Why purify proteins?

- Detailed studies on function
- Determination of structure
- Industrial/pharmaceutical applications
- Generate antibodies
- Amino acid sequence determination

Protein purification issues

- How much and how pure?
  - Application
  - Source
  - Feasibility
- Native configuration?
  - Functional/structural (yes)
  - Microsequence (no)
  - Antibody (maybe)
- Detection Method
  - Functional assay
  - Antigenic assay
  - Band on gel

Table of common methods of protein purification

Property	Methods
solubility	Precipitation with ammonium sulfate (salting out)*
Size / shape	Size-exclusion chromatography
Isoelectric point (charge)	Ion exchange chromatography
binding to small molecules	Affinity chromatography

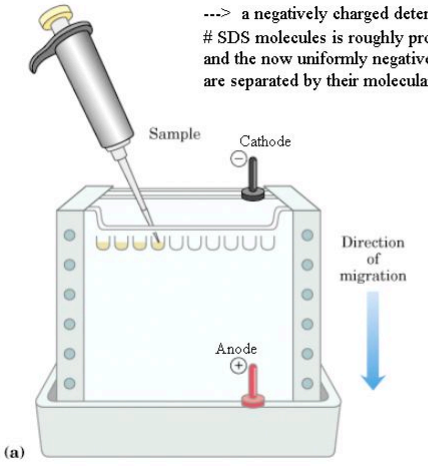
- Purification procedures attempt to maintain the protein in native form. Although some proteins can be re-natured, most cannot!
- To purify a protein from a mixture, biochemists exploit the ways that individual proteins differ from one another. They differ in:
  - Thermal stability*

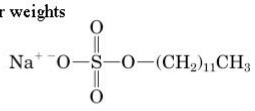
*Ammonium sulfate precipitation is cheap, easy, and accommodates large sample sizes. It is commonly one of the first steps in a purification scheme.

*For most protein purifications, all steps are carried out at ~5°C to slow down degradative processes.

Gel electrophoresis = separation in an electric field
often SDS-PAGE = SDS polyacrylamide gel electrophoresis,

---> a negatively charged detergent (SDS) binds the protein, # SDS molecules is roughly proportional to # of amino acids, and the now uniformly negatively charged proteins (anions) are separated by their molecular weights

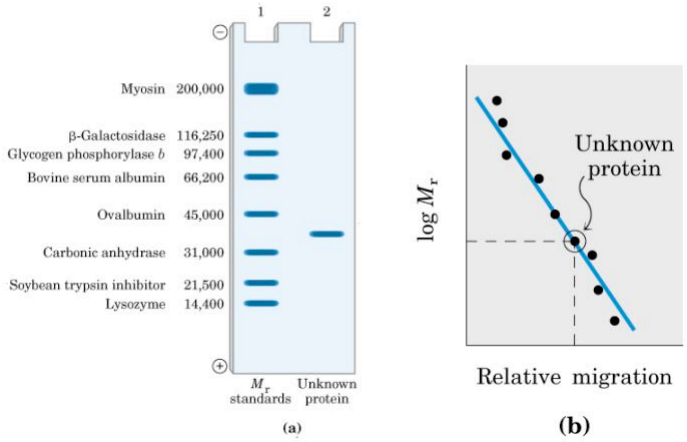




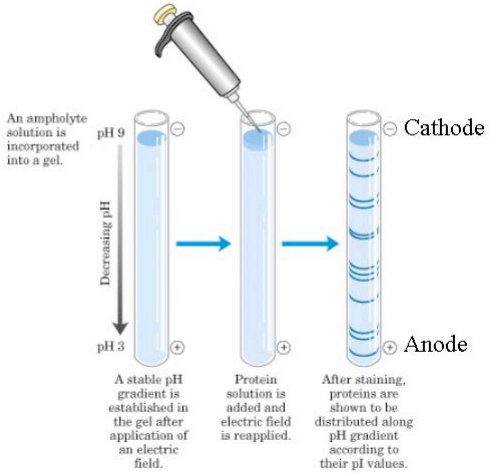
Sodium dodecyl sulfate (SDS)

Add to protein sample, often with a reducing agent like β-mercaptoethanol, heat, run on gel

Finding apparent (e.g. rough) molecular weight by running a gel with molecular weight standards:

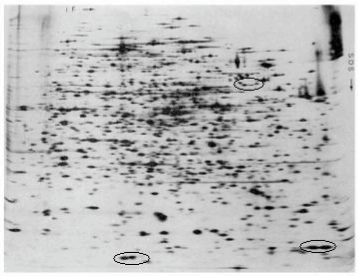


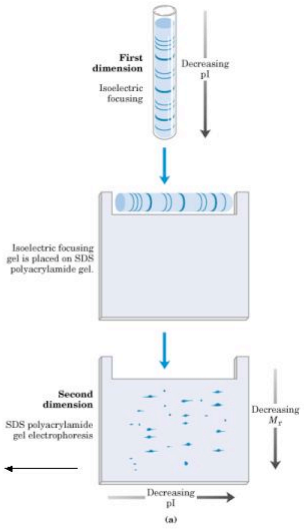
Isoelectric focusing (IEF) gel electrophoresis = separation by pI



Two-dimensional (2D) gels:

What might the circled proteins represent?





