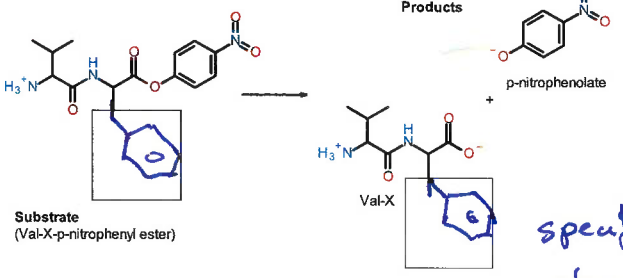
Lecture 21: Protein Purification.

Goals:

- Calculate specific activity
- Calculate net yield
- How separation techniques are applied
- How to develop a purification step

How to monitor purity: It is essential to have some method to evaluate purity, otherwise how do you know whether a specific step in the purification scheme has been successful in increasing the purity of the desired protein? To do so, we need to obtain two experimental measurements:

i) *Amount of **target** enzyme:* It is essential to have some method of determining the amount of the enzyme you are trying to purify at any given step of the purification scheme. Consequently, the most critical step in any purification scheme is to develop a ***specific*** assay for the enzyme that is being purified. This is usually done using enzymatic assays that produce colored products – allowing a spectrophotometric assay.



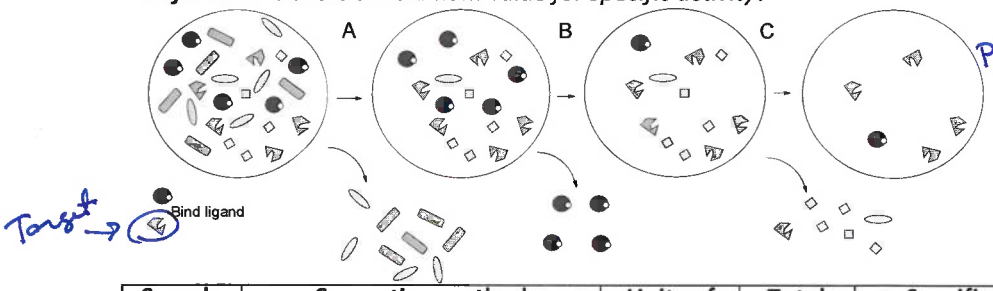
Reflection: If you were trying to purify chymotrypsin from pancreatic tissue, what substrate should you use?

ii) *Amount of total protein in the sample:* Simply measuring the absorbance at λ = 280 nm will often suffice. *What amino acids absorb at this wavelength?*

The purity of the enzyme during the purification scheme is generally monitored by measuring the specific activity:

Specific Activity: *The velocity of the enzyme catalyzed reaction for the desired enzyme divided by the **total** amount of **all protein** species in the sample. Typical units would be μmol/sec/mg total protein, where the μmol/sec refers to the amount of product produced (μmol) per unit time.*

Reflection: *Is there a maximum value for specific activity?*

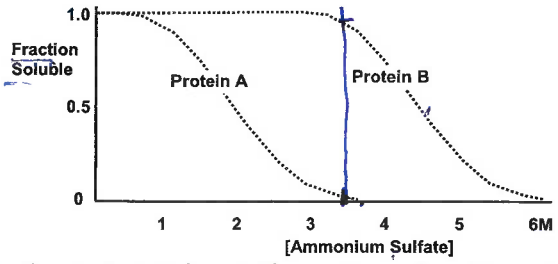


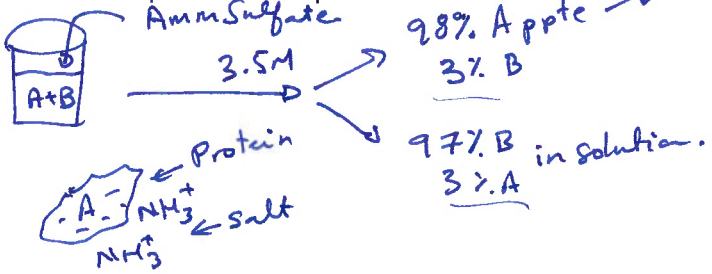
Sample	Separation method	Units of Activity	Total Protein	Specific Activity
Lysate		50	25	2.0
Step A	Size	50	16	3.1
Step B	Not spherical	50	12	4.2
Step C	Binding site for Ligand	40	5	8.0

Net yield (final amount of target/starting amount of target):

Increase in purity (final specific activity/initial specific activity):

