7.02/10.702
Protein Biochemistry Study Questions
Spring 2005

ANSWER KEY

Question 1

You have a crude lysate sample (CL) containing a mixture of six proteins (1, 2, 3, 4, 5, β-galactosidase), and your goal is to obtain purified β-gal. Some characteristics of these proteins are shown in the table below:

Protein	Concentration of ammonium sulfate (AS) required for precipitation	Molecular Weight (kDa)	Isoelectric point (pI)
1	45%	38	3.7
2	80%	22	4.8
3	65%	4	5.3
4	20%	75	6.8
5	30%	55	9.50
β-galactosidase	45%	115	5.3

You begin your purification by performing an ammonium sulfate (AS) precipitation. You add the appropriate concentration of AS to your CL sample, incubate overnight at 4˚C, then centrifuge to generate a supernatant (AS-S) and pellet (AS-P).

a) What concentration of AS will you use to precipitate β-galactosidase? **45%**

b) After addition of that concentration of AS and centrifugation, which protein(s) will be in the supernatant (AS-S)? **2, 3**

c) Which protein(s) will be in the pellet (AS-P)? **1, 4, 5, β-galactosidase**

d) After resuspending the AS-P in column buffer, you should use a PD-10 column to **desalt** your sample.

One way to purify β-galactosidase away from any contaminating proteins in the AS-P sample would be to separate them based on their molecular weight.

e) What type of column separates on this basis? **gel filtration (size exclusion)**

f) Which protein (from your AS-P) would elute first from this type of column? **β-gal**

Instead, you decide to use ion exchange chromatography to further purify β-galactosidase away from other proteins in your **AS-P** sample. You first run an **anion** exchange column equilibrated using column buffer with a **pH of 5.0.**

g) What charge does the matrix of an anion exchange column have? **positive**

h) At pH 5.0, which protein(s) from the AS-P stick to the anion exchange column? **1**

Question 1 (continued)

i) Explain your answer to part i) **in one or two sentences.**

Proteins are negatively charged when the pH>IEP, and positively charged when the pH is <IEP. Protein 1 has an IEP less than the pH of the column, and thus will stick to the positively charged column matrix. (Proteins 4, 5, and Bgal will be positively charged at this pH, and will flow through the column.)

j) State how you would elute a protein bound to an anion exchange column, and explain how this elution method works **in one or two sentences**.

Add increasing concentrations of salt (NaCl). The Cl- ions compete with the bound protein for binding to the positively charged matrix; when enough Cl- ions are added, the Cl- ions "exchange places" with the protein, which falls off the column.

You identify the fraction containing β-gal from your anion exchange column, and decide to run it over a **cation** exchange column to complete your purification.

k) Describe how you would use a **cation** exchange column to purify β-galactosidase away from any remaining contaminating protein(s). Be specific about: 1) the pH at which you'd equilibrate the column; 2) why you chose this pH; and 3) which protein(s) would bind and which protein(s) would flow through the column under these conditions, and why.

You should equilibrate the cation exchange column at a pH that is greater than 5.3 but less than 6.8 (5.3<pH<6.8). At this pH, β-galactosidase will have a negative charge (pH>IEP), but proteins 4 and 5 will both be positively charged (pH<IEP). Thus, proteins 4 and 5 will bind to the negatively charged matrix of the cation exchange column, whereas β-galactosidase will be repelled by the negatively charged matrix and will be found in the flowthrough.

Finally, you decide to run an SDS-PAGE gel to analyze your purification. You prepare your samples for loading as in lab, run your crude lysate (CL) sample (containing proteins 1-5 and β-galactosidase) on the gel, stain with Coomassie, and observe 6 bands.

l) Which protein will migrate closest to the dye front of the SDS gel? **3**
Explain your answer in one or two sentences.

Proteins are separated by size on an SDS-PAGE gel, with smaller proteins migrating more quickly through the pores of the gel than larger proteins. The dye front is found at the bottom of the gel (farthest from the wells), and thus the smallest protein should be found closest to the dye front.

Question 1 (continued)

You analyze a second sample of CL by SDS-PAGE, but this time, you do not add β-mercaptoethanol (β-ME) to your sample buffer. You find that protein 2 has the **same** relative mobility in both the presence and absence of β-ME.

