Size Exclusion Chromatography of Soybean Proteins and Isoflavones

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High-performance size exclusion chromatography was used to separate soybean extracts. The separation of the major storage proteins, including glycinin and β-conglycinin, was determined by analysis of the subunits on sodium dodecyl sulfate gel electrophoresis. The extracts of defatted flour and whole beans showed a high degree of polymerization of the storage proteins. Addition of a disulfide reducing agent (dithiothreitol) resulted in a decrease of the polymerized forms. The separation of the major glucoside isoflavones (genistin, daidzin, and glycitein 7-β-glucoside) was determined. The isoflavone glucosides eluted well past the elution time of a small totally included molecule, indicating that they are separating by mechanisms other than size exclusion. Size exclusion chromatography was used to monitor the separation of the soybean extract in an aqueous two-phase extraction system. The major storage proteins partitioned to the salt phase, and the isoflavones and other hydrophobic compounds partitioned to the poly(ethylene glycol) phase.

Keywords: *Size exclusion chromatography; soybean proteins; isoflavones; aqueous two-phase extraction*

INTRODUCTION

The new high-performance size exclusion chromatography (SEC) columns allow the rapid characterization of biomolecules and other polymers based on their mass. We have been using SEC to monitor separations of the complex mixture obtained from soybeans. Soybean extracts contain proteins, peptides, carbohydrates, lipids, isoflavones, and other low molecular mass components (Smith and Circle, 1972). Soybeans are an important source of high-quality cooking oil and a rich source of proteins. Isoflavones have attracted recent interest because of their possible effect on the prevention of certain types of cancer [reviewed in Messina and Barnes (1991) and Barnes et al. (1993)].

Soybean extracts analyzed by SEC columns had a number of peaks eluting past the elution time of a totally included molecule. Some of these peaks had absorption spectra suggesting they were isoflavones. We have characterized the SEC separation of the major proteins and isoflavones found in soybeans, to increase the utility of SEC to characterize this complex mixture.

EXPERIMENTAL PROCEDURES

Soybean Extracts. Soy proteins were obtained from defatted soy flour (Nutrisoy 7B, Archer Daniels Midland Co., Decatur IL). The soy flour was suspended in deionized water or buffer at a concentration of 0.5–10% w/w. The pH of the mixture was then adjusted with NaOH, and the mixture was allowed to mix for 1 h. The mixture was centrifuged at 6000*g* for 20 min at 25 °C. The supernatant was removed and used as a crude soybean extract. Extracts from whole soy beans (Bellatti Soybeans, Mt. Pulaski, IL) were obtained by grinding with a mortar and pestle to a fine powder. This powder was extracted as described above with an additional centrifugation step of 12000*g* for 5 min. The floating lipid layer was carefully avoided when the supernatant was removed.

An ethanol extract was prepared by refluxing 2 g of defatted soy flour in 50 mL of 80% v/v ethanol for 2 h with constant stirring. The ethanol extract was centrifuged at 6000*g* for 20 min to remove insoluble material.

Calculation of Molecular Mass and Standard Proteins. Molecular masses were determined by calibration of the SEC-3000 column using combinations of some of the following standard proteins: thyroglobulin, bovine (669 000); IgA, bovine (300 000); β-amylase, sweetpotato (200 000); IgG, bovine (150 000); alcohol dehydrogenase tetramer, yeast (141 000); serum albumin, bovine (68 000); alcohol dehydrogenase monomer, yeast (35 000); soybean trypsin inhibitor (20 100); myoglobin, horse heart (17 500); α-lactalbumin, bovine (14 400); and ribonuclease A, bovine pancreas (13 700). A linear regression was generated using the log of the molecular mass and the partition coefficient and the unknown masses calculated using the regression line.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was done according to the modifications of Fling and Gregerson (1986) on a 15% [total acrylamide, 2.6% bis(acrylamide) cross-linker] slab (1.5 mm thick). The gels were stained using colloidal Coomassie Brilliant Blue G-250 (Neuhoff et al., 1988; Anderson 1989). The gels were scanned and analyzed with a BioImage (Ann Arbor, MI) Visage 110 system. Band assignment on SDS–PAGE was facilitated by comparison to the pattern of Wolf et al. (1992). On our gel systems the α and α′ subunits were so closely spaced that they were integrated as a single band. The band identity was confirmed by comparison with purified storage proteins and molecular mass standards.

Size Exclusion Chromatography. The buffer was composed of 0.069 M dibasic sodium phosphate and 0.031 M monobasic sodium phosphate, final pH 7.2. The buffer was filtered through a 0.22 μm nylon filter. A Beckman System Gold HPLC, either a diode array or a single-channel detector, was run at 0.5 or 1.0 mL/min at ambient temperature. The column (300 × 7.8 mm) was a Biosep SEC-S3000 (Phenomenex, Torrance, CA) with a Phenomenex Biosep SEC-S guard column (75 × 7.8 mm).

