Use of a Scanning Densitometer or an ELISA Plate Reader for Measurement of Nanogram Amounts of Protein in Crude Extracts from Biological Tissues

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Protein contents of crude extracts from plant and animal tissues can be rapidly assayed using a Coomassie blue dye-binding procedure combined with scanning densitometry. Total protein is extracted from 100 mg of fresh-frozen or dried-ground tissue using 1 ml of extraction buffer. One-microliter aliquots of standard solutions or crude extracts are spotted in rows on a suitably sized sheet of Whatman 3MM chromatography paper. The dried samples are stained with Coomassie brilliant blue R-250 (0.2%, w/v, in acidified 50% MeOH) for 20 min and rinsed twice with acidified 20% MeOH. After drying, protein concentrations are read as reflectance using a scanning densitometer and peak heights or peak areas recorded using a digital integrator. In an alternative procedure, each spot is cut from the sample sheet and the dye-protein complex eluted in 1% sodium dodecyl sulfate (SDS) using an ultrasonic cleaner. Absorbance is subsequently read in a microwell sample holder at 590 nm with an enzyme-linked immunosorbent assay plate reader. Both procedures offer distinct advantages over previously reported methods. They are significantly faster when large numbers of samples are processed. They avoid interference by chlorophyll, dithiothreitol, SDS, 2-mercaptoethanol, Nonidet P-40, and phenylmethylsulfonyl fluoride (and other protease inhibitors) and they yield marked improvements in sensitivity, providing measurements of protein concentration below 100 and 200 ng · μl⁻¹, respectively.

KEY WORDS: Coomassie brilliant blue; protein measurement; dye binding.

Several colorimetric and fluorometric methods are currently available for the measurement of protein in biological extracts (1–7). Frequently, however, interfering substances are present in tissue samples and extraction buffers that prevent accurate measurement with these methods (4,7–9). Elimination of the interfering compounds usually requires time-consuming procedures that seriously detract from the convenience of even partially automated methods, particularly when large numbers of samples are being processed (10–13).

The Coomassie brilliant blue R-250 (CBBR-250)¹ dye-binding assay (14) generates a highly stable colored complex that is free from interference by many commonly used substances, but it includes a time-consuming step to elute the dye–protein complex from a support medium prior to spectrophotometric measurement. Moreover, accurate quantitation requires prepared samples that contain at least 0.05 mg of protein per milliliter of solution. The modified dye-binding method that we describe provides higher sensitivity and has a linear range

¹ Abbreviations used: AHA, 6-amino-n-hexanoic acid; CBBR-250, Coomassie brilliant blue R-250; BSA, bovine serum albumin; DTT, dithiothreitol; Epps, n-(2-hydroxyethyl)piperazine-n-3-propanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NP-40, nonidet P-40; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay; TCA, trichloroacetic acid.
from less than 100 to over 1300 ng·µl⁻¹ of protein. The procedure is exceptionally fast, requires only 1 µl of sample for an assay, is easily adapted for automation, and is readily performed without significant interference from chlorophyll or the components of commonly used buffers.

MATERIALS AND METHODS

Reagents and buffers. 6-Amino-n-hexanoic acid (AHA), benzamidine hydrochloride, bovine serum albumin (BSA), CBBR-250, dithiothreitol (DTT), EDTA, n-(2-hydroxyethyl)piperazine-n-3-propanesulfonic acid (Epps), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 2-mercaptoethanol, Nonidet P-40 (NP-40), and phenylmethylsulfonyl fluoride (PMSF) were obtained from Sigma and used as supplied. Sodium dodecyl sulfate (SDS) and Tris were obtained from Bio-Rad. All other reagents were of analytical grade.

Extractions and biological materials. Five extraction solutions were tested with the method (i) NaOH, 0.1 M (pH 12.8); (ii) NaOH, 0.1 M (pH 12.8) plus 2% (w/v) SDS; (iii) Epps buffer, 10 mM (pH 8.5) plus 0.4% (v/v) Nonidet P-40; (iv) Hepes buffer, 62.5 mM (pH 6.8); and (v) Tris buffer, 62.5 mM (pH 6.8) with the latter two made to 1 M with EDTA and PMSF, plus 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol or DTT (50 mM), and 2% (w/v) SDS. Tris buffer was also tested with AHA (5 mM) and benzamidine hydrochloride (1 mM) in conjunction with PMSF as protease inhibitors. Protein was extracted from 10-mg samples of freeze-dried livers of 3-month-old rats and from 10-day-old primary leaves of greenhouse-grown soybean using 1 ml of test buffer in 1.5-ml microcentrifuge tubes as outlined in Fig. 1. For chlorophyll interference experiments, dried leaf tissue was ground to 40-mesh in a Wiley mill, and 10 mg was extracted 3× with 1 × 3 ml of cold acetone followed by centrifugation for 10 min in an Eppendorf microfuge. The supernatants were pooled and absorbance determined at 645 and 663 nm before chlorophyll concentration was calculated (17). An aliquot was subsequently dried in a Speed Vacuum (Instruments, Inc., Model SVC100H) and re-dissolved in Tris buffer and amounts of chlorophyll ranging from 1 to 100 µg were added to solutions of protein standards.

