ARTICLES

Heterogeneity of Soybean Proteins: Two-Dimensional Electrophoretic Maps of Three Solubility Fractions

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Proteins were obtained by an exhaustive sequential extraction procedure from defatted soybean meal of *Glycine max* L. Merrill cultivar Dorman and subjected to two-dimensional polyacrylamide gel electrophoresis using isoelectric focusing (IEF) in the first dimension and sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis in the second dimension. Of the six solubility fractions, the water-soluble, 0.5 M NaCl soluble, and 60% (v/v) acetic acid soluble fractions were successfully separated by two-dimensional polyacrylamide gel electrophoresis. The two-dimensional polyacrylamide gel electrophoretic map of the water-soluble fraction contained at least 647 polypeptides (spots) detectable by Coomassie Blue R-250 staining while that of NaCl-soluble and acetic acid soluble fractions included at least 543 and 346 spots, respectively. Most of the major components on the two-dimensional polyacrylamide gel electrophoretic map of the water-soluble and acetic acid soluble fractions appeared to be the same polypeptides. These components corresponded to the subunits of the two major soybean protein components, namely, β-conglycinin (7S globulin) and glycinin (11S globulin).

Soybean (*Glycine max* L. Merrill) is one of the richest sources of vegetable protein (35–45%) among cultivated food and feed crops. However, like most proteins of vegetable origin, soybean protein is of low nutritional qulity for growing humans and other monogastric animals because of its low content of methionine and cysteine. The improvement of protein quality of soybean and other legumes by genetic means has recently received increased attention in view of their potential to become an important source of protein for humans. The standard methods (e.g., ion-exchange chromatography) for determining amino acid composition are too slow, expensive, and not very reproducible for methionine and cysteine (Radford et al., 1977). Therefore, they are not of much value in screening large numbers of cultivars and lines for genetic variants with high methionine and cysteine content. Radford et al. (1977) reported a method which utilizes the ratio of total nitrogen to total sulfur to evaluate the methionine and cysteine content of soybean seed. Although the total nitrogen to total sulfur ratio shows high correlation with methionine and cysteine, the method is somewhat tedious. Smartt et al. (1975) suggested increasing the relative proportion of polypeptides with the most desirable amino acid profile as a feasible way to improve protein quality in legumes. This approach would require the purification and characterization of major polypeptides that occurs in seeds of edible legume species and then develop a procedure to quantitatively screen for polypeptides with high methionine and cysteine content.

The major protein components of the soybean seed are reasonably well characterized (Catsimpoolas, 1969; Kitamura et al., 1976; Thanh and Shibasaki, 1976; Wolf, 1977; Moreira et al., 1979). However, there is very little information on the extent of protein heterogeneity in seeds since most electrophoretic investigations were either limited to specific protein fractions or used relatively low resolution techniques. Before initiating a comprehensive program to purify and characterize the major soybean seed proteins, specifically for determining their amino acid compositions, it is desirable to establish the extent of polypeptide heterogeneity. With this in mind, we (Hu and Esen, 1981) have recently extracted soybean seed meal sequentially with six different solvents and analyzed the resulting solubility fractions by sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis and isoelectric focusing (IEF). Here we report the results obtained by two-dimensional polyacrylamide gel electrophoresis.

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MATERIALS AND METHODS

Extraction of Soybean Proteins. Petroleum ether defatted soybean meal (*Glycine max* L. Merrill cv. Dorman) was extracted by exhaustive and sequential extraction with six solvents: water, 0.5 M NaCl, 70% 2-propanol, 60% (v/v) glacial acetic acid, 0.1 M NaOH, and 0.1 M sodium borate, pH 10, containing 1% (w/v) $NaDodSO_4$ and 1% (v/v) 2-mercaptoethanol. Details of extraction procedure are given elsewhere (Hu and Esen, 1981). This extraction schedule was a modified version of the one used for corn proteins by Landry and Moureaux (1970). The reason for performing this sequency of extractions was to obtain distinct protein fractions that are soluble in a particular solvent only and not in those preceding it.

