

BL4820 Biochemistry Techniques -- Lecture 1 -- Protein Assay

Text Reference: Expt #1, pages 5 to 13; Also useful to read Introduction on p. 3 & 4 and go over Table 1-1 - "Basic units used in biochemistry" - see also my notes on Help for Units (print a separate PDF file for the Help on Units and Standard Curve or use class hand out)

What you will do in Lab: first you will make a standard curve for Biuret Protein Assay Method since it is a very linear standard curve and good example of this type of biochemical tool. Then you will make a standard curve for the Folin/Lowry Protein Assay and analyze your unknown sample to determine the protein concentration in it.

What to put in the Lab Report: Follow the standard style for all Lab Reports and put Introduction, Methods (with Experimental Design section), Results and Discussion sections. In the Results, you must have the standard curve plots for both the Biuret and the Folin/Lowry Protein Assays. You also must report the number of your unknown and its average protein concentration in mg/ml.

1. Introduction - Recalling Beer's Law

You should read the Introduction to Expt #1 and the Protocols in the text. We will not be doing the qualitative part of the expt - we will do only the quantitative photometric assays. Each student will do this lab by themselves. You will be given a numbered sample of lysozyme with an unknown concentration for you to use in this Expt. You will want to report the concentration of the protein in your unknown in mg/ml along with its number in your Lab Report for Expt #1.

A. Beer's Law Review

The basic principle underlying all quantitative photometric assays is

Beer's Law: $A = \epsilon lc$

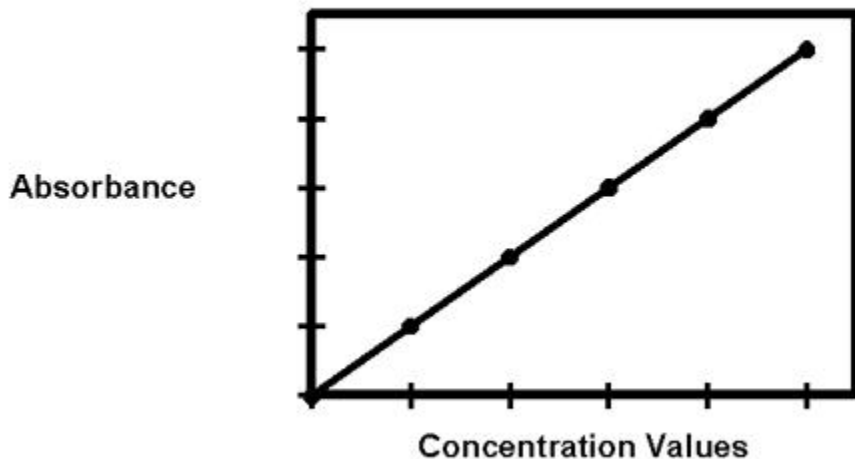
- A = absorbance (usually at the wavelength of maximum absorption)
- ϵ = Extinction Coefficient for the substance being analyzed (usually greek epsilon)
- l = path length of the cuvette (usually 1 cm)
- c = Concentration (in units related to extinction coefficient, ie. $1/\epsilon$)

If the ϵ is a molar extinction coefficient (cm/M), the concentration is M or moles/liter.

The most useful part of Beer's Law is Absorbance is proportional to concentration.

When Beer's Law applies: A plot of absorbance versus concentration will be

linear!



Plot of Absorbance Vs. Concentration - The slope of the line is the extinction coefficient (assuming $l = 1$ cm).

