Experiment #6a
Protein Concetration Determination

Introduction

It is often important to know the concentration of protein in your sample. For example, during the purification of an enzyme, the progress of purification may be tracked by comparing the total amount of enzymatic activity with the total amount of protein after each fractionation step. There are several conventional methods used to quantify protein concentration. Typically, they use a chromogenic assay to construct a standard curve from samples containing known amounts of a purified protein, usually bovine serum albumin (BSA). Then the amount of protein in the unknown sample is determined by comparison with the standard curve.

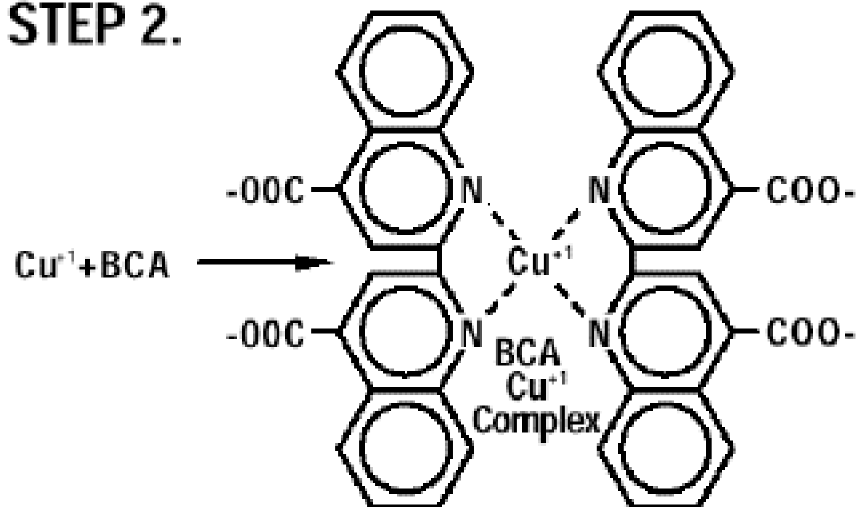
The concentration of protein can be estimated by measuring the absorbance at 280nm. This method is useful because it does not destroy the sample and is quick. As you may have noted in previous experiments, the absorbance at 280nm of the eluate from your chromatography columns was continuously measured. Most proteins have a maximum at 280 nm due to the aromatic residues: tyrosine (Y), tryptophan (W), and phenylalanine (F). Therefore, the absorptivity will vary depending upon the protein and its content of these amino acids. Furthermore, contaminants can be a problem for obtaining a good estimate of concentration. Nucleic acids absorb highly is this range; however, this can be corrected for.

The Lowry method (Lowry et. al. 1952) is one of the most commonly used methods of protein determination; it is inexpensive, easy to perform, very sensitive and highly reproducible. However, this assay suffers from serious limitations due to contaminants, standard curve is only linear at low protein concentrations, and the timing and mixing of reagents must be very precise.

The BCA reaction is similar to the Lowry reaction, except that BCA is used in place of another reagent. Copper(II) is reduced to copper(I) by protein in alkaline solution. Two molecules of BCA chelate to a cuprous ion, resulting in a purple color with absorption maximum at 562 nm. The sensitivity of the method is similar to that of the Lowry

method, but it isn't as susceptible to certain contaminants. The figure below illustrates how the BCA assay works.

STEP 1.



The binding of the dye Coomassie Brilliant Blue G-250 to proteins is also used to determine protein concentratin as it causes a shift in the adsorption maximum of the dye from 465 nm to 595 nm in acidic solutions. This dye forms strong, noncovalent complexes with proteins. The method is sensitive and accurate and a one-step procedure in which the dye reagent is added to the sample and the absorbance determined.

Experiment

1. Prepare a set of protein standards in microfuge tubes according to the table below. You may use 50 mM sodium phosphate, pH 7.0 as your diluent. **This is a very sensitive method, accurate pipetting will be critical.**

2. To prepare the working reagent you will need to mix 50 parts of BCA reagent A with 1 part of BCA reagent B. When BCA Reagent B is initially added to BCA Reagent A, precipitate is observed that quickly disappears upon mixing to yield a clear green working reagent. You will have 11 samples, and because each sample requires 2ml of working reagent you need a minimum of 22ml. It is advisable to make at least 24 ml to insure you do not run out of working reagent. Check with your TA to see if your ratio of reagent A

and B is correct.

3. Carefully pipet 0.1 ml of each standard into the appropriately labeled test tube. For use as a blank, pipet 0.1 ml of distilled water into one tube. Into the last two tubes pipet 50 μL and 100 μL of your unknown, respectively. Because our working volume of sample is 0.1 ml you will need to add into these tubes 50 μL and 0 μL of distilled water, respectively. Pipet 2.0 ml of the working reagent into 11 13x100 mm test tubes that are clean and dry.
4. Place parafilm firmly over each tube and vortex briefly for proper mixing.
5. Incubate all of the tubes in the water bath incubator for 30 minutes at 37°C.
6. Cool all tubes to room temperature and measure the absorbance at 562 nm of each tube vs. the blank you prepared. Note that the BCA reagent does not reach a true color endpoint, color development will continue even after cooling to room temperature. However, if all readings can be completed within 10 minutes, no significant error is introduced.

Name	Mix together:		Final BSA concentration (μg/mL)
	Volume of BSA solution (μL)	Volume of diluent (μL)	
-	300 of 'Stock'	0	2000
A	375 of 'Stock'	125	1500
B	325 of 'Stock'	325	1000
C	175 of 'A'	175	750
D	325 of 'B'	325	500
E	325 of 'D'	325	250
F	325 of 'E'	325	125
G	100 of 'F'	400	25

Data Analysis

Prepare a standard curve by plotting the average blank corrected reading for each BSA standard versus its concentration in (μg/ml). Using the standard curve,determine the protein concentration for each unknown sample.