Lecture 6 (9/20/23)

- 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

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 } Hydrodynamic properties
- Charge } Chemical properties
- Solubility
- Binding characteristics } Biological 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	Lab	←
	Organic extraction		
	Hydrophobic interaction Chromatography		
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?

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

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.

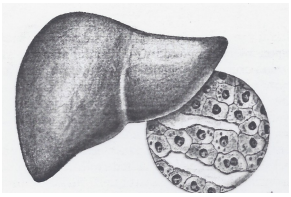
These are the two important criteria of purifications

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

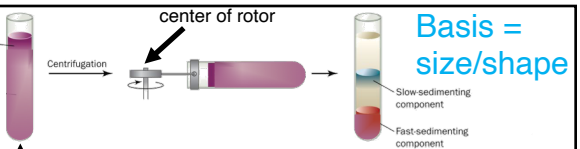
-What is the Yield?

Fractionation by Centrifugation

Differential



Homogenization



Basis = size/shape

Speed is ∝ to:

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/s^2)

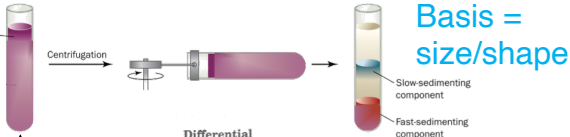
Time is ∝ to:

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

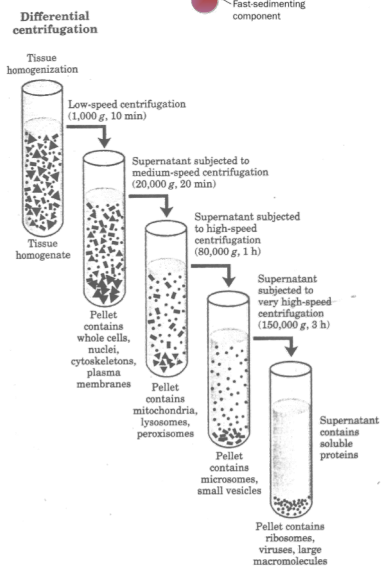
Fractionation by Centrifugation

Differential

Homogenization



Basis = size/shape



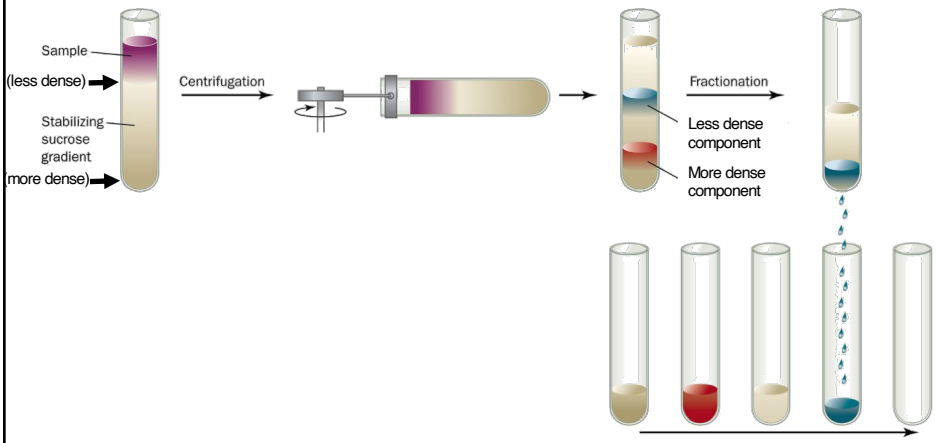
Fractionation by Centrifugation

Basis = density

Isopycnic (same density)

Equilibrium between two opposite forces:

