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Two-Dimensional Electrophoretic Analysis of Soybean Proteins

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Water-extracted proteins from seeds of soybeans (*Glycine max*) were analyzed by two-dimensional gel electrophoresis. Two closely related electrophoretic systems were used: for optimal separation of acidic and neutral proteins, isoelectric focusing was the first dimension; for optimal resolution of basic proteins, nonequilibrium pH gradient electrophoresis was the first dimension. The second dimension was, in each case, polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate. Of approximately 430 spots resolved in the two systems, 35 were identified by comparison with the electrophoretic patterns of purified proteins: glycinin (13 acidic components, 11 basic components), β-conglycinin (6 components), soybean lectin (4 components), and Kunitz trypsin inhibitor. Two-dimensional electrophoresis was used to examine the protein components in a $MgSO_4$–curd and its whey and in the coagulated and noncoagulated fractions produced by boiling a water extract for 30 min. A protein of unknown identity was found to be enriched in both the whey and the non-heat-coagulated fraction. Two acidic subunits of glycinin were also apparently enriched in the same samples. These results illustrate the usefulness of two-dimensional electrophoresis in analyzing complex mixtures of soybean proteins.

Two-dimensional gel electrophoresis in which isoelectric focusing (IEF) in cylindrical gels is followed by polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate (O'Farrell, 1975) is a very powerful technique for analyzing complex mixtures of proteins since it combines two entirely independent electrophoretic approaches that are each individually capable of excellent resolution. As originally formulated, the technique is limited to examining acidic and neutral polypeptides since the pH gradient in polyacrylamide focusing gels does not extend much above pH 7 even though basic Ampholines are used (O'Farrell, 1975). O'Farrell et al. (1977) overcame that limitation, however, through the introduction of nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension.

Soybean seed proteins have been analyzed by various electrophoretic techniques [e.g., Catsimpoolas et al. (1971), Kitamura et al. (1976), Thanh and Shibasaki (1976a), and Mori et al. (1981)] including two-dimensional gel electrophoresis, the first report of which (Hu and Esen, 1982) was published as this paper was in preparation. Our results confirm and extend the conclusion of Hu and Esen that two-dimensional electrophoresis appears to be a promising approach to studying genetic and nutritional aspects of soybean proteins. We have used a variety of electropho-

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resis conditions in this work. The most important variation is use of both IEF and NEPHGE in the first dimension.

The main purpose of the work we report here is clear-cut identification of proteins in the electrophoretograms. We have identified 35 spots by reference to the electrophoretic patterns of several purified proteins: glycinin, β-conglycinin, lectin, and Kunitz trypsin inhibitor. As examples of the usefulness of two-dimensional electrophoresis, we have examined several complex subsets of water-extracted soybean proteins, a $MgSO_4$-coagulated curd and its whey, and proteins that are coagulated during heat treatment of the sort used to inactivate soybean trypsin inhibitors. These analyses have shown that there are several proteins (or protein subunits) enriched in the soluble fractions from both treatments.

MATERIALS AND METHODS

Preparation and Treatments of Soybean Extract. Soybean seeds (Williams variety) grown in Riley County, Kansas, in 1978 were used for all water extracts, which were prepared from soaked beans as described by Lei et al. (1981). The protein concentration in such extracts has been estimated at 3.78% (Lei et al., 1981). To examine proteins coagulated by heating, an extract was boiled for 30 min. An extended treatment at 100 °C is frequently used to inactivate soybean trypsin inhibitors (Lei et al., 1981). The boiled extract was cooled on ice and lyophilized. After the solid material was suspended in water, the soluble and insoluble portions were separated by centrifugation as described in the American Oil Chemists' Society (1978) method for determining the nitrogen solubility index. To form a curd, an extract was brought to boiling and allowed to cool slightly. Then $MgSO_4$ was added as described by Wang (1967), to bring the extract to 2 mM $MgSO_4$. The resulting coagulated mass (the curd) was separated from the soluble material (whey) by filtering through muslin cloth.

