Optimization of Coomassie Staining for Quantitative Densitometry of Soybean Storage Proteins in Gradient Gel Electrophoresis

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ABSTRACT: Soybean (*Glycine max* L. Merr., cv. Dare) protein subunits were separated by gradient gel electrophoresis and analyzed by two-dimensional densitometry with computer-aided volume integration. Significant differences in the time required to achieve equilibrium staining with Coomassie Blue were revealed among the various polypeptides. Bands corresponding to lipoxygenase reached staining equilibrium in 2.7 h, whereas longer periods were required for polypeptides of β-conglycinin (5.5 to 6.7 h) and of glycinin (8.6 to 9.2 h). These differences among polypeptides could be attributed in part to changes in gradient concentration within the polyacrylamide gel. Optimal staining intensity among all soluble proteins extracted from soybean seed was reached after staining for 8 h. Shorter than optimal staining times lead to significant underestimation of parameters such as the percentage of β-conglycinin and glycinin of total soluble protein.

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KEY WORDS: β-Conglycinin, Coomassie Brilliant Blue, densitometry, *Glycine max*, glycinin, polyacrylamide gel electrophoresis, quantitative measurement, soybean storage protein.

Soybean storage protein consists of two main components, β-conglycinin and glycinin, which account for a majority of total seed protein. Glycinin is more abundant than β-conglycinin in nearly all soybean varieties, and their roles in nutrition and their functional properties differ. Their impact in food applications has been extensively reviewed (1,2). Knowing the precise quantity of these two proteins is helpful in selecting soybean cultivars with improved protein quality. For example, such quantitative determination of soybean storage proteins has led to the identification and characterization of the newly developed high-protein soybean cultivar, Prolina, with subtle changes in glycinin and β-conglycinin composition that resulted in enhanced functional properties of protein (3).

Quantitative estimates of storage protein composition in soybean may be obtained with gel electrophoresis using two-dimensional densitometry. Coomassie Brilliant Blue R250 (CBB) is commonly used to detect proteins on electrophoretic gels. In general, the stainability of proteins using CBB dye is time-dependent and also dependent on the pore size of a given polyacrylamide gel (4). Although the binding interaction of the dye and proteins is well understood on a molecular level (5), the conditions and optimal time required for equilibrium binding of CBB dye with soybean proteins have not been reported. The time interval routinely used in standard staining protocols with CBB on gradient sodium dodecyl sulfate (SDS)-polyacrylamide electrophoresis gels may explain apparent discrepancies in reproducibility or consistency of reports on soluble protein composition from given soybean genotypes. Therefore, we determined the optimal time required for equilibrium binding of dye with soy proteins and evaluated the impact of inadequate CBB staining on the analysis of proteins separated in different gradient concentrations of a polyacrylamide gel.

MATERIALS AND METHODS

Preparation of soybean protein. Protein was extracted for 30 min at room temperature from full-fat soybean (*Glycine max* L. Merr., cv. Dare) meal in a 1:20 (wt/vol) ratio with 0.03 M Tris-HCl buffer, pH 8.0, that contained 0.1 M β-mercaptoethanol (6). The mixture was centrifuged at 10,000 × *g* for 10 min at 4°C. Storage proteins and their polypeptides in the supernatant were dissociated in the same buffer containing 2% (wt/vol) SDS and 2% (vol/vol) β-mercaptoethanol in a boiling water bath for 10 min. Glycerol and bromphenol blue were added to each sample to the final concentrations of 10 and 0.025%, respectively.

Polyacrylamide gel electrophoresis (PAGE). Proteins and their polypeptides were separated in a Bio-Rad (Richmond, CA) Protean II vertical slab gel apparatus according to Chua (7) with the following modifications. Samples containing approximately 80–100 μg protein were loaded onto the gel and were separated by using a linear gradient of 10 to 20% polyacrylamide gel. Blank sample wells were left between loaded samples to prevent protein cross-contamination during electrophoresis and to facilitate accurate quantitation after electrophoresis. Electrophoresis of each protein sample was carried out in duplicate at a constant current of 10 mA/gel until the tracking dye reached the bottom of the gel.

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Staining and destaining. Gels were fixed in 40% (vol/vol) methanol and 10% (vol/vol) acetic acid for at least 1 h then stained in 0.25% CBB (w/w), 40% (vol/vol) methanol, and 10% (vol/vol) acetic acid on an orbit shaker. After 0.25, 0.5, 1, 2, 4, 8, 16, or 24 h, gels were destained in 40% (vol/vol) methanol and 10% (vol/vol) acetic acid on the same orbit shaker. The destaining solution was changed every 2 h for a minimum of four changes. The destaining was terminated when the background gels were almost visibly cleared of the dye. It was preferable in this experiment that the gels not be completely destained. Destained gels were soaked in deionized water for at least 5 min, sandwiched with two cellophane sheets, and then dried in a Bio-Rad GelAir dryer.