Define properties of target protein and critical impurities

- Know your protein's structure and function
  - pH, temperature stability (proteins may precipitate at pH=IEP)
  - Effects of salt, detergent, organic solvent, metal ions
  - Post-translational modifications that must be present—these will affect structure and properties

Strategy

1) Select a source
2) Break open cells, separate components
3) Keep the protein native (usually cold)
4) Develop an assay to follow the protein
5) Purification steps based on the properties of the protein

First Steps

1. Source. A good source is cheap and readily available. Many proteins are enriched in specific tissues, for example hemoglobin in blood. For this reason, these tissues may be excellent sources for your protein.
2. Assay. Most assays are chemical reactions catalyzed by specific enzymatic activities. Proteins that have no activity are usually assayed using SDS polyacrylamide gels.

First steps--Develop an Assay

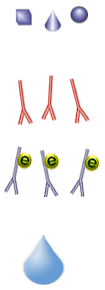
An assay for an enzyme is a method for quantifying its activity.

Since the assay is repeated many times, it is important that it be a simple procedure. Usually enzyme activity is monitored as a change in absorbance which can be measured using a spectrophotometer. For example an assay for ribonuclease measures the change in absorbance that accompanies the breakdown of RNA to ribonucleotides.

Develop analytical assays for your protein (and impurities)

- Protein concentration
  - UV/Vis
  - BCA, Bradford, Lowry
  - ELISA
- Protein purity/structure information
  - SDS PAGE, HPLC, IEF, Western blot
- Biological activity
  - *In vitro, in vivo*
- Impurities—allergens, immunogenic proteins, endotoxins, viruses, bacteria, etc.
  - e.g., PCR for viruses or bacteria; LAL for endotoxin; western blots or ELISAs for protein contaminants

The ELISA Method



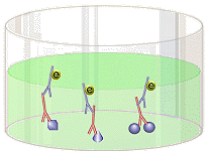
Partially purified, inactivated HIV antigens pre-coated onto an ELISA plate

Patient serum which contains antibodies. If the patient is HIV+, then this serum will contain antibodies to HIV, and those antibodies will bind to the HIV antigens on the plate.

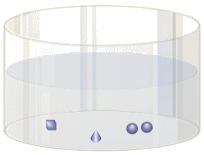
Anti-human immunoglobulin coupled to an enzyme. This is the second antibody, and it binds to human antibodies.

Chromogen or substrate which changes color when cleaved by the enzyme attached to the second antibody.

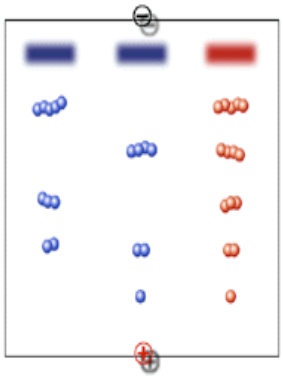
Positive ELISA Test



Negative ELISA Test

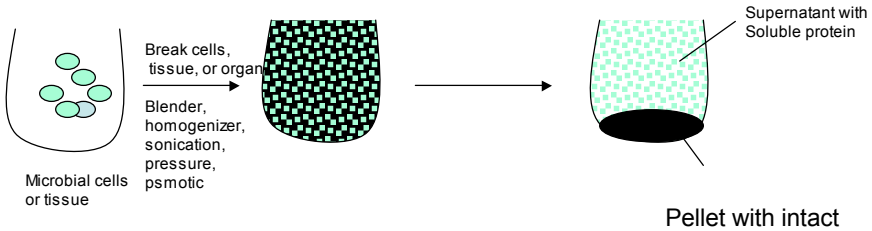


Western Blot Activity: Background



Whereas ELISA measures antibody to whole virus and gives a "positive," "negative" or indeterminate test result, western blotting is a more specific test. It allows one to visualize antibodies directed against each viral protein. For this reason, it is a confirmatory test for a positive HIV ELISA. In an HIV Western blotting, proteins are electrophoresed into a gel. As the proteins migrate through the gel they are separated based upon size and charge. Characteristically, smaller proteins migrate through the gel faster than larger protei

Preparing the sample—Crude extract. Protein from cells or tissue



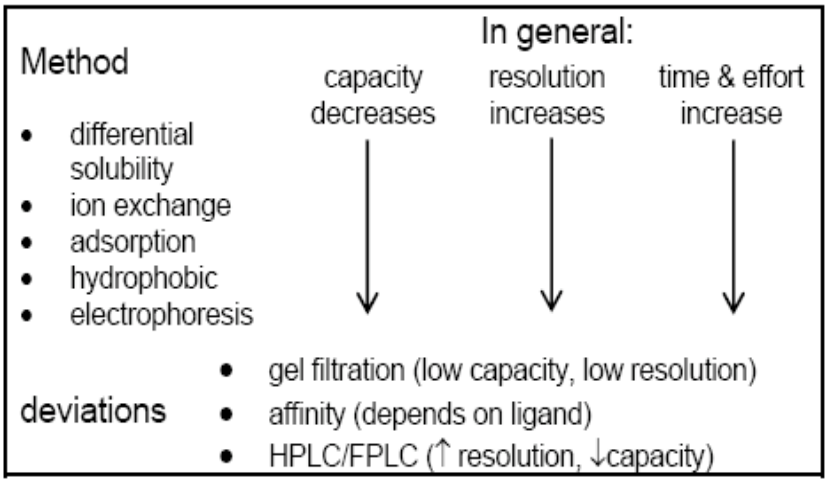
Pellet with intact cells, organelles, membranes, and membrane proteins

