Topic Introduction

Methods for Measuring the Concentrations of Proteins

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Determining the concentration of protein samples generally is accomplished either by measuring the UV absorbance at 280 nm or by reacting the protein quantitatively with dyes and/or metal ions (Bradford, Lowry, or BCA assays). For purified proteins, UV absorbance remains the most popular method because it is fast, convenient, and reproducible; it does not consume the protein; and it requires no additional reagents, standards, or incubations. No method of protein concentration determination is perfect because each is subject to a different set of constraints such as interference of buffer components and contaminating proteins in direct UV determination (A_{280}) or reactivity of individual proteins and buffer components with the detecting reagents in colorimetric assays. In cases in which protein concentration is critical (e.g., determination of catalytic rate constants for an enzyme), it may be advisable to compare the results of several assays.

A_{280} OF A GIVEN PROTEIN

An absorbance maximum near 280 nm (A_{280}) in the UV spectra of protein solutions is primarily due to the presence of aromatic tryptophan and tyrosine side chains (see Box 1). The A_{280} of a given protein solution is proportional to its concentration once corrected for the number, type, and environment of its amino acids. For most purposes, a sufficiently accurate extinction coefficient can be calculated from the amino acid sequence (Edelhoch 1967; Gill and von Hippel 1989; Pace et al. 1995), either manually as described in the box or using online tools (such as https://web.expasy.org/protparam/). In general, a 1 mg/mL solution of most proteins has an A_{280} of roughly 1 ± 0.6. For example, A_{280} values for 1 mg/mL solutions of some common protein standards are bovine serum albumin (BSA), 0.63; bovine, human, or rabbit IgG, 1.38; chicken ovalbumin, 0.70 (Fasman 1992); and 2.0 for the *Schistosoma japonicum* GST produced by most fusion vectors (GE Healthcare).

Measurements can be performed in a standard spectrophotometer with quartz or methacrylate cuvettes; glass and polystyrene cuvettes absorb at 280 nm and are not suitable. It is useful to scan the sample over a range of wavelengths (~230–310 nm) to monitor the quality of the spectrum and confirm that the wavelength of maximum absorbance of the protein solution occurs near 280 nm. Before measuring the spectrum of the protein solution, a matching buffer is scanned as a blank or baseline to correct for background absorbance of the spectrophotometer setup and buffer components. Most spectrophotometers automatically subtract the baseline from subsequent sample spectra. For accurate sample measurement, it is important to adjust the protein concentration to an absorbance value within the linear dynamic range of the instrument. This exact range depends on the instrument quality, slit width, and presence of attenuators; 0.1–1.0 absorbance units may be considered a safe range for most spectrophotometers, although many high-quality instruments claim to achieve much broader ranges (e.g., 0.001 to >3.0 for the Cary 4000 from Varian). Dilute samples

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BOX 1. CALCULATING PROTEIN EXTINCTION COEFFICIENTS

Each protein has a distinct UV spectrum and extinction coefficient (ε_{280}) based on its amino acid composition (Gill and von Hippel 1989). Major contributions arise from aromatic tryptophan and tyrosine residues with high extinction coefficients of 5500 and 1490 M^{-1} cm^{-1} (Ward 1923; Pace et al. 1995). In contrast, phenylalanine absorbs maximally at 260 nm but rather little at 280 nm (Ward 1923). Cystine (in the disulfide form) has a relatively low extinction coefficient of 125 M^{-1} cm^{-1} at 280 nm (Pace et al. 1995), whereas absorbance by reduced cysteine is negligible at wavelengths above 260 nm; thus, UV absorbance by cystine is diminished by the presence of reducing agents. Thus,

$$\varepsilon_{280} = (n_{Trp}) \times 5500 + (n_{Tyr}) \times 1490 + (n_{Cys}) \times 125, \quad (1)$$

where ε_{280} is the molar extinction coefficient at 280 nm, and n is the number of corresponding residues present in the protein. And

$$\text{Molar concentration} = A_{280} \times (\text{dilution factor})/\varepsilon_{280}, \quad (2)$$

$$\text{Concentration (in milligrams per milliliter)} = A_{280} \times (\text{dilution factor}) \times (\text{molecular weight in daltons})/\varepsilon_{280}. \quad (3)$$

should be concentrated (see Box 2), and more concentrated samples must be diluted before reliable UV measurements can be made. Accurate UV spectra of protein solutions also depend on the absence of interfering substances that absorb at 280 nm. These may include nucleic acids (DNA, RNA) or nucleotides (e.g., ATP, GTP), many small molecules (e.g., imidazole, nicotinamide adenine dinucleotide [NADH]), certain detergents (e.g., Triton X-100, Nonidet P-40), or proteins with prosthetic groups (e.g., heme) that absorb in the near-UV range. Most standard spectrophotometers require relatively large sample volumes of 0.5–1 mL for measurement, although specially designed cuvettes (e.g., ~10–100 µL for Sub-Micro Spectrophotometer Cells from Starna Scientific) or instruments (e.g., 0.5–2.0 µL for NanoDrop from Thermo Scientific) are amenable to smaller sample volumes (Desjardins et al. 2009).

