Separation of Lipoxygenase and the Major Soybean Proteins Using Aqueous Two-Phase Extraction and Poly(ethylene glycol) Precipitation Systems

Kenneth D. Cole

National Institute of Standards and Technology, Gaithersburg, Maryland 20899

This paper examines the separation of lipoxygenase and the major proteins in soybeans using aqueous two-phase extraction and poly(ethylene glycol) (PEG) precipitation systems. The aqueous two-phase systems were composed of poly(ethylene glycol) and the salt ammonium sulfate. The major proteins examined include glycinin, β-conglycinin, soybean agglutinin, lipoxygenase, and soybean trypsin inhibitor. Enzymatic activity was used to measure the partitioning and solubility of lipoxygenase. Lipoxygenase was found to partition almost exclusively to the top phase. Solubility of lipoxygenase in poly(ethylene glycol) systems was found to be dependent on ionic strength. High-resolution gel electrophoresis was used to examine the spectrum of proteins present in the top and bottom phases of two-phase systems and the supernatant of PEG systems. The proteins present in the acid-soluble fraction were also examined by two-dimensional gel electrophoresis.

INTRODUCTION

The aqueous extract of ground soybeans is a highly complex mixture of high molecular mass proteins, peptides, carbohydrates, oligosaccharides, and many low molecular mass compounds [reviewed in Smith and Circle (1972), Rackis (1972), and Wolf (1972)]. We are interested in studying the separation of complex mixtures of proteins by aqueous two-phase extraction systems (ATPS) and poly(ethylene glycol) (PEG) precipitation (Sikdar et al., 1991). One of the model systems we are studying is the behavior of the aqueous extract of soybeans. In a previous publication we studied the partitioning of the protease inhibitors present in this mixture (Cole, 1992). We observed the Kunitz soybean trypsin inhibitor (KSTI) and the Bowman–Birk trypsin–chymotrypsin inhibitor (BBI) to be present in high concentration in the top phase (PEG-rich) of the ATPS studied (Cole, 1992). We also noted the high solubility of KSTI and BBI in PEG solutions. When the top phase was analyzed by one-dimensional gel electrophoresis, a band with molecular mass of approximately 96 000 was noted. We suspected this band was lipoxygenase. To confirm this finding, we have measured the enzymatic activity of lipoxygenase in the two-phase and PEG systems. We also used high-resolution two-dimensional gel electrophoresis to determine the spectrum of proteins separated by the ATPS and PEG precipitation systems to characterize the separation.

EXPERIMENTAL PROCEDURES

Plant Materials. Defatted soy flour (Nutrisoy 7B) from Archer Daniels Midland Co. (Decatur, IL) was suspended in deionized water at a concentration of 10 g/100 g of total mass. The pH was adjusted to 8.0 with NaOH and the mixture stirred for 1 h at room temperature. The mixture was then centrifuged at 6000*g* (*g* is acceleration due to gravity) for 20 min at 25 °C. The supernatant was used as a crude source of soy proteins. An acid-soluble fraction was made by taking the above crude soy protein solution and adjusting the pH to 4.2 with 1 N HCl while constantly stirring the mixture. This solution was stirred for 20 min and then centrifuged at 6000*g* for 20 min. This supernatant was adjusted to pH 7.5 with 1 N NaOH with constant stirring. This solution was used as the acid-soluble fraction.

Size Exclusion Chromatography. Samples of crude extract, top phase, and bottom phase were analyzed on a size exclusion chromatography system composed of G1000PW and G3000SW TSK columns (7.8 × 300 mm) in tandem. The mobile phase was 25 mM tris(hydroxymethyl)aminomethane (Tris)/0.15 M NaCl (pH 7.5) at a flow rate of 0.5 mL/min. Detection was done by UV absorbance and refractive index.

ATPS and PEG Solubility. The partitioning of soy proteins in two-phase systems was done by adding the soybean protein extract supernatant (prepared as described above) to a phase system containing the final composition described in Table I (final total mass of 10 g). The appropriate amount of PEG 1000 (average molecular mass of 1000) was added gravimetrically from a 60 mass % stock solution (prepared by melting 600 g of PEG 1000 at 80 °C and adding it to 400 g of water). Ammonium sulfate and sodium chloride were added as powder or stock solutions. The appropriate amount of water was added, allowing space for sample to be added and the system mixed. The pH was adjusted with small volumes of HCl or NaOH. The sample was added to the phase system, the pH checked, and the sample gently mixed for 10–30 min at room temperature. The phases were separated by centrifugation (6000*g* for 10 min at 25 °C). The top phase was removed by aspiration and the bottom phase collected by puncturing the bottom of the tube and draining the phase into a fresh tube. Precipitated material at the interface was carefully avoided. Solubility in PEG 1000 solutions was determined by adding a solution of crude soybean protein extract (prepared as described above) to a solution containing PEG 1000 solution containing 25 mM Tris (pH 7.5) buffer. Final pH was adjusted to pH 7.5 by small additions of HCl or NaOH. The solutions were mixed, the pH was checked, and the samples were gently mixed at room temperature for 10–30 min and then centrifuged (12000*g* for 5 min).

Electrophoresis. Samples (0.2 mL) of top phase, bottom phase, and supernatant from PEG 1000 solutions were dialyzed in a microdialyzer using 6000 mass cutoff membrane and 0.5% ammonium bicarbonate at room temperature for 3–4 h and lyophilized (freeze-dried). Molecular mass standard proteins, BBI, and soybean agglutinin were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was done according to the modifications of Fling and Gregerson (1986). The final concentration of SDS in the samples was 0.05%. Two-dimensional gel electrophoresis (O'Farrell, 1975) was run using the general technology of the Anderson Iso-Dalt system (Anderson and Anderson, 1977). Samples were solubilized by addition to a 9 M urea/4% NP-40 detergent/2% ampholyte/0.1% dithiothreitol solution. Ampholytes used in the first dimension were a mixture of equal amounts of Pharmalyte (pH 3–10) and Ampholine (pH 3.5–10) (Pharmacia LKB Biotechnology, Uppsala, Sweden). The

