Problems Associated with Determining Protein Concentration:

A Comparison of Techniques for Protein Estimations

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Abstract

Although a range of methods are available for determining protein concentration, many scientists encounter problems when quantifying proteins in the laboratory. The most commonly used methods for determining protein concentration in a modern biochemistry laboratory would probably be the Lowry and/or the Bradford protein assays. Other techniques, including direct spectrophotometric analysis and densitometry of stained protein gels, are applied, but perhaps to a lesser extent. However, the reliability of all of the above techniques is questionable and dependent to some extent on the protein to be assayed. In this paper we describe problems we encountered when using some of the foregoing techniques to quantify the concentration of poly(adenosine diphosphate-ribose) polymerase-1 (PARP-1), a nuclear enzyme found in most eukaryotes. We also describe how, by using a fluorescence-based assay and amino acid analysis, we overcame the problems we encountered.

Index Entries: Bradford protein assay; Lowry protein assay; poly(ADP-ribose)polymerase-1.

1. Introduction

Our laboratory is currently investigating the relationship between structure and function in poly(adenosine diphosphate [ADP]-ribose) polymerase-1 (PARP-1). PARP-1 is a nuclear enzyme involved in a range of activities associated with DNA metabolism, and plays a key role in maintaining the integrity of DNA and chromatin structure ***(1,2)***. We have successfully produced and purified human PARP-1 with high specific activity by using the BAC-TO-BAC Baculovirus expression system (Invitrogen, Inc.) ***(3)***, work that required a reproducible and accurate assay to estimate protein concentration.

Many methods are available for determining protein concentration (**Table 1** provides a list of commonly used assays), and the criteria for a suitable assay usually include sufficient sensitivity, accuracy, and reproducibility. Several methods with the potential to meet these criteria were tested in the work described in this paper.

The most widely used protein assay in the literature, the Lowry protein assay ***(4)***, detects the phenolic group on tyrosine residues in proteins, and has a sensitivity of 2–100 µg/mL of protein ***(5)***. Because the Lowry protein assay detects tyrosine residues in a protein, and the number of tyrosine residues varies between proteins, it is important that the protein used as a standard have a similar proportion of tyrosine to the protein being assayed. Another critical component of the Lowry protein assay is the length of the incubation time to develop the product of the Lowry reaction; differences in incubation times between samples in a Lowry protein assay will lead to nonreproducible results. The Lowry protein assay is also subject to interference from a wide range of components such as Tris and ethylene diamine tetraacetic acid (EDTA) ***(6)***, both of which are components of many buffers used for the purification of recombinant proteins. However, if one assumes that there is sufficient protein, the effect of these

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Table 1
Comparisons of Routinely Used Methods for Quantitating Protein Concentration

Method	Detection Range	Active Residues Detected	Comments	Reference
Lowry protein assay	2–100 µg/mL	Phenolic group on tyrosine residues	• Choice of standard is critical • Incompatible with some detergents and some reducing agents • Tris and EDTA can interfere with assay	***(4)*** ***(6)***
Bradford protein assay	0.2–20 µg/mL	Basic amino acid residues, particularly arginine	• High protein to protein variation •Incompatible with some detergents	***(8)*** ***(10)***
Laser densitometry of SDS–PAGE gels				
Coomassie brilliant blue G250 staining	40–50 ng band	Detects basic amino acids on the same principle as the Bradford protein method	• Time consuming but can give reasonable estimate of protein concentration	***(11)***
SYPRO Orange staining[d]	1-ng band	Binds to SDS coat around proteins	• Low protein to protein variation • Detection not influenced by nucleic acids or other contaminants	***(18)*** ***(14,17)***
Silver staining	1–5 ng band	Relies on the reduction of ionic silver to its metallic form on binding to proteins	• Qualitative method • Time consuming	***(13)*** ***(12)*** ***(11)***
Amino acid analysis		Detects most amino acid residues	• Specialized techniques • Requires GC–MS or HPLC • Very accurate quantitation method	***(5)***

chemicals can be minimized by diluting the protein sample.