Purification of Individual Proteins. Glycinin and β-conglycinin were partially purified according to the pH precipitation method of Thanh and Shibasaki (1976b). Further purification of each protein was then achieved as follows. The final β-conglycinin fraction from the Thanh and Shibasaki procedure was reprecipitated at pH 4.8. That precipitate and, separately, the final glycinin precipitate were dissolved in 0.035 M sodium phosphate–0.4 M NaCl–10 mM 2-mercaptoethanol and adjusted to pH 7.6 with NaOH. Each sample was then chromatographed on concanavalin A–agarose (Bethesda Research Laboratories) as described by Kitamura et al. (1974). β-Conglycinin bound to that column whereas glycinin did not, thus allowing a facile removal of cross contaminations in the preparations of glycinin and β-conglycinin obtained by the procedure of Thanh and Shibasaki. Finally, the glycinin was subjected to chromatography on Sepharose 6B as described by Kitamura et al. (1974).

Purified soybean lectin and Kunitz trypsin inhibitor (catalog no. T9003) were obtained from Bethesda Research Laboratories and Sigma, respectively.

Two-Dimensional Gel Electrophoresis. To prepare samples for the first-dimension electrophoresis, the various components of the 9.5 M urea containing "lysis buffer" of O'Farrell (1975) were added directly to the water extract, the fraction not coagulated by heat treatment, and the heat-coagulated material resuspended in the same volume as the extract from which it was derived. The whey and purified samples of glycinin and β-conglycinin were concentrated for electrophoresis by precipitation at 25% trichloroacetic acid (on ice), collected by centrifugation, and washed with acetone. The washed precipitates were dissolved directly in lysis buffer. The curd was dissolved directly in lysis buffer; it dissolved fully.

We used either isoelectric focusing (IEF) or nonequilibrium pH gradient electrophoresis (NEPHGE) as the first dimension with only slight modifications of the procedures given by O'Farrell (1975) and O'Farrell et al. (1977), some of which have been described in a previous paper from our laboratory (Tyrell et al., 1982). Cylindrical gels for the first dimension were 2.5 × 180 mm. Ampholines (LKB) with pH ranges of 4–6 and 5–7 were used in a 1:1 mixture for IEF and 5–7 Ampholines were used alone for NEPHGE. The total Ampholine concentration was 1.5% in either case. The urea concentration was 9 M. In both IEF and NEPHGE, the upper (anode) reservoir solution was 10 mM phosphoric acid and the lower (cathode) reservoir solution was 0.2 M NaOH. All samples were applied at the tops of the gels. Note that this polarity for IEF is reversed from that used by O'Farrell (1975). We found that the reversed polarity greatly reduces streaking in the final electrophoretogram (for reasons that we do not understand). Electrophoresis was started at a voltage of 300 on the constant current mode of the Bio-Rad Model 2000A power supply. In IEF, after 45 min, when the voltage had risen to about 1000, the power supply was changed to constant voltage mode and electrophoresis was continued at 1000 V for 20 h. In NEPHGE, the initial voltage of 300 (on constant voltage mode) was increased to 1000 in several steps over a 1-h period. The voltage was then maintained at 1000 until a total of 3000 V h had elapsed.

The first-dimension gels were soaked in "equilibration buffer" for 1 h. The second-dimension slab gels were 210 × 150 × 0.8 mm. The slab gels were stained for 4 h in 0.2% Coomassie Blue R250–50% methanol–12% acetic acid and destained in 20% ethanol–10% acetic acid.

To measure the pH gradient, an IEF gel was cut into 1-cm sections. Each section was placed in a small test tube that contained 0.5 mL of deaerated 0.02 M KCl. The tubes were sealed with parafilm and shaken occasionally at room temperature for 4 h; then the pH was measured with a pH meter.