Protein assays. A stock solution of BSA (10 mg·ml⁻¹) was used to prepare a series of protein standards in each of the five salt and buffer mixtures. One-microliter aliquots of crude sample extract and a range of protein standards were spotted in duplicate rows, using 1-µl disposable micropipets, on a 9 × 16-cm sheet of Whatman 3MM chromatography paper placed over a grid on a light box. The spots were air-dried for 15 min and then stained with freshly prepared CBBR-250 (Fig. 1). At this point, a rapid estimate of protein in the test samples could be obtained by visual comparison with stained protein standards (Fig. 3). For accurate quantitation, however, each sample sheet (Fig. 2) was placed on a glass plate and the reflectance of the samples read using a Bio-Rad scanning densitometer (Model 1650) connected to a Hewlett-Packard digital integrator (Model 3390A). Calibration curves of standards were prepared by plotting peak areas or peak heights against total protein content.

In an alternative procedure, each spot was cut from the sample sheet (Fig. 2) and eluted in 1 ml of 1% SDS (w/v) in a disposable culture tube (12 × 75 mm) held in an ultrasonic cleaner for 60 min. A 200-µl aliquot of each sample was transferred to a microwell sample holder (Nunc, Model F-16) and absorbance measured with an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Tek, Model EL 4308) at 590 nm against a 1% SDS eluate of blank chromatography paper. Absorbance was plotted against protein content to obtain a standard curve.

Protein levels were also determined in liver and leaf tissue using standard protocols for the Bradford (4.18) and Lowry (1) methods.

RESULTS

The addition of SDS to the extraction solutions produced a halo effect at all medium
MEASUREMENT OF NANOGRAM AMOUNTS OF PROTEIN

**PLANT TISSUE**

- EXTRACT 10 mg DRIED-GROUND OR 100 mg FRESH-FROZEN TISSUE IN 1 ml COLD ACETONE.
- CENTRIFUGE 10 min AT 10,000 X g
- REPEAT 3 X
- PELLET, DRY UNDER VACUUM

**ANIMAL TISSUE**

- USE 10 mg DRIED-GROUND OR 100 mg FRESH-FROZEN TISSUE
- EXTRACT IN 1 ml BUFFER OF CHOICE, MIX FOR 1 min, 3 X
- PLACE IN BOILING WATER BATH 3 min
- CENTRIFUGE 1 min AT 10,000 X g
- SPOT 1 µl ON WHATMAN 3 MM CHROMATOGRAPHY PAPER, AIR DRY 15 min
- STAIN 20 min WITH 0.2% (W/V) CBBR-250 IN MEOH:H₂O:CH₃COOH, V/V/V (50/40/10)
- DESTAIN 1 h IN MEOH:H₂O:CH₃COOH, V/V/V (20/60/10)
- AIR DRY

READ SPOTS WITH SCANNING DENSITOMETER, COMPARE TO STANDARDS

**FLOW CHART FOR RAPID ASSAY OF PROTEIN IN CRUDE BIOLOGICAL EXTRACTS**

FIG. 1. Flow chart for rapid assay of protein in crude biological extracts.

and low concentrations of protein (Fig. 2). Although the halos produced split peaks during densitometer scans (Fig. 3), they did not detract from linearity or sensitivity, and the calibration curves for spots of all test solutions remained linear over the full range of concentrations tested (Fig. 4). Significant differences in color development of the dye-protein complex were observed in the presence of the different salts and buffers with the most intense response noted with Tris-SDS followed in order by NaOH-SDS, NaOH, and Epps. Heps and Tris buffers gave similar results in all tests, and only results for the latter, containing PMSF as a protease inhibitor, are presented.

Densitometer scans plotted as peak heights (Fig. 4A) or peak areas (data not shown) against protein concentration gave essentially identical calibration curves. Their baselines remained linear (Fig. 3) if sample sheets were kept small to avoid rippling and if edge
to edge distances between spots were not less than 5 to 10 mm (Fig. 2). They also had lower limits of detection and were generally more sensitive than the calibration curves of absorbance versus protein obtained with the ELISA plate reader (Fig. 4B). Minimum detection limits for linear plots of data collected with the densitometer and the ELISA reader were approximately 100 and 200 ng of total protein, respectively (Fig. 4). However, protein samples in the range of 20 ng could be detected visually on the sample sheets (Fig. 2), and the minimum detection limit with the densitometer could be extended to 60 or 80 ng given some loss in linearity (Fig. 3).

