BCH 372
Modern Concepts in Biochemistry Laboratory

Laboratory 15
SDS-Polyacrylamide Gel Electrophoresis

The purpose of this laboratory session is to use gel electrophoresis to analyze the proteins in the various fractions of the L-lactate dehydrogenase purification. In SDS-polyacrylamide electrophoresis (SDS-PAGE), proteins are denatured first by heating them in a sample buffer containing 2-mercaptoethanol, which breaks disulfide bonds, and a detergent called sodium dodecyl sulfate (SDS), which causes the proteins to unfold and coats them with a net negative charge. It has been shown that when these denatured proteins are then subjected to electrophoresis in a buffer containing SDS, they all migrate towards the positive electrode. The rate of movement is inversely related to the log of their molecular mass: the smaller proteins move faster than the larger proteins. The locations of the various proteins can then be determined by staining the gel with a colloidal version of Coomassie Blue. By comparing the pattern of proteins from your fractions to one another and to a series of molecular weight standards, you will be able both to see the extent of purification and to identify the sizes of the major proteins in each fraction.

I. PreLab Preparation

Before the lab, please read Chapter 9 in the lab manual **Experiments in Biochemistry: a hands-on approach** by S. O. Farrell and L. E. Taylor. For this lab, focus on sections 9.1, 9.3, 9.4, and 9.5. You will do a modification of Experiments 9b and 9c (SDS PAGE). However, rather than casting your own gels, you will use precast polyacrylamide gels that have a 8-16% gradient separating gel and a 4% stacking gel. For the SDS-PAGE, each group will run two gels under denaturing conditions, which will be loaded with mixtures of standard proteins and samples of the various fractions from the LDH purification. The gels will be stained overnight with a positive stain (colloidal Coomassie Blue) and then destained in water.

II. Laboratory Procedures

A. Preparation of Denaturing Polyacrylamide Gels

The purpose of this part of the experiment is to set up the systems for the SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

1. Each group will be provided with a BioRad mini-gel system. Each system holds two 8 cm x 5.8 cm x 1 mm minigels with 10 sample wells each. To save time, you will use precast gels that contain an 8-16% polyacrylamide separation gel and a 4% polyacrylamide stacking gel in a neutral buffer.

2. Wearing gloves, remove a precast **Pierce Precise™ 8-16% Gel** from its sealed pouch. Rinse the gel cassette with distilled water. Mount the gel in its cassette on one side of the central core assembly, with the shorter plate facing inwards as demonstrated by the instructor. Be sure the green gasket facing the gel is completely flat.

3. In the same way, remove a second precast **Pierce Precise™ 8-16% Gel** from its pouch, rinse it with water, and mount it on the other side of the core assembly. Mark one gel **A** and the other gel **B** with a Sharpie marker.

4. Clamp the gels into place as described in **Laboratory 9** and place core assembly with the gel cassettes in the buffer box.

5. Add **Tris-HEPES-SDS electrophoresis running buffer** to both the central (upper) buffer chamber and the lower buffer chamber. Fill the central buffer compartment until the buffer extends over the short plate and the wells are completely filled with buffer. Using a plastic transfer pipet, rinse out the wells with electrophoresis buffer so that there are no air bubbles in them.

B. Preparation of Denatured Protein Samples

The purpose of this part of the experiment is to prepare the samples for SDS-PAGE.

1. Retrieve the following fractions from the freezer box or from your set of refrigerated 15 ml centrifuge tubes.

crude extract (**CE**)
40% ammonium sulfate pellet (**40P**)
40% ammonium sulfate supernatant (**40S**)
65% ammonium sulfate pellet (**65P**)
65% ammonium sulfate supernatant (**65S**)
dialyzed resuspended 65% ammonium sulfate pellet (**D65P**)
Wash pools from the DEAE-Cellulose and CM-Cellulose ion-exchange columns
Elution pools from the DEAE-Cellulose and CM-Cellulose ion-exchange columns
AC pools from the Cibacron Blue affinity chromatography column

The actual number of samples will vary from group to group depending on the number of pools they collected during the ion-exchange or affinity chromatography experiments.

2. Based on the protein assays that were done as part of Laboratory 8 and Laboratory 14, start to fill in the following chart with the protein concentrations of each sample in **mg/ml**. **These data should be in the summary purification table that you prepared as part of Laboratory 14.**

fraction	protein conc. (mg/ml)	dilution factor to give 2 mg/ml	sample volume	water volume
CE	_____	_____	_____	_____
40P	_____	_____	_____	_____
40S	_____	_____	_____	_____
65P	_____	_____	_____	_____
65S	_____	_____	_____	_____
D65P	_____	_____	_____	_____
DEAE Wash Pool	_____	_____	_____	_____
DEAE Elution Pool	_____	_____	_____	_____
CM Wash Pool	_____	_____	_____	_____
CM Elution Pool	_____	_____	_____	_____
AC Pool	_____	_____	_____	_____

Some groups may have more than one Wash Pool or Elution Pool from a particular column. Add more lines as necessary so that you can use all of the fraction that you have.