RESULTS

Electrophoresis of the Water-Extracted Proteins and of β-Conglycinin, Soybean Lectin, and Kunitz Trypsin Inhibitor. The sample for the electrophoretograms shown in Figures 1 and 2 was total, water-extracted soybean proteins. The first-dimension electrophoresis was IEF in Figure 1 and NEPHGE in Figure 2. The electrophoretograms demonstrate a level of complexity—particularly in the relatively scarce proteins of the extract—that one-dimensional analyses had not revealed. Large amounts of protein were used in the analysis of Figures 1 and 2 to allow visualization of the less abundant components of the extract. As a result, resolution of the major proteins (particularly the acidic subunits of glycinin) is suboptimal. A number of individual spots in Figures 1 and 2 are identified on the basis of electrophoresis (to be discussed below) of purified soybean proteins. Note that all but the least basic of the basic subunits of glycinin are absent in Figure 1, in which the first dimension was IEF. This results from the general phenomenon of cathodic drift—the slow electrophoresis of basic ampholytes and basic proteins into the cathode reservoir (Righetti and Drysdale, 1976). By using NEPHGE in the first dimension, we have obtained excellent resolution of the basic proteins in the extract, but in such analyses (Figure 2) poor resolution of the acidic and neutral components is obtained.

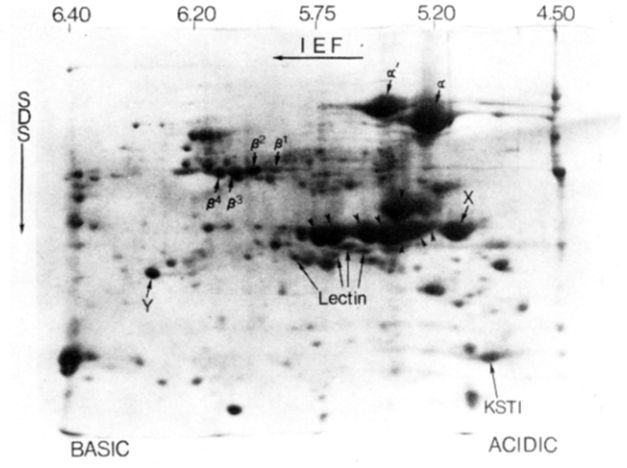

Figure 1. Two-dimensional electrophoresis of water-extracted soybean proteins, with IEF in the first dimension. Approximately 600 μg of protein was applied to the first dimension. The separating gel in the second dimension was polymerized from 10% acrylamide–0.27% bis(acrylamide). The subunits of β-conglycinin are labeled "α", "α′", "β^1", "β^2", "β^3", and "β^4". Soybean trypsin inhibitor (Kunitz) is labeled "KSTI". The four components of the soybean lectin are labeled "Lectin". Arrows lacking tails point to acidic subunits of glycinin. "X" indicates glycinin subunits that are preferentially enriched in soybean whey proteins (see Figure 6). The glycinin subunits were identified on the basis of results shown in Figure 3. "Y" identifies a protein that is prominent in soybean whey (see Figure 8).

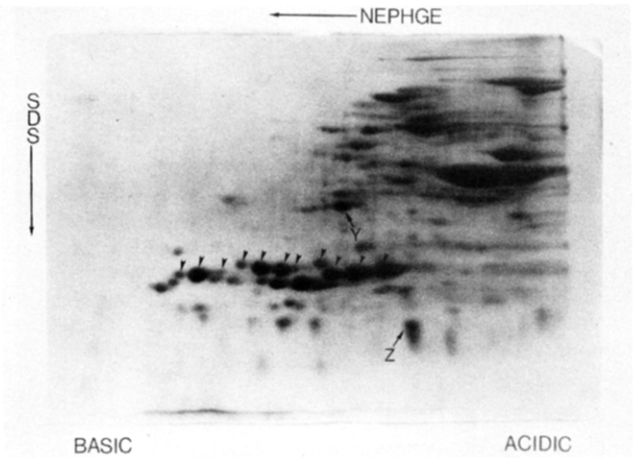

Figure 2. Two-dimensional electrophoresis of water-extracted soybean proteins, with NEPHGE in the first dimension. Second-dimension gel: 12% acrylamide–0.32% bis(acrylamide). Load: 600 μg. In NEPHGE, proteins do not generally reach positions corresponding to their p*I*'s (O'Farrell et al., 1977), so we indicate only the acidic and basic extremes for the first-dimension separation. Arrows lacking tails indicate the basic subunits of glycinin with molecular weight of about 20 000. The identification of the basic subunits of glycinin is based upon results shown in Figure 6. "Z" is possibly a low molecular weight subunit that has previously been described by Moreira et al. (1979). "Y", shown as a point of reference, is a protein that is strongly enriched in soybean whey (see Figure 8).