Enzymatic Digestion of Ethanol Extracts. The ethanol extract was concentrated (40–60 °C) under vacuum to remove the ethanol. The concentrate was suspended in 0.1 M sodium acetate, pH 5.0, up to the volume of the original extract and centrifuged at 6000*g* for 20 min. The discarded pellet of unknown composition did not contain the hydrophobic peaks. Alliquots of the supernatant had either β-glucuronidase (*Es-*

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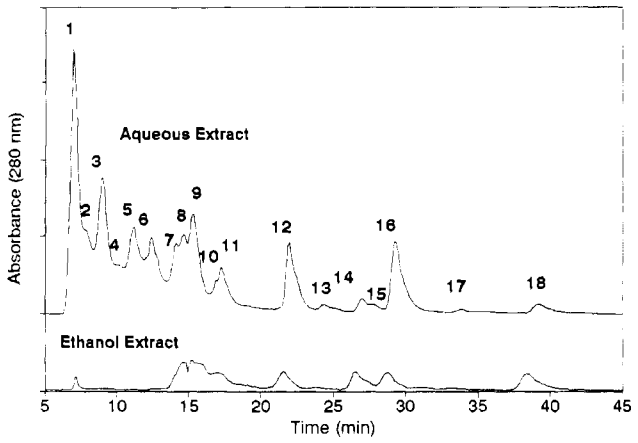


Figure 1. Size exclusion chromatography of aqueous and ethanol extract of soybeans. Samples (0.05 mL) are water extract (10% w/w pH 8.0, diluted 1:5) and ethanol extract (4% w/w, 1:2 dilution) run at 1.0 mL/min.

cherichia coli, Sigma Type VII-A) in 0.1 M sodium acetate, pH 5.0, buffer (final concentration 63 000 Sigma units/mL), β-glucosidase (almonds, Sigma no. G4511) in acetate buffer (25 units/mL), or buffer (blank). Samples were incubated at 37 °C for 18 h. The samples were centrifuged (12000*g* for 5 min) to remove any material (probably precipated protein from the enzyme extracts) and run on the HPLC as described.

Proteolytic Digestion of Soy Proteins. The aqueous extract was incubated at 37 °C for 2 h with trypsin (25 and 200 μg/mL, pH 8.0), chymotrypsin (25 and 200 μg/mL, pH 8.0); proteinase K (230 μg/mL in 0.01 M sodium phosphate, pH 7.2), and clostripain (100 μg/mL in 0.02 M sodium phosphate, pH 7.2, 1 mM dithithretol). At the end of the incubation period the samples were centrifuged (12000*g* for 5 min) before analysis.

Isolation of Isoflavones by High-Performance Liquid Chromatography. The reversed-phase HPLC method of Eldridge (1982a,b) was used to prepare soybean isoflavone standards. An extract was made by refluxing 1 g of Nutrisoy 7B in 25 mL of 80% (v/v) methanol for 4 h, followed by centrifugation at 12000*g* for 10 min. The extract was concentrated under vacuum to remove the methanol (60–80 °C) and suspended in 20% methanol. Samples (0.2 mL) were run on a 0.46 × 25 cm DuPont Zorbax ODS reversed-phase column with a flow rate of 1 mL/min. Initial condition were 80% pump A (15% methanol) and 20% pump B (65% methanol) and a linear gradient (over 20 min) was run to final conditions of 30% pump A. A commercial preparation of genistein (Calbiochem, La Jolla, CA) and genistin (obtained from Dr. Walter J. Wolf) were used to confirm the identity of the peaks from soybean extract. Enzymatic hydrolysis with β-glucuronidase (as described above) also confirmed the identity of the peaks.

Aqueous Two-Phase Extraction Systems. Aqueous two-phase extraction of the soybean extract was done according to the method of Cole (1993a,b). Aqueous soybean extract (1.5 g of a 10% w/w water extract) was added to a phase system of final total mass of 10 g. The two-phase system had a final composition of 15% (w/w) poly(ethylene glycol) (molecular mass of 1000), 14% (w/w) anhydrous ammonium sulfate, and the balance water and soy extract. The final pH was adjusted to 7.5 with NaOH. The phase systems are mixed for 30 min and centrifuged at 6000*g* for 20 min at 25 °C. After centrifugation, the top phase was collected by aspiration and bottom phases collected by puncturing the bottom of the tube. The samples were diluted with 0.1 M sodium phosphate buffer, pH 7.2, prior to being run on the HPLC.

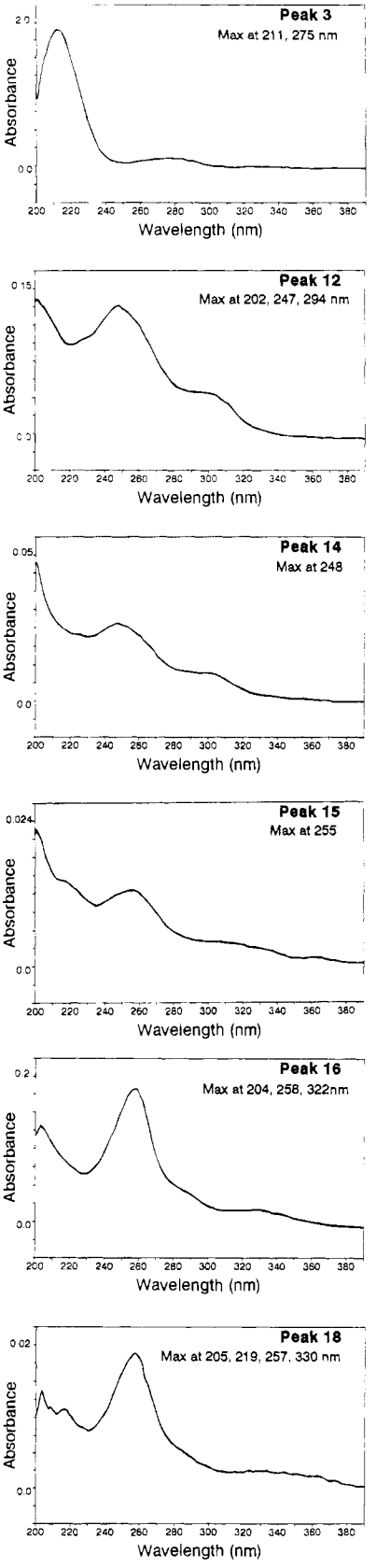


Figure 2. Absorbance spectrum of the peaks in the chromatograph shown in Figure 1.

