

EXERCISE 8B

Name _____

Isolating, purifying, and characterizing proteins

Day Two: What is the concentration of protein in your milk fractions?

Objectives

After completing this exercise, you should be able to:

- ◆ Explain what a protein assay is, and discuss why it is an important part of the purification process.
- ◆ Distinguish between a qualitative assay and a quantitative assay.
- ◆ Discuss the advantages and disadvantages of the following assays for protein concentration: A_{280} measurements, the biuret assay, the Lowry assay, and the Bradford assay.
- ◆ Perform a Bradford assay and make a standard curve to determine the concentration of protein in each of your milk fractions.

Prelab

Before you come to lab, read this entire exercise. You must also answer all questions and complete all assignments on the first 6 pages of this exercise. Your instructor will give you directions on when and where to turn in your work.

Last week, you used several techniques in an attempt to separate α -lactalbumin from the other molecules found in nonfat milk. At several points during the procedure, you saved samples of the milk fractions. These fractions will be analyzed using SDS-PAGE electrophoresis in order to assess the effectiveness of the various purification steps. Electrophoresis will separate the various proteins present in each fraction, allowing you to determine the number of proteins present and their molecular weights. Knowledge of molecular weights can then be used to help identify the various proteins present in each fraction.

Before you run gel electrophoresis, however, you must decide how much of each sample should be loaded onto the electrophoresis gel. This is important in order to avoid loading too much or too little protein onto the gel. The amount of sample needed will depend on the protein concentration of each sample. Therefore, your job today will be to estimate the total protein concentration of each milk fraction that you collected last week.

A protein **assay** is a test that is used to detect the presence of a protein. A **qualitative assay** simply indicates whether protein is present in your sample. Some qualitative assays will detect any protein, while others are designed to detect a specific protein. A **quantitative assay**, on the other hand, is used to determine how much protein is present. A quantitative assay may be used to measure the concentration of protein in a solution (e.g. in mg/mL), or, in the case of an enzyme, the assay may be designed to measure the rate of enzyme activity. During protein purification, it is useful to have a quantitative assay to determine how much protein is present after each step of the purification process. This allows scientists to monitor the concentration and/or activity of the protein as it is being purified.

In today's lab, you will determine the concentration of protein in each of the 8 milk fractions that you prepared during Lab 8A:

- Skim milk
- Pellet
- Supernatant
- The 5 column fractions obtained from size exclusion chromatography of the whey proteins

There are several methods for determining the concentration of protein in a solution. One method, which was mentioned in the previous lab, is **absorbance of light at 280nm (A₂₈₀)**. This assay has the advantage of being quick and easy; you simply place the sample in the spectrophotometer and read the absorbance. In addition, this method is nondestructive, so it does not use up any of the sample that is being analyzed. Unfortunately, this assay is not very accurate because it depends on the concentration of amino acids with rings in their R-groups, and different proteins have different amounts of those amino acids.

The **biuret** and **Lowry assays** are both based on the binding of copper ions (Cu²⁺) to the nitrogen atoms present at the peptide bonds of proteins. This reaction causes the protein solution to turn purple (biuret) or blue (Lowry). The color intensity is proportional to the concentration of protein in the sample. The color intensity is measured using a spectrophotometer, and the results are compared to a **standard curve** prepared using known concentrations of a purified protein standard. Each of these assays has advantages and disadvantages. The biuret assay is the most reliable assay for protein because other substances present in the solution rarely interfere with the accuracy of the results. Unfortunately, it takes about 20-30 minutes for the color to develop, so it isn't nearly as fast as the A₂₈₀ assay, and it can only detect relatively high concentrations of protein (1 mg/mL or more.) The Lowry assay, in which a second reagent is added to the biuret reagent, is much more sensitive and can be used to measure protein concentrations as low as 5µg/mL. However, the Lowry assay takes even longer to complete, requiring 40-60 minutes for the color to develop. Reliability is another disadvantage of the Lowry assay because other substances in the solution can greatly affect its accuracy.