β-Conglycinin, purified as described under Materials and Methods, was subjected to two-dimensional gel electrophoresis with IEF in the first dimension (Figure 3). This made possible the identification in Figure 1 of the α, α′, and four β subunits. [The nomenclature is that of Thanh and Shibasaki (1976a, 1977)]. Our preparations apparently contained nothing that corresponded to the minor γ component of Thanh and Shibasaki. We observed four β subunits expected on the basis of the work of Thanh and

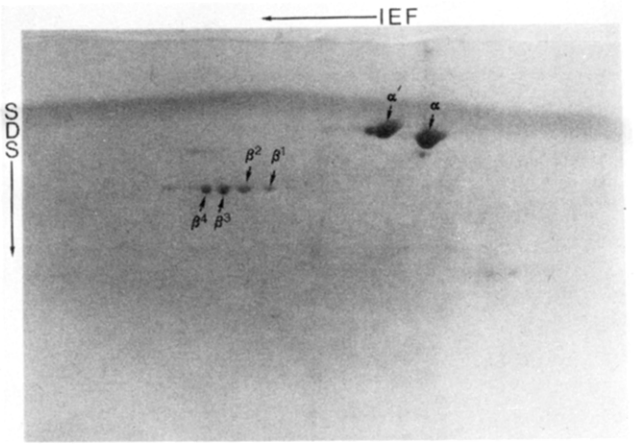

Figure 3. Two-dimensional electrophoresis of purified β-conglycinin, with IEF in the first dimension. Second-dimension gel: 10% acrylamide–0.27% bis(acrylamide). Load: 20 μg.

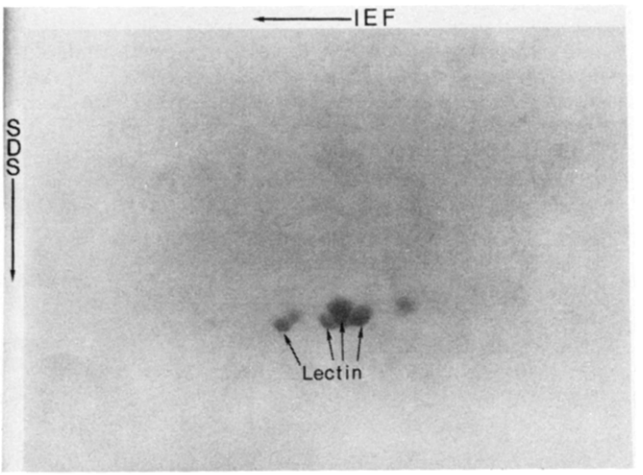

Figure 4. Two-dimensional electrophoresis of purified soybean lectin, with IEF in the first dimension. Second-dimension gel: 10% acrylamide–0.27% bis(acrylamide). Load: 20 μg.

Shibasaki. Purified Kunitz trypsin inhibitor [presumably corresponding to Ti[a] in the nomenclature of Orf and Hymowitz (1979)] gave a single spot on two-dimensional electrophoresis. When this was mixed with the extract, a spot in Figure 1, labeled KSTI (Kunitz), was identified as the inhibitor (not shown). Soybean lectin that had been purified by affinity chromatography and that was stated by the supplier to exhibit a single band on polyacrylamide gel electrophoresis gave rise to several closely grouped spots on two-dimensional gel electrophoresis (Figure 4). Electrophoresis of the extract supplemented with the purified lectin (not shown) allowed identification in Figure 1 of the four major components of the lectin. The heterogeneity in the affinity-purified lectin presumably is a reflection of the heterogeneity in the soybean lectin that was reported by Lis et al. (1966).

