Preparative Reversed-Phase High-Performance Liquid Chromatography of Soybean Proteins and Characterization of Fractions by Gel Electrophoresis

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Preparative (100–200 mg) reversed-phase high-performance liquid chromatography was conducted on water-extractable, acid-precipitated, and whey proteins plus purified β-conglycinin and glycinin from soybeans. Chromatographic fractions were collected, freeze-dried, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Polypeptide profiles obtained by electrophoresis of the fractions indicated that glycinin and β-conglycinin, the two major storage proteins, separated only partially. Glycinin subunit G5 (A_3B_4) was the first of these two proteins to elute followed by three other glycinin subunits. As elution of glycinin subunits continued, they were accompanied by β-conglycinin. Apparently, glycinin subunits and β-conglycinin do not differ sufficiently in hydrophobicity to permit their complete separation. Elution behaviors of minor proteins including Kunitz trypsin inhibitor and agglutinin were also determined.

INTRODUCTION

Soybean proteins are a complex mixture consisting of two major storage proteins, β-conglycinin and glycinin, plus numerous minor proteins with biological activities, e.g., trypsin inhibitors, lipoxygenases, and lectins. The storage proteins have multimeric structures and are high in molecular weight. β-Conglycinin has a trimeric structure, and three different subunits, α′, α, and β, have been identified. Glycinin has a hexameric structure, and each subunit consists of an acidic polypeptide linked to a basic polypeptide by a single disulfide bond. Five genes, Gy_1, Gy_2, Gy_3, Gy_4, and Gy_5, encoding the predominant subunits of glycinin have been characterized (Nielsen et al., 1989). The respective glycinin subunits encoded by these genes are G1 ($A_{1a}B_2$), G2 (A_2B_{1a}), G3 ($A_{1b}B_{1b}$), G4 ($A_5A_4B_3$), and G5 (A_3B_4). Designations in parentheses refer to the nomenclature used in earlier work to specify acidic and basic polypeptide chains purified from each subunit (Nielsen, 1985a,b; Nielsen et al., 1989). However, not all soybean cultivars contain all five of these glycinin subunits; Raiden cultivar, for example, lacks subunit G4 ($A_5A_4B_3$) (Staswick and Nielsen, 1983; Scallon et al., 1987). Because of the overall complexity of soybean proteins, it would be desirable to have available rapid methods for quantitative analysis of mixtures such as crude extracts for screening purposes. One such technique that has been very useful with other plant proteins, particularly for analysis of cereal proteins, is reversed-phase high-performance liquid chromatography (RP-HPLC).

RP-HPLC was first reported as a separation technique for cereal proteins in 1983 (Bietz, 1983, 1986). It has been used successfully on wheat (Bietz, 1983, 1986; Huebner and Bietz, 1988; Burnouf and Bietz, 1989; Sutton et al., 1989; Wieser and Belitz, 1989), barley (Wieser and Belitz, 1989), rye (Wieser and Belitz, 1989), oats (Wieser and Belitz, 1989), corn (Paulis and Bietz, 1988), and sorghum (Sastry et al., 1986). However, its application to other plant proteins such as those of soybeans has been more limited. RP-HPLC has been used for analysis of protein isolates in beef–soy protein blends (Ashoor and Stiles, 1987; Parris and Gillespie, 1988), scale-up from analytical to preparative RP-HPLC of glycinin (Peterson and Wolf, 1988), optimization of conditions for analysis of total proteins from soybean seeds (Buehler et al., 1989b), and screening of soybean cultivars for identification purposes (Buehler et al., 1989a). It was also used to separate subunits of β-conglycinin, γ-conglycinin, and the basic 7S globulin of soybeans, but no data on this separation were published (Hirano et al., 1987).

Except for our earlier preparative work on partially purified glycinin (Peterson and Wolf, 1988), there has been no identification of the various fractions obtained by RP-HPLC of soybean proteins. We have therefore extended our earlier scale-up study to unfractionated soybean proteins, two crude fractions, and purified preparations of β-conglycinin and glycinin. Our primary objectives were to obtain sufficient quantities of the chromatographic fractions for analysis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and to identify the proteins in the various RP-HPLC fractions. The results of our experiments are described here and present an overview of how the various soybean proteins behave on RP-HPLC.

MATERIALS AND METHODS

Protein Samples. Proteins were prepared from Raiden soybeans after flaking and defatting with hexane. Water-extractable, acid-precipitated, and whey proteins were prepared as described elsewhere (Wolf et al., 1966). All preparations were dialyzed against distilled water and freeze-dried.

Crude glycinin and β-conglycinin were prepared essentially according to the method of Thanh and Shibasaki (1976). A pH 8.0, 30 mM Tris-HCl extract (containing 10 mM 2-mercaptoethanol) of defatted flakes was adjusted to pH 6.4 with 2 N HCl, cooled to 3–5 °C overnight, and centrifuged at 4 °C. The precipitate was washed with 30 mM Tris-HCl (pH 6.4), dissolved in standard buffer (pH 7.6, 0.5 ionic strength, 33 mM K_2HPO_4, 2.6 mM KH_2PO_4, 0.4 M NaCl, 0.02% NaN_3, and 10 mM 2-mercaptoethanol), dialyzed against distilled water, and freeze-dried to yield crude glycinin. The supernatant remaining after

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removal of the initial glycinin precipitate from the 30 mM Tris-HCl extract (described above) was adjusted to pH 4.8 with 2 N HCl to precipitate β-conglycinin. The precipitate was removed by centrifugation and dispersed in 30 mM Tris-HCl (pH 7.6). The pH was then adjusted to 6.2, and the dispersion was centrifuged to remove polymerized β-conglycinin. The supernatant was dialyzed against water and freeze-dried to yield crude β-conglycinin.

β-Conglycinin was further purified by dissolving 2 g of crude protein in 25 mL of standard buffer and applying it to a column (12.5 × 2.6 cm i.d.) of concanavalin A–Sepharose 4B (Sigma, St. Louis, MO). After the unadsorbed fraction was washed through the column, the adsorbed fraction was eluted with 0.2 M methyl α-mannoside. The eluted material was dialyzed against water and freeze-dried. A typical ultracentrifugal analysis revealed 91% 7S and 9% 11S.