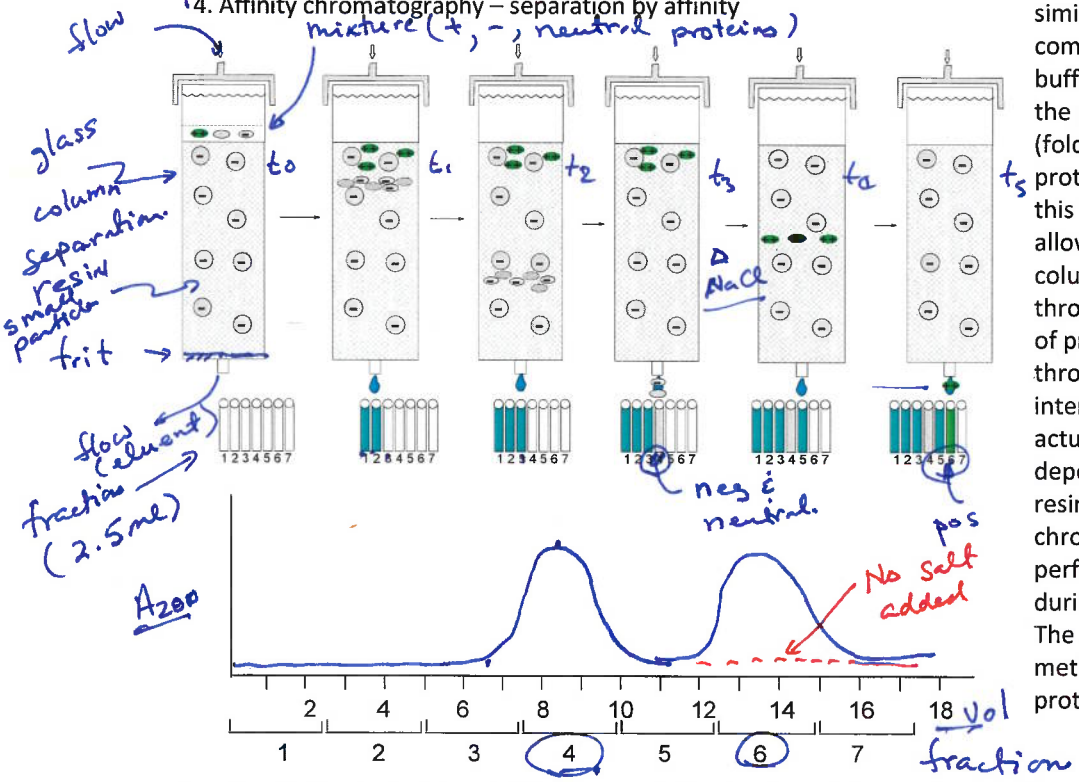
Separation by Solubility.: High concentrations of ammonium sulfate can precipitate proteins by reducing the ability of water to interact with the protein. Different proteins have different levels of solubility at any given concentration of ammonium sulfate. After separation, the precipitated proteins *usually* remain active and can be re-dissolved in buffer and carried to the next purification step.





Separation using Column Chromatography: There are four common methods of separation by column chromatography.

1. Size exclusion (gel filtration) – separation by size
2. Anion exchange – separation by charge
3. Cation exchange. – separation by charge
4. Affinity chromatography – separation by affinity



Chromatography is performed in long glass tubes filled with a matrix or resin (particle size similar to a fine sand) that is completely immersed in a buffered salt solution to keep the proteins in their native (folded) form. The mixture of proteins is added to the top of this column and buffer is allowed to flow through the column. As the buffer flows through the column the mixture of proteins is drawn down through the column and interacts with the resin. The actual mode of separation depends on the nature of the resin. Usually several different chromatographic steps are performed with different resins during a purification scheme. The actual order of separation methods will depend on the protein being purified.

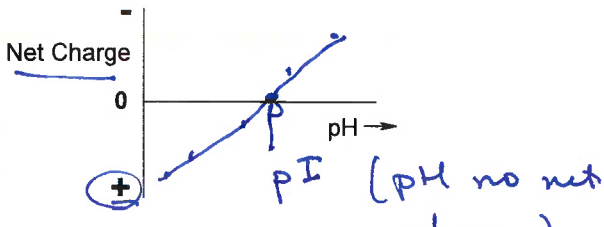
Fraction: Collection of liquid that drips out the bottom of the column – i.e. the total amount of liquid is fractionated into separate fractions. Fractions containing the protein of interest are pooled (combined) before next step.

Effect of pH on net Charge of Protein:

pI (Isoelectic pH)- pH at which proteins have no *net* charge:

- Proteins become positively charged for pH < pI
- Proteins have a negative charged if pH > pI.

Therefore, the charge on a protein can be changed by altering the pH, increasing or decreasing the binding to ion exchange resins.