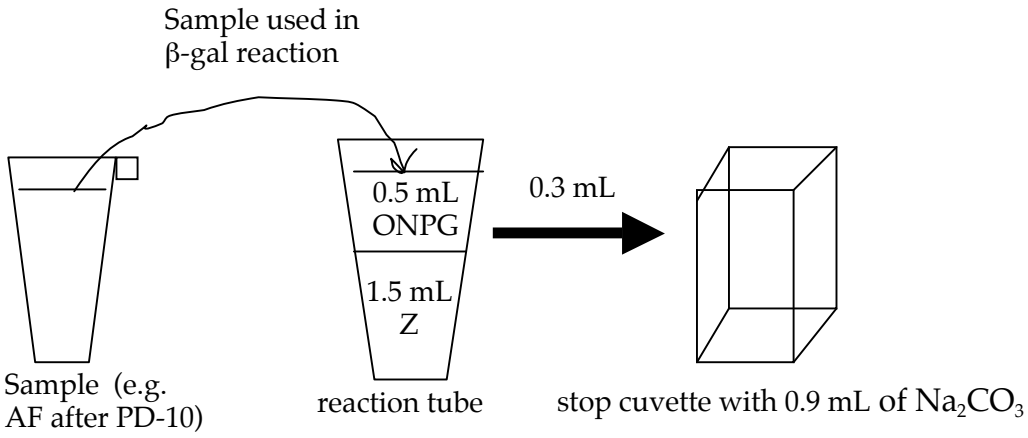
m) What do these results allow you to conclude about protein 2? Justify your answer **in one or two sentences**.

β-ME breaks disulfide bonds, and thus a protein containing them would change mobility on an SDS-PAGE gel when β-ME was added. Since protein 2 does not change mobility +/- β-ME, it must not contain disulfide bonds.

Question 2

You are interested in purifying β-galactosidase from the bacterium *S. typhimurium,* and decide to use a purification scheme similar to the one you used in 7.02 laboratory.

At each step of the purification, you perform a quantitative β-galactosidase by assaying a sample of your protein in a "reaction tube" containing 1.5 mL of Z buffer and 0.5 mL of 10 mM ONPG. When the color has developed sufficiently, you transfer 0.3 mL of your reaction to a cuvette containing 0.9 mL of Na_2CO_3. This assay setup is diagrammed below:



Question 2 (continued)

You obtain the following data for your AF after PD-10 sample:

sample name	total sample volume (mL)	[protein] (mg/mL)	volume of sample used in reaction tube(μL)	stop time (min.)	A_{420} at stop
AF after PD-10	1.5	0.133	30	6	0.333

a) Calculate the total activity of the "AF after PD-10" sample. **SHOW YOUR CALCULATIONS, and be sure to include UNITS at each step!**

1. **Start with A420 in the cuvette: A_{420} = 0.333**
2. **Therefore, in reaction tube: 0.333 x 4 (dilution factor) = 1.332 A_{420}**
3. **Calculate reaction rate: $\frac{1.332\ A_{420}}{6\ \text{minutes}} = \frac{0.222\ A_{420}}{\text{minute}}$**
4. **Determine amount of total activity in the reaction tube:**

$$\frac{0.222 A_{420}}{\text{minute}} \times \frac{1\ \text{nmol ONP/mL}}{0.0045\ A_{420}} \times 2.030\ \text{mL} = \frac{100.1\ \text{nmol ONP}}{\text{minute}} = 100.1\ \text{U}$$

5. **Determine units of activity per microliter of enzyme used in reaction:**

$$\frac{100.1\ \text{U}}{30\ \mu\text{L}} = \frac{3.34\ \text{U}}{\mu\text{L}}$$

6. **Calculate total units of activity:**

$$\frac{3.33\ \text{U}}{\mu\text{L}} \times 1.5\ \text{mL} \times \frac{1000\ \mu\text{L}}{\text{mL}} = 5 \times 10^3\ \text{U}$$

b) **Define** and **determine** the specific activity (SA) of the "AF after PD-10" sample. **SHOW YOUR CALCULATIONS, and don't forget UNITS!**

$$\text{SA of AF after PD10} = \frac{\text{TA of AF after PD-10}}{\text{TP in AF after PD-10}}$$

$$= \frac{5 \times 10^3\ \text{U}}{(\text{protein concentration})(\text{volume})} = \frac{5 \times 10^3\ \text{U}}{(0.133\ \text{mg/mL})(1.5\ \text{mL})}$$

$$= 2.5 \times 10^4\ \text{U/mg}$$

Question 2 (continued)

c) You have obtained a 12.5X fold purification and a yield of 2% at the "AF after PD-10" step. What was the protein concentration of your crude lysate (CL) sample, if its volume was 20 mL? **SHOW YOUR CALCULATIONS, and don't forget UNITS!**

Yield = TA of AF/TA of CL

$$0.02 = \frac{5 \times 10^3 \text{ U}}{\text{TA CL}}$$

TA of CL = 2.5×10^5 U

FP = SA of AF/ SA of CL

$$12.5 = \frac{2.5 \times 10^4 \text{ U/mg}}{\text{SA of CL}}$$

SA of CL = 2×10^3 U/mg

$$\text{SA of CL} = \frac{\text{TA of CL}}{(\text{volume of CL})(\text{protein concentration})}$$

$$\frac{2000 \text{ U}}{\text{mg}} = \frac{2.5 \times 105 \text{ U}}{(\text{protein concentration})(20 \text{ mL})}$$

protein concentration = 6.25 mg/mL

You repeat the quantitative assay shown above, but use 60 μL of protein in your reaction tube instead of 30μL.

d) Assuming that ONPG is in excess in these reactions, how long will it take (in minutes) to obtain an A420 of 0.500 **in the cuvette**? **Justify your answer.**

If ONPG is in excess, then the rate of the reaction is proportional to the amount of protein in the reaction; thus, if you double the amount of protein, you double the reaction rate.