Electrophoresis in the First Dimension. Proteins extracted with water, 0.5 M NaCl, and 60% acetic acid (which will be hereafter referred to as H_2O-soluble, NaCl-soluble, and HAc-soluble, respectively) were separated by isoelectric focusing (IEF) in 3 × 115 mm cylindrical polyacrylamide gels in the first dimension. Acrylamide concentration of the gel (5% T; 3% C) was the same as that used by Davies (1975) except that it included 8 M urea and 1% (v/v) Triton X-100 (O'Farrell, 1975) to perform the separation under denaturing conditions. The pH gradient was formed by using a mixture of ampholytes (LKB) pH 3.5–10, 4–6, 5–7, 5–8, and 8–9.5 in a ratio of 1:1:1:1:1 at a final concentration of 2% (w/v). The anodic and cathodic electrolytes were 0.01 M H_3PO_4 and 0.02 M NaOH, respectively, following the procedure of O'Farrell (1975). Freeze-dried protein powders were prepared in the lysis buffer at 10 mg/mL and routinely about 400 μg of protein was applied onto a gel at the anodic end. IEF was performed under regulated voltage, starting with 200 V and increasing the voltage in steps (100 V/h) to 500 V in 3 h and then maintaining it at 500 V for 3 h. The IEF gels

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were extruded from tubes at the end of the run, frozen immediately, stored in the freezer at −20 °C, and usually subjected to electrophoresis in the second dimension within 1 week. No attempt was made to determine the isoelectric points of polypeptides since such determinations are of questionable accuracy in polyacrylamide gels containing urea.

The IEF gels were equilibrated with 0.125 M Tris-HCl buffer, pH 6.8, containing 10% (v/v) glycerol, 2% $NaDodSO_4$ (w/v), and 5% 2-mercaptoethanol (v/v) for 15 min at room temperature before being loaded onto the second-dimensional gels.

Electrophoresis in the Second Dimension. Separation in the second dimension was performed by $NaDodSO_4$–discontinuous polyacrylamide gel electrophoresis essentially following the procedures of Laemmli (1970) and O'Farrell (1975). The second-dimension resolving gel slab (125 × 0.75 × 155 mm, $l \times d \times h$) contained a linear gradient of 10–13% polyacrylamide, with a final concentration of 0.8 M Tris-HCl (pH 8.8) and 0.24% (w/v) $NaDodSO_4$. The large-pore (stacking) gel (20 × 0.75 × 155 mm) contained 5% acrylamide in a final concentration of 0.3 M Tris-HCl (pH 6.8) and 0.24% $NaDodSO_4$ (w/v). The concentration of buffer components in both resolving and stacking gels was about twice as that used in the standard $NaDodSO_4$–polyacrylamide gel electrophoretic systems. Soon after polymerization of the stacking gel, the equilibrated IEF gel was loaded onto the stacking gel and stabilized in place with 1% agarose solution containing 0.125 M Tris-HCl buffer (pH 6.8) and 1% $NaDodSO_4$. Electrophoresis was carried out under constant current at 30 mA/slab until the tracking dye front entered the resolving gel and at 20 mA/gel thereafter. Under these conditions electrophoresis required 9 h. After electrophoresis, the gel slab was stained with 0.1% Coomassie Brilliant Blue R-250 in acetic acid–2-propanol–water (10:25:65 v/v) for 12–16 h and destained with the same solution minus the dye. The two-dimensional profile of each solubility fraction was photographed and also directly traced onto a transparent sheet placed on a wet gel sandwiched between two glass plates. This procedure yields an exact replica of spots on wet gels.

