BCH 372
Modern Concepts in Biochemistry Laboratory

Laboratory 11
Ion Exchange Chromatography - Part A

The purpose of this laboratory session is to continue the purification of the L-lactate dehydrogenase from your crude extract. In this experiment, you will use ion-exchange chromatography to further separate the proteins in the dialyzed resuspended 65% ammonium sulfate pellet fraction (**fraction D65P**). You will begin the experiment by preparing two small ion-exchange columns. You will then load a small portion of your **fraction D65P** onto the top of each column and wash the proteins through columns with buffer. You will test the material that elutes from the columns for LDH activity using the standard enzyme assay and pool those fractions that contain the most LDH activity. You will continue the experiment next week by eluting those proteins which bound to the columns using buffers containing increasing concentrations of salt. By the end of this experiment, you should have several more highly purified samples of LDH.

I. PreLab Preparation

Before the lab, please read Chapter 5 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 5a of the lab manual. At the end of today's lab, you will save the partially purified fractions containing LDH activity that did not bind to the ion-exchange columns. You will also save the columns to which other proteins bound (perhaps including more LDH) for next week's lab.

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 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 activity of the solution.

1. Retrieve the 15 conical centrifuge tube with your **D65P fraction**. The material from the dialysis sac was placed in one of these tubes by the instructor after three cycles of dialysis against 0.03 M bicine buffer, pH 8.5. Measure and record the **volume** of the solution in your notebook. Keep this solution cold in an ice bucket.

2. Look at the solution carefully. If there is an obvious pellet of salt crystals at the bottom of the tube, it will be necessary to do a short centrifugation to remove it. If there is no obvious salt, you do not need to do steps 3, 4, and 5.

3. Obtain another 15 ml conical centrifuge tube and fill it with water to the same height as your fraction.

4. Place the tubes opposite one another in the refrigerated table top centrifuge in CLCC 367 and centrifuge them at 3000 rpm for 5 minutes.

5. Decant the liquid from your fraction into a clean 15 ml conical centrifuge tube. Label the tube **CD65P** (centrifuged dialyzed 65% ammonium sulfate pellet) and measure the volume again.

6. Measure the activity in your **D65P** or **CD65P** 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

7. 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.

8. 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 resuspended 65% ammonium sulfate pellet as part of Laboratory 8. This may be 5 or 10 µl of a 1/10 dilution or something similar**.

9. 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.

10. 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.

11. 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 50 µl to 450 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**.

12. 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 Ion Exchange Columns

The purpose of this part of the experiment is to prepare small columns of a weak anion exchange resin called DEAE (diethylaminoethyl) Cellulose and a weak cation exchange resin called CM (carboxymethyl) Cellulose. These resins are listed in Table 5.2 of the lab manual. The DEAE Cellulose has a positively-charged group as the functional ligand and so will preferentially bind those proteins with a net negative charge at pH 8.5. The CM Cellulose has a negatively-charged group as the functional ligand and so will preferentially bind those proteins with a net positive charge at pH 8.5. **To do this experiment most efficiently, one member of each group should prepare and run one column and another member of each group should prepare and run the other column.**

1. Obtain two (2) BioRad Poly-Prep chromatography columns. 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 each column and place a two-way plastic stopcock on the end. Using a two-fingered clamp, mount one of the columns on a small ring stand. Then mount the other column another small ring stand. Label the columns **D** and **C** with a Sharpie marker.

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

3. You will be provided with tubes containing about 5 ml of each of the resins. The resins 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 with the DEAE Cellulose resin to resuspend the particles. With a plastic transfer pipet, add about 1 ml of the DEAE Cellulose resin to the **D** column. Open the stopcock and allow the liquid to drip slowly into the beaker. Add more DEAE Cellulose 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 10 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.

5. In the same way, prepare the **C** column with a 2 ml resin bed of CM Cellulose. Again, wash the column with 0.03 M bicine buffer, pH 8.5, but never let it run dry.