The **Bradford assay** is the most commonly used protein assay because of its accuracy, sensitivity, and convenience. The Bradford assay is much more accurate than A₂₈₀ measurements, few other substance interfere with its accuracy, it is sensitive to protein concentrations as low as 1µg/mL, and it is very rapid, producing a color change within five minutes. The Bradford assay is based on the binding of **Coomassie Blue dye** to proteins. The dye alone has a brownish color and absorbs light most efficiently at 465nm. When the dye combines with protein, however, the resulting protein-dye complex has an intense blue color whose maximum absorption is at 595nm.

In today's lab, you will use the Bradford assay to determine the protein concentration of the 8 milk fractions you prepared last week. In order to determine the protein concentration of these milk fractions, you will need to prepare a **standard curve**, which shows the exact relationship between the A₅₉₅ value of each fraction and its protein concentration.

To prepare a standard curve for the Bradford assay, first prepare a series of solutions of KNOWN protein concentration using **bovine serum albumin (BSA)**, a protein commonly used as a protein standard. Next, add Bradford's Reagent (containing Coomassie Blue dye) to the BSA solutions and measure the A₅₉₅ value of each solution. Next, prepare a scatter diagram of your data by plotting protein concentration of the BSA solutions on the x-axis and A₅₉₅ values on the y-axis. Now, use linear regression to determine the equation of the best-fit straight line for your data points. This equation is the equation of your standard curve; it shows the exact relationship between protein concentration and the A₅₉₅ value of a solution.

Once you have the linear regression equation for your standard curve, you can use the Bradford assay to determine the protein concentrations of the 8 milk fractions you prepared last week. First, dilute the 8 milk fractions so their A₅₉₅ values will lie within the range of your standard curve. Next, add Bradford's Reagent to each diluted milk fraction and measure its A₅₉₅ value. Now, substitute these A₅₉₅ values into your linear regression equation for "y" and calculate the corresponding values for "x". The "x" values equal the protein concentrations of the diluted milk fractions. Finally, calculate the protein concentrations of the undiluted milk fractions.

Main steps involved in purifying 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.

Predict the types and amounts of protein that you expect to find in your fractions

As a reminder, the major proteins found in milk, and their molecular weights, are listed in the following table:

<u>Milk Protein</u>	<u>Molecular Weight (daltons)</u>
α -lactalbumin	14,437
β -lactoglobulin	18,000
Caseins	~19,000-30,000
Blood serum albumin	68,000
Lactoferrin	87,000
Immunoglobulins	~160,000-1,000,000

In the previous lab, you saved 8 samples during the purification of α -lactalbumin: the original nonfat milk, the pellet after acid-heat treatment and centrifugation, the supernatant from the centrifugation (the whey), and five of the chromatography column fractions. Each step of this purification procedure was designed to separate α -lactalbumin from the other milk proteins. Based on your knowledge of the purification process, list the proteins you expect to find in each of the following milk fractions:

Skim milk: _____

Pellet: _____

Whey: _____

Five column fractions (combined): _____

Which do you predict will have a higher concentration of protein, the milk or whey? Explain your reasoning.

Which do you predict will have a higher concentration of protein, the whey or column fractions? Explain your reasoning.

Calculate the dilutions of BSA

In order to prepare a standard curve for the Bradford assay, you will make 0.1 mL of each of the following BSA dilutions: 0 mg/mL, 0.1 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL, 1.0 mg/mL, 1.2 mg/mL, and 1.4 mg/mL. To make these dilutions, you will be provided with a stock solution containing 2 mg/mL of BSA.