If:

$$\text{30 } \mu\text{L} \rightarrow \frac{0.333 \text{ A420}}{6 \text{ minutes}} = \frac{0.0555 \text{ A420}}{\text{minute}}$$

Then:

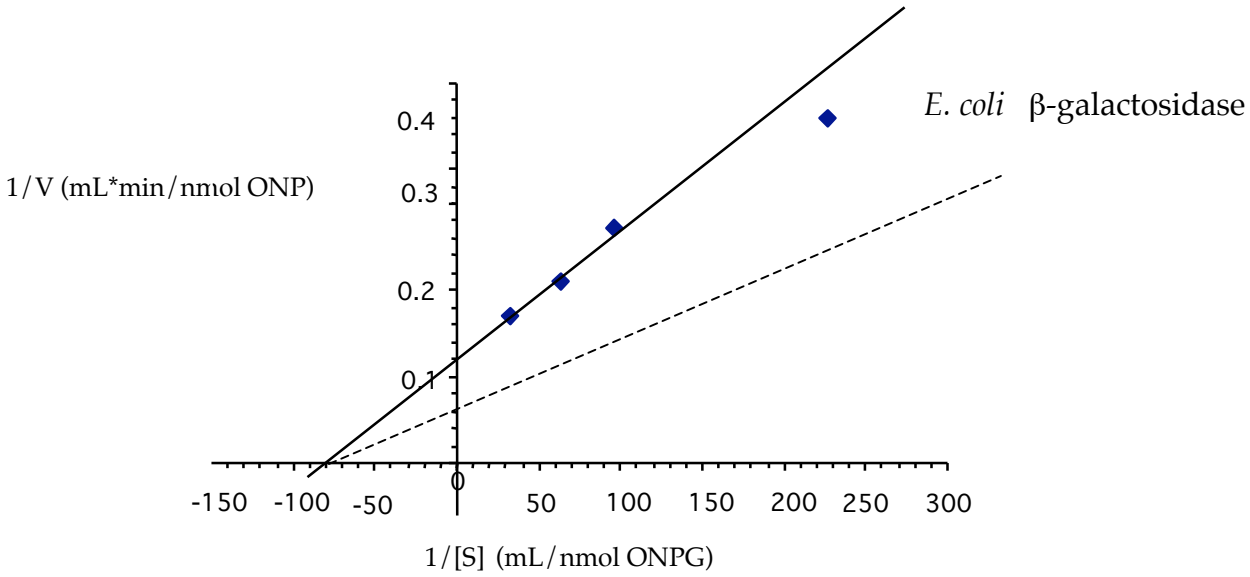
60 μL --> rate will be 0.111 A420/minute

Therefore:

$$0.500 \text{ A420} \times \frac{1 \text{ minute}}{0.111 \text{ A420}} = 4.5 \text{ minutes}$$

Question 2 (continued)

After purifying the *S. typhimurium* β-galactosidase, you decide to compare its kinetic parameters to those of *E. coli* β-galactosidase. You perform enzyme kinetics experiments with both *E. coli* and *S. typhimurium* β-galactosidase using varying concentrations of ONPG as in 7.02 lab. You obtain the following plot for *E. coli* β-galactosidase:



You compare *S. typhimurium* β-galactosidase to *E. coli* β-galactosidase , and find that *S. typhimurium* β-galactosidase can bind to ONPG **exactly as efficiently** as *E. coli* β-galactosidase.

e) What must be the Km of *S. typhimurium* β-galactosidase? **Show your calculations, and include units!**

***S. tymphimurium* Bgal binds ONPG exactly as efficiently as *E. coli* Bgal; therefore Km of *E. coli* Bgal = Km of *S. tymphimurium* Bgal.**

X intercept = -1/Km = -80
therefore Km = 0.0125 nmole ONPG/mL

Question 2 (continued)

You performed both the *S. typhimurium* and *E. coli* β-galactosidase enzyme kinetics experiments using the **same** quantity of β-galactosidase: 2.5×10^{-11} moles/mL.

f) Determine Kcat for *E. coli* β-galactosidase. **Show your calculations, and include units!**

Kcat (*E. coli*) = Vmax *E. coli*/[Etot] ; Y intercept = 1/Vmax = 0.2 mL*min/nmol ONP
Vmax = 5 nmol ONP/mL*min

$$\textbf{Kcat} = \frac{\textbf{5 nmol ONP/mL*min}}{\textbf{2.5 x } 10^{-11} \textbf{ moles/mL}} = \textbf{2 x } 10^{11} \textbf{ U/moles}$$

g) On the plot above, draw the results you'd expect if *S. typhimurium* β-galactosidase is **more efficient** at catalyzing the conversion of ONPG to ONP + galactose than *E. coli* β-galactosidase. **(same Km, higher Vmax, therefore lower 1/Vmax)**

Question 3

In the Protein Biochemistry module, you analyzed the results of your β-galactosidase purification using SDS-PAGE and Western blotting.