Preparative Electrophoresis and Amino Acid Analysis. Water- and NaCl-soluble fractions were separated by preparative $NaDodSO_4$–polyacrylamide gel electrophoresis in 3 mm thick gels containing 10% polyacrylamide. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 for 15 min which permitted the visualization of predominant bands. Bands corresponding to α' and α subunits of β-conglycinin and 11S-2 (acidic) and 11S-1 (basic) subunits of glycinin from H_2O-soluble fractions (Figure 1) and two bands, designated as Glb 1 and Glb 2, from the NaCl-soluble fraction were cut in strips with a razor blade. The gel strips were placed in a mortar, ground in liquid nitrogen with a pestle, and transferred to glass centrifuge tubes. The resulting gel homogenate was extracted with methanol (100%) several times to remove the dye. The homogenate was then extracted twice with 60% HAc to recover the protein. The extracts were then combined, dialyzed, lyophilized, and subjected to (automated) amino acid analysis by ion-exchange chromatography.

RESULTS

Electrophoresis in the First Dimension. Three solubility fractions, H_2O-, NaCl-, and HAc-soluble proteins, were successfully separated by isoelectric focusing into 70–80 components (Hu and Esen, 1981). Proteins in other solubility fractions consistently gave poor resolution perhaps because of nonprotein impurities. Consequently, these fractions were not subjected to two-dimensional analysis. The IEF profiles of H_2O- and HAc-soluble fractions were similar while that of NaCl-soluble fraction differed considerably. The pH range in the gradient was from 2.9 to 8.7 based on measurements on gel slices. The IEF profile of a given fraction was reproducible under standardized conditions.

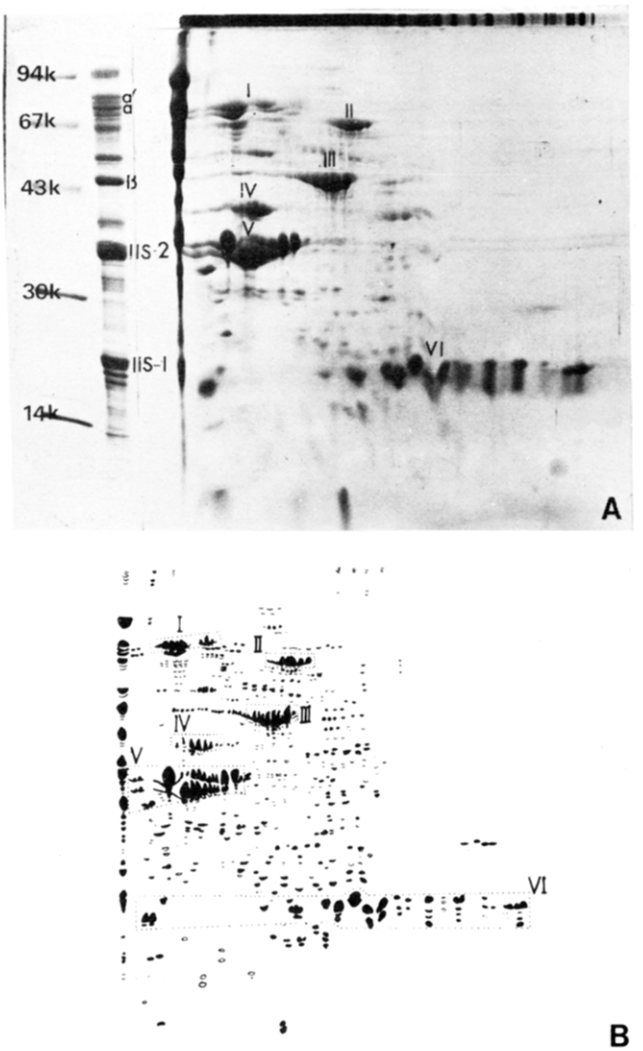

Figure 1. (A) Two-dimensional gel electrophoretic map of H_2O-soluble proteins from soybean seed. One-dimensional IEF gel at top, anode on the left, and cathode on the right. One-dimensional $NaDodSO_4$–polyacrylamide gel electrophoretic gel including marker proteins and H_2O-soluble soybean proteins on the left in which the cathode and origin are at top. Clusters of major polypeptides are indicated with roman numerals I–VI on the two-dimensional map. (B) shows a replica tracing of the wet two-dimensional gel, details not visible in (A), and boundaries of six major polypeptide clusters (marked with dotted rectangles).