Table I. Compositions of ATPS and PEG Systems

system	PEG 1000 mass, %	salt type	salt mass,[a]	pH	phase volume ratio[b]
A1	15.0	ammonium sulfate	14.0	7.5	0.8
A2	18.5	ammonium sulfate	16.0	7.5	0.8
A3	21.0	ammonium sulfate	20.0	7.5	0.8
P30[c]	30.0			7.5	
P40[c]	40.0			7.5	
P40S[c]	40.0	sodium chloride	5.0	7.5	

[a] Concentration of anhydrous salt. [b] Ratio of the volume of top phase divided by the volume of bottom phase. [c] These systems contained 25 mM Tris (pH 7.5) buffer.

second-dimension acrylamide slab gels were poured as a linear gradient of 10–20% total acrylamide [2.6% of total, bis-(acrylamide)]. Increased Tris concentrations in the gel and running buffers (Fling and Gregerson, 1986) were used to improve resolution of low molecular mass proteins. The second-dimension gels contained 0.75 M Tris (pH 8.85) and 0.1% SDS. The running (tank) buffer contained 0.05 M Tris, 0.19 M glycine, and 0.1% SDS. The gels were fixed for 4 h in 2% H_3PO_4/50% ethanol and stained for 40–48 h in 2% H_3PO_4/10% ammonium sulfate/20% methanol with Coomassie Brilliant Blue G-250 added as a powder in excess (Neuhoff et al., 1988; Anderson, 1989). Alternatively, the gels were silver stained by fixing overnight in 10% acetic acid/40% ethanol, washed with water, and cross-linked in 0.5% glutaraldehyde for 1 h and then silver stained [Oakley et al., 1980; modifications as in Edwards et al. (1990)]. The gels were scanned and analyzed with a BioImage (Ann Arbor, MI) Visage 110 system.

Lipoxygenase Assay. The activity of the lipoxygenase 1 was determined by the increase in absorbance at 234 nm after addition of linoleic acid in 0.2 M borate buffer (pH 9.0) (Axelrod et al., 1981). An equal amount of blank solution (buffer, PEG, top phase or bottom phase) was assayed to determine the blank activity.

The partitioning of the lipoxygenase 1 in two-phase systems was done by adding the soybean protein extract supernatant (prepared as described above) to a phase system containing the final composition described in Table I. The phase systems were mixed for 10 min at room temperature and centrifuged (6000*g* for 10 min) at 25 °C. The top phase was removed by aspiration and the bottom phase collected by puncturing the bottom of the tube. Precipitated material at the interphase was carefully avoided. The activity of lipoxygenase in the top and bottom phases was measured as described above. Recovery was calculated by measuring the activity in the phase or supernatant and multiplying by the respective volume and dividing by the total units of activity originally added to the system.

Solubility in PEG solutions was determined by adding the soybean protein extract (prepared as described above) to PEG solutions containing 25 mM Tris (pH 7.5) buffer. The solutions were mixed at room temperature for 30 min and centrifuged (12000*g* for 5 min). Activity remaining in the supernatant was measured as described above.

RESULTS AND DISCUSSION

UV-Visible Spectrum. The UV-visible spectrum of the diluted soybean aqueous extract is shown in Figure 1. The diluted top and bottom phases from system A1 containing soy extract are also shown in Figure 1. The ratio of absorbances at 260/280 nm of the extract is equal to 1.2. There is also a broad absorbance in the range 300–360 nm. This yellow material is also present in the top phase of system A1 as seen by the absorbance spectrum of the top phase (Figure 1) and visual inspection. A large amount of the material absorbing at 260 nm was present in the top phase of system A1 as shown by the 260/280 ratio of 1.6. The bottom phase has a 260/280 ratio of 0.9, more characteristic of a protein fraction. There is also a large amount of precipitated material at the interphase

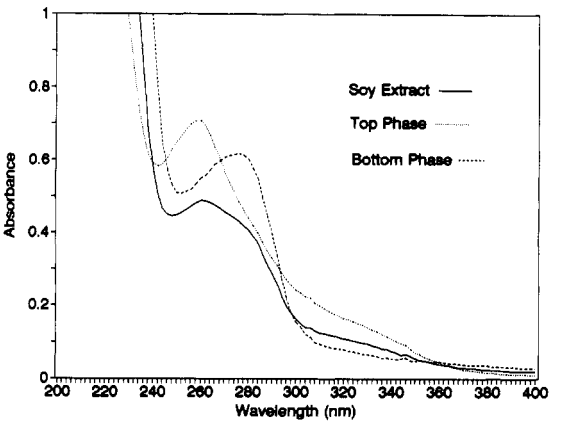

Figure 1. Ultraviolet–visible spectrum of soybean extract, isolated top phase and isolated bottom phase. Soy extract (prepared as described in the text) was diluted 1:200 with water and the spectrum measured. The isolated top and bottom phases from system A1 (1 g of extract in total mass of 10 g) were diluted with water 1:100 and 1:50, respectively, and the spectra measured.

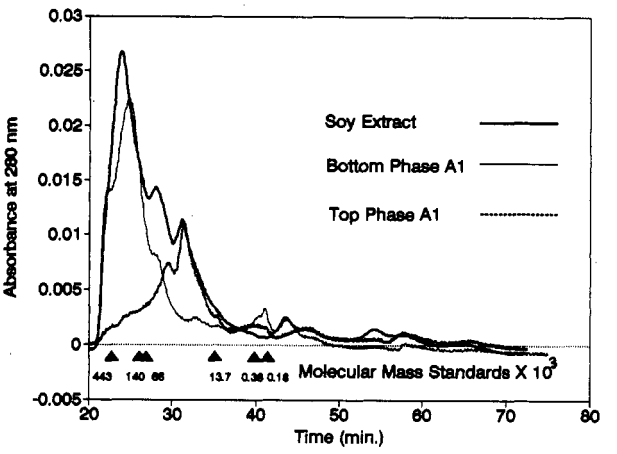

Figure 2. Size exclusion chromatography of soy extract and isolated phases of system A1. Solid triangles mark the retention time of molecular mass standards run on the column under conditions described in the text. The standards and masses used are horse spleen apoferritin (443 000), yeast alcohol dehydrogenase (140 000), bovine serum albumin (68 000), bovine pancreas ribonuclease A (13 700), sucrose (380), and fructose (180).

region that forms a layer upon centrifugation. This material is uncharacterized at this time.

