Solubilization of Protein–Dye Complexes on Nitrocellulose to Quantify Proteins Spectrophotometrically

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Proteins absorbed directly onto nitrocellulose membranes were stained with amido black, ponceau S, colloidal silver, or Coomassie blue and solubilized in dimethyl sulfoxide and the absorbance was measured spectrophotometrically. The optimal wavelength of each dye/protein/nitrocellulose solution was found to be at 625, 529, 420, and 600 nm, respectively. A linear relationship was found between the protein concentration and absorbance at the appropriate wavelength for all the stains with individual purified proteins or protein mixtures. Protein (0.2–0.8 μg) can be determined with the colloidal silver and 2–30 μg with the other stains. Coomassie blue produced variable background staining of the nitrocellulose and is therefore not recommended. Proteins transferred electrophoretically to nitrocellulose from a sodium dodecyl sulfate–polyacrylamide gel were also stained with the above dyes and solubilized in dimethyl sulfoxide. Amido black was the most sensitive stain, detecting proteins in the range of 1–10 μg. Components of the gel interfered with silver staining. © 1996 Academic Press, Inc.

Quantification of proteins bound to nitrocellulose can be achieved by four methods. Proteins bound to nitrocellulose can be stained using a range of organic dyes including amido black (1, 2), ponceau S (2), Coomassie blue (3), colloidal silver/gold (4, 5), india ink (3, 6, 7), ferridye (7–9), and aurodye (7, 9). Quantitation can then be realized either by eluting the dye into solution in the presence of sodium hydroxide (10), SDS isopropanol (11), pyridine, or acetonitrile (12) and determining the dye eluted or by using densitometry. The opaque nature of the nitrocellulose can be overcome by soaking the nitrocellulose in organic solvents such as xylene (13), liquid paraffin (14), toluene (2), or microscope immersion oil (15) and the blot can be scanned with a transmission densitometer. The density of the stain is directly proportional to the amount of protein bound to the nitrocellulose.

The third method uses metabolically labeled proteins or radiolabeled antibodies (16–18) followed by liquid scintillation counting. The last method is to detect the protein with an antibody–enzyme complex and then use chromogenic substrates for the enzyme (19). The color intensity of the product of the enzyme catalyzed reaction is proportional to the amount of protein on the nitrocellulose.

In this report we describe a different approach where proteins on the nitrocellulose were stained with a range of dyes and then the protein/dye complex solubilized in dimethyl sulfoxide. The resulting solution was read spectrophotometrically at the appropriate wavelength for each dye and the absorbance was found to be proportional to the quantity of protein originally absorbed to the nitrocellulose. We explored the technique further and found that it was also appropriate for the quantitation of protein bands that had been transferred electrophoretically from a sodium dodecyl sulfate–polyacrylamide gel to nitrocellulose.

MATERIALS AND METHODS

A 0.45-μm nitrobind (nitrocellulose) transfer membrane (MSI, West Boro) was used throughout. Bovine serum albumin (BSA)[2] (M_r 67,000), lysozyme (M_r 14,700), carbonic anhydrase (M_r 30,000), and ponceau S were all purchased from Sigma. Amido black, Coomassie blue R-250 and dimethyl sulfoxide (DMSO) were purchased from Unilab, Univar, and British Drug House (BDH), respectively. All other chemicals and solvents were analytical grade. Gloves were worn when handling dimethyl sulfoxide.

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[2] Abbreviations used: SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin.

Determination of the Optimum Wavelength for Each Dye/Protein/Nitrocellulose Solubilized Mixture

A range of 0–30 μg of BSA, solubilized in 0.15 M phosphate-buffered saline, pH 7.2, was spotted onto a nitrocellulose membrane and stained with one of the protein stains as described below. Once stained and allowed to air dry, each protein spot on the membrane was cut out (1 cm^2) and solubilized in 1.0 ml of DMSO with vortexing for 20 s. A Shidmadzu Spectrophotometer was used to scan the absorption spectrum of each of the dye/protein/nitrocellulose solutions from 200 to 800 nm. Nitrocellulose exposed to each dye and solubilized in DMSO served as a blank.

The protein staining was performed as follows. Amido black: 0.1% w/v amido black was dissolved in 10% acetic acid and 25% isopropanol. Nitrocellulose-bound proteins were stained for 5 min in the dye and destained for 5–10 min in the solvent, rinsed in distilled water. Ponceau S: 0.2% ponceau S was dissolved in 3.0% trichloroacetic acid. Bound proteins were stained for 2 minutes and then destained by rinsing in distilled water until the background was constant. Coomassie blue staining was performed as described by Hancock and Tsang (3). The colloidal silver stain protocol described by Kovarik *et al.* (20) was performed as modified by Draber (21). When the method was used to compare BSA, lysosyme, and carbonic anhydrase, proteins were solubilized in 0.15 M phosphate-buffered saline, pH 7.2, or SDS sample buffer.