The Bradford protein assay is both rapid and accurate ***(7)***. Ausubel et al. ***(5)*** proposed that the Bradford protein assay is "the method of choice" for accurately determining protein concentration. Practical advantages of this method are that the Bradford protein reagent is simple to prepare and that the color develops rapidly and is stable. The assay relies on the binding of Coomassie Brilliant Blue G250 (CBB) to protein ***(8)***. The dye binds in its anionic form to basic amino acids within the protein (particularly arginine residues), and when bound produces a complex that has an absorbance peak at 595 nm ***(9)***. The amount of dye that binds depends on the content of basic amino acid residues in the protein. Thus, the proportion of basic residues in the protein standard for the assay should be similar to that in the protein to be assayed. The sensitivity range for the Bradford assay is between 0.2 and 20 µg/mL of protein ***(10)***.

Protein concentration can also be estimated through laser densitometry of stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels; for some stains the intensity of a stained band of protein in such a gel is

proportional to the concentration of protein in the band. CBB is one of the most commonly used stains for protein gels. Although this stain can be used to detect relatively small amounts of protein in an SDS-PAGE gel (it can detect as little as 40–50 ng of protein in a band on a gel ***[11]***), it is generally regarded as at best semiquantitative (Personal communication: John Walker, Hertfordshire University, Hatfield, UK).

Silver staining provides a significant advantage over the traditional CBB stain, since it is reported to be between 20 and 200 times more sensitive ***(12)***, and thus able to detect as little as 1–5 ng of protein in a band on an SDS-PAGE gel ***(11,13).*** However, the chemical interactions between the protein and the silver salts in silver staining are unknown ***(12,13)***, and silver staining is generally regarded as nonquantitative.

It is claimed that fluorometric methods for quantifying protein concentration outperform all existing routine methods for determining protein concentration ***(14)***. The newly developed SYPRO Orange fluorescent stain (Bio-Rad Laboratories Pty. Ltd.) for SDS-PAGE gels can detect as little as 1 ng of protein in a band on a gel ***(15)***.

The most accurate and sensitive method for determining protein concentration is amino acid analysis. Although access to this approach in the past was restricted to specialized laboratories, it is now readily available, usually as a service provided by specialist laboratories. Additionally, significant advances in the technologies underpinning this approach have improved the precision and the sensitivity of amino acid analysis ***(5)***. Amino acid analysis requires proteins to be quantitatively broken down to their constituent amino acids by chemical treatments that lead to peptide-bond hydrolysis; this is most commonly achieved by acid hydrolysis. The amino acids are then resolved and quantitated with high-pressure liquid chromatography or gas chromatography–mass spectrometry.

In our study, we used amino acid analysis to get an accurate estimation of the protein concentration of a stock solution of purified human PARP-1. This stock solution was then used to test a range of other assays for their sensitivity, accuracy, and reproducibility. From the results obtained it is clear that two of the most commonly used protein assays, the Lowry and the Bradford methods, should be used with caution, at least when determining PARP-1 concentration. In contrast to this, determining human PARP-1 concentration from stained SDS-PAGE gels when using bovine serum albumin (BSA) as a standard gave accurate estimates of protein concentration. An added advantage of estimating protein concentration from SDS-PAGE gels is that one also gets information about the quality of the protein.

2. Materials and Methods

2.1. Stock Solution of Human PARP-1

Human PARP-1 was prepared from the BAC-TO-BAC Baculovirus expression system as described by Knight and Chambers ***(3)***. A stock solution of human PARP-1 was made by dissolving purified human PARP-1 in 50 m*M* Tris HCl, pH 8.0, 1 m*M* EDTA, 1 m*M* NaN_3, 1 m*M* glutathione and 0.5 m*M* dithiothreitol (DTT).

2.2. Amino Acid Analysis

Amino acid analysis was performed by the Australian Proteome Analysis Facility at Macquarie University, Sydney, Australia.