Two-Dimensional Electrophoresis of Purified Glycinin. From earlier electrophoretic and chromatographic analyses, it was clear that there is considerable heterogeneity in the subunits of glycinin. Those subunits fall into two major classes: acidic polypeptides with molecular weights of approximately 40 000 and basic polypeptides with molecular weights of about 20 000. Using two-dimensional gels (Figures 5 and 6), we examined the subunit heterogeneity of glycinin that had been purified as described under Materials and Methods. We used both IEF and NEPHGE in the first dimension in order to obtain optimal separations among the acid and basic subunits, respectively. In the insets of Figure 5 we present schematic

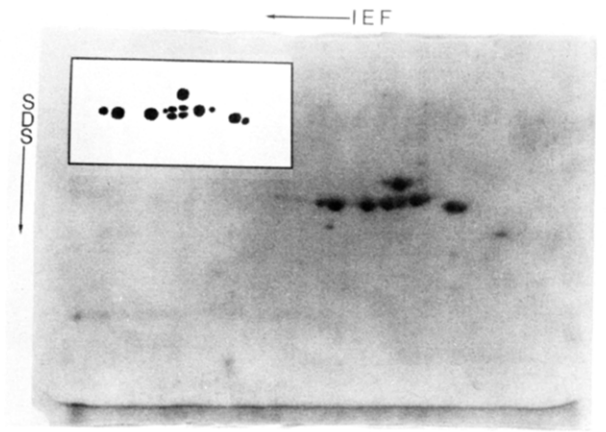

Figure 5. Two-dimensional electrophoresis of purified glycinin, with IEF in the first dimension. Second-dimension gel: 10% acrylamide–0.27% bis(acrylamide). Load: 50 μg. The inset is a somewhat idealized diagram of the glycinin acidic subunits that is drawn to the scale of this electrophoretogram but that is based on results obtained under a variety of electrophoresis conditions. There are several faint spots in the electrophoretogram that could conceivably be very minor glycinin subunits but whose identification as such remains uncertain.

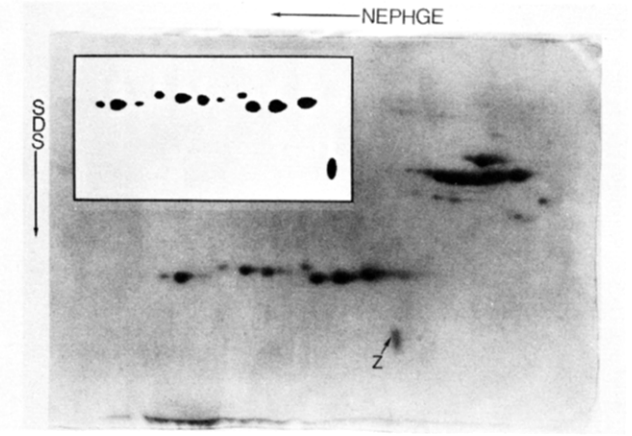

Figure 6. Two-dimensional electrophoresis of purified glycinin, with NEPHGE in the first dimension Second-dimension gel: 12% acrylamide–0.32% bis(acrylamide). Load: 50 μg. A schematic illustration of the basic subunits of glycinin is shown in the inset.

diagrams of the electrophoretic patterns that summarize the results obtained at a variety of loading levels and at different acrylamide concentrations. The heterogeneity in both the acidic and basic subunits that is revealed by the two-dimensional gels is greater than that previously detected by one-dimensional electrophoretic approaches. There are at least 13 acidic subunits and 11 basic subunits separable by two-dimensional electrophoresis. Note that only some of the basic proteins in the extract having molecular weights in the range of 20 000 appear to be constituents of glycinin (compare Figures 2 and 6).

Electrophoretic Analysis of Soybean Curd and Whey Proteins. We used two-dimensional electrophoresis to examine the polypeptide components of a $MgSO_4$-coagulated curd and the corresponding soluble (whey) proteins. Figures 7 and 8 show electrophoretograms of the curd and whey proteins, respectively. The vast majority of glycinin and β-conglycinin, the major soybean globulins, was coagulated into the curd, as were the Kunitz trypsin inhibitor and the subunits of the soybean lectin.