RESULTS AND DISCUSSION

Chromatography of Soybean Extracts and Identification of Proteins. Figure 1 shows the chromatograph of extracts of soybean defatted flour run on the size exclusion column. A number of peaks elute past the retention time of sodium acetate (which elutes with peak 7 on Figure 1). The peaks in Figure 1 that elute past acetate are extracted in ethanol (refluxed for 2 h in 80% ethanol) indicating their hydrophobic nature. Figure 2 shows the absorbance spectrum of selected peaks of the chromatograph shown in Figure 1. Peaks

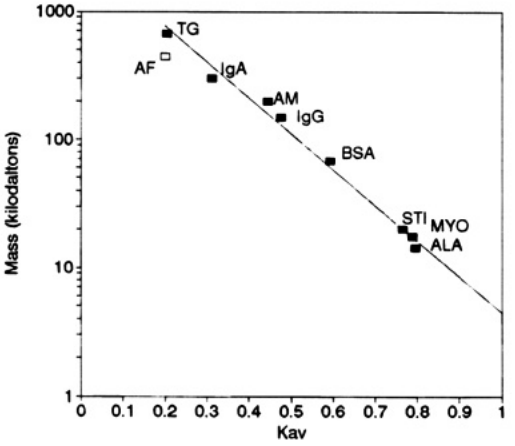


Figure 3. Typical standard curve used to calculate molecular masses. The standard proteins used to calculate the linear regression shown are (TG) thyroglobulin, (IgA) immunoglobulin IgA, (AM) β-amylase, (BSA) bovine serum albumin, (IgG) immunoglobulin IgG, (STI) soybean trypsin inhibitor, (MYO) myoglobin, and (ALA) α-lactalbumin. Apoferritin (AF) was not used in the regression.

1–6 have absorbance spectrum that are typical of proteins (illustrated by Figure 2, peak 3).

The apparent molecular masses of the peaks were measured by calibrating the column using standard proteins. Peaks 1 and 2 are large aggregates and elute near the exclusion limit of the column. Peaks 3, 5, and 6 had apparent molecular masses (in kilodaltons) of 348 $\pm$ 26 ($N = 5$), 59.4 $\pm$ 3.1 ($N = 5$), and 24.0 $\pm$ 4.0 ($N = 5$), respectively. The regression lines had r^2 values of 0.97 or greater (Figure 3).

Preparative runs were done by running 10- or 20-fold more extract on the column and collecting fractions (described in Figure 4A). Flow rate was reduced to 0.5 mL/min to allow more fractions (0.5 min) to be collected. The fractions were run on SDS–PAGE to identify the proteins. Figure 4 show the elution of the major soybean proteins on the SEC column. The storage protein glycinin (also known as 11S, made up of six basic polypeptides and six acidic polypeptides) was identified by the location of the polypeptide bands on SDS–PAGE. Band A3 (containing the acidic polypeptidet A_3), band A1–2 (containing the polypeptides subunits A_{1a}, A_{1b}, and A_2), and band B (containing the basic polypeptides) shown in Figure 4A,C, indicate that glycinin is present mainly in peak 3 and also as higher mass aggregates (peaks 1 and 2). β-Conglycinin (also known as 7S, made up of three subunits) was identified by the location of the alpha band (containing both the α and α' subunits) and the beta band (containing the β subunits) (Figure 4B). The locations of the lipoxygenase band, the soybean trypsin inhibitor band, and soybean agglutinin elution time are also shown on Figure 4A.

It is known that at low ionic strengths the 7S protomer of β-conglycinin forms a 9S dimer (Naismith, 1955; Wolf, 1972; Nielsen, 1985). Gel filtration on Sephadex does not resolve glycinin and β-conglycinin (Haseqawa et al., 1963; Koshiyama, 1968, 1969) indicating the two proteins have similar Stokes radii. We did an extraction in a high ionic strength buffer (0.1 M sodium phosphate, 0.5 M NaCl, pH 7.2), conditions that should minimize β-conglycinin dimer formation. The increased ionic strength did not have a significant effect on the elution profile (Figure 5, trace C, compared to Figure 1). A reducing agent (1 mM dithiothreitol) was added to this extract (Figure 5, traces B and C). The area of peaks 1 and 2 were reduced and the area of Peak

