BCH 372
Modern Concepts in Biochemistry Laboratory

Laboratory 8
Ammonium Sulfate Fractionation of L-Lactate Dehydrogenase, Part B

The purpose of this laboratory session is four-fold: 1) to measure the LDH activity in the 65% Ammonium Sulfate pellet fraction (**fraction 65P**) and the 65% Ammonium Sulfate supernatant fraction **(fraction 65S**) which were prepared last week; 2) to determine the protein concentrations of the various samples made as part of the Ammonium Sulfate fractionation of the crude extract; 3) to calculate the specific activities of the various fractions and to summarize the LDH purification; and 4) to prepare fraction 65P for further purification through chromatography by dialyzing it to remove the Ammonium Sulfate.

For the first part of the lab, you will do L-lactate dehydrogenase assays using the standard protocol you have used in the last few weeks. For the second part of the lab, you will prepare a protein standard curve using bovine serum albumin as the standard protein and the Bradford Reagent. You will then test varying volumes of the crude extract, the 40% Ammonium Sulfate pellet, the 40% Ammonium Sulfate supernatant, the 65% Ammonium Sulfate pellet, and the 65% Ammonium Sulfate supernatant for their protein contents. For the third part of the lab, you will prepare a summary purification table and do several additional calculations. For the fourth part of the lab, you will transfer fraction 65P to a dialysis sac so that it can be dialyzed against a large volume of 0.03 M bicine buffer, pH 8.3.

I. PreLab Preparation

Before the lab, please review **Chapter 3** on Spectrophotometry in the lab manual **Experiments in Biochemistry: a hands-on approach** by S. O. Farrell and L. E. Taylor. Pay particular attention to Sections 3.3, 3.4, 3.5, and 3.6. In this lab, you will just do the first part of Experiment 3a on the Ammonium Sulfate fractions. You will make another protein standard curve and test the various fractions from the chromatography experiments later in the semester. Also read **Chapter 4** in the lab manual on Enzyme Purification again. Pay particular attention to Sections 4.3 and 4.5 and be sure you understand the calculations. Finally, review the Summary of Basic Calculations Used in the Measurement and Purification of L-Lactate Dehydrogenase which was provided as part of the handout for **Laboratory 4.**

II. Laboratory Procedures

A. Assays of the 65% Ammonium Sulfate Fractions for LDH Activity

The purpose of this part of the experiment is to determine the L-lactate dehydrogenase activity in the resuspended 65% Ammonium Sulfate pellet fraction (**fraction 65P**) and the 65% Ammonium Sulfate supernatant fraction **(fraction 65S**).

You will use the same procedure you have done before, so refer back to Laboratory 4, 5, 6, and 7 if necessary. Turn on the Genesys 20 spectrophotometer and allow it to warm up for 15 minutes. Set the wavelength to 340 nm. Again, you will be provided with the following stock solutions for the LDH assay:

Assay Buffer Solution = 0.15 M CAPS, pH 10.0
NAD^+ = 6 mM
L-lactate = 150 mM, pH 7.0

To set up a standard assay, add 1.9 ml of the Assay Buffer to a 4.5 ml methacrylate cuvette. Then add 0.5 ml (500 µl) of the NAD^+ solution and 0.5 ml (500 µl) of the L-lactate solution. Place a piece of Parafilm over the top of the cuvette and invert the solution several times to mix everything together. Insert the cuvette into the spectrophotometer and set the instrument to zero absorbance. Then remove the cuvette and add sufficient enzyme and water to give a total volume of 3.0 ml. Rapidly place a piece of Parafilm over the top of the cuvette and invert the solution several times to mix everything together. Place the cuvette back in the instrument and read the absorbance at 340 nm at 15 second intervals for a total of 3 minutes. Record the absorbance values in your lab notebook.

1. Retrieve your resuspended 65% AS pellet fraction (**fraction 65P**). Invert the centrifuge tube several times to mix the suspension completely. Measure the LDH activity using the standard protocol. This fraction should have relatively high activity so you might start with just 5 or 10 µl. If the reaction is very rapid, make a 1/10 dilution of this fraction by adding 50 µl to 450 µl of 0.03 M bicine buffer, pH 8.3 in a 1.5 microcentrifuge tube. Remember to add water to the reaction mixture so that the total volume is **3.0 ml**.

