BCH 372
Modern Concepts in Biochemistry Laboratory

Laboratory 14
Completion of LDH Purification

The purpose of this laboratory session is to determine the protein concentrations of the pooled fractions from the ion-exchange and affinity columns. You will prepare a new BSA standard curve and assay the fractions as described in Laboratory 8 for the ammonium sulfate fractions. The protein values will be used to complete a purification table for LDH from your crude extract. They will also be needed for the next gel electrophoresis lab.

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 and **Laboratory 8 (Ammonium Sulfate Fractionation of LDH, Part B).** Also look at the summary of the Basic Calculations Used in the Purification of L-Lactate Dehydrogenase which was included as part of Laboratory 4 (Assay for L-Lactate Dehydrogenase). Review Chapter 4 in the lab manual on Enzyme Purification, paying particular attention to Sections 4.3 and 4.5.

II. Laboratory Procedures

A. Measurement of LDH activity in Affinity Chromatography Pools

The purpose of this part of the experiment is to finish up Laboratory 13. If you did not measure the L-lactate dehydrogenase activity in your affinity chromatography pool(s) at the end of the last lab session, do so at the beginning of this lab.

1. Retrieve your AC pool (or pools) from the last experiment and do three replicate assays on each pool. You may need to adjust the volume to get a good initial velocity, but 50 µl may still be sufficient.

2. Plot the data as before and calculate the initial velocities for each of the three runs. Then calculate the average initial velocity for the pooled fraction. Convert the ΔA_{340}/min rates into µmole/min using the formula presented in Chapter 4. Then express the results in **µmoles/min ml** of enzyme solution by correcting for the volume used in the assay.

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. You will follow the same general protocol given in Laboratory 8.

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 again 1) add water 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; 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

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 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. You can put the tubes directly in the cuvette holder in the sample compartment. Use tube # 1 to set the instrument to zero absorbance since this "blank" contains only water and Bradford Reagent.

8. Calculate the average absorbance value for each of the duplicate samples. Then plot these average values as a function of the amount of BSA in each tube. 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. You can also make the graph using Excel.

C. Protein Concentrations of Column Fractions

The purpose of this part of the experiment is to determine the protein concentrations of various fractions obtained during the ion exchange and gel filtration chromatography experiments.

1. Retrieve the following samples from the LDH purification process:

   a) **D65P** – this is the 65% ammonium sulfate pellet that was resuspended in buffer, dialyzed against bicine buffer, and then used for the ion exchange and affinity chromatography experiments.

   b) **IE wash pools** - the pools of material that did not absorb to the DEAE-Cellulose or CM-Cellulose columns

   c) **IE elution pools** – the pools of material that flowed through the DEAE Cellulose or CM-Cellulose columns during elution with buffers containing increasing concentrations of NaCl.

   d) **AC pool** - the pool of material that was obtained from the Cibacron Blue Agarose column

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.

3. Make 2 serial 1/10 dilutions of each fraction in the following way. Add 900 µl of 0.03 M bicine buffer, pH 8.5 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 13 x 100 mm tubes as shown in the following table for the D65P fraction.

tube	water	sample	sample volume	Bradford Reagent
1	100 µl	none	0 µl	3.0 ml
2	90	undiluted D65P	10	3.0
3	70	undiluted D65P	30	3.0
4	90	D65P 1/10	10	3.0
5	70	D65P 1/10	30	3.0
6	90	D65P 1/100	10	3.0
7	70	D65P 1/100	30	3.0

5. Note that by following this protocol, you will be testing 10 µl and 30 µl volumes of the undiluted fraction and 10 µl and 30 µl volumes of each of the 1/10 and 1/100 dilutions. The goal is to find several samples of each fraction that falls within the range of the standard curve.

6. Following this general protocol, plan to add more tubes to the table so that you can determine the protein concentration of each of your other pools. The number of tubes involved will vary from group to group, depending on how many different pools it has. Again plan to test 10 µl and 30 µl volumes of the undiluted fraction and 10 µl and 30 µl volumes of 1/10 and 1/100 dilutions.

7. 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 proteins together.

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

9. 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.

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

11. Measure the absorbance of the solution in each tube at 595 nm. Use tube # 1 to set the instrument to zero absorbance.

12. 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. Calculate the amount of protein in µg in each of the usable sample.

13. Correct for the volume used in each sample and the dilution factor and 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 this solution. If none of the samples for a particular fraction are usable, change the volume or dilution factor and repeat the assay.

