Lecture 6 (9/17/25)

- Reading: Ch3; 83–87, 89–90
  Ch1; Fig 7
  Ch9; 313–314
- Homework #6

NEXT

- Review session

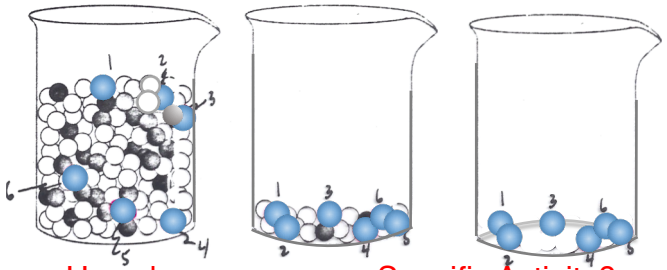
OUTLINE

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

Protein Purification

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

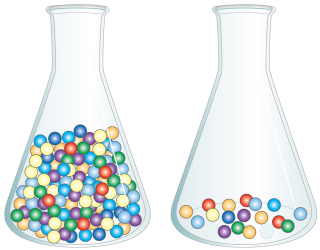


How do you measure Specific Activity?

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?

(of red proteins?)



From [protein] (mg/mL)

How do you measure specific activity?

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?

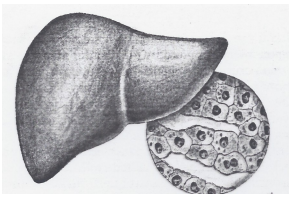
Protein Purification Procedures

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	
Binding Specificity	Affinity Chromatography	Lab

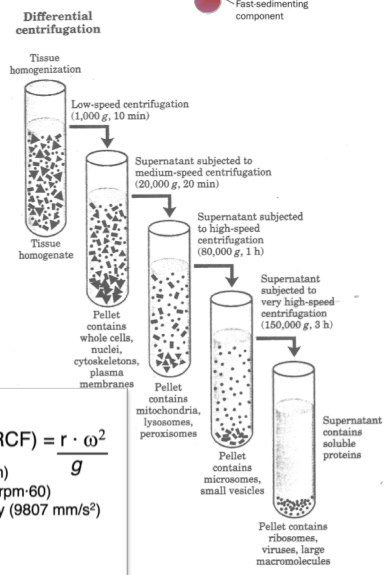
Fractionation by Centrifugation

Basis = size/shape

Differential



Homogenization



Speed is ∝ to:

$$\text{Relative Centrifugal Force (RCF)} = \frac{r \cdot \omega^2}{g}$$

r = radius from center of rotor (mm)
ω = angular velocity (radians/sec)(rpm·60)
g = standard acceleration of gravity (9807 mm/s²)

Time is ∝ to:

$$\omega_1^2 \cdot t_1 = \omega_2^2 \cdot t_2$$

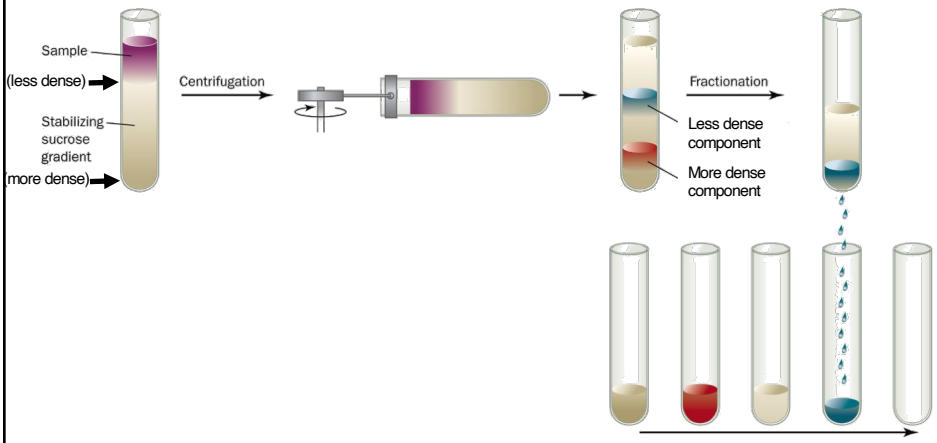
Fractionation by Centrifugation

Basis = density

Isopycnic (same density)

