Lecture 6 (9/18/20)

- Reading: Ch3; 89-93, 95-96
  Ch1; Fig 1-9
  Ch9; 332-333
- Problems: Ch3 (text); 9, 10, 14, 15, 22
  Ch1 (text); 9

NEXT MON-none

NEXT WED-AFTER EXAM

- Reading: Ch3; Fig 3-23(p. 96)
  Ch4; 117-119
- Problems: Ch3 (text); 5, 8, 17, 21
  Ch4 (text); 1, 5, 6, 12

Lecture 6 (9/18/20)

OUTLINE

Electrophoresis

Protein Purification

A. Introduction; what is the basis
B. Goals; Specific Activity
C. Methods
   1. Centrifugation
      a. Differential
      b. Isopycnic
   2. Precipitation
      a. Salting-out; ammonium sulfate
      b. dialysis
   3. Chromatography
      a. Gel filtration
      b. Ion exchange
      c. Affinity
D. Summary

Electrophoresis for Protein Analysis

- For proteins to separate, they have to have a charge.
- The charge on a protein will depend on the pH.

But once they are moving, what does the velocity depend on?

$$\text{Velocity} = \frac{E \bullet z}{f}$$

Electric field

charge on the protein

friction

$f \propto$ mass, shape, viscosity of media

$f = 6\Pi \bullet \eta \bullet r$

where:

η = coefficient of viscosity

r = Stokes radius (mass and shape)

[this is from actual radius and specific volume (cm^3/g)]

$$\text{Velocity} = \frac{E}{6\Pi \bullet \eta} \bullet \frac{z}{r}$$

This is essentially the charge:mass ratio if all proteins are roughly the same shape (globular).

Electrophoresis for Protein Analysis

- For proteins to separate, they have to have a charge.
- pH and pI dependence:
  - At pH near the pI, not much movement
  - At pH below the pI, proton concentration is higher, so charge becomes positive ⊕
  - At pH above the pI, protons will be titrated off, so the charge will become ⊖

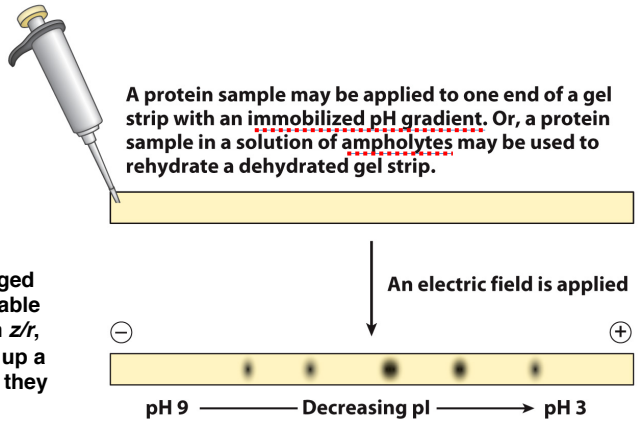
Isoelectric Focusing Takes advantage of the pI differences in Proteins for Separation

Can also be used to determine the pI values

Isoelectric Focusing

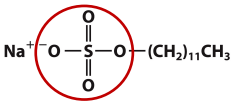
$$\text{Velocity} = \frac{E}{6\Pi \bullet \eta} \bullet \frac{z}{r}$$

Ampholytes are highly charged small MW polymer with variable pI values. Due to their high *z/r*, they migrate rapidly setting up a buffered pH gradient. Once they reach the pH equal to their individual pI values, they STOP migrating, thus creating an **immobilized pH gradient**.



Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS PAGE) Separates Proteins by Molecular Weight

- SDS – sodium dodecyl sulfate – a detergent



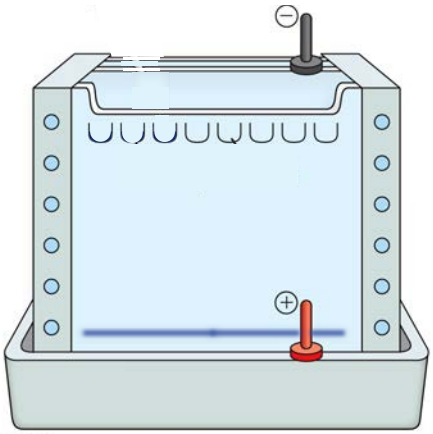
Sodium dodecyl sulfate (SDS)