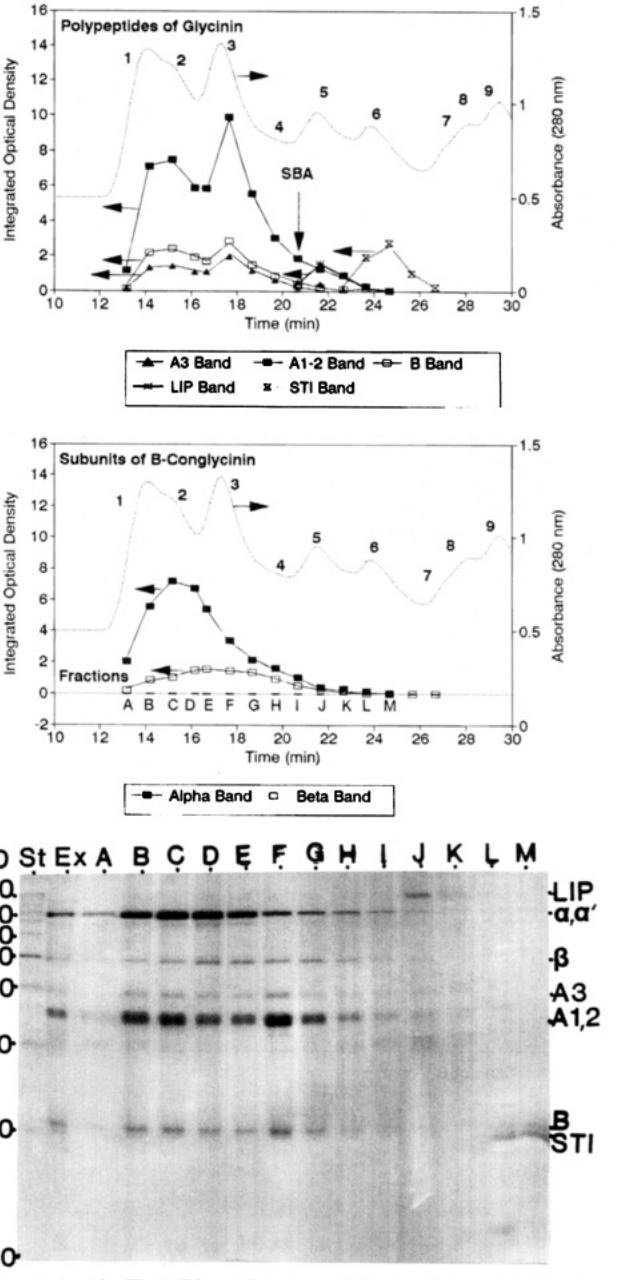


Figure 4. (A, Top) Identification of the soybean proteins in the water extract of Nutrisoy 7B. Injection volume was 0.2 mL of the aqueous extract (1:2 dilution of 10% w/w water extract pH 8.0) with a flow rate of 0.5 mL/min. Fractions (at 0.5 min intervals) were collected and analyzed by SDS–PAGE. (A3) Integrated area of A_3 polypeptide band of glycinin; (A1–2) integrated area of the A_{1a}, A_{1b}, A_2 acidic polypeptides bands of glycinin; (B) integrated optical density of the basic polypeptide band of glycinin; (LIP) integrated optical density of the lipoxygenase band; (STI) integrated optical density of the soybean trypsin inhibitor band; (SBA) elution time of purified soybean agglutinin. (B, Middle) Identification of location of subunits of β-conglycinin water extract of Nutrisoy 7B. (Alpha) Integrated area of the α and α' band; (beta) integrated optical density of the β subunit. Locations of fractions (0.5 min) are indicated by letters along x-coordinate. (C, Bottom) Identification of the proteins in fractions by 15% SDS–PAGE. Fractions indicated in (B) were analyzed by SDS–PAGE, the gel was stained, and bands were scanned as described in the text.

3 was greatly increased. The reduction occurred in a time-dependent manner. A preparatory run was done on the reduced high ionic strength buffer extract and the fractions analyzed (Figure 6). Glycinin (peak 3) area was greatly increased and the polymerized glycinin forms (peaks 1 and 2) were greatly reduced (Figure 6A).

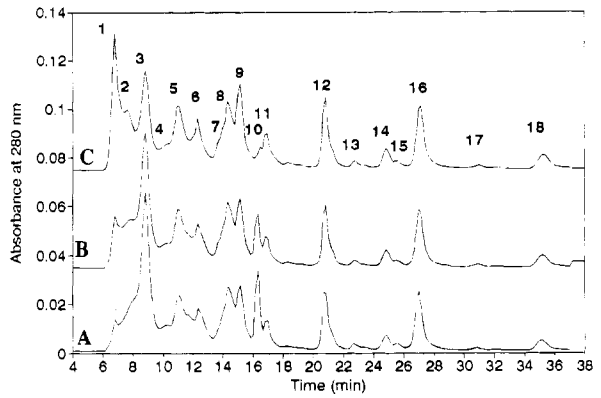
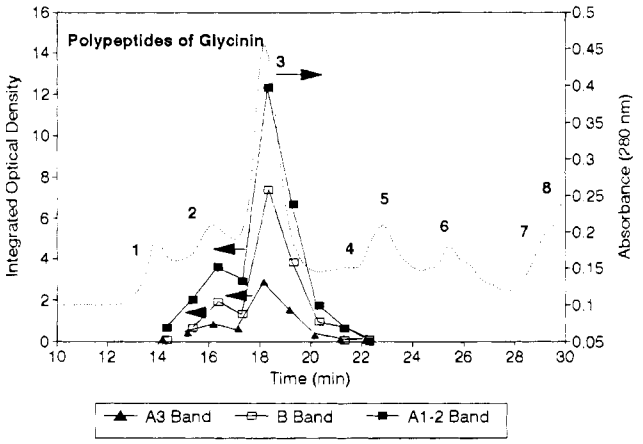


Figure 5. Effect of a reducing agent on the profile of an extract in high ionic strength buffer. An extract of Nutrisoy 7B (0.5% w/w) was done in high ionic strength buffer (0.1 M sodium phosphate, 0.5 M NaCl, pH 7.2) and analyzed (C). Dithiothreitol (1 mM) was added to the extract and analyzed after 1.5 (B) and 3.5 h (A) at ambient temperature.