Glycinin was purified by dissolving freeze-dried, crude glycinin (2 g) in 25 mL of the above pH 7.6, 0.5 ionic strength buffer and applying it to a column (12.5 × 2.6 cm i.d.) of concanavalin A–Sepharose 4B equilibrated with the same buffer. Buffer was passed through the column until absorbance at 280 nm returned to the baseline. The unadsorbed protein fraction was either dialyzed against water and freeze-dried or concentrated by pervaporation and applied to two Sepharose 6B (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) columns (93 × 2.6 cm i.d.) connected in tandem. The solvent used for gel filtration was standard buffer described earlier. Fractions (10 mL/h per tube) were collected, and absorbance at 280 nm was measured. Fractions for the main peak were pooled, analyzed in the ultracentrifuge, dialyzed against distilled water, and freeze-dried to yield purified glycinin. A typical ultracentrifugal analysis revealed 4% 7S and 96% 11S.

Chromatography. *Equipment.* The chromatographic system included a Spectra-Physics Autolab Division (San Jose, CA) 8100XR automated liquid chromatograph; a Hewlett-Packard (Palo Alto, CA) 8450A diode array UV–spectrophotometer, an Omniscribe (Houston Instrument, Austin, TX) recorder, and a ModComp (Modular Computer Systems, Inc., Fort Lauderdale, FL) 32/85 computer system. The chromatograph's injection valve was fitted with a 10-mL injection loop connected externally to the chromatograph with 150 × 1.0 mm i.d. stainless steel tubing.

Solvents. Trifluoroacetic acid (TFA) (Sequanal grade) was from Pierce Chemical Co. (Rockford, IL). HPLC grade acetonitrile was purchased from various suppliers (Fisher Scientific, Springfield, NJ; J. T. Baker Chemical Co., Phillipsburg, NJ; and EM Science, Cherry Hill, NJ). Distilled water was treated in a NANOpure water system (Barnstead/Thermolyne Corp., Dubuque, IA). Solvents were sparged vigorously with helium for 5 min and held under a low-pressure (1–2 psig) positive helium atmosphere during chromatography.

Chromatographic Conditions. Separations were performed at 60 °C on a Rainin Instrument Co. Inc. (Woburn, MA) Dynamax 300-Å 12 μm C18 (250 × 21.4 mm i.d.) column. A Brownlee Labs (Santa Clara, CA) RP-2 cartridge (30 × 4.6 mm i.d.) served as guard column. The column was equilibrated for 20 min with 20:80 acetonitrile/water containing 0.1% TFA prior to injection. A 90-min gradient of 20–45% acetonitrile in water (both containing 0.1% TFA) was then used. The final 45% acetonitrile concentration was maintained for 20 min, yielding a 110-min chromatogram. Flow rate was 6 or 8 mL/min. The effluent was passed through a diode array detector where it was scanned from 200 to 800 nm every 10 s. Detector outputs were transmitted to the ModComp computer for subsequent graphic representations.

Sample size was 100 or 200 mg dissolved in 4 or 8 mL of pH 7.6, 0.5 ionic strength standard buffer. Protein fractions were collected manually as shown in the chromatograms. Fraction 1, which contained buffer salts and 2-mercaptoethanol, was dialyzed against water and freeze-dried. All other fractions were freeze-dried directly.

Polyacrylamide Gel Electrophoresis. SDS–PAGE was done by a Hoefer Scientific Instruments (San Francisco, CA) Model SE 600 unit on 1.5-mm polyacrylamide gels. Two gel systems were used: (A) 12.3% gels with an acrylamide/bis ratio of 36:1 and (B) 12.5% gels with an acrylamide/AcrylAide (FMC BioProducts, Rockland, ME) ratio of 36:1.5. System B was used in later stages of the study to stabilize gels on GelBond PAG film (FMC BioProducts) for drying. The Fling and Gregerson (1986) modification of the Laemmli (1970) procedure was used. Proteins were dissolved (2 mg/mL) in sample buffer and heated for 5 min at 100 °C. Gels were stained with Coomassie Brilliant Blue R-250 (Sigma) as per Fling and Gregerson (1986). Low molecular weight protein standards (Bio-Rad Laboratories, Richmond, CA) (phoshorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme) were run with each gel. After a preliminary analysis using 50–60 μg/sample, a second gel was run and sample loads were adjusted, if necessary, to give approximately equal intensities for the major protein bands. The SDS–PAGE bands were identified on the basis of the behavior of purified β-conglycinin, glycinin, Kunitz trypsin inhibitor, agglutinin (Sigma) and results in the literature (Hirano et al., 1987; Nielsen, 1985b; Coates et al., 1985; Staswick and Nielsen, 1983; Sathe et al., 1987).

For identification of the acidic polypeptides of glycinin in RP-HPLC fractions of the water-extractable proteins, the samples were run on a 12% polyacrylamide gel in a Mini-Protean II cell and then blotted onto a PVDF membrane in a Mini Trans-Blot transfer cell (Bio-Rad Laboratories). The blotted bands were excised and subjected to N-terminal amino acid microsequencing.

N-Terminal Amino Acid Microsequencing. Blotted acidic polypeptide bands of glycinin separated from the water-extractable proteins were microsequenced for the first five residues on an Applied Biosystems, Inc. (Foster City, CA), Model 477A sequencer by the University of Illinois Biotechnology Center, Genetic Engineering Facility (Urbana, IL).

Double-Diffusion Immunoassay. Assays were performed using the procedure of Kuo et al. (1984). Rabbit antiserum to Kunitz soybean trypsin inhibitor was provided by T. M. Kuo of this Center. The antiserum was diluted 1:4 with 0.1 M barbital buffer, pH 8.5, prior to use.