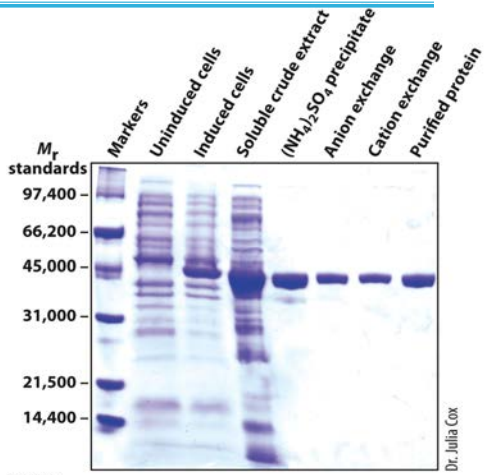
$$\text{Velocity} = \frac{E}{6\Pi \bullet \eta} \bullet \frac{z}{r}$$

- SDS micelles bind to proteins and facilitate unfolding.
  - SDS gives all proteins a uniformly negative charge and shape (micelle)
  - The native shape is perturbed; de-natured.
  - SDS binds proteins at a constant ratio of mass (1.4g SDS/g protein), coating them with a negative charge.
  - So much charge is added that all proteins have nearly the same charge:mass ratio, and the rate of movement will only depend on the sieving properties of the gel: small proteins will move farther.

SDS PAGE Separates Proteins by Molecular Weight

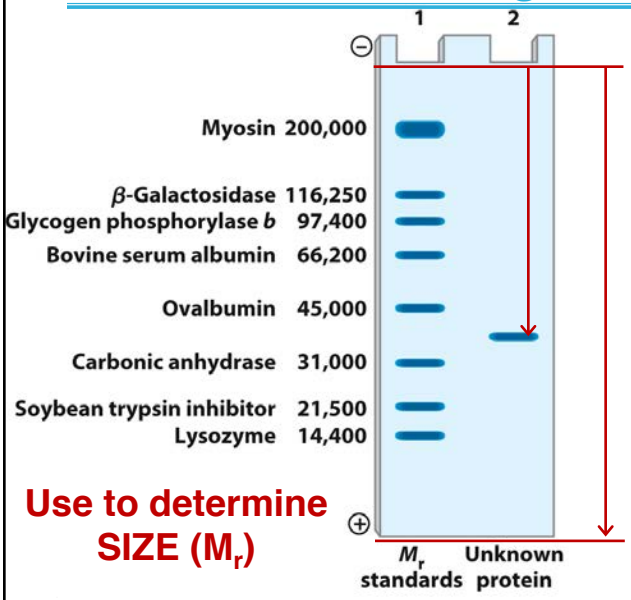
SDS-PAGE

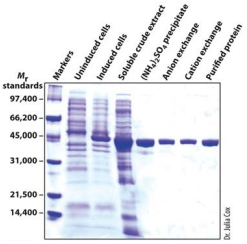




Use to check for purity

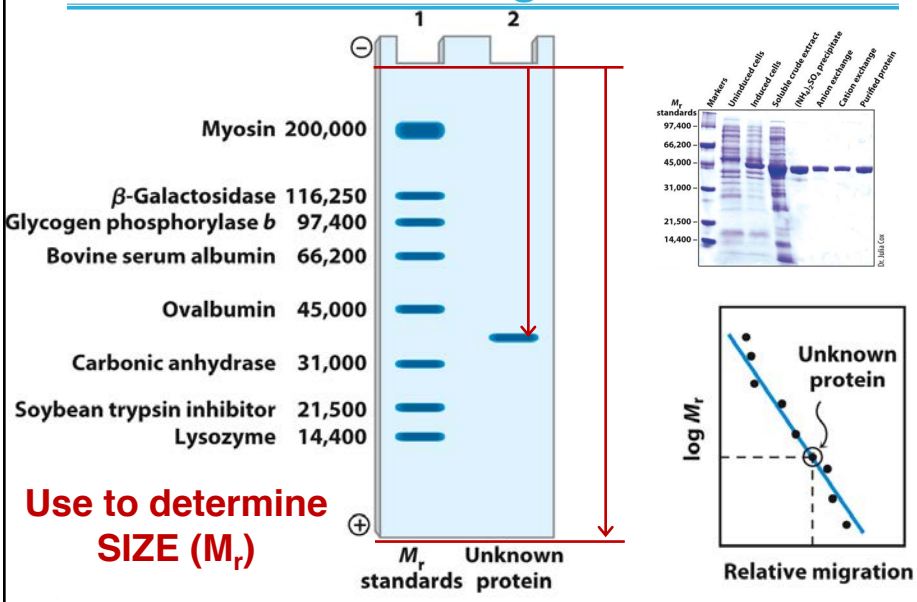
SDS PAGE Can Be Used to Calculate the Molecular Weight of a Protein



