Macromolecules: Experiments with Protein

This exercised is designed to introduce you to the study of macromolecules. Proteins, DNA, RNA, and polysaccharides such as starch, glycogen, and cellulose are all macromolecules. Macromolecules are formed by connecting many smaller molecules together. The individual components of a macromolecule are referred to as monomers. Proteins are composed of monomers called amino acids. All amino acids have a carboxyl group, an amino group, and a central or alpha carbon. The central carbon of each amino acid contains a side chain that is often referred to as an R group. Amino acids form polymers when the carboxyl group and amino group of two amino acids form a peptide bond as shown in Figure 1. Water and a dipeptide are formed in the reaction. More amino acids can be added to the carboxyl group of this dipeptide until a polypeptide is formed.

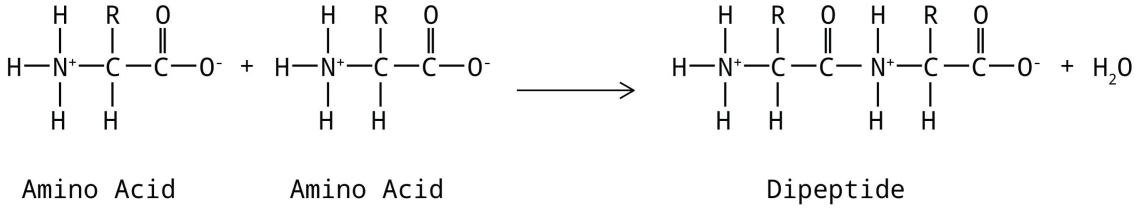

Figure 1

There are 20 different amino acids that are found in proteins, and each one has a different R-group. These side chains are very important because they impart each amino acid with different characteristics. Amino acids can be characterized as polar, nonpolar, or charged. Charged amino acids are further characterized as acid or basic. Uncharged amino acids can be considered neutral. Three different amino acids are shown in Figure 2. Aspartic acid is acidic, lysine is basic, and alanine is neutral. The sequence of amino acids that make up a polypeptide are referred to as the primary structure of the protein. The primary structure determines how the protein will fold, which will determine its function. The shapes within a polypeptide are referred to as the secondary structure. The three dimensional structure of an entire polypeptide is referred to as its tertiary structure.

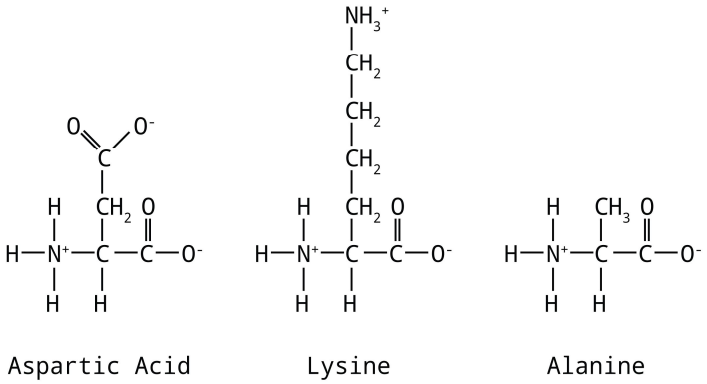

Figure 2

In this exercise you will use the Bradford assay to determine the protein content of two samples. The Bradford assays is an extremely sensitive assay for protein. The Bradford reagent contains a dye called Coomassie G-250 that can interact with the R-group of specific amino acids. One of your samples is milk. The dominant protein in milk is called casein, which is composed of 224 amino acids. Thirteen

of these amino acids react with the dye in the Bradford reagent. These amino acids include one tryptophan, four arginines, four tyrosines, and four histidines.

When the dye in the Bradford reagent interacts with these specific amino acids it turns the solution blue. The greater the concentration of protein in solution the deeper the color will be. If a set of known protein concentrations are allowed to react with a known concentration of Bradford reagent, we can measure the absorbance of the resulting solutions to create a standard curve. When a graph of absorbance *vs.* concentration is plotted for the standard solutions, a direct relationship should result, as shown in Figure 3. The direct relationship between absorbance and concentration for a solution is known as Beer's law. To determine the protein concentration of an unknown solution, we can measure its absorbance and see where it falls on the standard curve. Because the relationship is linear, we could also calculate the protein concentration using the formula for the standard curve.

