Quantitative Analysis of Soybean Proteins by Densitometry on Gel Electrophoresis

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ABSTRACT

Cereal Chem. 63(6):493-496

Quantitative analyses of soybean globulins were made by densitometry in a sodium dodecyl sulfate (SDS)-polyacrylamide slab gel using lysozyme as an internal standard protein and Coomassie Brilliant Blue as a staining reagent. The slopes of standard curves for soybean globulins showed a considerable difference, depending on their proteins. The densitometric intensity for the 7S globulins of γ-conglycinin, basic-7S globulin, and β-conglycinin was higher than for glycinin and Kunitz trypsin inhibitor. Proteins extracted with both water and 0.5*M* sodium chloride contained glycinin (36.5%), β-conglycinin (27.8%), γ-conglycinin (6.2%), Kunitz trypsin inhibitor (2.9%), basic-7S globulin (3.0%), and other proteins (23.6%) that included whey protein. The relative concentrations of the major components (glycinin and β-conglycinin) were evaluated to accuracies better than ±15%, and the other minor components were evaluated to ±20%.

Polyacrylamide gel electrophoresis (PAGE) is a superior technique presently available for separating proteins, and Coomassie Blue is a sensitive protein stain. Much recent work in quantitative PAGE is concerned with stains and staining procedures. Coomassie Blue has been reported to follow Beer's law over only a low concentration range (Chrambach et al 1967, Fazekas de St. Groth et al 1963), and there are difficulties in accurately determining the amount of protein within a gel. Fishbein (1972) has shown the major sources of error in quantitative densitometry.

In this study, we adopted an internal standard method for quantitative densitometry on PAGE. This method is not necessary to accurately determine the amounts of protein loaded on a gel, but to determine the proportion of different protein components in a mixture.

The quantities of some soybean globulins were reported from ultracentrifugal analysis by Wolf and Briggs (1956) and immunological analysis by Koshiyama and Fukushima (1976). Recently, Hughes and Murphy (1983) and Murphy and Resurreccion (1984) investigated the major soybean globulins by analysis of eluted Coomassie Blue dye and an immunological method, respectively. Ultracentrifugal analysis cannot distinguish β-conglycinin from γ-conglycinin. The immunological method requires an individual antibody, and one sample can measure only one protein species. Densitometric analysis on PAGE can analyze all the proteins in one measurement. This paper describes the quantitative analysis for not only the major proteins glycinin (11S globulin) and β-conglycinin (7S globulin) but also the minor proteins γ-conglycinin (7S globulin), basic-7S globulin, and Kunitz trypsin inhibitor.

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MATERIALS AND METHODS

Materials

Defatted soybean meal was prepared (*Glycine max* var. Raiden, from 1982 and 1983 crops in Iwanuma, Miyagi, Japan). The soybean seeds were dehulled, flaked, and defatted with hexane. The β-conglycinin (7S globulin), glycinin (11S globulin) (Thanh and Shibasaki 1976), γ-conglycinin (7S globulin) (Sato et al 1984), and basic-7S globulin (Yamauchi et al 1984) were isolated by the cited methods. All the proteins were pure, as checked by column chromatography and PAGE. Kunitz trypsin inhibitor was purchased from Miles Laboratories (Pty) Ltd. Lysozyme and bovine serum albumin were obtained from Sigma Chemical Co. All reagents were of the highest grade.

Protein Determination

The yields of water- and NaCl-soluble proteins were determined by Lowry's method (Lowry et al 1951) as referred to bovine serum albumin. For the standard curves of the peak area, the concentrations of lysozyme and soybean proteins were taken gravimetrically.

Sample Preparation

The water- and NaCl-soluble fractions were prepared by the modified procedure of Hu and Esen (1981) (Fig. 1). Defatted soybean meal was agitated with 20 volumes (v/w) of distilled water for 1 hr at room temperature and centrifuged (15,000 × *g*, 5°C, 25 min). The precipitate was extracted with distilled water in the same way three times. The water- and NaCl-soluble fractions were dialyzed against water and lyophilized after measurements of each volume and the protein content. Acid-precipitated protein was obtained from the first water-soluble fraction adjusted to pH 4.8 and dialyzed against water and lyophilized.

