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Application Note

Fluorometric Quantitation of Protein using the Reactive Compound Fluorescamine

Total protein content is a measurement common to many applications in basic science and clinical research. Several different fluorescent techniques are available that eliminate many of the problems associated with the traditional absorbance-based colorimetric methods to measure total protein content. Here we describe the use of fluorescamine to quantitate total protein using the BioTek FL600 fluorescence microplate reader.

Introduction

Quantitation of total protein content is a measurement common to many applications in basic science and clinical research. Most biochemical studies that involve the measurement of a biological activity require the normalization of that activity to the protein content. For instance, the specific activity of an enzyme is of particular importance when proteins are being purified or different samples are being compared. Regardless of the method of protein determination, laboratories requiring high throughput have often adapted the described protocol to a 96-well microplate based format.

Over the years, many different absorbance-based colorimetric methods to quantify protein have been developed, the most utilized of which rely on the reduction of copper in the presence of a chromogenic reagent (1, 2). While these methods work well, they are subject to interference by many compounds commonly used in protein purification, namely detergents and reducing agents. Alternatively, simple absorbance measurements of protein solutions at 280 nm (A280) can be performed, but are subject to interference from any nucleic acid contamination. In response to these difficulties, dye-binding protein assays were developed, the most commonly used being the method described by Bradford (3). This assay is subject to the formation of aggregates leading to a loss of signal over time.

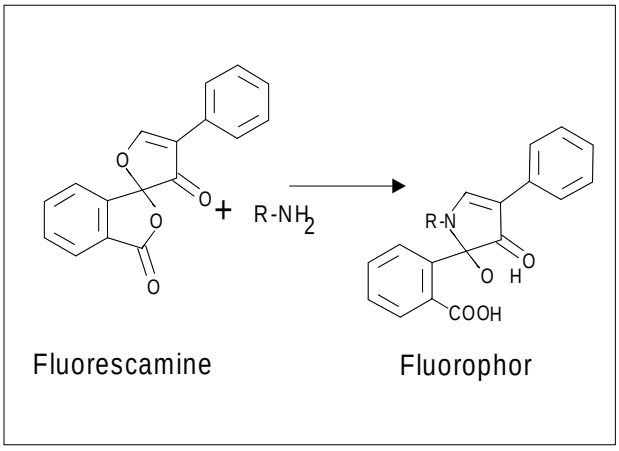

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Figure 1. Reaction of fluorescamine and primary amino groups. Fluorescamine reacts with the primary amino groups found in terminal amino acids and the e amine of lysine to form fluorescent pyrrolinone type moieties.

Several fluorescent assays for protein quantitation have been developed in an attempt to alleviate the difficulties presented when using absorbance based assays. One such fluorescence-based assay, first described by Udenfriend (4), is based on the rapid reaction of fluorescamine (4-phenyl-spiro [furan-2(3H), 1¢-phthalan] -3,3¢ -dione) with proteins. The non-fluorescent compound, fluorescamine, reacts rapidly with primary amines in proteins, such as the terminal amino group of peptides and the e-amino group of lysine, to form highly fluorescent moieties (Figure 1).

Here we describe a fluorescence assay using fluorescamine to quantitate total protein in the 96-well microplate based format using the BioTek FL600 fluorescence microplate reader in conjunction with KC4 data reduction software.

Materials and Methods

Fluorescamine, BSA protein standard, phosphate buffer saline (PBS) powder packets (catalogue number 1000-3), and acetone (HPLC grade) were obtained from Sigma Chemical Company (St Louis, MO). The 96-well black microplates with clear bottoms, catalogue number 3603, were purchased from Costar, (Cambridge, MA).

The protein assay was performed according to Udenfriend (4), and modified for microplates as described by Lorenzen (5). A series of dilutions of Bovine Serum Albumin (BSA) ranging from 0 to 1000 mg/ml were made using phosphate buffered saline (PBS) pH 7.4 as the diluent. After dilution, 150 ml aliquots of samples and standards were pipetted into microplate wells in replicates of eight. The microplate was placed on a microplate shaker and 50 ml of 10.8 mM (3mg/ml) fluorescamine dissolved in acetone was added to each well. Following the addition of fluorescamine the plate was shaken for one minute. The fluorescence was then determined using a FL600 fluorescence plate reader (BioTek Instruments, Winooski, VT) with a 400 nm, 30 nm bandwidth, excitation filter and a 460 nm, 40 nm bandwidth emission filter. The sensitivity setting was at 29 and the data collected from the bottom with a 5 mm probe using static sampling with a 0.35 second delay, 50 reads per well

Results

As demonstrated in Figure 2, protein concentration shows a direct correlation with fluorescence. The fluorescence intensity was determined for BSA protein concentrations ranging from 0.0 to 1000 mg/ml. Over this range the fluorescence intensity increased in a hyperbolic fashion. Using KC4 data reduction software (BioTek Instruments), a 4-parameter nonlinear equation describing the standard curve was generated. Although the curve begins to plateau at a protein concentration of 500 mg/ml (Figure 2), determinations can be made with a high level of confidence ($r^2 = 0.999$). Determinations in the middle portion of the curve offer the greatest degree of accuracy with a 4-parameter logistic fit due to the greater change in signal verses change in protein concentration.