RESULTS

Water-Extractable Proteins. Unfractionated proteins extracted from defatted soybean meal with water separated upon RP-HPLC as shown in Figure 1A. Two small, sharp peaks eluted early, but most proteins emerged after 56 min in four major unresolved complex fractions. Eluted proteins were pooled into 10 fractions as indicated in Figure 1A and analyzed by SDS–PAGE (Figure 1B). Water-extractable proteins are shown for comparison and consisted primarily of lipoxygenase (Hirano et al., 1987), the β-conglycinin complex (α′-, α-, and β-subunits), A_3 and other acidic glycinin polypeptides, and basic glycinin polypeptides. Fraction 1 contained minor proteins of 32 to <10 kDa; none of the bands had detectable counterparts in the water-extractable proteins.

Fraction 2 consisted of a dark diffuse band of 17 kDa, plus two lighter bands of 11 and <10 kDa. Fraction 3 consisted of a major band of 14 kDa plus lesser bands of 13 and <10 kDa. Fraction 4, which contained the minor proteins between fractions 3 and 5 (Figure 1A), was a series of bands from about 40 to <10 kDa. Fraction 5, a pool of several minor RP-HPLC peaks, contained a distinct band of 40 kDa that coincides with glycinin's A_3 polypeptide chain. Identity of A_3 was confirmed by microsequencing the electroblotted band which yielded the expected N-terminal sequence: Ile-Thr-Ser-Ser-Lys- (Nielsen et al., 1989). The other major band of fraction 5 is likely the B_4 chain of glycinin, the basic polypeptide partner in glycinin subunit A_3B_4 (Nielsen, 1985b). This interpretation was confirmed by nonreducing SDS–PAGE that revealed a 57-kDa band in place of A_3 and the presumed B_4 band, indicating that glycinin subunit G5 (A_3B_4) was present in fraction 5.

Fraction 6 contained five or six bands. The faint, uppermost band of 50 kDa migrated slightly slower than the β-subunit of β-conglycinin and may be β-amylase (Hirano et al., 1987). Traces of glycinin polypeptide A_3

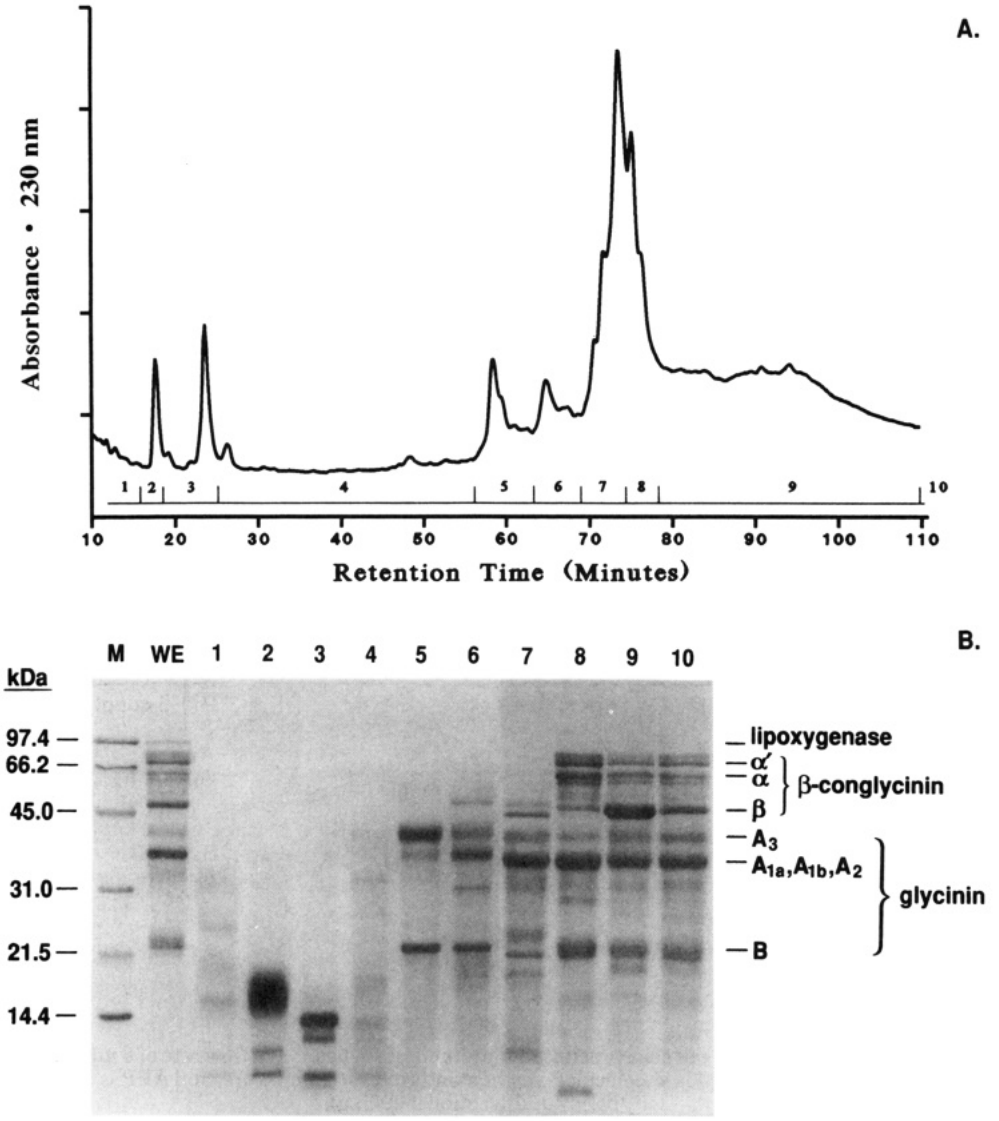

Figure 1. RP-HPLC chromatogram of 100 mg of water-extractable proteins of soybeans at a flow rate of 8 mL/min (A) and SDS–PAGE (system A) of RP-HPLC fractions 1–10 (B). M is a molecular weight standard protein mixture, and WE is water-extractable soybean proteins. Sample loads: 50 μg/lane except for standards (2 μg/band), fraction 2 (25 μg), and fraction 6 (63 μg). In this and following figures protein bands were identified as described under Materials and Methods (Polyacrylamide Gel Electrophoresis).