Centrifugal Force ⇄ Buoyancy Force

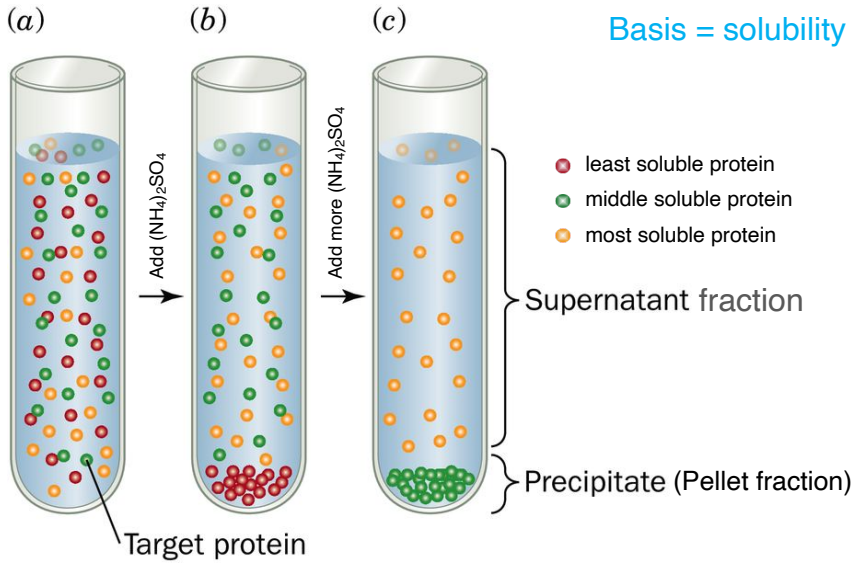


Protein Purification Procedures

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

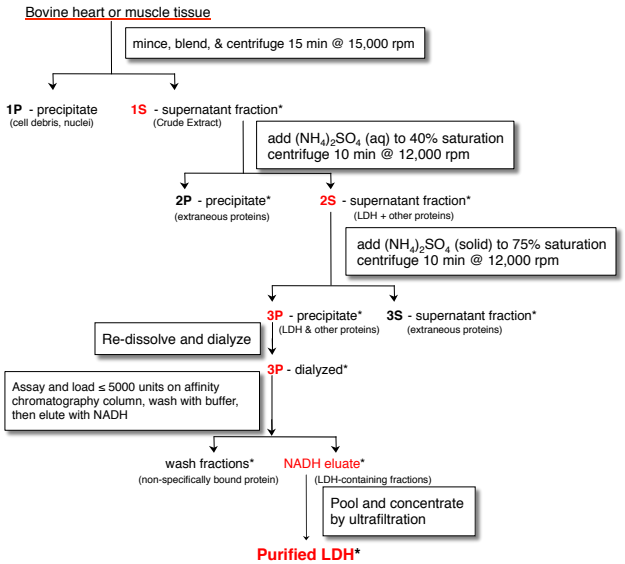
Ammonium Sulfate Fractionation: Fractionation by Salting Out