C. Ion Exchange Chromatography on DEAE Cellulose

The purpose of this part of the experiment is to determine if LDH from your organ source binds to the anion exchange resin DEAE Cellulose.

1. Transfer **1.0 ml** of your dialyzed resuspended 65% ammonium sulfate pellet fraction (**D65P or CD65P**) to a clean tube.

2. Set up a test tube rack containing six (6) 13 x 100 mm glass tubes. Number the tubes 1-6. 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 will be collecting the material that elutes from the column as a series of **3 ml fractions** in tubes # 2-6. 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 **D** 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 1 ml sample of the **D65P** or **CD65P 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 2 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 10 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 5 fractions while washing the column with 0.03 M bicine, pH 8.5 (that is, through tube # 6).

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

8. When all of the fractions have been collected, close the stopcock. Any proteins that cannot bind to DEAE Cellulose should be found in these wash fractions. Any proteins that can bind to DEAE Cellulose should still be on the column.

D. Ion Exchange Chromatography on CM Cellulose

The purpose of this part of the experiment is to determine if LDH from your organ source binds to the cation exchange resin CM Cellulose. You will follow the same protocol you have just used for the DEAE Cellulose column.

1. Transfer **1.0** ml of your dialyzed resuspended 65% ammonium sulfate pellet fraction **(D65P or CD65P)** to a clean tube.

2. Set up another test tube rack containing six (5) 13 x 100 mm glass tubes. Number the tubes 1-6. 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 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 **C** 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 1 ml sample of the **D65P** or **CD65P 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 2 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 10 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 and collect a total of 5 fractions while washing the column with 0.03 M bicine, pH 8.5 (through tube # 6).

7. Again, you may find that the flow rate through the column is relatively slow. If this is the case, use the stopper and syringe provided with your set-up to put a moderate amount of pressure on the column to increase the flow rate.

8. When all of the fractions have been collected, close the stopcock. Any proteins that cannot bind to CM Cellulose should be found in these wash fractions. Any proteins that can bind to CM Cellulose should still be on the column.

E. Measurement of LDH Activity in the Wash Fractions from the DEAE Cellulose and CM Cellulose Columns

The purpose of this part of the experiment is to measure the LDH activity in the fractions that eluted from DEAE Cellulose resin and from the CM Cellulose resin during washing stage. **You should start to do these assays while the columns are being washed with 0.03 M bicine, pH 8.5 as described in Parts C and D.**

1. Measure the LDH activity of each of the column fractions # 2 to # 6 from the DEAE Cellulose (**D**) column. Use the standard protocol for the LDH assay that you used earlier today in Part A**.** 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: it will either have some LDH activity or it will not.** Express the activity in ΔA_{340}/min and then in µmoles/min.

2. In the same way, measure the LDH activity in each of the column fractions # 2 to # 6 from the CM Cellulose (**C**) column. Express the activity in ΔA_{340}/min and in µmoles/min.

3. Make an elution profile for each of the columns in which you plot the activity in µmoles/min as a function of the fraction number. You can just connect the points to indicate the relative activity in each of the fractions.

4. You may find that none of the five fractions from a particular column has any activity. What does that tell you? Alternatively, you may find that the LDH activity from a particular column is spread out over several fractions.

5. For the column(s) in which the LDH activity was eluted during this washing step, **pool the two or three adjacent fractions with the highest activity.** Transfer the liquid to a 15 ml plastic centrifuge tube and measure the total volume. Label the tube with the type of resin (**D or C**) and **IEwash**. If you observe that there are several peaks of activity among the fractions during the washing step, you should create two separate pools.

6. Place the pooled wash fractions in designated rack for storage in the refrigerator until the next lab. During the next lab, they will be assayed for LDH activity. They will also be saved for another protein assay and for gel electrophoresis.

F. Storage of Columns

The purpose of this part of the experiment is to save the columns to which the LDH appeared to bind.

1. Close off the stopcock and cover the top of each column with a plastic cap or with Parafilm.

2. Label the columns with your initials and place in it the designated beaker for storage in the refrigerator until next week. The enzyme should remain stable during this time.