Calculate how much stock BSA (2 mg/mL) and how much dH₂O you need to make 0.1 mL of each of the dilutions shown in the table below. Enter the required amounts in this table and in the table at the top of p. 8 in the Procedures section. **Be sure to include appropriate units of measure.**

Preparation of 0.1 mL of each BSA dilution from a 2 mg/mL stock solution of BSA			
Cuvette #	Final BSA concentration (mg/mL)	Amount of stock BSA needed	Amount of dH₂O needed
1	0		
2	0.1		
3	0.2		
4	0.4		
5	0.6		
6	0.8		
7	1.0		
8	1.2		
9	1.4		

After you prepare these 9 BSA dilutions, you will add 3.0 mL of Bradford's Reagent to each cuvette and measure the A₅₉₅ values. You will then plot a scatter diagram of your data and use linear regression to determine the standard curve for the Bradford assay. The standard curve will show the exact relationship between protein concentration and A₅₉₅ values. Using the standard curve, you will calculate the protein concentration of the 8 milk fractions that you saved during your purification steps on Day 1.

Calculate the dilutions of the eight milk fractions that you collected during Lab 8A

Previous experience has shown that the protein concentrations of some of the milk fractions you collected during Lab 8A exceed the linear range of the Bradford assay. Therefore, those milk fractions must be diluted before they are assayed.

Calculate how much of each milk fraction and how much dH₂O you will need to make 0.1 mL of each of the dilutions shown in the table below. Enter the required amounts in this table and in the table at the bottom of p.8 in the Procedures section. **Be sure to include appropriate units of measure.** (Note that a 1% dilution is one part of the milk fraction and 99 parts of dH₂O.)

Preparation of 0.1 mL of each diluted milk fraction			
Cuvette #	Milk fraction (dilution)	Amount of milk fraction needed	Amount of dH₂O needed
10	Nonfat milk (2.0 %)		
11	Nonfat milk (3.0 %)		
12	Pellet (3.0 %)		
13	Pellet (5.0 %)		
14	Whey (50.0 %)		
15	Whey (100.0 %)		
A	Column fraction # _____ (100 %)		
B	Column fraction # _____ (100 %)		
C	Column fraction # _____ (100 %)		
D	Column fraction # _____ (100 %)		
E	Column fraction # _____ (100 %)		

After you prepare these 11 milk dilutions, you will add 3.0 mL of Bradford's Reagent to each cuvette and then measure the A₅₉₅ values. You will then substitute these A₅₉₅ values into the equation for your standard curve (prepared from the BSA dilutions) in order to calculate the protein concentrations of the diluted milk fractions. Finally, you will use the protein concentrations of the diluted milk fractions to calculate the protein concentrations of the undiluted milk fractions.

Lab Procedures:

Turn on the Spec-20 and set the wavelength to 595 nm. Allow the Spec-20 to warm up for at least 15 min before taking any readings. Review the procedure for operating the Spec-20 in Appendix D, if necessary.

Place 20 Spec-20 cuvettes in a rack and use tape to label them “1” through “15” and “A” through “E”. Consult your instructor if insufficient Spec-20 cuvettes are available.

I. Prepare the samples that you will analyze by gel electrophoresis next week

1. Use a permanent marker to label 8 clean Eppendorf tubes as follows: “Milk”, “Pellet”, “Whey”, and “C” followed by the fraction number of each of the 5 column fraction that you saved (e.g. column fraction 9 would be labeled “C9.”). Also label each tube with your group name. Write directly on the Eppendorf tubes; do not use label tape
2. Add 50 μ L of the appropriate milk fraction to each Eppendorf tube. Save the remainder of each milk fraction for use later during this lab.
3. Locate the Eppendorf tube labeled “2X Sample Buffer” at your workstation and add 50 μ L of “2X Sample Treatment Buffer” to each of the 8 Eppendorf tubes.
4. Close the lids on the Eppendorf tubes tightly and mix the contents by repeatedly tapping the side of each tube with your finger.
5. Insert all 8 Eppendorf tubes into a holder and place them in a boiling water bath. Heat for 5 minutes, then carefully remove the tubes and bring them back to your work area.
6. With their caps securely closed, place all 8 Eppendorf tubes into a small container for storage. Have your instructor check the contents of your container to make sure that you have everything you will need for next week’s lab.
7. Cover the container with a piece of plastic wrap and secure the plastic wrap with a piece of label tape that goes all the way around the container. On the label tape, write the following:
 - **Your instructor’s name (very important!)**
 - **Your lab day and time**
 - **Your group name**
 - **Today’s date**
8. Give the container containing your milk fractions to your instructor, who will store them at -20° C until the next lab period.