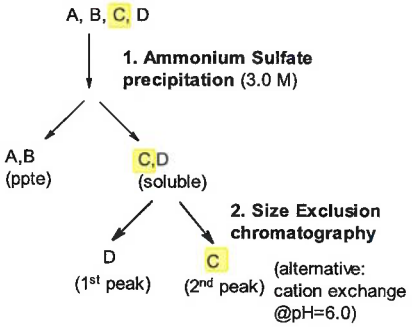


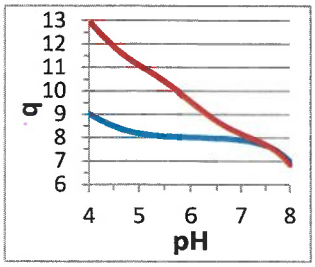
Example – Purification of Protein C

Step 1 – ppt using 3 M amm. Sulfate

- Set concentration of salt (3M)
- Perform step
- Assay fractions (ppte & super.)
- Collect supernatant

Protein	[Ammonium Sulfate] that precipitates 50% of protein*	# Residues (Mol Wt)	#Asp (pK_a=4.0)	#His (pK_a=6.0)	#Lys (pK_a=9.0)
A	1.0 M	120 (13,200 Da)	2	0	10
B	1.5 M	120 (13,200 Da)	4	4	12
C	4.0 M	120 (13,200 Da)	4	3	12
D	6.0 M	240 (26,400 Da)	2	0	10





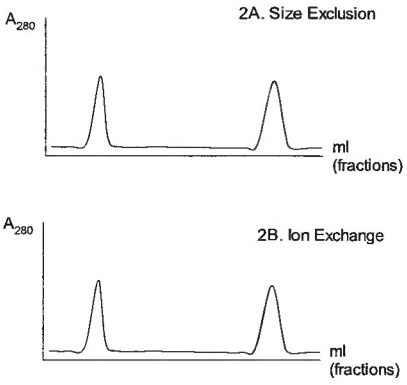
Step 2A – Size exclusion (gel filtration)

- Run column
- Assay one fraction each peak
- Select all fractions with activity
- Collect samples that contain C.

Alternative Step 2B – cation exchange @ pH=6.0

q_C = -4 + 1.5 +12 = +9.5, will elute 2nd peak.

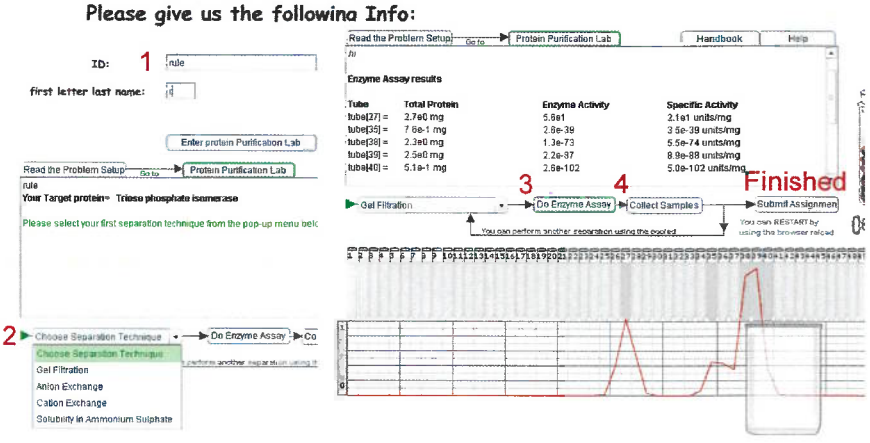
q_D= -2 + 0 + 10 = +8.0, would elute 1st peak