In the second part of this exercise, you will compare the Bradford reagent to the Biuret Reagent. The Biuret reagent is also used to detect proteins in solution, but it is not nearly as sensitive as the Bradford assay. The Biuret reagent will turn a purplish color in the presence of protein. The Biuret reagent contains copper ions that can interact with strands of protein molecules. These complexes form between the peptide bonds of different polypeptide chains. As a result, the intensity of the color change will be directly related to the amount of protein in solution.

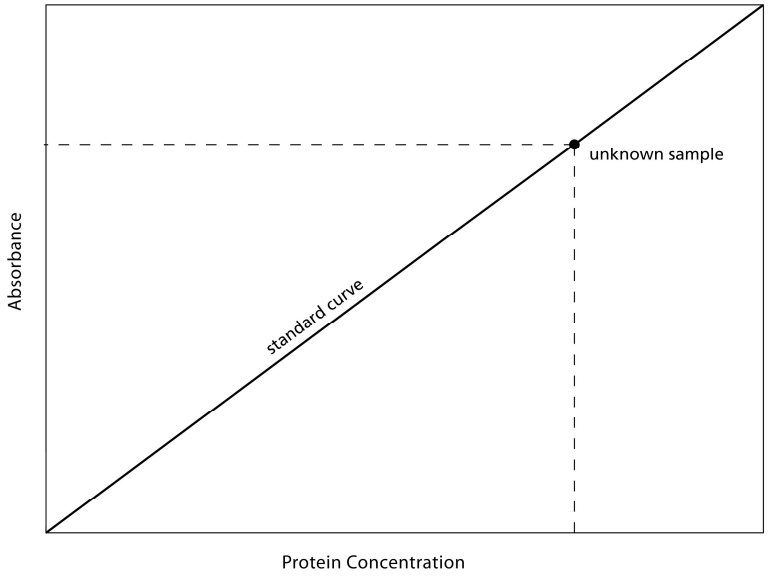

Figure 3

OBJECTIVES

In this experiment, you will

- Create a standard protein curve using the Bradford assay.
- Determine the protein concentration of milk and a high protein drink.
- Determine if the Bradford assay can detect both proteins and amino acids.
- Determine if the Biuret assay can detect both proteins and amino acids.

MATERIALS

computer
Vernier computer interface
Logger *Pro*
Colorimeter or Spectrometer+
twenty 1.5 mL cuvettes with caps*
20–200 µL micropipette**
100–1000 µL micropipette**
200 µL micropipette tips (1 box)
1000 µL micropipette tips (1 box)
two 1.5 mL microtubes
six 15 mL centrifuge tubes
1% tryptophan solution
1% nonfat milk protein solution
Biuret reagent mixture
Quick Start Bradford Reagent
Phosphate buffered saline (PBS)
Bovine γ-globulin standard set
milk (lowfat or nonfat)
high protein drink

+ No interface is required if using a Spectrometer.
* If using a Colorimeter, use 3 mL cuvettes supplied by Vernier and double the volume of all reagents.
** Appropriate graduated transfer pipettes (1 and 5 mL) may be substituted.

PROCEDURE

Part I Determination of protein content in different samples using the Bradford Assay

Both Colorimeter and Spectrometer Users

1. Obtain and wear goggles and gloves.
2. Obtain three 15 mL centrifuge tubes.
   - Label one tube **PB** and add 10 mL of PBS.
   - Label the other two tubes **BR** and add 15 mL of Quick Start Bradford Reagent to each.
3. Obtain two 1.5 mL microtubes.
   - Label one tube **M** and add 980 µL of PBS. Then add 20 µL of milk.
   - Label the other tube **HP** and add 980 µL of PBS. Then add 20 µL of high protein drink.
4. Obtain seven empty cuvettes with caps and a set of Bovine γ-globulin standards to create a new set of protein standards.
   a. Fill each cuvette with 1 mL of Bradford reagent.
   b. Label one cuvette **2.0** and add 20 µL of solution from the 2.0 mg/mL standard. Cap the cuvette and gently invert the cuvette three times.
   c. Label the next cuvette **1.5** and add 20 µL of solution from the 1.5 mg/mL standard. Cap the cuvette and gently invert the cuvette three times.
   d. Label the next cuvette **1.0** and add 20 µL of solution from the 1 mg/mL standard. Cap the cuvette and gently invert the cuvette three times.
   e. Label the next cuvette **0.75** and add 20 µL of solution from the 0.75 mg/mL standard. Cap the cuvette and gently invert the cuvette three times.
   f. Label the next cuvette **0.5** and add 20 µL of solution from the 0.5 mg/mL standard. Cap the cuvette and gently invert the cuvette three times.
   g. Label the next cuvette **0.25** and add 20 µL of solution from the 0.25 mg/mL standard. Cap the cuvette and gently invert the cuvette three times.
   h. Label the next cuvette **0.125** and add 20 µL of solution from the 0.125 mg/mL standard. Cap the cuvette and gently invert the cuvette three times.