Equilibrium between two opposite forces:

Centrifugal Force ⇄ Buoyancy Force

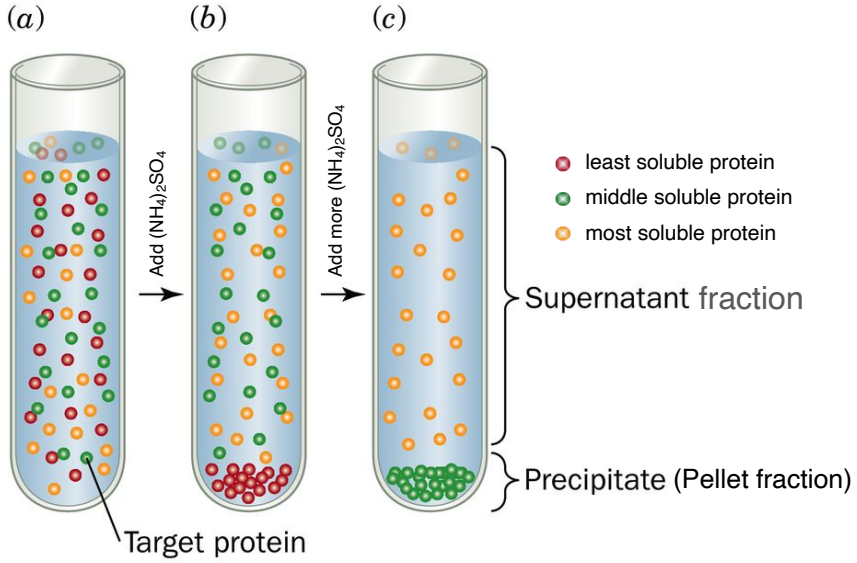


Protein Purification Procedures

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	
Binding Specificity	Affinity Chromatography	Lab

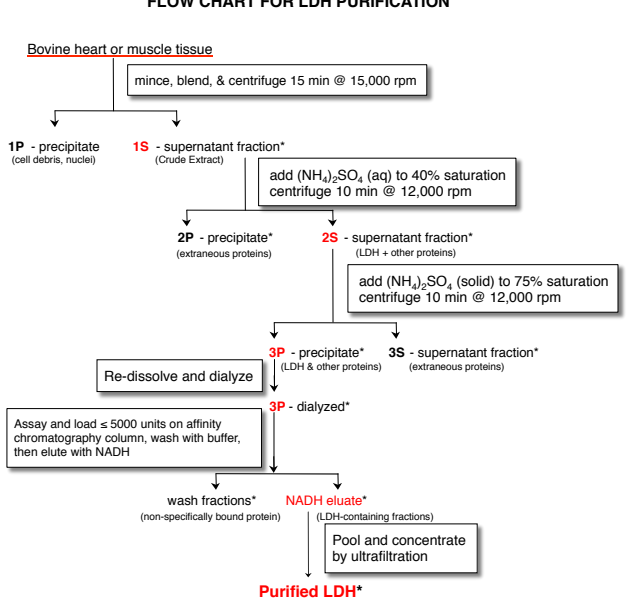
Ammonium Sulfate Fractionation: Fractionation by Salting Out

Basis = solubility



Purification in Laboratory begins with Fractionation by Salting Out

Basis = solubility



Proteins are Least Soluble at Their Isoeletric Point

Basis = solubility

pH at:	Cation →	Zwitterion →	Anion
	<pI	pI	>pI

TABLE 5-3 Isoelectric Points of Several Common Proteins

Protein	p*I*
Pepsin	<1.0
Ovalbumin (hen)	4.6
Serum albumin (human)	4.9
Tropomyosin	5.1
Insulin (bovine)	5.4
Fibrinogen (human)	5.8
γ-Globulin (human)	6.6
Collagen	6.6
Myoglobin (horse)	7.0
Hemoglobin (human)	7.1
Ribonuclease A (bovine)	9.4
Cytochrome *c* (horse)	10.6
Histone (bovine)	10.8
Lysozyme (hen)	11.0
Salmine (salmon)	12.1