Basis = solubility



Purification in Laboratory begins with Fractionation by Salting Out

FLOW CHART FOR LDH PURIFICATION



Proteins are Least Soluble at Their Isoeletric Point

TABLE 5-3 Isoelectric Points of Several Common Proteins

Protein	pI
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

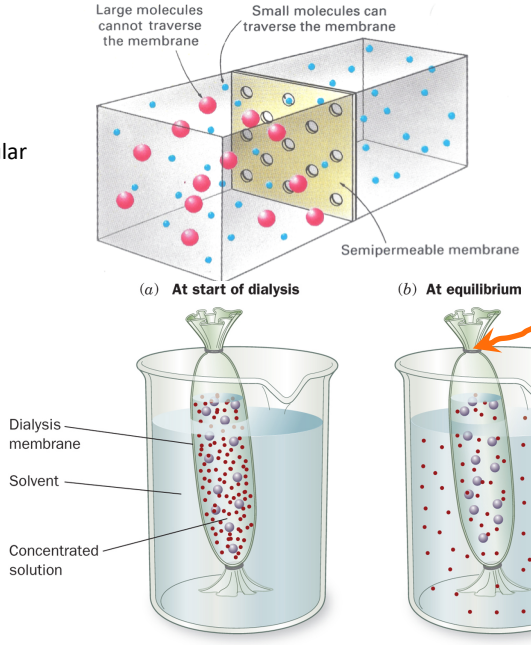
Basic proteins

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

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	
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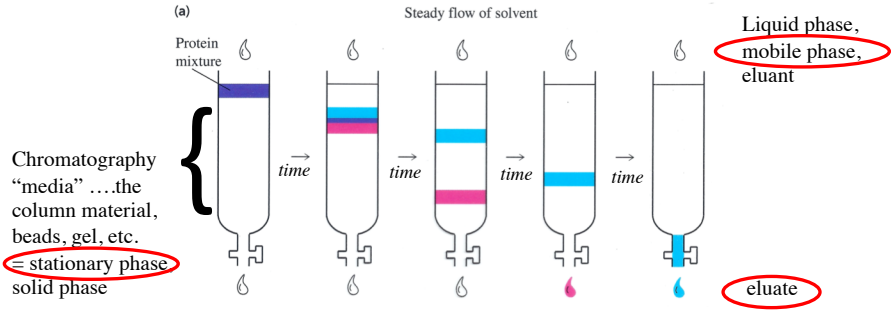
Solubility	Salting out Organic extraction Hydrophobic interaction Chromatography	Lab
Binding Specificity	Affinity Chromatography	Lab

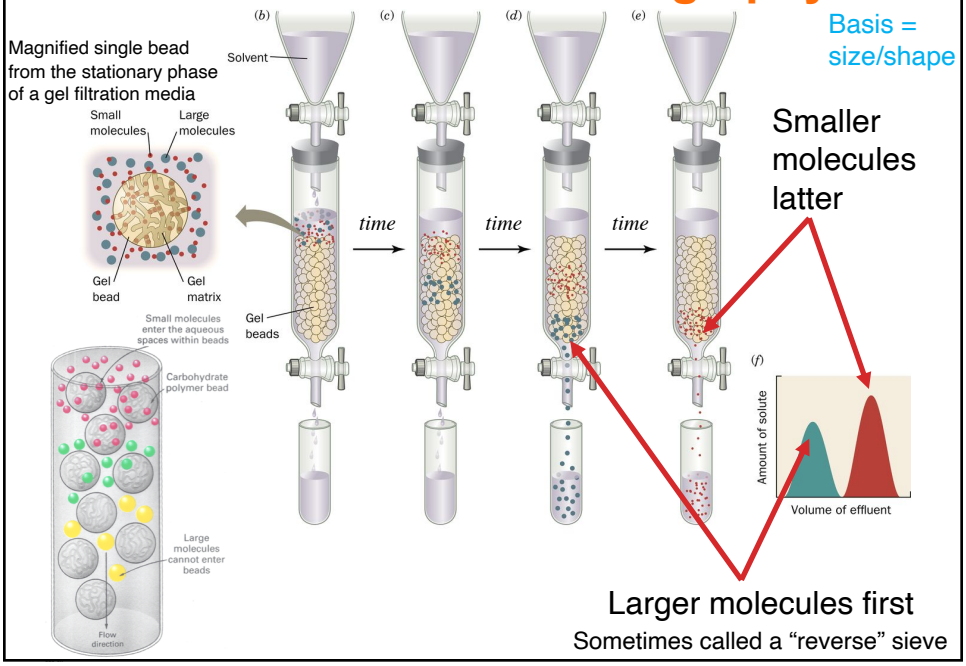
Basic Chromatography



Three Types of Chromatography:

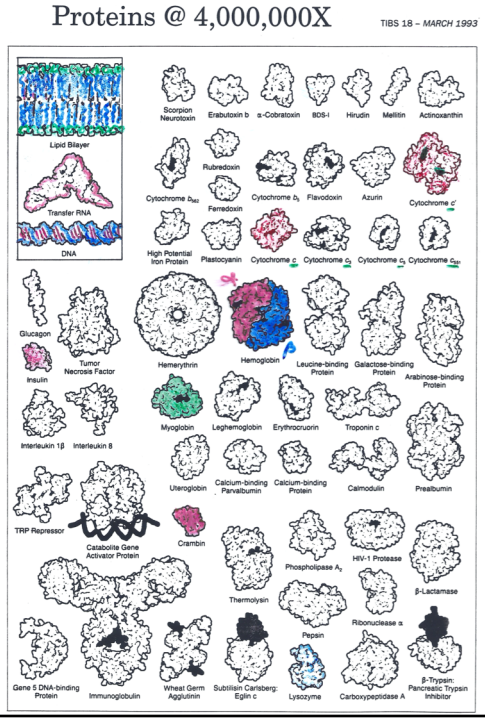
1) **Gel Filtration** (size/shape)
2) **Ion exchange** (charge)
3) **Affinity** (function)

Gel Filtration Chromatography



Shapes of Proteins: all same scale

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



Protein Purification Procedures

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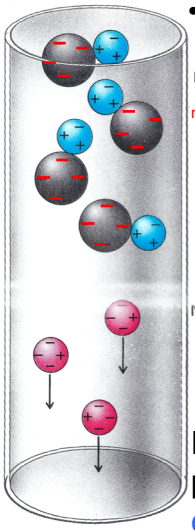
Ion Exchange Chromatography

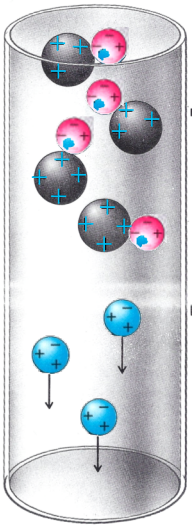
Basis = charge

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

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

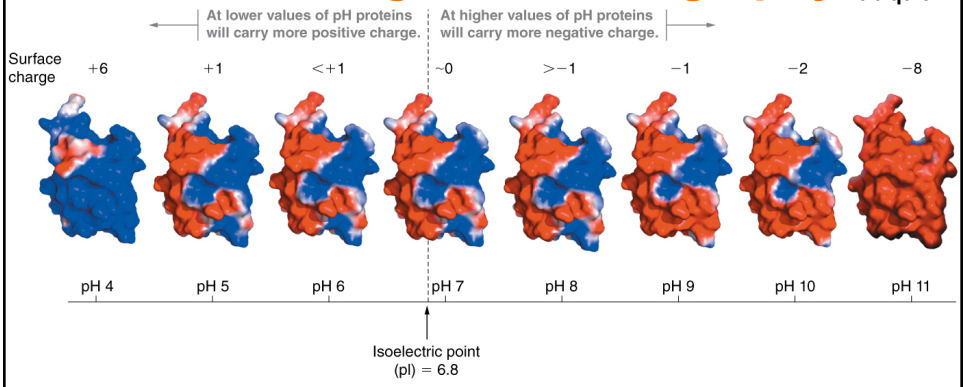


Negatively charged particles/beads = **cat-ion exchange**



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

Ion Exchange Chromatography



Will bind to Cation exchanger

Will bind to Anion exchanger

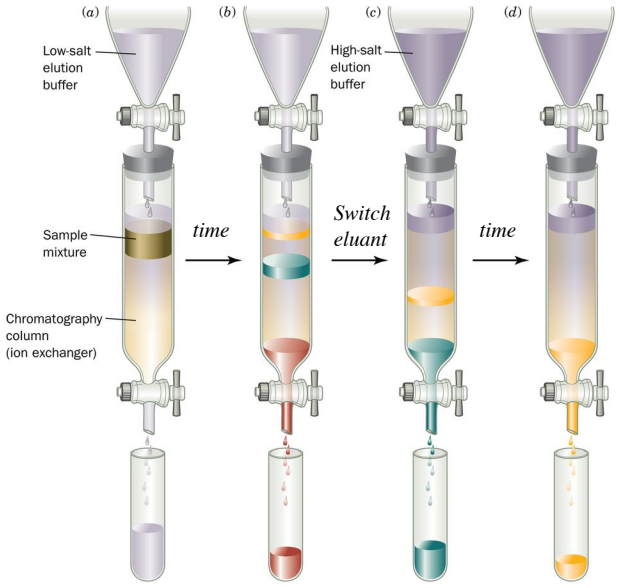
If pH < pI POSITIVE charge

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?