Size Exclusion Chromatography. The size exclusion chromatography profile of the crude extract of soybeans is shown in Figure 2. The extract and the bottom phase sample have major peaks in the 400 000–150 000 mass range as expected for the storage proteins glycinin and β-conglycinin. The protein spectrum for the upper phase is more abundant in the lower molecular mass range, with a peak at approximately 20 000. There is a large amount of material eluting at and past the included volume of the column (position of 180 mass standard, Figure 2). The material eluting past the included volume of the column indicates interactions besides size exclusion are occurring between the compounds and the columns.

Lipoxygenase Partitioning and PEG Solubility. The 96 000 mass band on SDS-PAGE is present in the top phase of system A1 and not detectable in the bottom phase (Figure 3). When low amounts of soy extract or top phase are run on the gels, this band resolved into two closely spaced bands. These results indicate that all of the lipoxygenase isoforms are present in the top phase.

We measured the enzymatic activity of lipoxygenase 1 in these two-phase systems (Table II). The activity is

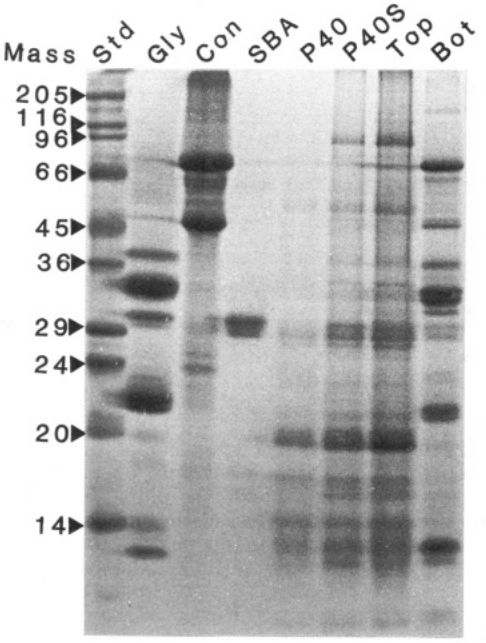

Figure 3. 15% Polyacrylamide SDS–PAGE of soybean extract samples. Lanes contain the following samples: Std, molecular mass standard proteins; Gly, purified soybean glycinin; Con, purified soybean β-conglycinin; SBA, purified soybean agglutinin; P40, sample of the supernatant of a 40% PEG 1000 solution; P40S, sample of the supernatant of a 40% PEG 1000 and 5% sodium chloride solution; Top, sample of the top phase of system A1; Bot, sample of bottom phase of system A1. One milliliter of soy extract was added to a total mass of 10 g for systems A1, P40, and P40S. The compositions of the systems are described in Table I. Sample preparation and electrophoresis conditions are described in the text. The identities and molecular masses of the standard proteins used are (starting from the top of gel): rabbit muscle myosin (205 000); *Escherichia coli* β-galactosidase (116 000); rabbit muscle phosphorylase (96 000); bovine serum albumin (66 000); egg albumin (45 000); rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 000); bovine carbonic anhydrase (29 000); bovine pancreas PMSF treated trypsinogen (24 000); soybean trypsin inhibitor (20 000); and α-lactalbumin (14 200).

Table II. Lipoxygenase 1 Activity in Phase Systems

system[a]	% recovery in phase[b]	partition coefficient[c]
A1 top phase	104.3 ± 3.3 (3)	50.1 ± 3.2 (3)
A1 bottom phase	2.1 ± 0.5 (3)	
A3 top phase	49.0 ± 0.4 (3)	235.7 ± 11.3 (3)
A3 bottom phase	0.2 ± 0.1 (3)	

[a] Compositions given in Table I. [b] Determined as percent of total activity added to system. Shown is the mean ± the standard error of the mean (three determinations). [c] Defined as concentration in top phase divided by concentration in bottom phase. Shown is the mean ± the standard error of the mean (three determinations).

present almost exclusively in the top phase. One important variable in these systems is the concentration of PEG and salt. Increasing the PEG and salt concentrations increases the length of the tie line (line that connects the compositions of the top and bottom phases) on the phase diagram. This has the effect of increasing the difference between the top and bottom phases. The systems with longer tie lines (such as A3) have an increased concentration of PEG and a decreased amount of salt (ammonium sulfate) present in the top phase compared to systems with a shorter tie-line length (such as A1). These systems also have increased concentrations of salt (ammonium sulfate) and decreased amounts of PEG in the bottom phase compared to systems with shorter tie-line lengths. We have measured the phase diagrams for these and other salt–PEG two-phase systems (Snyder et al., 1992). Figure 4 shows the

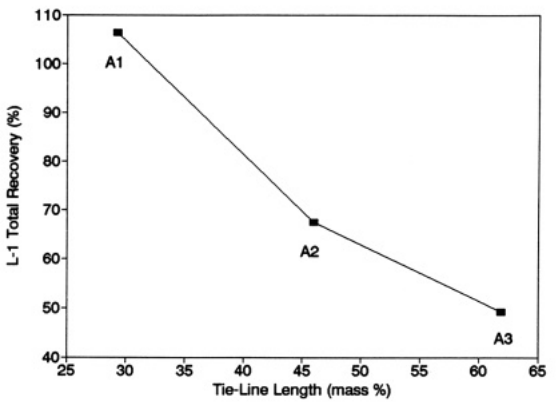

Figure 4. Influence of tie-line length on the total recovery of lipoxygenase 1 activity in aqueous two-phase systems. Soy extract (1 mL) was added to phase systems (total mass 10 g) of compositions described in Table I. The tie-line lengths of the indicated systems were calculated using the phase diagram data in Snyder et al. (1992).