Electrophoresis

The polyacrylamide thin slab-gel system of Swank and Munkres

(1971) was used with an acrylamide concentration of 10% and a sodium dodecyl sulfate (SDS) concentration of 0.1%. The gel dimensions were 140 × 140 × 2.0 mm with nine sample wells, each of 10-mm width. Electrophoresis was conducted at a constant voltage of 100 V for 5 hr.

Staining

Coomassie Brilliant Blue G-250 was used at a concentration of 0.25% in a solution of methanol, acetic acid, and water (45:10:45, v/v) for 1 hr. Destaining took two to three days employing changes in the ratio of methanol, acetic acid, and water (20:7:73, v/v). Gels were considered completely destained when there was no visible background stain remaining in the gel.

Densitometric Scanning of the Protein Patterns

For protein quantification by densitometric scanning, gels were scanned at 570 nm (reference, 730 nm) using the scanning attachment for a Shimadzu dual-wavelength CS-900 TLC scanner with a scanning speed of 10 mm/min and a chart speed of 20 mm/min. Peak areas were measured by planimetry.

Standard curves of each protein were determined according to the internal standard method. These standard curves were used to evaluate unknown concentrations in each fraction from peak ratios to a known concentration of lysozyme, which was the internal standard protein. The standard proteins were made as follows: 0–11.10 mg/ml (β-conglycinin), 0–13.59 mg/ml (glycinin), 0–3.72 mg/ml (γ-conglycinin), 0–2.61 mg/ml (Kunitz trypsin inhibitor), and 0–9.88 mg/ml (basic-7S globulin). Each sample for the standard curve was made in duplicate or triplicate, and in the case of quantification for unknown samples, each sample was repeated for four determinations or more.

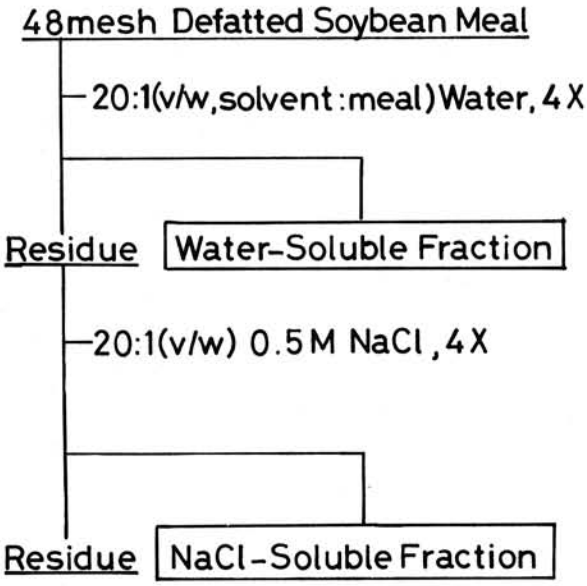

Fig. 1. Preparation of the water-soluble fractions and NaCl-soluble fractions. Extractions with water and 0.5*M* NaCl were performed at room temperature.

TABLE I
Protein Content of Each Fraction in Terms of Total Soybean Meal Constituents and Total Protein

Fraction	Soybean Meal % (w/w)	Total Protein % (w/w)
Water-soluble		
1st	27.8	69.4
2nd	8.4	20.9
3rd	1.6	4.0
4th	0.5	1.1
NaCl-soluble		
1st	1.1	2.8
2nd	0.5	1.2
3rd	0.2	0.4
4th	0.1	0.2
Total	40.1	100.0

RESULTS

Extraction of Protein from Defatted Soybean Meal

The total protein content of defatted soybean meal amounted to 40.1% (w/w), and about 90% of the total protein was contained in the first and second water-soluble fractions (Table I).

