difference in the intrinsic zinc and extrinsic ^{65}Zn exchanges of soy flour previously mentioned (Table III).

The in vitro ^{65}Zn values from the Tris–His treatment may be useful for predictive ranking of values for zinc bioavailability from other protein foods. Further investigation is needed to validate the procedure fully for a wider range of foods.

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Registry No. Zn, 7440-66-6; L-His·HCl, 645-35-2; Na_2EDTA, 139-33-3; tris(hydroxymethyl)aminomethane, 77-86-1.

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Effect of Phytate Removal Treatments upon the Molecular Weight and Subunit Composition of Major Soy Protein Fractions

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Soy protein extracts were prepared from commercially defatted flakes and treated by alkaline pH or ion exchange phytate removal processes. Control and phytate-reduced soy protein extracts were characterized for phosphorus content and for changes in molecular weight profile and subunit composition of the major protein fractions by Sepharose 6B gel filtration chromatography and SDS gel electrophoresis. The alkaline pH phytate removal treatment only removed 62% of the total phosphorus content of soy extract but caused major amounts of aggregation of its glycinin and β-conglycinin-containing Sepharose protein fractions. In contrast, the ion exchange phytate removal treatment removed 86% of the total phosphorus content of soy extract and resulted in only minor alteration of the molecular weight profile and subunit composition of the Sepharose protein fractions. This experimental approach could be used to evaluate the effects of different processing treatments upon the physicochemical properties of the major soy protein components.

INTRODUCTION

The high quality and protein content of soy protein products make them attractive as ingredients in formulated food products (Erdman, 1979). As soy proteins become more extensively utilized in our food supply, modification and improvement of their nutritional and functional properties will become more important (Hartman, 1979).

Phytate, the hexaphosphate salt of myoinositol, is the chief storage form of phosphorus in the soybean (Okubo et al., 1975; Cosgrove, 1966). Phytate is coextracted and recovered with the proteins during soy protein isolate manufacture, resulting in phytate contents in the range of 1.5–2.5% (Brooks and Morr, 1982; Hartman, 1979).

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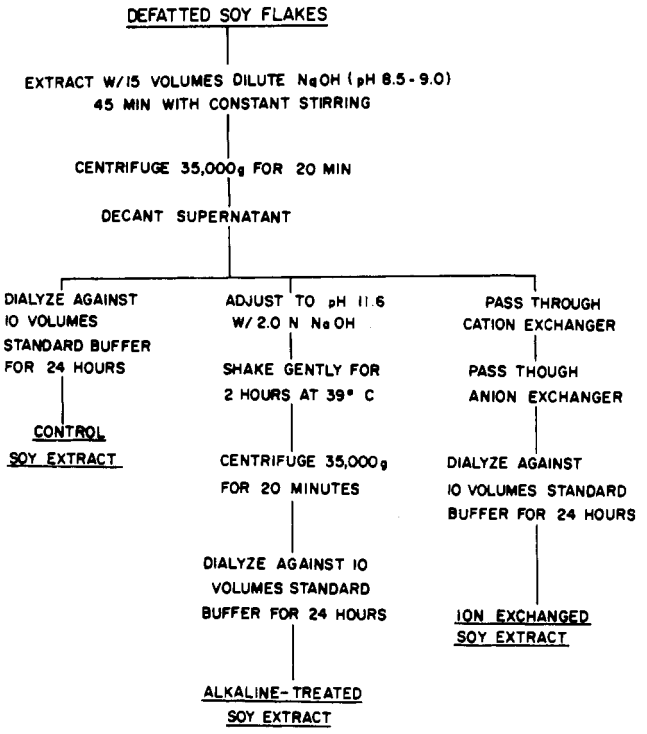

Figure 1. Procedure for producing soy protein extracts.

High concentrations of phytate in soy protein products result in formation of soy protein–phytate complexes that inhibit protein digestibility (Hartman, 1979; Ritter and Morr, 1985) and interfere with essential mineral bioavailability (Cheryan, 1980; Erdman, 1979; Hartman, 1979; Okubo et al., 1975; Maddaiah et al., 1964). In addition, phytate may adversely affect the solubility and related functionality of soy proteins in commercial food applications (Okubo et al., 1975; Chen and Morr, 1985). It may also interfere with the fractionation and characterization of the major soy protein components, glycinin and β-conglycinin, and this prevent accurate determination of their molecular weight, subunit composition, and other chemical and physicochemical properties. This study was conducted to determine the changes in the soy protein molecular weight profile and subunit composition of major Sepharose gel filtration protein fractions of soy extract resulting from treatment by the alkaline pH (Hartman, 1979) and ion exchange (Brooks and Morr, 1982) processes to remove phytate.