Before you start

- Define objectives for purity, activity, and quantity
- Define properties of target protein and critical impurities
- Develop analytical assays for your protein (and impurities)
- Minimize sample handling at every stage
- Minimize use of additives—they will have to be removed
- Remove harmful contaminants early—e.g., multiple barriers for pathogens
- Use a different technique at each step (multiple barriers)
- Minimize number of steps (within reason)

Developing a purification scheme

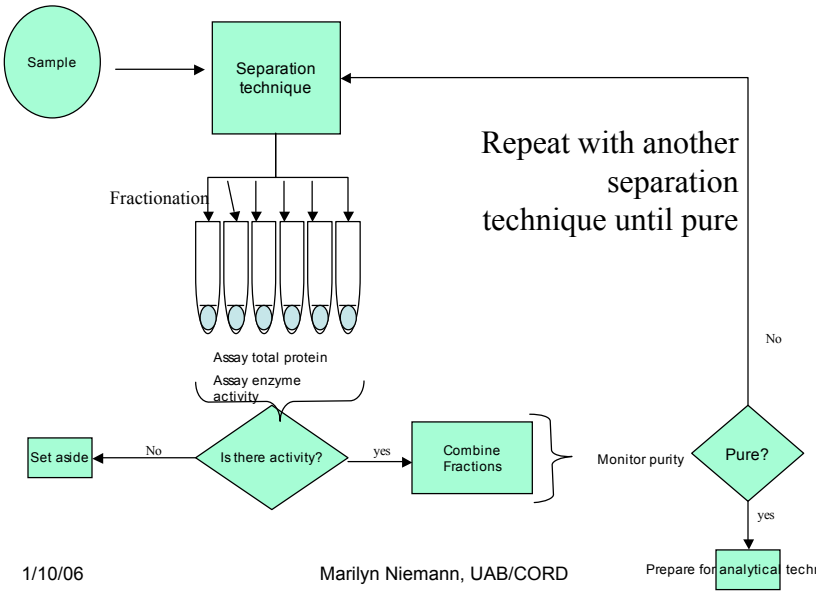
- Carry out small pilot experiments to evaluate various separation techniques
- Start with rapid high capacity techniques (which are generally low resolution) and progress to high resolution low capacity techniques
- Minimize time and number or manipulations whenever possible
- Arrange methods to minimize buffer changes if other factors are equal
- Exploit unique features



Different approaches to purity

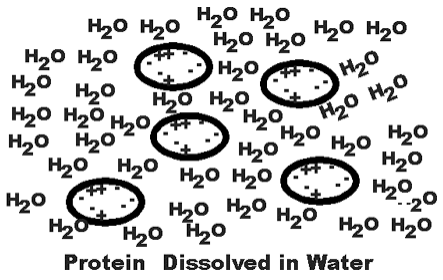
- Purification by removing the target molecule from the contaminants
  - Affinity chromatography techniques are very specific for the target molecule or for a group of molecules with closely related biological properties. This makes them capable of “fishing out” the target molecule (or the group), leaving all contaminants behind.
  - When applicable, these techniques are to be preferred, since they drastically simplify the purification protocol
- Purification by removing the contaminants from the target molecule
  - When a suitable affinity chromatography technique is not at hand, one has to rely on a sequence of general chromatography techniques to remove the contaminants.
  - A typical purification protocol when nothing is known about the target protein employs the IEX-HIC-GF sequence of purification steps.

Purification is a multi-step procedure.

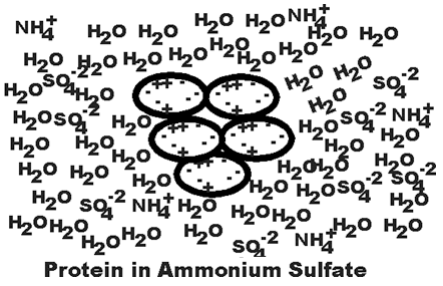


Solubility of Proteins

- Solubility of a protein depends on ionic strength of the solution
- Ionic strength is defined as $I = (1/2) \sum_i C_i Z_i^2$
- The sum is over all ionic species
- Because the charge Zi is squared, divalent and trivalent ions contribute greatly to I.
- Ci is the molar concentration of species i
- Solubility of proteins increases as salt conc is increased, but at high salt conc. the solubility eventually decreases.
- Explanation: Salt (ions) helps to diminish the charge interactions between protein molecules that might cause aggregation or precipitation.
- At very high salt, too much water might be used to solvate the ions, leaving too little to solvate the protein, causing a loss of protein solubility.