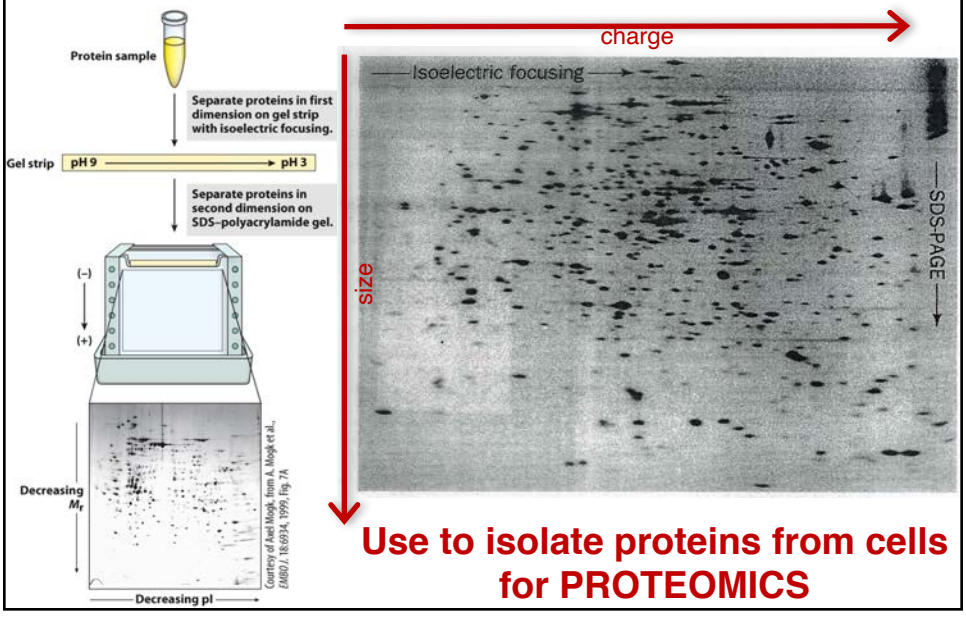


Use to determine SIZE (M_r)

SDS PAGE Can Be Used to Calculate the Molecular Weight of a Protein



SDS-PAGE + Isoelectric Focusing Can Separate nearly ALL the Proteins in *E. coli*



Protein Purification

Protein Purification

Proteins are separated from each other (along with other macromolecules) due to the vast variability they have. The basis of the separation can be put into 4 categories:

- Size, shape, density
- Charge

} Hydrodynamic properties

- Solubility
- Binding characteristics

} Chemical properties

Protein Purification Procedures

Basis	Procedure	Covered	
Hydrodynamics (size, shape, density	Gel filtration Chromatography	Lab	←
	SDS-PAGE ✔	Lab	
	Centrifugation	Lab	←
Charge	Ion exchange Chromatography		←
	Isoelectric focusing ✔		
	Native electrophoresis ✔	Lab	
Solubility	Salting out Organic extraction Hydrophobic interaction Chromatography	Lab	←
Binding Specificity	Affinity Chromatography	Lab	←

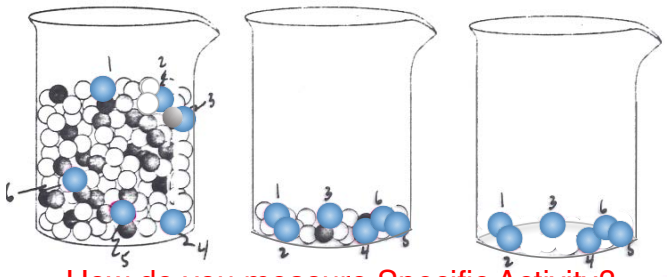
Before you can separate "your favorite protein (YFP)" from all the thousands of others, you need a way to "see" it…..... An Assay!

- enzymes ➟ activity assay
- binding proteins ➟ binding assay
- other proteins ➟ immunoblot or ELISA

How do you monitor the purification?

- Specific Activity = $\frac{\text{Activity of YFP}}{\text{Total protein}}$

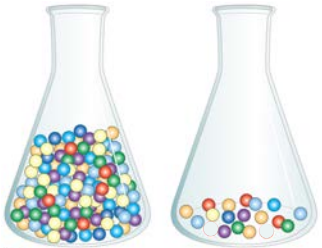
- Specific Activity = $\frac{\text{Activity of YFP}}{\text{Total protein}}$



How do you measure Specific Activity?