MATERIALS AND METHODS

General Methods. Ion exchange resins were Rexyn 101 cation exchanger and Rexyn 202 anion exchanger from Fisher Scientific, Atlanta. Dialysis tubing was Spectrapore, 32-mm flat diameter, with a 12000 molecular weight cutoff (Fisher Scientific, Atlanta). Demineralized, distilled water was prepared by passing glass distilled water through a Barnstead Model BD-2 demineralizer. Standard pH 7.6 phosphate buffer was prepared to contain 35 mM potassium phosphate and 0.4 M sodium chloride according to Wolf and Briggs (1959) and also 0.05% sodium azide as preservative. Immediately before using the buffer, 0.001 M dithioerythritol (DTE) was added as disulfide reducing agent and 0.0002 M (phenylmethyl)sulfonyl fluoride (PMSF) was added as protease inhibitor.

Preparation of Soy Protein Extract. Commerical, low temperature processed, defatted soy flakes with high protein solubility, obtained from Ralston Purina Company, St. Louis, MO, were extracted with 15 volumes of dilute sodium hydroxide solution at pH 8.5–9 for 45 min with mild agitation as in Figure 1. The extract was clarified by centrifuging at 35000*g*.

Phytate Removal. Phytate was removed from the above soy extract by treating with the alkaline pH process of Hartman (1979). The extract was adjusted to pH 11.6 with 2 N sodium hydroxide and gently mixed by shaking in a 39–40 °C water bath for 2 h to precipitate phytate. The resulting extract was then centrifuged at 35000*g* and the supernatant fraction was dialyzed against standard phosphate buffer in the cold at 4 °C (Figure 1). Phytate was removed from a second portion of soy extract by passing it through 2.5 cm i.d. × 20 cm cation and anion exchange columns as in Figure 1 (Brooks and Morr, 1982). Flow rates through the columns were controlled to provide approximately 30-min residence time for the extract (J. T. Baker Chemical Company, 1958). The control and phytate-reduced extracts were then dialyzed 24 h against separate 10-volume quantities of standard phosphate buffer at 4 °C with a buffer change after 12 h (Figure 1).

Regeneration of Ion Exchange Columns. The cation exchange column was regenerated by first passing 5 bed volumes (BV) of 1.5 N hydrochloric acid with a 30-min residence time as before. The column was then flushed with 3 BV of distilled water. The resin was removed from the column, adjusted to pH 8.0–8.5 with 1.5 N sodium hydroxide to place it in the Na^+ form, repacked into the column, and rinsed with 1 BV of distilled water. The anion exchange column was regenerated by passing 5 BV of 1.5 N sodium hydroxide as above to remove bound protein, pigments, and phenolic compounds (Brooks and Morr, 1982). After the column was flushed with 3 BV of distilled water, the resin was removed from the column, adjusted to pH 4.0 with 1.5 N hydrochloric acid to place it in the Cl^- form, adjusted to pH 8–8.5 with 1.5 N sodium hydroxide, repacked into the column, and flushed with 1 BV of distilled water.

Analytical Methods. The protein content of soy extracts and their Sepharose gel filtration chromatographic fractions were determined by the Coomassie Blue dye-binding method (Bio-Rad Laboratories, 1976) by using bovine plasma γ-globulin as reference protein. Total phosphorus was determined by the method of Allen (1940), as modified by Brooks and Morr (1982), since these latter samples did not contain sufficient phytate to permit its determination. Inorganic phosphorus was also determined by the method of Allen (1940) with the following modifications. The sample was added to a centrifuge tube containing perchloric acid and 35 mL of distilled water, mixed, and centrifuged at 35000*g* for 20 min to sediment the finely dispersed protein precipitate, and the phosphorus content of the supernatant fraction was determined without digestion.