To compare the efficacy of the two assay procedures for the measurement of protein in chlorophyllous tissue, total protein, as a
The densitometric scans were more sensitive than absorbance measurements over the full range of protein concentrations, with the greatest advantage observed at the low end of the curve. Removal of leaf pigments with an acetone wash prior to extraction with a buffer did not have any significant effect on results (Fig. 5), nor was quantitation affected when 1–100 µg of chlorophyll extract was added to BSA standards prior to measurement (data not shown). Thus acetone extraction of leaf pigments is an unnecessary step, but it does not alter the accuracy of the protein measurement. However, when protein spots on sample sheets of chromatography paper (Fig. 2) were washed with acetone to remove putative interferences, losses of up to 30% were recorded, presumably caused by the physical effects of the washing process.

The type of extraction solution used did not have a significant effect on the precision of the protein assays or on the absolute values obtained with plant or animal tissue (Table 1). The results obtained with the scanning densitometer and the ELISA plate reader were similar. Moreover, no significant differences were observed when microgram quantities of protein in trichloroacetic acid (TCA) precipitates from extracts of liver and leaf tissue were analyzed and compared (Table 3) using scanning densitometry and the Lowry (1) and Bradford methods (4,18).

### TABLE 1

<table>
<thead>
<tr>
<th>Protein concentration (µg µl⁻¹)</th>
<th>Scanning densitometer</th>
<th>ELISA plate reader</th>
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<tbody>
<tr>
<td></td>
<td>Liver</td>
<td>Leaf</td>
</tr>
<tr>
<td>0.1 M NaOH</td>
<td>2.69 ± 0.05</td>
<td>1.32 ± 0.03</td>
</tr>
<tr>
<td>0.1 M NaOH + 2% SDS</td>
<td>2.71 ± 0.06</td>
<td>1.31 ± 0.01</td>
</tr>
<tr>
<td>Tris + 2% SDS</td>
<td>2.66 ± 0.06</td>
<td>1.33 ± 0.06</td>
</tr>
</tbody>
</table>

**Note:** Three extraction solutions and two modes of quantitation are compared. Each value is the mean of duplicate readings of three samples ±1 SE.
Table 2

Assays of TCA-Precipitated Protein from 10-mg Samples of Freeze-Dried Rat Liver and Primary Leaves of Soybean Extracted with Tris-SDS

<table>
<thead>
<tr>
<th>Method</th>
<th>Rat liver</th>
<th>Soybean leaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>CBBR-250, scanning</td>
<td>2.76 ± 0.12</td>
<td>1.03 ± 0.04</td>
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<tr>
<td>densitometer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bradford</td>
<td>2.95 ± 0.33</td>
<td>1.00 ± 0.04</td>
</tr>
<tr>
<td>Lowry</td>
<td>2.83 ± 0.40</td>
<td>1.05 ± 0.03</td>
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</tbody>
</table>

Note. Each value is the mean of duplicate readings of three samples ± 1 SE.

Discussion

The use of CBBR-250 in a quantitative protein assay was previously described by Esen (14) and adapted for use by Marder et al. (15). The dye-binding action was shown to be intensified by SDS, and the method provided a linear range from 0.05 to 4 mg·ml⁻¹ of protein although variability among replicates was unacceptably high at protein concentrations of less than 0.2 mg·ml⁻¹. The present modification of the dye-binding procedure offers several distinct advantages over these and other methods of protein analysis, including (i) a linear response from less than 100 to at least 1300 ng·µl⁻¹, with coefficients of variation not exceeding 8.9 and 3.4% at the low and high ends, respectively; (ii) only 1 µl of protein solution is required for a reliable measurement with a scanning densitometer; (iii) semiquantitative visual estimates of protein are obtained at concentrations as low as 20 ng·µl⁻¹; (iv) freedom from significant interference from chlorophyll and other leaf pigments and from reagents commonly included in buffers used for protein extraction and gel electrophoresis, including AHA, benzamidine hydrochloride, DTT, EDTA, 2-mercaptoethanol, NP-40, PMSF, and SDS; (v) crude extracts are readily and reliably quantified, thus eliminating the need for isolation of a TCA precipitate or other forms of sample cleanup; (vi) fast and precise quantitation of large numbers of plant or animal samples using a scanning densitometer or ELISA plate reader, with the option of interfacing either instrument to a computer; and (vii) the excision and elution of the dye–protein complex required for quantitation with an ELISA reader or with a spectrophotometer (14) is eliminated with the scanning densitometer option.

The CBBR-250 dye-binding method is several times more sensitive than the Bradford (4.18) or Lowry (1) method and it has the added advantage of long-term stability of the dye–protein complex. This allows sample sheets to be filed for repeat measurements at any time over a period of several months. Thus, if samples are dried rapidly with a hair dryer or in a microwave oven, they can be measured 1 min after spotting or at any convenient time thereafter. In addition to its obvious advantages of speed, sensitivity, and the stability of the dye–protein complex, its freedom from interference by components of buffer systems simplifies the measurement of protein in tissue extracts prior to polyacrylamide gel electrophoresis. For example, with the Bradford (4.18) and Lowry (1) assays, a TCA-precipitation step is required to eliminate interference by SDS in order to ensure subsequent loading of equal-sized protein samples for electrophoretic separation and for quantitative comparisons of the protein bands. The TCA-precipitation step is not required with the dye-binding procedure.

References