

## BC 367 Experiment 2 Comparison of Protein Assays

### Introduction

Proteins perform a range of complex functions in nature, including roles in structure, transport, and catalysis. When studying proteins, a biochemist often needs to determine protein concentration in a complex mixture of other molecules. Since there are numerous methods to measure protein content, it is critical to understand the advantages and disadvantages of each method available. In this exercise, you will use three different assays to determine the concentration of a standard protein. By sharing data for other proteins with other groups, you will compare the relative merits of each assay and establish how protein composition affects the sensitivity of a particular assay.

Absorbance Spectroscopy All protein concentration determination methods depend upon measuring the absorbance of a solution. Absorbance (A) is a logarithmic measurement of the Transmittance (T) of light through a sample (Equation 1). Because Absorbance is a logarithmic function, values greater than 1.0 (10% T) or smaller than 0.1 (80%T) make it very difficult for the instrument to accurately determine the intensity of transmitted light. Solutions that do not absorb between 1.0 - 0.1 should be diluted or concentrated to give accurate data.

$$A = -\log T \quad \text{Equation 1}$$

Chromophores in dilute solutions typically show a linear dependence between concentration and Absorbance (Equation 2). This functional dependence, known as the “Beer-Lambert law”, indicates that Absorbance is dependent upon the path length of light (l, in cm) through the sample, the concentration of the chromophore (c, in M), and the molar extinction coefficient ( $\epsilon$ , in  $M^{-1}cm^{-1}$ ). The path length and concentration can easily be changed, but the extinction coefficient is an intrinsic physical property of the chromophore.

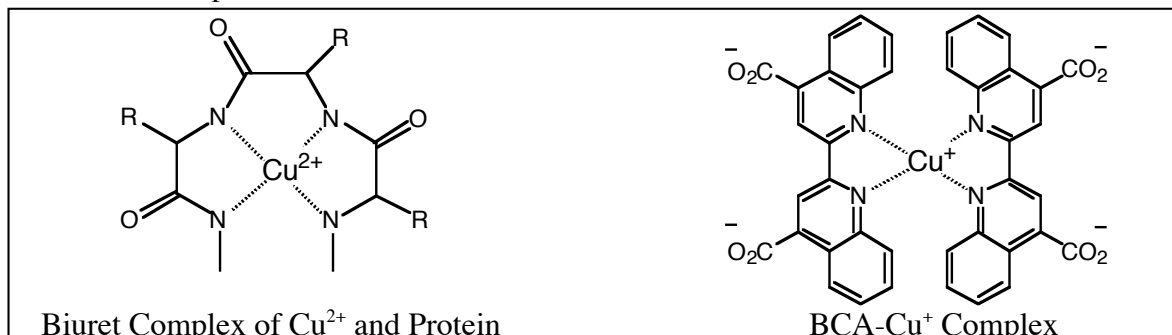
$$A = \epsilon c l \quad \text{Equation 2}$$

Because the relationship between the absorbance of the chromophore and its concentration is linear, it is possible to construct a calibration curve of absorbance versus concentration. An unknown concentration can then be determined from the absorbance of solution in question and the equation of the best-fit line. It is important to note that the unknown concentration should lie in the concentration range of the standard solutions for an accurate concentration determination.

Protein Concentration Assays Brief descriptions of a few of the more commonly used methods of protein determination and some of their limitations are given below.

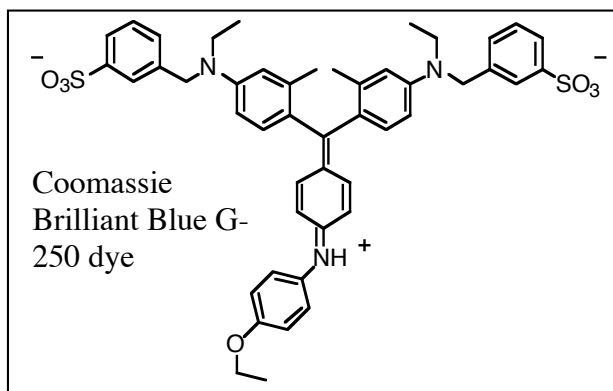
**1. BCA Assay:** In alkaline solutions,  $Cu^{2+}$  binds to peptide bonds of proteins. Cys, Trp, and Tyr, are capable of reducing the bound  $Cu^{2+}$  to  $Cu^{+}$ , resulting in formation of a moderate purple color proportional to the protein concentration. This color is used in the rather insensitive “Biuret Assay” to determine protein concentration. The sensitivity can be increased by addition of bicinchoninic acid (BCA). When BCA binds  $Cu^{+}$ , an intense purple color proportional to the protein concentration is observed. However, because the composition of reducing amino acids

varies substantially among proteins, the color yield per milligram of protein is not a constant between different proteins.