2.3. Lowry Protein Assay Determination of PARP-1 Concentration

A modified version of the Lowry method ***(4)*** was used for this work. A calibration curve was prepared by using a series of solutions containing 10, 20, 30, 40, 50, 75, and 100 µg of standard protein (BSA, Fraction V, cat. no. A-7906 [Sigma]) in a total volume of 500 µL of double-distilled water. A reagent blank and dilutions of the PARP-1 stock solution were also prepared to a final volume of 500 µL of double- distilled water. A volume of 500 µL of Lowry protein assay solution A [1 mL 5% (w/v) $CuSO_4$, 9 mL 1% (w/v) potassium tartrate, and 100 mL 10% (w/v) Na_2CO_3 in 0.5 *M* NaOH] was added to each of the standards and PARP-1 dilutions, and mixed thoroughly into them by vortexing. The standards and the PARP-1 dilutions were then incubated at 37°C for 10 min. After incubation, 1.5 mL of Lowry protein

assay solution B (1 mL Folin–Ciocalteau's reagent and 10 mL double-distilled water) were added to each of the standards and the PARP-1 dilutions, and mixed thoroughly into them by vortexing. The standards and the PARP-1 dilutions were then incubated at 52°C for 20 min. Absorbances of standards and the PARP-1 dilutions were determined at 680 nm.

2.4. Bradford Protein Assay Determination of PARP-1 Concentration

The Bradford protein assay for determining protein concentration was performed as described by Bradford *(8)*. A calibration curve was prepared by using a series of solutions containing 10, 20, 40, 70, and 100 µg of standard protein (BSA) in a total volume of 100 µL double-distilled water. A reagent blank and appropriate dilutions of the PARP-1 stock solution were prepared to a final volume of 100 µL with double-distilled water as the diluent. A volume of 5.0 mL of Bradford protein reagent (100 mg CBB dissolved in 50 mL of 95% ethanol, mixed with 100 mL of 85% orthophosphoric acid and made up to a final volume of 1.0 L with distilled water) was added to each tube and then mixed thoroughly with the standards and PARP-1 solutions in the respective tubes by inversion. Absorbances for the PARP-1 dilutions and the standards were determined at 595 nm.

2.5. Estimating Protein Content with CBB, Silver, and SYPRO Orange-Stained SDS–PAGE Gels

Standards and dilutions of the stock PARP-1 solution were run on 7.5% SDS-PAGE gels as described in the Bio-Rad Laboratories (Mini Protean II Dual Slab Cell Instruction Manual). After electrophoresis, gels were stained either CBB, silver, or SYPRO Orange.

CBB staining was performed according to the method described in the LKB-Pharmacia laboratory manuals *(16)*. The gel was placed in fixing solution (80 mL ethanol and 20 mL glacial acetic acid, made up to a final volume of 200 mL with distilled water) for a minimum of 30 min. The gel was then placed in CBB staining solution (1.25 g CBB dissolved in 230 mL methanol and 230 mL distilled water). The solution was stirred for an hour, and 40 mL glacial acetic acid was then added. If any particles appeared, the solution was filtered through Whatman 3M filter paper. The gel was left in the stain for 30 min and was then destained in fixing solution until the background was clear. The gel was then washed several times with distilled water.

Silver staining was performed according to the method described in the LKB Pharmacia laboratory manuals *(16)*. Gels were placed in fixing solution (80 mL ethanol and 20 mL glacial acetic acid, made up to a final volume of 200 mL with distilled water) for a minimum of 30 min to allow the SDS to diffuse out of the gel and the proteins to precipitate. The gel was then placed in incubation solution (60 mL ethanol, 13.6 g sodium acetate·$3H_2O$, 0.4 g sodium thiosulfate, and 1.04 mL glutaraldehyde [added immediately before use], made up to a final volume of 200 mL with distilled water) for a minimum of 30 min, after which it was washed several times with distilled water (at least 10 min per wash for three washes), placed in silver solution (0.2 g silver nitrate and 40 µL of formaldehyde [added immediately before use], made up to a final volume of 200 mL with distilled water) and allowed to shake in the latter for 40 min. The gel was then placed in developing solution (5.0 g sodium carbonate and 20 µL of formaldehyde [added immediately before use], made up to a final volume of 200 mL with distilled water) until the protein bands became intensely dark. To stop further color development via the reaction, the gel was placed in stop solution (2.92 g EDTA dissolved in distilled water to a final volume of 200 mL) for 15 min.

