EXAM 4: Form A   Name:___ANSWER KEY_____

153L Fall 2005   TA:_________Section:______

For TA use:

Q.1.______ + Q.2._______ + Q.3.________ = Total Score: ________

Write your name on each page.

Question 1: (25 pts.)

You carry out an ammonium sulfate survey experiment to determine the range for a two-step ammonium sulfate procedure. Each addition is an increase of 10% saturation of salt, and at each step you resuspend the pellet to determine enzyme and protein amounts. Note: you take the supernatant from each step and add the next 10% saturation salt addition. That result of the survey experiment is shown in the table to the left.

Pellet Data at % saturation	Protein (mg)	Enzyme (units)
10%	30	0
20%	50	0
30%	60	0
40%	100	0
50%	300	500
60%	400	600
70%	200	400
80%	100	10
90%	20	0
100%	0	0

a) Assuming you could lose no more than 20% of the enzyme from this procedure, design a two-step salting out procedure, provide the expected purification factor and the approximate percent yield from the crude extract.

(14 pts) **Since we are not to lose more than 20% of enzyme, we must collect *at least* (500+600+400) = 1500 units out of the total (500+600+400+10) = 1510 units of enzyme. The % yield of enzyme units must be > 80%.**

In a 2-step salting out procedure, enzymes that precipitate into the 1st cut pellet is lost; so the 1st cut must be at the 40% (or earlier) saturation step in order not to lose the 500 enzyme units. The 2nd cut, then, should be at the 70% step so 500+600+400 units of enzyme all get collected.

So for a 40% to 70% cut procedure, % yield = 1500 units / 1510 units = 99.3 %
Purification factor = S.A. of 40%-70% pellets / S.A. of crude extract.
S.A. of 40%-70% pellets = (500+600+400)units enzyme / (300+400+200) mg protein = 1.67 units/mg.
S.A. of crude extract = (sum of enzyme units from all % steps) / (sum of mg protein from all % steps) = 1510 units / 1260 mg = 1.20 units/mg. So purification factor = 1.67 / 1.20 = 1.39 fold.

b) You carry out another two-step ammonium sulfate procedure with 40% and then 60% saturation. You split your 60% solution for centrifugation, and obtain two 60% pellets. You will take only one of your 60% pellets and resuspend it in a volume that is 1/8th volume of the 40% supernatant. Your initial enzyme assays require a 300 fold dilution in order to obtain a reliable slope for both the crude sample and the 40% supernatant. What dilution factor do you predict to work for the enzyme assay of the 60% resuspended pellet? Show all work.

(11 pts) **To find the dilution factor, consider how much more concentrated is the 60% pellet compared to the 40% supernatant.**

The 60% pellet is dissolved in a volume 8x smaller, but since only ½ of the 60% pellets were used, its concentration would be 4x greater than the 40% supernatant.

But that's assuming <u>all</u> the enzyme in the 40% supernatant precipitated into the 60% pellets. From the table above, we see while the 40% supernatant has 1510 units of enzyme, a 40%-60% cut would collect only (500+600) = 1100 units of enzyme. So instead of being 4x more concentrated, it's only 4 x (1100 / 1510) = 2.91x more concentrated.

So the 60% redissolved pellet should be diluted 2.91x *more* than the 40% supernatant: 300 x 2.91 = 873 fold.

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Question 2: **(25 pts.)**

Limit your answers to 3-4 sentences.

a) Why do we choose AMP on our affinity column's agarose beads rather than pyruvate?

AMP does have a greater number of interactions to LDH than pyruvate.

The AMP must bind to LDH first to have a stable enough interaction with pyruvate. AMP binding allows for LDH to undergo a conformational change in the active site, which allows for pyruvate to bind. Pyruvate alone, would not significantly bind to LDH, and thus most LDH would elute during the collection of the load fractions. (5 pts.)

b) A pH 6.0 buffer is used in our column procedure rather than a pH 7.2 buffer. Explain why this buffer is acceptable for our column, but not for our enzyme assay? How do we avoid the adverse effect of the pH 6.0 buffer on our enzyme assay when assaying the enzyme concentration in our 60% pellet and column fractions?

The pH 6.0 buffer keeps the R-groups (such as histidine and aspartate) protonated which increases LDH's binding affinity to AMP or the adduct. The tighter binding lowers the catalytic rate of LDH (primarily due to the increase binding affinity – less release of substrate/product). (5 pts.)

We avoid the loss of activity by the significant dilution of the LDH samples with pH 7.2 buffer. The diluted sample will have a pH that is essentially at a pH of 7.2. (5 pts.)

c) We can elute LDH from our AMP-agarose beads using either buffer alone, AMP buffered solution, or NAD-pyruvate adduct buffered solution. Why can we use all three to elute our enzyme, but would prefer adduct over the other two solutions? Explain.

Since the interaction between LDH and AMP is via noncovalent interactions, there is constant binding and release, and so any of these three solutions will allow LDH to elute from the column. (4 pts.) **Buffer alone will elute LDH along with other AMP binding protein contaminants very slowly from the column.** (2 pts.) **An AMP buffered solution will elute LDH faster, but also with AMP binding protein contaminants.** (2 pts.) **Adduct will elute LDH specifically from AMP binding protein contaminants because LDH has the greatest number of interactions to the adduct than any other AMP binding protein.** (2 pts.)

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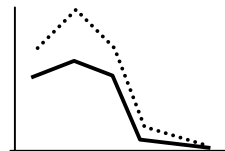
Question 3: (30 pts.)

You perform an AMP-affinity column on your resuspended 60% pellet. In doing so, you made various mistakes forcing you to repeat the procedure a few times.

a) In your first attempt to carry out this column, you poured the agarose beads into the column, and as they were packing, you allowed the buffer to elute entirely from the column. You still loaded your sample and began to collect fractions. In 1-2 sentences, explain the nature of the mistake. Which assay will first indicate that such a mistake was made? What would your result look like on an elution profile (Draw a basic plot of this elution profile)?

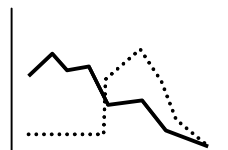
The agarose beads dried out causing the beads to shrink which resulted in crack formations throughout the column. This causes all LDH along with other proteins to nonspecifically flow through cracks and elute in the load and rinse fractions. (3 pts.) The tetrazolium spot plate will detect the presence of enzymes in the load and rinse fractions. (2 pts.) Drawing of plot: solid line – protein, dashed line –enzyme (5 pts.)



b) In your second attempt, you properly packed your column, and then loaded your sample. You then added the buffer containing adduct after the load had almost entirely flowed into the beads. In 1-2 sentences, explain the nature of the mistake. What will your elution profile look like (Draw a basic plot)?

There rinse step was skipped and as a result, non-AMP binding protein contaminants and LDH will elute from the column simultaneously. (5 pts.)

Drawing of plot: solid line – protein, dashed line –enzyme (5 pts.)



c) In your third attempt, you had to make an entirely new crude extract. But in your haste, you only performed <u>one</u> 60% salting out step. You then resuspended your 60% pellet, and loaded it into a properly packed column. What will happen to your elution profile? In 1-2 sentences, explain how your mistake caused this result?

The 40% step was skipped and thus the final 60% pellet will contain fats and lipids that will clog the column which will prevent free flow of the sample through the column. As a result, there will be no elution of any protein. (10 pts.)