Proteins that are relatively rich in reducing amino acids give higher absorbance values than those relatively poor in these amino acids. Nevertheless, the method is very useful for following changes in protein content, for example, during protein purification. When the BCA assay is carried out at  $60^\circ\text{C}$ , variability between proteins is often less than that observed at room temperature. Non-protein reducing compounds in buffers can be a source of error with the BCA method if they can reduce  $\text{Cu}^{2+}$  to  $\text{Cu}^{+}$ , so appropriate controls should be run with “buffer-only” samples.

**2. The Bradford Dye Binding (Bio-Rad) Protein Assay:** This method is a dye-binding assay based on the differential color change of a dye in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 dye (shown below) shifts from 465 nm to 595 nm when noncovalent electrostatic and van der Waals interactions with proteins occur. Since the dye is anionic, it is more sensitive to proteins with high Arg, Lys, and His content. This method has gained popularity in research and other applications since it is a simple, quick, one-step procedure that forms a relatively stable colored complex and is free of many of the interferences that limit the application of other assays. The dye, however, can bind strongly to the cuvettes used for measurements and is somewhat difficult to remove from glassware. A constant volume of 1 M NaOH can be used with this assay if protein precipitation is observed upon adding the dye.



**3. The Warburg-Christian Assay:** This direct spectrophotometric method is based on the fact that aromatic amino acids Tyr, Trp and Phe absorb ultraviolet light maximally at  $\sim 280$  nm. The  $\epsilon_{280}$  for Trp =  $5690 \text{ M}^{-1}\text{cm}^{-1}$ , Tyr =  $1280 \text{ M}^{-1}\text{cm}^{-1}$ , and Phe =  $10 \text{ M}^{-1}\text{cm}^{-1}$ . Many pure protein solutions containing  $\sim 1$  mg/mL of protein exhibit an absorbance at 280 nm of about 1.0 in a 1 cm cuvette. This assay is the only method that is non-destructive to protein samples (since the protein is not tied up in a colorimetric reaction). However, since different proteins contain varying amounts of aromatic amino acids, the method is very sensitive to amino acid composition, and two different proteins can have widely varying extinction coefficients ( $\epsilon_{280}$ ).

Therefore, this method is not ideal for determining concentrations of mixtures of proteins. Protein structure can also affect the UV absorbance of aromatic side chains. Therefore, any conditions that alter the structure (pH, temperature, ionic strength, detergents) can affect the ability of aromatic residues to absorb light at 280nm and change the value of the protein's extinction coefficient. With a pure protein, however, it is possible to determine its unique extinction coefficient ( $\epsilon_{280}$ ) under specific buffer and temperature conditions in order to use the  $A_{280}$  as a measure of the absolute amount of that protein.

Pipette Usage You will use automatic microliter pipettes extensively in the biochemistry laboratory. We have four sizes of pipettors in the lab: 2 microliters, 20 microliters, 200 microliters, and 1000 microliters. Please make sure that you never dial the pipettor past the maximum volume, or you will damage the pipettor. To draw up the sample into the pipette tip, push the button down to the first notch, immerse the tip in the sample, and slowly release the button. Check the tip to make sure you didn't capture any air bubbles. To dispense the sample, push the button all the way down. For all dilutions that you perform, make sure that you add the protein solution to the bottom of the tube (not the side, where it might stick) and vortex your mixtures well after adding the appropriate volume of distilled water.

**If you have any questions about the micropipettors, please ask your instructor before use.** Micropipettors can be severely damaged if they are improperly used. Furthermore, in order to obtain quantitative data in the biochemistry lab, it is essential to master good pipetting.

Cuvettes We will use plastic cuvettes that fit into the Ocean Optics spectrophotometers for the various assays. To minimize scratches on the surface, only hold these cuvettes by the side that will not be in the light path, and do not insert anything sharp inside the cuvette. Be sure that the cuvettes you use are clean (this is true in general of all glassware). If any appear to be stained blue, try rinsing them with a small volume of ethanol, or dispose of them. Even though cuvettes may LOOK clean, you may wish to rinse all tubes with deionized water. When finished rinsing, invert the cuvettes on a paper towel to dry them before use.

Note that there are two different kinds of cuvettes in the lab, regular ones and UV-transparent ones. Please use the UV cuvettes only for the Warburg-Christian assay; they are very expensive, and the blue dye stains them.