Scanning densitometry. Dried gels were scanned with a Molecular Dynamics Personal Densitometer SI (Sunnyvale, CA) equipped with a HeNe laser light source. ImageQuant (Molecular Dynamics) software for volume integration was used in data analysis to determine total absorbance of entire protein bands. Apparent absorbance of each protein was obtained by subtracting the local average background absorbance from the total absorbance of the protein bands within the same gel volume. All data are reported as means of two replications. Minimum staining time for individual polypeptides separated from soy storage protein was determined from first-order rate constants for each fraction. This rate constant was determined from the regression of ln[(observed protein concentration)(staining time)$^{-1}$] against ln(staining time, h). The staining time required to achieve dye binding equilibrium was approximated from the derived intercept (slope)$^{-1} \times 2.713$.

RESULTS AND DISCUSSION

Soluble soybean protein consists of two major constituents, β-conglycinin and glycinin. β-Conglycinin is composed of molecular species with trimeric combinations of α′, α, and β polypeptides (8,9). Glycinin is composed largely of two polypeptides, one with an acidic and the other with a basic isoelectric point (10). These fractions account for the majority of soluble storage proteins in soybean. A typical pattern of separation of these and other soluble polypeptides on 10 to 20% (w/w) linear gradient SDS-polyacrylamide gels stained for 1, 8, and 24 h is shown in Figure 1. Routinely, detection of proteins in SDS-polyacrylamide electrophoretic gels is achieved by staining for 1 h or less in 0.25% (w/w) CBB. This staining protocol may be sufficient for semiquantitative protein analyses, but appears to be inadequate for quantitative analyses of the protein composition in soybean. As shown in Table 1, considerable experimental variation in the concentration of respective polypeptides evidently was a function of staining time. In addition to the obvious differences in polypeptide concentration, staining for 1 h leads to a significant underestimation of the contribution of the β-conglycinin and glycinin to total soluble protein. In the 1-h treatment, the sum of β-conglycinin plus glycinin accounted for 73.8% of the soluble protein, whereas these constituents accounted for 80.6% of the protein in the 8 h treatment. The proportion of each protein fraction also varied with time. This was evident in a statistically significant change in the glycinin to β-conglycinin ratio from 1.61 in the 1-h treatment to 1.69 in the 8 h treatment (least significant difference at the 0.05 level: 0.03).

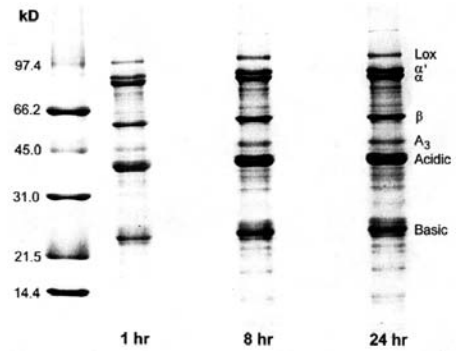

FIG. 1. Typical protein profile of soluble proteins extracted from soybean (*Glycine max* L. Merr., cv. Dare) on a linear gradient (10–20%) sodium dodecyl sulfate-polyacrylamide gel showing β-conglycinin polypeptides (α′, α, and β), glycinin polypeptides (acidic and basic), Lox, (lipoxygenases). They were stained for 1, 8, and 24 h, respectively.

TABLE 1
Effect of Staining Time on Estimates (% total soluble protein) of Soluble Storage Protein Composition in Soybean Seed[a]

Time		β-Conglycinin			Glycinin			
(h)	LOX	α′	α	β	Acidic	Basic	Other	Total
0.25	3.3	6.9	10.6	9.2	25.0	17.4	27.6	100.0
0.50	3.2	7.1	11.1	9.6	25.6	18.8	24.7	100.0
1.0	3.1	7.3	11.3	9.8	26.1	19.4	23.1	100.0
2.0	2.7	7.5	22.5	9.9	26.9	20.6	21.0	100.0
4.0	2.5	7.7	11.7	9.9	27.7	21.4	19.0	100.0
8.0	2.4	7.7	12.0	10.2	28.4	22.3	17.0	100.0
16.0	2.2	7.7	12.1	10.5	28.6	22.8	16.1	100.0
24.0	2.2	7.7	12.1	10.5	28.7	22.8	16.1	100.0
$LSD_{0.05}$	0.2	0.2	0.3	0.3	1.3	1.2	3.3	