The spots labeled "X" in Figure 7 are of particular interest. (The region labeled X resolves into two spots when electrophoresis is carried out at a lower loading level.) By mixing whey with purified glycinin, we found that the X

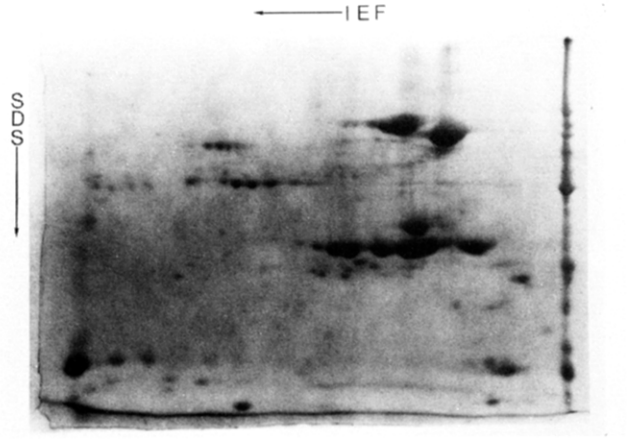

Figure 7. Two-dimensional electrophoresis of $MgSO_4$–soybean curd proteins, with IEF in the first dimension. Load: 500 μg. Second-dimension gel: 10% acrylamide–0.27% bis(acrylamide).

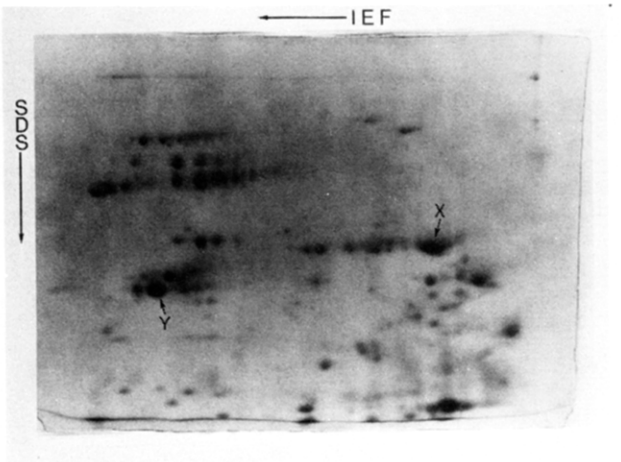

Figure 8. Two-dimensional electrophoresis of soybean whey proteins, with IEF in the first dimension. The whey was that which resulted in forming the curd analyzed in Figure 6. Second-dimension gel: 10% acrylamide–0.27% bis(acrylamide). Load: 120 μg. "X" indicates acidic subunits of glycinin that are preferentially enriched in the whey. "Y" is a priminent whey protein that is a minor component of the total water-extracted proteins (Figure 1).

spots in the whey comigrate with the two most acidic spots diagrammatically shown in the inset of Figure 5. It therefore seems that two of the acidic subunits of glycinin coagulated to a lesser extent than did the other glycinin acidic subunits. On the other hand, the whey was depleted to comparable extents in all of the basic subunits of glycinin (NEPHGE–$NaDodSO_4$ analysis, not shown).

The spot labeled "Y" in Figure 8 is particularly distinctive in that it is markedly enriched in the whey. It is a minor component of the extract (see Figure 1) but one of the most abundant polypeptides in whey.

Analysis of Heat-Coagulated Proteins and Those Not Coagulated by Heating. We have also examined the proteins coagulated by boiling the extract for 30 min. This is a heat treatment typical of those used to inactivate soybean trypsin inhibitors. About 50% of the protein in the extract is recovered as a precipitate after boiling, lyophilizing, suspending in water, and centrifuging. The insoluble protein dissolved fully in lysis buffer for two-dimensional electrophoresis. Figure 9 shows that the insoluble protein is largely glycinin and β-conglycinin, which are present in the precipitate in approximately the same relative proportions in which they occur in the extract (compare to Figure 1).

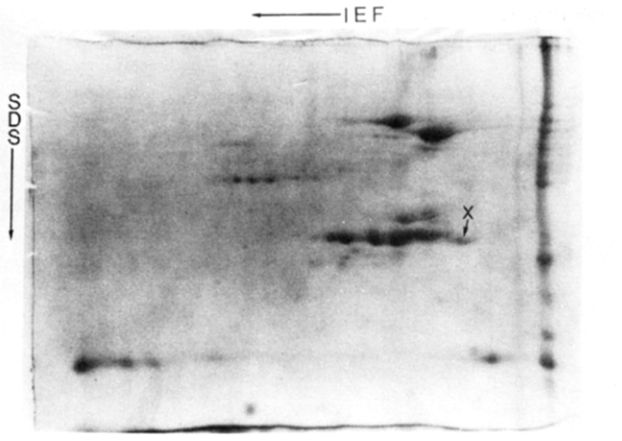

Figure 9. Two-dimensional electrophoresis of heat-coagulated soybean proteins, with IEF in the first dimension. Second-dimension gel: 10% acrylamide–0.27% bis(acrylamide). Load: 100 μg. "X" indicates acidic subunits of glycinin that are depleted in this sample compared to their level in the water extract (Figure 1).