Table III. Ionic Strength Dependence of Solubility of Lipoxygenase 1 in 30% PEG 1000/25 mM Tris (pH 7.5) Solutions

sodium chloride mass,[a] %	soy extract mass,[b] %	% recovery in supernatant[c]
0.0	0.95	1.70 ± 0.03 (3)
1.0	0.95	69.4 ± 7.5 (3)
1.9	0.95	84.0 ± 4.4 (3)
2.9	0.95	90.6 ± 2.0 (3)
4.8	0.95	103.2 ± 3.8 (3)
4.8	2.14	110.4 ± 2.8 (3)
4.8	3.33	115.0 ± 3.6 (3)
4.8	5.48	112.7 ± 1.9 (3)

[a] Final concentration of anhydrous sodium chloride added to system P30 (Table I). [b] Final concentration of soy extract calculated by adding varying amounts of 10% extract. [c] Calculated as percent of the total added to system. Shown is the mean ± the standard error of the mean (three determinations).

effect of increasing the concentration of PEG and ammonium sulfate in these systems. As can be seen from the data shown in Table II, increasing the tie-line length results in an increased partition coefficient and a decreased yield. The decreased yields indicate precipitation of the protein is occurring.

The solubility of the band corresponding to lipoxygenase in PEG solutions is very dependent upon the presence of salt. All of the PEG systems contained 25 mM Tris (pH 7.5) to control the pH. We used a non-phase-forming salt (sodium chloride) to investigate if the increased solubility was dependent upon ionic strength or upon specific ions such as ammonium or sulfate. We examined the soy proteins present in the supernatant of a 40% PEG solution when soy extract is added. The band at 96 000 is not present in the supernatant of 40% PEG without added salt (Figure 3, lane P40). When 5% NaCl is added to a 40% PEG solution, the band is present (Figure 3, lane P40S). We investigated the recovery of lipoxygenase 1 in the supernatant of PEG solutions. The data in Table III show the influence of added NaCl on the recovery of lipoxygenase 1 activity in the supernatant of 30% PEG 1000 solutions. Lipoxygenase 1 showed a rapid increase in solubility at low ionic strength. Increasing amounts of extract were added to a 30% PEG 1000 system containing 4.8% NaCl. Table III shows that the solubility of the system of lipoxygenase 1 in 30% PEG containing 4.8% sodium chloride was not exceeded even when over half of the system was added soy extract. Ingham (1978) showed that the addition of salts at pHs above the isoelectric point (4.5) of human serum albumin increased its solubility in

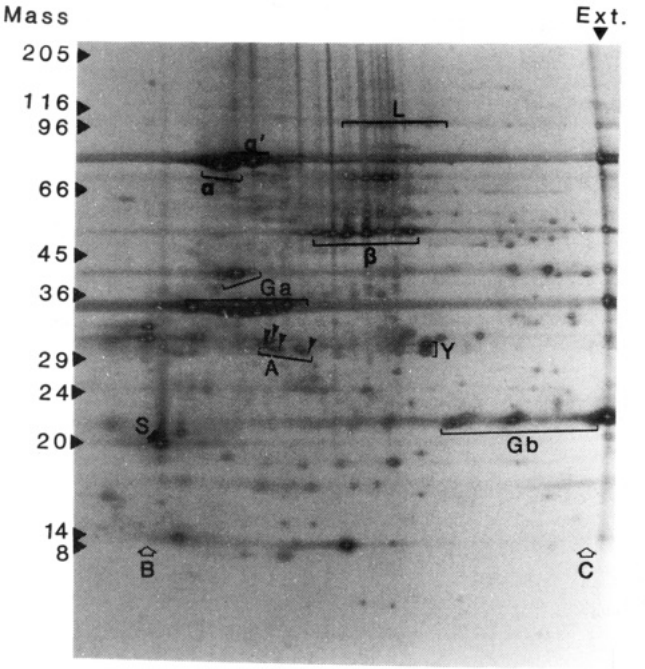

Figure 5. Two-dimensional gel electrophoresis of soy extract stained with Coomassie Brilliant Blue G-250. Soy extract was prepared, electrophoresed, and stained as described in the text. The locations of the proteins are indicated by brackets and arrows as follows: L, lipoxygenase; α, α', and β, respective subunits of β-conglycinin; Ga, acidic subunits of glycinin; Gb, basic subunits of glycinin; A, soybean agglutinin; Y, prominent whey protein(s) identified in Lei et al. (1983); S, Kunitz soybean trypsin inhibitor. The open arrows indicate the first-dimension focusing of Bowman–Birk inhibitor (B) and rabbit muscle creatin kinase (C). The molecular mass standards are the same as described in the legend of Figure 3 with the addition of Bowman–Birk inhibitor (mass of 8000). A sample of the extract was run in the second dimension on the extreme right of the gel (Ext.).

PEG solutions. He found that when the solution was at pH below the isoelectric point of albumin, the solubility was decreased. Our results show that the solubility of lipoxygenase in PEG solutions is strongly influenced by the ionic strength of the solution.

Soybean Protein Detected by Two-Dimensional Gel Electrophoresis. Soybean extract run on high-resolution two-dimensional gel electrophoresis resolved over 300 spots when the gels were stained with the Coomassie Brilliant Blue G method (Figure 5). Silver staining (Figure 6) reveals even more spots and detects proteins present in low quantities. The source of soybeans used in this study was a mixed preparation of strains, and a number of the spots could be due to genetic polymorphisms. We are currently investigating this by running extracts from defined stains of soybeans. Our two-dimensional gel electrophoresis patterns are similar to those obtained by others (Lei et al., 1983; Lei and Reeck, 1987; George and de Lumen, 1991). We used gradient gels and increased concentrations of Tris in the gel and tank buffer (Fling and Gregerson, 1986). Lei et al. (1983) also used the nonequilibrium technique (NEPHGE) to resolve a large number of the basic subunits of glycinin that do not resolve using the standard condition of focusing in the first-dimension pH gradients. Fling and Gregerson (1986) observed a sharp break in the mobility vs log of molecular mass at approximately 14 000. We observe a similar transition in mobility vs molecular mass based on the mobility of the standard at 14 200 (α-lactalbumin) and the 8000 molecular mass standard (Bowman–Birk inhibitor) (Figure 5). A number of proteins and peptides below 14 000 are detected, closely spaced and well resolved.

