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Application Note

Quantitation of Total Protein using OPA

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 the compound o-phthaldialdehyde (OPA) 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.

Several fluorescent assays for protein quantitation have been developed in an attempt to alleviate the difficulties presented when using absorbance-based assays. The compound, o-phthaldialdehyde (OPA) in conjunction with reduced sulfhydryl groups reacts with primary amines to form fluorescent moieties (Figure 1). Although OPA reacts in the same fashion as fluorescamine, it has the advantages of being more water soluble and having greater sensitivity (6). Here we describe a fluorescent assay with (OPA) 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.

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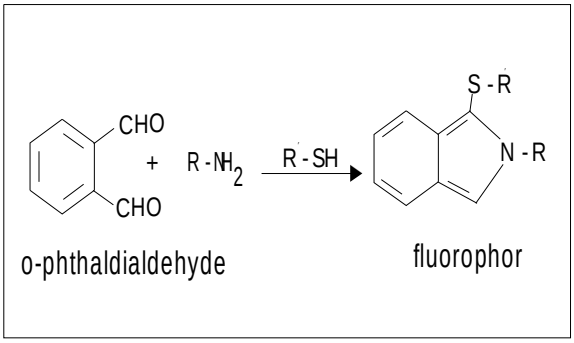

Figure 1. Reaction of OPA and primary amino groups. o-Phthaldialdehyde, in the presence of reduced sulfhydryl groups, reacts with the primary amino groups found in terminal amino acids and the e-amino group of lysine to form fluorescent moieties.

Materials and Methods

o-Phthaldialdehyde reagent solution (incomplete) catalogue no. P-7914, b-mercaptoethanol, and PBS powder packets (catalogue no. 1000-3) 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) and clear 96 well microplates catalogue number CFCPN9650 were obtained from PerSeptive Biosystems.

A series of dilutions ranging from 0.0 to 1000 µg/ml of Bovine Serum Albumin (BSA) were made using phosphate buffered saline (PBS) pH 7.4 as the diluent. Samples and standards were placed in microplate wells (10 µL per well) and 300 µL of OPA reagent solution (complete) was added to each well. OPA reagent solution (complete) was made by the addition of 0.2 ml of ß-mercaptoethanol to 100 ml of OPA reagent solution (incomplete) immediately prior to use. Samples were allowed to incubate for 2 minutes with moderate shaking at room temperature and the fluorescence was determined using a FL600 fluorescent plate reader with a 360 nm, 40 bandwidth excitation filter and a 460 nm, 40 nm bandwidth emission filter. The sensitivity setting was at 38 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.

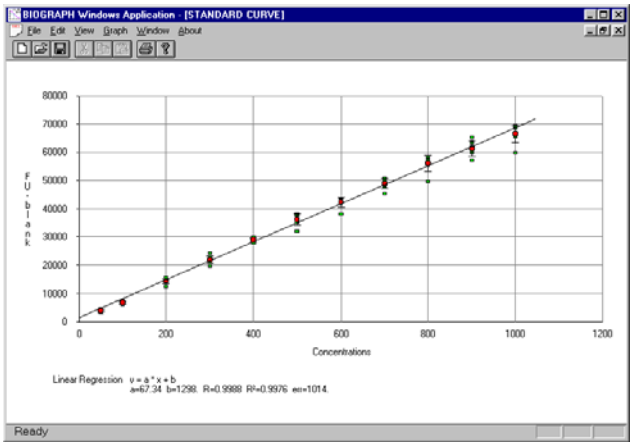

Figure 2. Linearity of the Assay. Concentration curve from 0.0 to 1000 µg/ml of BSA with linear regression analysis. Data indicated has been corrected by subtraction of the 0 µg/ml blank. Note that the equation describing the regression curve is provided along with statistics concerning the curve.

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 µg/ml. Over this range the fluorescence intensity increased in a linear fashion. Using KC4

data reduction software (BioTek Instruments), least means squared linear equation describing the standard curve was generated. The high correlation coefficient (r2=0.999) indicates that determinations can be made with a high level of confidence over the entire concentration range tested. The average coefficient of variance (% CV) of the standards was less than 6%, with the greatest percent variation-taking place in the lowest protein concentrations tested (data not shown).

In terms of sensitivity, the assay was found to be sensitive to the nanogram level. The lowest concentration tested (50 µg/ml) representing 500 ng per well, was found to be statistically different ($P<0.0001$) from the PBS only blank. When lower concentrations of protein were examined, fluorescent response remained linear in regards to protein concentration (Figure 3). Concentrations as low as 7.8 µg/ml were found to be statistically different from the 0 µg/ml control. This represents less than 80 ng per well when sample volume is accounted for.