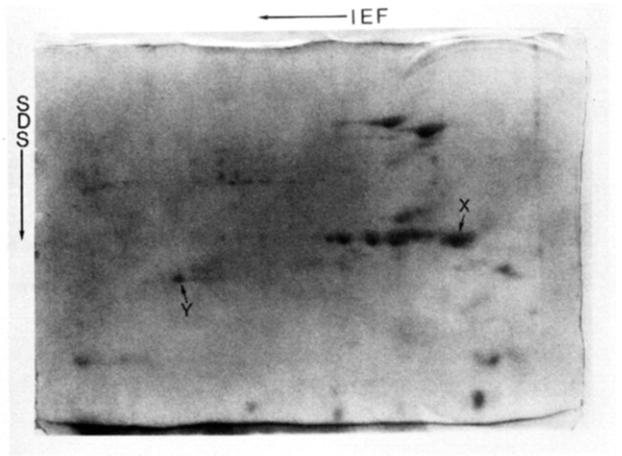

Figure 10. Two-dimensional electrophoresis of soybean proteins not coagulated by heating, with IEF in the first dimension. Second-dimension gel: 10% acrylamide–0.27% bis(acrylamide). Load: 100 μg. "X" indicates acidic subunits of glycinin that are enriched in this sample compared to their level in the water extract (Figure 1). "Y" is the protein indicated in Figure 8 that is markedly enriched in soybean whey.

There are two notable similarities between the soluble proteins of Figure 10 and the whey proteins shown in Figure 8. Protein Y is enriched in the proteins not coagulated by heating (Figure 10), as it was in the whey. Protein Y thus appears exceptionally able to remain soluble after extended heating as well as in the presence of a solute ($MgSO_4$) that coagulates many of the soy proteins. In addition, spots labeled X are enriched in the fraction not coagulated by heating (Figure 10). This enrichment is not nearly as marked as the enrichment of the same spots in the whey (Figure 8), but that is to be expected since approximately half of each of the glycinin acidic subunits is in the soluble fraction.

DISCUSSION

The water-extracted soybean proteins are a very complex mixture, with several hundred detectable components. They vary widely in p*I*'s, in molecular weights, and in abundance in the extract. Therefore, no single set of conditions for two-dimensional electrophoresis can provide a comprehensive, high-resolution analysis of all of the polypeptides in the extract. Both IEF and NEPHGE must be used as the first-dimension electrophoresis to allow analysis of acidic, neutral, and basic proteins. A wide range in the amount of protein loaded onto the gels is necessary: the high loading level (600 μg of protein) necessary to visualize the less abundant proteins gives poor separation of the most abundant proteins. A loading level of about 100 μg is best for resolution of the subunit of the major proteins. Use of more than one acrylamide concentration (8–15%) is helpful in the second dimension because of the wide range of molecular weights that the soybean polypeptides possess.

Despite its uniquely high-resolving power, the O'Farrell approach to two-dimensional gel electrophoresis (O'Farrell, 1975; O'Farrell et al., 1977) has not been widely applied in the study of seed proteins. To our knowledge, our two-dimensional analysis of soybean proteins has been preceded only by that of Hu and Esen (1982). There is agreement in general terms between our conclusions and those of Hu and Esen: two-dimensional gel electrophoresis should prove to be very useful in analyzing the genetic background of soybeans and in studying the nutritional contributions of individual soybean polypeptides. Our identification of numerous protein components should aid such endeavors. Presumably, the technique will prove suitable for similar applications in the study of proteins from other seeds including cereal grains.