BOX 2. CONCENTRATING PROTEIN SOLUTIONS

After the initial protein concentration is determined, it may be necessary to further increase the concentration of the proteins in solution for downstream applications or storage. This can be achieved by several techniques, including centrifugal concentration (a form of ultrafiltration), ammonium sulfate precipitation, and lyophilization. The main objective in concentrating protein is to decrease the volume of the sample by removing water (and in some cases, soluble salts, buffers, and other small molecules) while minimizing protein loss and retaining an active protein conformation. For these reasons, the optimal concentration method will depend on the specific characteristics of the target protein.

Salting-Out Proteins

One of the earliest methods for concentrating proteins was derived from the work of Hofmeister and coworkers in the development of the Hofmeister series (Hofmeister 1888). This series represents an analysis and categorization of ions according to their effects on the solubility of proteins, which ranged from precipitation to increased solubility ("salting-in"). Using this categorization, Hofmeister concluded that the salt's affinity for water deprives the protein of the solvating water molecules necessary to keep it in solution. It was later determined that the extent of protein precipitation also depends on the amount of salt present, the concentration of the protein, the pH, and the temperature (Chick and Martin 1913). More than a century later, high concentrations of salts (mainly ammonium sulfate) still are used as a means to purify and concentrate proteins through precipitation (Kent 1999; Burgess 2009; Moore and Kery 2009).

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BOX 2. *Continued*

Ammonium sulfate is the salt of choice for "salting-out" proteins because it is effective and highly soluble, has a versatile pH range and a low heat of solution, and is inexpensive. Precipitation usually is achieved by slowly adding solid or a saturated aqueous solution (~4.1 M at room temperature) of ammonium sulfate, while stirring the protein solution on ice. To use ammonium sulfate fractionation for partial purification of a protein of interest, ammonium sulfate is initially added until the majority of undesired proteins precipitate while leaving most of the target protein in solution. After the precipitated contaminants are removed by centrifugation, addition of ammonium sulfate to the remaining solution continues until a concentration is reached that precipitates the target protein. The fractional saturation of ammonium sulfate required for precipitation varies widely among different proteins and must be determined empirically. Because the addition of ammonium sulfate changes the final volume, and hence the saturation of the solution, it is most convenient to refer to tables (Englard and Seifter 1990; Burgess 2009) or online calculators (http://www.encorbio.com/protocols/AM-SO4.htm) to determine how much ammonium sulfate is needed to achieve the desired saturation. The final precipitated protein is resuspended in buffer, and residual ammonium sulfate is removed by dialysis, ultrafiltration (mentioned below), or with a desalting column. Although ammonium sulfate precipitation is often reversible, it is not guaranteed that the structure or function of the target protein will not be affected. If integrity of the protein is adversely affected by ammonium sulfate precipitation (as determined by an activity assay), other options should be explored.

Freeze-Drying or Lyophilization

Perhaps one of the most straightforward protein concentration techniques is freeze-drying or lyophilization. This process extracts water from a frozen sample (through sublimation and desorption) under a vacuum (for review, see Gatlin and Nail 1994; Roy and Gupta 2004) to partial or complete dryness. Samples that are to be freeze-dried often need to be supplemented with additives. During the initial freezing stages, cryoprotectants (such as ethylene glycol or glycerol, etc.) can be used to protect the protein from denaturation, but lyoprotectants (sugars such as sucrose, mannitol, or trehalose, etc.) are needed to prevent protein damage caused during the drying process (Ward et al. 1999; Chang et al. 2005). In the absence of cryoprotectants and lyoprotectants, the native secondary structure of the protein has been shown to be altered in both the frozen and freeze-dried states (Schwegman et al. 2007).

The presence of freeze-drying protectants can prevent large conformational changes (caused by α-helical and unstructured regions changing to β-sheet secondary structures) and aggregation, but suitable lyoprotectants must be identified and optimized empirically. Another drawback to freeze-drying is that the nonvolatile salts and buffers in the protein solution are concentrated along with the proteins. This may require that an additional buffer exchange step be added to the protocol or that only volatile buffers be used. Although freeze-drying may be suitable in cases in which structural integrity is not paramount (e.g., production of antigens, denatured proteins), more sophisticated methods, such as ultrafiltration, are typically preferred.

Ultrafiltration

Ultrafiltration is far gentler on proteins and is more efficient because it simultaneously concentrates the protein and removes small molecules in the solutes. This process is a variation of membrane filtration that uses pressure (generated by a mechanical pump, pressurized gas, or centrifugation) to force a liquid sample against a semipermeable membrane. Selective membrane permeability is controlled by the pore size or molecular weight cutoff (MWCO) of the membrane. Molecules that are larger than the MWCO are retained upstream of the filter in the retentate, while water, salts, and molecules that are smaller than the MWCO flow through the membrane to form the filtrate.