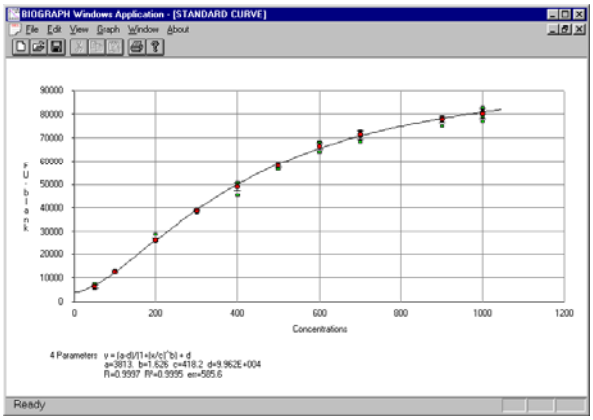

Figure 2. Analysis of the assay. Concentration curve from 0.0 to 1000 mg/ml of BSA with 4-parameter regression analysis. Circles represent the mean of eight determinations with the 0 mg/ml blank subtracted. Note that the equation describing the regression curve is provided along with statistics concerning the curve.

When lower protein concentrations (0-500 mg/ml) are examined the reaction was found to be linear (Figure 3). Using a least means squared regression analysis a straight line can be generated and utilized for the determination of protein concentrations. This allows for easy determination of an equation describing the standard curve. Routine dilution of each sample would be expected to provide determinations at an appropriate concentration.

In these experiments the plates were read immediately, but if they remained sealed and protected from light, the reaction was found to be stable for several hours. Similar results were also obtained when data was collected from the top. However, when data was collected from the top, proper adjustment of the probe to bring the detector as close to the sample as possible was necessary for best results.

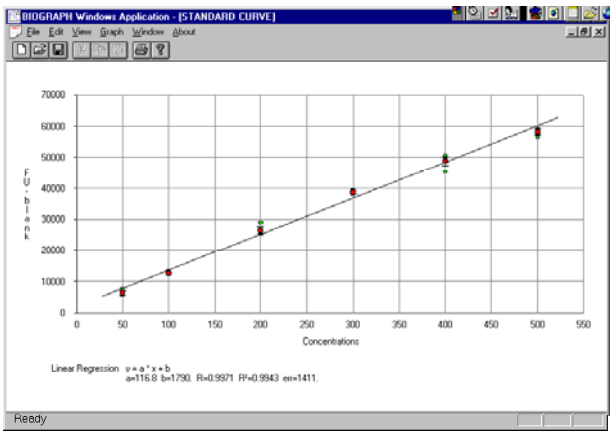

Figure 3. Analysis of low BSA concentrations. Linear regression analysis was performed on BSA concentrations of 0 to 500 mg/ml using the data presented in figure 2. Note that the equation describing the regression curve is provided along with statistics concerning the curve.

Discussion

This report demonstrates that ability to quantitate total protein from samples using fluorescamine in a 96-well format. The easy addition of one reagent with a short incubation time allows for rapid determination of samples. The non-fluorescent compound fluorescamine reacts with primary amino groups in a matter of milliseconds to produce a fluorescent product, while unreacted fluorescamine hydrolyzes in a matter of seconds to non-fluorescent products effectively removing it from the reaction.

Due to its poor water solubility and high reactivity, fluorescamine must be added to the assay samples dissolved in water miscible nonhydroxylic solvent. Acetone has been found to be particularly suitable due to the commercial availability of grades low in fluorogenic impurities (6). Other solvents that have been found to be acceptable include dioxane, dimethylsulfoxide, tetrahydrofuran, and acetonitrile (6). In some instances, acetonitrile has the added advantage of being used to stop enzymatic reactions when multiple fluorometric assays are being performed simultaneously.

The use of nonaqueous solvents to dissolve fluorescamine requires that solvent resistant materials be used. This presents a set of unique problems when using many microplate accessories. Commonly used polystyrene disposable pipettes and reagent troughs are unacceptable due to their low solvent resistance characteristics. Glass or polypropylene materials, with their much higher resistance to many organic solvents, are much more acceptable. The dilution of the solvent obtained when adding the fluorescamine to the protein sample (1:4) is adequate to prevent the microplate from dissolving.

Several factors can influence the reactivity of fluorescamine to proteins. The use of a buffer with a basic pH results in greater fluorescence. Fluorescamine fluorophors emit maximally at pH 9 and primary amino groups are more likely to be protonated and thus more reactive in pH levels above pH 7 (6). Fortunately, less than optimal pH levels including those around the physiologic range (pH 6-9) provide quite acceptable results. Many buffer systems in the pH range of 6-9, such as PBS or sodium borate, are suitable for this reaction; however, they should not contain amines (e.g., Tris or glycine). Hydroxylic solvents such as methanol or ethanol have been found to react with fluorescamine to form addition products and should be avoided as they drastically reduce the reactivity of fluorescamine toward primary amines (6, 8).

The assay described in this report relies on the interaction of fluorescamine and primary amines to form a fluorescent moiety. Primary amines exist in proteins at the amino terminus and at the e-position of the amino acid, lysine. Although all proteins contain an amino terminus, they have varying amounts of lysine residues depending on the protein and as such, different proteins would be expected to return different amounts of fluorescent signal from equivalent amounts of protein. Although it is optimal to use a calibration curve generated from the protein to be measured, this may not always be possible or warranted. However, in order to make comparisons between assays, it is important to be consistent with the protein used to make the calibration curve.

The use of KC4 software to control the reader allows the user a great deal of flexibility in regards to data reduction capabilities. The software allows the user to define any configuration of plate map necessary. With several different curve fit algorithms to choose from, regression analysis of the standards and the subsequent concentration determinations of samples can be accomplished with a high degree of confidence. Likewise, the software is capable of performing statistical analysis on sample groups, as well as any mathematical calculation required by the user.

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