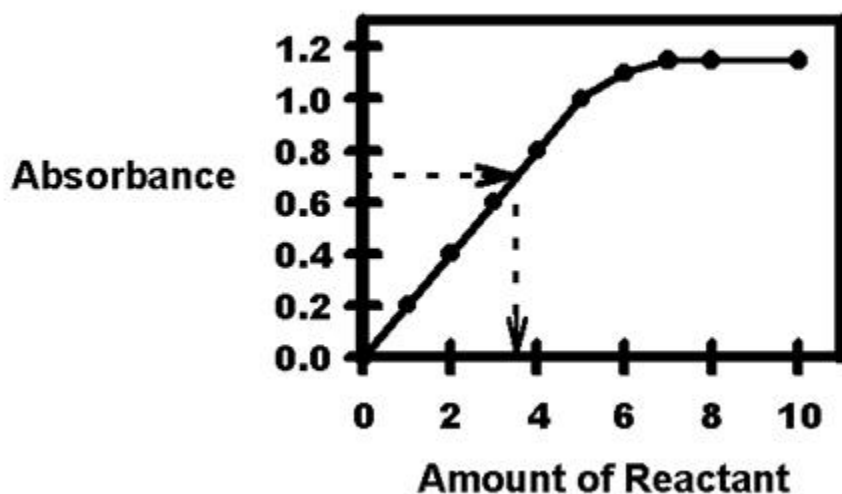
2. Standard Curves

For substances where the extinction coefficient is not known, but a pure sample or a "standard" sample is available, Beer's law can still be used by constructing a **STANDARD CURVE**. This is done by measuring the absorbance of several concentrations of the pure substance or the "standard" substance for which unknowns will be compared. This can even be done for substances which do not strongly absorb light, if the substance can be reacted with a dye which will produce a unique color when combined with the substance of interest.

For Example

- Reactant + Dye (in excess) = Colored Complex or Compound

A STANDARD CURVE can be made using known amounts of the Reactant:



Plot Used to Make the Standard Curve

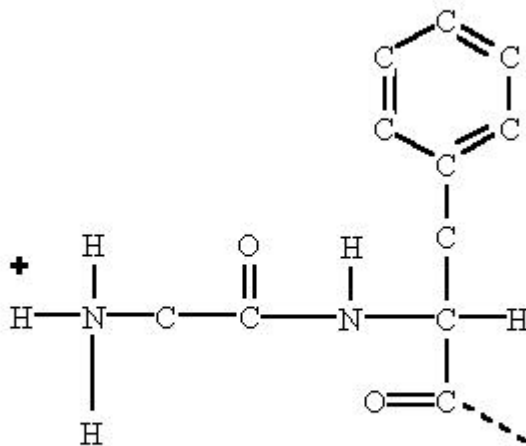
The slope of the linear part of the curve can be used like an extinction coefficient to determine the amounts of reactant in an unknown solution. There are two ways to do this:

1. Use the Absorbance of the Unknown and going along the Standard Curve find the corresponding Amount of Reactant. This is illustrated in the above Standard Curve Plot example by the dashed lines starting on the Y-Axis or Absorbance Axis and going out to intersect the Standard Curve (must be in the linear portion of the line) and then continuing down to the X-Axis or Amount of Reactant Axis. After finding the Amount of Reactant, the concentration of the reactant in the original solution can be found by dividing the amount of reactant in the sample by the volume of the sample used in the reaction that gave the absorbance of the unknown.
2. Determine the slope of the line formed by the points in the linear part of the standard curve and use the slope to determine the Amount of Reactant using the absorbances found for the unknowns. This approach is best when done using a statistical program to calculate the linear regression fit for the equation of a line to the data set used to make the Standard Curve. The linear regression draws the "best-fit" line to the data and gives you the equation of the line. The way to apply this method is discussed at the very end of the "Help Notes on Units and Standard Curve".

3. Protein Assays

How do you determine the amount of protein in an unknown solution?

First, let's discuss what a protein is. Proteins are polymers of amino acids, where the amino acid units are joined by peptide bonds:



Structure of a Protein - Only the first 2 amino acid units of the protein are shown.

Peptide bonds have partial double bond character and absorb light at 220 nm in the far UV. Proteins also often contain the amino acids with aromatic rings in the side chains (ie. Trp, Tyr & Phe) which absorb at about 280 nm in the near UV. The presence of protein therefore can be detected using its UV absorbance: all proteins can be detected by absorbance at 220 nm (but the solution must be free of other UV absorbing substances - for example in the output stream of an HPLC where the background from the buffer or mobile phase can be subtracted from the protein peaks in the output) or by absorbance at 280 nm if the protein contains an aromatic amino acid.