There is, however, considerable difference in the details of our results and those of Hu and Esen (1982). To a large extent those differences result from our having electrophoresed several purified soybean proteins and to our having used both IEF and NEPHGE in the first dimension of our gels. (From the description of their methods and the appearance of their electrophoretograms, it is clear that Hu and Esen employed NEPHGE in the first dimension, even though their first dimension was referred to as IEF.) Our use of true IEF resulted in much better separation of neutral and acidic proteins compared to that given by NEPHGE. This is expected to be the case in general (O'Farrell et al., 1977). In addition, the peculiar shape of many spots in the earlier study (Hu and Esen, 1982) and a lack of correspondence in some features between those patterns and ours suggest some spots in the earlier work may be artifactual.

Apart from the identifications of proteins, our most interesting findings pertain to subunit heterogeneity in glycinin. It has been clear for some time that both the acidic and basic subunits are heterogeneous. On the basis of work from several laboratories, Moreira et al. (1981) have suggested the existence of six acidic and six basic subunits. By two-dimensional electrophoretic analyses, we have found 11 basic subunits and 13 acidic subunits. On the basis of the work of Moreira et al. (1979, 1981) and of Iyengar and Ravestein (1981), the heterogeneity in the acidic subunits can be attributed in part to the existence of a family of glycinin genes. We have, however, identified more spots than there have been unique amino acid sequences found. Our results are therefore consistent with the suggestion of Moreira et al. (1981) of heterogeneity within subfamilies of acidic subunits. One possible source of such heterogeneity that does not seem to have been raised before is variability in the sites of proteolytic cleavage within the polypeptide translation product of a given gene. Slight variations in the apparent molecular weights of the acidic and basic subunits (see our Figures 5 and 6) would be consistent with such a possibility.

There is a polypeptide with an apparent molecular weight considerably less than 20 000 that is in our glycinin preparations (labeled "Z" in Figures 2 and 6). Although

we do not know its identity with certainty, its electrophoretic mobilities appear consistent with its being the 10 000-dalton acidic subunit that has been reported by Moreira et al. (1979). Spot Z diffused rather readily from our gels and was erratic in amount.

Moreira et al. (1979) reported that with the exception of acidic subunit A4, the acidic subunits of glycinin are joined to basic subunits by disulfide linkages. A4 had the lowest p*I* among the acid subunits (Moreira et al., 1981). We have found two spots that appear to be acidic subunits of glycinin that are preferentially enriched in whey and, to a lesser extent, in the fraction not coagulated by heating. Those spots are the most acidic of the glycinin subunits. Thus, we can tentatively identify our spots "X" with the A4 subunit of Moreira et al. (1979). If that identification is correct, it suggests that the absence of a disulfide linkage between the A4 subunits and the basic subunits could contribute to the enrichment of the A4 subunits in the whey proteins.

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Amidation or Esterification of Bovine β-Lactoglobulin To Form Positively Charged Proteins

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Bovine β-lactoglobulin was amidated or esterified to yield modified proteins with 55–83% of the free carboxyl groups blocked and with mean isoionic points of 7–10 compared to 5.2 for the native protein. Upon gel electrophoresis at acidic pH values or in sodium dodecyl sulfate, the amidated protein was polydisperse and contained small quantities of dimer, whereas esterified proteins were less polydisperse and not dimerized. A more random structure as a result of amidation or esterification was evident from examination of the modified proteins using circular dichroism.

Whey, the liquid remaining after the process of cheese making, contains most of the salts, lactose, and water-soluble proteins of the milk. The world production of cheese whey has been estimated to be approximately 72 000 million kg (Kosikoski, 1979), much of which is disposed of without being utilized. Undenatured whey proteins can be separated from the liquid by ultrafiltration, ion-exchange chromatography, or gel filtration chromatography and are marketed as whey protein concentrates. Besides having excellent nutritional quality, the undenatured whey proteins are soluble over a wide pH range, have good emulsification capacity and whipping ability, and form gels when subjected to heat treatments under proper conditions (Marshall, 1979).

With the advent of ultrafiltration, more whey processors are marketing whey protein concentrates. The result is an excess of the products on the market with an attendant decline of price and, therefore, profitability. This factor along with competition from plant protein concentrates necessitates additional research for the development of new markets for whey proteins.

Alteration or extension of the functional properties of whey proteins may result by changing the net charge of the proteins from negative to positive at pH 7. It should be possible to prepare positively charged proteins by

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