LITERATURE CITED
Alexander, A. R.; Whanger, P. D.; Miller, L. T. *J. Nutr.* **1983**, *113*, 196.
Baker, S. S.; Lerman, R. H.; Krey, S. H.; Crocker, K. S.; Hirsh, E. F.; Cohen, H. *Am. J. Clin. Nutr.* **1983**, *38*, 769.
Bjorn-Rasmussen, E.; Hollberg, L.; Walker, R. B. *Am. J. Clin. Nutr.* **1973**, *26*, 1311.
Cantor, A. H.; Scott, M. L.; Noguchi, T. *J. Nutr.* **1975a**, *105*, 96.
Cantor, A. H.; Langevin, M. L.; Noguchi, T.; Scott, M. L. *J. Nutr.* **1975b**, *105*, 106.
Douglass, J. S.; Morris, V. C.; Soares, J. H.; Levander, O. A. *J. Nutr.* **1981**, *111*, 2180.
Food and Nutrition Board *Recommended Dietary Allowances*, 9th revised ed.; National Academy of Science, National Research Council: Washington, DC, 1980.
Gabrielsen, B. O.; Opstvedt, J. *J. Nutr.* **1980**, *110*, 1096.
Hadjimarkos, D. M.; Bonhorst, C. W. *J. Pediatrics* **1961**, *59*, 256.
Hoffman, I.; Westerby, R. J.; Hidirogloue, M. *J. Assoc. Off. Anal. Chem.* **1968**, *5*, 1039.
Horowitz, W. *Official Methods of Analysis*, 14th ed.; Association of Official Analytical Chemists: Washington, DC, 1984; p 153.
Kien, C. L.; Ganther, H. E. *Am. J. Clin. Nutr.* **1983**, *37*, 319.
Layrisse, M. C.; Martinez-Torres, C.; Roche, M. *Am. J. Clin. Nutr.* **1968**, *21*, 1175.
Levine, S. E.; Weaver, C. M.; Kirleis, A. W. *J. Food Sci.* **1982**, *47*, 1283.
Mason, A. C.; Weaver, C. M. *J. Nutr.* **1986**, *116*, 1883.
Meyer, N. R.; Stuart, M. A.; Weaver, C. M. *J. Nutr.* **1983**, *113*, 1255.
Morris, V. C.; Turnlund, J. R.; King, J. C.; Levander, O. A. *Fed. Proc.* **1985**, *44*, 1670.
Quercia, R. A.; Korn, S.; O'Neill, D.; Dougherty, J. E.; Ludwig, M.; Schweizer, R.; Sigman, R. *Clin. Pharmacol.* **1984**, *3*, 531.
Stuart, S. M.; Ketelsen, S. M.; Weaver, C. M.; Erdman, J. W. *J. Nutr.* **1986**, *116*, 1323.

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Phytate Hydrolysis in Soybean and Cottonseed Meals by *Aspergillus ficuum* Phytase

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Microbial phytase was produced by solid-state cultivation of *Aspergillus ficuum*, the enzyme (crude culture filtrate) was applied to soybean and cottonseed meals, and factors affecting the hydrolysis of phytate were studied. Soybean and cottonseed meals contain about 60% and 50% of total phytate as water-soluble forms, respectively. The water-insoluble portion of phytate was further hydrolyzed and removed by application of microbial phytase. Treatment at higher temperature (50 °C), pH 4–5.5, and heating the substrate (1 h at 121 °C) prior to enzyme treatment facilitated the hydrolysis of phytate by the microbial phytase. The heat treatment also reduced the level of total extractable phytate from these substrates. Hydrolysis and removal of phytate in soybean meal was more effective than that for cottonseed meal. About 85% of phytate in soybean meal was hydrolyzed by the microbial phytase whereas only 67% of the phytate in cottonseed meal was destroyed by the same enzyme treatment.

Phytic acid is the principal storage form of phosphate in plants, particularly in cereal grains and legumes. The interaction of phytic acid with protein, vitamins, and several minerals is considered to be one of the primary factors limiting the nutritive values of cereal grains and legume seeds. For instance, soybean and cotton seed meals are the major protein supplement in poultry feeds and also are a source of phosphorus. However, two-thirds of soybean meal phosphorus is bound as phytate and unavailable for poultry (Whitaker and Brunnert, 1977). Hydrolyzing plant phytate prior to animal consumption would increase the availability of inositol and inorganic phosphorus in animal diet. Thus, attempts have been made to hydrolyze dietary phytate by microbial phytase to improve the feed quality (Chang et al., 1977; Liener, 1977; Nelson et al., 1968; Whitaker and Brunnert, 1977). Although beneficial effects of the enzyme treatment were evident, the high cost of enzyme production and lack of a practical method for enzyme application were cited as limiting factors in using the enzyme in animal diets.