Figure 6–3 Activity versus specific activity. The difference between these two terms can be illustrated by considering two jars of marbles. The jars contain the same number of blue marbles (representing an unknown protein), but different amounts of marbles of other colors. If the marbles are taken to represent proteins, both jars contain the same *activity* of the protein represented by the blue marbles. The second jar, however, has the higher *specific activity* because here the blue marbles represent a much higher fraction of the total.

What is the Yield?



How do you measure specific activity?

From [protein] (mg/mL)

From [activity] (U/mL)

Purification of a hypothetical protein

Procedure or step	Fraction volume (ml)	Total protein (mg)	Activity (units)	Yield (%)
1. Crude cellular extract	1,400	10,000	100,000	100
2. Precipitation	280	3,000	96,000	96
3. Ion-exchange chromatography	90	400	80,000	80
4. Size-exclusion chromatography	80	100	60,000	60
5. Affinity chromatography	6	3	45,000	45

* All data represent the status of the sample *after* the procedure indicated in the first column has been carried out.

These are the two important criteria of purifications

-If protein was "pure" after step #5, what would the Specific Activity be after you performed a step #6?

-What is the Yield?

How do you measure specific activity?

From [protein] (mg/mL)

From [activity] (U/mL)

Purification of a hypothetical protein

Procedure or step	Fraction volume (mL)	Total protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)
1. Crude cellular extract	1,400	10,000	100,000	10	100
2. Precipitation	280	3,000	96,000	32	96
3. Ion-exchange chromatography	90	400	80,000	200	80
4. Size-exclusion chromatography	80	100	60,000	600	60
5. Affinity chromatography	6	3	45,000	15,000	45

* All data represent the status of the sample *after* the procedure indicated in the first column has been carried out.

These are the two important criteria of purifications

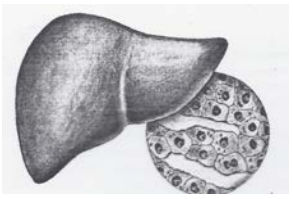
-If protein was "pure" after step #5, what would the Specific Activity be after you performed a step #6?

-What is the Yield?

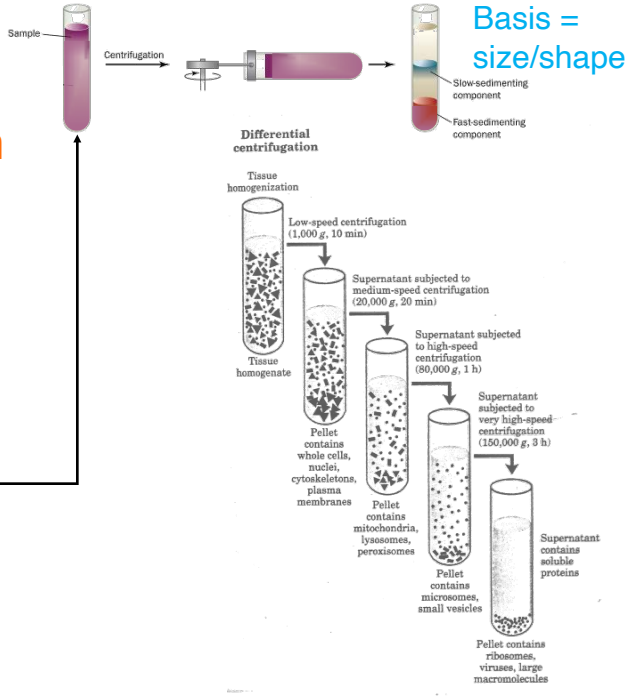
Fractionation by Centrifugation

Basis = size/shape

Differential



Homogenization



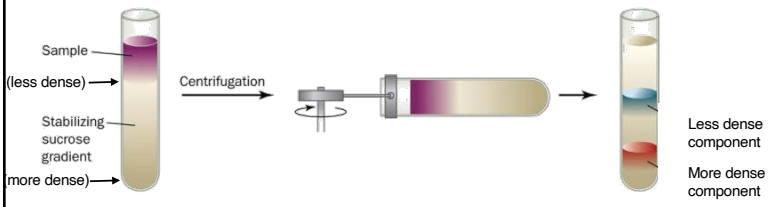
Fractionation by Centrifugation

Basis = density

Isopycnic (same density)

Equilibrium between two opposite forces:

Centrifugal Force ⇄ Buoyancy Force



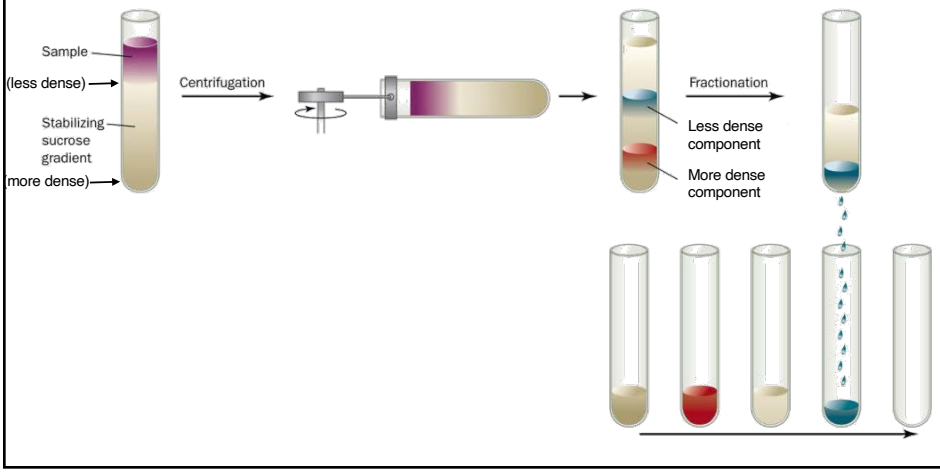
Fractionation by Centrifugation

Basis = density

Isopycnic (same density)

Equilibrium between two opposite forces:

Centrifugal Force ⇄ Buoyancy Force

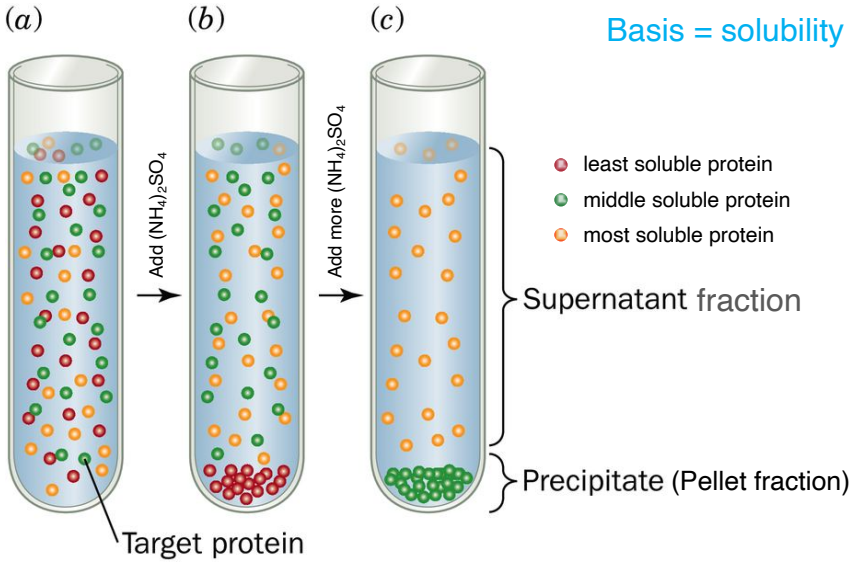


Protein Purification Procedures

Basis	Procedure	Covered
Hydrodynamics (size, shape, density	Gel filtration Chromatography	Lab
	SDS-PAGE	✔ Lab
	Centrifugation	✔ Lab
Charge	Ion exchange Chromatography	
	Isoelectric focusing	✔
	Native electrophoresis	✔ Lab
Solubility	Salting out Organic extraction Hydrophobic interaction Chromatography	Lab
Binding Specificity	Affinity Chromatography	Lab

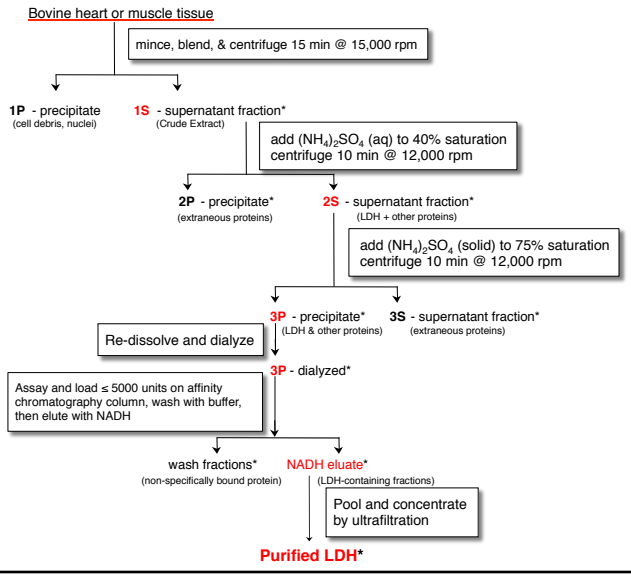
Ammonium Sulfate Fractionation: Fractionation by Salting Out