Electrophoretic Profiles and Densitometric Patterns of Water- and NaCl-Soluble Fractions

The SDS-PAGE patterns of the extracted proteins are shown in Figure 2. The water-soluble fractions include the two major soybean protein components of β-conglycinin (7S globulin) and glycinin (11S globulin) and some of the minor components of γ-conglycinin (7S globulin) and Kunitz trypsin inhibitor. Glycinin and β-conglycinin are composed of 12 subunits of acidic and basic subunits and three subunits of α′, α, and β-subunits, respectively. The NaCl-soluble fraction showed two main bands, which were the low-molecular-weight and high-molecular-weight subunits of basic-7S globulin (Fig. 2). Figure 3 shows the standard curves of each protein by the internal standard method. The color ratios to β-conglycinin determined from the inclination of curves were as follows: 100.0 (β-conglycinin), 67.0 (glycinin), 80.8 (γ-conglycinin), 51.7 (Kunitz trypsin inhibitor), and 88.2 (basic-7S globulin). The correlation coefficients of the standard curves used in the internal standard method were more than 0.99.

Quantitative Estimation of Soybean Proteins

The linear relationship between the protein concentrations and peak areas was obtained in various protein ranges as shown in

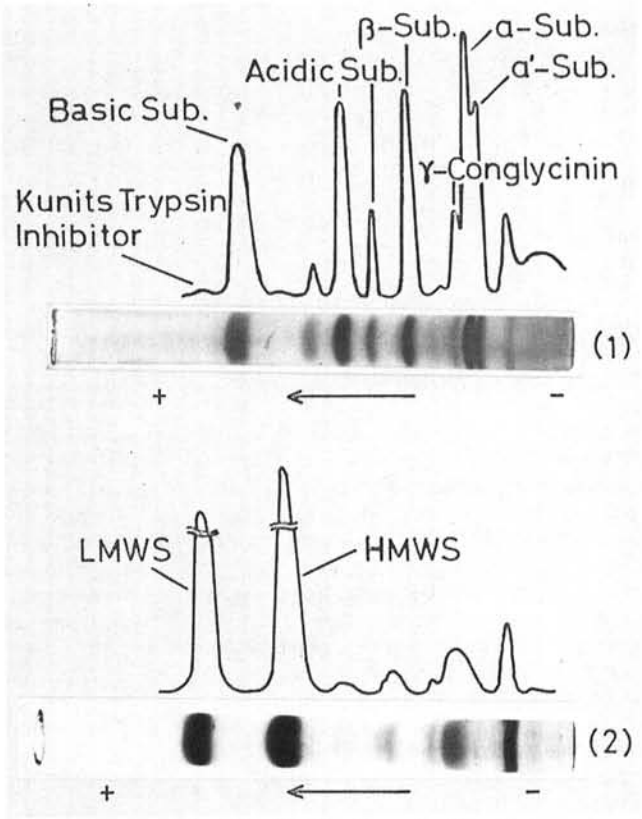

Fig. 2. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoretic patterns and densitometric scans of water- and NaCl-soluble fractions. 10% SDS-polyacrylamide gel electrophoresis was performed at a constant voltage of 100 V for 5 hr. The gel was stained with Coomassie Brilliant Blue G-250. HMWS, high-molecular-weight subunit; LMWS, low-molecular-weight subunit.

Figure 3. The percentage of each protein in total protein in each soluble fraction is shown in Table II. Although the amounts of β-conglycinin and glycinin contained in each water-soluble fraction were considerable, the percentages of γ-conglycinin and Kunitz trypsin inhibitor in relation to total protein were 6.2 and 2.9%, respectively. The amounts of Kunitz trypsin inhibitor were so small in the water-soluble fractions that only the first water-soluble fraction could be quantitated. In the NaCl-soluble fractions, the amount of basic-7S globulin was about 3.0% of total protein. The amounts of each acid-precipitated protein that were removed in the whey fraction were also calculated and are reported in Table III. The total amounts of β-conglycinin and glycinin were about 90% (w/w) of the acid-precipitated proteins. The other related data are listed in Table III.