Gel Electrophoresis. SDS gel electrophoresis (SDS-PAGE) was performed in 12% gels with a Model 220 vertical unit (Bio-Rad Laboratories, Richmond, CA) and the method of Laemmli (1970). Soy extracts and Sepharose chromatographic fractions were diluted with SDS sample buffer to a protein concentration of about 1 mg/mL and 30 μL of the resulting solution was added to each well. Low molecular weight protein standards (Bio-Rad Laboratories, Richmond, CA) were simultaneously electrophoresed as reference markers. SDS concentrations were increased in all solutions to 0.2% (w/v) and 0.01 M DTE was used in place of mercaptoethanol as reducing agent (Brooks and Morr, 1984). The 0.75-mm thick gels were electrophoresed at a constant current of 35 mamp. Gels were fixed overnight in solution containing 11.5% trichloroacetic acid and 3.5% sulfosalicylic acid (w/v) and

Table I. Phosphorus Removal from Soy Protein Extract

	protein content, mg/mL	total P content, mg/100 mg protein	total P removal, %
control extract	54.8	0.424	
alkaline-treated extract	56.7	0.159	62.5
ion exchanged extract	25.8	0.053	86.3

stained in a 0.12% Coomassie Blue R-250 solution that also contained 25% ethanol and 8% acetic acid (w/v). Gels were destained in a 25% ethanol and 8% acetic acid (w/v) destaining solution.

Sepharose Gel Filtration Chromatography. Aliquots of soy extract containing 300–350 mg of protein were applied to the 2.6 cm i.d. × 89 cm Sepharose 6B column (Pharmacia Fine Chemicals, Piscataway, NJ) that were previously equilibrated with standard phosphate buffer. Absorbance was monitored at 280 ηm by using a Model UA-5 absorbance/fluorescence monitor (Instrumentation Specialties Company, Lincoln, NB) and the effluent was collected in 6-mL fractions with an automatic fraction collector (Instrumentation Specialties Company, Lincoln, NB). The elution volume was determined at the midpoint of each elution peak and expressed as the ratio of elution volume to void volume, determined by chromatographing Blue Dextran (Pharmacia Fine Chemicals, Piscataway, NJ). Effluent fractions were combined for each peak and concentrated by ultrafiltration with a 50-mL unit equipped with a YM-10 membrane (Amicon Corp., Danvers, MA) prior to examination by SDS-PAGE.

RESULTS AND DISCUSSION

Phytate Removal from Soy Extract. Since Ca^{+2}, Mg^{+2}, and other mineral ions, along with the polyanionic phytate ions, are effectively bound by the cationic and anionic exchangers during treatment of soy extracts, this approach has proven simple, efficient, and rapid for removing phytate (Brooks and Morr, 1982). An insignificant amount of protein is lost during the process treatment due to adsorption onto the ion exchanger resins (Brooks and Morr, 1982). Although phytate-reduced soy protein isolates are more soluble than control soy protein isolates over a broad pH range (Brooks and Morr, 1982) and especially at pH values below their isoelectric point (Chen and Morr, 1985), information is lacking on the general physicochemical changes, if any, that may be produced in soy proteins as a result of the phytate removal process treatment. It is also of interest to compare the effects of alkaline pH phytate removal process of Hartman (1979) upon the physicochemical properties of soy proteins with those produced by the ion exchange process which maintains them at pH values of ≤8.5–9.0.

Total phosphorus values for control and ion exchanged soy extracts, expressed per 100 mg of protein in Table I, are slightly lower than those previously reported for similar extracts (Brooks and Morr, 1982), which is probably due to the higher centrifugal force of 35 000g used to initially clarify the extracts in this study. Since there was an insufficient quantity of Sepharose gel filtration fractions to determine their phytate content and since phytate accounts for a nearly constant 70% of the total phosphorus content of the soybean (Okubo et al., 1975), changes in total phosphorus content were used to indicate the relative effectiveness of the two phytate removal processes.

Although both processes effectively removed phytate from soy extract, the ion exchange process removed 86% of the total phosphorus compared to a value of only 62% by the alkaline pH phytate removal process. Although the percentage of phosphorus, e.g., phytate, removal provided by ion exchange processing is less than previously reported (Brooks and Morr, 1982), this difference is largely due to the above indicated lower phosphorus content of the control extract which was a result of the higher centrifugal force mentioned above. The residual total phosphorus contents of ion exchange processed soy extracts were comparable for this study and those of the previous study (Brooks and Morr, 1982).

Table II. Elution Properties of Sepharose 6B Fractions of Soy Protein Extracts

	elution volume ratio[a]		
peak	control extract	alkaline-treated extract	ion exchanged extract
1	1.0	1.1	1.0
1a			1.4
2	1.7	1.4	1.6
3	1.8	1.9	1.8
3a			1.9
4	2.1	2.1	2.1
5	2.2	2.2	2.2
6	2.6	2.5	
7	2.8	2.8	

[a] Ratio of elution volume (mL) to void volume (mL).