II. Prepare BSA dilutions for your standard curve

1. You have been provided with a stock solution containing 2 mg/mL of BSA.
2. Dilute the BSA stock solution as shown in the table below. Accuracy is **CRITICAL**, so make sure you use an appropriate measuring device and you adjust it correctly.

Preparation of 0.1 mL of each BSA dilution from a 2 mg/mL stock solution of BSA			
Cuvette #	Final BSA concentration (mg/mL)	Amount of stock BSA needed	Amount of dH₂O needed
1	0		
2	0.1		
3	0.2		
4	0.4		
5	0.6		
6	0.8		
7	1.0		
8	1.2		
9	1.4		

III. Prepare dilutions of the 8 milk fractions you collected in Lab 8A

1. Dilute samples of your milk fractions as shown in the table below. Accuracy is **CRITICAL**, so make sure you use an appropriate measuring device and you adjust it correctly. Also, be sure not to cross-contaminate your samples. Use a clean measuring device each time you switch to a different type of sample. This includes using a separate, clean, measuring device for each of your column fractions.

Preparation of 0.1 mL of each diluted milk fraction			
Cuvette #	Milk fraction (dilution)	Amount of milk fraction needed	Amount of dH₂O needed
10	Nonfat milk (2.0 %)		
11	Nonfat milk (3.0 %)		
12	Pellet (3.0 %)		
13	Pellet (5.0 %)		
14	Whey (50.0 %)		
15	Whey (100.0 %)		
A	Column fraction # _____ (100 %)		
B	Column fraction # _____ (100 %)		
C	Column fraction # _____ (100 %)		
D	Column fraction # _____ (100 %)		
E	Column fraction # _____ (100 %)		

IV. Perform the Bradford assay on your BSA dilutions and your diluted milk fractions

Important note: After the Bradford reagent is added to your protein samples, the mixture will develop sufficient color for an accurate reading after about five minutes. The color change will give accurate measurements for up to an hour. However, for the measurements to be consistent, each of the protein samples must have reacted with the dye for approximately the same period of time. That is, if the A₅₉₅ of **all** of your samples is measured 10 minutes after the dye is added, then the assay is accurate. If the A₅₉₅ of **all** of your samples is measured 20 minutes after the dye is added, then the assay is accurate. However, if some samples are measured 10 minutes after the dye is added and others are measured 20 minutes after the dye was added, then the results will not be reliable.

An efficient way to make sure all of your samples have reacted with the dye for about the same amount of time is the following:

1. Before adding Bradford reagent to any of the cuvettes, make sure your Spec-20 is warmed up, set to A₅₉₅, and ready to go. Also, all of your cuvettes (1-15 and A-E) should contain the proper dilutions of BSA and milk fractions.
2. Cut 20 small squares of Parafilm—just large enough to cover the tops of your cuvettes when stretched.
3. Make a table for recording the absorbance values for your 20 cuvettes.
4. Add 3.0mL of Bradford reagent to the first cuvette and cover with a small square of Parafilm. Note the time. Mix the contents of the tube by holding your thumb over the Parafilm-covered top of the cuvette and **gently and quickly** inverting it 2-3 times. Replace the tube in the rack.
5. Add 3.0mL of Bradford reagent to your second cuvette, cover and mix as you did the first cuvette. Replace it in the rack.
6. Repeat with the remaining cuvettes, adding 3.0mL of Bradford reagent to each cuvette, one after another, in numerical order. Try to keep a steady pace as you move from one cuvette to the next.
7. After you have mixed the Bradford reagent with the contents of all 20 cuvettes, check the time. When at least five minutes have passed since you added Bradford reagent to cuvette #1, proceed to the next step.
8. Remove the Parafilm from the tops of the cuvettes. Wipe the sides of cuvette #1 (your blank) with a Kimwipe and use it to calibrate the Spec-20. **Have your instructor check your calibration before you proceed.**
9. Wipe the sides of Cuvette #2 with a Kimwipe, insert it into the Spec-20, and read and record the A₅₉₅.
10. Keeping a steady pace (try to keep about the same pace as when you were adding and mixing the Bradford reagent to your cuvettes), read and record the A₅₉₅ values of your remaining cuvettes in numerical order. **Have your instructor check your data before continuing.**
11. After your instructor has checked your data, pour the contents of the cuvettes into the sink. Place the cuvettes in the fume hood and fill them with ethanol. Allow them to soak in ethanol for at least 10 minutes to remove any adhering blue dye. After the tubes have soaked for at least 10 minutes, pour the ethanol into the designated waste container. Rinse the cuvettes 3 times with tap water and then 3 times with dH₂O. Finally, place the cuvettes upside down in a test tube rack to drain.

Remember! Never use brushes, scouring pads, or abrasive cleansers on Spec-20 cuvettes! Rinse with water only!
12. Leave the Spec-20 on and keep all of your samples, the Bradford reagent, cuvettes, etc. handy – you may have to repeat the assay for one or more samples after examining your standard curve.

V. Plot the standard curve for your Bradford assay

1. Using a sheet of graph paper or a computer with spreadsheet program, make a scatter diagram of the data from your BSA dilutions (**tubes 1 through 9 only**) by plotting the A₅₉₅ values of these dilutions on the y-axis and the protein concentration of these dilutions on the x-axis. **Make sure your graph has a title and you adequately label both axes of your graph.**
2. Examine the 9 points on your scatter diagram. They should form a straight line. Using a hand-held calculator or a computer with spreadsheet program, carry out linear regression to determine the equation of the “best fit” straight line for your data points. Remember, **protein concentration** is the x value at each point and the corresponding **A₅₉₅ value** is the y value.
3. After you complete linear regression, examine the **linear correlation coefficient** for your data. As mentioned in previous labs, most scientists will conclude that 2 variables are linearly related if the absolute value of the linear correlation coefficient is greater than 0.95. If the absolute value of your linear correlation coefficient is less than 0.95, this may indicate that the linear relationship is “breaking down” at the highest protein concentrations. (Why might this happen?) If this is the case, the points on your scatter diagram at highest protein concentrations will seem to “flatten out”. If they do, you may need to repeat the linear regression without these points. However, you should not arbitrarily omit points in the middle of your curve or at lower protein concentrations. Ask your instructor for help if you omit the points at the highest protein concentrations and the absolute value of the linear correlation coefficient remains below 0.95.
4. Write the **equation** for the “best fit” straight line on your scatter diagram. Next to the equation, write down the **linear correlation coefficient**. Finally, plot the “best fit” straight line in the “linear region” of your scatter diagram. This is your **standard curve** for the Bradford assay.

Do not clear the entries in your calculator yet! You will use them in Part VI.

VI. Use your standard curve to determine the protein concentrations of your diluted milk fractions

1. For your BSA standard curve, enter the range of absorbance readings where the relationship between A₅₉₅ values and protein concentration remains linear:

The relationship between A₅₉₅ values and protein concentration remains linear for A₅₉₅ values from zero _____ to _____.

2. Examine the table where you recorded the A₅₉₅ values for your diluted milk fractions (cuvettes 10-15 and A - E). Circle the A₅₉₅ values that fall within the linear range of your BSA standard curve. Make sure the A₅₉₅ value for at least one milk fraction of each type (nonfat milk, pellet, whey, and column fractions) falls within the linear range of the standard curve. If not, repeat the Bradford assay using a greater or lower dilution of that milk fraction.