D. Protein Concentrations of Other Fractions

The object of this part of the experiment is to repeat any of the protein assays from Laboratory 8 which gave ambiguous or highly variable results.

1. Look back at the BSA protein standard curve that you prepared as part of Laboratory 8 and compare it with the one you made today. If the earlier version showed more scatter in the data points or poor linearity compared to today's curve, you may want to repeat some of the assays.

2. As part of Laboratory 8, you determined the protein concentrations of the crude extract (**fraction CE**), the 40% ammonium sulfate supernatant (**fraction 40S**), the 65% ammonium sulfate supernatant (**fraction 65S**), and the resuspended 65% ammonium sulfate pellet (**fraction 65P**). Go back and look at your measurement of the protein concentrations of these fractions. If the results were highly variable or if the calculations appeared to be inconsistent, you can repeat the protein assays today.

3. If you need to, make 1/10 and 1/100 dilutions of the stored fractions in 0.05 M sodium phosphate buffer, pH 7. Then carry out a protein assay using 10 µl or 30 µl volumes as described above. Using the absorbance values that fall within the range of the standard curve, calculate the amount of protein in µg in each fraction. Finally, correct for the volume used in each sample and the dilution factor calculate the protein concentration of each fraction in mg/ml.

E. Completion of Purification Table

The object of this part of the experiment is to complete a purification table for L-lactate dehydrogenase from bovine heart muscle. A blank summary table is given on page 7 of this handout. You may need to add additional lines to include the various pools that you obtained with your particular extract. Table 4.3 in Chapter 4 gives an example of such a table and a similar chart is shown on page 205 of the lab manual.

1. Go back to your notebook and find the total volume in ml, the LDH activity in **µmoles/min ml**, and total number of enzyme units in **µmoles/min** in each fraction or pool you have obtained. Add the results to the table shown on pages 7 and 8.

2. Go back to your notebook and find the protein concentrations in **mg/ml** and specific activities in **µmoles/min mg** of the crude extract and ammonium sulfate fractions. Add the results to the table.

3. Now add your new calculated protein concentrations for the D65P fraction, the IE wash pools, IE elution pools, and AC pools to the same table.

4. Calculate the specific activity of the IE wash pools, the IE elution pools, and the AC pool. Add these values to the same table.

5. The recovery in a particular fraction is usually expressed as a percentage of the total number of units in a fraction to the total number of units in the crude homogenate. **Since you only added 1.0 ml of the dialyzed ammonium sulfate fraction (D65P) to each of the ion exchange chromatography columns or 0.5 ml to the affinity chromatography column, calculate the recovery on the basis of actual volume loaded. We will assume that the recovery would have been the same if the entire fraction had been loaded onto a larger column and the enzyme eluted in the same way.** Fill in all of these values in the table. You may find that the activity was distributed among several pools.

6. The extent of purification is usually expressed as the ratio of the specific activity in a particular fraction to that of the specific activity in the crude homogenate. Calculate these values and add them to the table as well.

fraction	volume (ml)	LDH activity (µmol/min ml)	total units	recovery (percent)	protein (mg/ml)	specific activity (units/mg)	fold purification
crude extract	_____	_____	_____	_____	_____	_____	_____
Ammonium Sulfate Fractionation							
40% AS pellet	_____	_____	_____	_____	_____	_____	_____
40% AS supernatant	_____	_____	_____	_____	_____	_____	_____
65% AS pellet	_____	_____	_____	_____	_____	_____	_____
65% AS supernatant	_____	_____	_____	_____	_____	_____	_____
dialyzed 65% AS pellet	_____	_____	_____	_____	_____	_____	_____
DEAE-cellulose column							
IE wash pool A	_____	_____	_____	_____	_____	_____	_____
IE wash pool B	_____	_____	_____	_____	_____	_____	_____

IE elution pool A	_____	_____	_____	_____	_____	_____	_____
IE elution pool B	_____	_____	_____	_____	_____	_____	_____
CM-cellulose column							
IE wash pool A	_____	_____	_____	_____	_____	_____	_____
IE wash pool B	_____	_____	_____	_____	_____	_____	_____
IE elution pool A	_____	_____	_____	_____	_____	_____	_____
IE elution pool B	_____	_____	_____	_____	_____	_____	_____
Cibacron Blue Column							
AC pool	_____	_____	_____	_____	_____	_____	_____