Acidic proteins

Example of this at the end

Basic proteins

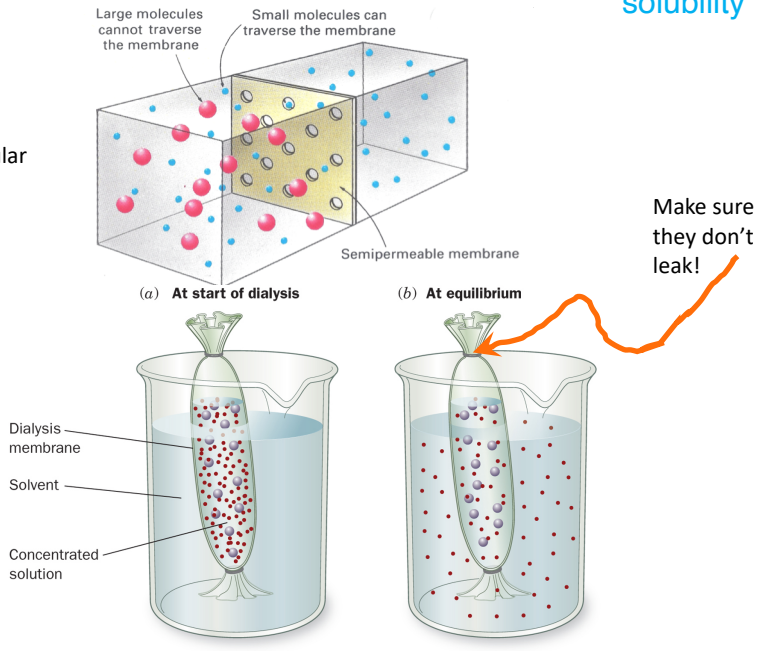
Notice that between 7.1 – 9.4 most of these proteins are charged

If you are performing a salting-out procedure prior to most other procedures, you will need to remove the Molar concentrations of salt. How?

Dialysis: Diffusion of Solutes

Basis = solubility

MWCO=molecular weight cutoff

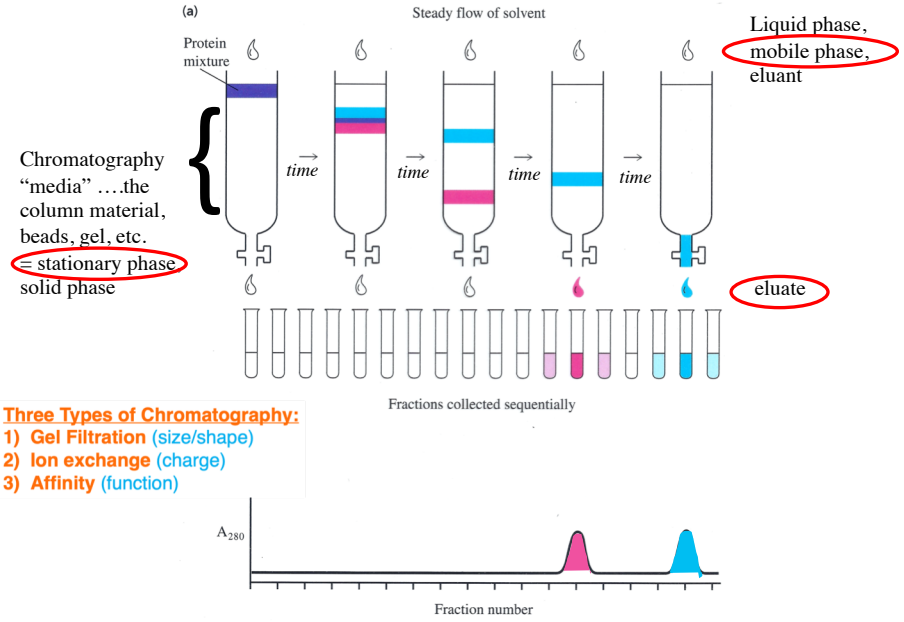


Make sure they don't leak!

