BCH 372
Modern Concepts in Biochemistry Laboratory

Laboratory 13
Affinity Chromatography

The purpose of this laboratory session is to continue the purification of L-lactate dehydrogenase from your particular organ extract. In this experiment, you will use affinity chromatography to separate the proteins in the dialyzed resuspended 65% ammonium sulfate pellet fraction (**fraction D65P**). You will begin by preparing a small affinity column containing the resin Cibacron Blue agarose. You will then load a **0.5 ml** portion of your **fraction D65P** onto the top of the column and wash the proteins through column with buffer. You will test the material that elutes from the columns for LDH activity using the standard enzyme assay. You will then elute the LDH bound to the column using buffers containing increasing concentrations of salt. By the end of this experiment, you should have another more highly purified sample of LDH from your organ sample.

I. PreLab Preparation

Before the lab, please read Chapter 6 in the lab manual **Experiments in Biochemistry: a hands-on approach** by S. O. Farrell and L. E. Taylor. Because we do not have fraction collectors and UV absorbance monitors for each student group, you will carry out a modification of the experiment described in Experiment 6 of the lab manual.

II. Laboratory Procedures

A. Measurement of LDH Activity in the Dialyzed Resuspended 65% Ammonium Sulfate Pellet Fraction

The purpose of this part of the experiment is to determine the current enzyme activity in the dialyzed resuspended 65% ammonium sulfate pellet fraction (**fraction D65P**). You will use the same procedure you have done before to measure the LDH activity of this fraction.

1. Retrieve the 15 conical centrifuge tube with your **D65P fraction**. Invert the tube to mix the sample completely. Keep this solution cold in an ice bucket.

2. Measure the activity in this fraction using the standard protocol you have used in the previous labs. 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

3. 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. Note that the total volume at this point is **2.9 ml**. It will be easiest if you use a 5 or 10 ml pipet to add the buffer and a P-1000 micropipetter to add the NAD^+ and L-lactate solutions. Be sure to use a clean tip for each solution.

4. Since you do not know how much activity is in the dialyzed resuspended 65% ammonium sulfate pellet fraction, you may need to try several volumes. **The final total volume in the cuvette should always be 3.0 ml**, so you can add up to **100 µl (0.1 ml)** of the enzyme solution and water. **Start by assaying the same volume that you found to be suitable when you assayed the dialyzed resuspended 65% ammonium sulfate pellet as part of Laboratory 11.** This may be **5-10 µl of a 1/10 dilution** of the stock **solution** or something similar.

5. Add the buffer, substrates, and water to the cuvette first. Then 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 of the enzyme solution. 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.

6. If the reaction occurs at a reasonable rate (for example, an initial increase in A_{340} of about 0.1 or 0.2 per minute), repeat the assay two more times. Plot the results for each reaction on a piece of graph paper as before. Identify the linear portion of the rate curve and use it to determine the initial velocity (V_o) of the reaction. Calculate the average initial velocity.

7. If the rate of the reaction is too slow, try adding a larger volume to the reaction. **Be sure to adjust the amount of water, so that the final total volume 3.0 ml in each case**. If the rate of the reaction is too fast, make a 1/10 dilution of the enzyme solution by adding 10 µl to 90 µl of 0.03 M bicine buffer, pH 8.3 in a microcentrifuge tube. Then assay 10, 25, or 50 µl volumes using the standard protocol. Again, the total volume should be **3.0 ml**.

8. Once a suitable volume of the enzyme solution has been identified, do three replicate assays. Plot the data, determine the initial velocities (V_o), and calculate the average **initial velocity** of the reaction. Then calculate the **activity** of the fraction in **µmoles/min ml** as you have done before.

B. Preparation of an Affinity Chromatography Column

The purpose of this part of the experiment is to prepare a small column of the affinity resin Cibacron Blue Agarose (type 3000-CL, Sigma-Aldrich). This is one of the resins listed in **Table 6.1** of the lab manual, which has a ligand with the structure shown in **Figure 6.4**.

1. Obtain a BioRad Poly-Prep chromatography column. Remember that each column has a small porous bed support and is designed to hold about 2 ml of resin material. Note that both the lower part of the column, which will hold the resin bed, and the upper part of the column, which will hold extra buffer, are calibrated in milliliters. Snap off the tip of the column and place a two-way plastic stopcock on the end. Using a two-fingered clamp, mount the column on a small ring stand.

2. Place a small 100 ml beaker under the end of the column and close off the stopcock. Add about 5 ml of 0.03 M bicine buffer, pH 8.5, to the column. Open the stopcock and allow the liquid to drain into the beaker. Close the stopcock and add another 5 ml of buffer to the column. Open the stopcock and allow the buffer to drain down until there is about 1 ml in the lower portion of the column.

3. You will be provided with a tube containing about 3 ml of the resin. The resin will have been equilibrated with 0.03 M bicine buffer, pH 8.5, and degassed under vacuum to remove air bubbles.

4. **Gently invert** the tube to resuspend the particles. With a plastic transfer pipet, add about 1 ml of the resin to the column. Open the stopcock and allow the liquid to drip slowly into the beaker. Add more resin as needed until you have created a resin bed of about 2 ml. The idea is to let the resin beads slowly settle through the buffer and to pack evenly in the bottom part of the column. NEVER LET THE RESIN BED RUN DRY. After the resin bed has been formed, wash the resin bed with about 5 ml of 0.03 M bicine buffer, pH 8.5. BE SURE THAT THERE IS ALWAYS AT LEAST 1 ML OF BUFFER ON TOP OF THE RESIN BED.