Staining of SDS-PAGE gels with SYPRO Orange was performed according to the Bio-Rad Laboratories SYPRO Orange Protein Gel Stain Instruction Manual *(17)* with some minor modifications. The gel was placed in SYPRO Orange staining reagent (10 µL of SYPRO Orange protein stain, dissolved in 50 mL of 7.5% glacial acetic acid) for 30 min and then destained in 7.5% glacial acetic acid for 30 min to 1 h.

The staining intensities of the CBB-, silver-, and SYPRO Orange-stained protein gels were

captured with a Fujifilm LAS1000 charged-coupled device (CCD) and analyzed by using ImageGauge densitometry analysis software, version 3.121 (Fujifilm Ltd., 1998).

Estimates of protein concentration from stained SDS-PAGE gels were based on densitometry of duplicate gels. For the CBB- and SYPRO Orange-stained gels, the standard curve was generated from mean optical densities (ODs) for duplicate gels. All experiments were repeated at least once and gave similar results. The total amount of protein in a band was estimated by measuring the intensity across the whole area of the band, thus compensating for differences in band size.

3. Results and Discussion

We used a stock solution of human PARP-1, expressed from a cloned source and purified as described in Knight and Chambers ***(3)***, to compare several different quantitative protein assays so as to determine which assay would be most reliable for accurate determination of PARP-1 concentrations for future work in our laboratory. We determined the concentration of the stock PARP-1 solution used in this comparison by amino acid analysis, and was estimated to be 10 µg/mL. This was consistent with the level of PARP-1 enzyme activity associated with the preparation (data not shown). This estimate was therefore assumed to be a reasonable measure of the PARP-1 concentration in the stock solution.

The Lowry protein assay detects tyrosine residues in a protein, and because the number of tyrosine residues varies among proteins, it is important that the protein used as a standard has a similar proportion of tyrosine residues to the protein being assayed. The percentages of tyrosine residues in BSA and human PARP-1 are 3.5% and 3.3%, respectively, and BSA should therefore be a suitable standard for assaying PARP-1 concentration.

The Lowry protein assay is also subject to interference from a range of chemicals, such as Tris and EDTA ***(6)***, both of which are components of the buffer used to make the standard stock solution of human PARP-1 that was used in our study. However, the concentration of these components in the PARP-1 buffer was minimal and was therefore unlikely to have interfered with the assay. This was shown to be the case in an experiment in which a range of concentrations of BSA was dissolved in PARP-1 buffer and in water, and the two sets of solutions were subjected to the Lowry protein assay. The resultant absorbance values were not affected by the buffer (data not shown).

The Lowry protein assay was performed in duplicate and repeated at least once, and gave similar results. With BSA used as a standard, the stock solution of human PARP-1 used for this work was estimated to have a protein concentration of 2.6 mg/mL (**Fig. 1**). Thus, the modified Lowry protein assay gave an estimate of protein concentration that was two orders of magnitude higher than the concentration of the stock solution of human PARP-1 as determined by amino acid analysis. Therefore, the Lowry protein assay is clearly not a suitable method for determining the concentration of PARP-1 when BSA is used as a standard.

The Bradford protein assay detects the amount of CBB dye that binds to base amino acids in proteins; consequently, the relative proportions of basic amino acids in the standard (BSA) and the protein being assayed are of critical importance. The percentages of basic amino acid residues in BSA and PARP-1 are 17.0% and 17.5%, respectively. Therefore, BSA was considered to be a suitable standard when determining the protein concentration of PARP-1.

The Bradford protein assay was performed in duplicate and repeated at least once and gave similar results. With BSA used as the standard, the Bradford protein assay gave a protein concentration for the stock human PARP-1 solution of 220–240 µg/mL (**Fig. 2**), a value that is 10–20 times greater than that obtained by amino acid analysis. Although this result is better than that given by the Lowry assay, it is still more than an order of magnitude greater than the expected value. Therefore, the Bradford assay, is not suitable for estimating PARP-1 concentration when BSA is used as a standard.