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	
Binding Specificity	Affinity Chromatography	Lab

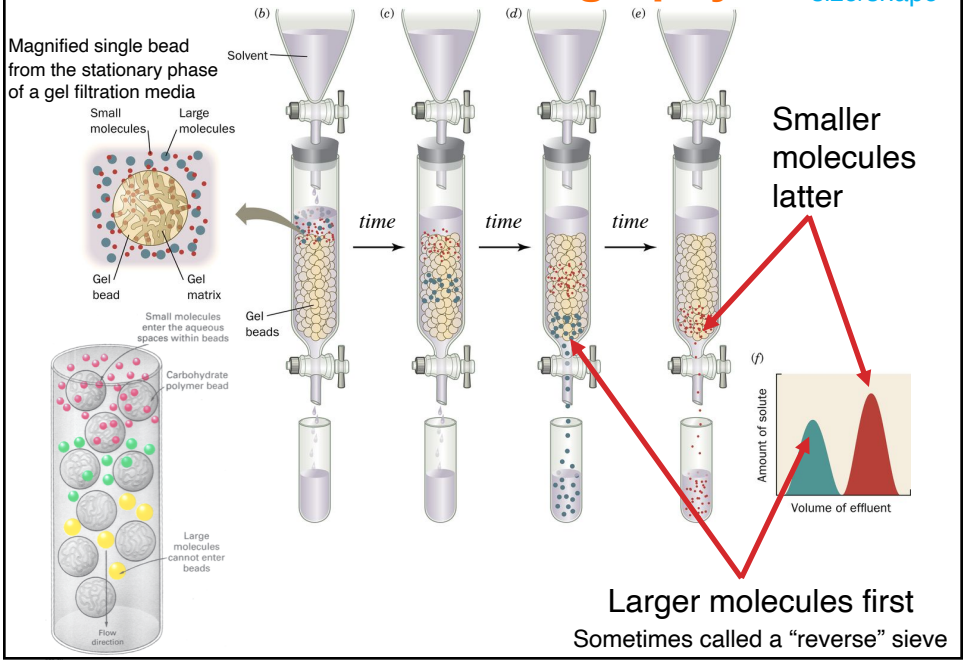
Basic Chromatography



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

Sometimes called a "reverse" sieve

Shapes of Proteins: all same scale

Proteins @ 4,000,000X

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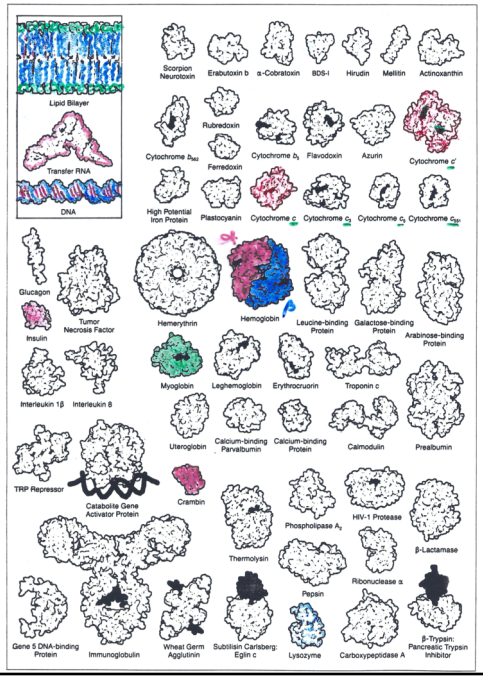

Basis = size/shape

Recall our picture of all the different sizes and shapes of proteins!

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	
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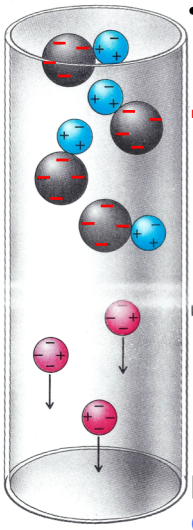
Ion Exchange Chromatography

Basis = charge

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

pH at:	Cation →	Zwitterion →	Anion
	<pI	pI	>pI

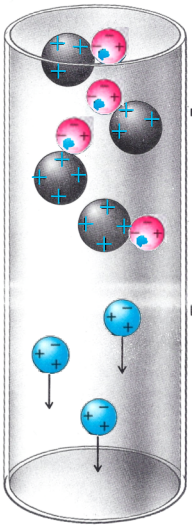
- Positive (+) when pH < pI
- Negative (–) when pH > pI