Ocean Optics Spectrometer. You will use an Ocean Optics spectrophotometer to record absorbance data. These instruments need to warm up for ~5 minutes prior to use, so make sure they have been turned on when you start preparing your protein solutions. Recall that Absorbance (A) is a logarithmic measurement of the Transmittance (T) of light through a sample (Equation 1). Any samples that do not absorb between 0.1 and 1.0 are not likely to be quantitative. Follow the Ocean Optics instructions carefully. This is a separate document posted on the BC367 webpage. Make sure you print out this document and bring it to lab.

Protein Solutions. Each group will be assigned one of the three standard proteins on which to do all three protein assays. Write your name and this standard protein on the board to facilitate sharing of data between groups.

Protein Standard Solutions:           1.00 mg/mL bovine alkaline phosphatase  
   1.00 mg/mL bovine serum albumin (BSA)  
   1.00 mg/mL hen egg white lysozyme

Each group will also be given two solutions containing a known concentration of their particular protein, one at 0.100 mg/mL and one at 10.0 mg/mL. You will use your standard curves for each assay to experimentally verify the concentrations of these "known proteins," thereby checking the reliability of each assay.

## Experimental Procedure

### **I. Protein Standard Dilutions**

For your specific protein, you will create a separate standard curve for each type of assay. Each standard curve will contain absorbance data for the following protein concentrations: 0, 10, 25, 50, 100, 250, 500, 1000  $\mu\text{g/mL}$ .

**Before lab**, prepare a plan for diluting your 1.00 mg/mL protein solution to obtain 3.0 mL of each protein concentration (10, 25, 50, 100, 250, 500, 1000  $\mu\text{g/mL}$ ) to use in your protein assays.

Remember that a convenient way to calculate dilutions is to use the formula:

$$\text{concentration}_1 \times \text{volume}_1 = \text{concentration}_2 \times \text{volume}_2 \quad (c_1V_1 = c_2V_2)$$

Because concentration (moles/L) times volume (L) results in units of "moles," this equation really just states that the number of moles remains constant during a dilution. You should review your general chemistry text if this is unclear.

**NOTE\*\*** For standards less than 100  $\mu\text{g/mL}$ , you should first prepare a 100  $\mu\text{g/mL}$  solution and then dilute this solution again to obtain the final 0-50 $\mu\text{g/mL}$  concentration.

A dilution example:

To prepare 3.0 mL of a 200  $\mu\text{g/mL}$  protein solution, you should combine:  
 0.6 mL standard protein solution + 2.4 mL water

This was calculated by using  $c_1V_1 = c_2V_2$ :

$$1000 \mu\text{g/mL} \times \text{unknown volume} = 200 \mu\text{g/mL} \times 3.0\text{mL}$$

$$\text{unknown volume} = 0.6\text{mL or } 600\mu\text{L}$$

1. Prepare a table of  $\mu\text{g/mL}$  concentration,  $\mu\text{L}$  standard protein solution (at 1.00 mg/mL or 100  $\mu\text{g/mL}$ ), and  $\mu\text{L H}_2\text{O}$  to be added for each 3.0 mL dilution **before you come to lab**.

Before you start diluting your standard samples, check your calculations with your instructor to make sure you have the correct dilutions.

2. Prepare 3.0 mL of each standard protein concentration (10, 25, 50, 100, 250, 500, and 1000  $\mu\text{g/mL}$ ) in separate labeled test tubes. Your dilutions will be made up with deionized water. This 3.0 mL of each concentration should be sufficient to carry out the whole experiment.

For all dilutions, make sure that you add the protein solution toward the bottom of the test tube (not at the top where it might not be mixed well) and mix your samples well after adding the appropriate volume of distilled water. These proteins are robust, and solutions can be vortexed, although in general, it is unwise to vortex proteins.