The accuracy of all standard curves was ±10%. On the other hand, the relative concentrations of the major components (glycinin and β-conglycinin) in each fraction and acid-precipitated protein were evaluated to accuracies better than ±15%, and the minor components (γ-conglycinin, Kunitz trypsin inhibitor, and basic-7S globulin) were evaluated to ±20%. These accuracies may be considered satisfactory for this type of experiment. The error may include sample preparation, efficiency of staining and destaining, instrument errors in recording (regarded as small), and errors in planimetry.

DISCUSSION

Fazekas de St. Groth et al (1963) first described the application of staining with Coomassie Brilliant Blue R-250 for the quantitation of protein by zone electrophoresis. We were able to show a linear relationship in a given range between the amount of a protein loaded on the gel and the staining intensity of the band binding with Coomassie Brilliant Blue G-250. Because different proteins have different affinities for Coomassie Brilliant Blue G-250 (Fig. 3), quantitating the concentration from the area ratio of a peak to total peaks may result in large errors without standard curves. Further, the technique of using an internal standard minimizes their errors, because it is not necessary to strictly determine the amount of protein on SDS gel. One must be careful, however, to select an internal standard protein that does not overlap other bands. The contents of Kunitz trypsin inhibitor, β-conglycinin, γ-conglycinin, and glycinin in acid-precipitated protein were 4.1, 40.7, 5.0, and 49.3%, respectively.

We chose the soybean variety Raiden for densitometric analysis. Staswick and Nielsen (1983) reported that Raiden has the glycinin subunit of A_6. However, A_6 contains a smaller amount, and two or three complicated bands are observed around A_6 in the gel electrophoretic pattern reported by Staswick and Nielsen (1983). The spot, moving faster than the acidic subunit in Figure 2A, is considered to be mainly a lectin (Lei et al 1983) together with A_6. We did not identify those. Some cultivars other than Raiden have other types of glycinin subunits, for example, A_5. If necessary, the internal standard of lysozyme should be changed for another suitable standard, because the glycinin subunit of A_5 has a comparable molecular weight.

The results could not be directly compared with immunological and ultracentrifugal analyses because of the difference in varieties and method. However, immunological analysis by Murphy and