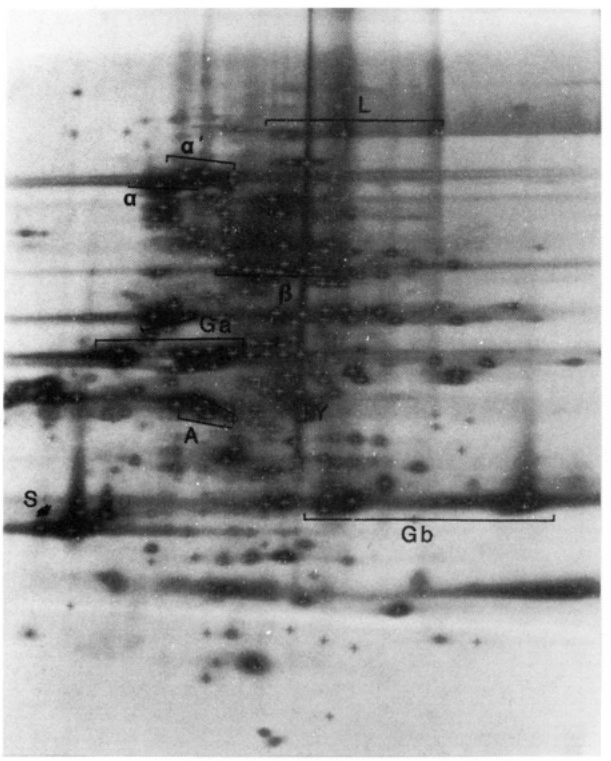

Figure 6. Two-dimensional gel electrophoresis of soy extract stained with silver. Soy extract was prepared, electrophoresed, and stained as described in the text. The identities of the proteins are as described in the legend of Figure 5.

Lipoxygenases. The commercial preparations of lipoxygenase tested gave a large number of bands on SDS–PAGE. The band at 96 000 was enriched but made definitive identification difficult. When soybean extract was run on two-dimensional gel electrophoresis, three to five spots were observed at molecular mass of approximately 96 000. The major spots observed are indicated in Figures 5 and 6. Soybean lipoxygenase has been separated into four isoforms termed L-1, L-2, L-3a, and L-3b (Axelrod et al., 1981). L-3a and L-3b are very similar, and differences are believed to be due to posttranslational modifications [reviewed in Siedow (1991)]. The isoelectric points for the three isozymes are 5.68 for L-1, 6.25 for L-2, and 6.15 for L-3 (Christopher et al., 1972). The deduced molecular masses of the three isozymes are 94 038 for L-1 (Shibata et al., 1987), 97 053 for L-2 (Shibata et al., 1988), and 96 541 for L-3 (Yenofsky et al., 1988).

The spots of lipoxygenase are present in the top-phase sample of system A1 and absent in the bottom phase (Figure 7). The lipoxygenase spots are also present in the supernatant of 40% PEG 1000 plus 5% NaCl (system P40S) and absent in the supernatant of 40% PEG (system P40; Figure 8). Samples of the acid-soluble fraction also contain the lipoxygenase spots (Figure 9). Until purified isozymes become available and identified on two-dimensional gel electrophoresis, the spots observed can only be tentatively assigned as lipoxygenase.

Storage Proteins. The spots associated with the storage proteins, β-conglycinin, and glycinin were identified by running purified proteins (provided by Dr. W. J. Wolf, USDA-ARS, Peoria, IL). The major spots associated with β-conglycinin and glycinin are indicated in Figures 5 and 6. A number of minor spots associated with the storage proteins are also present in the soybean extracts. The α, α', and β polypeptides of β-conglycinin have been shown to contain glucosamine and mannose (Thanh and Shibasaki, 1977). The α, α', and β subunits of β-conglycinin

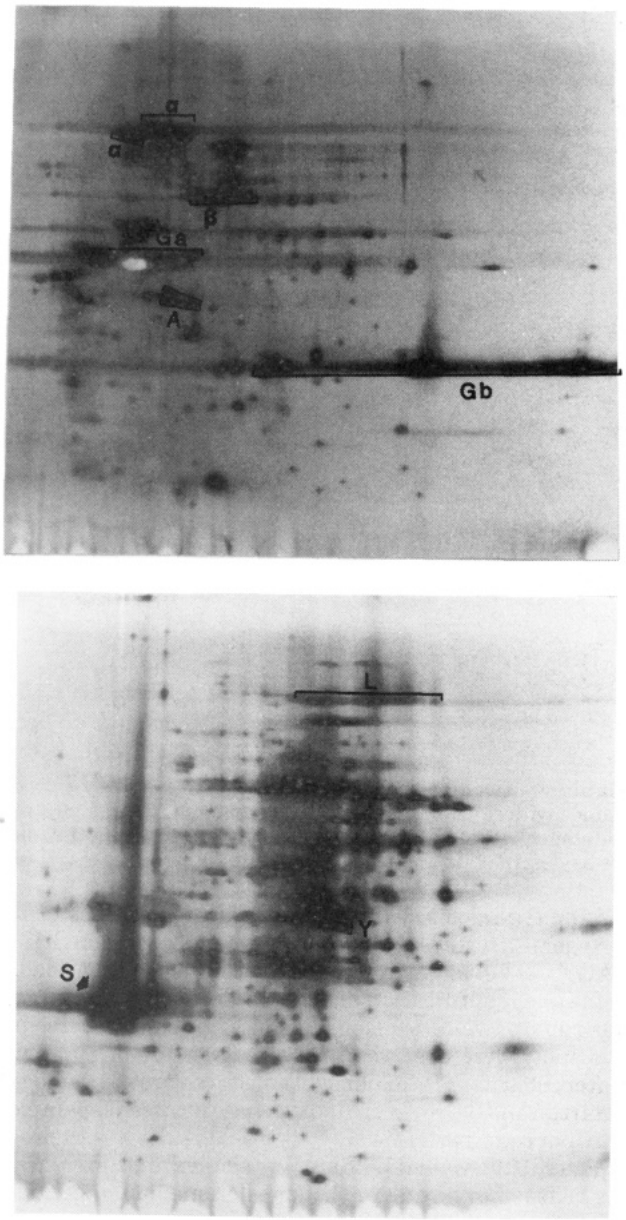

Figure 7. Two-dimensional gel electrophoresis of isolated phases stained with silver. Soy extract (2 mL) was added to system A1 (total mass 10 g; Table I), and the phases were isolated, prepared, electrophoresed, and stained as described in the text. (A, Top) Sample of bottom phase of system A1. (B, Bottom) Sample of top phase of system A1.