Protein concentration can be estimated through laser densitometry of stained SDS-PAGE gels, and CBB is undoubtedly one of the most com-

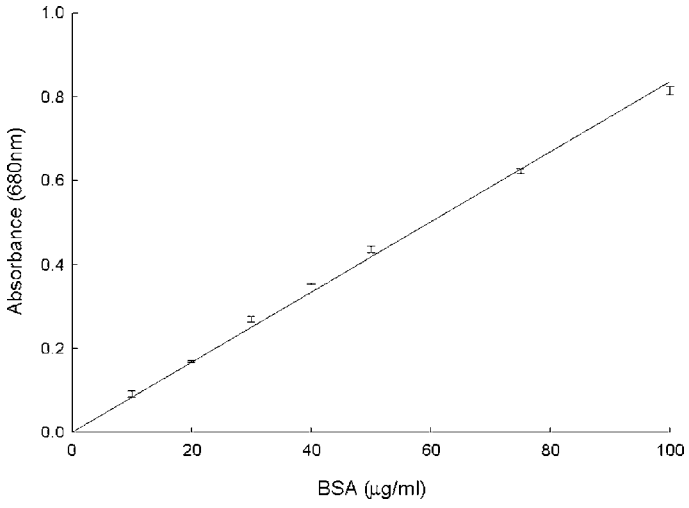

Fig. 1. Standard curve used for the Lowry protein assay.

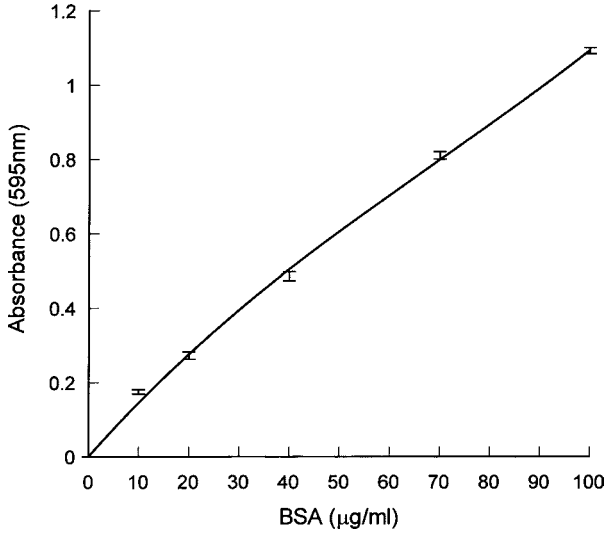

Fig. 2. Standard curve used for the Bradford protein assay.

monly used stains for this purpose. A range of BSA standards (50–400 ng) was resolved on a 7.5% SDS-PAGE gel and the gel was stained with CBB. The CBB-stained protein gel image was captured on Fujifilm LAS1000 CCD (**Fig. 3A**), and the OD of the stained bands of BSA was determined with ImageGauge Analysis densitometry software, version 3.121. The intensity of CBB-stained BSA protein bands was found to be proportional to the concentration of protein in the respective bands, and from approximately 50–300 ng of BSA, the relationship between intensity and protein concentration was almost linear (**Fig. 3B**).

The concentration of PARP-1 stock solution appeared to be at the detection limits for CBB-stained gels. Nonetheless, ImageGauge densitometry analysis, using the complete area of each band on the gel (**Fig. 3B**), gave a value of 60 ng of protein. This would equate to a concentration of approximately 6 μg/mL in the PARP-1 stock solution, a value that more closely reflects that determined by amino acid analysis. Thus, the estimated concentration of PARP-1 in the stock solution was considerably lower than had previously been estimated with the Lowry and Bradford protein assays for the same preparation.

Silver stain can detect as little as 1 ng of protein in a band on an SDS-PAGE gel, and is therefore much more sensitive than the CBB stain ***(13)***. However, silver stain does not have a large linear dynamic range for quantifying protein concentration, and there is huge protein-to-protein variation that makes silver staining generally a poor choice of method for estimating protein concentration ***(15)***. Nonetheless, silver staining can be useful as a means of obtaining crude estimates of protein concentration. From visual inspection of the silver-stained gel used for work described in this paper, we estimated the amount of human PARP-1 present in the band representing this protein to be 50–100 ng (**Fig. 4**). This would equate to 5–10 μg/mL in the stock solution of PARP-1, a result that is consistent with that obtained from the CBB stained gel.

