation
        - Ribosomal Binding Site
        - in-frame cloning of the coding sequence
        - translation termination

The "DNA transfer Method" ie. how are you gonna get the DNA into host cells ?

- chemical transformation
- viral transduction
- protoplast fusion
- liposomes
- electroporation
- microprojectile
- microinjection

The Production Method ie. how are you gonna mass produce the protein ?

- shake cultures
- fermentors
- bioreactors
- the plant/animal

The Purification System ie. how are you gonna get pure protein ?

- Protein chemistry approaches
- rDNA approaches