The diagrams on the next few pages indicate "expected results" for two different steps in the SDS-PAGE and Western blotting process, along with examples of "bad" results obtained by different student groups at each step. For simplicity, only the following lanes of the gel or Western are shown: MW standards, CL, CL-S, CL-P, AS-S, AS-P.

For each "bad" result:

1. **Identify the reagent or experimental step that was forgotten/performed incorrectly ("Problem")**
2. **State the specific observations that led you to this conclusion ("Evidence")**
3. **State how this reagent/step—when used/performed correctly—allows you to obtain the "expected results" ("Explanation")**

(see Study Questions for Diagrams)

Question 3 (continued)

Group	"Problem"	"Evidence"	"Explanation"
1	Group 1 failed to destain their Coomassie gel	The background of the gel is dark when it should be clear	Destain removes Coomassie blue dye that is not bound to protein and results in a clear background
2	Group 2 failed to add Coomassie Blue to their gel	There is no staining of protein bands other than the MW standards, which are prestained	Coomassie Blue binds nonspecifically to proteins and allows them to be visualized on the gel

Group	"Problem"	"Evidence"	"Explanation"
3	Group 3 forgot to add Blotto	The entire membrane is dark, suggesting that primary and secondary antibody has stuck everywhere (leading to NBT/BCI ppt everywhere)	Blotto "blocks" the membrane and prevents antibody from sticking to the membrane. Thus, you will only get "bands" where the Bgal is located.
4	One (or both) of the antibodies were not added	No bands were obtained on their gel	Primary antibody is required to bind to Bgal, and the secondary antibody binds to the primary antibody. The secondary Ab has an enzyme, AP, attached, which cleaves BCIP to BCI—leading to a purple NBT/BCI ppt at the location of Bgal.

Question 4

Below are seven amino acids. Indicate all characteristics that apply to each amino acid by writing letter(s) in the blanks provided.

Amino acid	
lysine	**b, h**
arginine	**b, h**
proline	**e, d**
aspartic acid	**b, h**
tryptophan	**e, f, c**
cysteine	**a, g**
glutamic acid	**b, h**

Question 5

In 7.02 this term, you purified and characterized β-galactosidase from *E. coli* cells. Some groups purified the wild type enzyme (CSH36), while others purified the mutant enzyme (HIS461). Next semester, we would like to have students purify a mutant *E. coli* β-galactosidase (ARG388) that is known to be severely deficient in substrate binding.

Which two experiments that we did this year in class will reveal to the students the nature of the ARG388 defect? Explain your reasoning.

Experiment 1: **Enzyme Kinetics (Day 6)**

A severe defect in substrate binding (as described for ARG388) would result in an effect on the Km of the enzyme as compared to wild type. Specifically, you'd expect that the Km of ARG388 would be greater than that of wild type as a high Km indicates reduced affinity for substrate (substrate binding). You'd expect that there would be no effect on the Vmax or Kcat of this enzyme as compared to wild type.

Experiment 2: **Affinity Chromatography**

The affinity column step of purification depends on the binding of Bgal to its substrate, APTG. WT β-gal should stick to the APTG column until eluted with sodium borate, while other proteins should flow through and be separated from βgal. Thus, you would expect that the affinity column would help purify wt β-gal (increased SA from DEAE-AF LOAD to AF after PD-10).

ARG388, however, would not stick to the column, and would be found in the column Flowthrough. Therefore, you'd expect no further purification of the ARG388 sample using the APTG column (SA DEAE AF-LOAD = SA of AF after PD-10).

Question 6

After 7.02 lab, you decide to do a UROP in a protein biochemistry lab. Your lab is interested in the AB complex, which is formed by two proteins ("A"+"B"). Your project is to see how mutations in A or B affect complex formation.

You first decide to purify the wild type complex. After a number of purification steps, you have two bands left in your most pure sample (as judged by Coomassie staining of an SDS-PAGE gel). To identify the bands, you decide to perform a western blot.

You probe your western blot with primary antibody (rabbit anti-"A"), then a secondary antibody (goat anti-rabbit IgG) conjugated to alkaline phosphatase. Your NBT-BCIP reaction worked great—and you see that one of the bands in your most pure sample is definitely protein A.

Next, you want to check if the other band is protein B.

a) Your postdoc had protein B injected into chickens to obtain protein B-- specific antibodies (IgG). Antibodies made in chickens are concentrated in the egg yolk. What is a simple way to purify protein B—specific IgG molecules away from other proteins in the egg yolk? (Hint: this can be accomplished in ONE column).

Use an affinity column with protein B bound to the matrix of the column.

You probe your Western blot with your purified chicken anti—"B" primary antibody. You then use the same secondary antibody as before (goat anti-rabbit IgG, alkaline phosphatase conjugate) and develop your blot. You see no bands on your blot.

b) Explain what went wrong, and how you would change your protocol to fix the problem. (Assume that the second protein in your most pure sample is indeed protein B, and that both antibodies are "functional"—i.e. not denatured.)