Sepharose Gel Filtration Fractionation of Soy Extracts. Sepharose 6B gel filtration chromatography fractionated soy extract proteins into 7 broad and incompletely resolved peaks (Figure 2), indicating a highly size-heterogeneous molecular weight distribution. Protein peaks from the three soy extracts reveal similar elution volume to void volume ratios (Table II), indicating generally similar molecular weight distributions. One exception was the slight shift of elution volume to void volume ratio of peak 2 from the alkaline pH treated soy extract compared with those of control and ion exchanged extracts.

Additionally, both phytate removal processes resulted in minor shifts in soy protein molecular weight distribution. For example, a larger portion of alkaline pH treated extract proteins eluted as a broad peak 1 and correspondingly smaller peaks 2 and 3 (Figure 2, part B) and these peaks were less completely resolved than those from the other extracts. This shift toward higher apparent molecular weight protein components is indicative of protein denaturation and/or aggregation. Ion exchanged extract proteins eluted with shoulder regions on the trailing edges of peaks 1 and 3 (labeled 1a and 3a in Figure 2 part C). Although the amount of peak 1 was similar to that of the control extract, the major reduction in the size of peak 2 and the appearance of peak 3a indicate a shift toward smaller sized protein molecules or aggregates in ion exchanged soy extract compared to the control. Also, the ion exchanged extract elution pattern revealed that peaks 6 and 7 were missing. Although these peaks represent large amounts of pigment, but small amounts of protein, their loss does not represent a significant reduction in protein recovery by the ion exchange process.

Figure 3 contains the SDS-PAGE patterns for the subunits of the three soy extracts as well as their corresponding Sepharose gel filtration chromatographic fractions. Combining information from these two fractionation techniques allows for some general conclusions on the effects of the two phytate removal treatments upon soy extract proteins. Sepharose 6B gel filtration chromatographic fraction 1, e.g., peak 1, from control and ion exchanged extracts consists of a low concentration of highly turbid protein components that apparently formed aggregates during their separation from the other proteins on the column. SDS-PAGE patterns reveal that fraction

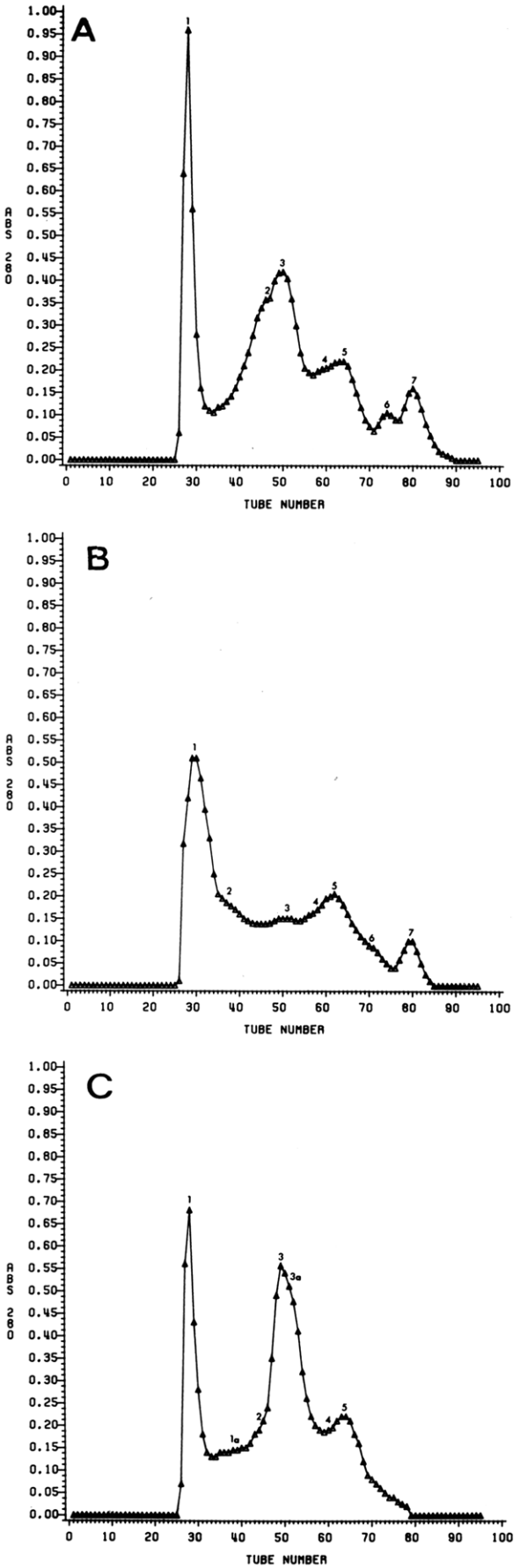

Figure 2. Sepharose 6B elution patterns for (A) control soy extract, (B) alkaline pH treated soy extract, and (C) ion exchanged extract.