Basis = solubility



Purification in Laboratory begins with Fractionation by Salting Out

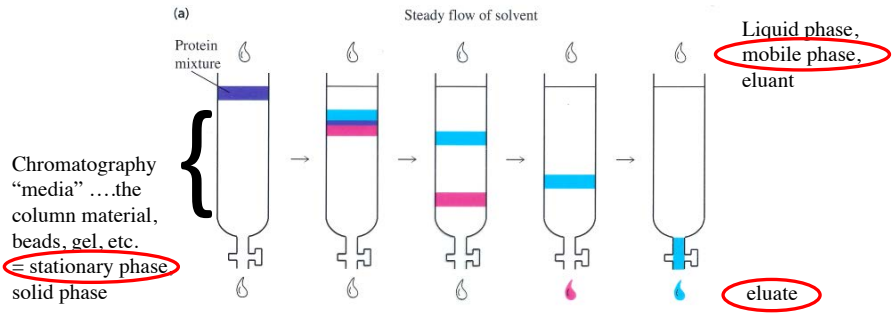
FLOW CHART FOR LDH PURIFICATION



Protein Purification Procedures

Basis	Procedure	Covered	
Hydrodynamics (size, shape, density	Gel filtration Chromatography	Lab	←
	SDS-PAGE	✔ Lab	
	Centrifugation	✔ Lab	
Charge	Ion exchange Chromatography		←
	Isoelectric focusing	✔	
	Native electrophoresis	✔ Lab	
Solubility	Salting out	✔ Lab	
	Organic extraction		
	Hydrophobic interaction Chromatography		
Binding Specificity	Affinity Chromatography	Lab	←

Basic Chromatography

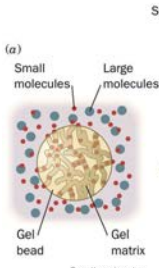


Three Types of Chromatography:

1) **Gel Filtration** (size/shape)
2) **Ion exchange** (charge)
3) **Affinity** (function)