The secondary antibody was a goat anti-rabbit IgG, while your protein B-specific primary antibody was raised in chickens. Thus, the secondary antibody will not recognize the constant domain of chicken IgG, and you'd have no way to visualize your protein B on the blot. (Remember that alkaline phosphatase is conjugated to the secondary antibody.) To fix the problem, use a goat (or any animal but chicken) anti-chicken IgG conjugated to alkaline phosphatase to visualize the primary antibody, and thus protein B.

Question 7

After 7.02 ends, you decide to do a UROP in the lab of Dr. Jean Tics. Dr. Tics wants to see how much you learned about protein purification in 7.02, so she asks you to purify a well characterized protein (PHD Isomerase) using the lab's standard protocol.

Starting from crude lysate (CL), you performed the following steps (and created these samples to be assayed):

1. Precipitation by ammonium sulfate (yielding AS-P)
2. Desalting on PD-10 column (yielding DEAE-Load)
3. DEAE column (eluted fractions pooled as DEAE total)
4. Affinity column followed by PD-10 desalting (yielding AF after PD10)

You then assayed each sample for PHD Isomerase activity, and obtained the following results:

sample	Volume (ml)	Protein conc. (mg/ml)	Total protein (mg)	Total Activity (U)	Specific Activity	Fold Purification
CL	25	4	100	200000	**2000**	**1X**
AS-P	4	10	40	120000	**3000**	**1.5X**
DEAE-Load	2	17.5	35	105000	**3000**	**1.5X**
DEAE-Total	2	11	22	68200	**3100**	**1.55X**
AF after PD-10	1	3	3	60000	**20000**	**10X**

a) Fill in the specific activity and fold purification for each step of the purification on the chart above (include units where appropriate).

Specific Activity units= U/mg; Fold Purification has no units

b) Which step of the protocol gave the best purification? How do you know?

The affinity column (or Step #4) gave the best purification. You can tell this because this step had the best fold purification.

c) If Dr. Tics asked you to improve this protocol, which step might you choose to eliminate? Why?

You could improve the protocol by eliminating the DEAE column (Step #3). This column gives you no real increase in SA or fold purification, and results in a significant loss of protein.

Question 8

As part of your UROP you decide to try to purify human β-galactosidase (β-gal) from gut epithelial cells. You first need to develop an assay to track your human β-gal through the purification process. Prior experiments by a postdoc in your lab have indicated that human β-gal is present at ~0.5 mM in crude lysate from these cells.

a) You plan to set up your human β-gal assay by placing a sample of protein in Z buffer, then adding an appropriate substrate. Which of the following would be the most useful substrate for following the purification of human β-gal? Circle one and explain your reasoning.

i. lactose at 10mM ii. **ONPG at 10 mM**

iii. Xgal at 0.5 mM iv. ONPG at 0.5 mM

First, you want the substrate used to follow the purification of a protein to be in excess of that protein's concentration in your samples (so product production is proportional to the amount of enzyme present). Since human β-gal is present at 0.5 mM, you want the substrate concentration to be higher than this (eliminating iii and iv). You also need a substrate whose conversion to product is easily quantifiable. ONPG cleavage produces a yellow product whose production can be measured at A420; lactose cleavage produces two clear products.

Armed with your assay, you are ready to begin your purification. You detect enzyme activity in your CL-S sample, and decide to next perform an Ammonium Sulfate precipitation. You don't know the concentration of AS at which human β-gal precipitates, so you decide to try three different concentrations: 10%, 35%, and 55%.

b) You start with 15 ml of CL-S. How much 100% ammonium sulfate would you need to add to bring the concentration of the CL-S to 55% AS? **Show your calculation**.

a. (100)(volume) = 55 (volume + 15 ml)
b. 100(volume) = 55(volume) + 55(15 ml)
c. 45 (volume of AS) = 825 ml
d. (volume of AS) = 18.3 ml

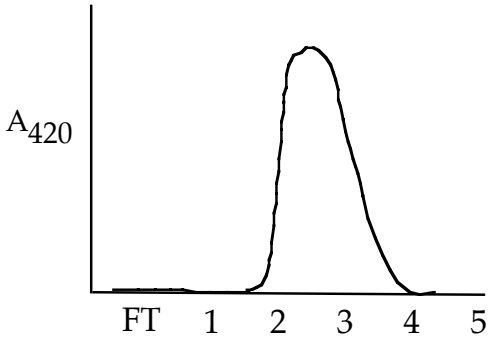
c) Another UROP in your lab (who didn't take 7.02!) thinks you are wasting your time trying to determine how much AS to use. He asks: "Why don't you just use 90% AS?" What would you tell him?

If you used 90% AS, you would precipitate your protein of interest plus many other proteins that you are NOT interested. Thus, your sample would be less pure to begin with (lower SA). Your goal with AS precipitation is to both concentrate AND purify your sample.