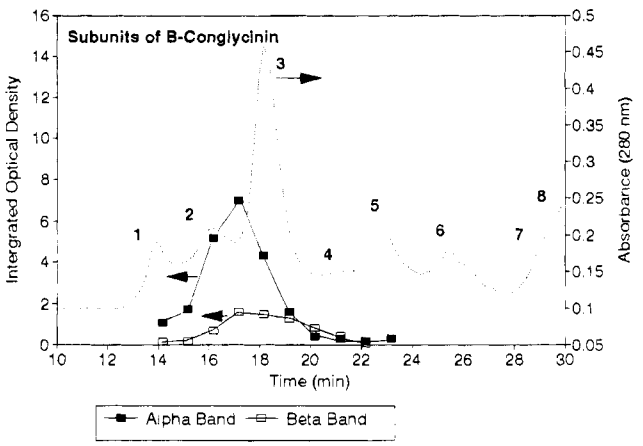
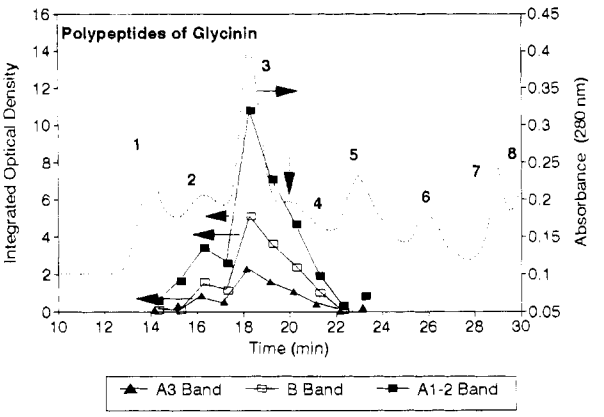


Figure 6. (A, Top) Identification of polypeptides of glycinin in reduced high ionic strength extract. Sample (0.2 mL) was 0.5% Nutrisoy 7B in 0.1 M sodium phosphate, 0.5 M NaCl, pH 7.2, 1 mM dithiothreitol. Preparatory run and analysis were done as described in Figure 4. (B, Bottom) Identification of subunits of β-conglycinin in reduced high ionic strength extract. Preparatory run and analysis were performed as described in Figure 4.

The apparent mass of β-conglycinin was shifted to a lower average mass in the reduced high ionic strength extraction buffer (Figure 6B compared to Figure 4B).

Analysis of β-conglycinin is further complicated by multiple forms based on their subunit composition (Thanh and Shibasaki, 1977, 1978). The β-conglycinin peak is relatively broad, and the ratio of α and β subunits changes with retention time. Thanh and



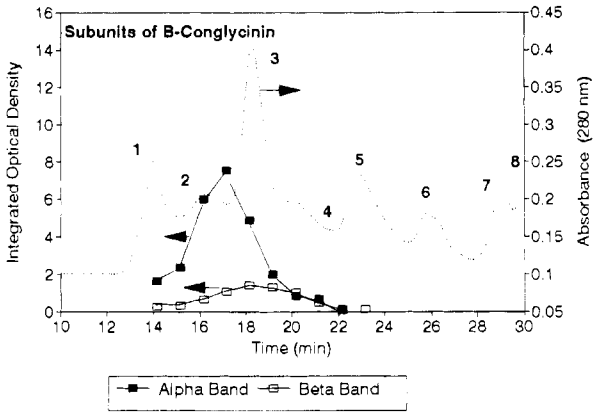


Figure 7. (A, Top) Identification of polypeptides of glycinin in reduced water extract. Sample (0.2 mL) was 0.5% Nutrisoy 7B in water, pH 8.0, 1 mM dithiothreitol. Preparatory run and analysis were done as described in Figure 4. (B, Bottom) Identification of subunits of β-conglycinin in reduced water extract. Preparatory run and analysis were done as described in Figure 4.

Shibasaki (1978) described six forms of β-conglycinin (with different subunit composition) with three classes of different masses. Group C forms (containing three α subunits), group B (containing two α subunits and a β subunit), and group A (containing an α subunit and two β subunits) had calculated masses of 171 000, 156 000, and 141 000, respectively (Thanh and Shibasaki, 1978). Isolation and characterization of a β' type subunit indicates even greater heterogeneity in β-conglycinin (Coates et al., 1985). The β-conglycinin subunit composition seen in separation in Figure 6B is consistent with the separation of these molecular mass classes.

A water extract containing a reducing agent (Figure 7) gave similar results with the exception that lower mass forms of glycinin was observed. A new peak appears with lower molecular mass (indicated by arrow in Figure 7A). This is made up of glycinin subunits but lower mass indicates that reduction in low ionic strength resulted in some partial disassembly of glycinin. It has been shown that glycinin will dissociate in half molecules at low ionic strength (Wolf and Briggs, 1958). β-Conglycinin eluted at a similar time compared to the high ionic strength extract (Figure 7B).