Gel Filtration Chromatography

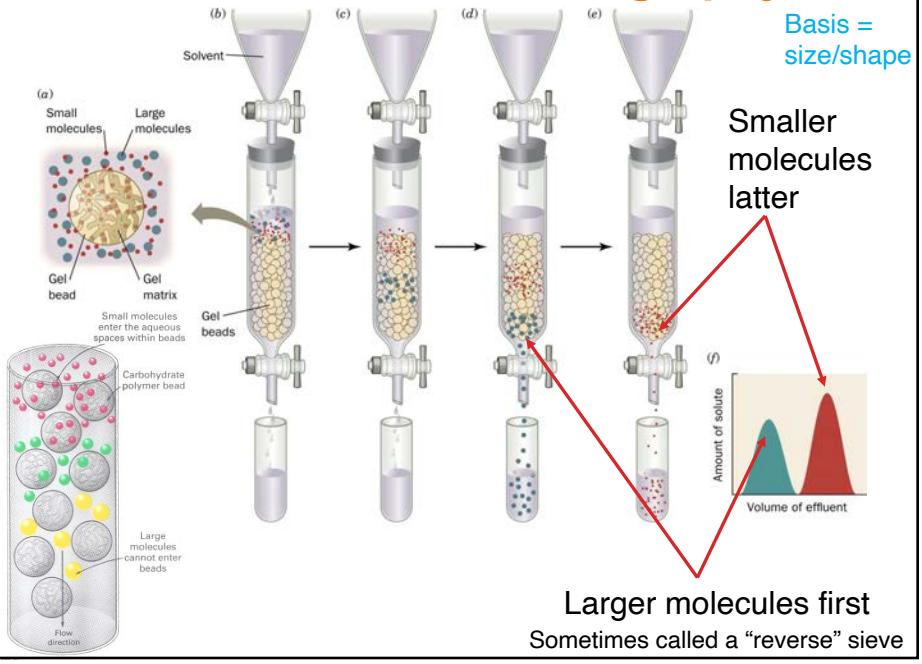
Basis = size/shape



Smaller molecules latter

Larger molecules first

Gel Filtration Chromatography



Basis = size/shape

Smaller molecules latter

Larger molecules first

Sometimes called a "reverse" sieve

Protein Purification Procedures

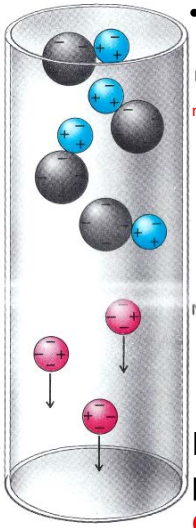
Basis	Procedure	Covered
Hydrodynamics (size, shape, density	Gel filtration Chromatography	✔ Lab
	SDS-PAGE	✔ Lab
	Centrifugation	✔ Lab
Charge	Ion exchange Chromatography	
	Isoelectric focusing	✔
	Native electrophoresis	✔ Lab
Solubility	Salting out	✔ Lab
	Organic extraction	
	Hydrophobic interaction Chromatography	
Binding Specificity	Affinity Chromatography	Lab

Ion Exchange Chromatography

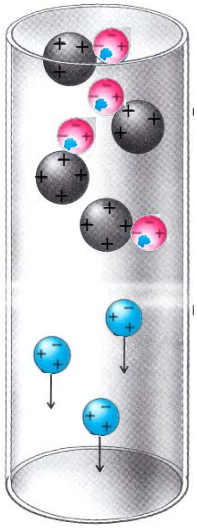
Basis = charge

At certain pH values, molecules will have a net charge:

- Positive (+) when pH < pI
- Negative (–) when pH > pI



Negatively charged particles/beads = **cation exchange**



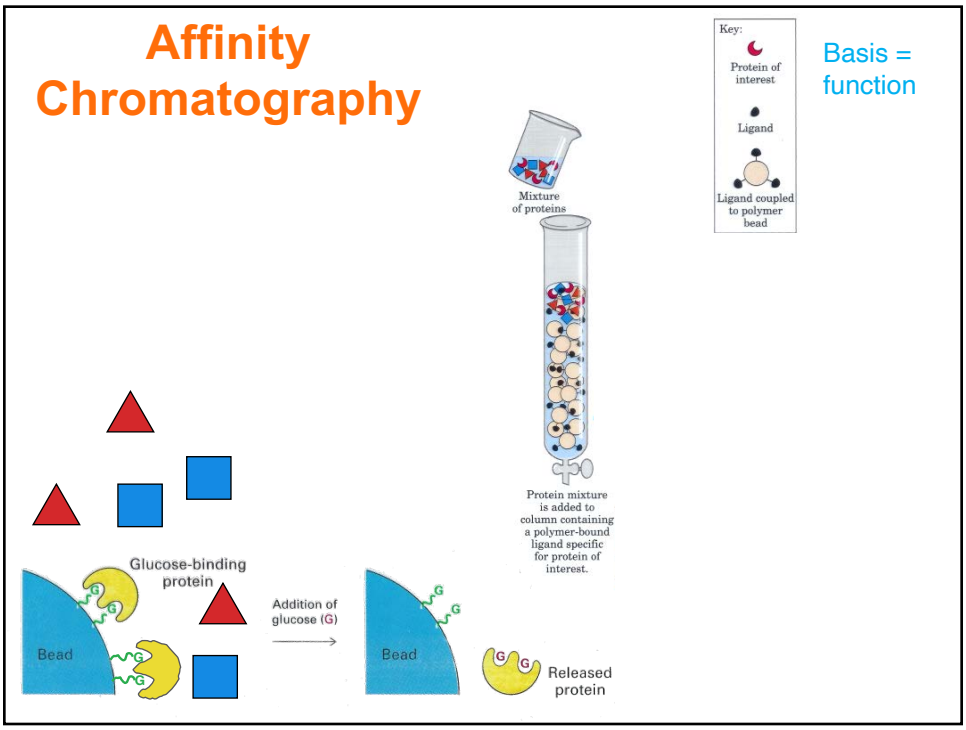
Positively charged particles/ beads = **anion exchange**

Protein Purification Procedures

Basis	Procedure	Covered
Hydrodynamics (size, shape, density	Gel filtration Chromatography	✔ Lab
	SDS-PAGE	✔ Lab
	Centrifugation	✔ Lab
Charge	Ion exchange Chromatography	✔
	Isoelectric focusing	✔
	Native electrophoresis	✔ Lab
Solubility	Salting out Organic extraction Hydrophobic interaction Chromatography	✔ Lab
Binding Specificity	Affinity Chromatography	Lab