Positively charged protein binds to negatively charged bead

Negatively charged protein flows through

Negatively charged particles/beads = **cat-ion exchange**



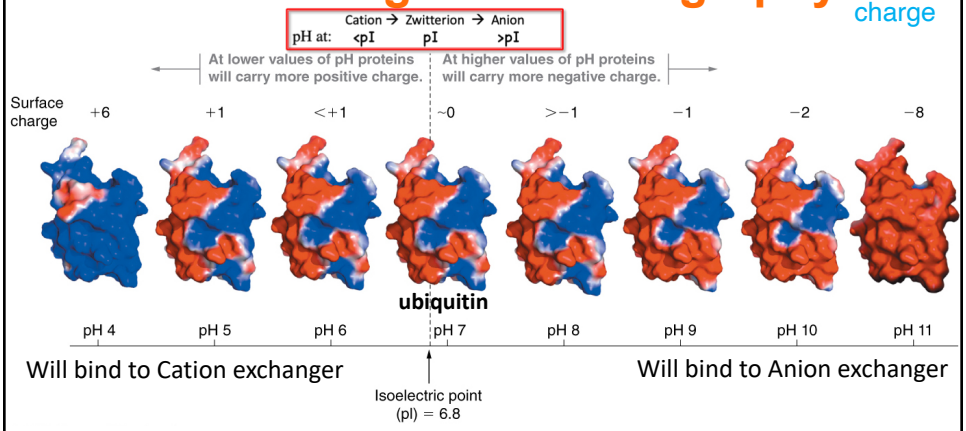
Negatively charged protein binds to positively charged bead

Positively charged protein flows through

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

Ion Exchange Chromatography

Basis = charge



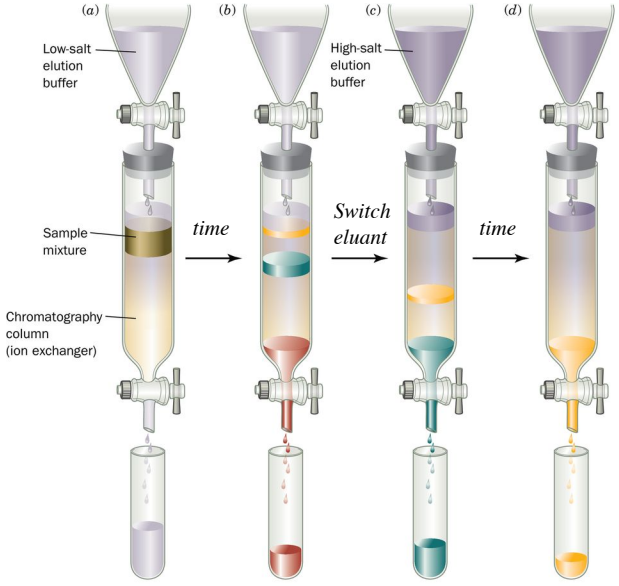
If pH < pI POSITIVE charge

pH	< pI	> pI
Net charge of protein	+	−
Sticks to	− (cation exchanger)	+ (anion exchanger)
Types	Dowex-50, carboxyl-methyl (CM), Phosphocellulose, mono-S (methyl-sulfite)	Dowex-1, Diethylaminoethyl (DEAE), mono-Q (quaternary amine)

If pH > pI NEGATIVE charge

Ion Exchange Chromatography

Basis = charge



What can you use to elute proteins that stick?

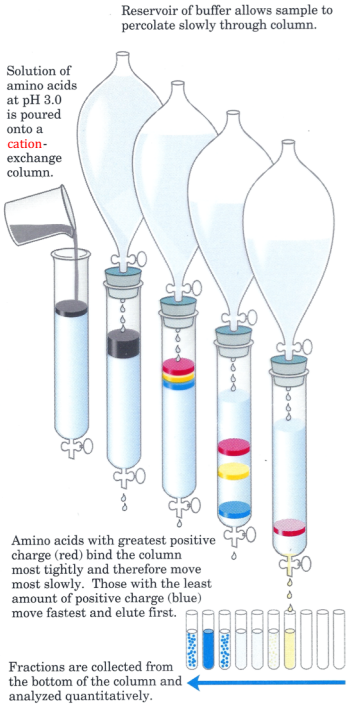
Elute with salt or pH gradient

Ion Exchange Chromatography: EXAMPLE of an amino-acid mixture

Basis = charge

If this were a mixture of G, K, and D, what would the order of elution be?