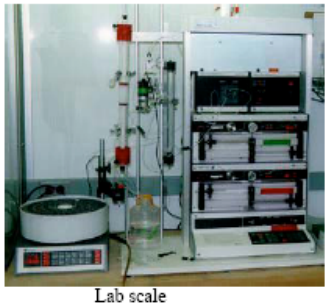


Ammonium Sulfate Fractionation of Protein Mixtures

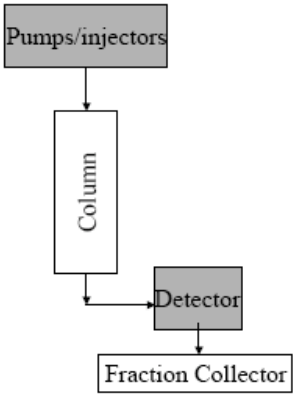


Equipment

- Pump
- Column
- Detector – usually UV/Vis
- Fraction collector

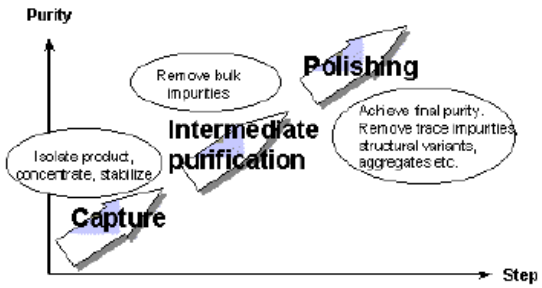


Lab scale



Combining purification steps

Three Phase Strategy

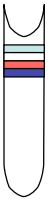


CIPP

- **The capture (C) step** aims at concentrating the sample and removing the bulk of the contaminants. Emphasis is primarily on *speed and load capacity*. Media with high load capacities and good flow properties are used often under step elution conditions.
- **The intermediate purification (iP) step(s)** aims at separating the components of the now concentrated partially purified sample. Emphasis here is on *maximum resolution* since the remaining contaminants have rather closely related chromatographic properties. This is done by *combining techniques of independent selectivities* run in high resolution mode (gradient elution, smaller bead sizes etc.).
  Flow rates and load capacities have to be restricted or resolution will suffer.
- **The polishing (P) step(s)** serves to achieve the final purity and to eliminate contaminants such as polymers etc. and is also often used to condition the final product, e.g. to remove salts, when the final product is to be lyophilised.

Load Sample (4 protein mix)

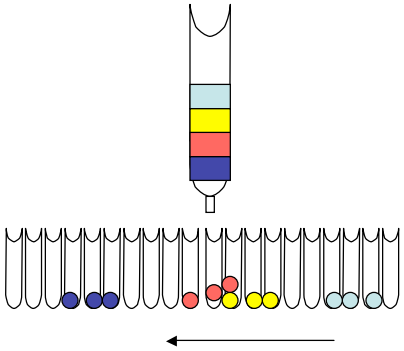
Image of apparatus with protein mixture on top.



The crude extract is placed on top of the solid matrix. In this case we are using a mixture of 4 proteins, indicated by different colors.

The proteins move at different rates through the matrix based on the properties of the proteins and the type of column beads.

Fractionation



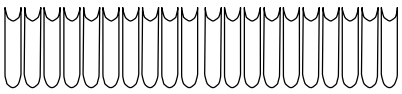
As the column separates the proteins in the mixture, the effluent drips into a series of fraction tubes that are moving at a specific rate of speed. These tubes are called *fractions.*

Here we are showing 20 tubes. Fraction collectors in most labs have about 75-200 tubes.

Be sure to remove color from column as it drips into the tubes below! If the sample is spread over three tubes, the center tube will be darker in color.

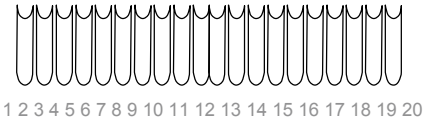
In reality, proteins aren't color-coded so we must ask ourselves three questions:

1. How do we know which fractions contain protein?
2. Which of those fractions contain the desired protein?
3. How do we asses the purity?



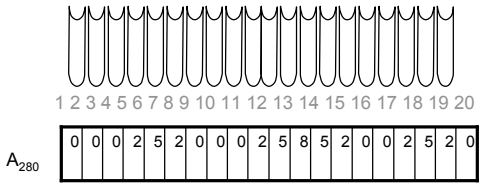
???

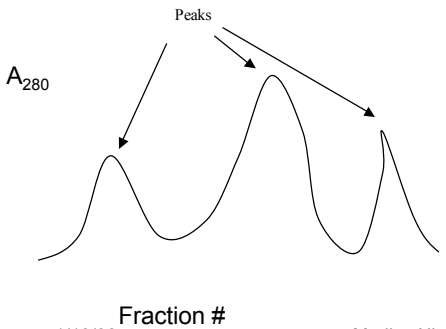
Question 1. How do we know which fractions contain protein?



Total protein a can be estimated by taking the absorbance at 280 nm in a spectrophotometer. Aromatic amino acids absorb light at this wavelength causing all proteins to have absorbance at 280nm. Many fraction collectors measure the A280 as the column is running.

Question 1. How do we know which fractions contain protein?