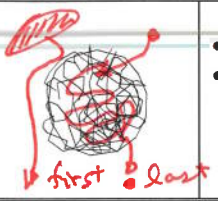
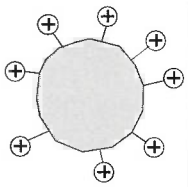
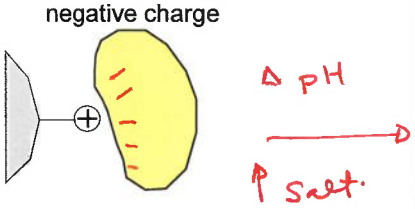
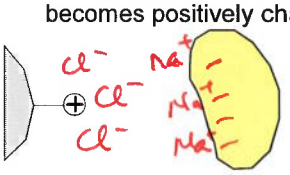
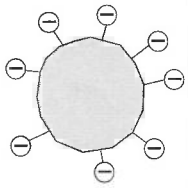
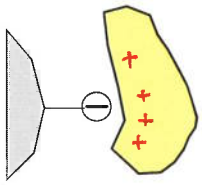
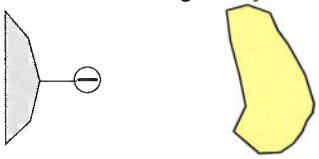
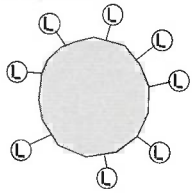
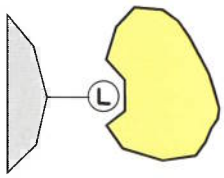
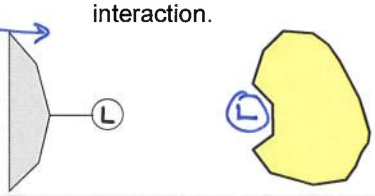
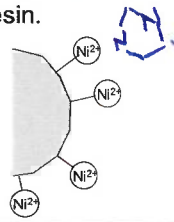
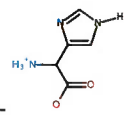
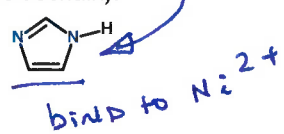
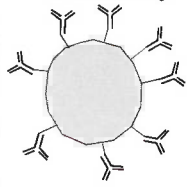
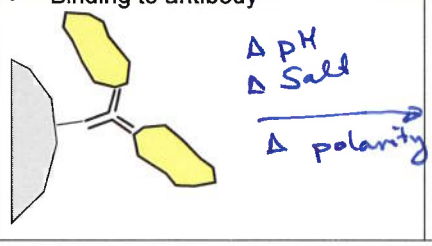


Demo - Protein Purification Simulation:

http://www.andrew.cmu.edu/user/rule/prot_pure/

1. Select protein (determined during login for problem set)
2. Select the purification scheme from the drop-down menu. If you select anything else but gel filtration, you will also need to define the pH of the separation (ion exchange) or the amount of ammonium sulfate (M).
3. After the separation occurs, you need to find out where your enzyme is. In the case of column chromatography, click on the tubes that you think will have your enzyme. In the case of ammonium sulfate, the two fractions will be the precipitate (tube 1) or the supernatant (tube 2). After you have selected the fractions that you want to assay, click on the "Do Enzyme Assay" button. The simulator will return the total amount of protein, the activity of your enzyme, and the specific activity of each fraction. *In the above example, tubes 27, 35, 38, 39, and 40 were assayed for enzyme activity. Tube 27 showed a high level of activity, indicating that the first peak off of the column corresponds to the target protein.*
4. Once you have determined where your enzyme is, you want to tell the program which fractions to pool to carry forward to the next step. Select those fractions by clicking on them, you must to this, even if you had selected the fractions for enzyme assays. Once you have selected the fractions you want, click on the "Collect Samples" button, this will pool all of your samples into the beaker. You can either do another separation step or if your protein is pure, click on the "Submit Assignment" button. *In this example, I would have selected tube 25, 26, 27, 28, and 29.*



Type	Type of Resin	Principal of Separation	How to Elute the Protein
Size Exclusion (gel filtration) *Separation by size*		• Proteins don't "stick". • Small proteins enter the interior of the beads, and therefore take longer to wash off of the column	• Simply washing the column with buffer will eventually wash the proteins out of the column. • Smaller proteins elute last.
Anion Exchange *Separation by charge.*	• Beads with a **positive** charge 	Protein stick to resin because of: • Overall **negative** charge (*anions*) • Proteins have patches of negative charge 	• Wash, bound proteins **may** elute. • Increase salt concentration to weaken electrostatic interaction. • Change of pH to pH < pI (protein becomes positively charged) 
Cation Exchange *Separation by charge.*	• Beads with a **negative** charge 	Protein stick to resin because of: • Overall **positive** charge (*cations*) • Proteins have patches of positive charge 	• Wash, bound proteins **may** elute. • Increase salt concentration to weaken electrostatic interactions. • Change of pH to pH > pI (protein becomes negatively charged) 
Affinity Chroma-tography *Separation by affinity, either ligand affinity, or antibody..*	• Beads with a ligand:. 	Protein stick to resin because of: • Binding site for ligand 	• Excess ligand • Change in pH, Salt, solvent to weaken protein-ligand interaction. 
	Common: Ni^{+2} ions attached to resin. 	Protein has 6 His residues on N or C-terminus. "His-tag": HisHisHisHisHisHis---------- 	Eluted with imidazole (similar to His sidechain). 
	• or Antibody 	Protein stick to resin because of: • Binding to antibody 	• Changes in solution conditions (pH, Salt, solvent) to weaken protein-antibody interaction.