2. When you find a volume that gives you a reasonable rate of reaction, repeat the assay two more times. Plot the results for each reaction on a piece of graph paper as before and determine the initial velocity (V_o) for each reaction. Calculate the average initial velocity in ΔA_{340}/min and then in µmoles/min. Finally, calculate the activity of the fraction in µmoles/min ml.

3. In the same way, retrieve your 65% AS supernatant fraction (**fraction 65S**). Mix it carefully by inverting the bottle several times. Measure the LDH activity using the standard protocol. This fraction should have relatively little activity, so you might want to start with 50 µl or 100 µl of enzyme. Remember to add water so that the total volume in the reaction mixture is **3.0 ml**.

4. When you find a volume that gives you a reasonable rate of reaction, repeat the assay two more times. Plot the results for each reaction on a piece of graph paper as before and determine the initial velocity (V_o) for each reaction. Calculate the average initial velocity in ΔA_{340}/min and then in μmoles/min. Finally, calculate the activity of the fraction in μmoles/min ml.

5. Transfer 0.5 ml of **fraction 65P** to a 1.5 ml microcentrifuge tube for use in the protein assays. Keep the rest of the fraction on ice in the 15 ml centrifuge tube for the dialysis treatment in Part E.

6. Transfer 0.5 ml of **fraction 65S** to a 1.5 ml microcentrifuge tube for use in the protein assays. Keep the rest of the fraction on ice for storage.

B. Preparation of a Protein Standard Curve

The purpose of this part of the experiment is to prepare a protein standard curve, using bovine serum albumin as the protein standard and the Bradford Reagent.

1. You will be provided with a 1.0 mg/ml stock solution of bovine serum albumin (BSA). To improve the accuracy of the standard curve, you will 1) add water to each tube to ensure that the total sample volume is 100 μl; 2) do all of the assays in duplicate; 3) carefully wipe the outside of the pipet tips with a Kim-Wipe; and 4) invert rather than vortexing the solutions with the Bradford Reagent.

2. Set up 17 13 x 100 mm glass tubes as shown in the following table.

tube	water	1.0 mg/ml BSA	Bradford Reagent
1	100 μl	0 μl	3.0 ml
2	95	5	3.0
3	95	5	3.0
4	90	10	3.0
5	90	10	3.0
6	85	15	3.0
7	85	15	3.0
8	80	20	3.0
9	80	20	3.0
10	70	30	3.0
11	70	30	3.0
12	60	40	3.0
13	60	40	3.0
14	50	50	3.0
15	50	50	3.0
16	40	60	3.0
17	40	60	3.0

Note that tubes 2 and 3 are duplicates, tubes 4 and 5 are duplicates, and so on. Using duplicate samples will allow you to make a more accurate standard curve.

3. Using micropipetters, add the water to the tubes first. Then add the BSA solution. It will help the accuracy if you use a new tip for each sample and wipe off the outside of the tip quickly with a Kim-Wipe. Also, when you add the BSA, draw the liquid up and down several times to rinse the inside of the tip and to mix the water and BSA together.

4. When all of the samples have been prepared, add **3.0 ml** of Bradford Reagent to each tube using the Repipetter.

5. Cover each tube with part of a square of Parafilm and invert it several times. This is better than vortexing the samples because it does not generate a lot of foam.

6. Allow the tubes to sit at room temperature for 10 minutes.

7. Measure the absorbance of each tube at 595 nm. The tubes will fit directly into the sample compartment of the spectrophotometer Use tube # 1 to set the instrument to zero absorbance since this "blank" contains only water and Bradford Reagent. Calculate the average absorbance value for each of the duplicate samples.

8. Plot the average absorbance values as a function of the amount of BSA in each tube. Note that because the concentration of the standard is 1.0 mg/ml, 1 µl contains 1 µg of protein. Draw a "best fit" line through data points. It should go through the origin (0 BSA = 0 Absorbance) and be linear through at least some of the points. You might find, however, that the standard curve becomes nonlinear at high protein concentrations. Graph paper will be provided so you can make as accurate a graph as possible but you can also make your graph with Excel.