3. Now calculate how much each fraction must be diluted to give a final protein concentration of 2 mg/ml. **If a fraction has a protein concentration of < 2 mg/ml, it does not need to be diluted.**

For example, if a particular fraction has a protein concentration of 13.4 mg/ml, the dilution factor would be calculated as follows:

$$\frac{\text{initial protein concentration}}{\text{final protein concentration}} = \frac{13.4 \text{ mg/ml}}{2 \text{ mg/ml}} = 6.7$$

dilution factor = 1/6.7

That is, the original solution is 6.7 times more concentrated that you want the final solution to be. A particular volume of the 13.4 mg/ml solution would need to be diluted 1/6.7 to give a final concentration of 2 mg/ml of protein. Put the dilution factors into the chart shown in step # 2.

4. After doing this calculation for each of your fractions, make an appropriate dilution of each fraction in a 1.5 ml microcentrifuge tube so that you have a total volume of 100 µl at a concentration of 2 mg/ml. Put your calculations into the table in step # 2 and label the tubes carefully.

   For example, suppose a certain fraction has a protein concentration of 5.6 mg/ml. You can calculate how much to use to make 100 µl of solution in two ways:

   A. The first is to use the dilution factor you calculated in step # 4

   $$\frac{\text{initial concentration}}{\text{final concentration}} = \frac{5.6 \text{ mg/ml}}{2.0 \text{ mg/ml}} = 2.8$$

   The dilution factor is 1/2.8, so:

   $$\frac{1}{2.8} \times 100 \text{ µl} = 35.7 \text{ µl}$$

   For 100 µl, you need to combine 35.7 µl of the stock solution and (100 – 35.7) or 64.3 µl of water.

   B. The second is to calculate how much protein you need to add:

   $$100 \text{ µl} \times \frac{2.0 \text{ mg}}{\text{ml}} \times \frac{1 \text{ ml}}{1000 \text{ µl}} = 0.2 \text{ mg}$$

   $$X \text{ µl} \times \frac{5.6 \text{ mg}}{\text{ml}} \times \frac{1 \text{ ml}}{1000 \text{ µl}} = 0.2 \text{ mg}$$

   $$0.0056X = 0.2, \text{ so } X = 35.7 \text{ µl}$$

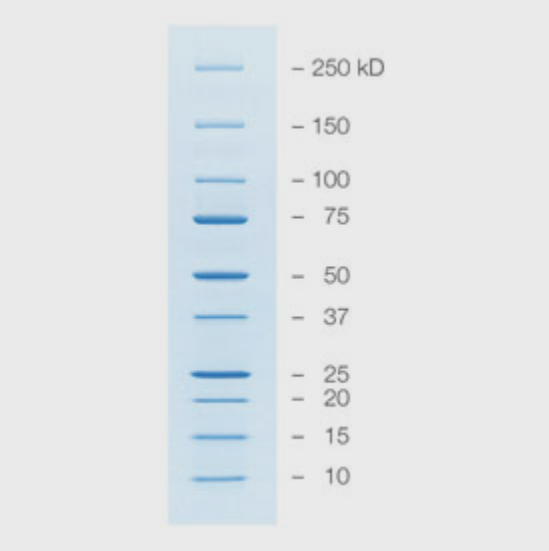
   If you combine 35.7 µl with 64.3 µl of water, you will have 100 µl at the correct concentration.

5. After you have made up all of the samples, add 100 µl of 2X SDS-sample buffer to each of the tubes. This buffer contains SDS, 2-mercaptoethanol, and bromphenol blue. This will give a final protein concentration of 1 mg/ml and a total sample volume of 200 µl.

6. You will be provided with tubes containing 50 µg of bovine heart LDH (**HLDH**) or 50 µg of bovine muscle LDH (**MLDH**) in a volume of 25 µl. Add 25 µl of 2X SDS-sample buffer to each of these tubes to give a total sample volume of 50 µl.

7. Each group also will be provided with microcentrifuge tubes containing 25 µl of a

mixture of unstained proteins standards of known molecular weights (**MWM**).



The mixture contains 10 recombinant proteins that vary in size from 10,000 to 250,000 daltons in a sample buffer. You do **not** need to add anything to this tube.

8. Place your samples from the LDH purification along with the purified LDH samples and the MWM mixture in a circular rack (you may need to do this in two batches). Heat them in a large beaker of boiling water for 5 minutes to denature the proteins.

9. Allow the samples to cool briefly and centrifuge the tubes in a microcentrifuge briefly to collect the liquid at the bottom.

C. Loading and Electrophoresis of Denatured Protein Samples

The purpose of this part of the experiment is to load the samples into the wells of the polyacrylamide gels and to subject the proteins to electrophoresis. You will be able to see the samples and to follow the electrophoresis by looking at the bromphenol blue tracking dye that is in the sample buffer.