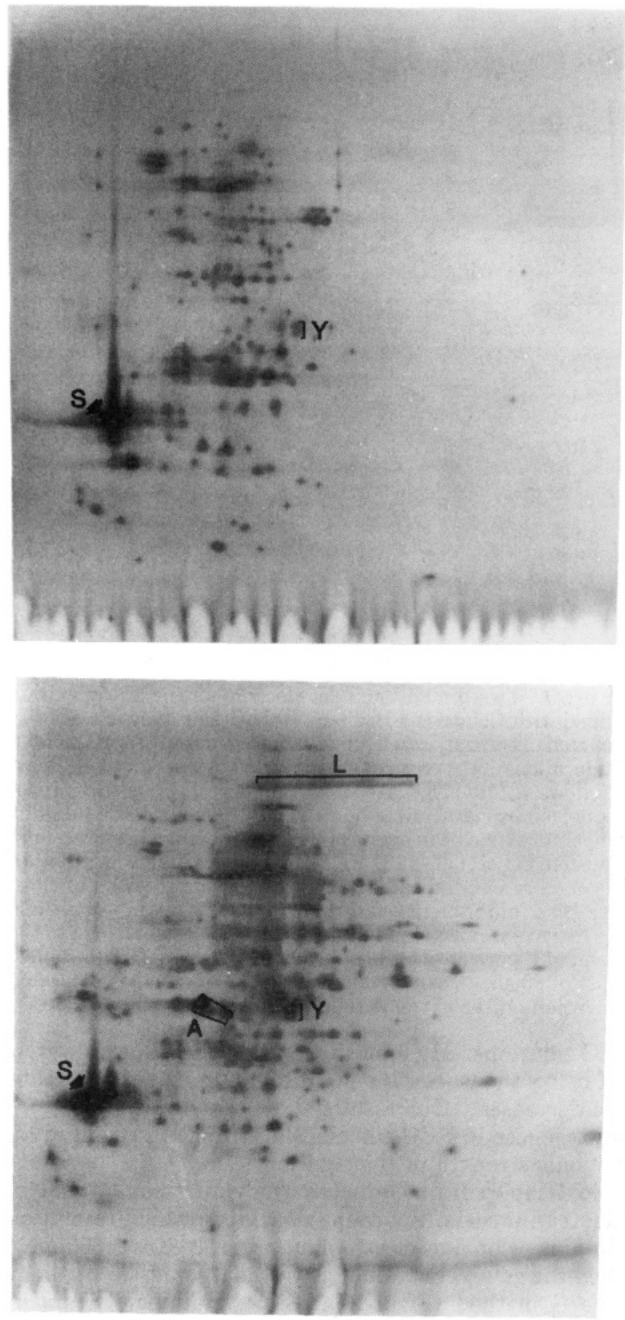

Figure 8. Two-dimensional gel electrophoresis of supernatants of PEG 1000 systems stained with silver. Soy extract (2 mL) was added to PEG 1000 systems (total mass 10 g; Table I) and the supernatant isolated as described in the text. (A, Top) Sample of supernatant of system P40. (B, Bottom) Sample of supernatant P40S top phase of system A1.

are indicated in Figures 5 and 6. The storage proteins were present in the bottom phase of system A1 but not detectable in the top phase (Figure 7). The storage proteins are not detectable in the P40 or P40S supernatants (Figure 8). The acid-soluble fraction contains a significant amount of conglycinin (Figure 9).

Soybean Agglutinin. Soybean agglutinin (SBA) is a tetrameric protein with subunits of approximately 30 000 and a measured isoelectric point of 5.81 (Lotan et al., 1974). SBA is a glycoprotein containing significant amounts of D-mannose and *N*-acetyl-D-glucosamine (Lis et al., 1966). Catsimpoolas and Meyer (1969) resolved four forms of SBA using isoelectric focusing. Two-dimensional gel electrophoresis resolved purified SBA into four closely resolved spots with mass of approximately 30 000 (Figure 5). SBA is in the bottom phase of system A1 and not detectable in the top phase (Figure 7). SBA is in the supernatant of PEG 1000 containing 5% NaCl (P40S) and absent in the supernatant of P40 (Figure 8). The substitution of 5% NaCl for an equivalent amount of water to phase system A1 results in SBA being present in the top phase and undetectable in the bottom phase (results not shown). The addition of NaCl to system A1 would be expected to increase the ionic strength of both top and bottom phases. The addition of NaCl could also directly affect the protein structure as well as change the chemical composition of the phases. Additional research will be required to distinguish between the two. It is clear that the addition of NaCl has specific effects in increasing the partition coefficient of SBA in two-phase systems and increasing its solubility in PEG. SBA was also present in the acid-soluble fraction (Figure 9).

Trypsin Inhibitors. Purified KSTI resolves as one major spot (indicated in Figures 5 and 6) along with a

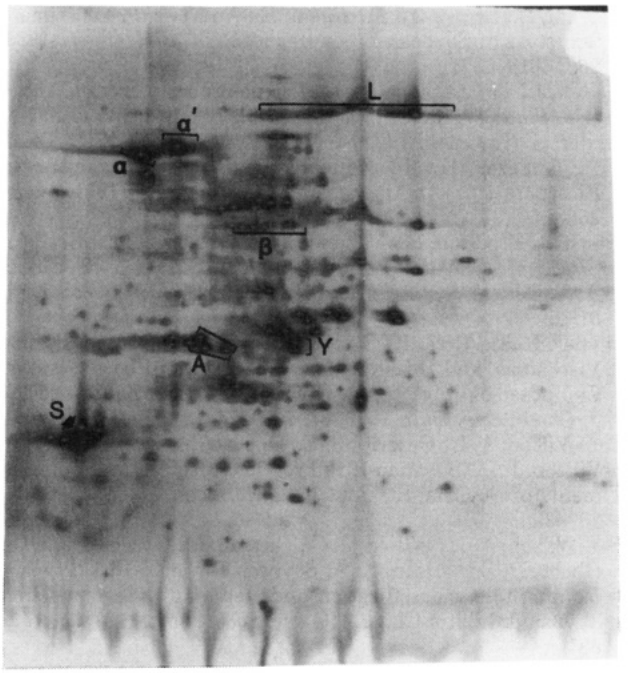

Figure 9. Two-dimensional gel electrophoresis of soy acid-soluble supernatant stained with silver. The acid-soluble supernatant was prepared, electrophoresed, and stained as described in the text.