Fluorescent stains are reported to provide the sensitivity of silver staining, but with the added advantage of a large linear dynamic range for determining protein concentration (Product Information: NanoOrange Protein Quantitation Kit). The newly developed SYPRO Orange fluorescent stain has been reported to detect as little as 1 ng of protein in a band on an SDS-PAGE gel ***(18)***. The SYPRO Orange reagent becomes fluorescent upon binding to the SDS coat that surrounds proteins in SDS-PAGE gels, and the amount of SYPRO Orange that becomes bound is directly proportional to the amount of SDS-coated protein. Thus, there is little protein-to-protein variation,

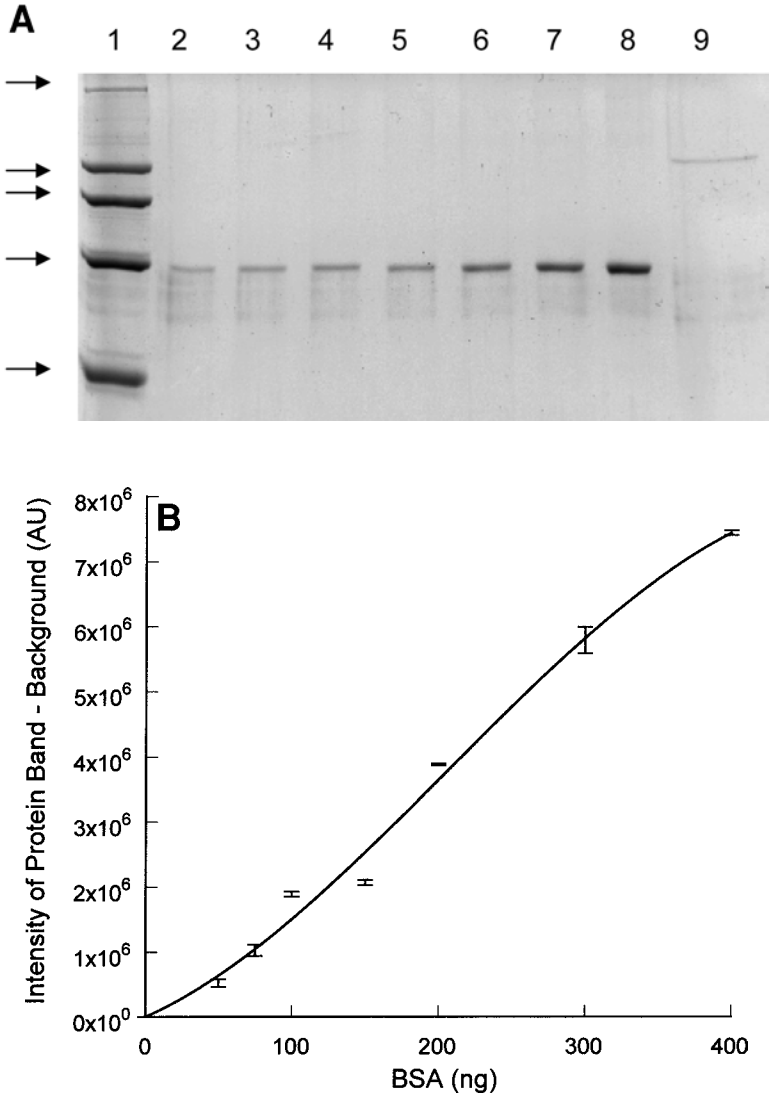

Fig. 3. CBB-stained SDS-PAGE gel (7.5%). Lane 1: Molecular-weight markers (myosin 205 kDa, β-galactosidase 116 kDa, phosphorylase B 97.4 kDa, BSA 66 kDa, and egg albumin 45 kDa). Lanes 2–8: 50, 75, 100, 150, 200, 300, and 400 ng BSA, respectively. Lane 9: 10 µL of PARP-1 stock solution. The contaminants that migrated just below the BSA, PARP-1, and molecular-weight standards were artifacts of electrophoresis (**A**). The OD across the whole area of stained bands of BSA was determined with ImageGauge densitometry analysis software, version 3.121. A standard curve was generated to calculate the concentration of human PARP-1 in the stock solution, using mean ODs obtained from duplicate gels (**B**). All experiments were repeated at least once and gave similar results.

allowing accurate quantitation of purified proteins or protein mixtures ***(17)***.