Electrophoresis in the Second Dimension. The two-dimensional map of each of the three solubility fractions displayed tremendous polypeptide heterogeneity (Figures 1–3), confirming the high resolving power of two-dimensional polyacrylamide gel electrophoresis reported by other investigators (O'Farrell, 1975; Anderson and Anderson, 1977; Basha, 1979). The best resolution was obtained when separation in the second dimension was performed in gradient gels. Equilibration time for the IEF gel was found to be critical to the detection of minor polypeptides. Prolonged equlibrium (e.g., 45–60 min) led to the loss of minor spots from two-dimensional gels, obviously by diffusion into equilibration buffer. Therefore, equilibration time was reduced to 15 min so as to detect these minor polypeptides. However, overall resolution

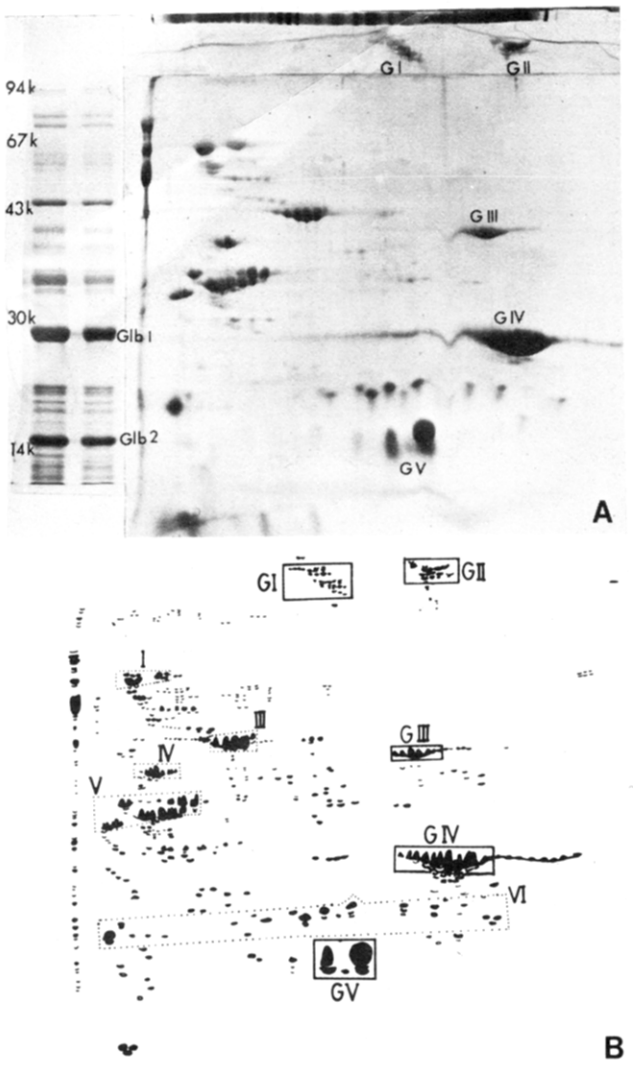

Figure 2. (A) Two-dimensional gel electrophoretic map of 0.5 M NaCl soluble proteins from soybean seed. Major groups of polypeptides unique to the NaCl-soluble fraction are indicated with roman numerals I–V after the letter G (globulin). Glb 1 and Glb 2 in one-dimensional $NaDodSO_4$–polyacrylamide gel electrophoretic profile on the left correspond to GIV and GV, respectively, on the two-dimensional map. (B) shows a replica tracing of the wet two-dimensional gel and details not visible in (A). Direction and polarities of electrophoretic separations are the same as those in Figure 1.

