BCH 372
Modern Concepts in Biochemistry Laboratory

Laboratory 12
Ion Exchange Chromatography - Part B

The purpose of this laboratory session is to continue the purification of the L-lactate dehydrogenase from the **D65P fraction** by ion-exchange chromatography. During last week's lab, you assembled two small ion-exchange columns, one containing DEAE-Cellulose as the resin and one containing CM-Cellulose as the resin. You added 1.0 ml of your dialyzed 65P fraction to each column and washed the proteins through the columns with 0.03 M bicine buffer, pH 8.5. You collected a series of fractions, tested them for LDH activity, and pooled those fractions with the most activity. In the week's lab, you will determine more precisely the LDH activity in these pools. You will also elute the other proteins that bound to the ion-exchange columns by adding bicine buffers with contain increasing concentrations of NaCl. You will then assay these fractions for LDH activity and pool those that contain the most activity. All of the pooled fractions will be saved for further analysis.

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. As indicated in the handout from last week, you will carry out a modification of the experiment described in Experiment 5a of the lab manual.

II. Laboratory Procedures

A. Measurement of LDH Activity in the Ion-Exchange Wash Pools

The purpose of this part of the experiment is to measure precisely the L-lactate dehydrogenase activity in the pools created from the fractions that eluted from the DEAE-Cellulose and CM-Cellulose columns last week during the initial washing step.

1. Retrieve your pooled fractions and determine the total volume of each fraction in its 15 ml plastic centrifuge tube.

2. Measure the LDH activity in each pool using the standard LDH assay. If the fractions that were pooled gave a reasonable rate of reaction with 50 µl (0.1-0.2 ΔA_{340}/min) , measure the activity with this same volume three time. If the fractions that were pooled gave a higher activity, use a smaller volume such as 5, 10, or 20 µl so that you get a ΔA_{340}/min of 0.1-0.2.

3. Once you have calculated an average initial velocity as ΔA_{340}/min, express the velocity as μmoles/min. Then calculate the activity in μmoles/min ml.

4. Multiply the activity of each pool by its total volume to calculate the number of units of LDH activity in that pool. Based on the total number of units that were in the 1.0 ml sample of the D65P fraction which was loaded on each column, calculate the recovery of the enzyme activity in each of your pools.

5. Save the pooled fractions in the refrigerator. They will be used later for a protein assay.

B. Elution of Additional Proteins from the Ion-Exchange Columns

The purpose of this part of the experiment is to elute additional proteins from the ion-exchange columns that you prepared last week. To do this, you will step-wise add 0.03 M bicine buffers containing 0.2 M, 0.4 M, 0.6 M, 0.8 M, and 1.0 M NaCl. The added cations or anions will compete with the proteins for binding sites on the resin and cause the proteins to come off. **You should do this with both the DEAE-Cellulose column and the CM-Cellulose column**. As the fractions are collected from the columns, assay them for LDH activity as described in Part C. If you find that most of the activity has been eluted from the column at an intermediate salt concentration, you can stop collecting fractions.

1. Retrieve your columns and mount them on the rings stands as you did last week. Remove the caps and place a two-way stopcock at the bottom of each column.

2. Place ten (10) 13 x 100 tubes in the test tube racks you used before to collect fractions from the columns and number them fractions **7-16.** This will just continue the numbers from last time so that all of the fractions can be included in the same elution profile. Fill another tube with 3 ml of 0.03 M bicine, pH 8.5 and mark the height with a Sharpie marker. This will allow you to see how much liquid to collect in each tube.

3. With a transfer pipet, remove most of the excess buffer on top of the resin bed in each column and discard it. Open the stopcock and allow the buffer to run into the resin bed until it just reaches the top of the resin. Start to collect the liquid as part of fraction # 7.

4. 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 fraction # 7 and fraction # 8. Each fraction should contain a total of 3.0 ml, so with each new buffer, you should obtain about two new fractions.

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

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

7. 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.

8. 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.

9. 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. At the end of this process, you should have two sets of tubes with the additional fractions from each column.

C. Measurement of LDH Activity in the Eluted Fractions from the DEAE Cellulose and the CM Cellulose Columns

The purpose of this part of the experiment is to determine which the fractions obtained by increasing the salt concentration in the buffer contain LDH activity. You can start doing this part of the experiment as the fractions are collected from the ion-exchange columns.

1. Measure the LDH activity of each of the column fractions you collect in Part B. Use the standard protocol for the LDH assay that you used last week and in the previous labs. Since the samples will be diluted somewhat during the chromatography, use **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 in µmoles/min.

2. Make an elution profile for each column in which you plot activity as a function of fraction number.

3. As before, you may find that the LDH activity is concentrated in one tube or spread out over several fractions.

4. With the instructor's help, decide which additional fractions to pool. You can collect two or three additional pools of activity from each column.

5. Transfer the liquid to plastic centrifuge tubes and measure the total volume in each case. Label the tube with your group's name, the column, and the fraction numbers.

D. LDH Activity in Ion-Exchange Chromatography Elution Pools

The purpose of this part of the experiment is to determine the LDH activity in the pools of material that eluted from the column in the presence of added salt.

1. Use the standard LDH protocol to do three replicate assays on the new pools as you did in Part A. You may need to adjust the volumes to get a good initial velocity.

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. Then express the results in μmole/min ml of enzyme solution by correcting for the volume used in the assay. Multiply by the volume and calculate the total number of units in each pool. Finally, calculate the percentage of the LDH units that were recovered in each of these new pools.

3. Save these new pools for use in later experiments.

E. Preparation of Elution Profiles and Purification Summary

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

1. On one piece of graph paper, make an comprehensive elution profile for the DEAE-Cellulose ion-exchange column. Use the data from last week's lab along with the data from this week's lab. Plot the LDH activity in μmoles/min ml as a function of the fraction number. You can also make this graph with Excel. Connect the points to show how LDH was eluted from the column.

2. On a second piece of graph paper, make an elution profile for the CM-Cellulose ion-exchange column. Use the data from last week's lab along with the data from this week's lab. Plot the LDH activity in μmoles/min ml as a function of the fraction number. You can also make this graph with Excel. Connect the points to show how LDH was eluted from the column.

3. Prepare a table summarizing the results of the ion-exchange chromatography such as that shown below. Some groups may have only one wash pool and others may have only one elution pool.

Column	Total Units Loaded	Percentage of Units Recovered			
		Wash Pool 1	Wash Pool 2	Elution Pool 1	Elution Pool 2
DEAE-Cellulose	_____	_____	_____	_____	_____
CM-Cellulose	_____	_____	_____	_____	_____