C. <u>Protein Concentrations of Ammonium Sulfate Fractions</u>

The purpose of this part of the experiment is to determine the protein concentrations of various ammonium sulfate fractions using the BSA standard curve.

1. Retrieve the following samples from the class freezer box or from your ice bucket: 1) crude extract (**fraction CE**), 2) resuspended 40% Ammonium Sulfate pellet (**fraction 40P**), 3) 40% Ammonium Sulfate supernatant (**fraction 40S**), 4) resuspended 65% Ammonium Sulfate pellet (**fraction 65P**), and 5) 65% Ammonium Sulfate supernatant (**fraction 65S**).

2. Since you do not know the protein concentrations of these fractions, you will need to make several dilutions so that some of your protein samples will fall within the range of the standard curve when you do the assay.

3. Make 2 serial 1/10 dilutions of each fraction in the following way. Add 900 µl of 0.05 M potassium phosphate buffer, pH 7.0 to each of two 1.5 ml microcentrifuge tubes. Mix one of the fractions by inversion and add 100 µl of solution to the first tube. Close the cap and invert several times to mix. Then add 100 µl of the 1/10 dilution to the second tube to make a 1/100 dilution. Again, close the cap and invert to mix. Repeat the process with each of the other fractions.

4. Set up a new protein assay with 31 tubes as shown in the following table.

tube	water	sample	sample volume	Bradford Reagent
1	100 µl	none	0 µl	3.0 ml
2	90	fraction CE	10	3.0
3	70	fraction CE	30	3.0
4	90	fraction CE 1/10	10	3.0
5	70	fraction CE 1/10	30	3.0
6	90	fraction CE 1/100	10	3.0
7	70	fraction CE 1/100	30	3.0
8	90	fraction 40P	10	3.0
9	70	fraction 40P	30	3.0
10	90	fraction 40P 1/10	10	3.0
11	70	fraction 40P 1/10	30	3.0
12	90	fraction 40P 1/100	10	3.0
13	70	fraction 40P 1/100	30	3.0
14	90	fraction 40S	10	3.0
15	70	fraction 40S	30	3.0
16	90	fraction 40S 1/10	10	3.0
17	70	fraction 40S 1/10	30	3.0
18	90	fraction 40S 1/100	10	3.0
19	70	fraction 40S 1/100	30	3.0
20	90	fraction 65P	10	3.0
21	70	fraction 65P	30	3.0
22	90	fraction 65P 1/10	10	3.0
23	70	fraction 65P 1/10	30	3.0
24	90	fraction 65P 1/100	10	3.0
25	70	fraction 65P 1/100	30	3.0
26	90	fraction 65S	10	3.0
27	70	fraction 65S	30	3.0
28	90	fraction 65S 1/10	10	3.0
29	70	fraction 65S 1/10	30	3.0
30	90	fraction 65S 1/100	10	3.0
31	70	fraction 65S 1/100	30	3.0

Note that by following this protocol, you will be testing 10 µl and 30 µl volumes of each of the concentrated fractions and each of the 1/10 and 1/100 dilutions. The goal is to find several samples of each fraction that fall within the range of the standard curve. Some tubes will probably be too dark to read and some tubes will be too light to read, but some should be usable.

5. Using micropipetters, add the water to the tubes first. Then add the unknown protein solutions. Again, it will help the accuracy if you use a new tip for each sample, wipe the outside of the tip quickly with a Kim-Wipe, and draw and liquid up and down in the water to rinse the inside of the tip and to mix the water and sample together.

6. When all of the samples have been prepared, add **3.0 ml** of Bradford Reagent to each tube using the Repipetter.

7. Cover each tube with part of a square of Parafilm and invert several times. This is better than vortexing the samples because it does not generate a lot of foam.

8. Allow the tubes to sit at room temperature for 10 minutes.

9. Measure the absorbance of the solution in each tube at 595 nm. Again, you can put the tubes directly into the cuvette holder of the spectrophotometer.

10. Calculate the amount of protein in µg in each of the usable samples. **Use only those absorbance values that fall within the linear range of your BSA standard curve for the analysis.** You can either interpolate directly along the line of the standard curve or create a simple conversion factor derived from it. Refer back to Laboratory 4 for examples of this type of calculation.