## II. Protein Assays

### A. BCA Assay for Total Protein

1. Place 100  $\mu\text{L}$  of each of your protein standard dilutions (concentrations of 10, 25, 50, 100, 250, 500, and 1000  $\mu\text{g/mL}$ ) into separate test tubes. Also place duplicate 100- $\mu\text{L}$  samples of water (for the reference solutions) and duplicate 100- $\mu\text{L}$  samples of your 0.100 mg/mL and 10.0 mg/mL protein samples into separate test tubes. (Hint: you should also prepare one of these solutions as a 10-fold dilution. Think about why this is true.)
2. A repipetter is a device that delivers an exact volume of solution. A repipetter containing the BCA reagent has been calibrated to deliver 2.0 mL of reagent. All you need to do is to push the lever. Using this repipetter, add 2.0 mL of the BCA reagent to each of the above test tubes (including the water blanks), cover the test tubes with a small piece of parafilm, and mix by vortexing.
3. Incubate the test tubes for 40 minutes in a water bath at 60°C.
4. After cooling the samples to room temperature, transfer samples to cuvettes, and calibrate the spectrophotometer with the appropriate reference solution (the “blank”): one of the samples containing distilled  $\text{H}_2\text{O}$  plus BCA reagent. Record the  $A_{562}$  of each sample (including the second reference solution, which will probably not read exactly zero) in your notebook.
5. Using the  $A_{562}$  data from the standard protein solutions, construct a standard curve of  $A_{562}$  versus protein concentration ( $\mu\text{g/mL}$ ) in an EXCEL spreadsheet. Include the second water blank as a data point. Be certain to use at least four significant figures in your linear fit equation. Include an  $R^2$  value on your graph. If the data are nonlinear at high concentrations, generate another plot using only the data that are within the linear range.
6. Use the equation of the resulting “best-fit” line to determine the protein concentration of your known protein samples based on their average absorbance values. One of these samples will probably give a more accurate value as the 10-fold dilution (why?).
7. Discard solutions in the “Basic Metal Waste” container in the fume hood.

**B. The Bradford (Bio-Rad) Assay**

1. Place 100  $\mu\text{L}$  of each of your protein standard dilutions (concentrations of 10, 25, 50, 100, 250, 500, and 1000  $\mu\text{g}/\text{mL}$ ) into separate cuvettes. Also place duplicate 500- $\mu\text{L}$  samples of water (for the blanks) and duplicate 100- $\mu\text{L}$  samples of your 0.100 mg/mL and 10.0 mg/mL protein samples into separate cuvettes. (Hint: you should also prepare one of these solutions as a 10-fold dilution. Think about why this is true.)
2. Add 400  $\mu\text{L}$  of water to all cuvettes except for the water blanks.
3. A repipetter is a device that delivers an exact volume of solution. A repipetter containing the Bio-Rad reagent has been calibrated to deliver 2.5 mL of reagent. All you need to do is to push the lever. Using this repipetter, add 2.5 mL of the Bio-Rad dye reagent to all samples with a repipetter. Cover with parafilm and mix by inverting several times.
4. Wait 5 minutes and then calibrate the spectrometer with one of the water samples. Record the  $A_{595}$  for each sample into your notebook.
5. Prepare your standard curve in EXCEL as described for the BCA assay.
6. Determine the concentrations of your knowns based on their average absorbance values and the standard curve. One of these samples will probably give a more accurate value as the 10-fold dilution (why?).
7. Discard your solutions in the "BioRad" waste container in the fume hood.

**C. The Warburg-Christian Method**

1. You may use either the Ocean Optics or the NanoDrop (ND-1000) spectrophotometer for this assay.
  - a) For the Ocean Optics: you must use the special UV-transparent cuvettes to measure at 280 nm. Add 1 mL of each sample to a separate cuvette (each of your standard protein concentrations, two water blanks, duplicate samples of each of your known proteins, and duplicate samples of a 10-fold dilution of one of the known proteins).
  - b) For the NanoDrop: simply take the test tubes containing your protein solutions to the spectrophotometer. You will use 2  $\mu\text{L}$  of each sample. No cuvettes are needed.
2. Calibrate the spectrometer with a water blank, and record the  $A_{280}$  for each sample into your notebook.
3. Prepare graph(s) in EXCEL as described for the BCA assay.
4. Find the molecular weight of your known protein in the appendix. Calculate the molar concentrations for your protein samples, and make a new graph using the Beer-Lambert law ( $A = \epsilon l C$ ) to determine the  $\epsilon_{280}$  for your protein.

### III. Amino Acid Sequence Analysis

Sequences for each of the proteins are included in the appendix. Analysis of sequence content can be done on the web at <http://bioinformatics.org/sewer>.

1. Select >Protein from the menu on the left side of the SeWeR webpage.
2. Select the ProtParam program from the pull-down menu.
3. Cut and paste your protein sequence from the appendix into the analysis window.
4. Select Submit button for an analysis of the protein's amino acid content.

#### Analysis

In your notebook, be sure to include clearly labeled copies of all your standard curves. Report the most reliable assay(s) for determining the concentration of your protein. That is, which assay gave you the most accurate concentration determination? Explain.

Although each group performed all three assays on only one protein, **each group will obtain data from other laboratory groups** for the other two proteins. Therefore, you must post your data (protein assayed, equation of each standard curve,  $R^2$  values, absorbance values of samples of known concentration for each assay, and your name) on the fileserver within 24 hours after the completion of lab. Note that if you fail to report your data correctly and within the time limit, your own grade will be negatively impacted.