1. Prepare a **protocol** for loading the protein samples from the LDH purification, the purified LDH, and the MWM into the wells of the two gels as suggested in the following tables. It is suggested that you load 10 µl of each fraction, but if a fraction has a protein concentration < 2 mg/ml, you can increase the volume to 20-30 µl. Note that this protocol has five unused wells. You can use these for other pool samples or for larger volumes (20-30 µl) of the samples with low protein concentrations.

Sample Loading Protocol - Gel A

well:	1	2	3	4	5	6	7	8	9	10
volume:	10	10	10	10	10	10	10	10	10	10
sample:	CE	40P	MWM	40S	65P	65S	D65P	HLDH	DEAE	DEAE

Sample Loading Protocol - Gel B

well:	1	2	3	4	5	6	7	8	9	10
volume:	10	10	10	10	10					
sample:	CM Wash Pool	CM Elution Pool	AC	ML	DH	MW	M			

	Wash Pool	Elution Pool

2. Have your protocol checked by the instructor. When it is approved, load the samples into the wells of the gels. Note that you will load the samples through the buffer and between the glass plates. The wells are numbered from 1-10. You can place a yellow plastic block over the central compartment as a guide. It will take a little practice to see the wells and to load the samples, but once the first sample is loaded, the rest will be quite easy. When the gel on one side of the system has been fully loaded, carefully turn the system around and load the wells of the second gel.

3. **Remove the yellow block** and carefully place the lid on the system so that the electrodes are correctly aligned. Insert the leads from the lid into the power supply. Check to see that the power supply is set to run at **150 Volts** and **400 mA** for at least **45 minutes**.

4. Run the gels at 150 Volts for about 35-45 minutes. Note the position of the bromphenol blue tracking dye throughout the run.

5. When the tracking dye gets to within **1 mm** of the bottom (this is <u>below</u> the lower edge of the green gasket), disconnect the leads from the power supply, turn off the power, and carefully remove the lid.

6. Pour off the buffer from the upper and lower buffer chambers and remove the cassettes with the gels from the system. Decant the buffer into the designated waste container.

D. <u>Staining of Gels with Coomassie Blue</u>

1. Using the edge of the green plastic tool that comes with your gel system, crack open one of the cassette and carefully pry the two plastic plates apart. The gel will usually stick to one of the plates. Place the plate with the gel side up in a plastic staining dish. With a piece of label tape, label the dish gel A or gel B as appropriate along with your initials.

2. Repeat the process with the other gel cassette.

3. Add about 50 ml of deionized water to each of the staining dishes. Shake the staining dishes gently for about 5 min on the Belly Dancer platform shaker to begin to extract the SDS from the gel. The gel will float off of the plastic plate during this process, so you

can remove the plate from the staining tray. Decant the water and add 50 ml of fresh water. Shake the gel again for about 5 min. Decant the water and add 50 ml of fresh water. Shake again for 5 min. Be careful not to discard the gel as you decant the washing solutions.

4. Add 20 ml of Pierce Gel-Code Blue Stain Reagent to each of the gel staining dishes.

5. The instructor will allow the gels to stain over the weekend to allow maximal development of blue-stained bands.

6. He will then decant the stain, wash the gels with two portions of deionized water, and allow them to destain in distilled water.

E. Analysis of Gels

The purpose of this part of the experiment is to analyze the gels.

1. Because there are no more formal lab sessions, you will need to retrieve the scanned image of your gel from the course website.

2. For each lane in each of the two gels, measure the distance from the **bottom of the well** to the **center of each protein band**. This is called the **mobility**.

3. Identify each of the proteins in the MWM mixture based on its apparent size and mobility. Make up a chart in which you summarize the mobilities of the proteins in Gel A and Gel B. Then calculate the average mobility for each of the standard proteins.

4. Use a piece of semi-log graph paper to construct a standard curve for the 8-16% gradient polyacrylamide gel. Plot the average mobility of each of the standard proteins in **cm** on the **X axis** and the **log of its molecular mass** on the **Y axis**. Draw the best-fit line through all of the data points. See Figure 9.5 in the lab manual as an example of this type of graph. You can also make this graph in Excel.

5. Label all of the visible proteins in each of the fractions from the LDH purification by number (1, 2, 3, 4, etc.), starting from the top of the gel and moving towards the bottom of the gel. You can use either the bands from any of your samples if you used two different volumes. You will probably find that some bands are clearer in one sample or another.
6. Use the standard curve to determine the denatured molecular mass of each of the proteins in each of the fractions from the LDH purification.

7. Compare the total number of proteins in the various fractions. Is there evidence of purification? Does the total number of protein bands decrease? Which columns seemed to give the greatest purification?

8. The denatured molecular mass of LDH is about 37,000. Can you see a band of this size

in the partially purified heart and muscle LDH samples? Is there a band with this size in any of the fractions? Does it persist through the purification process?

8. Place your graph (or an image of it if you manually produce it) and your labeled gel-image into a Word document and email it to ty@asu.edu. This will serve as the final datum sheet.