Sodium chloride (0.5 M) was added the 0.1 M sodium phosphate, pH 7.2, column buffer to increase the ionic strength. Figure 8 shows the effect of the increased ionic strength on the profile of the reduced high ionic strength soy extract. The ionic strength of the regular and high ionic strength buffer used had calculated ionic strengths of 0.21 and 0.71 M. The increased ionic

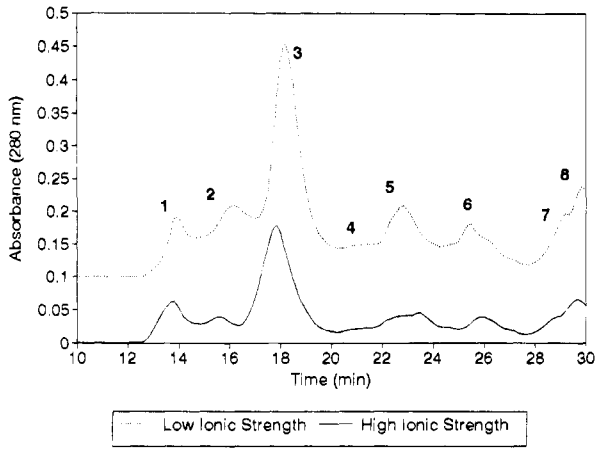


Figure 8. Comparison of high ionic strength buffer (0.1 M sodium phosphate, 0.5 M NaCl, pH 7.2) to low ionic strength buffer (0.1 M sodium phosphate, pH 7.2). The sample (0.2 mL) was 0.5% Nutrisoy 7B extracted with 0.1 M sodium phosphate, 0.5 M NaCl, 1 mM dithiothreitol, pH 7.2 buffer. The flow rate was 0.5 mL/min.

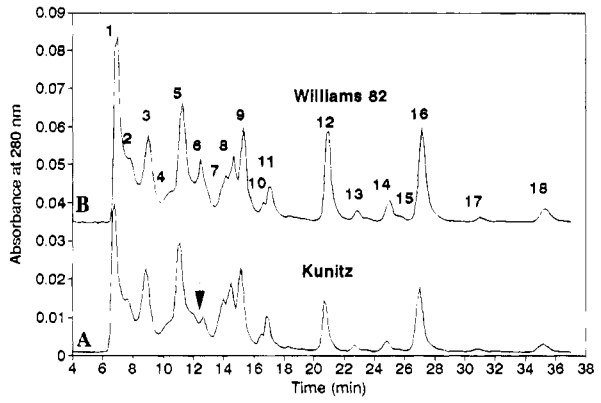


Figure 9. Comparison of profiles obtained from whole beans of Kunitz (A) and Williams 82 (B) cultivars. Sample (0.05 mL) was 1.0% (w/w) extracted in 0.1 M sodium phosphate, pH 7.2, at a flow rate of 1.0 mL/min.

strength of the column buffer had only minor effects on the profile.

The profiles obtained from whole soybeans in shown in Figure 9. The buffer extracts (0.1 M sodium phosphate, pH 7.2) prepared from two cultivars show that the profiles were similar to the defatted soy flour (Figures 9 and 1). The Kunitz cultivar is a null mutant for Kunitz soybean trypsin inhibitor (Orf and Hymowitz, 1979). The Kunitz profile compared to the Williams 82 profile shows the absence of the major peak 6 identified by the arrow in Figure 9. Peak 6 elutes with purified soybean trypsin inhibitor.

Proteolytic Digestion. Figure 10 shows the results of the incubation of the soybean extract with proteolytic enzymes. Incubation of the soybean extract with low amounts of trypsin and chymotrypsin had little effect on the protein elution pattern (results not shown), due to the large amounts of trypsin and chymotrypsin inhibitors present in soybeans. Increased amounts of trypsin or chymotrypsin resulted in hydrolysis of peaks 1 and 2 (Figure 10, traces B and C). Kim et al. (1990) found that the action of trypsin resulted in more extensive hydrolysis of β-conglycinin and glycinin was less effected. Clostripain digestion also resulted in extensive digestion of peaks 1 and 2 and reduced peak 3 to a lower molecular mass (Figure 10, trace D). The more general proteinase, proteinase K, resulted in extensive hydrolysis of peaks 1–6 (Figure 10, trace E).

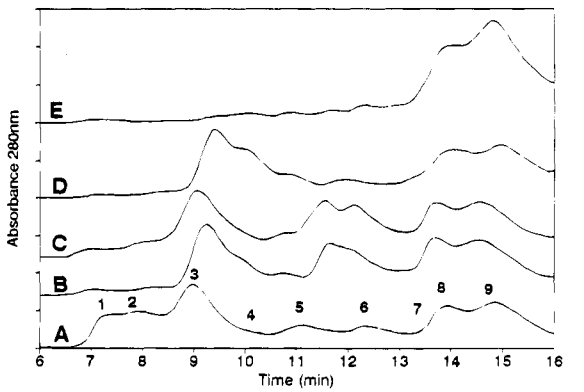


Figure 10. Effect of proteases on the soybean size exclusion chromatography profile: (A) soybean extract with no added protease; (B) soybean extract with trypsin added; (C) soybean extract with chymotrypsin added; (D) soybean extract with clostripain added; (E) soybean extract with proteinase K added. Samples (0.05 mL) were run at 1.0 mL/min.

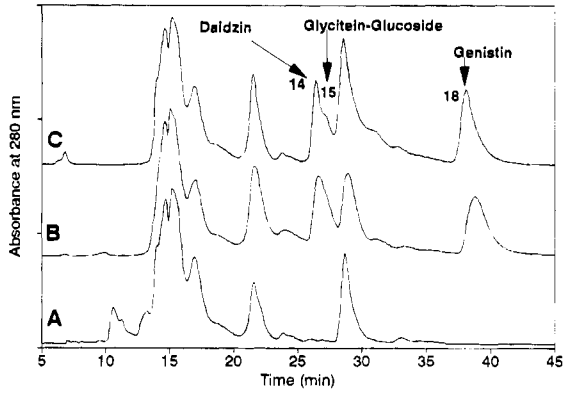


Figure 11. Effect of glycolytic enzymes on the soybean ethanol extract profile: (A) ethanol extract incubated with β-glucosidase; (B) ethanol extract incubated with β-glucuronidase; (C) ethanol extract incubated without added enzyme. Samples (0.05 mL) were run at 1.0 mL/min.