appeared, but major bands were of the other acidic polypeptides of glycinin plus their basic polypeptide partners. Microsequencing of the main acidic polypeptide band indicated that it was a mixture of polypeptides, but results were consistent with the sequences Phe-Ser-Ser-Arg-Glu- for A_{1a} and Leu-Arg-Glu-Glu-Ala- for A_2 plus minor amounts of amino acid residues corresponding to polypeptide A_{1b}. Fraction 7 contained a faint band (β-amylase?) slightly above and a darker one slightly below the β-subunit of β-conglycinin (absent in fraction 7 but present in fractions 8–10). The latter may be the β′-subunit of β-conglycinin described by Coates et al. (1985). Also present were small amounts of glycinin polypeptide A_3 accompanied by a much higher concentration of one or more acidic polypeptides of glycinin with possibly three basic polypeptide bands. Microsequencing of the major acidic polypeptide band of fraction 7 indicated the presence of polypeptides A_{1a} and A_2 plus small amounts of A_{1b}. A small amount of a 12-kDa band was also observed. In fraction 8 we see the abrupt appearance of β-conglycinin subunits, particularly the α′- and α-subunits, still accompanied by acidic and basic polypeptides of glycinin. The major acidic polypeptide band was sequenced and again revealed that A_{1a} and A_2 plus low concentrations of A_{1b} were present. The basic polypeptides of glycinin, however, appear more diffuse than in fractions 5–7. Also present is a band of <10 kDa.

Fraction 9 was dominated by the β-subunit of β-conglycinin, with small amounts of other β-conglycinin subunits and acidic and basic polypeptides of glycinin. However, there was no band in the <10-kDa region. Fraction 10 had a composition almost identical to that of water-extractable proteins except for the lipoxygenase band. Surprisingly, none of the fractions contained a detectable lipoxygenase band. As noted for fractions 6–8, the dark acidic polypeptide band in fraction 10 had sequences corresponding to A_{1a} and A_2 along with a low level of A_{1b}. The presence of glycinin subunits in fractions 6–10 was confirmed by SDS–PAGE under nonreducing conditions, which revealed a 57-kDa band instead of the acidic and basic polypeptides.

Acid-Precipitated Proteins. Adjusting a water extract of defatted soybean meal to pH 4.5 precipitates the globulin fraction that contains about 90% of the total nitrogen in the water extract (Smith et al., 1955). The RP-HPLC chromatogram of the acid-precipitated proteins (Figure 2A) is similar to that of water-extractable proteins (Figure 1A) as expected, because globulins constitute most of the

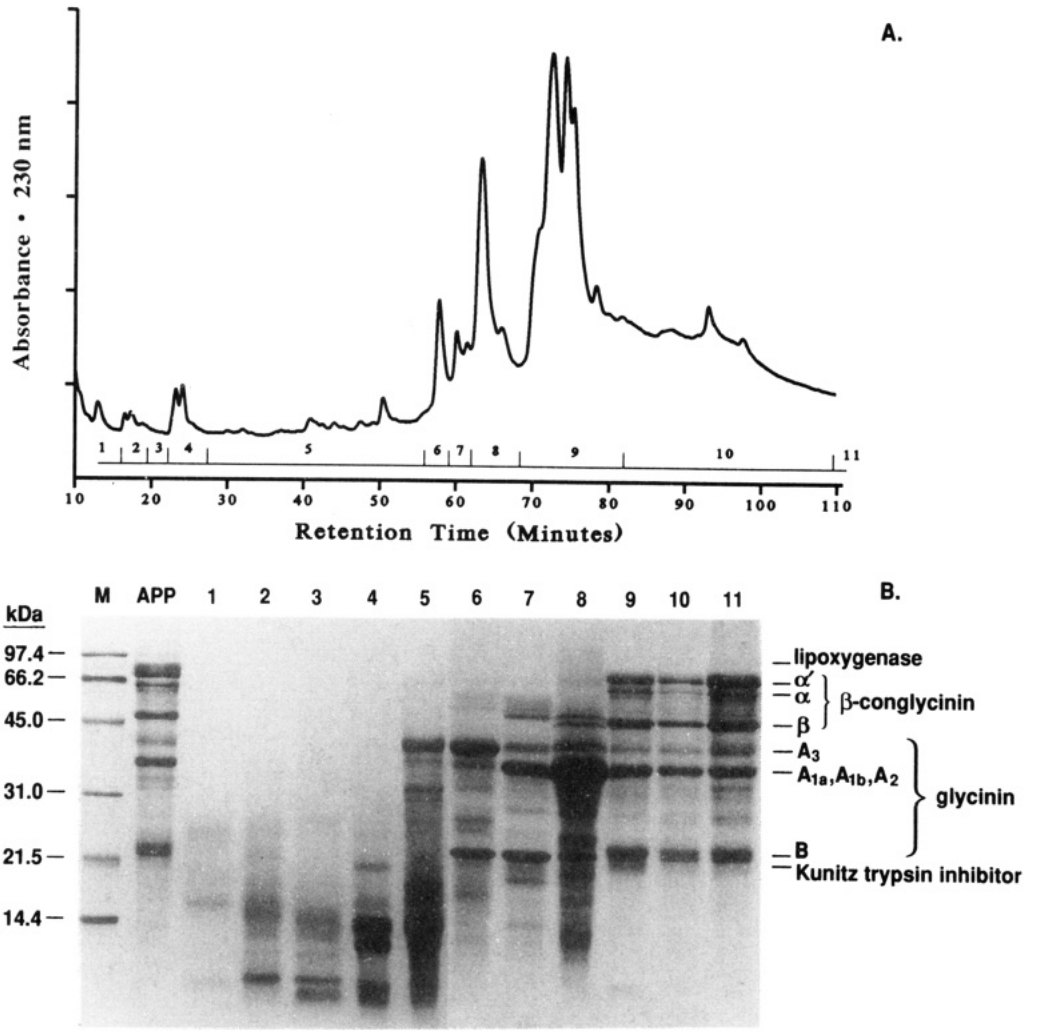

Figure 2. RP-HPLC chromatogram of 200 mg of acid-precipitated proteins of soybeans at a flow rate of 8 mL/min (A) and SDS–PAGE (system A) of RP-HPLC fractions 1–11 (B). M is a molecular weight standard protein mixture, and APP is acid-precipitated proteins. Sample loads: 50 μg/lane except for standards (2 μg/band) and fraction 1 (90 μg).

water-extractable proteins. As with water-extractable proteins, the early portion of the chromatogram (0–56 min) has a series of small peaks, and most proteins eluted after 56 min.