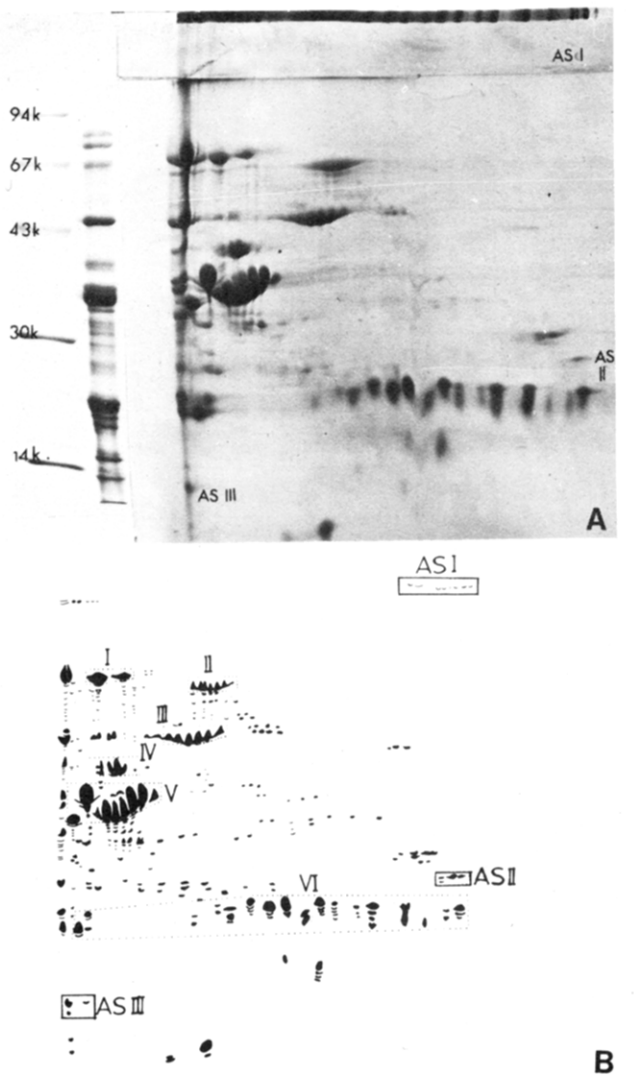

Figure 3. (A) Two-dimensional gel electrophoretic map of HAc-soluble proteins from soybean seed. Polypeptide clusters unique to this fraction are designated as ASI and ASII, AS standing for acid soluble. Note (cf. Figures 1 and 3) considerable similarity between the two-dimensional maps of H_2O- and HAc-soluble proteins. (B) shows a replica tracing of the wet two-dimensional gel and details not visible in (A) as well as clusters of major polypeptides shared by HAc- and water-soluble fractions. Direction and polarities of electrophoretic separations are the same as those in Figure 1.

deteriorated due to localized pH disturbances, caused by carrier ampholytes retained in the IEF gel and their entry into the two-dimensional gel. Under these conditions, the tracking dye front was broad and wavy. Increasing (doubling) the molarity of stacking and resolving gel buffers corrected this problem considerably and improved the resolution.