Ultrafiltration devices come in several different formats (including pressurized ultrafiltration cells or centrifugal devices), but the design of choice is often a centrifugal concentrator because they are quick, efficient, and compatible with standard centrifuges. Several companies now manufacture conical tubes coupled to semipermeable membranes (Amicon centrifugal filters; Millipore, Vivaspin ultrafiltration spin columns; Sartorius Stedim Biotech) of various sizes and MWCOs (2–300 kDa) to facilitate the concentration of a broad range of sample volumes and protein sizes.

In general, ultrafiltration techniques produce more predictable yields than traditional methods without requiring the addition of any stabilizing or precipitating agents. Repeated concentration and dilution of the protein with a new buffer also can be used to exchange the buffer of the solution. However, proteins

BOX 2. *Continued*

concentrated by centrifugal concentration sometimes aggregate or bind to the membrane during concentration (Boyd and Zydney 1998). Protein aggregation has been reported to result from rapid supersaturation and high solvent velocity (generating shear forces) in the sample immediately adjacent to the membrane surface (Kim et al. 1993). The shear forces created by the solvent flow at the membrane surface can unfold protein molecules and promote nonspecific binding to the membrane or other particles. To prevent a supersaturated layer from forming at the membrane, samples should be centrifuged for relatively short durations (~5 min) with thorough but gentle mixing between centrifugations. If loss of protein during concentration is significant, there are other measures that can be taken. These may include stopping the process at a lower protein concentration, pretreating the membrane with 5% Tween 80 or 5% benzalkonium chloride (Lee et al. 2003), or using another method to concentrate the protein.

COLORIMETRIC ASSAYS

Colorimetric assays such as the Bradford, BCA, or Lowry protein assays that measure absorbance >550 nm (see below) are preferred, given these considerations, for determining the concentrations of partially purified, lower-abundance proteins or cell lysates. Color changes of the sample are monitored using a spectrophotometer set at an appropriate wavelength and compared with those of a dilution series (standard curve) of a control protein with a known concentration (usually BSA or IgG, sometimes ovalbumin). If available, standard curves generated using the protein of interest at known concentrations are more accurate, because color yields often vary among individual proteins (Pierce and Suelter 1977; van Kley and Hale 1977). All of these assays provide a reasonable estimate of the total protein content of a solution, although each is subject to its own limitations in terms of protein concentration, incubation time, and interference from buffer components.

Bradford Assay

The Bradford assay (Bradford 1976) is a quick (~10 min) and fairly sensitive method based on the shift in absorbance maximum from 465 to 595 nm of Coomassie Brilliant Blue G-250 dye following binding to denatured proteins in solution. The method is relatively sensitive, with a linear range of ~1–20 µg for an assay volume of 1 mL. The results of the assay can vary considerably among individual proteins because of differences in the degree of dye binding. Compared with other colorimetric assays, relatively few buffer components interfere with the Bradford method (Compton and Jones 1985). For example, the reducing agents DTT and β-mercaptoethanol are compatible at commonly used concentrations (respectively, up to 5 mM or 1 M). Detergents are an exception; for example, relatively low concentrations of >0.125% (v/v) Triton X-100 or >0.06% (v/v) Tween 20 interfere with the Bradford assay, compared with the common use of these detergents at ~1% (v/v) in protein solutions. Given these advantages, this method is described in detail in Protocol: **Bradford Assay for Determining Protein Concentration** (Kielkopf et al. 2020).

Lowry Assay

The Lowry assay is based on reduction of copper ion from Cu^{2+} to Cu^{1+} in a manner that depends on the number of peptide bonds as well as the tyrosine and tryptophan content of the protein (Lowry et al. 1951). The oxidized Cu^{1+} produces a colored blue product through reaction with a phosphotungstic acid and phosphomolybdic acid mixture in phenol, called the Folin's phenol or Folin–Ciocalteu reagent. This product is detected by its absorbance at 750 nm and shows less variability among different proteins than the Bradford assay. The Lowry assay is quite sensitive, with a linear range of 1–100 µg for an assay volume of 1 mL. However, a larger number of substances interfere with this assay than with the Bradford method (e.g., low concentrations of detergents, Tris, EDTA, sugars, and alcohols) (Peterson 1983). Many of these interfering substances can be

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removed with a precipitation step before the assay, but this further lengthens an already relatively slow protocol (~40 min of incubation).

BCA Assay

The bicinchoninic acid assay, or more commonly the BCA assay (Smith et al. 1985), is a variation on the Lowry assay that is susceptible to fewer interfering substances. The purple-colored reaction product (detected at 562 nm) is formed by chelating of the reduced Cu^{1+} by two BCA molecules. The BCA method is not a true end-point method; that is, the color continues to develop indefinitely, but at a sufficiently slow rate at room temperature to allow even large numbers of samples to be assayed in concert. Accordingly, the linear range can be manipulated from 1 to 30 µg for a 30-min incubation at 37°C to 0.1–10 µg for a 2-h incubation at 50°C for an assay volume of 1 mL. This assay tolerates detergents well and is the preferred method for the detection of membrane proteins, which usually are dissolved in 1% SDS before color development. Reducing agents (DTT, β-mercaptoethanol), chelating agents, and strong acids or bases interfere with Cu^{2+} reduction and should be equivalent between buffer controls, standards, and protein samples.

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