Upon SDS–PAGE (Figure 2B), acid-precipitated proteins gave a pattern similar to that of water-extractable proteins (Figure 1B). Fraction cuts in Figure 2A were made at times different from those of Figure 1A. A cut was made at 20–23 min, resulting in an additional fraction. RP-HPLC fraction 1 consisted of minor bands similar to those of fraction 1 in Figure 1B. Fraction 2 also contained ill-defined material ranging from about 27 to <10 kDa, with a major band at 10 kDa. Fraction 3 was similar to fraction 2 except for an additional band of ≪10 kDa. Fraction 4 had two dark bands of 15 and 13 kDa, plus two bands of <10 and ≪10 kDa as in fraction 3. Fraction 5 contained the two major bands in fraction 4, plus a prominent band corresponding to acidic polypeptide A_3 of glycinin; its basic polypeptide partner, B_4, does not appear to be present.

In fraction 6, however, we see acidic chain A_3 and a basic partner, presumably B_4, as major bands, plus a small amount of one other acidic polypeptide. Fraction 7 contained minor bands in the β-conglycinin region but showed a pronounced decrease in the ratio of A_3 to the other acidic polypeptides and a major basic glycinin polypeptide band.

Fraction 8 consisted of two minor bands, one just above and one just below the position of the β-subunit of β-conglycinin. The latter may again be the β′-subunit of β-conglycinin (Coates et al., 1985), as noted in water-extractable proteins (fraction 7, Figure 1B). A small amount of acidic chain A_3 still appears, but the major band corresponds to the other acidic glycinin chains, accompanied by their corresponding basic chains. The ratio of acidic:basic chains appears much higher than that observed in the pattern for acid-precipitated proteins (lane 2). In fraction 9, β-conglycinin subunits appear, with appreciable amounts of glycinin. Three bands are in the basic glycinin polypeptide region, compared to one in fractions 6 and 7. Fractions 10 and 11 were similar to fraction 9.

Whey Proteins. Whey proteins, soluble at pH 4.5 after removal of acid-precipitated proteins, consist of several minor proteins that sediment in the 2–6S range in the ultracentrifuge (Eldridge et al., 1966). Figure 3A shows the RP-HPLC chromatogram for whey proteins. The chromatogram has a series of sharp peaks of decreasing size up to about 60 min, followed by peaks of increasing height. SDS–PAGE of whey (Figure 3B) revealed two bands of 97 and 91 kDa (probably isozymes of lipoxygenase) and a band of 55 kDa that probably is β-amylase (Hirano et al., 1987). The three bands of 36–34 kDa correspond to soybean agglutinin (identified in a separate experiment). The dark band of 21 kDa is Kunitz trypsin inhibitor.

