Lodz University of Technology
Institute of Materials Science and Engineering

Laboratory 9
Protein assay

Instruction
for the Laboratory
of Biophysics

HR EXCELLENCE IN RESEARCH

I. THEORETICAL INTRODUCTION

This experiment is conducted to learn the principles of protein assays. This exercise introduces students to methods of determining protein concentrations and shows shows the differences between the methods to determine the amount of protein.

A protein is any of numerous, large, complex, naturally occurring molecules that is composed of one or more chains of amino acids. They are found in all animal and plant tissue as well as in viruses and are necessary for a wide variety of activities, including muscular growth and cell repair.

Proteins are also a functional component of enzymes, hormones, antibodies, etc. they are used for energy only when carbohydrates and fats are not available. An enzyme is any protein that acts as a catalyst in living organisms. A catalyst is a chemical that mediates or speeds up a specific chemical reaction without being destroyed or altered upon completion of the reaction. Proteins differ from one another primarily in their sequence of amino acids, which is dictated by the nucleotide sequence of their genes, and which usually results in folding of the protein into a specific three-dimensional structure that determines its activity.

II. EXPERIMENTAL PART

A. Standard procedure of 2-D Quant Kit

The 2-D Quant Kit is designed for the accurate determination of protein concentration in samples to be analyzed by high resolution electrophoresis techniques such as 2-D electrophoresis, SDS-PAGE or IEF. Many of the reagents used in the preparation of such samples, including detergents, reductants, chaotropes and carrier ampholytes, are incompatible with other protein assays. The procedure works by quantitatively precipitating proteins while leaving interfering substances in solution. The assay is based on the specific binding of copper ions to protein. Precipitated proteins are resuspended in a copper-containing solution and unbound copper is measured with a colorimetric agent. The color density is inversely related to the protein concentration. The assay has a linear response to protein in the range of 0–50 μg.

The procedure is compatible with such common sample preparation reagents as 2% SDS, 1% DTT, 8 M urea, 2 M thiourea, 4% CHAPS, 2% Pharmalyte™ and 2% IPG Buffer.

1. Prepare a standard curve according to Table 1 using the 2 mg/ml Bovine serum albumin (BSA) standard solution. Set up six tubes and add standard solution according to Table 1. Tube 1 is the assay blank, which contains no protein.

Table 1: Preparation of standard curve

Tube number	1	2	3	4	5	6
Volume of 2 mg/ml BSA standard solution	0 µl	5 µl	10 µl	15 µl	20 µl	25 µl
Protein quantity	0 µg	10 µg	20 µg	30 µg	40 µg	50 µg

2. Take two tubes of the sample with unknown concentration.

3. Add 500 µl precipitant to each tube (including the standard curve tubes). Vortex briefly and incubate the tubes 2–3 min at room temperature.

4. Add 500 µl co-precipitant to each tube and mix briefly by vortexing or inversion.

5. Centrifuge the tubes at a minimum of $10\,000 \times g$ for 5 min. This sediments the protein.

6. Remove the tubes from the centrifuge as soon as centrifugation is complete. A small pellet should be visible. Decant the supernatants. Proceed rapidly to the next step to avoid resuspension or dispersion of the pellets.

7. Carefully reposition the tubes in the microcentrifuge as before, with the cap-hinge and pellet facing outward. Centrifuge the tubes again to bring any remaining liquid to the bottom of the tube. A brief pulse is sufficient. Use a micropipette to remove the remaining supernatant. There should be no visible liquid remaining in the tubes.

8. Add 100 µl of copper solution and 400 µl of distilled or de-ionized water to each tube. Vortex briefly to dissolve the precipitated protein.

9. Add 1 ml of working color reagent to each tube. Ensure instantaneous mixing by introducing the reagent as rapidly as possible. Mix by inversion.

10. Incubate at room temperature for 15–20 min.

11. Read the absorbance of each sample and standard at 480nm using water as the reference. The absorbance should be read within 40 min of the addition of working color reagent

Note: Unlike most protein assays, the absorbance of the assay solution decreases with increasing protein concentration. Do not subtract the blank reading from the sample reading or use the assay blank as the reference.

12. Generate a standard curve by plotting the absorbance of the standards against the quantity of protein. Use this standard curve to determine the protein concentration of the samples.

B. Protein determination using absorbance at 280 nm, 260 nm, and 235 nm.

Determination of protein concentration by ultraviolet absorption (260 nm to 280 nm or 235 nm to 280 nm) depends on the presence of aromatic amino acids in proteins. Tyrosine and tryptophan absorb at approximately 280 nm. Higher orders of protein structure also may absorb UV light or modify the molar absorptivities of tyrosine and tryptophan, and thus the UV detection is highly sensitive to pH and ionic strength at which measurement is taken. Many other cellular components, and particularly nucleic acids, also absorb UV light. The ratio of A_{280}/A_{260} is often used as a criterion of the purity of protein or nucleic acid samples during their purification. The real advantages of this method of determining protein

concentration are that the sample is not destroyed and that it is very rapid. Although different proteins will have different amino acid compositions and thus different molar absorptivities, this method can be very accurate when comparing different solutions of the same protein.

Estimate protein concentration:
 1. Adjust wavelength to 280 nm
2. Calibrate the spectrophotometer with ddH_2O
3. Measure absorbance of the protein solution
4. Repeat point 1-3 for the wavelengths 260 nm and 235 nm
5. Calculate the protein concentration using equations:

 [Wartburg and Christian method] (mg/mL) = $1.55*A_{280} - 0.76*A_{260}$
[Whitaker and Granum method] (mg/mL) = $2.50*(A_{235} - A_{280})$

C. Bradford method:
1. Prepare 0,9% NaCl solution
    0,9%   -0,9 g +100ml ddH_2O

2. Prepare BSA solutions:
    2 mg/ml BSA   -200 mg BSA dissolve in 100 ml ddH_2O

1.	2 mg/ml	2 mg/ml
2.	0,5 ml (1) + 0,5 ml NaCl	1 mg/ml
3.	0,5 ml (2) + 0,5 ml NaCl	0,5 mg/ml
4	0,5 ml (3) + 0,5 ml NaCl	0,25 mg/ml
5	0,5 ml (4) + 0,5 ml NaCl	0,125 mg/ml
6	0,5 ml (5) + 0,5 ml NaCl	0,0625 mg/ml
7	0,5 ml (6) + 0,5 ml NaCl	0, 03125mg/ml
8	0,5 ml (7) + 0,5 ml NaCl	0,015625 mg/ml

    Generate a standard curve and equation
   3. Prepare dye solution:
        1 : 4   dye: ddH_2O
   4. Mix 1 ml of dye solution with 20 μl of BSA solution
   5. Incubate 10 min in dark
   6. Measure absorbance at 595 nm

III. REPORT
1. Show a calibration curves for a measurement method in part A and C
2. Calculate the concentration of protein using the formulas given in Part B.
3. Compare the results of protein concentration
4. Discuss possible differences in the calculated concentrations of the protein