The enzyme phytase, which hydrolyzes phytic acid, is widely distributed among plant and animal tissues. It is also produced by a variety of microorganisms, and its characteristics have been studied (Cosgrove et al., 1970; Greaves et al., 1967; Han et al., 1987; Shieh et al., 1969; Yamada et al., 1968). Although the enzyme activity has been previously studied with pure phytic acid or its derivatives as a substrate, the factors affecting hydrolysis of phytate in native seeds are not well elucidated. In the native state, phytate exists in close association with other plant components that make it difficult to be hydrolyzed by microbial enzyme. Thus, the present investigation was conducted to establish the factors affecting hydrolysis and removal of phytate in soybean and cottonseed meals by *Aspergillus ficuum* phytase.

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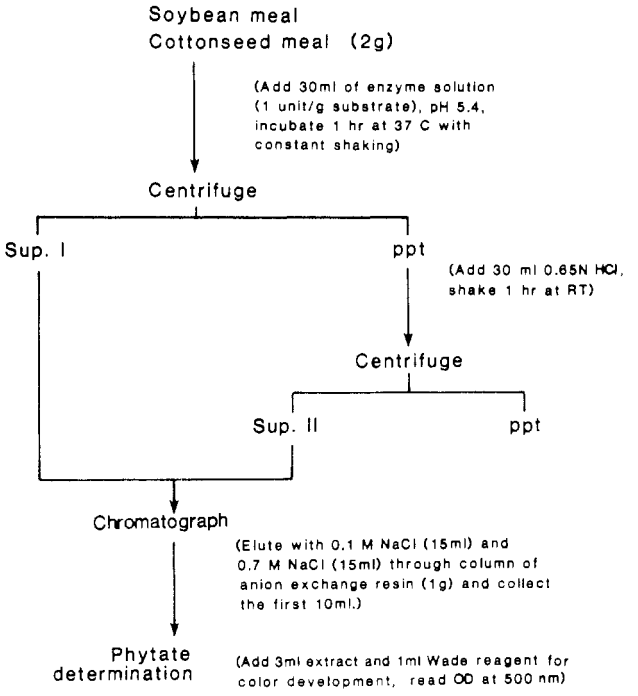
MATERIALS AND METHODS

Materials. Wheat bran, soybean meal, and cottonseed meal were obtained from a local feed store and stored at 18 °C and 50% relative humidity. The moisture content of the meals was about 11%, and the total phosphorus content was 0.24 and 0.34% for soybean meal and cottonseed meal, respectively. Commercial phytase (6-phytase; phytate 6-phosphatase; myoinositol hexakisphosphate 6-phosphohydrolase; EC 3.1.3.26) prepared from wheat by Sigma Chemical Co. (St. Louis, MO) was used as a standard for phytase activity.

Phytase Production and Enzyme Assay. *A. ficuum* (NRRL 3135) was grown and maintained on potato dextrose agar at 30 °C. The conidia formed on the agar

Scheme I. Schematic Diagram of Hydrolysis, Extraction, and Determination of Phytate in Soybean and Cottonseed Meals



surface was scraped and collected in distilled water. A portion of spore suspension were washed and resuspended in distilled water and used as an inoculum. For phytase production, steam-sterilized wheat bran containing about 40% moisture was inoculated with the spore suspension. The initial pH of the substrate was 6.3. In a typical fermentation run, 50 g of wheat bran in a 400-mL glass container was inoculated with 0.5 mL of spore suspension and fermented at room temperature (about 25 °C) for 1–2 weeks. The fermentation was carried out in either a stationary culture or an agitative mode in which the fermentation mixture was agitated by rotating the jars on a plate rotating vertically at 1 rpm. When the enzyme level reached its peak (about 40 units/g of substrate), water was added to the fermentation mixture and the enzyme extracted. The crude culture filtrate was used as an enzyme source. Details of the phytase production on solid substrate and the characteristics of the enzymes are reported elsewhere (Han et al., 1987). Phytase activity was assayed by following the release of orthophosphate from phytate. The liberated inorganic phosphate was determined by the method of Heinonen and Lahti (1981). The enzyme reaction mixture contained 0.1 mL of suitably diluted culture filtrate, 3.0 mL of 0.1 M acetate buffer (pH 5.4), and 0.5 mL of 15 mM sodium phytate. The reaction mixture was incubated for 30 min at 37 °C. The color developed was measured by reading the optical density at 420 nm. One unit of enzyme is defined as the amount of enzyme required to liberate 1 μmol of inorganic phosphate/min under the assay condition.