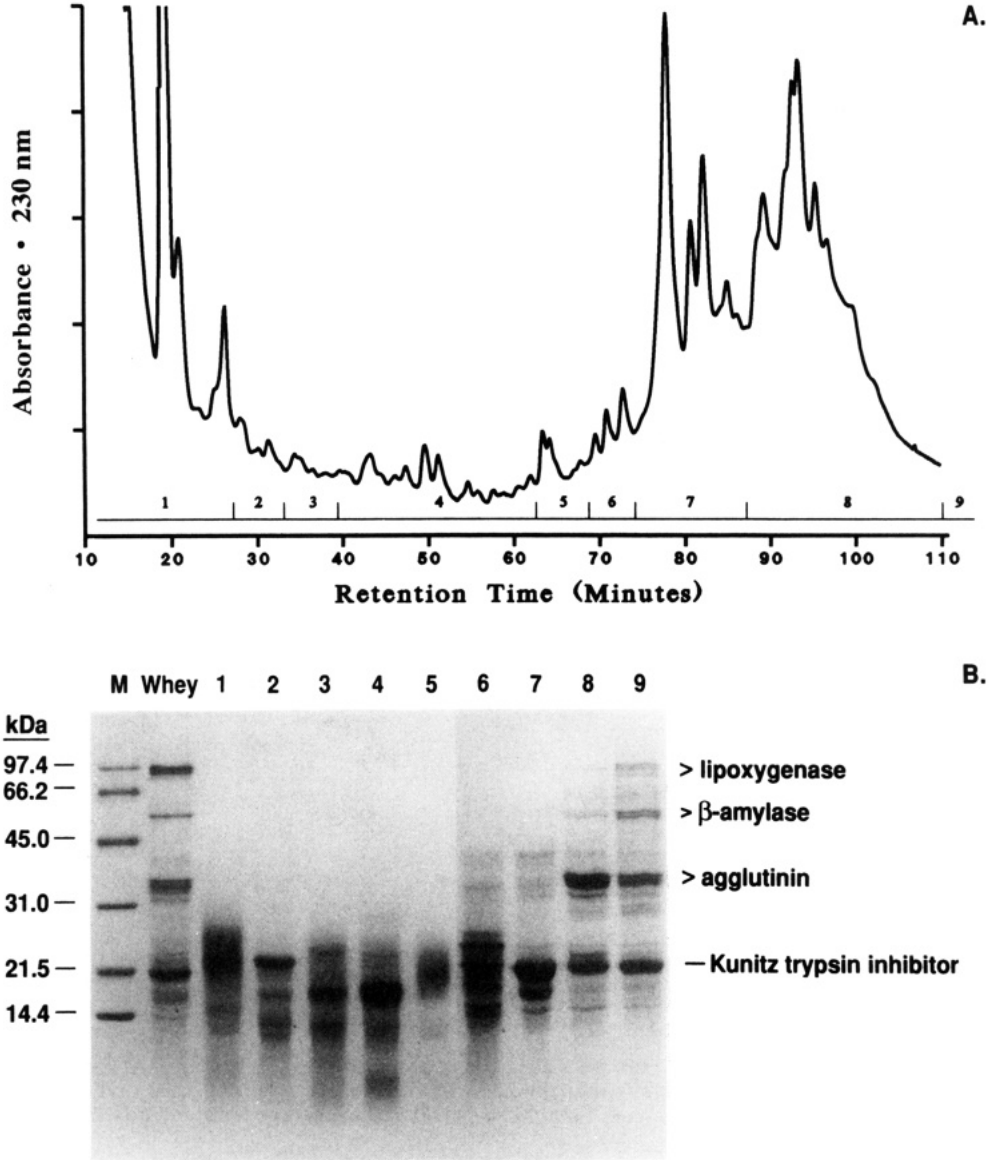

Figure 3. RP-HPLC chromatogram of 200 mg of soybean whey proteins at a flow rate of 8 mL/min (A) and SDS–PAGE (system B) of RP-HPLC fractions 1–9 (B). M is molecular weight standard mixture. Sample loads: 60 μg/lane except for standards (2 μg/band), fraction 5 (30 μg), and fraction 7 (30 μg).

Fraction 1 consisted of poorly resolved protein bands in the <10–27-kDa range. Fraction 2 contained a mixture of about the same size range as fraction 1 but also contained a distinct band of 23 kDa and several bands in the <10–15-kDa region; the 23-kDa band was a minor band in unfractionated whey. Minor proteins ranging from <10 to 25 kDa made up fraction 3 with a dark band of 19 kDa. A mixture of low relative molecular mass (M_r) bands also occurred in fraction 4, but a 19-kDa band was most prominent. In contrast, fraction 5 consisted of a diffuse major band of about 22 kDa plus traces of lower M_r proteins.

In fraction 6 there are several bands with M_r values of 24, 21, 15, and 14 kDa; the 21-kDa band corresponds to Kunitz trypsin inhibitor (one of the standards). Fraction 7 is predominantly trypsin inhibitor plus two lower M_r bands. Fractions 8 and 9 were similar and did not differ greatly from the pattern for whey. The presence of Kunitz trypsin inhibitor in fractions 6–10 was confirmed by Ouchterlony double-diffusion immunoassays; all formed a precipitin line when diffused against anti-Kunitz trypsin inhibitor serum. Fraction 8 contained a relatively high level of agglutinins and one band near the top of the gel likely to be an isomer of lipoxygenase. Fraction 9 and whey contained a doublet of 91 and 100 kDa at the top of the gel; these bands may be isozymes of lipoxygenase (Hirano et al., 1987).

β-Conglycinin. When crude and purified β-conglycinins were subjected to RP-HPLC, we noted only minor differences between the chromatograms for the two preparations. Crude β-conglycinin yielded four minor peaks in the range 15–45 min (data not shown), whereas these peaks were absent in the purified globulin (Figure 4A). However, SDS–PAGE of the fractions from crude β-conglycinin revealed the presence of glycinin as a contaminant that eluted over the range 55–110 min corresponding to fractions 5–11 of Figure 4A.

SDS–PAGE of purified β-conglycinin and its RP-HPLC fractions is shown in Figure 4B. The major constituents of β-conglycinin, the α′-, α-, and β-subunits, are in the purified starting preparation with traces of proteins with higher and lower M_r. Freeze-drying of fractions 1–5 yielded less than 1 mg per fraction, and SDS–PAGE showed a series of minor proteins with no obvious counterparts in the starting material. Fraction 1 contained a heavy doublet of 29 and 28 kDa, plus many other components from 22