1st= D, 2nd = G, 3rd = K



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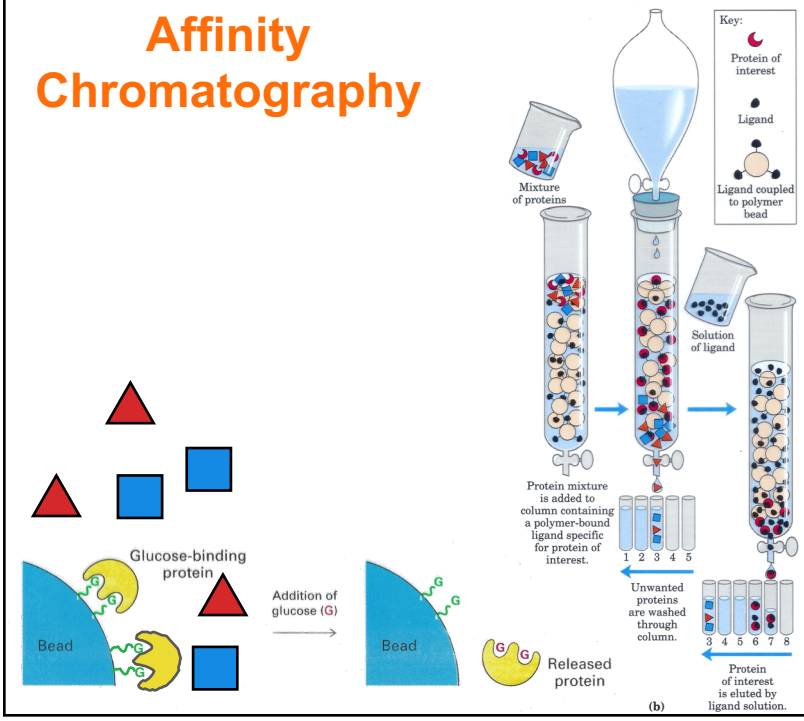
Binding Specificity	Affinity Chromatography	Lab

Affinity Chromatography

Basis = function



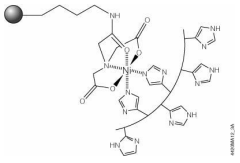
Affinity Chromatography

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:	Column beads have attached:
Maltose-binding protein	Maltose
Chitin-binding protein	Chitin
Glutathione-S-transferase	Glutathione (γ-Glu-Cys-Gly)
His-His-His-His-His-His	Ni-chelate



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Binding Specificity	Affinity Chromatography	Lab

Purification of Myoglobin (Mb)

Step	Total Protein (mg)	Mb (µmol)	Specific Activity (µmol Mb/mg Total Protein)	% Yield	Overall Fold Purification
1. Crude extract	1550	0.75		100	1
2. DEAE-cellulose chromatography	550	0.35			1.3
3. Affinity chromatography	5.0	0.28			117

Calculations:

Specific Activity		Yield		Fold Purification	
0.75 ÷ 1550 =	0.00048				
0.35 ÷ 550 =	0.00064	0.35 ÷ 0.75 =	0.47	0.00064 ÷ 0.00048 =	1.3
0.28 ÷ 5.0 =	0.056	0.28 ÷ 0.75 =	0.37	0.056 ÷ 0.00048 =	117

Purification of a hypothetical protein

Procedure or step	Fraction volume (ml)	Total protein (mg)	Activity (units)	Specific activity (units/mg)	Fold increase in SA	Yield (%)	% loss in Yield
1. Crude cellular extract	1,400	10,000	100,000	10		100	
					3x		4%
2. Precipitation	280	3,000	96,000	32		96	
					6x		17%
3. Ion-exchange chromatography	90	400	80,000	200		80	
					3x		25%
4. Size-exclusion chromatography	80	100	60,000	600		60	
					25x		25%
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.

Calculate fold increase in SA for each step ➔ helps determine if step is effective.
Which is the best step? .....step 5
Which is the worst step? .....step 2 or step 4

Look at yield…... Calculate fraction (%) of YFP lost at each step.
Which of step 2 or step 4 resulted in loss of more YFP? .....step 4