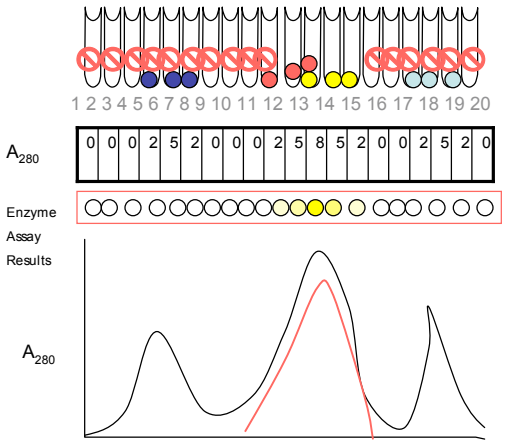


Total protein a can be estimated by taking the absorbance at 280 nm in a spectrophotometer.

The values can be plotted against the fraction number in is what is called an *elution profile*.

Notice the peaks on the graph. These indicate where the fractions are that contain protein.

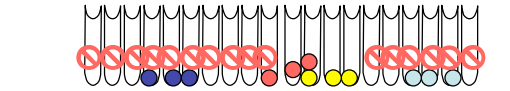
Question 2. Which fractions contained the desired protein?



Enzyme activity can be determined by performing an enzyme assay on each fraction that contains protein.

Notice the results of the enzyme assay. Notice that the highest activity corresponds to one of the peaks.

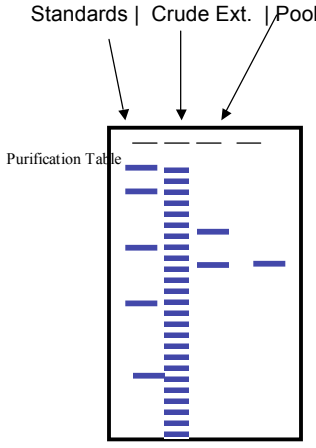
Combine (pool) the fractions with activity



1. NEXT We want to pool the fractions that have enzyme activity

It may be useful to consider more than just the activity of a fraction. Specific activity is a measure of the amount of enzyme activity per amount of protein (units/mg). The higher the specific activity, the higher the purity. When pooling fractions, judgement is needed as to whether to optimize yield or specific activity.

Question 3: Is this pooled sample pure? How do you monitor the progress?



1. Look at it on a gel. A monomer should have <u>one</u> band.
2. Calculate the *specific activity* by doing a careful quantitative assay for enzyme activity/total protein.

Purification Table

Procedure	Fraction vol (ml)	Total Prot (mg)	Activity (units)	Specific activity Units/mg
Crude cellular extract	1400	10000	100000	10
Separation method 1	90	400	80000	200
Separation method 2	**8**	**4**	**60000**	**15000**

Results:
1. Gel shows one band
2. Specific activity is 15000. Looks good

Your protein seems pure. YOU'RE DONE!!!.

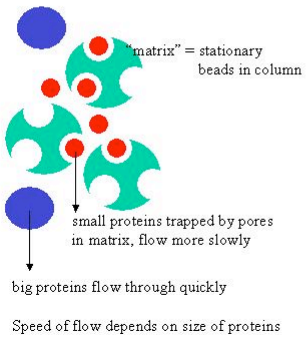
Size Exclusion

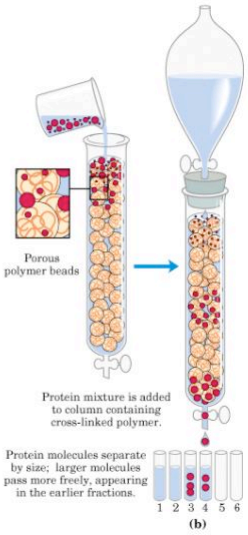
	LC	FPLC	HPLC
<u>Sta phase</u>	agarose	agarose	silica gel
<u>Part size</u>	50-200 micron varying pore sizes	15 micron 2 pore sizes	5 micron 3 pore sizes
<u>Elution</u>	Isocratic. Separations achieved on the basis of partition, i.e., the speed at which proteins move through the column is inversely related to their ability to penetrate the gel particles in the stationary phase. Thus large proteins are eluted quickly, small ones later in an order governed by their molecular weight or hydrodynamic volume.		

Concentration Methods

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

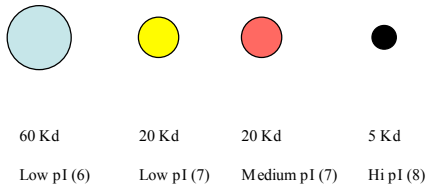
Size exclusion chromatography (also called "gel filtration") = separate by size, not charge



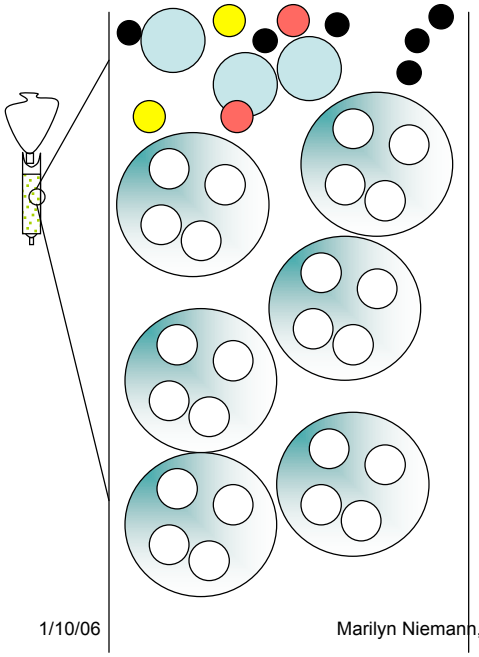


Gel Filtration--Basis

Here's our sample mix of proteins.
Our goal is to purify protein #….