NOTE: If you must repeat the Bradford assay for one or more milk fractions, make sure you use a dilution that will give you 0.1 mL of a sample that contains a *sufficiently lower* concentration of protein than your original dilutions. If making this dilution requires measuring less than 2 μL of the milk fraction, you will have to make more than 0.1 mL and then transfer 0.1 mL to your cuvette to be analyzed by the Bradford assay.

3. The linear regression equation for your BSA standard curve should have the following general formula,

$$y = mx + b$$

where “y” represents A₅₉₅ values and “x” represents protein concentrations.

Substitute the A_{595} value of each diluted milk fractions (cuvettes 10-15 and A - E) into your linear regression equation for “y” and then calculate the value of “x”. The value of “x” equals the protein concentration of the diluted milk fraction. Record your results in a table. **NOTE: When calculating the protein concentrations of the diluted milk fractions, make sure you use only the A_{595} values that fall within the linear region of the BSA standard curve.**

VII. Calculate the protein concentrations of your undiluted milk fractions

1. To calculate the protein concentration of each **undiluted** milk fraction, simply take the protein concentration of the corresponding **diluted** milk fraction and divide it by the dilution percent. For example, if a 1% dilution of nonfat milk had a protein concentration of $380 \mu\text{g/mL}$, then to calculate the protein concentration of the **undiluted** nonfat milk simply divide $380 \mu\text{g/mL}$ by 1% (0.01). This means the undiluted nonfat milk had a protein concentration of $38,000 \mu\text{g/mL}$ or 38 mg/mL .

NOTE: When performing the Bradford assay, you used 2 different dilutions of 3 milk fractions: the nonfat milk, the pellet, and the whey. In each case, calculate protein concentrations using only the dilution that produced an A_{595} value that lies within the linear region of your standard curve. If **both** dilutions of a given milk fraction gave an A_{595} value that lies within the linear region of your standard curve, then calculate the protein concentration of both dilutions, and use both results to calculate the protein concentration of the undiluted milk fraction. Obviously, both dilutions should give you the same answer for the concentration of the undiluted milk fraction; if the answers are different, average them.

Clean up

Dispose of your excess solutions as directed by instructor.

Remove label tape and any marks made with a marking pen from all glassware. Wash and rinse all glassware, give it a final rinse with dH_2O , and leave it inverted at your work area in order to drain. Remember, never use brushes, scouring pads, or abrasive cleansers on Spec-20 cuvettes! Rinse with water only!

All disposable glassware goes into the special glass disposal receptacle.

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. Prepare a clearly labeled table which shows the names of the 11 **diluted** milk fractions (cuvettes 10-15 and A - E), the A_{595} values for the 11 **diluted** milk fractions, and the calculated protein concentrations for the 11 **diluted** milk fractions. **Make sure you include appropriate units of measurement.** NOTE: If the A_{595} value for a given diluted milk fraction lies outside the linear region of your BSA standard curve, then leave the calculated protein concentration for that dilution blank.
2. Prepare a clearly labeled table which shows the names of the 8 **undiluted** milk fractions along with the calculated protein concentrations of the 8 **undiluted** milk fractions. **Make sure you include appropriate units of measurement.**
3. The nutritional label on a container of nonfat milk indicates that it contains 9 g of protein per cup (240 mL). Express the protein concentration of nonfat milk in terms of mg/mL. How does this value compare with the protein concentration of nonfat milk that you calculated using the Bradford assay?
4. Describe the purpose of protein assays in protein purification and characterization procedures.
5. List the advantages and disadvantages of each of the following types of protein assays: A_{280} measurements, the biuret assay, the Lowry assay, and the Bradford assay.
6. Describe the effects of each step of protein purification on the concentration of protein in the fractions that you saved. Which step had the most radical effect on protein concentration? Which step had the smallest effect on protein concentration?
7. What might you do to improve the precision of your Bradford assay?