Effect of Western Transfer Conditions on Protein Quantitation

BSA (1–2 μg) was spotted onto nitrocellulose and stained with amido black, ponceau S, or silver stain and solubilized with DMSO before reading at 625, 529, or 420 nm, respectively. This was then compared to spotting the same range of protein concentrations, placing the nitrocellulose in contact with a 15% discontinuous SDS–polyacrylamide gel, (22) and exposing the gel/membrane to Western transfer conditions overnight (23). The membrane was then stained with amido black or silver, the protein stain was solubilized, and the absorption was read appropriately.

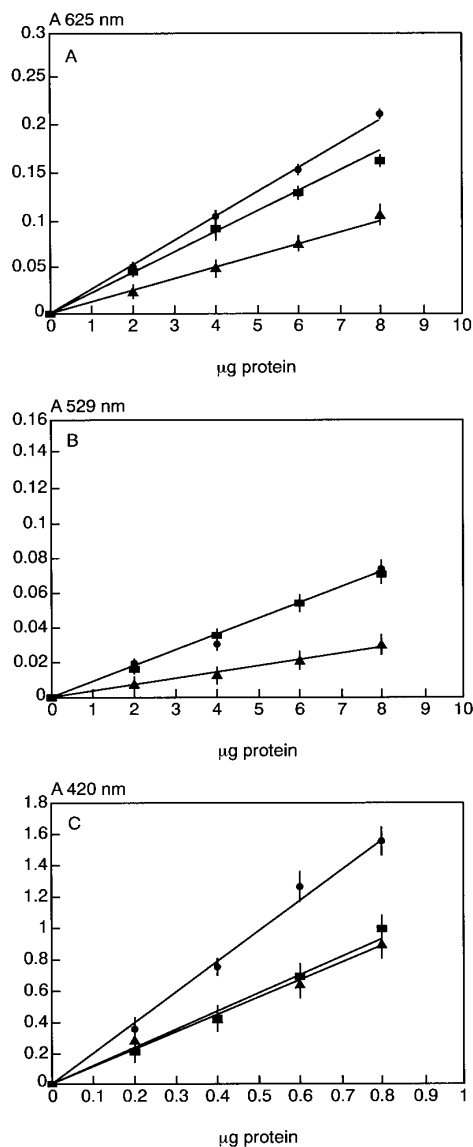## Quantifying Proteins Which Have Been Transferred from an SDS–Polyacrylamide Gel to Nitrocellulose

A discontinuous 15% SDS–polyacrylamide gel system was prepared as described by Laemmli (22). Solutions containing 1–10 μg of BSA, lysozyme, or carbonic anhydrase (molecular mass standards) were run on a 15% SDS–polyacrylamide gel and transferred to nitrocellulose (23). The nitrocellulose was stained with amido black or silver and the bands were excised and

solubilized in DMSO before reading the absorbances at 625 or 420 nm, respectively.

RESULTS

Comparing the Variation in Absorbance for Different Proteins

All three stains, amido black, ponceau S, and silver (Figs. 1A–1C) exhibit a linear relationship between

FIG. 1. Plot of absorbance versus protein concentration for different proteins and protein stains on nitrocellulose solubilized with dimethyl sulfoxide. BSA (●), lysozyme (▲), and carbonic anhydrase (■) in SDS sample buffer were absorbed onto nitrocellulose and stained with (A) amido black, (B) ponceau S, and (C) colloidal silver. The nitrocellulose was solubilized with dimethyl sulfoxide and the absorbance of each solution was measured. Each absorbance point is the mean of three readings in one experiment with standard deviations (vertical bars).