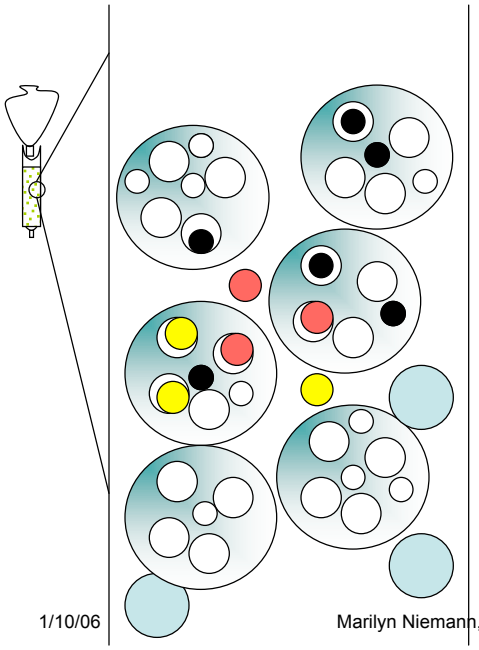


- Gel filtration column chromatography separates proteins on the basis of size.
- We will start with 4 proteins.
- You will want to purify the "yellow one"



Gel filtration 2:
Close-up of beads

- The matrix of a size-exclusion chromatography column is porous beads.

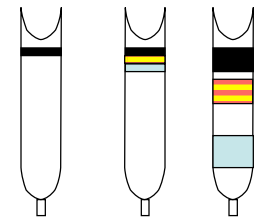


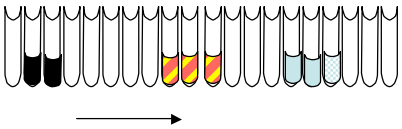
Gel filtration 3:

Run close-up of column

- The matrix of a gel filtration column are beads with pores.
- The large green proteins can't fit in pores so flows faster.
- The red/yellow medium sized proteins get trapped in the pores.
- The black small proteins stay trapped in pores longer.

Gel filtration 4 - zoom out

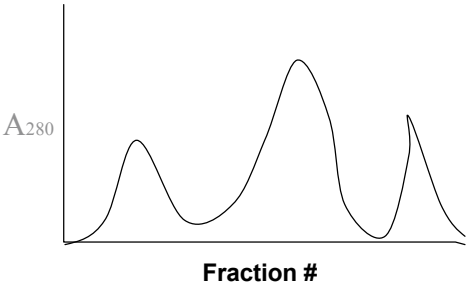




Tubes march in from left

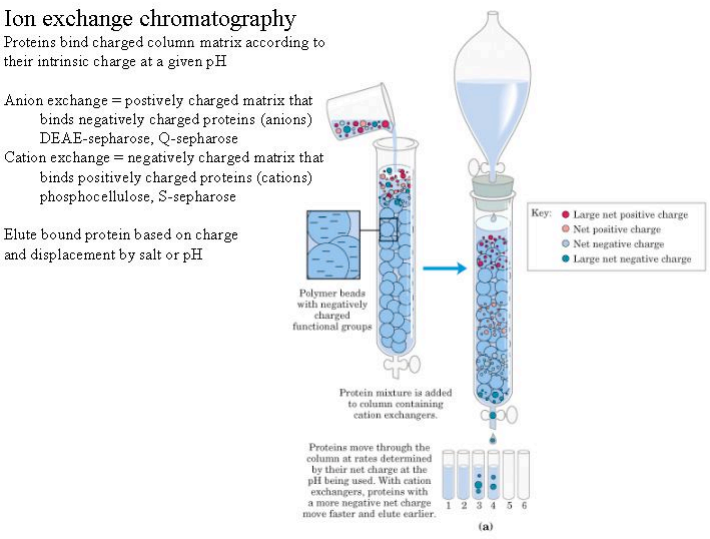
Click on the peak that represents the protein of the largest molecular weight.

Be sure to keep in mind that the colors will either be in the column or in the tubes, not both!