number of minor spots. Three isoforms of KSTI have been described (Orf and Hymowitz, 1977, 1979; Freed and Ryan, 1980). These isoforms were shown to have different electrophoretic mobility and to bind trypsin with different affinities. KSTI is present in the top phase of system A1 but not detected in the bottom phase (Figure 7). KSTI is detected in the supernatant of P40 and P40S (Figure 8) as well as the acid-soluble fraction (Figure 9).

Purified Bowman–Birk inhibitor (BBI) resolves as one major spot (location indicated in Figure 5) with a number of minor spots when stained with the Coomassie Brilliant Blue-G (CBB-G) method. A large number of forms of the BBI family have been described (Hwang et al., 1977; Tan-Wilson et al., 1985, 1987). BBI was not reliably detected when soy extract samples were stained with the CBB-G method. This indicated that the staining procedure was not very sensitive for BBI. The silver staining procedure used in this paper did not detect the BBI isoforms at all. We are currently studying various staining and electrophoretic procedures to optimize the detection of the isoforms of BBI.

Conclusions. The ATPS and PEG precipitation systems used in this study have significant advantages for the fractionation of the soy proteins. The separations are rapid and require no special equipment. The separations are also suitable for large scale. The systems do not expose the proteins to low pH as in the isoelectric precipitation methods. In the ATPS, the storage proteins remain in solution and are cleanly separated from a number of the other proteins, such as the protease inhibitors and lipoxygenase. The protease inhibitors are of great interest because of their effects on human nutrition and possible role in cancer prevention [reviewed in Messina and Barnes (1991)]. Lipoxygenase is believed to be responsible for the development of bitter off-flavors in soybean products. These methods that allow the rapid and economical separation of the storage proteins free from the protease inhibitors and lipoxygenase should facilitate such studies. Iwabuchi and Yamauchi (1987) showed that the globulin (acid-precipitated) fraction is contaminated by significant amounts of storage proteins along with lipoxygenase, agglutinin, trypsin inhibitor, and β-amylase. The acid-soluble fraction in this study also contained a significant amount of β-conglycinin (Figure 9). A number of variables in the ATPS and PEG precipitation systems can be changed to suit the particular separation. The ionic strength and composition appear to be particularly important. PEG precipitation at low ionic strength (system P40) gives a fraction enriched in the trypsin inhibitors and other specific proteins (such as Y; Figure 7). PEG precipitation at moderate ionic strength (system P40S) gives a fraction enriched in protease inhibitors, lipoxygenase, SBA, and other specific proteins (Figure 7). Partitioning the soy proteins in system A1 yields a top-phase fraction containing protease inhibitors, lipoxygenase, and other specific proteins. The bottom phase contains the storage proteins, SBA, and other specific proteins in a soluble form. The addition of sodium chloride to system A1 moves SBA from the bottom to the top phase.

The silver-stained gels were extremely useful for detecting proteins present in low concentrations. These gels resolve a large number of rare spots that are present in either the top or bottom phase or both. The utility of two-dimensional gel electrophoresis to monitor the separation of the complex mixture of soy proteins will increase as more proteins become mapped.

ACKNOWLEDGMENT

We acknowledge Dr. W. J. Wolf, USDA ARS, Northern Regional Research Center, Peoria, IL, for purified storage proteins and suggestions; Dr. J. J. Edwards, National Institute of Standards and Technology, Gaithersburg, MD, for help with silver staining and suggestions; and Steve Snyder for technical assistance.

LITERATURE CITED

Anderson L. Adaptation of the colloidal Coomassie Blue stain to quantitative, multi-gel 2-D work. *2D News* **1989**, *3*, 3.

Anderson, L.; Anderson, N. G. High Resolution Two-Dimensional Electrophoresis of Human Plasma Proteins. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5421–5425.

Axelrod, B.; Cheesebrough, T. M.; Laakso, S. Lipoxygenase from Soybeans. *Methods Enzymol.* **1981**, *71*, 441–451.

Catsimpoolas, N.; Meyer, E. W. Isolation of Soybean Hemagglutinin and Demonstration of Multiple Forms by Isoelectric Focusing. *Arch. Biochem. Biophys.* **1969**, *132*, 279–285.

Christopher, J. P.; Pistorius, E. K.; Axelrod, B. Isolation of a Third Isoenzyme of Soybean Lipoxygenase. *Biochim. Biophys. Acta* **1972**, *284*, 54–62.

Cole, K. D. Purification of Soybean Low Molecular Mass Protease Inhibitors using Aqueous Two-Phase Extraction and Polyethylene Glycol Precipitation. In *Bioseparation Technologies*; Ataai, M. M., Sikdar, S. K., Eds.; American Institute of Chemical Engineers: New York, 1992 (in press).

Edwards, J. J.; Reeder, D. J.; Atha, D. H. Separation and Characterization of Fibronectin Domains by Two-Dimensional Electrophoresis. *Appl. Theor. Elec.* **1990**, *1*, 207–212.

Fling, S. P.; Gregerson, D. S. Peptide and Protein Molecular Weight Determination by Electrophoresis Using a High-Molarity Tris Buffer System without Urea. *Anal. Biochem.* **1986**, *155*, 83–88.

Freed, R. C.; Ryan, D. S. Isolation and Characterization of Genetic Variants of the Kunitz Soybean Trypsin Inhibitor. *Biochim. Biophy. Acta* **1980**, *624*, 562–571.