Question 8 (continued)

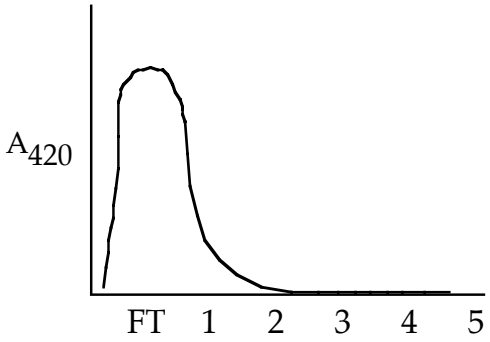
You decide to try anion exchange chromatography (DEAE column) next. To do this, you first equilibrate your column with column buffer (0.1M NaCl, pH 7.5). You then apply your desalted AS-P sample to the column, and collect the flowthrough (FT). Next, you elute the column with increasing concentrations of NaCl, and collect fractions (#1-5). Finally, you perform a quantitative assay of each fraction for activity.

d) Below are three possible (independent) outcomes of this experiment. Explain what each outcome tells you about the properties of human β-gal under the conditions you ran the column. (You may assume that the AS-P fraction had activity before you loaded it.)



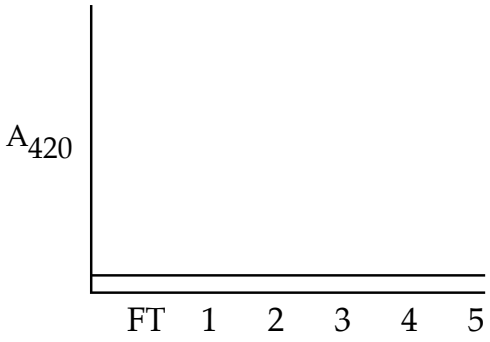
Properties of human β–gal indicated:

Human Bgal is negatively charged at pH 7.5 because it sticks to the column. This means that human Bgal's pI must be less than 7.5.



Properties of human β-gal indicated:

Human Bgal is positively charged or neutral at pH 7.5 because it flows through the column. This means that human Bgal's pI must be greater than or equal to 7.5.



Properties of human β-gal indicated:

Human Bgal does not elute off the column at the salt concentrations you used for elution (very negatively charged; pI << 7.5)
OR
Human Bgal is unstable at pH 7.5. Thus, once loaded on the column, it denatures and loses activity.

Question 8 (continued)

e) After finishing your purification, you pour an SDS polyacrylamide gel containing both stacking and separating portions, prepare your samples as in 7.02 lab, and load the gel. While the gel is running, you notice that the sample band is very **diffuse**. What might have gone wrong in the preparation of your gel, leading to the observation of a diffuse band?

The stacking gel was prepared at the incorrect pH (i.e. not 6.8). If the pH is too high, the glycine ions will not be neutral/positively charged, and will fail to help "stack" the proteins at the interface between the stacking and separating gels. Alternatively, forgetting glycine in the stacking gel completely may have a similar effect.

Finally, you are interested in performing enzyme kinetics on the human β-gal protein. After attempting to purify the protein using anion exchange (DEAE), cation exchange (CM) and affinity columns (Affinity), you run your samples on a Coomassie stained gel and perform a Western blot. You observe the following:

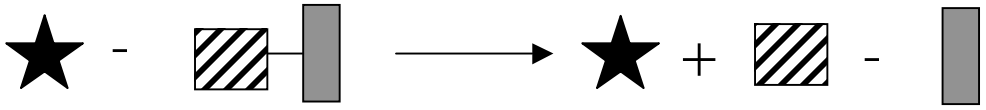
(See SQ for figures)

f) For enzyme kinetics, you want to use the sample with the highest β-gal specific activity--but you've run out of BioRad assay reagents! Which sample do you predict will have the highest β-gal specific activity? Explain your reasoning.

Affinity-total. This sample contains Bgal and has the least other proteins. (It is the most pure sample). Since SA= TA/TP, this sample will have the highest SA.

Question 9

The fictitious enzyme NMDase carries out the following reaction:



NMDase XY NMDase X Y (BLUE)

Experimentally, the progress of the reaction can be measured by looking at the production of product Y. Product Y is BLUE, and its accumulation can be measured in the spectrophotometer at A_{595}.

Question 9 (continued)

