Methods in Molecular Biology

Volume 1

PROTEINS

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Preface

In recent years there has been a tremendous increase in our understanding of the functioning of the cell at the molecular level. This has been achieved in the main by the invention and development of new methodology, particularly in that area generally referred to as "genetic engineering". While this revolution has been taking place in the field of nucleic acids research, the protein chemist has at the same time developed fresh methodology to keep pace with the requirements of present day molecular biology. Today's molecular biologist can no longer be content with being an expert in one particular area alone. He/she needs to be equally competent in the laboratory at handling DNA, RNA, and proteins, moving from one area to another as required by the problem he/she is trying to solve. Although many of the new techniques in molecular biology are relatively easy to master, it is often difficult for a researcher to obtain all the relevant information necessary for setting up and successfully applying a new technique. Information is of course available in the research literature, but this often lacks the depth of description that the new user requires. This requirement for in-depth practical details has become apparent by the considerable demand for places on our Molecular Biology Workshops held at Hatfield each summer. This book is therefore an attempt to provide detailed protocols for many of the basic techniques necessary for working with DNA, RNA, and proteins. This volume gives practical procedures for a wide range of protein techniques. A companion volume (Volume 2) provides coverage for nucleic acids techniques. Each method is described by an author who has regularly used the technique in his or her own laboratory. Not all the techniques described necessarily represent the state-of-the-art. They are, however, dependable methods that achieve the desired result.

Each chapter starts with a description of the basic theory behind the method being described. However, the main aim of this book is to describe the practical steps necessary for carrying out the method successfully. The Methods section therefore contains a detailed step-by-step description of a protocol that will result in the successful execution of the method. The Notes section complements the Methods section by indicating any major problems or faults that can occur with the technique, and any possible modifications or alterations.

This book should be particularly useful to those with no previous experience of a technique, and, as such, should appeal to undergraduates (especially project students), postgraduates, and research workers who wish to try a technique for the first time.

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Chapter 1

The Lowry Method for Protein Quantitation

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Introduction

The most accurate method of determining protein concentration is probably acid hydrolysis followed by amino acid analysis. Most other methods are sensitive to the amino acid composition of the protein and absolute concentrations cannot be obtained. The procedure of Lowry et al. (*1*) is no exception, but its sensitivity is moderately constant from protein to protein, and it has been so widely used that Lowry protein estimations are a completely acceptable alternative to a rigorous absolute determination in almost all circumstances where protein mixtures or crude extracts are involved.

Materials

1. *Complex-forming reagent*: prepare immediately before use by mixing the following 3 stock solutions A, B, and C in the proportion 100:1:1, respectively.

Solution A: 2% (w/v) Na_2CO_3 in distilled water
Solution B: 1% (w/v) $CuSO_4 \cdot 5H_2O$ in distilled water
Solution C: 2% (w/v) sodium potassium tartrate in distilled water

2. *2N NaOH*
3. *Folin reagent* (commercially available): Use at 1*N* concentration.
4. *Standards*: Use a stock solution of standard protein (e.g., bovine serum albumin fraction V) containing 4 mg/mL protein in distilled water stored frozen at − 20°C. Prepare standards by diluting the stock solution with distilled water as follows:

Stock solution	μL	0	1.25	2.50	6.25	12.5	25.0	62.5	125	250
Water	μL	500	499	498	494	488	475	438	375	250
Protein concentration	μg/mL	0	10	20	50	100	200	500	1000	2000

Method

1. To 0.1 mL of sample or standard, add 0.1 mL of 2*N* NaOH. Hydrolyze at 100°C for 10 min in a heating block or a boiling water bath.
2. Cool the hydrolyzate to room temperature and add 1 mL of freshly mixed complex-forming reagent. Let the solution stand at room temperature for 10 min.
3. Add 0.1 mL of Folin reagent, using a Vortex mixer, and let the mixture stand at room temperature for 30–60 min (do not exceed 60 min).
4. Read the absorbance at 750 nm if the protein concentration was below 500 μg/mL or at 550 nm if the protein concentration was between 100 and 2000 μg/mL.
5. Plot a standard curve of absorbance as a function of initial protein concentration and use it to determine the unknown protein concentrations.

Notes

1. If the sample is available as a precipitate, then dissolve the precipitate in 2*N* NaOH and hydrolyze as in step 1. Carry 0.2 mL aliquots of the hydrolyzate forward to step 2.
2. Whole cells or other complex samples may need pretreatment, as described for the Burton assay for DNA (See Vol. 2). For example, the PCA/ethanol precipitate from extraction I may be used directly for the Lowry assay or the pellets remaining after the PCA hydrolysis (step 3 of the Burton assay) may be used for Lowry. In this latter case, both DNA and protein concentrations may be obtained from the same sample.
3. Rapid mixing as the Folin reagent is added is important for reproducibility.
4. A set of standards is needed with each group of assays, preferably in duplicate. Duplicate or triplicate unknowns are recommended.

References

1. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.

Chapter 2

Determination of Protein Molecular Weights by Gel Permeation High Pressure Liquid Chromatography

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Introduction

An essential part of the characterization of any protein is the determination of its molecular weight. The method of choice for these determinations, because of its simplicity and rapidity, has most frequently been sodium dodecyl sulfate (SDS) gel electrophoresis (*See* Chapter 6). The alternative method of gel filtration under denaturing conditions (*1,2*) has not been so widely used, in part as a result of the longer times required for a single run. How-

ever, recent developments in gel filtration supports for high pressure liquid chromatography (HPLC) have made more rapid separations possible, and thus gel permeation HPLC is becoming a widely used technique for molecular weight determinations (*3,4*). Gel permeation HPLC, in addition to taking less time than SDS gel electrophoresis, allows easier quantitation and recovery of separated proteins, and the resolution is better than that achieved by gel filtration with conventional materials.

The volume accessible to a protein in gel filtration supports depends on both its size and shape. Thus in order to determine molecular weight the sample protein must have the same shape as the proteins used for calibration. In the presence of denaturants, such as 6*M* guanidine hydrochloride, all proteins in their reduced state adopt a linear random coil conformation whose molecular radius is proportional to molecular weight (*5*). Under these conditions the molecular weight of a protein can be expressed in terms of its elution volume from the column (*1–3*).

Denaturation causes an increase in the intrinsic viscosity of the protein, and hence an increase in its molecular dimensions. Thus, under denaturing conditions the molecular weight exclusion limits of gel filtration matrices are lower than those in the absence of denaturant. Of the column supports used for gel permeation HPLC under denaturing conditions those of the TSK-G3000 SW type are suitable for proteins of less than 70,000 molecular weight (mw), whereas the TSK-G4000 SW type can be used for proteins up to 160,000 mw.

Gel permeation HPLC in guanidine hydrochloride has proved to be a reliable and convenient method for accurate molecular weights determinations (*3,4*). A wide range of sensitivities can be covered, from femtomolar to nanomolar amounts. Because of the high absorbance of guanidine hydrochloride at < 220 nm, the eluate cannot be monitored at this wavelength, therefore proteins in the nanomolar range are detected by absorbance at 280 nm. The sensitivity of detection can be increased by incorporating radioactive label into the protein, for instance by reduction and [^{14}C]-carboxymethylation of cysteine residues.