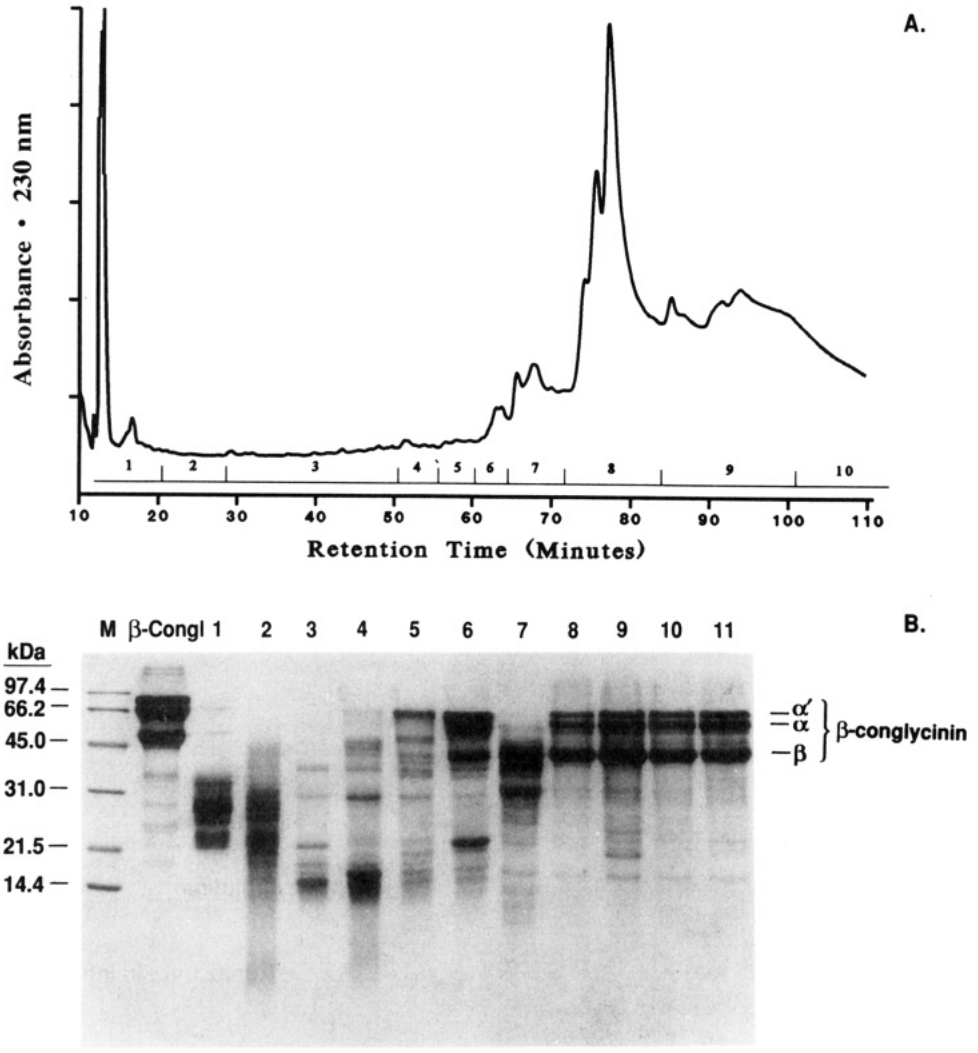

Figure 4. RP-HPLC chromatogram of 200 mg of purified β-conglycinin at a flow rate of 6 mL/min (A) and SDS–PAGE (system B) of RP-HPLC fractions 1–11 (B). Fraction 11 [not shown in (A)] was the column wash fraction collected after 110 min. M is a molecular weight standard mixture, and β-Congl is β-conglycinin. Sample loads: 50 μg/lane except for standards (2 μg/band).

to 35 kDa. Fraction 2 streaked from 45 to ≪10 kDa, with a dozen or more minor bands. Fraction 3 had about six bands, the major one being at 16 kDa. Fraction 4 had a wider distribution of proteins, ranging from a minor one (68 kDa) between the α′- and α-subunits of β-conglycinin down to <10 kDa; the major protein was 17 kDa. Fraction 5 contained a dark 68-kDa band, plus a dozen or more minor bands of lower M_r. Fraction 6 resembled fraction 5 except for dark bands of 56, 42, and 24 kDa. Fraction 7 also was complex: it had a dark band in the region of the β-subunit of β-conglycinin and two other dark bands at 43 and 36 kDa. Fractions 8–11 were essentially the same and had the α′-, α-, and β-subunits of β-conglycinin. However, ratios of the three subunits differed from those in the starting sample.

Glycinin. Earlier we published an RP-HPLC chromatogram for crude glycinin (Peterson and Wolf, 1988). When crude glycinin was purified by concanavalin A–Sepharose 4B chromatography (to remove β-conglycinin) followed by gel filtration on Sepharose 6B, we obtained the results shown in Figure 5A. Although essentially homogeneous by ultracentrifugation, purified glycinin yielded a complex chromatogram. Four small peaks eluted at 45–60 min, but the bulk of the protein eluted as several poorly resolved peaks in the range 60–110 min.

SDS–PAGE (Figure 5B) of purified glycinin revealed the absence of the β-subunit of β-conglycinin, which is the main contaminant of crude glycinin (Peterson and Wolf, 1988). Fractions 1–5 yielded only traces of protein upon freeze-drying, and these were primarily proteins of <10 to 27 kDa. Fraction 6 contained mainly >10 and 13 kDa components. Fraction 7 contained primarily 31-kDa protein, which was also present in later fractions. This protein appears to correspond to minor acidic polypeptide A_6 previously isolated by Staswick and Nielsen (1983) from glycinin separated from Raiden cultivar which was also used in this study. Fraction 8 contained a heavy band of 31-kDa protein plus glycinin acidic chain A_3; as noted earlier, it was not accompanied by a distinct band corresponding to B_4, but faint bands were in the region corresponding to basic chains. The 31-kDa band was predominant in fraction 9, with another heavy band of <10 kDa. A small amount of glycinin A_3 was noted, with a more distinct band likely corresponding to B_4.

In fraction 10 there are clearly two acidic bands, the upper one being A_3, plus a dark basic band; 31-kDa material is still present, as is a <10-kDa band. In fraction 11, A_3 has almost disappeared, and a prominent A_{1a}–A_2 acidic band is present; there appear to be three basic bands. Fractions 12–15 contain little A_3 but major amounts of the other acidic polypeptides plus a heavy basic polypeptide band. The ratio of acidic to basic chains appears to be constant.

