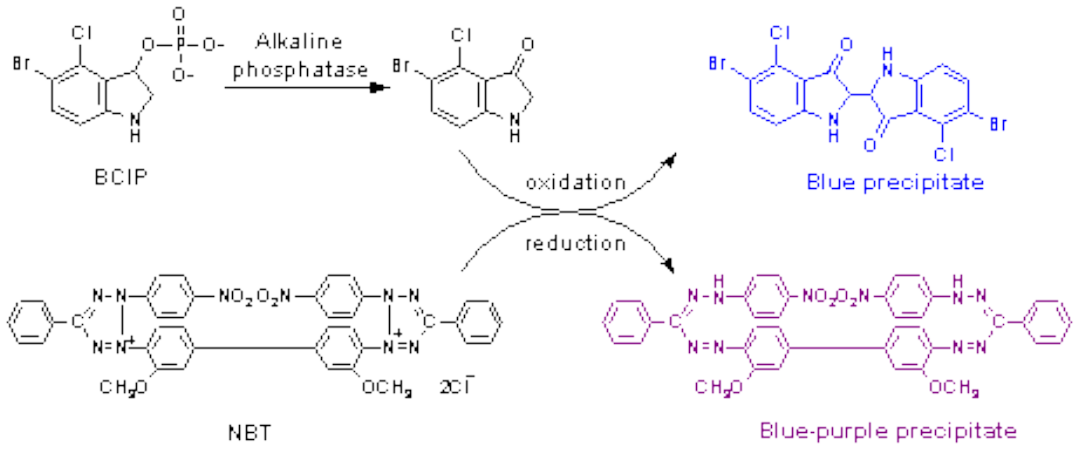
BCH 372
Modern Concepts in Biochemistry Laboratory

Laboratory 10
Western Blot Analysis of L-Lactate Dehydrogenase, Part B

During the last lab session, the proteins in the samples from the initial stages of the LDH purification and two purified samples of L-lactate dehydrogenase were run on two nondenaturing gels. The proteins in one gel were stained with Coomassie Blue. The proteins in the other gel were transferred electrophoretically out of the gel onto a PVDF (polyvinylidene difluoride) membrane. The membrane was placed in a blocking solution and stored in the refrigerator. The purpose of this laboratory session is to examine the stained gel and to finish the Western blot analysis. The stained gel will be scanned and the various proteins characterized in terms of their mobilities. The PVDF membrane will be treated with a series of reagents including a primary antibody directed specifically against LDH and a secondary antibody conjugated to an enzyme. This will allow the bands that correspond to this particular protein to be revealed.

I. PreLab Preparation

Before the lab, please read again Chapter 10 in the lab manual **Experiments in Biochemistry: a hands-on approach** by S. O. Farrell and L. E. Taylor. Pay particular attention to Western Blot Theory and Antibodies. Rather than using a horseradish peroxidase conjugate as the secondary antibody, you will use an alkaline phosphatase conjugate as part of a kit from Bio-Rad Laboratories. The following image shows the reaction that will occur.



Chromogenic reaction of NBT/BCIP by alkaline phosphatase

II. Laboratory Procedures

A. Analysis of Stained Gel

The purpose of this part of the experiment is to analyze the stained gel of the fractions from the LDH purification and to determine numbers and sizes of the proteins in each fraction. You can do this part of the experiment while you are doing all of the washing and incubation steps associated with the Development of the Western Blot (Part C).

1. Scan the first of your stained gels (the one not used for the Western blot) using the HP optical scanner and save the image as a JPG file. You can transfer the file to a flash drive and print it later in larger format.

2. For each lane, measure the distance from the **bottom of the well** to the **center of each protein band**. This distance will be called the **mobility** of each protein.

3. Identify as many of the proteins in the molecular weight marker (MWM) mixture as possible. Note that the 25,000, 50,000, and 75,000 dalton proteins should stain darker than the others. Fill in the following table with the sizes of the proteins and their apparent mobilities.

Protein	Size	Mobility
1	250,000	_____
2	150,000	_____
3	100,000	_____
4	75,000	_____
5	50,000	_____
6	37,000	_____
7	25,000	_____
8	20,000	_____
9	15,000	_____
10	10,000	_____

4. Make two graphs of these data which might be used as standard curves. For the first graph, plot mobility on the X axis and the size of the protein on the Y axis. For the second graph, plot mobility on the X axis and the log of the size of the protein on the Y axis. Draw the best-fit line through each set of the points which you can. This line might be a straight line or a curve.