The two-dimensional map of the H_2O-soluble fraction (Figure 1) contained at least 647 polypeptides (spots) while that of the NaCl-soluble fraction included a minimum number of 543 spots (Figure 2). As for the HAc-soluble fraction, its map (Figure 3) contained at least 346 spots. Again, as was observed with IEF profiles, the two-dimensional maps of the H_2O- and HAc-soluble fractions contained essentially the same major polypeptides (cf. Figures 1 and 3). However, they differed considerably in their composition of minor polypeptides; the HAc-soluble fraction contained about half as many minor polypeptides, most of which appeared to be different from those in the H_2O-soluble fraction. The major spots (polypeptides) in the map of the H_2O-soluble fraction formed six clustered groups, indicated in Figure 1B with roman numerals I–VI and boundaries marked with dotted rectangles. Cluster I included about 10 spots (M_r 79 000), one of which was believed to be the α' subunit of β-conglycinin (7S globulin) described by Thanh and Shibasaki (1976, 1977). There appeared to be at least seven spots within cluster II (M_r 71 000), and one of them was thought to be the α subunit of β-conglycinin. Cluster III, 12 spots (M_r 45 000), was presumed to include the β subunit of β-conglycinin. There were about 9 spots (M_r 41 000) within cluster IV and 35 spots (M_r 37 000) within cluster V; some of these spots were thought to correspond to the acidic subunits of glycinin, 11S globulin (Kitamura et al., 1976), or to 11S-2 of Beachy et al. (1978). Cluster VI consisted of about 50 spots (M_r 22 000); some of these were very likely the basic subunits of glycinin (Kitamura et al., 1976) and corresponded to the 11S-1 of Beachy et al. (1978). The remaining spots (those which were not included within these six major clusters) that appeared in the two-dimensional map of the H_2O-

Table I. Amino Acid Composition of Various Soybean Protein Subunits Isolated by Preparative $NaDodSO_4$-Polyacrylamide Gel Electrophoresis

amino acid	α′ [a]	α [a]	11S-2 [b]	11S-1 [b]	Glb 1 [c]	Glb 2 [c]
Lys	6.1	5.8	5.5	4.4	4.5	2.4
His	1.6		1.8	1.9	2.5	4.5
Arg	6.3	4.1	5.9	5.6	2.7	3.3
Asp	13.0	13.5	11.8	13.2	9.7	10.1
Thr	3.2	3.6	4.0	4.7	5.7	8.2
Ser	8.6	8.4	6.3	7.6	11.1	7.4
Glu	17.8	18.7	23.3	12.9	13.5	12.3
Pro	5.7	5.8	6.4	5.6	5.7	8.4
Gly	6.5	7.2	9.0	7.8	12.0	7.8
Ala	5.8	6.5	5.1	7.7	7.4	6.7
Cys	0.3	0.3	1.0	1.1	1.3	2.4
Val	4.8	5.4	4.2	7.1	6.4	6.5
Met	0.5	0.7	1.2	1.2	1.4	1.2
Ile	4.0	4.2	3.5	4.0	2.8	3.0
Leu	8.4	8.7	5.4	8.3	7.6	10.3
Tyr	2.4	1.8	2.1	2.5	1.7	1.1
Phe	5.1	5.3	3.7	3.9	3.9	4.5
TSAA [d]	0.8	1.0	2.2	2.3	2.7	3.6

[a] Subunits of β-conglycinin. [b] Subunits of glycinin. [c] Components from the 0.5 M NaCl soluble fraction. See Figures 1A and 2A for the position of these subunits in one-dimensional $NaDodSO_4$-polyacrylamide gel electrophoretic profiles. [d] Total sulfur amino acids.

soluble fraction were assumed to belong to the "whey protein" fraction described by Rackis et al. (1959). These six clustered groups of polypeptides appeared also in the two-dimensional maps of the HAc-soluble fraction. Likewise, the salt-soluble fraction maps contained five of those clusters and lacked cluster II. However, similarities between the H_2O- and NaCl-soluble fractions were not striking. Furthermore, both NaCl- and HAc-soluble fractions had spots which were unique to these fractions (Figures 2B and 3B). In the case of the NaCl-soluble fraction, these unique spots formed five clusters, which were designated as GI through GV (G stands for globulin) and boundaries marked with solid line rectangles (Figure 2B). Polypeptides within the cluster GI (~31 spots) and GII (~17 spots) had estimated molecular weights above 150000 and failed to enter the resolving gel (Figure 2). The cluster GIII (M_r 45000), GIV (M_r 26000), and GV (M_r 16000) included 7, 21, and 6 spots, respectively. Of these, the cluster GIV was the predominant one and accounted for 22.2% of the protein in the NaCl-soluble fraction based on quantification in a one-dimensional gel by densitometric scanning. Furthermore, there were hundreds of minor spots in the two-dimensional map of each solubility fraction. Whether or not these minor polypeptides were common to all three functions or unique to one of them could not be ascertained conclusively in all cases. Finally, it should be mentioned that each two-dimensional map included a train of spots on its left-hand margin (Figures 1A, 2A, and 3A) which corresponded to the anodic end of the first-dimensional IEF gel. It appears that these spots resulted from protein which precipitated on top of IEF gel and thus failed to enter it.

