EXERCISE 8D

Name ____________________________

Isolating, purifying, and characterizing proteins

Day Four: *How can gel electrophoresis be used to analyze the milk fractions that were collected during your purification of α-lactalbumin?*

Objectives

After completing this exercise, you should be able to:

- Determine the molecular weight of a protein using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE).
- Evaluate a SDS-PAGE gel to assess how effectively a protein has been purified.

Prelab

Before you come to lab, read this entire exercise.

During Lab 8A, you collected 8 milk fractions during purification of α-lactalbumin from nonfat milk (nonfat milk, pellet, whey, and 5 column chromatography fractions.) Last week, you used gel electrophoresis to separate the different proteins present each milk fraction. In this lab, you will examine and analyze your gel in order to assess the effectiveness of the various purification steps.

Because gel electrophoresis separates the different proteins present in each milk fraction into separate bands, counting the number of bands in each lane of your gel will allow you to determine the number of proteins present in each milk fraction. Furthermore, you can estimate the relative concentrations of the various proteins in each milk fraction based on how dark or light each band is. The higher the concentration of a particular protein, the darker its band will be.

In addition, when the SDS-coated proteins travel through the polyacrylamide gel during SDS-PAGE, their rate of movement toward the anode is determined almost entirely by size. Previous experience has shown that there is a linear relationship between the log of the molecular weight of each protein and its migration distance. Because of this, SDS-PAGE can be used to estimate the molecular weight of a protein based on its migration distance. In order to do this, a standard curve that shows the relationship between the migration distance through the gel and the log of the molecular weight must be generated. The standard curve is generated by measuring the migration distance of several proteins of known molecular weight (MW standards), plotting a scatter diagram of migration distance vs. log of MW, and then using linear regression to determine the equation of the "best fit" straight line for the data points. Once this is done, you can substitute the migration distance of any protein on your gel into the linear regression equation and then calculate the log of MW for that protein. Knowledge of molecular weights can then be used to help identify the various proteins present in each fraction.

Main steps in purifying & analyzing α-lactalbumin

1. Set aside a sample of nonfat milk to assay later
2. Precipitate the casein milk proteins using heat and low pH. The other milk proteins should remain in solution. Centrifuge the heat and acid treated milk to separate the precipitated casein proteins from the soluble proteins.
3. Set aside a sample of the pellet of precipitated proteins to assay later.
4. Remove any remaining precipitated proteins from the supernatant (whey) using ultrafiltration.
5. Set aside a sample of the whey to assay later.
6. Separate α-lactalbumin from the other proteins that remain in the whey using size exclusion chromatography.
7. Set aside the five chromatography fractions most likely to contain α-lactalbumin to assay later.
8. Prepare a standard curve for the Bradford assay using solutions of known protein concentration.
9. Use the Bradford assay and your standard curve to determine the protein concentrations of the 8 milk fractions that you set aside on Day1.
10. Calculate the amount of each milk fraction that should be loaded into your SDS-PAGE gel so that each lane contains an appropriate amount of protein.
11. Load, run, and stain SDS-PAGE gels.
12. **Analyze your gel to determine the number of different proteins that are present in each milk fraction and the molecular weights of these proteins.**
13. **Use your gel analysis to evaluate how effectively you isolated and purified α-lactalbumin from the other components of milk.**

Lab Procedures:

I. Determine log of MW and migration distance of the 7 molecular weight standards

1. Remove your gel from the Petri dish and wrap it between layers of plastic so it does not dry out.

   Transparency film used for overhead projectors cut to a size slightly larger than your SDS-PAGE gel works well for fishing these gels out of a Petri dish without tearing them. You can also use two pieces of transparency film to sandwich your gel for long term storage by sealing the edges of the "sandwich" with tape to keep the gel from drying out.

   Place the gel so the cut corner is to the lower right, which orients the gel in the same way it was oriented when you cut off the corner. Recall that the proteins migrated down through the gel, so the top edge represents the origin (start) of separation.