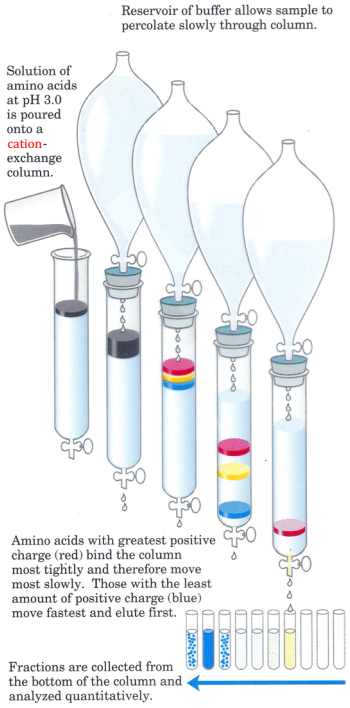
Elute with salt or pH gradient

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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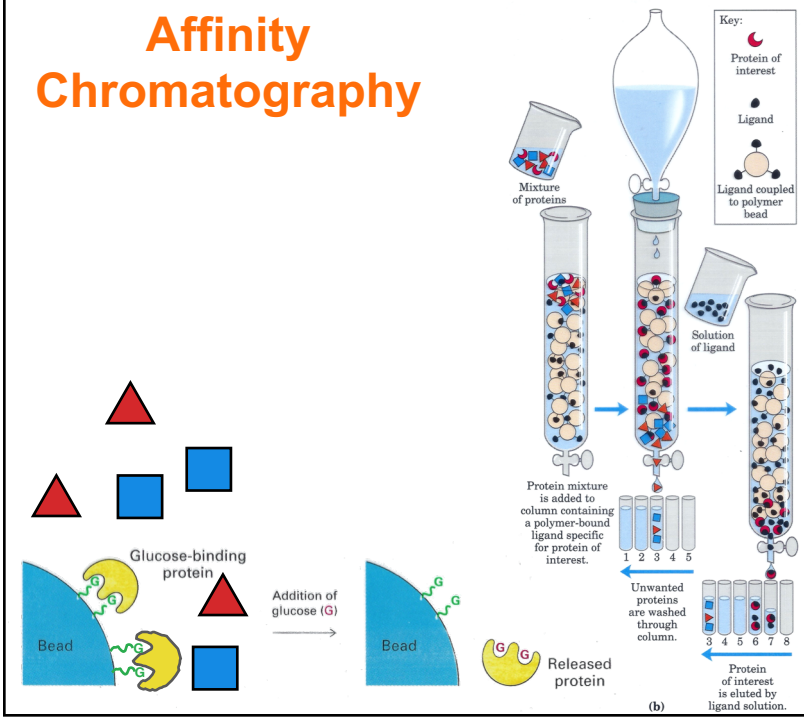
Affinity Chromatography

Basis = function



Affinity Chromatography

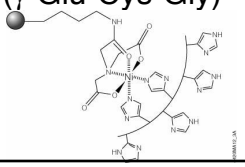
Basis = function

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



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	Organic extraction	
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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

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 (%)
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	1500x	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.

Always increasing until pure

Overall purification = 1500x

Estimate Approximate Expression Level:

Yield of 45% means you can calculate the mg if 100%: It would be 3/0.45 = 6.7 mg of YFP
If total protein was 10,000 mg, then fraction that is YFP in crude is: 6.7/10000=0.00067=0.07%
Well……. 1/(overall purification) gives the same result (1/1500=0.07%)