The proteolytic enzymes had no apparent effect on the peaks associated with hydrophobic substances.

Identification of Isoflavones. The absorbance spectrum of the more hydrophobic peaks that elute after acetate suggested they were phenolic type compounds (Figure 2, peaks 12, 14–16, and 18). Soybeans are known to contain relatively large amounts of the isoflavone glucosides, genistin, daidzin, and glycitein 7β-*O*-glucoside and lower amounts of the corresponding aglycons genistein, daidzein, and glycitein, respectively (Eldridge, 1982b, 1983). Since commercial preparations of the isoflavone glucosides are not available, standards were prepared by the reversed phase HPLC method of Eldridge (1982a). The isoflavones glucosides, genistin, daidzin, and glycitein 7β-glucoside, eluted as peaks 18, 14, and 15, respectively. The aglycons were not detected and based on their higher hydrophobicity, probably bound to the guard column, and did not elute.

The identities of the isoflavone glucosides were confirmed by their disappearance when the ethanol extract were incubated with the glycolytic enzyme β-glucosidase (Figure 11, traces A and C). Incubation of ethanol extracts with β-glucuronidase did not result in any reduction of the peaks (Figure 11, traces B and C). We observed no discernible change in the other hydrophobic peaks upon incubation of these enzymes. The absorbance spectrum of peaks 18, 14, and 15 had a high correlation coefficient (greater than 0.97) compared to

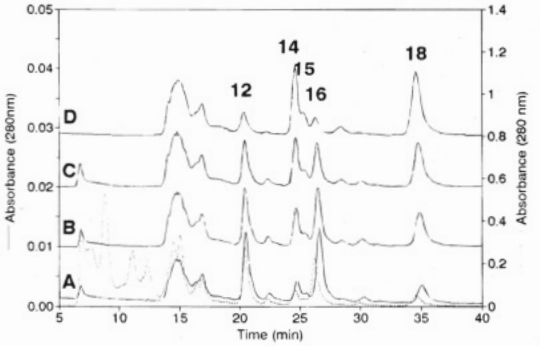


Figure 12. Effect of reflux time on recovery of hydrophobic peaks from defatted soy flour: (A) ethanol extract from defatted soy flour mixed for 10 min at room ambient temperature; (B) ethanol extract from defatted soy flour refluxed for 1 h; (C) ethanol extract from defatted soy flour refluxed for 2 h; (D) ethanol extract from defatted soy flour refluxed for 6 h. Nutrisoy 7B was suspended in 80% ethanol at a concentration of 4 g/100 mL. The dotted line shows the profile of an aqueous extract of defatted soy flour displayed on a scale so that equivalent amounts of material are the same. Ethanol extracts were centrifuged for 5 min (12000*g*) and diluted (1:5) with 0.1 M sodium phosphate, pH 7.2, buffer, and 0.05 mL was injected and run at a flow rate of 1.0 mL/min.

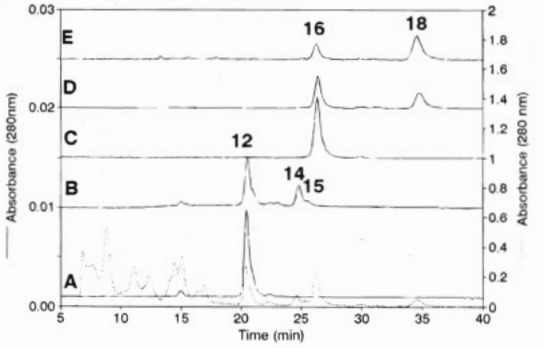


Figure 13. Effect of heating on peaks 12 and 16. Peaks 12 and 16 were collected from an aqueous extract (shown by the dotted line). (A) Peak 12 stored at 4 °C for 16 h before analysis; (B) peak 12 incubated at 45 °C for 16 h before analysis; (C) peak 16 incubated at 4 °C for 16 h before analysis; (D) peak 16 incubated at 45 °C for 16 h before analysis; (E) peak 16 incubated at 45 °C for 16 h and 20 min at 100 °C before analysis. Samples (0.05 mL) were run at a flow rate of 1.0 mL/min.

the absorbance spectrum of the genistin, daidzin, and glycitein 7β-*O*-glucoside standards, respectively.

Kudou et al. (1991) has shown the presence of 6″-*O*-malonylglucoside conjugates of the isoflavones in soybeans. Farmakakalidis and Murphy (1985) have also shown the presence of 6″-*O*-acetyldiaidzin and 6″-*O*-acetylgenistin in toasted soy flakes. Kudou et al. (1991) and Coward et al. (1993) suggest that the acetylglucoside isoflavones may form from the decarboxylation of the malonylglucoside isoflavones, a reaction presumably promoted by heating of soy products. In Coward et al. (1993) experiments are discussed that extraction of soy products with hot alcohols causes deesterification of 6″-*O*-malonylglucoside and 6″-*O*-acetylglucoside isoflavones to form the glucoside isoflavones. Unfortunately, standards of the 6″-*O*-malonyl or acetyl isoflavone glucosides are not available.