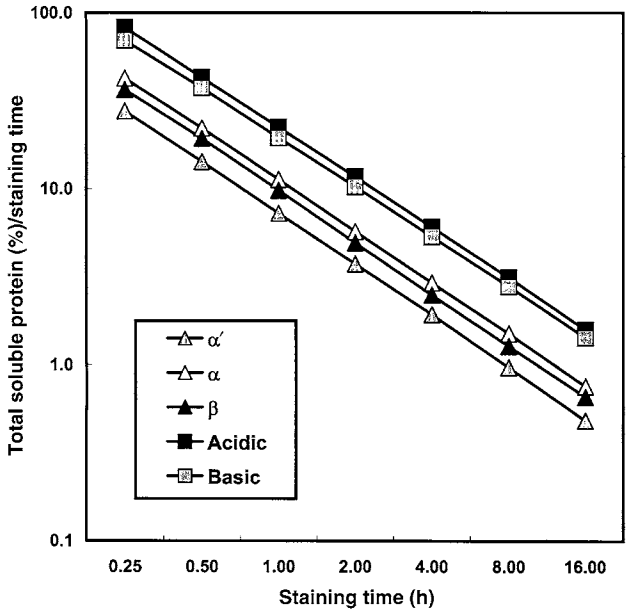

FIG. 2. Staining equilibrium for subunits of β-conglycinin and glycinin proteins.

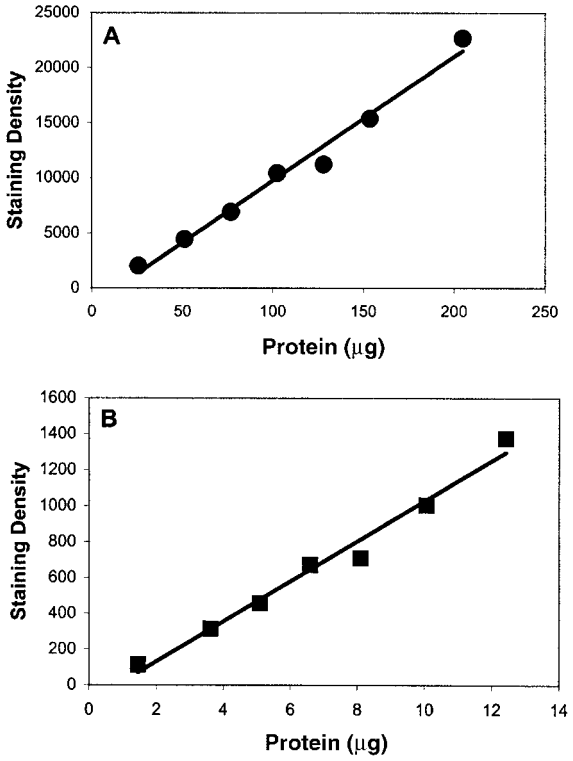

FIG. 3. Staining intensity at equilibrium as a function of protein concentration. (A) Total soluble protein; (B) β-subunit.

This change was observed with the polypeptides of both β-conglycinin and glycinin (Table 1).

The cause of variation in these measurements among treatments was attributed to differential rates of reaction between specific polypeptides and CBB. In that regard, the staining reaction for each protein was a function of first-order kinetics. Estimates of the time required to achieve equilibrium staining were derived from these relations (see the Materials and Methods section). Equilibrium staining of lipoxygenase bands was reached at 2.7 h (data not shown). Among glycinin polypeptides, equilibrium staining occurred at 9.2 and 8.6 h for acidic and basic polypeptides, respectively. Among β-conglycinin polypeptides, equilibrium staining was at 5.5, 6.7, and 6.3 h for α′, α, and β polypeptides, respectively (Fig. 2). Thus, the overall staining rate of the glycinin fraction was slower than the β-conglycinin fraction. Although the binding affinity of CBB dye may be related to the polarity of a given protein, the apparent differences in staining equilibrium could be influenced by pore size or concentration of polyacrylamide where such polypeptide band is present. The time required for staining equilibrium could also be dependent on the amount of each polypeptide present in the crude protein extract. As seen in Figures 3A and 3B for total soluble protein and β subunit of the β-conglycinin, respectively, the staining intensity at equilibrium at 8 h or longer is a linear function of protein concentration. Nevertheless, this staining differential, especially at shorter staining periods, may lead to significant misrepresentation of protein composition.

Thus, the glycinin to β-conglycinin ratio may be regarded as a predictive indicator of relative protein nutritional quality and functionality. In the event that ratio is used as a selection index for screening progeny within breeding populations, minor fluctuations due to experimental error could seriously impede breeding progress. At least, this is one example to help demonstrate the general point that precision in protein analysis can be jeopardized by inattention to factors as seemingly trivial as the staining time employed to develop SDS-PAGE gels.

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