   Make sure you understand and use the correct terms when discussing your gel:
   - **Band** – a single blue-stained line on the gel
   - **Lane** – the area of the gel below a single well. Usually, each lane will have several protein bands.
   - **Protein** – a molecule with a specific molecular weight. Each band usually contains one protein, but the same protein may appear in several different lanes.

2. Make a pencil sketch of your gel, indicating where each protein band is located with a horizontal pencil line. Try your best to make the darkness and thickness of these pencil lines accurately represent the darkness and thickness of the stained bands. If a Polaroid camera is available, you can substitute a photograph of the gel for the pencil sketch. Alternatively, you can photocopy the gel if you seal it between 2 sheets of transparency film.

3. Prepare a table consisting of 4 columns. Title the columns of your table as follows: Protein, Molecular Weight, log MW, and Migration Distance.

4. In **the first two columns** of your table, list the names of the proteins used as molecular weight standards and their molecular weights. This information is shown below. The molecular weights are given in units of kilodaltons or kD. (Note: Most of these proteins are not milk proteins. They were chosen because they are readily available, easy to isolate, and they have a broad range of molecular weights.)

MW Marker Protein	Molecular Weight
Bovine serum albumin	66.0 kD
Egg white albumin	45.0 kD
Gly-3-P dehydrogenase	36.0 kD
Carbonic anhydrase	29.0 kD
Trypsinogen	24.0 kD
Trypsin inhibitor	20.1 kD
α-lactalbumin	14.2 kD

5. In the third column of your table, list the log of the molecular weight for each protein standard. Just as when you prepared the standard curve for your gel exclusion chromatography column, you will prepare the standard curve for the electrophoresis gel by plotting the log of the molecular weights rather than molecular weights themselves. This is done so that the points on your standard curve fall on, or close to, a straight line.

6. Examine the bands in the 2 lanes of your gel containing the molecular weight standards (lanes 2 and 9). These lanes were loaded with the same sample type and should look identical. Furthermore, because there are 7 molecular weight standards, each of these lanes should contain 7 bands. During SDS-PAGE, the smallest proteins migrate through the gel fastest. Use this fact to determine which protein is present in each band of the lanes containing the molecular weight standards.

   Now, measure the distance from the origin to the middle of each protein band in one of the lanes containing the molecular weight standards (lane 2 or 9). Use whichever lane has the sharpest and straightest bands. Record the migration distance of each MW standard in the fourth column of your table.

   NOTE: You may be able to see the bands more clearly if you place your gel on a light box.

II. Use log of MW and migration distance of the 7 molecular weight standards to prepare a standard curve

1. Using either a sheet of graph paper or a computer with spreadsheet program, make a scatter diagram that shows the relationship between log of MW and migration distance for the 7 molecular weight standards. Plot migration distance on the x-axis and log of molecular weight on the y-axis.

   Make sure your scatter diagram is big enough so that it fills an entire 8.5" x 11" sheet of paper. See Appendix E for more detailed descriptions of graphing techniques.

2. Using either a hand-held calculator or a computer with spreadsheet program, carry out linear regression to determine the equation for the "best fit" straight line for your data points. Write the equation below your graph. Next to the equation, write down the linear correlation coefficient. If the absolute value of the linear correlation coefficient is less than 0.95, ask your instructor for help.

   If you used a calculator, do not clear it at this point. You will use the stored data in Part III.

III. Estimate the molecular weights of the proteins found in the 8 milk fractions, and use this information to tentatively identify these proteins

1. Prepare a table to record and analyze the data from the milk fraction that was loaded into the first lane of your gel (nonfat milk.). Your table should have 4 columns: title the first column "Protein Band", the second column "Migration Distance", the third column "Estimated MW", and the fourth column "Protein Identified".