5. Obtain two empty cuvettes with caps and the microtubes labeled with M and HP.
   a. Fill each cuvette with 1 mL of Bradford reagent.
   b. Label one cuvette with an **M** and add 20 µL from microtube M. Cap the cuvette and gently invert the cuvette three times.
   c. Label the other cuvette **HP** and add 20 µL from microtube HP. Cap the cuvette and gently invert the cuvette three times.

6. Prepare a blank by filling an empty cuvette with 1 mL of Bradford reagent and 20 µL of PBS. Label it **B**. To correctly use cuvettes, remember:
   - Wipe the outside of each cuvette with a lint-free tissue.
   - Handle cuvettes only by the top edge of the ribbed sides.
   - Dislodge any bubbles by gently tapping the cuvette on a hard surface.
   - Always position the cuvette so the light passes through the clear sides.

Spectrometer Users Only (Colorimeter users proceed to the Colorimeter section)

7. Use a USB cable to connect the Spectrometer to your computer. Choose New from the File menu.

8. Calibrate the Spectrometer.
   a. Place the blank cuvette into the cuvette slot of the Spectrometer.
   b. Choose Calibrate ▶ Spectrometer from the Experiment menu. The calibration dialog box will display the message: "Waiting 90 seconds for lamp to warm up." After 90 seconds, the message will change to "Warmup complete."
   c. Click Finish Calibration and allow the calibration to finish. Click OK.

9. Determine the optimum wavelength for examining the absorbance of the Bradford reagent when it is bound to protein and set up the data-collection mode.
   a. Remove the blank cuvette. Gently invert Cuvette M twice and then place it into the Spectrometer.
   b. Click ▶ Collect. A full spectrum graph of the solution will be displayed. Note that one area of the graph contains a peak absorbance. Click ■ Stop to complete the analysis.
   c. Store the data by choosing Store Latest Run from the Experiment menu.
   d. To set up the data-collection mode and select a wavelength for analysis, click Configure Spectrometer Data Collection, .
   e. Select Abs *vs.* Concentration as the Set Collection Mode. The wavelength of maximum absorbance (λ max) will be selected. Verify that the maximum absorbance is close to 595 nm.
   f. Enter **Protein Concentration** as the Column Name. Enter **Pr. Conc**. as the Short Name. Enter **mg/mL** as the Units.
   g. Click OK. Remove the cuvette from the Spectrometer and proceed to Step 10.

Colorimeter Users Only

7. Connect the Colorimeter to the computer interface. Prepare the computer for data collection by opening the file "17 Macromolecules" from the *Advanced Biology with Vernier* folder of Logger *Pro*.

8. Open the Colorimeter lid, insert the blank, and close the lid.

9. To calibrate the Colorimeter, press the < or > button on the Colorimeter to select the wavelength of 635 nm (Red). Press the CAL button until the red LED begins to flash and then release the CAL button. When the LED stops flashing, the calibration is complete. Remove the cuvette from the Colorimeter and proceed to Step 10.

Both Colorimeter and Spectrometer Users

10. You are now ready to collect absorbance data for the seven protein standards. Click Collect. Obtain the cuvette labeled 2.0. Wipe the outside with a tissue and place it in the device (close the lid if using a Colorimeter). Wait for the absorbance value displayed on the screen to stabilize, then click Keep. Enter **2.00** as the concentration, and then press ENTER or click OK. The data pair you just collected will now be plotted on the graph. Remove the cuvette from the device.

11. Obtain the cuvette labeled 1.5. Wipe the outside and place it in the device. When the absorbance value stabilizes, click Keep, enter **1.50**, and press ENTER.

12. Repeat the Step 11 procedure for the remaining protein standards. When you have finished with the 0.125 mg/mL standard solution, click Stop.

13. In Table 1, record the absorbance values.

14. Examine the graph of absorbance *vs.* concentration. To see if the curve represents a direct relationship between these two variables, click Linear Fit, . A best-fit linear regression line will be shown for your data points. This line should pass near or through the data points.

15. In Table 1, record the equation of this line in the space provided.

16. You are now ready to collect absorbance data for your unknowns. Obtain the cuvette labeled M. Wipe the outside of the cuvette and place it in the device (close the lid if using a Colorimeter). When the displayed absorbance value stabilizes, record the value in Table 2. **Important:** The reading on the screen is live, so it is **not** necessary to click Collect to read the absorbance value.