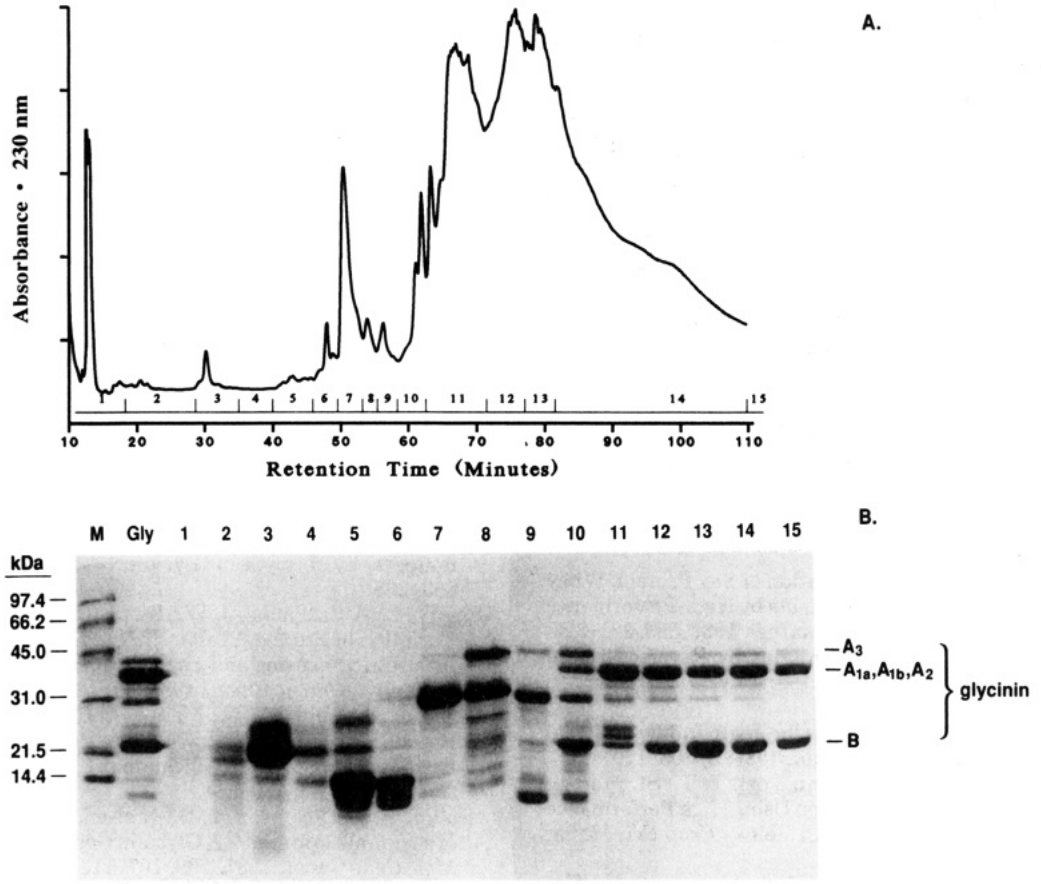

Figure 5. RP-HPLC chromatogram of 200 mg of purified glycinin at a flow rate of 6 mL/min (A) and SDS–PAGE (system B) of RP-HPLC fractions 1–15 (B). M is a molecular weight standard mixture, and Gly is glycinin. Sample loads: 50 μg/lane except for standards (2 μg/band).

DISCUSSION

By scaling up our earlier analytical RP-HPLC procedure (Peterson and Wolf, 1988), we succeeded in obtaining sufficient material to characterize all of the proteins eluting from the column by SDS–PAGE. Although it was necessary to pool some of the minor fractions, all of the proteins were collected and examined by SDS–PAGE.

β-Conglycinin and glycinin, the two major storage proteins in soybeans, constitute about 23 and 32%, respectively, of the total extractable proteins in Raiden cultivar (Iwabuchi and Yamauchi, 1987) which was used in this study. These two proteins comprise most of the peaks that elute between 55 and 110 min upon RP-HPLC of the water-extractable proteins (Figure 1) and the acid-precipitated globulin fraction (Figure 2). The complexity of these two storage proteins is reflected in their elution behaviors. Of the two proteins, glycinin begins to elute first. Fraction 5 of the water-extractable proteins (Figure 1) contains glycinin subunit G5 (A_3B_4), but small amounts of this subunit continued to elute in later fractions. Emergence of G5 (A_3B_4) by itself in fraction 5 indicates that glycinin is at least partially dissociated into subunits under the conditions of RP-HPLC. In fraction 6 we found predominantly subunits G1 ($A_{1a}B_2$) and G2 (A_2B_{1a}) plus small amounts of G3 ($A_{1b}B_{1b}$). These subunits likewise continued to elute in later fractions.

On continued elution of the water-extractable proteins, β-conglycinin begins to elute in fraction 8 accompanied by acidic and basic polypeptides of glycinin. Elution of β-conglycinin before all of the glycinin is eluted is in agreement with our earlier analytical scale studies (Peterson and Wolf, 1988). The present work demonstrates this behavior in the water-extractable proteins as well as in the acid-precipitated protein fraction. Overlapping elution is also apparent when the chromatograms for β-conglycinin (Figure 4) and glycinin (Figure 5) are compared.

Elution of a portion of glycinin before β-conglycinin suggests that the former is less hydrophobic than β-conglycinin. This suggestion is in agreement with calculated hydrophobicities (Yamauchi et al., 1985) as well as measured hydrophobicities (Kato et al., 1987; Yuno et al., 1988) for the two proteins. However, the differences in hydrophobicities do not appear large enough to allow a complete separation of glycinin from β-conglycinin under the conditions we employed. Therefore, in its present form, our preparative RP-HPLC method does not offer any advantages over the traditional techniques such as affinity chromatography and gel filtration for purification of glycinin and β-conglycinin. Further studies are needed to find more optimal conditions for separation of these two storage proteins.

As noted with glycinin, β-conglycinin also appears to dissociate into subunits during chromatography. This is illustrated by comparing SDS–PAGE of fractions 8 and 9 of the water-extractable proteins (Figure 1). The α′- and α-subunits are concentrated in fraction 8, while the β-subunit is concentrated in fraction 9.

The presence of acidic polypeptide A_3 without its basic partner, B_4, in fraction 5 of acid-precipitated proteins (Figure 2B) and in fraction 5 of glycinin (Figure 5B) suggests that the disulfide bond between A_3 and B_4 is cleaved either during preparation of the fractions or during RP-HPLC. However, both fractions 5 are small; hence, there does not appear to be extensive cleavage of this interchain disulfide bond. Fractions 6 and later fractions contain both A_3 and B_4 as expected. Further work on

glycinin would be desirable, particularly with isolated acidic and basic polypeptides, to ascertain chromatographic behavior of the polypeptides when the interchain disulfide bond is cleaved.

Information about RP-HPLC of minor soybean proteins was obtained primarily by analysis of whey proteins (Figure 3). While several minor components eluted in fractions 1–5, lipoxygenase, β-amylase, agglutinin, and Kunitz trypsin inhibitor are more hydrophobic and did not elute until fractions 6–9 (65–100 min). This behavior of the whey proteins further complicates the separation of glycinin and β-conglycinin by RP-HPLC of a crude mixture such as the water-extractable proteins.

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