We found that use of the SYPRO Orange stain in conjunction with SDS–PAGE was well suited to estimating PARP-1 concentration. Using densitometry and ImageGauge analysis software version 3.121, we estimated the amount of human PARP-1 present in the SYPRO Orange-stained protein band to be 100 ng, which would equate to a concentration of 10 µg/mL in the PARP-1 stock solution (**Fig. 5A**,**B**), a value similar to that provided by densitometry analysis of CBB-stained

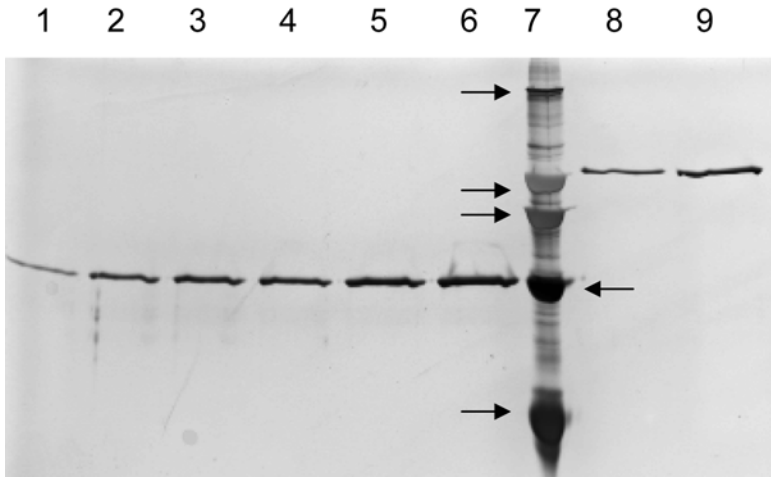

Fig. 4. Silver-stained SDS-PAGE gel (7.5%). *Lanes 1–6:* 25, 50, 75, 100, 150, and 200 ng BSA, respectively. *Lane 7:* Molecular-weight markers (myosin 205 kDa, β-galactosidase 116 kDa, phosphorylase B 97.4 kDa, BSA 66 kDa, and egg albumin 45 kDa. *Lanes 8 and 9:* 10 µL and 20 µL pf PARP-1 stock solution, respectively. The gel was silver stained and captured with a Fujifilm LAS 1000 CCD.

protein gels (*see* **Fig. 3A**,**B**) and consistent with a visual comparison with silver-stained protein gels (*see* **Fig. 4**) and with the results of amino acid analysis.

We also tried to determine the protein concentration in the PARP-1 stock solution by using the NanoOrange Protein Quantitation Kit (Molecular probes, Eugene, OR). However we found that the levels of fluorescence obtained were extremely variable for both the stock solution of PARP-1 and the BSA standards at concentrations of 10–50 ng/mL (data not shown), thus making it difficult to accurately determine the concentration of human PARP-1 in the stock solution.

Table 2
Protein Concentration of Stock Human PARP-1 Solution as Determined with Several Different Assays

Type of Protein Assay	Estimated Concentration of Standard PARP-1 Solution (µg/mL)	Magnitude Difference Compared to Amino Acid Analysis
Amino acid analysis	10	—
Lowry assay	2600	260-fold
Bradford assay	220–240	22–24-fold
CBB gel[a]	6	0.6-fold
SYPRO Orange gel[b]	10	1.0-fold

[a]Coomassie Brilliant Blue stained SDS–PAGE gel.
[b]SYPRO Orange-stained SDS–PAGE gel.