Amino Acid Composition of Preparative $NaDodSO_4$-Polyacrylamide Gel Electrophoretic Fractions. Three-millimeter thick preparative gels yielded 1.5–4 mg of protein/band for amino acid analysis. The amino acid composition data (Table I) shows that the subunits of β-conglycinin are extremely low in methionine (0.5–0.7%) and total sulfur amino acids (TSAA; methionine plus cysteine) while those of glycinin contain about twice as much methionine (1.2%) and TSAA as found in β-conglycinin components (Table I). These data are in close agreement with those reported for α and α′ subunits of β-conglycinin by Thanh and Shibasaki (1977) and for acidic (11S-2) and basic (11S-1) subunits of glycinin by Kitamura and Shibasaki (1975). The two major components (Glb 1 and Glb 2) from the NaCl-soluble fraction contained more methionine (1.2 and 1.4%, respectively) and TSAA (2.7 and 3.6%, respectively) than any of the β-conglycinin and glycinin components. Unfortunately, the component with highest TSAA content (3.6%) had the lowest lysine content (2.4%) among all preparative $NaDodSO_4$-polyacrylamide gel electrophoretic fractions (Table I).

DISCUSSION

The data presented in this paper show that soybean seed proteins are highly heterogeneous. The total number of spots detected by staining on two-dimensional maps of three solubility fractions was at least 1536. If polypeptides that occur in the remaining solubility fractions (alcohol, NaOH, and $NaDodSO_4$ soluble) could be analyzed by two-dimensional polyacrylamide gel electrophoresis, the minimum number would considerably exceed this number. However, it should be pointed out that the H_2O- and HAc-soluble fractions contained essentially the same major polypeptides, and nearly one-third of the NaCl-soluble polypeptides appeared to be homologous to those soluble in H_2O and HAc. In addition, the train of spots (Figures 1A, 2A, and 3A) on the left-hand margin of each two-dimensional map very likely resulted from protein precipitated on the top of the IEF gel. They mostly appeared to be the same as those that occurred in the main body of two-dimensional maps. However, when allowance is made for homologous polypeptides shared by the three solubility fractions, one is still left with at least 1000 different polypeptides. This is about 30 times more than the highest number, 32, reported by Catsimpoolas et al. (1968), previously based on one-dimensional analysis of the whole H_2O extracts.

The occurrence of what appeared to be the same components in two-dimensional maps of the three solubility fractions was not expected because extraction with each solvent was repeated 8 times using high solvent to meal ratios (e.g., 30:1). In addition, the completeness of extraction with each solvent was monitored by the assay of protein after each extraction. Then the next solvent in the sequence was introduced only after the preceding one was no longer able to extract any detectable amount of protein. Some of apparent homology among different fractions might be due to polypeptides with similar isoelectric points and sizes but differing in primary structures. However, this alone would not be a satisfactory explanation of the observed similarities. As mentioned before, H_2O was the first solvent in sequence, followed by NaCl, 2-propanol, HAc, and others. It is conceivable that H_2O extraction failed to solubilize either preexisting or newly formed oligomers and polymers of normally H_2O-soluble proteins. These multimeric complexes may be soluble partially in 0.5 M NaCl and completely in 60% HAc, accounting for similarities among the three solubility fractions. We examined the pellet by light microscopy after H_2O, NaCl, alcohol, and HAc extractions and by transmission electron microscopy after H_2O and HAc extractions. The results (not shown) indicated that the cell wall and cell integrity remained essentially intact both after grinding of the seed and repeated extractions of the meal successively with different solvents. Moreover, protein bodies, and smaller spherical inclusions that were thought to arise from protein bodies (Tombs, 1967), were present in all intact cells examined. The electron microscopic studies showed well-