The absorbance at 280 nm ($A_{280\text{ nm}}$) is most useful for detecting proteins in biochemical expts. In 1934, it was shown that for a mixture of yeast proteins, the $A_{280\text{ nm}}$ for a 1 mg/ml solution was 1.0. This is a useful fact to remember and can be applied often to estimate the amount of protein present in a solution. You will use this in Expt #3 for estimating the amount of protein in the fractions you obtain when purifying the GOT enzyme.

4. Commonly Used Protein Assay Methods

The common methods for determining protein depend on using a standard protein. There is no absolute method for determining the protein concentration when you have a mixture of proteins in a solution (see part 5 for discussion of pure proteins). So in general, a specific protein which is readily available in quite pure form and not too expensive; for example many biochemical labs

use bovine serum albumin (BSA). In the expt this week in the lab, you will use both BSA and the enzyme lysozyme, which is one of the major proteins in egg white. By using a commonly available purified protein, it is easy for you and others to reproduce the results you obtain.

The following methods are in wide spread use in biochemistry:

Method	How it Works	Sensitivity Range
Biuret	Cu ²⁺ -Peptide Bond Complex	1 to 10 mg Protein
Folin (Lowry)	Heavy Metal Complex with Aromatic Amino Acids	20 to 300 µg Protein
Bradford (Bio-Rad)	Dye Reaction with Amino Group Side Chains (Lys)	1 to 100 µg Protein

Description of Methods:

- **Biuret** - This method is the most linear because its color depends on a direct complex between the peptide bonds of the protein and Cu²⁺ ion. It is not highly sensitive since the complex does not have a high extinction coefficient. You will use this method to learn making a standard curve - your Biuret Standard Curve should be nearly perfectly linear!
- **Folin (Lowry)** - The Folin assay (also called Lowry method after the scientist who discovered it and published a description of the method in 1951) is dependent on the presence of aromatic amino acids in the protein. First, a cupric/peptide bond complex is formed and then this is enhanced by a phosphomolybdate complex with the aromatic amino acids. Overall, about 10 to 50 times more sensitive than the Biuret method. The Folin Standard Curve is usually not perfectly linear, but in your expt in this class your results are usually quite linear if the assay is properly done. Many substances interfere with the Folin assay for protein.
- **Bradford** - Bradford assay is based on a blue dye (Coomassie Brilliant Blue) that binds to free amino groups in the side chains of amino acids, especially Lys. This assay is as sensitive as the Folin assay and can be done so that it is more sensitive, especially using the commercial kits available from Bio-Rad and some other chemical companies. This method can yield quite linear standard curves with BSA but often is found to be not quite linear. Few substances interfere with this assay. The assay can also be done in the presence of detergent which makes it useful for determining protein concentrations for membrane proteins.

Summary - There is no universal assay for protein mixtures. It can be expected that new methods will be developed in the future.

5. Dealing with Pure Proteins

Although it is beyond the expt you will do in the lab this week, it is important to discuss the problem of determining the amount of a protein present in solution when it has been completely purified. The methods discussed in this lecture are really only effective for mixtures of proteins. Since all these methods (Folin/Lowry or Bradford) depend on the presence of specific amino acids (aromatic or lysine) in the proteins being assay, a pure protein may differ significantly from the standard protein being used to make the standard curve. So the amount of protein found may be off by 50% or more.

This is not a simple problem to overcome. If the protein contains a prosthetic group or cofactor bound to it, for example like iron in hemoglobin, then the amount of cofactor present can be used to quantify the amount of pure protein. If the protein contains no cofactor, then its UV spectrum can be determined and the peak absorbance in the near UV used to find the protein's unique extinction coefficient. Most proteins absorb near 280 nm and the extinction coefficients of many pure proteins can be found in Biochemical Handbooks and perhaps also on some WEB sites.

A protein's extinction coefficient for its UV peak (about 280 nm) can be calculated by quantifying the amount of protein in a solution of known absorbance by doing its amino acid composition or amino acid sequence. In the old days, the protein was dialyzed against deionized water until no salt was left and then the protein dried and weighed. This of course requires a lot of protein and today, more sensitive methods are used like the quantitative results from amino acid sequencing using an automated Sequencer based on the Edman method of N-terminal sequencing.

Also print PDF file on Help for Units and Standard Curve or use the class handout for help.

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