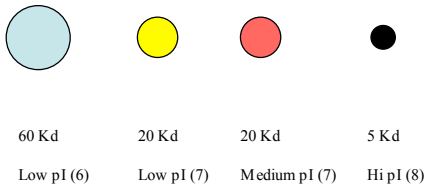


Ion Exchange

	LC	FPLC	HPLC
Sta phase	CM-cellulose DEAE-cellulose	Sulfonated agarose TEAE-agarose	Sulfonated SG Amine SG
Part size	200-400 microns	35-50 microns	5 microns
Elution	Gradient delivering eluant of increasing ionic strength. Counter ions in eluant compete with bound protein for sites on stationary phase.		
Capacity	grams	milligrams	1-10 mg- Micrograms

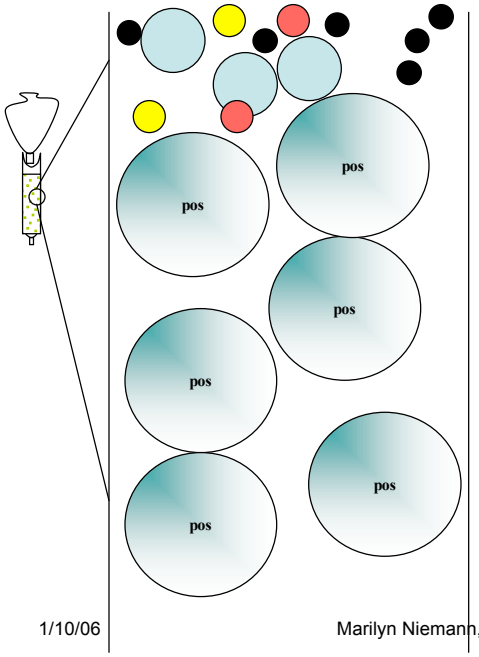


ION –EXCHANGE 1

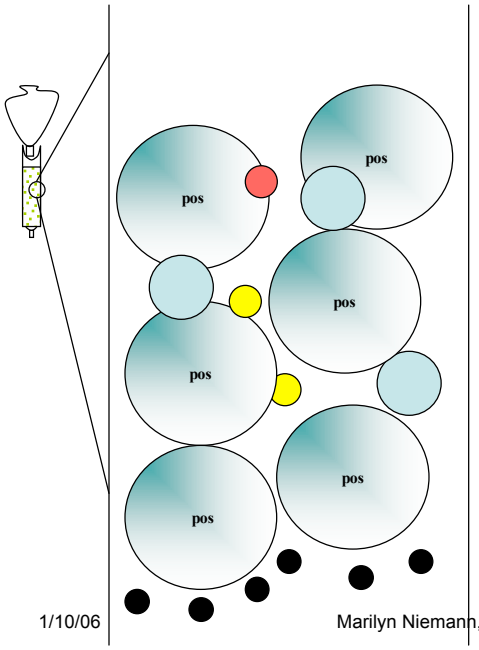


- Ion-exchange column chromatography separates proteins on the basis of charge.
- We will start with 4 proteins.
- pH 7.2
- pos charged column

ION EXCHANGE 2

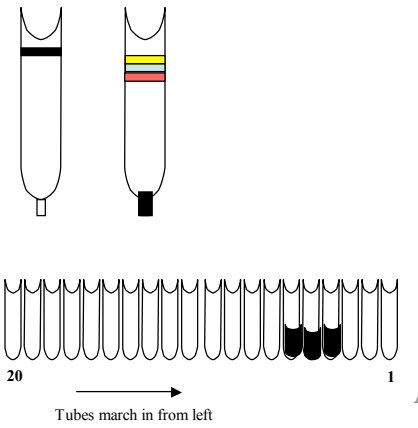


- The matrix of an ion exchange is positively charged.
- What do you think will happen?



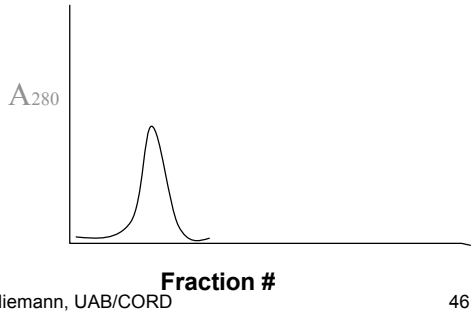
COLUMN RUN

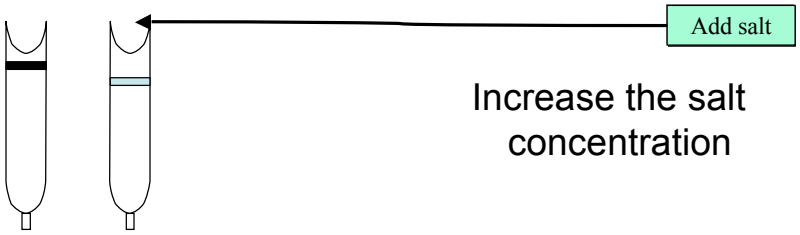
- The matrix of an ion exchange is positively charged.
- Only the pos charged proteins run through the pos charged column. The others "stick" to the column.



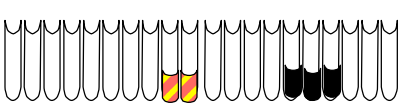
Only the POS charged proteins run through the column.

How can we elute the other proteins?