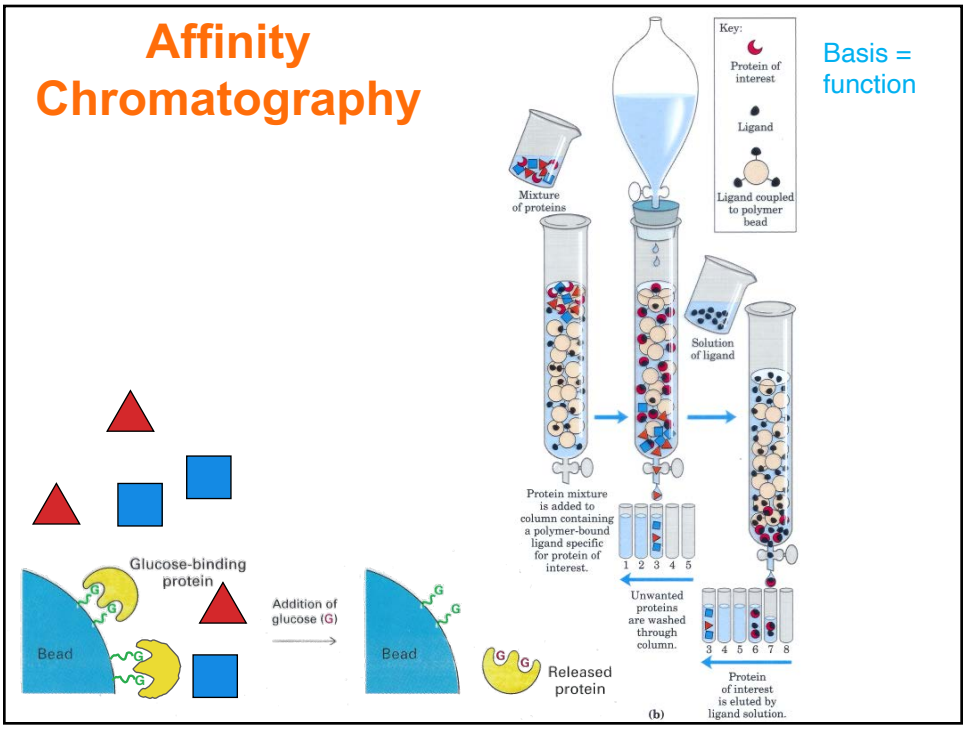
Affinity Chromatography

Basis = function



Affinity Chromatography

Basis = function



Affinity Chromatography

Basis = function

Biotechnology (recombinant DNA technology) has revolutionized protein purification.

At the level of the DNA sequence, the DNA sequence encoding such binding proteins or "tags" can be "fused" to the sequence encoding YFP. In this way, a chimeric protein is produced that has the binding function, which allows the use of affinity chromatography.

Common "tags" are:
- Maltose-binding protein
- Chitin-binding protein
- Glutathione-S-transferase
- His-His-His-His-His-His

Column beads have attached:
- Maltose
- Chitin
- Glutathione (γ-Glu-Cys-Gly)
- Ni-chelate

How to Calculate the pI of a

Estimate the pI value of the following hexapeptide:

Phe-Lys-Asp-Cys-Thr-Tyr

Step 1: Determine the total positive charge on the peptide when all acidic and basic groups are fully protonated (at low pH).
Step 2: Determine the total negative charge on the peptide when all the groups are titrated (at high pH).
Step 3: List the pK_a values of all acidic and basic groups in order from lowest (pK_{a1}) to highest.
Step 4: Calculate the pI as the average of the values for pK_a value of the proton dissociation forming a neutral species from a +1 species, and pK_a value of the proton dissociation forming a –1 species from the neutral species.

So for this peptide
Step 1: charge when fully protonated +2
Step 2: charge when fully de-protonated -4
Step 3: pK_a values are:
9.0(N-term), 10.5(Lys), 3.9(Asp), 8.4 (Cys), 10.5(Tyr), 3.5(C-term)
List from lowest to highest

pKa		3.5	3.9	8.4	9.0	10.5	10.5
Charges	+2 ⇆	+1 ⇆	0 ⇆	-1 ⇆	-2 ⇆	-3 ⇆	-4

Step 4: The pI is (3.9 + 8.4)/2 = 6.2