   IMPORTANT: You will prepare a total of 8 tables, one for each milk fraction (nonfat milk, pellet, whey, and the 5 column chromatography fractions.) Make sure each table has a title that identifies the milk fraction being analyzed.

2. Examine the protein bands in the first lane of your gel. This lane was loaded with a sample of nonfat milk.

   In the first column of your table, identify each protein band by number, counting from the top of the gel downwards.

   In the second column of your table, record the migration distance of each band.

   Next, use the equation of your standard curve to calculate the MW of the protein in each band, and record that information in column 3 of your table.

Finally, review the molecular weights of the major milk proteins listed in the table below. Compare these molecular weights to the molecular weights of the proteins found in lane # 1 of your gel and assign the identities of as many as possible by their molecular weights. Record this information in column 4 of your table.

Milk Protein	Molecular Weight (daltons)
α-lactalbumin	14,437
β-lactoglobulin	18,000
κ-casein	19,000
α-casein	23,000
β-casein	24,000
γ-casein	30,650
blood serum albumin	68,000
lactoferrin	87,000
various immunoglobulins	~160,000-1,000,000

You should be filling in your table in the following manner (with your measurements rather than x, y, and z in the second column, of course).

Major Proteins Found during Electrophoresis of Nonfat Milk			
Protein Band	**Migration distance**	**Estimated MW**	**Protein Identified**
1st protein	*x* mm	223,000 daltons	immunoglogulin
2nd protein	*y* mm		
3rd protein	*z* mm		
etc.			

3. Repeat steps 1 and 2 for the other 7 milk fractions that you loaded onto your gel. Remember that Lanes 2 and 9 contain your MW markers, so leave those lanes out of this analysis of milk proteins.

IV. Evaluate the effectiveness of your purification steps

1. Prepare a table that shows which of the 9 major milk proteins were present in each milk fraction. List the names of the 8 milk fractions across the top of the table, and the names of the 9 major milk proteins down the left side. If a particular protein was not present in a purification fraction, indicate this with a "-" sign. If the protein was present, indicate the relative amount of protein by using "+" for a light band, "++" for a darker band, and "+++" for the darkest bands.

Clean up

Dispose of your gel and excess solutions as directed by instructor.

Remove label tape and any marks made with a marking pen from all glassware. Wash and rinse all used glassware, give it a final rinse with dH_2O, and leave it inverted at your work area in order to drain.

All instruments should be turned off and unplugged.

Wipe off your workspace with a damp paper towel.

Make sure everything that you have used is clean, put away, or discarded. Leave your work area in the same order that you found it in.

Ask your instructor to check your work area before you leave.

Postlab

1. The bands of α-lactalbumin and BSA that are visible in several of your milk fractions are particularly easy to identify. Why?

2. In which milk fractions would you expect to find α-lactalbumin? Explain why. Is α-lactalbumin present in the corresponding lanes of your gel?

3. Study the pencil sketch or photograph or photocopy of your SDS-PAGE gel, along with the table you prepared in section IV of the lab procedures, to evaluate each step of the purification procedure.

   a. How efficient was each step in the purification procedure? How many bands were eliminated by each purification step?

   b. Which milk fraction seems to contain the highest concentration of α-lactalbumin?

   c. Which column chromatography fraction seems to contain the highest concentration of α-lactalbumin? Is this what you predicted in Exercise 8A, based on the standard curve for your size exclusion chromatography column? If the actual result was different from your prediction, give some possible reasons for the discrepancy.

   d. Examine the relative amounts of α-lactalbumin and β-lactoglobulin in the 5 column chromatography fractions. Did the highest concentration of α-lactalbumin occur in the same fraction as the highest concentration of β-lactoglobulin, in an earlier fraction, or in a later fraction? Is this what you would expect? Explain why or why not.

   e. Were you able to obtain a sample that contains only a single protein with the same molecular weight as α-lactalbumin? If not, what might you do to further purify α-lactalbumin?