C. Affinity Chromatography on Cibacron Blue Agarose

The purpose of this part of the experiment is to bind LDH to the column and then to elute it with buffers containing increasing concentrations of NaCl.

1. Transfer **0.5 ml** of your dialyzed resuspended 65% ammonium sulfate pellet fraction (**D65P**) to a clean tube.

2. Set up a test tube rack containing 14 of the 13 x 100 mm glass tubes. Number the tubes 1-14. Fill the first tube (# 1) with **3 ml** of 0.03 M bicine, pH 8.5. Mark the height with a Sharpie marker. In this experiment, you again will be collecting the material that elutes from the column as a series of **3 ml fractions**, and by using tube # 1 as a blank, you will be able to see how much liquid needs to collected in each tube.

3. Open the stopcock on the column and slowly allow the buffer to drain into the beaker until it just gets to the top of the resin bed. Close the stopcock. Position the test tube rack under the stopcock so that the liquid can be collected into tube # 2. Carefully add the sample of the **D65P fraction** to the top of the resin bed. Open the stopcock and allow the liquid to drip into tube # 2. Allow the sample to run into the resin bed until the liquid just reaches the top of the resin bed. Close the stopcock. DO NOT LET THE COLUMN RUN DRY.

4. Now add about 3 ml of 0.03 M bicine, pH 8.5, to the top of the resin bed. Open the stopcock and allow the liquid to run into the column. Continue to collect the liquid that elutes from the column into tube # 2. Close off the stopcock.

5. Now add about 6 ml of 0.03 M bicine, pH 8.5 to the reservoir on top of the resin bed. Open the stopcock and allow the liquid to flow through the column. Continue to collect the liquid into tube # 2 until it reaches the height corresponding to 3 ml. Then move the test tube rack over one position and collect the liquid as part of fraction # 3.

6. Add more buffer as necessary to the reservoir and collect a total of 4 fractions while washing the column with 0.03 M bicine, pH 8.5 (that is, through tube # 5). Close the stopcock. Any proteins that cannot bind to Cibacron Blue should be found in these wash fractions. Any protein that can bind to Cibacron Blue should still be on the column.

7. You may find that the flow rate through the column is relatively slow. If this is the case, you can use the stopper and syringe provided before to put a moderate amount of pressure on the column to increase the flow rate.

D. <u>Elution of Proteins from the Affinity Chromatography Column</u>

1. Now add **6.0** ml of 0.03 M bicine, pH 8.5 containing 0.2 M NaCl to the top of the resin bed. The increased salt concentration will allow some of the proteins that have bound to the resin to detach from the ligand and to flow through the column. Open the stopcock and allow the buffer to wash through the column. Collect the liquid as part of fractions # 6 and # 7. Each fraction should contain a total of 3.0 ml, so with each new buffer, you should obtain about two new fractions.

2. When the buffer just reaches the top of the resin bed, close off the stopcock. DO NOT LET THE COLUMN RUN DRY.

3. Now add **6 ml** of 0.03 M bicine, pH 8.5 containing 0.4 M NaCl to the column. Open the stopcock and continue to collect the liquid that elutes from the column. When the buffer just reaches the top of the resin bed, close off the stopcock.

4. Add **6 ml** of 0.03 M bicine, pH 8.5 containing 0.6 M NaCl to the column. Open the stopcock and continue to collect the liquid that elutes from the column. When the buffer just reaches the top of the resin bed, close off the stopcock.

5. Add **6 ml** of 0.03 M bicine, pH 8.5 containing 0.8 M NaCl to the column. Open the stopcock and continue to collect the liquid that elutes from the column. When the buffer just reaches the top of the resin bed, close off the stopcock.

10. Add **6 ml** of 0.03 M bicine, pH 8.5 containing 1.0 M NaCl to the column. Open the stopcock and continue to collect the liquid that elutes from the column. When the buffer just reaches the top of the resin bed, close off the stopcock.

11. **As with the ion exchange chromatography, it may not be necessary to use all of the salt-containing buffers to elute the LDH from the column. Once the enzyme has come off of the column, you can stop.**

12. Again, you can use a cap and syringe to put a moderate amount of pressure on the column to increase the flow rate.

E. Measurement of LDH Activity in the Eluted Fractions from the Affinity Chromatography Column

The purpose of this part of the experiment is to determine which the fractions obtained from the Cibacron Blue column contain the LDH activity. You can start doing this part of the experiment as the fractions are collected from the column.

1. Measure the LDH activity of each of the column fractions you collect in Parts C and D. Use the standard protocol for the LDH assay that you used for the ion exchange chromatography. Since the samples will be diluted somewhat during the chromatography, try using **50 µl** of each fraction for the initial assays. You only need to assay each fraction once at this point since either it will have activity or it will not. Express the activity in ΔA_{340}/min and then in **µmoles/min ml**.

2. You may find that the LDH activity is spread out over several fractions. If this is the case, pool the two or three fractions with the highest activity. Measure the total volume and transfer the liquid to a plastic centrifuge tube. Measure the total volume and label the tube **AC pool**.

3. Then do three replicate assays on this pool. You may need to adjust the volume to get a good initial velocity. 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.

4. Save the AC pool for use in later experiments.

F. Preparation of an Elution Profile

The purpose of this part of the experiment is to summarize the results of the affinity chromatography as an elution profile. An elution profile is a graph in which enzyme activity (μmoles/min) is plotted as a function of fraction number.

1. On a piece of graph paper, make an elution profile for the Cibacron Blue affinity chromatography column. Plot the LDH activity in μmoles/min ml as a function of the fraction number. Indicate on the profile which fractions were combined to make the AC pool.