17. Choose Interpolation Calculator from the Analyze menu.

18. Check that the absorbance value that you recorded is displayed. If it is not, enter the absorbance value in the correct space. The protein concentration in mg/mL will be displayed. Record the concentration value in Table 2. Click OK. A point on the graph will be displayed for this protein sample.

19. Obtain the cuvette labeled with a HP. Wipe the outside of the cuvette and place it in the device. When the displayed absorbance value stabilizes, record the value in the Data and Calculations table. **Important:** The reading on the screen is live, so it is **not** necessary to click Collect to read the absorbance value.

20. Repeat Steps 17–18, then proceed to Step 21.

Part II Comparison of Bradford and Biuret Reagents

21. Obtain another 15 mL centrifuge tube and label the tube with **BI**. Add 10 mL of the Biuret Reagent.

22. Obtain two more 15 mL centrifuge tubes.
    - Label one tube **NFM** and add 4.0 mL of 1% non-fat milk protein solution.
    - Label the other tube **TRP** and add 4.0 mL of 1% tryptophan solution.
23. Obtain three empty cuvettes with caps.
    a. Fill each cuvette with 1 mL of Bradford reagent.
    b. Label the first cuvette **BR T** and add 100 µL of 1% tryptophan solution. Cap the cuvette and gently invert the cuvette three times.
    c. Label the next cuvette **BR M** and add 100 µL of 1% non-fat milk protein solution. Cap the cuvette and gently invert the cuvette three times.
    d. Label the next cuvette **BR C** and add 100 µL of PBS, the control.
24. Obtain three more empty cuvettes with caps.
    a. Fill each cuvette with 1 mL of Biuret reagent.
    b. Label the first cuvette **BI T** and add 500 µL of 1% tryptophan solution. Cap the cuvette and gently invert the cuvette three times.
    c. Label the next cuvette **BI M** and add 500 µL of 1% non-fat milk protein solution. Cap the cuvette and gently invert the cuvette three times.
    d. Label the next cuvette **BI C** and add 100 µL of PBS, the control.

DATA AND CALCULATIONS

Table 1	
Protein Concentration (mg/mL)	Absorbance
2.00	
1.50	
1.00	
0.750	
0.500	
0.250	
0.125	
Linear Fit for Standard Curve _____	

Table 2				
Samples	Absorbance	Protein conc. (mg/mL)	Actual conc. (mg/mL)	Concentration from label (mg/mL)
Milk				
High Protein				
Calculated protein concentrations from linear fit				
Milk				
High Protein				

Table 3		
Sample	Bradford reagent	Biuret reagent
Tryptophan		
Non-Fat milk protein		
Control		

PROCESSING THE DATA

Part I Determination of protein content in different samples using the Bradford Assay

1. Determine the actual protein concentration of your samples. Remember that you diluted your original milk and high-protein samples by a factor of 50 before conducting the Bradford assay. Multiply the protein concentration that you observed for each sample by 50 to get the actual protein concentration of your sample. Record this value in the space provided in Table 2.
2. Obtain the published protein values for each sample from your instructor. These values are typically found on the nutrition labels of the milk or protein drink container. Convert the published values to mg/mL of protein. To do this, divide the amount of protein per serving by the volume of each serving. You may need to convert from fluid ounces to mL. Write these values down in the space provided in Table 2.
3. Calculate the actual protein concentration of each sample using the formula for the standard curve. Use the absorbance that you observed for each sample as your y value and then solve for x. Multiply the resulting value by 50 to get the actual protein concentration. Write these two numbers down in the space provide in Table 2.

Part II Comparison of the Bradford and Biuret Reagents

4. Examine the cuvettes that contain the Bradford reagent from Part II. Did the cuvette contents turn a blue color? Write down your observations in the space provided in Table 3.
5. Examine the cuvettes that contain the Biuret reagent from Part II. Did the cuvette contents turn a purplish color? Write down your observations in the space provided in Table 3.

QUESTIONS

Part I Determination of protein content in different samples using the Bradford Assay

1. Compare the protein values that you observed for each sample to the published protein values. Are the values close? If they are not, can you think of any reason why they would be different?

2. Compare your observed protein values to the protein values that you calculated using the formula for the standard curve. Are the values different or are they the same? Is there any reason why they should be different?