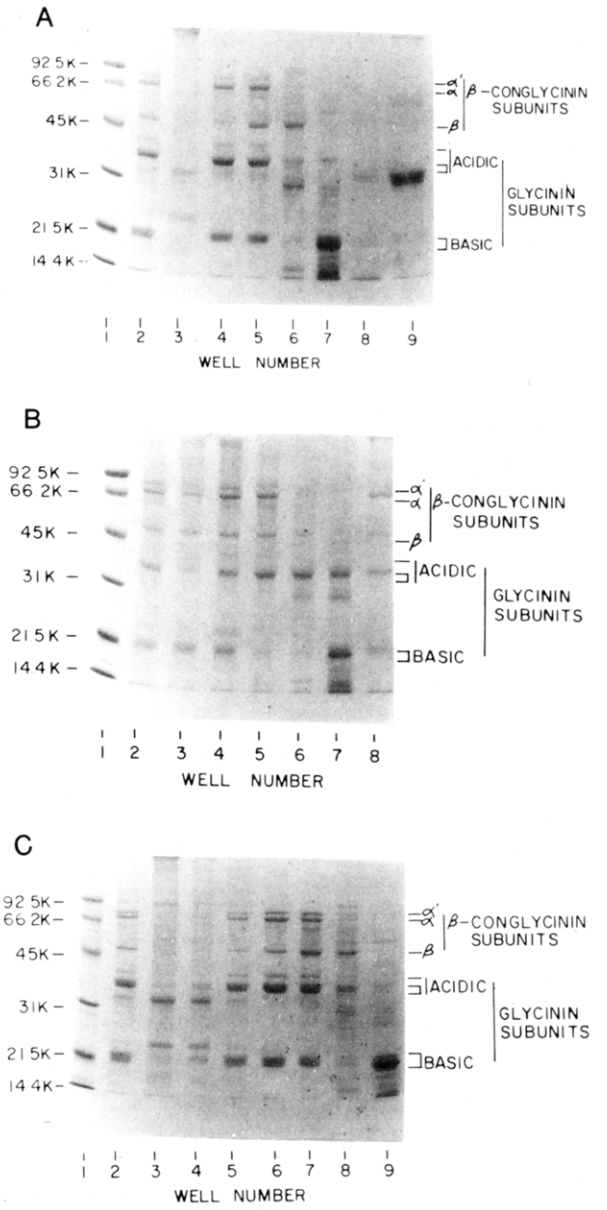

Figure 3. A. SDS-PAGE patterns for control soy extract proteins and their Sepharose 6B protein fractions: (well 1) Bio-Rad low molecular weight standards (phosphorylase B 92.5K, bovine serum albumin 66.2K, ovalbumin 45K, carbonic anhydrase 31K, soy trypsin inhibitor 21.5K, and lysozyme 14.4K); (well 2) control soy extract; (well 3) Sepharose protein fraction 1; (well 4) Sepharose protein fraction 2; (well 5) Sepharose protein fraction 3; (well 6) Sepharose protein fraction 4; (well 7) Sepharose protein fraction 5; (well 8) Sepharose protein fraction 6; (well 9) Sepharose protein fraction 7. B. SDS-PAGE patterns for alkaline pH treated soy extract proteins and their Sepharose 6B protein fractions: (well 1) Bio-Rad low molecular weight standards as in part A; (well 2) alkaline pH treated soy extract; (well 3) Sepharose protein fraction 1; (well 4) Sepharose protein fraction 2; (well 5) Sepharose protein fraction 3; (well 6) Sepharose protein fraction 4; (well 7) Sepharose protein fraction 5; (well 8) Sepharose protein fraction 7. C. SDS-PAGE patterns for ion exchange treated soy extract proteins and their Sephrose 6B protein fractions: (well 1) Bio-Rad low molecular weight standards as in part A; (well 2) ion exchange treated soy extract; (well 3) Sephrose protein fraction 1; (well 4) Sepharose protein fraction 1a; (well 5) Sepharose protein fraction 2; (well 6) Sepharose protein fraction 3; (well 7) Sepharose protein fraction 3a; (well 8) Sepharose protein fraction 4; (well 9) Sepharose protein fraction 5.