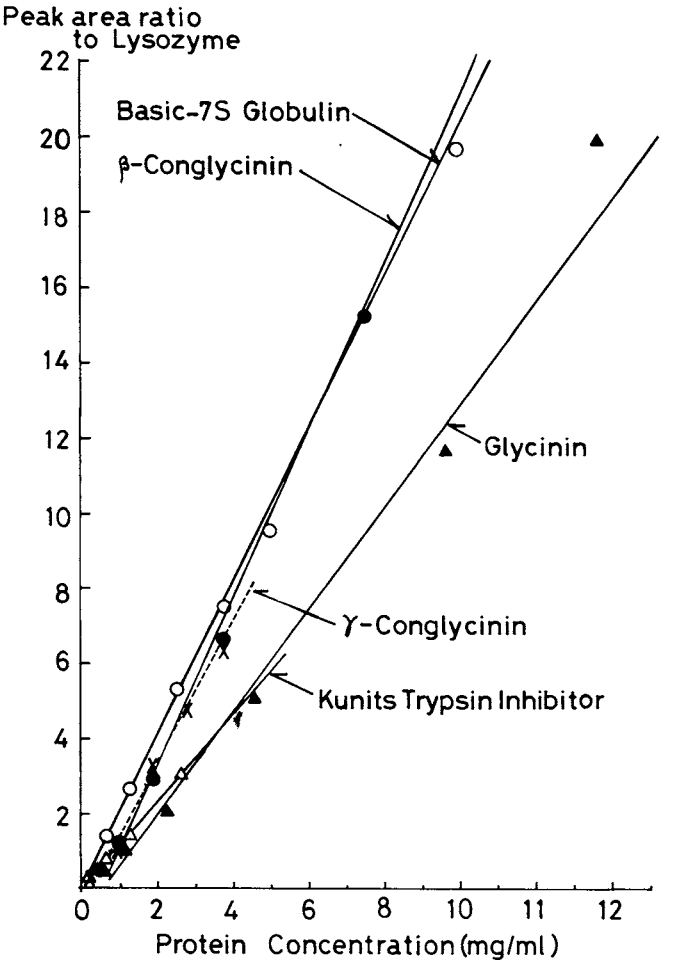

Fig. 3. Standard curves of five soybean globulins. Glycinin, β-conglycinin, γ-conglycinin, Kunitz trypsin inhibitor, and basic-7S globulin were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels, stained with Coomassie Brilliant Blue G-250, and scanned at 570 nm using a Shimadzu dual-wavelength CS-900 TLC scanner. Peak area ratio, peak ratios of a known concentration of the standard proteins to a known concentration of lysozyme.

TABLE II
Proportion of Soybean Proteins in Water- and NaCl-Soluble Fractions

	Protein % (w/w)					
Fraction	**β-Conglycinin**	**γ-Conglycinin**	**Glycinin**	**KTI[a]**	**Basic-7S Globulin**	**Total**
Water-soluble						
1st	28.2	5.6	34.1	4.2	···	72.0
2nd	31.0	9.1	48.8	···	···	88.9
3rd	35.7	9.4	54.0	···	···	99.1
4th	28.1	7.1	41.0	···	···	76.2
NaCl-soluble						
1st	···	···	···	···	50.0	50.0[b]
2nd	···	···	···	···	34.3	34.3[b]
3rd	···	···	···	···	17.6	17.6[b]
4th	···	···	···	···	12.3	12.3[b]
Total extracted proteins	27.8	6.2	36.5	2.9	3.0	76.4

[a] Kunitz trypsin inhibitor.
[b] The rest of the protein was contaminated with other unidentified water-soluble proteins. They were omitted because of their negligible amounts compared to those of the water-soluble fractions as shown in Table I.

TABLE III
Comparison of Soybean Globulin Contents
by Densitometric, Ultracentrifugal, and Immunological Analyses

Soybean Globulin	% of Total Extracted Proteins (including whey)			% of Acid-Precipitated Proteins	
	Densitometric[a]	Immunological[b]	Immunological[c]	Densitometric[c]	Ultracentrifugal[d]
2S Globulin	...	...	...	...	11
(KTI)[e]	2.9	...	...	4.1	...
7S Globulin	37.6	30.9	...	45.7	...
(β-conglycinin)	27.8	27.9	18.5	40.7	...
(γ-conglycinin)	6.2	3.0	...	5.0	...
(basic-7S globulin)	3.6	...	...	...	...
11S Globulin (glycinin)	36.5	...	51.0	49.3	48
15S Globulin	...	...	...	...	7
Total	76.4	...	69.5	99.1	99

[a] Data from this work.
[b] Data from Koshiyama and Fukushima (1976).
[c] Data from Murphy and Resurreccion (1984).
[d] Data from Robert and Briggs (1963).
[e] Kunitz trypsin inhibitor.

Resurreccion (1984) reported a remarkably low β-conglycinin and high glycinin contents as shown in Table III. They used renatured glycinin and β-conglycinin as the standard. Renaturation of β-conglycinin would give a good yield, although not complete. However, renaturation of glycinin gives a remarkably low yield. Kitamura et al (1977) reported only a 20% yield from the reductively denatured state. We observed that this renaturation yield would influence the protein contents. One of us (Iwabuchi and Yamauchi, *unpublished*) measured glycinin (34%) and β-conglycinin (27%) by an immunological method in water-extracted proteins using native proteins as the standard. These results are in close agreement with those shown in Table III.

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[Received April 16, 1985. Accepted June 6, 1986.]