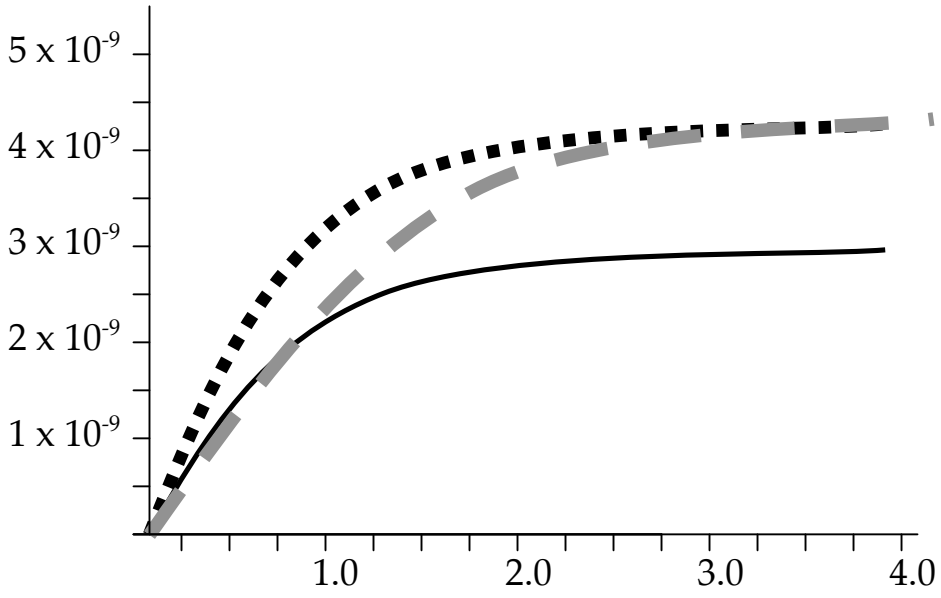
An important first step in the conversion of XY to X + Y by NDMase is the binding of enzyme to substrate. One residue suspected to be important for substrate binding is Trp999. To test this hypothesis, you have created mutant NMDase enzymes with the following amino acid substitutions at position 999: His999 (Trp999◊His999); Asp999 (Trp999◊Asp999); Gly999 (Trp999◊Gly999); and Lys999 (Trp999◊Lys999).

a) Draw the **side chains** of the following amino acids at pH 7.0. Indicate any charges expected in aqueous solution (positive, negative, neutral).

(See any biochem text for structures)

Tryptophan Charge **neutral**	Histidine Charge **pos/neutral**	Aspartic Acid Charge **negative**
Glycine Charge **neutral**	Lysine Charge **positive**	Any additional aa Name: _____ Charge **varies with student's choice**

You perform enzyme kinetics on wild type NDMase and two of the mutants (Asp999 and Gly999) that have been purified for you by a colleague in your laboratory. You obtain the following Michaelis-Menton curves for the three enzymes:



Question 9 (continued)

b) Compare the **kinetic parameters** of the Asp999 and Gly999 enzymes to those of the wild type NDMase. If a parameter has changed, be sure to say **how** it has changed (increased or decreased). If a parameter is the same as wild type, indicate this as well.

Asp999: Km has increased, Vmax has stayed the same
Gly999: Km has stayed the same, Vmax has decreased

c) Which part(s) of the enzymatic reaction (generalized as E + S ◊ ES ◊ E + P) is affected in the Asp999 mutant? In the Gly999 mutant? Explain your reasoning.

The Asp999 mutant has a defect in the formation of the ES comples (E + S◊ ES). You know this because the Km has increased (requires more substrate to reach 1/2 Vmax), while Vmax stays the same (same maximal velocity given excess substrate). Mathematically, since Km = $\frac{k2 + k\text{-}1}{k1}$, a decreased k1 will lead to the observed increased Km.
The Gly999 mutant has a defect in catalysis once the ES complex has formed (that is, ES◊ E + P). Defects in catalysis result in a lower Vmax, which is observed, with no change in Km (~binding affinity).

In order to compare the **Kcats** of the three enzymes (wt, Asp999, Gly999). you need information from the enzyme kinetics experiment described above as well as **<u>two</u>** additional experiments.

d) **Name** these two additional experiments, and describe what piece(s) of information needed for your Kcat calculation each provides.

Kcat = $\frac{Vmax}{[E]tot}$

1. **SDS-PAGE stained with Coomassie: purity and molecular weight**
2. **Bradford Assay: protein concentration**

After all these experiments, you've run out of your enzymes, and need to purify some more protein.

e) Using the Michaelis-Menton graphs on the previous page, identify the best concentration(s) of XY to use to **<u>follow the purification</u>** of wild type NDMase, Asp999, and Gly999. Justify your answer.

wild type: 3.0 μM (or greater); Asp999: 3.0 μM (or greater); Gly999: 2.0 μM (or greater)

When following the purification of an enzyme, you want the concentration of substrate to be in excess, such that the amount of activity measured (here, compound Y produced) is proportional to the [E]. Thus, you'd want to use a [substate] at or higher than that required to reach Vmax.

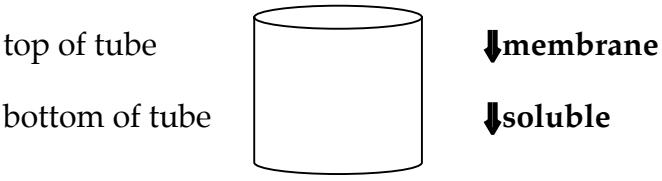
Question 10

P22 phage adheres to the surface of the bacteria *Salmonella typhimurium* (*S. tymphimurium)* by binding to a membrane bound protein on the cell surface. In order to understand more about this P22/*Salmonella* interaction, you decide to purify this membrane protein using a density gradient centrifugation technique.

a) Name the type of density gradient centrifugation you would use to purify this protein and state the composition of the gradient (material and density range).