1 from control and ion exchanged soy extracts have similar subunit compositions that contain no glycinin or β-conglycinin. The shoulder region of ion exchanged extract

peak 1 (1a in Figure 2 part C) contains similar subunit compositions as the parent peak and thus represents a smaller size, less aggregated form of Sepharose fraction 1 proteins.

Sepharose 6B fractions 2 and 3 (peaks 2 and 3) from control extract and fractions 2 through 3a from ion exchanged extract contained a typically high concentration of glycinin and β-conglycinin subunits as shown in Figure 2 part A and C. Thus, the appearance of the shoulder region on peak 3 (peak 3a in Figure 2 part C), coupled with the smaller size peak 2 for ion exchanged extract proteins, indicates a shift toward smaller sized protein molecular complexes as a result of the ion exchange treatment. It was also observed that peak 3 from all three extracts contained a higher concentration of β-subunits from β-conglycinin than in peak 2. This observation is consistent with work of Sykes and Gayler (1981), who reported that a β-conglycinin fraction consisting of 3 β-subunits was isolated from the trailing edge region of the glycinin-containing peak separated by Sepharose 6B gel filtration chromatography.

Although Sepharose 6B gel filtration chromatography effectively separates glycinin (11S) and β-conglycinin (7S) from the other soy proteins, it does not separate these two proteins from each other. It is therefore difficult to determine the separate effects of the ion exchange and alkaline pH phytate removal treatments upon each of these proteins. However, based upon the observation that 11S soy protein is essentially phytate free (Brooks and Morr, 1984), it is likely that the phytate removal process treatments would be most effective for altering the β-conglycinin (7S) protein components.

Physicochemical changes in the alkaline pH treated soy extract proteins were more severe than those for ion exchange treated soy extract. Sepharose peak 1 (Figure 2 part B) was not only slightly displaced with respect to control extract peak 1 in terms of elution volume to void volume ratio (Table II), but it was also broader and contained a greater amount of protein. As indicated above, these latter changes, coupled with the reduction in size of peaks 2 and 3, indicate that the alkaline pH treatment (Hartman, 1979) causes a shift toward larger sized protein complexes. Further, SDS-PAGE data (Figure 3) show that alkaline pH treated soy protein components of peak 1 contain significant amounts of glycinin and β-conglycinin subunits, which were absent from peak 1 of the other two extracts. Hartman (1979) reported no apparent denaturation of soy proteins upon holding soy extract at pH 11 for up to 7 h on the basis of disulfide group and lysinoalanine criteria. He concluded that temperatures in the range of ≥50 °C were more damaging to soy proteins than pH, per se. Our results disagree and demonstrate that the highly alkaline pH treatment results in substantial amounts of protein aggregation, which may or may not involve denaturation of the glycinin and β-conglycinin-containing protein components. These differences are probably due to the increased sensitivity of the electrophoretic and gel filtration methods used in this study.

In conclusion, it has been confirmed that the ion exchange process effectively removes phytate from soy protein extracts with a minimum of protein aggregation. Extracts prepared by this procedure should retain their functionality in most food product applications better than those processed by the alkaline pH treatment.

Registry No. Phytic acid, 83-86-3.

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Formation of 2-(1-Pyrrolidinyl)-2-cyclopentenones and Cyclopent(*b*)azepin-8(1*H*)-ones as Proline Specific Maillard Products

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By means of MS, IR, and ^{1}H NMR spectroscopy eight 2-(1-pyrrolidinyl)-2-cyclopentenones (**1–8**) and eleven cyclopent(*b*)azepin-8(1*H*)-ones (**9–20**) were characterized in proline/monosaccharide and proline/cyclic enolone model experiments. The pyrrolidines possess bitter adstringent taste and the azepine derivates bitter taste qualities. Both classes of compounds are formed by a Strecker-type reaction of proline and cyclic enolones.

It has been demonstrated that the Maillard reaction of L-proline with monosaccharides produce a complex mixture of proline specific compounds with bread, cracker-like, roasty aromas and bitter taste qualities (Hunter et al., 1969; Shigematsu et al., 1975; Mills and Hodge, 1976; Tressl et al., 1981; Pabst et al., 1984). During our investigation of the compounds produced by the reaction of L-proline with D-glucose we isolated a nitrogen containing

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