From the entire laboratory's data, determine the most reliable assay or assays for each protein. Some criteria you may want to consider in these decisions are assay reproducibility between groups (precision), accuracy of the experimental determinations of the known concentrations, and the sensitivity of each assay for each protein (hint: what is the slope of each standard curve telling you?). Note that you do not need to include all your classmates' standard curves. Instead, you can simply make a summary table containing all the relevant information.

Make a table that displays percent amino acid composition for *reducing*, *cationic*, and *aromatic* amino acids for each of the protein standards. Compare the percent content of responsive amino acids for each of the assays. For the Warburg-Christian Assay, recall that  $\epsilon_{280}$  for Trp =  $5690\text{M}^{-1}\text{cm}^{-1}$ , Tyr =  $1280\text{M}^{-1}\text{cm}^{-1}$ , and Phe =  $10\text{M}^{-1}\text{cm}^{-1}$ . How does the percent composition data compare to the slopes for each standard protein? Relate this information to the assay sensitivity for each protein, which is your ultimate goal for this exercise. For each assay, discuss the characteristics that make a protein suitable for using it.

#### References

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## Appendix: Amino acid sequences of relevant proteins<sup>1</sup>

### **Lysozyme, Chicken: 129 amino acids; $M_r=14,313$**

KVFGRCELAAAMKRHGLDNRYRGYSLGNWVCAAKFESNFNTQATNRNTDGSTDYGILQINSRW  
WCNDGRTPGSRNLCNIPCSALLSSDITASVNC AKKIVSDGNGMNAWVAWRNRCKGTDVQAWIR  
GCRL

### **Bovine Serum Albumin: 583 amino acids; $M_r=66,433$**

DTHKSEIAHRFKDLGEEHFKGLVLIAFSQYLQQCPFDEHVKLVNELTEFAKTCVADESHAGCEKS  
LHTLFGDELCKVASLRETYGDMADCCEKQEPERNECFLSHKDDSPDLPKLKPDPNTLCDEFKAD  
EKKFWGKYL YEIARRHPYFYAPELLYANKYNGVFQECCQAEDKGACLLPKIETMREKVLASS  
ARQLRCASIQKFGERALKAWSVARLSQKFPKAEFVEVTKLVTDLTKVHKECCHGDLLECADD  
RADLAKYICDNQDTISSKLKECCDKPLLEKSHCIAEVEKDAIPENLPPLTADFAEDKDVCCKNYQE  
AKDAFLGSFLYEYSRRHPEYAVSVLLRLAKEYEATLEECCA KDDPHACYSTVFDKLKHVLVDEPQ  
NLIKQNC DQFEKLG EYGFQNALIVRYTRKVPQVSTPTLVEVSRSLGKVGTRCCTKPESERMPCTE  
DYL SLILNRLCVLHEKTPVSEKVTKCCTESLVNRRPCFSALTPDETYVPKAFDEKLFTHADICTL  
PDTEKQIKKQTALVELLKHKPKATEEQLKTVMENFVAFVDKCCAADDKEACFAVEGPKLVVST  
QTALA

### **Bovine Alkaline Phosphatase: 486 amino acids; $M_r = 52, 373$**

LVPVEEEDPAFWNRQAAQALDVAKKLQPIQTA AKNVILFLGDGMGVPTVTATRILKGQMNGKL  
GPETPLAMDQFPYVALSKTYNVDRQVPDSAGTATAYLCGVKGNRYRTIGVSA AARYNQCKTTRG  
NEVTSVMNRAKKAGKSVGVT TTRVQHASPAGAYAHTVNRNWYSDADLPADAQMNGCQDIA  
AQLVNNMDIDVILGGGRKYMFPVGTDPPEYPDDASVNGVRKRKQNLVQAWQAKHQGAQYVW  
NRTALLQAADDSSVTHLMGLFEPADMKYNVQQDHTKDPTLQEMTEVALRVVSRNPRGFYLFVE  
GGRIDHGHDDKAYMALTEAGMFDNAIAKANELTSELDTLILVTADHSHVFSFGGYTLRGT SIF  
GLAPSKALDSKSYTSILYGNPGYALGGSRPDVNDSTSEDPSYQQQA AVPQASETHGGEDVAV  
FARGPQAHLVHGVEEETFVAHMAFAGC VEPYTD CNLPAPTTATSIPD

<sup>1</sup> data obtained from the Swiss Prot Data Base