5. Label all of the visible proteins in each of the fractions from the LDH purification by number, starting from the top of the gel and moving towards the bottom of the gel. For example, CE1, CE 2, CE3, etc. or 40P1, 40P2, 40P3, etc. Do the same with the visible proteins in the purified heart LDH and muscle LDH samples.

6. Using standard curves of the proteins of known molecular weight, determine the nondenatured or native molecular mass of each of the proteins in each of the fractions from the LDH purification and the purified samples of LDH.

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 steps in the ammonium sulfate fractionation seemed to give the greatest purification?

8. The nondenatured molecular mass of LDH is about 150,000. Is there a band with this size in the fractions? Does it persist through the purification process? Are there other proteins that persist through the purification process?

9. Discard the gel in the designated container.

B. Analysis of Stained Gel from the Western Blot

The purpose of this part of the experiment is to analyze the stained gel from the Western blot and to assess the efficiency of protein transfer to the PVDF membrane.

1. In the same way, scan the stained gel from the Western blot using the HP optical scanner and save the image as a JPG file. You can transfer the file to a flash drive and print it later in larger format.

2. You do not need to do a complete analysis of this image, but compare it to the other stained gel. Are most of the bands still apparent or have they disappeared? Is there any pattern to which bands are present and which are not?

C. <u>Development of the Western Blot</u>

The purpose of this part of the experiment is to visualize the bands corresponding to LDH on the PVDF membrane. All of the washing and treatment steps will be done at room temperature with gentle shaking on the Belly Dancer shaker.

1. Retrieve your staining dish with the PVDF membrane in blocking solution. When the instructor turned off the system, he cut off one corner of the membrane so that it would correspond to the cut-off corner of your gel. He also marked the side of the membrane that was facing the gel with a black dot using a Sharpie permanent marker.

2. Remove the membrane from the staining dish and discard the blocking solution. Place the membrane back in the dish and wash it twice (2X) with 20 ml portions of deionized water for 5 minutes.

3. Wash the membrane twice (2X) with 20 ml portions of Tris-Buffered Saline (20 mM Tris, 500 mM NaCl, pH 7.4) containing 0.05% Tween 20 for 5 minutes. Carefully pour off the last of the solution.

4. Add 20 ml of the primary antibody solution. This is a Mouse Monoclonal Antibody to Human LDH-B (heart) from Santa Cruz Biotechnology. The antibody at a concentration of 100 μg/ml was diluted 1/500 in Tris-Buffered Saline containing 0.05% Tween 20 and 1% gelatin. Shake the membrane in the solution for 20 minutes.

5. Discard the antibody solution and wash the membrane three times (3X) with 20 ml of Tris-Buffered Saline containing 0.05% Tween 20 for 10 minutes.

6. Add 20 ml of the secondary antibody-enzyme conjugate solution. This is a Goat Anti-Mouse Antibody linked to Alkaline Phosphatase in Tris-Buffered Saline containing 0.05% Tween 20 and 1% gelatin. Shake the membrane in this solution for 20 minutes.

7. Discard the antibody-conjugate solution and wash the membrane two times (2X) with 20 ml of Tris-Buffered Saline containing 0.05% Tween 20 for 5 minutes.

8. Wash the membrane with 20 ml of Tris-Buffered Saline without Tween 20 for 5 minutes.

9. While the membrane is washing, add 200 μl of AP Color Reagent A [nitroblue tetrazolium in aqueous dimethylformamide (DMF) containing $MgCl_2$] and 200 μl AP Color Reagent B (5-bromo-4-chloro-3-indoyl phosphate in dimethylformamide (DMF)] to a tube containing 20 ml of color development buffer.

10. Immerse the membrane in 20 ml of the complete color development solution. Shake the membrane at room temperature until color development is complete.

11. Stop the color development by washing the membrane in 20 ml of deionized water for 5 minutes. Decant the washing solution and wash the membrane again in 20 ml of deionized water for 5 minutes.

12. Let the membrane air dry on a piece of filter paper.

13. If you like, you can scan the dried membrane just as you would a gel and save the image as a JPG file.

14. Compare the Western blot to the image of the Coomassie Blue stained gel. Are there visible bands in the gel corresponding to LDH? Where do these bands appear in each of your samples?