defined electron-dense protein bodies in sections from the intact seed. After H_2O extraction, protein bodies became electron transparent with small amorphous electron-dense particles or regions scattered within their boundary. Often the protein bodies were irregular in shape, having vesicular projections which appeared to form smaller protein bodies by a budding-like process. After HAc extractions, cells contained protein body "ghosts" only, and no electron-dense particles or areas within their boundary were discernible. Moreover, protein bodies were more numerous and smaller in size after HAc extraction than after H_2O extraction. Apparently, the electron-dense areas or particles observed in protein bodies after H_2O extraction represent protein aggregates which are insoluble in H_2O but soluble in HAc. There is circumstantial evidence in support of this interpretation. The evidence is that the H_2O-soluble fraction is not completely soluble in H_2O and NaCl after it has been dialyzed and freeze-dried. However, both freeze-dried powder and its insoluble residues after H_2O and NaCl extractions are readily soluble in 60% HAc. Thus, the similarities between the two-dimensional profiles of H_2O- and HAc-soluble fractions are likely to be due to the existence or formation of H_2O-insoluble but HAc-soluble polymers or aggregates of normally H_2O-soluble proteins.

Although soybean proteins were shown to be highly heterogeneous, about 50–60 major polypeptides (spots) out of over 1000 contributed most of the total protein. Moreover, nearly all of these major polypeptides were included in the H_2O-soluble fraction. Assuming that H_2O- and HAc-soluble fractions are essentially identical especially with respect to the major polypeptides, the H_2O- and NaCl-soluble fractions represent over 90% of the total protein. Of 50–60 major polypeptides present in the H_2O-soluble fraction, about one-fourth appeared to represent the subunits of the two major soybean proteins (7S globulin and 11S globulin). The remaining major polypeptides represent either some as yet unidentified subunits of these two major proteins or the components of the whey protein fraction. Amino acid analysis data (Table I) show that the TSAA content of 7S globulin components is extremely low. Thus, decreasing the level of 7S globulin and other major proteins with low TSAA content by genetic means would considerably improve the quality of soybean protein. Alternatively, increasing the levels of the two major polypeptides in the NaCl-soluble fraction which are relatively rich in TSAA and of other major proteins with high TSAA content would accomplish the same goal. Efforts to improve protein quality by increasing the level of polypeptides with the most desirable amino acid profile or decreasing the level of those with the least desirable profile should be directed at the H_2O- and NaCl-soluble fractions, specifically their major polypeptides. In either case, it is essential that the major polypeptides that occur in the soybean seed be purified and characterized especially with respect to amino acid composition before any meaningful genetic manipulation could be undertaken.

Two-dimensional electrophoretic analysis can have a variety of applications in soybean protein biochemistry and genetics. First, two-dimensional polyacrylamide gel electrophoresis can provide information as to the degree of polypeptide heterogeneity in different solubility and chromatographic fractions as well as the number of major polypeptides and their relative amounts in such fractions. Second, it may be possible to recovery homogeneous protein from individual major spots or groups of spots of preparative two-dimensional gels in quantities sufficient for amino acid analysis. This would allow identification of polypeptides or groups of polypeptides of interest without resorting to lengthy purification protocols. Third, two-dimensional polyacrylamide gel electrophoresis may serve as an invaluable tool in genetic studies concerned with identification of altered gene products after mutagen treatment. In this regard one would also find two-dimensional polyacrylamide gel electrophoresis to be useful in defining a molecular basis at the gene product level for certain variations detected at the morphological level. Finally, two-dimensional polyacrylamide gel electrophoresis may be used for cultivar identification and fingerprinting of genotypes as well as for taxonomic studies.

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