Part II Comparison of the Bradford and Biuret Reagents

3. Did the Bradford reagent turn a blue color in the presence of non-fat milk protein? If it did, can you explain why it would do this? What about the 1% solution of the amino acid tryptophan? Did the Bradford reagent react with this amino acid even though it is not a protein? Can you explain why this would happen?

4. Did the Biuret reagent turn a purplish color in the presence of non-fat milk protein? If it did, can you explain why it would do this? What about the 1% solution of the amino acid tryptophan? Did the Biuret reagent react with this amino acid even though it is not a protein? Can you explain why this would or would not happen?

5. How are the results from this part of the exercise related to the primary and/or secondary structure of a given protein?

EXTENSIONS

1. Serial dilution is the typical method that is used to create a standard curve. Follow the instructions below to create a new standard curve and then repeat Part I of this exercise. **Note:** The new protein standards do not include a 1.5 mg/mL standard.
   a. Obtain the 2.0 mg/mL solution from the bovine γ-globulin standard set.
   b. Obtain four empty microtubes and one microtube with 1 mL of PBS.
   c. Add 100 µL of PBS to each empty microtube.
   d. Label the first tube **1.0** and add 100 µL of the 2.0 mg/mL γ-globulin standard. Cap and invert the tube three times.
   e. Label the next tube **0.5** and add 100 µL from the tube labeled 1.0. Cap and invert the tube three times.
   f. Label the next tube **0.25** and add 100 µL from the tube labeled 0.5. Cap and invert the tube three times.
   g. Label the next tube **0.125** and add 100 µL from the tube labeled 0.25. Cap and invert three times.

2. The dominant protein in milk is called casein and it is composed of 224 amino acids. Thirteen of these amino acids react with the dye in the Bradford reagent to turn the solution blue. Your standard curve is based on the protein bovine γ-globulin, which is not composed of the same number and/or ratio of amino acids. Non-fat milk powder does contain the protein casein. Use the non-fat milk protein solution from Part II to create a new standard curve. Your current stock of non-fat milk protein solution is at 10 mg/mL (1%). Dilute the stock by a factor of 5 in PBS to get a 2 mg/mL solution. Repeat the steps in Extension 1 to

generate your set of protein standards. Then repeat Part I of this exercise and compare your results.

3. Compare the Biuret reagent to the Bradford reagent. To do this, repeat Part I of this exercise but use the Biuret reagent instead of the Bradford reagent. You will need to create 1 mL of each protein standard to create a standard curve. Add 500 µL of each protein standard to an appropriately labeled cuvette and then add 1 mL of Biuret reagent. Measure the absorbance of each cuvette at 540 nm to create your standard curve. Determine the protein concentration of your samples by adding 500 µL of each diluted sample to a cuvette and then add 1 mL of Biuret reagent. Measure the absorbance of each cuvette at 540 nm. Then use the formula for the standard curve or the interpolation calculator to determine the protein concentration of your samples. Compare your results using this method to results you obtained using the Bradford assay.

4. Design an experiment that quantifies the qualitative differences you observed in Part II of this exercise. You can start by repeating Part II of this exercise. Then measure each cuvette at the proper absorbance. For the Bradford reagent measure the absorbance at 595 nm. For the Biuret regent measure the absorbance at 540 nm.

5. The Bradford reagent is a very sensitive method for determining protein concentrations. Design an experiment to determine the sensitivity of this method. You can start by creating a set of protein standards that are in the 1–10 µg/mL range. Create 1 mL of each standard. Start by determining if you can use the same ratio of Bradford reagent to protein standard to create a standard curve in this new range. If you cannot, try increasing the volume of protein solution that you add to each cuvette.

6. Amino acids are classified as polar, non-polar, basic or acidic. This classification is based on their R-groups or side chains. Most polar and non-polar amino acids are considered neutral. We can easily determine if an amino acid is basic, acid or neutral by using Logger *Pro*, a pH Sensor, some distilled water, and a magnetic stirrer. You can start with the amino acids arginine, tyrosine, and aspartic acid.

   a. Place a pH Sensor in 250 mL of distilled water. Make sure that the magnetic stir bar is turning at a moderate speed.
   b. Collect pH data using Logger *Pro* and a Vernier computer interface.
   c. After a few seconds have elapsed, add a very small amount of aspartic acid to the distilled water.
   d. Record the pH for at least one minute.
   e. Dispose of the distilled water and then repeat the experiment for tyrosine and arginine.
   f. Determine if each amino acid is basic, acidic or neutral.
   g. Compare your results with the description of each amino acid in a textbook. Do your data support what is in your textbook?