11. Then, correct for the volume used in each sample and the dilution factor to calculate the protein concentration of each of the fractions in mg/ml. If you have several usable values for a particular fraction, average them to get a single protein concentration for that solution.

D. Calculation of Specific Activities and Purification Factors

As noted in Section 4.3 of Chapter 4 of the lab manual, the purification of an enzyme such as L-lactate dehydrogenase can be followed by measuring the activity of each fraction, by calculating the total number of enzyme units in each fraction, and by determining the specific activity in each fraction. **Refer to the calculations described at the end of the handout for Laboratory 4.**

Specific activity is expressed as the units of enzyme activity in µmol/min per unit of protein in mg. In a typical calculation, you determine the amount of activity in µmol/min per ml of each fraction and the amount of protein in mg per ml of each fraction. The specific activity can be then determined as follow:

$$\text{specific activity} = \frac{\text{activity}}{\text{protein}} = \frac{\mu\text{mol/min ml}}{\text{mg/ml}}$$

The volumes in ml cancel out, so specific activity has units of μmoles/min mg.

You can calculate this by multiplying the activity by the inverse of the protein concentration:

$$\frac{\mu\text{moles}}{\text{min ml}} \times \frac{\text{ml}}{\text{mg}} = \frac{\mu\text{moles}}{\text{min mg}}$$

$$= \text{units/mg}$$

1. Go back to your notebook and find the volume and LDH activity in each fraction in μmol/min ml. Add the results to the summary table on the next page.

2. The total amount of activity in enzyme units (μmoles/min) can be determined by multiplying the activity of each fraction by its volume. For example,

$$\frac{2.46\ \mu\text{moles}}{\text{min ml}} \times 8\text{ ml} = \frac{19.7\ \mu\text{moles}}{\text{min}} = 19.7\text{ units}$$

3. The recovery of enzyme activity as you go through a purification process is calculated by dividing the amount of activity in each fraction by the amount of activity in the initial homogenate. For example, suppose that you had a total of 45.9 enzymes units (μmoles/min) in the homogenate and recovered a total of 3.89 enzyme units in the 65% ammonium sulfate supernatant fraction. The recovery in this fraction is thus:

$$\frac{3.89\text{ units}}{45.9\text{ units}} \times 100\% = 8.45\%$$

4. Now add your calculated protein concentrations to the same table.

5. Calculate the specific activity of each fraction and add these values to the table. Again, the specific activity is the amount of activity per unit of protein (μmole/min mg). Which fraction has the highest specific activity?

6. Finally, calculate the extent of purification. This is usually expressed as the ratio of the specific activity in a particular fraction to the specific activity in the crude homogenate. Calculate these values and add them to the table.

Summary of Ammonium Sulfate Fraction of LDH Activity in Crude Extract

fraction	volume (ml)	LDH units/ml	total units	% recovery	protein mg/ml	LDH sp. act.	fold purification
crude extract							
40% AS pellet							
40% AS supernatant							
65% AS pellet							
65% AS supernatant							

E. Dialysis of 65% Ammonium Sulfate Pellet

The purpose of this part of the experiment is to remove the excess salt from **fraction 65P**. This is necessary because it will interfere with the binding of proteins to the resins in the subsequent chromatography experiments.

1. Segments of washed dialysis tubing will be available. Use a plastic clamp to seal off one end of an 8 inch piece of tubing.

2. With a plastic transfer pipet, carefully transfer the rest of your resuspended 65% AS pellet (**fraction 65P**) to the dialysis sac. You should have about 5-6 ml of solution, but if you have more, you can use another piece of dialysis tubing. Place another clamp over the other end. Note the numbers on the clamps so you can distinguish your sample from those of the other groups.

3. Place the dialysis tubing sac in a large (2,000 ml beaker) of 0.03 M bicine buffer, pH 8.3. The samples with be kept in this buffer overnight with continuous stirring. The next day, the instructor will transfer the dialyzed solutions to plastic centrifuge tubes for storage in the refrigerator at 4^{o}C and use in next week's laboratory.

F. Storage of Samples

The purpose of this part of the lab is to save samples for the gel electrophoresis lab next week.

1. Before you leave the lab, be sure to place the samples of the crude extract, the 40P fraction, the 40S fraction, the 65P fraction, and the 65S fraction in either the freezer box or the rack for storage until the next lab.