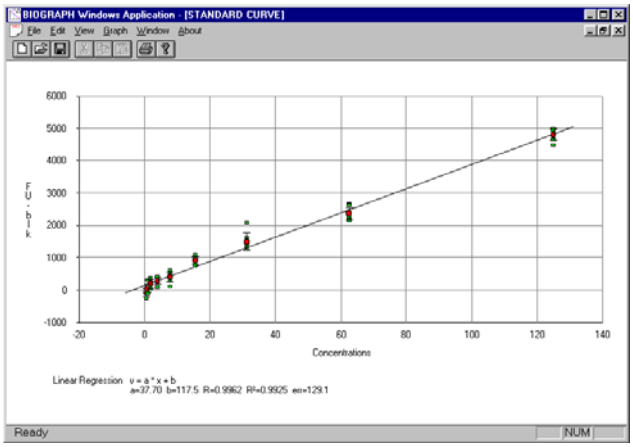

Figure 3. Linearity of low protein concentrations. Concentration curve from 0.0 to 125 µg/ml of BSA. Samples were assayed using low background fluorescence microplates from PerSeptive Biosystems and linear regression analysis performed. Data indicated has been corrected by subtraction of the 0 µg/ml blank.

Discussion

This report demonstrates that ability to quantitate total protein from samples using reactive compound o-phthaldialdehyde in a 96-well format. The easy addition of one reagent with a short incubation time allows for rapid determination of samples. o-Phthaldialdehyde reacts with primary amino groups in a matter of seconds to produce a highly fluorescent product.
The assay described in this report relies on the interaction of OPA 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. In order to make comparisons between assays, it is important to be consistent with the protein used to make the calibration curve.

Several factors can influence the reactivity of OPA to proteins. The use of a buffer with a basic pH (pH 9.0 is optimal) results in greater fluorescence as primary amino groups are more likely to be protonated and thus more reactive. 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). For these experiments PBS with a pH of 7.4 was used as a buffering agent.

The accessibility and availability of lysine residues in native proteins can also affect reactivity. The use of SDS and b-mercaptoethanol in the reagent effectively solubilizes most proteins and allowing access to all non-protected lysine residues. More samples that are resistant can be solubilized by boiling in SDS and b-mercaptoethanol prior to addition of the reagent (8). The e-amino position of lysine residues of native proteins is generally found in a non-conjugated state. However, in samples that have been heated, alkali treated or lyophilized, significant amounts of lysine residues have their e-amino group conjugated and therefore inaccessible (8). Naturally, this would, in turn, lead to a reduction in fluorescence.

o-Phthaldialdehyde offers several advantages over fluorescamine for protein quantitation. The aqueous solubility and stability of OPA is distinctly superior to fluorescamine (7, 8). Fluorescamine requires a stock solution using nonhydoxylic solvents to be added to aqueous protein samples, while OPA can be added in aqueous solution. OPA is also stable for long periods while in solution. In addition to its aqueous stability, OPA exhibits greater fluorescent quantum yields (nearly an order of magnitude) than fluorescamine.

Protein quantitation using OPA requires very little sample (5-10 µL), allowing the investigator sufficient material for other necessary assays. With the increased sensitivity along with small sample volumes, samples containing as little as 80 ng in a 10 µL volume can be assayed in a matter of minutes with confidence.

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.

Like most assays that are read in microplates, the ability to read all of the samples simultaneously greatly reduces the manual labor required to obtain the data. The microplate format also lends itself to “off the shelf” automation for laboratories with high volume requirements. The smaller reaction volumes in microplates will lead to lower per assay costs by reducing the amount of expensive reagents necessary to perform the assay.

References

(1) Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall (1951) Protein Measurement with the Folin Phenol Reagent, J. Biol. Chem. 193:265-275.
(2) Smith, P.K., et al. (1985) Measurement of Protein Using Bicinchoninic Acid, Anal. Biochem. 150:76-85.
(3) Bradford, M.M. (1976), A Rapid and Sensitive Method for Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-dye Binding, Anal. Biochem. 72:248-254.
(6) Benson, J.R. and P.E. Hare, (1975), o-Phthaladehyde: Fluorogenic Detection of Primary Amines in the Picomole Range. Comparison with Fluorescamine and Ninhydrin, Proc. Nat. Acad. Sci. USA 72:619-622.
(7) Lorenzen, A. and S.W. Kennedy (1993), A Fluorescence-Based Protein Assay for Use with a Microplate Reader, Anal. Biochem. 214:346-348.
(8) Goodno, C.C., H.E. Swaisgood, and GG.L. Catignani (1981) A Fluorimetric Assay for Available Lysine in Proteins, Anal. Biochem. 115:203-211.

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