TABLE 1

Optimum Wavelength for Each Protein/Dye/Nitrocellulose
DMSO-Solubilized Complex

Protein stains	Peak wavelength[a] (nm)
Amido black	625
Ponceau S	529
Coomassie blue	600
Colloidal silver	420

[a] For each protein stain, 10, 20, and 30 μg of protein (BSA) in PBS were spotted onto nitrocellulose and stained with amido black, ponceau S, Coomassie blue, and colloidal silver, respectively. The protein/dye/nitrocellulose was solubilized in dimethyl sulfoxide and the solution was scanned from 200 nm to 800 nm.

protein concentration and absorbance at their optimum wavelength (Table 1). The linearity on the absorbance versus protein concentration graph was maintained up to 30 μg of protein for amido black and ponceau S (data not shown). The silver stain is the most sensitive of the stains as indicated by a greater change in absorbance per unit protein. The silver stain (Fig. 1C) also detects lower quantities of protein, i.e., 0.1 μg of protein, compared to 1.0 μg with both ponceau S and amido black. The silver stain, though sensitive, becomes saturated at increasing protein concentrations. Coomassie blue has a similar limit of sensitivity to amido black (not shown), but destaining after the Coomassie blue results in inconsistent background. This observation is congruous with that reported by Hancock and Tsang (3).

For each protein, i.e., BSA, lysozyme, and carbonic anhydrase, a different slope was obtained for the solubilization method with each of the dyes. When these proteins were compared using the methods of Bradford (24) and Lowry *et al.* (25) for protein determination, individual slopes were also obtained. We found less variation in absorbance readings when proteins were in SDS sample buffer compared to phosphate-buffered saline. We have used solubilized red cell proteins and plasma proteins and established that the technique is applicable to mixtures of proteins. We have not included the data to keep the diagrams uncluttered.

Quantifying Proteins Which Have Been Transferred from an SDS–Polyacrylamide Gel to Nitrocellulose
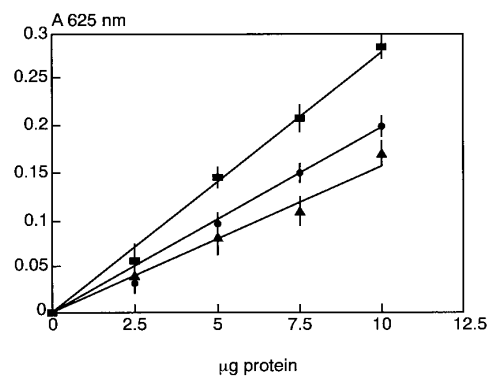
When we employed the DMSO solubilization method to proteins that had been transferred to nitrocellulose from an SDS–polyacrylamide gel, we found that there was a linear relationship between the concentration of each protein and the absorbance value obtained (Fig. 2). Carbonic anhydrase gave the steepest slope followed by BSA and then lysozyme. This result is different from that presented in Fig. 1A, where BSA produced a steeper slope than carbonic anhydrase. We attribute

this difference to the purity of the proteins. We used SDS–polyacrylamide molecular mass standards for this experiment because the proteins used in the first experiment (Fig. 1A) produced several minor protein bands on SDS–polyacrylamide gels. When the molecular mass standards were placed directly onto nitrocellulose, stained with amido black, and solubilized, similar but marginally steeper absorbance profiles were obtained with each protein. We found the ponceau S stain to be less sensitive than the amido black stain and the silver staining method to be nonspecific. By placing nitrocellulose-bound proteins against a polyacrylamide gel under electrophoretic conditions we established that the silver method stains the nitrocellulose nonuniformly due to chemical interactions between silver and polyacrylamide on the nitrocellulose.

DISCUSSION

We present a method for quantifying proteins bound to nitrocellulose membranes. The method involves detecting protein on nitrocellulose with conventional dyes and then solubilizing the protein/dye/nitrocellulose in dimethyl sulfoxide. Proteins can then be quantitated spectrophotometrically by measuring the dye in the final solution. The method is therefore appropriate for use in laboratories without access to densitometers and has the advantage of not requiring the use of radiolabeled proteins or antibodies.

We found that for the protein dyes amido black, ponceau S, and silver (Fig. 1) there was a linear relationship between protein bound to the nitrocellulose and the absorbance values obtained when the protein/dye/nitrocellulose was solubilized in dimethyl sulfoxide.

FIG. 2. Quantitation of protein on nitrocellulose after transfer from a SDS polyacrylamide gel. BSA (●), lysozyme (▲), and carbonic anhydrase (■) molecular mass markers were run on a 15% discontinuous SDS–polyacrylamide gel and transferred electrophoretically onto nitrocellulose paper. Proteins were stained with amido black, the protein band on the nitrocellulose was cut out and solubilized with dimethyl sulfoxide, and the absorbance was determined. Each point is the mean of three readings in one experiment with standard deviations (vertical bars).

Ponceau S was the easiest and quickest stain to perform. The stain had little nonspecific binding to the nitrocellulose paper and therefore low background. Although this stain has a low sensitivity (an absorbance change of 0.05 units over $1–5$ μg protein), it is a reversible stain and therefore commonly used to detect proteins before using an immunodetection system (26). In comparison we found that Coomassie blue stained the nitrocellulose membrane leading to high background staining as has been reported by others (2) and therefore is not useful in this method.