4. Conclusions

In this paper we have highlighted the potential for obtaining enormous variation in estimates of protein concentration for a specific protein solution with a range of commonly used techniques when using a different protein as a standard (**Table 2**). This highlights the importance of testing more than one technique for one's research or for routine analytical work. Amino acid analysis provides one of the most sensitive and accurate approaches for estimating protein concentration, but the technology required for this is probably not suitable or appropriate for routine analysis in most laboratories. The next best approach would be to use amino acid analysis to determine the concentration of a stock solution of the protein to be estimated in future work, and to then use this stock solution as a standard. If, however, preparing a stock solution of a known standard is not a viable option, one should consider testing and comparing a range of techniques and possible standards to determine which combination is best suited to the work that is to be undertaken. This report highlights the problem of assuming that any one of the

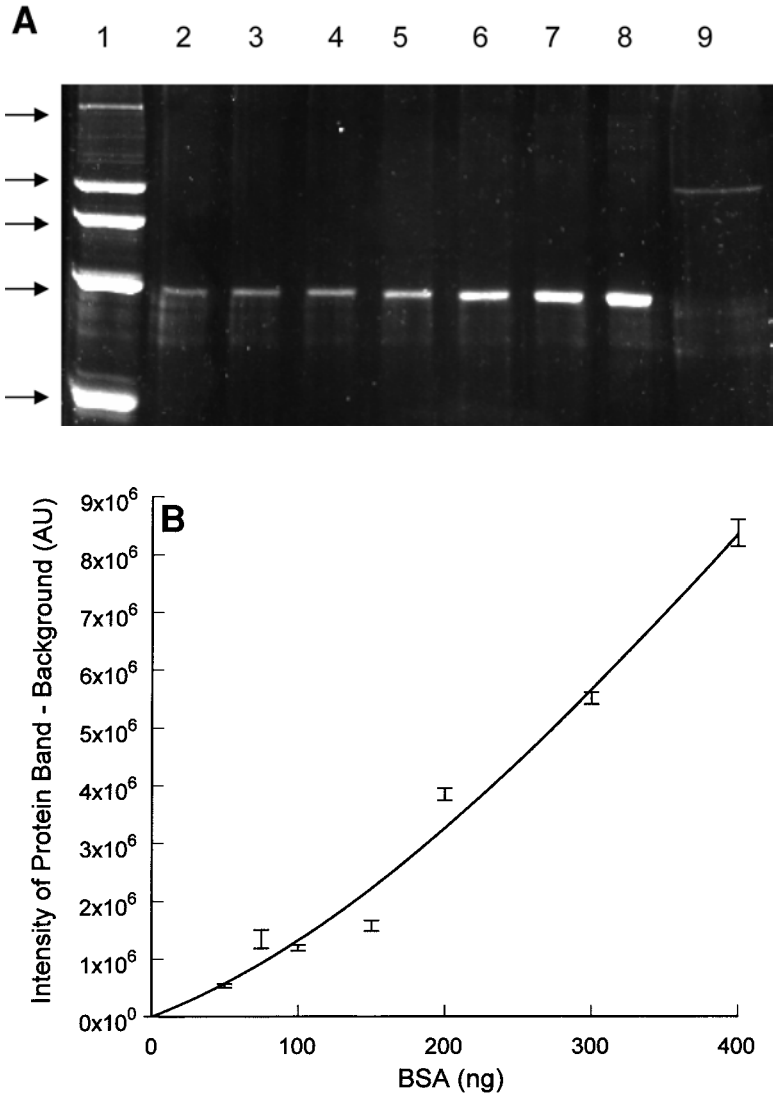

Fig. 5. SYPRO Orange-stained SDS-PAGE gel (7.5%). *Lane 1:* Molecular-weight markers (myosin 205 kDa, β-galactosidase 116 kDa, phosphorylase B 97.4 kDa, BSA 66 kDa, and egg albumin 45 kDa). *Lanes 2–8:* 50, 75, 100, 150, 200, 300, and 400 ng BSA, respectively. *Lane 9:* 10 µL of PARP-1 stock solution. The contaminants that migrated just below the BSA, PARP-1, and molecular weight markers were artifacts of electrophoresis (**A**). The OD across the whole area of stained bands of BSA was determined with ImageGauge densitometry analysis software, version 3.121. A standard curve was generated to calculate the concentration of human PARP-1 in the stock solution, using the mean ODs obtained from duplicate gels (**B**). All experiments were repeated at least once and gave similar results.

commonly used approaches will suffice. On the basis of our results, we suggest that for laboratories lacking access to amino acid analysis, the best method for determining low concentrations of human PARP-1 is quantitation from an SDS–PAGE protein gel stained with SYPRO Orange.

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