George, A. A.; de Lumen, B. O. A Novel Methionine-Rich Protein in Soybean Seed: Identification, Amino Acid Composition, and N-Terminal Sequence. *J. Agric. Food Chem.* **1991**, *39*, 224–227.

Hwang, D. L.-R.; Lin, K. T.-D.; Yang, W.-K.; Foard, D. E. Purification, Partial Characterization, and Immunological

Relationships of Multiple Low Molecular Weight Protease Inhibitors of Soybean. *Biochim. Biophys. Acta* **1977**, *495*, 369–382.

Ingham, K. C. Precipitation of Proteins with Polyethylene Glycol: Characterization of Albumin. *Arch. Biochem. Biophys.* **1978**, *186*, 106–113.

Iwabuchi, S.; Yamauchi, F. Electrophoretic Analysis of Whey Proteins Present in Soybean Globulin Fractions. *J. Agric. Food Chem.* **1987**, *35*, 205–209.

Lei, M. G.; Reeck, G. R. Two-Dimensional Electrophoretic Analysis of the Proteins of Isolated Soybean Protein Bodies and the Glycosylation of Soybean Proteins. *J. Agric. Food Chem.* **1987**, *35*, 296–300.

Lei, M. G.; Tyrell, D.; Bassette, R.; Reeck, G. R. Two-Dimensional Electrophoretic Analysis of Soybean Proteins. *J. Agric. Food Chem.* **1983**, *31*, 963–968.

Lis, H.; Sharon, N.; Katchalski, E. Soybean Hemagglutinin, a Plant Glycoprotein. *J. Biol. Chem.* **1966**, *241*, 684–689.

Lotan, R.; Siegelman, H. S.; Lis, H.; Sharon, N. Subunit Structure of Soybean Agglutinin. *J. Biol. Chem.* **1974**, *249*, 1219–1224.

Messina, M.; Barnes, S. The Role of Soy Products in Reducing Risk of Cancer. *J. Natl. Cancer Inst.* **1991**, *83*, 541–546.

Neuhoff, V.; Arold, N.; Taube, D.; Ehrhardt, W. Improved Staining of Proteins in Polyacrylamide Gels Including Isoelectric Focusing Gels with Clear Background at Nanogram Sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **1988**, *9*, 255–262.

Oakley, B. R.; Kirsch, D. R.; Morris, N. R. A Simplified Ultrasensitive Silver Stain for Detecting Proteins in Polyacrylamide Gels. *Anal. Biochem.* **1980**, *105*, 361–363.

O'Farrell, P. H. High Resolution Two-Dimensional Electrophoresis. *J. Biol. Chem.* **1975**, *250*, 4007–4021.

Orf, J. H.; Hymowitz, T. Inheritance of a Second Trypsin Inhibitor in Seed Protein of Soybeans. *Crop Sci.* **1977**, *17*, 811–813.

Orf, J. H.; Hymowitz, T. Inheritance of the Absence of the Kunitz Trypsin Inhibitor in Seed Protein of Soybeans. *Crop Sci.* **1979**, *19*, 107–109.

Rackis, J. J. Biologically Active Components. In *Soybeans: Chemistry and Technology*; Smith, A. K., Circle, S. J., Eds.; AVI Publishing: Westport, CT, 1972; Vol. 1, pp 158–202.

Shibata, D.; Steczko, J.; Dixon, J. E.; Hermodson, M.; Yazdanparast, R.; Axelrod, B. Primary Structure of Soybean Lipoxygenase-1. *J. Biol. Chem.* **1987**, *262*, 10080–10085.

Shibata, D.; Steczko, J.; Dixon, J. E.; Andrews, P. C. Primary Structure of Soybean Lipoxygenase-2. *J. Biol. Chem.* **1988**, *263*, 6816–6821.

Siedow, J. N. Plant Lipoxygenase: Structure and Function. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1991**, *42*, 145–188.

Sikdar, S. K.; Cole, K. D.; Steward, R. M.; Szlag, D. C.; Todd, P.; Cabezas, H., Jr. Aqueous Two-Phase Extraction in Bioseparations: An Assessment. *Bio/Technology* **1991**, *9*, 253–256.

Smith, A. K.; Circle, S. J. Chemical Composition of the Seed. In *Soybeans: Chemistry and Technology*; Smith, A. K., Circle, S. J., Eds.; AVI Publishing: Westport, CT, 1972; Vol. 1, pp 61–92.

Snyder, S. M.; Cole, K. D.; Szlag, D. C. Phase Compositions, Viscosities, and Densities for Aqueous Two-Phase Systems Composed of Polyethylene Glycol and Various Salts at 25 °C. *J. Chem. Eng. Data* **1992**, *37*, 268–274.

Tan-Wilson, A. L.; Cosgriff, S. E.; Duggan, M. C.; Obach, R. S.; Wilson, K. A. Bowman-Birk Proteinase Isoinhibitor Complements of Soybean Strains. *J. Agric. Food Chem.* **1985**, *33*, 389–393.

Tan-Wilson, A. L.; Wilson, J. C.; Duggan, M. C.; Chapman, C.; Obach, R. S.; Wilson, K. A. Soybean Bowman-Birk Trypsin Isoinhibitors: Classification and Report of a Glycine-Rich Trypsin Inhibitor Class. *J. Agric. Food Chem.* **1987**, *35*, 974–981.

Thanh, V. H.; Shibasaki, K. Beta-Conglycinin from Soybean Proteins Isolation and Immunological and Physicochemical Properties of the Monomeric Forms. *Biochim. Biophys. Acta* **1977**, *490*, 370–384.

Wolf, W. J. Purification and Properties of the Proteins. In *Soybeans: Chemistry and Technology*; Smith, A. K., Circle, S. J., Eds.; AVI Publishing: Westport, CT, 1972; Vol. 1, pp 93–143.

Yenofsky, R. L.; Fine, M.; Liu, C. Isolation and Characterization of a Soybean (*Glycine Max*) Lipoxygenase-3 Gene. *MGG, Mol. Gen. Genet.* **1988**, *211*, 215–222.

Received for review August 28, 1992. Accepted November 18, 1992. The National Institute of Standards and Technology does not endorse any particular brand of product or company. Commercial names are used only for the precise description of experimental materials and procedures.