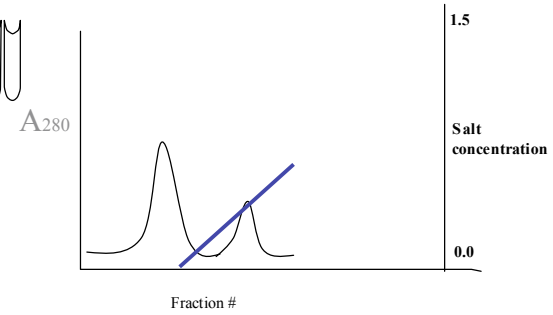




Increase the salt concentration



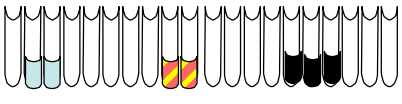
Tubes march in from left



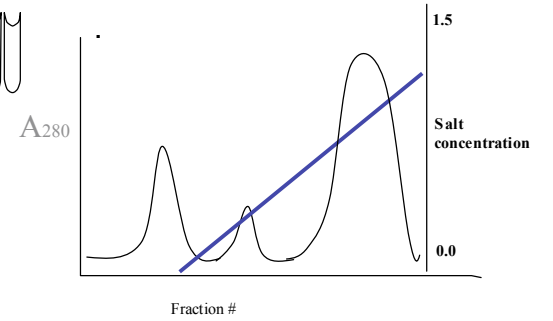
RESULTS

Notice that 2 of the proteins eluted at the same time. Why?

Is our protein pure? We were supposed to purify the red one.



Tubes march in from left



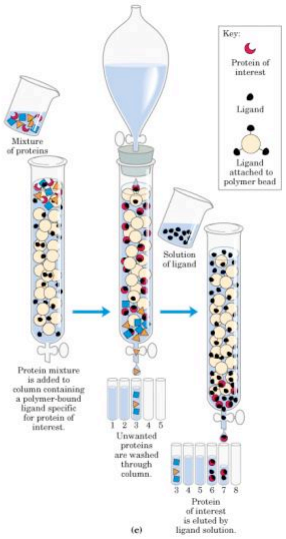
Affinity Chromatography

	LC
Sta phase	crosslinked agarose derivatized with appropriate ligand Derivatization usually obtained by activating agarose with CNBr, which allows covalent attachment of amine-containing compounds to the agarose polymer.
Part size	200-400 microns
Elution	Isocratic. Using solvent capable of dissociating desired material from the ligand causing its retention on the column.

Affinity chromatography = column matrix specifically binds proteins (often recombinant proteins with genetically manipulated "tags")

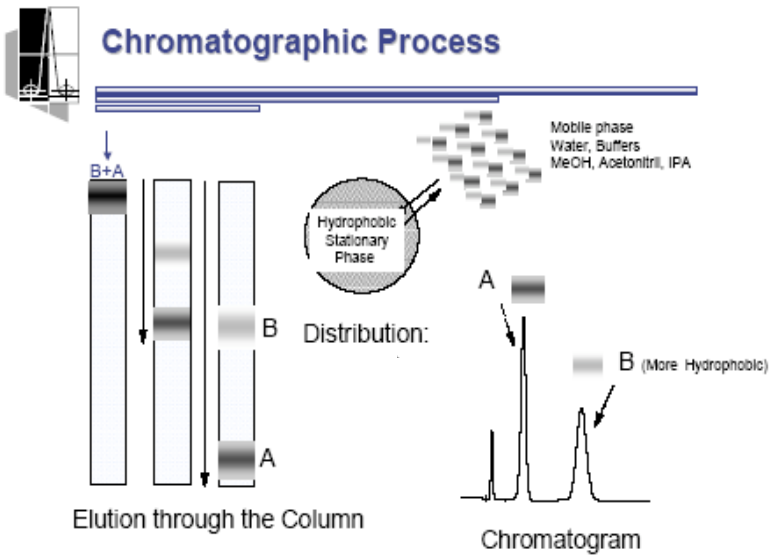
One common type is the nickel affinity column, which binds to a 6 histidine sequence in a protein (e.g. His-His-His-His-His-His…)

Other common "tags":
-chitin binding protein
-maltose binding protein
-glutathione-S-transferase



Reverse-phase Chromatography

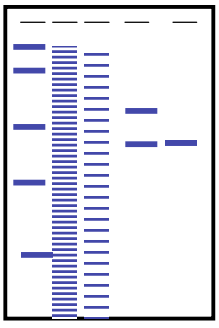
	HPLC
Sta phase	silica gel derived with C (4) chains (for small number of large peptides) derived with C (8) chains derived with C (18) chains (for large number of small peptides) derived with diphenyl groups
Part size	5-10 microns
Elution	Gradient of increasing hydrophobic character, allowing hydrophobic interactions between protein and stationary phase to become increasingly less favorable. E.g., 5% acetonitrile in water>>50% acetonitrile in water.



Evaluation of protein purification

- qualitative (gel electrophoresis)
- quantitative
  - recovery (% yield)
    - divide total activity recovered by total starting activity
  - fold-purification
    - divide the recovered specific activity by the starting specific activity
- recovery and fold-specific activity can be calculated for each step or from the original starting material

Picture of protein gel with lanes showing sequential purification steps



Purification Table

Procedure	Fraction vol (ml)	Total Prot (mg)	Activity (units)	Specific activity Units/mg	Purification factor	Yield
Crude cellular extract	1400	10000	100000	10	1	100%
Size-exclusion	90	400	80000	200	20	80%
Ion exchange	80	100	60,000	600	60	60%

Note: The type and order of steps are customized for each protein to be purified. An effective purification step results in a high yield (minimal loss of enzyme activity) and large purification factor (large increase in specific activity).