With these results in mind, we examined the effect of extraction time in hot ethanol (reflux) upon the recovery of the hydrophobic peaks. Figure 12 shows the result of this experiment. The highest recovery of peaks 12 and 16 was obtained in extraction with 80% ethanol at ambient temperature (Figure 12, trace A). The recovery of the hydrophobic peaks by extraction in ethanol with no reflux was similar to the recovery from aqueous solutions (Figure 12, trace A and dotted line). Figure 12 shows that prolonged refluxing in ethanol resulted in increasingly low recovery of peaks 12 and 16 and increased recovery of peaks 14, 15, and 18 (the isoflavone glucosides). These results suggested that peaks 12 and 16 were being converted to the isoflavone glucosides. To confirm this, peaks 12 and 16 were collected from an aqueous extract. Figure 13 shows that peak 12 fraction held at 45 °C for 16 h approximately 50% of the peak area was converted to peaks 14 and 15 (daidzein and glycitein 7β-glucoside, respectively). The peak fractions were stable when held at 4 °C overnight. Peak 16 was converted to peak 18 (genistin) when held at 45 °C for 15 h (Figure 13, traces C and D). Further heating of this fraction (100 °C) resulted in almost complete conversion of peak 16 to genistin (Figure 13, trace E). These fractions were in 0.1 M sodium phosphate buffer, pH 7.2. The conversion therefore occurred

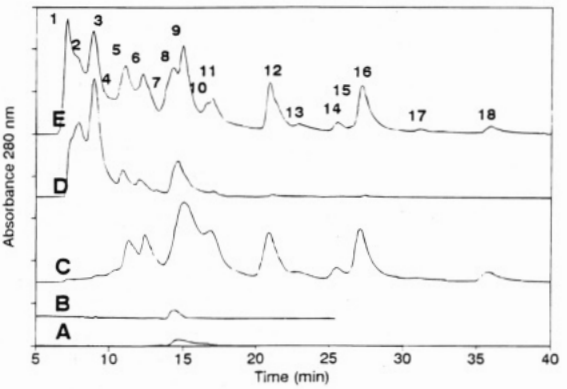


Figure 14. SEC of the top and bottom phases from the partitioning of the aqueous extract in the aqueous two-phase system: (A) Sample of top phase without added soybean extract; (B) sample of bottom phase without added soybean extract; (C) sample of top phase from system with added soybean extract; (D) sample of bottom phase from system with added soybean extract; (E) sample of soybean extract that was added to the aqueous two-phase system. Samples (0.05 mL) were run at 1.0 mL/min.

under essentially neutral conditions. Deesterification reactions would be accelerated by extremes of pH.

Aqueous Two-Phase Extraction Systems. Figure 14 shows the results of partitioning soybean extract in an aqueous two-phase system composed of poly(ethylene glycol) 1000 and ammonium sulfate. This result confirms (Cole, 1993a,b) that the high molecular mass storage proteins are present in the bottom (ammonium sulfate rich) phase (Figure 14, trace D) and other lower molecular mass proteins are present in the top [poly(ethylene glycol) rich] phase (Figure 14, trace C). These results also show that the isoflavones and other more hydrophobic peaks are present in the top phase and are not detected in the bottom phase. The partitioning of the isoflavones would be expected to partition to the more hydrophobic polyethylene glycol phase.

Conclusions. The chemical and physical properties of the major soybean storage proteins have been carefully studied and reviewed (Wolf, 1972; Nielsen, 1985). The form of glycinin and β-conglycinin is dependent upon the pH, ionic strength, and presence of a reductant. It has been shown that gel filtration does not resolve these two major storage proteins (Haseqawa et al., 1963; Koshiyama, 1969), indicating the similar Stokes radii of the two proteins. Our results using SEC

media confirm that the two molecules have similar Stokes radii. The packing material used in these SEC columns is spherical silica particles with a bonded hydrophilic coating and a pore size of 290 Å for the SEC-S3000 (Ahmed and Modrek, 1992). These columns gave very linear results using standard proteins with the exception of bovine apoferritin (Figure 3). Ahmed and Modrek (1992) found that carbonic anhydrase, another metal-binding protein, did not fall on the standard curve line. Additional studies are necessary to determine if this is a general effect with other metal-binding proteins. Our estimate of 348 000 mass for glycinin is very close to the mass measured by sedimentation, gel filtration, and gel electrophoresis (Badley et al., 1975). β-Conglycinin revealed heterogeneity on SEC and the major form appeared to have a mass slightly larger than glycinin, even under conditions designed to minimize dimer formation.

The SEC column reveals the high degree of polymerization of the storage proteins present in either defatted soy flour or whole beans. Our results with whole beans, done with a minimum of manipulation (extraction and centrifugation), show that reduction sensitive polymers of the storage proteins are present in the whole beans. Similar results have been obtained using ultracentrifugation studies and defatted flour (Wolf and Sly, 1967; Wolf, 1972).

The use of these high-performance SEC columns allows the rapid and easy profiling of the major proteins of soybeans. This is shown by the profiles obtained from the Kunitz and Williams 82 cultivars. The amount and degree of polymerization of the storage proteins can also be determined rapidly. The large number of peaks eluting past the included volume of the column illustrates the complexity of this mixture. The glucoside forms of the isoflavones can be easily and rapidly measured using these SEC columns. These results indicate that the main forms of the isoflavones present in soybeans are heat-labile forms, most likely the 6″-*O*-malonylglucosides as discussed in Coward et al. (1993).

This study has shown the utility of SEC to rapidly analyze complex mixtures. The samples require a very minimal amount of preparation, increasing the confidence that additional preparation steps are not resulting in selective losses or changing the sample. The ability of SEC to analyze soybean extract is shown by the separations obtained by the aqueous two-phase extraction (Figure 14) and the various extraction conditions used in this study.

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