Name: **Sucrose buoyant density centrifugation**
Gradient: **Sucrose, density range of 10-40%**

b) Draw the relative positions of membrane bound proteins and soluble proteins in the gradient tube below.

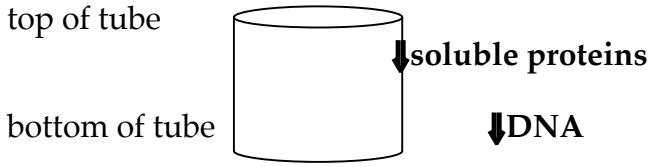


c) Name two characteristics that determine where a protein will migrate in this density gradient.

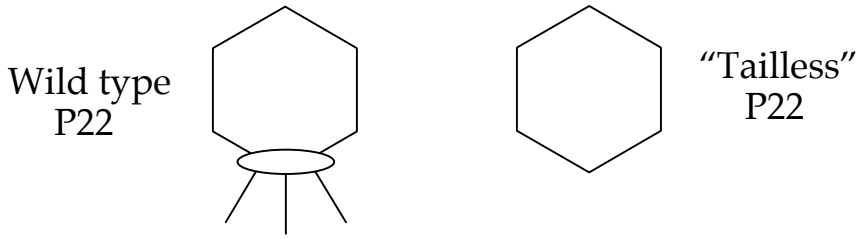
1. mass 2. buoyant density 3. frictional coefficient (choose two)

d) The expression of the *S. tymphimurium* membrane protein that P22 attaches to is regulated by a DNA binding protein. What type of gradient would you use if you wanted to purify this DNA binding protein? Draw the relative positions of DNA and soluble proteins in this gradient.

Use a CsCl gradient!



One of your colleagues has identified a mutant P22 ("tailless") that cannot lyse bacterial cells on its own. When she looks at these mutant P22 under the electron microscope, she sees that they have no tails!



Question 10 (continued)

Your colleague asks you to help her determine what P22 structural protein(s) are missing in the "tailless" P22. She gives you two tubes--one containing "tailless" P22 and one containing wild type P22--to work with, and you may assume that you have any equipment/reagents used in the PBC module at your disposal.

e) Describe an experiment that will allow you to determine how many protein(s) are missing in the "tailless" P22 mutant. **(BE BRIEF—3 sentences MAX!)**

1. **Denature samples of wild type and "tailless" P22 with SDS/heat/BME**
2. **Run an SDS gel and stain with Coomassie.**

f) In your experiment described above, what observation(s) will you make that let you know that a protein is "missing" in the "tailless" P22.

A protein band that is present in the wild type lane but absent in the "tailless" lane corresponds to a "missing" tail protein.

g) What additional piece of information can you gain about the P22 structural protein(s) from this experiment? **molecular weight (size)**

h) From your experiment in part e), you find that there are four proteins missing in the P22 tailless mutant. Name **five** properties that will likely differ between these proteins, and which could possibly be used to separate them during purification.

Any five:

1. **size**
2. **shape**
3. **function/activity**
4. **charge**
5. **hydrophobicity**
6. **affinity**
7. **cellular location**
8. **solubility (AS ppt)**

Question 11

a) In the 7.02 PBC module, you used two different colorometric assays: the Bradford (BioRad) assay and the βgal assay. Complete the following chart to compare and contrast these two assays:

	Bradford (BioRad) Assay	βgal Assay
What is this assay used for?	**determine total [protein]**	**determine Bgal activity**
What are the components of each reaction? (i.e. what goes in the reaction tube?)	**1. BioRad reagent 2. protein sample 3. column buffer**	**1. ONPG 2. protein sample 3. Z buffer**
Which of the above components was used to "start" the reaction?	**Biorad reagent (Coomassie blue)**	**ONPG**
What did you observe after the reaction had proceeded for a while? What happened to give this result?	**Observe: solution turned blue Reason: Biorad binds proteins and is goes from protonated to deprotonated**	**Observe: solution became yellow Reason: ONPG is cleaved by Bgal to give ONP (yellow) + galactose**
How was the assay quantitated (nm)?	**A595**	**A420**
What did you use as a standard, and what did you do with this data?	**Standard: BSA Use: generate a standard curve used to determine [protein] of your samples**	
What reagent did you use to stop the reaction, and how did this work?		**Na2CO3 high pH denatures Bgal**

b) Two other experiments performed in 7.02 lab give you similar information as the Bradford and βgal assays, but do so in a qualitative rather than quantitative way. Name these two experiments, and explain your choices in a sentence or two.

Experiment 1: SDS-PAGE with Coomassie is the qualitative equivalent of Bradford assay. SDS-PAGE with Coomassie allows you to determine how much protein is in a sample by looking at the number of stained bands on a gel and their intensity.

Experiment 2: Western blot is qualitative equivalent of Bgal assay. Western blot allows you to look specifically at Bgal in a sample through the use of an anti-Bgal antibody, and can tell you if you have Bgal in a sample (presence of a band) and how much (intensity of that band).