Amido black was found to be a more sensitive stain than ponceau S giving a 0.13 units absorbance change over a range of $1–5$ μg protein. This stain can also be followed by immunostaining techniques. The stain requires fewer steps than the silver stain and therefore is the most convenient method to use in the microgram range.

The silver staining was the most sensitive of the protein stains examined. The stain detected proteins in the range of 0.1 to 0.8 μg with a change in absorbance of 1.6 units. The method is restricted to low quantities of proteins and reaches saturation as protein concentration increases.

The absorbance values obtained for the proteins BSA, lysozyme, and carbonic anhydrase were each found to be different for each dye. This difference was also found when the standard protein detection methods of Lowry *et al.* (25) and the Bradford (24) were used with these proteins. This result emphasizes the comparative nature of most protein determination methods and may be due to the inherent differences in the hydrophobic nature and molecular masses of different proteins as pointed out by Nakamura *et al.* (2). Additionally different absorbance versus protein profiles for each protein could be attributed to the variation in the number of basic amino acids present in the proteins. The dyes used are all acidic dyes and have been shown to bind more strongly to basic proteins than to acidic proteins (27, 28).

We compared the DMSO solubilization method with a technique using sodium hydroxide to decrease the ionic interaction between protein and dye (1). The concentration of dye in solution can then be related to the amount of protein bound on the nitrocellulose. We found that the DMSO solubilization method was more sensitive and simpler to run than the alkali method (data not shown).

We then extended the DMSO solubilization method to include proteins transferred electrophoretically from a SDS–polyacrylamide gel to nitrocellulose (Fig. 2). Smith *et al.* (29) showed that SDS increases the binding of protein to nitrocellulose by increasing the hydrophobicity of the protein. In this quantitation method, the presence of SDS in the protein solution is thus an advantage rather than an interference. There was a de-

crease in the absorbance values obtained for BSA, and an increase in the values obtained for carbonic anhydrase when comparing the data in Fig. 1A to those in Fig. 2. We attribute these differences to the purity of each protein sample. The BSA and carbonic anhydrase used in the initial experiments produced several minor protein bands on an SDS–polyacrylamide gel indicating the presence of contaminating and broken protein segments. We therefore used SDS–polyacrylamide gel molecular mass standard proteins for the experiment presented in Fig. 2. The lysozyme we purchased was much "cleaner" and hence similar absorbance values were obtained in the two figures. We repeated the first experiment with molecular mass standards and obtained plots that were marginally higher than those in Fig. 2. We suggest that this decrease in protein absorbance is due to the efficiency of electrophoretic transfer of the proteins (30).

Because of the comparative nature of this method emphasis must be placed on treating the standard protein the same as the unknown protein sample, i.e., subjecting both to polyacrylamide gel electrophoresis and electrophoretic transfer to nitrocellulose.

After the gel and Western transfer procedure the proteins were stained with amido black, ponceau S, or colloidal silver. The protein bands were excised and solubilized in dimethyl sulfoxide for spectrophotometric analysis. We were able to quantify proteins using the amido black and ponceau S stains in the range of $1–10$ μg.

With the silver stain, nonspecific and nonuniform staining of the blot was observed when the nitrocellulose had been in contact with SDS polyacrylamide gels. Hashimoto *et al.* (31) have indicated that the addition of iodoacetamide to the sample buffer or ultrafiltration of the gel mix before polymerization could reduce artifacts seen in gels with the silver stain. Hochstrasser *et al.* (32) suggest that the methylene bisacrylamide used as a crosslinker in a gel may be partially responsible for the high background staining with the silver stain because the amide groups in the crosslinker actively bind silver ions. We found that the nonuniform staining of the nitrocellulose occurred in the region of contact between gel and membrane. Because of the sensitivity of the silver staining method, we found that we were unable to reduce this general staining sufficiently to produce reliable results for quantification.

The method we have presented is versatile and can be used with a range of common protein dyes to quantify proteins bound to nitrocellulose. The method is also applicable to quantifying individual protein bands from Western blots. An advantage to this approach is that individual protein bands can be identified before being cut out and solubilized in dimethyl sulfoxide. The technique can be employed to determine individual protein loss during a purification procedure, to determine the

levels of expression of a recombinant protein, and to investigate interactions between proteins and nitrocellulose membranes in the presence of ions and detergents.

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