Extraction and Determination of Phytic Acid. Phytic acid in soybean and cottonseed meals was extracted and determined according to the modified method of Latta and Eskin (1980). A schematic presentation of hydrolysis, extraction, and determination of phytic acid is shown in Scheme I. A 2-g portion of substrate was mixed with 30 mL of enzyme solution containing 2 units of phytase, and the mixture was adjusted to pH 5.4. The mixture was then incubated at 37 °C for 1 h with constant shaking. At the end of reaction time, the mixture was centrifuged and the supernatant collected (Sup. 1). To the precipitate was added 30 mL of 0.65 N HCl and the residual phytate extracted by constant shaking for 1 h at room temperature (about 25 °C). The mixture was centrifuged and the supernatant again collected (Sup. 2). The two supernatants were then eluted through an anion-exchange resin (Dowex 1-X8, 200–400 mesh, chloride form; Biorad Laboratory, Richmond, CA) first with 0.1 M NaCl (to remove inorganic phosphorus and other interfering compounds) and then with 0.7 M NaCl (to collect phytic acid). The phytate content was measured by using the modified Wade reagent (0.03% $FeCl_3 \cdot 6H_2O$ and 0.3% sulfosalicyclic acid in distilled water). The degree of color change in the Wade reagent due to the sample was measured by reading the optical density at 500 nm and correlated to the concentration of phytic acid. Sodium phytate (Sigma, St. Louis, MO) was used as a standard for estimating phytic acid. Acetate buffer was used through out the experiment as the citrate and phosphate buffer interfered with the color development in the Wade reagent. The total phytate in soybean and cottonseed meals was determined by repeated extraction of phytate from the substrates with acetate buffer (0.1 M, pH 5.4) and 0.65 N HCl, according to Scheme I. Total phosphorus was determined by the X-ray fluorescence method of Knudsen et al. (1981).

Table I. Extraction of Phytic Acid from Soybean and Cottonseed Meals[a]

	soybean meal		cottonseed meal	
solvent	% solid	% total phytate[b]	% solid	% total phytate[b]
H_2O	1.35 ± 0.13	60.5	2.21 ± 0.33	49.8
0.65 N HCl	1.94 ± 0.19	86.9	3.78 ± 0.15	85.3
3% TCA	1.45 ± 0.10	65.0	3.69 ± 0.19	83.3

[a] Seed meals were extracted with different solvents for 1 h at 28 °C with constant shaking. Values are means and standard deviations. [b] The value of the total phytate removed by repeated extractions with water and 0.65 N HCl, as shown in Table II, was used as 100%.

RESULTS AND DISCUSSION

Analyses of phytic acid in foods and feedstuffs are generally based on the phytate extractable by dilute HCl or TCA. In this work, the effectiveness of these two extractants in removing phytic acid from soybean meal and cottonseed meal was investigated. About 60% of phytate in soybean meal and 50% of phytate in cottonseed meal existed as water-soluble forms and were easily extracted in water by shaking 1 h at room temperature (Table I). Of the two extractants, 0.65 N HCl extracted more phytate (87% from soybean meal and 85% from cottonseed meal) than 3% TCA (65% from soybean meal and 83% from cottonseed meal). The extent of phytate removal from soybean meal by 3% TCA was almost the same as by water, whereas about 60% more phytate was removed from cottonseed meal by TCA than by water. For cottonseed meal, the level of phytate removal by 3% TCA and 0.65 N HCl was about the same. The results obtained in this study were somewhat different from that of Chang et al. (1977), who reported that 3% TCA extracted more phytate than 0.5 N HCl from California small white beans. This could probably be due to a different analytical method or due to the inherent difference in the substrate used.

Temperature of extraction (25–50 °C), extraction time (1–24 h), and comminuting the particle size (by Waring blender) had little effect on the total amount of phytate extracted from these substrates. A small amount (7–11%) of residual phytate was further removed by repeated extraction with 0.65 N HCl, after the first two extractions with water and the acid (Table II). However, the amount

Table II. Enzymatic Hydrolysis and Extraction of Phytate in Soybean and Cottonseed Meals

treatment[a]	phytate extracted (% of solid)[b] Sup. I	Sup. 2	Sup. 3	total	phytate hydrolyzed: % original
soybean meal control	1.49	0.56	0.18	2.23	0
phytase treated	0.66	0.56	0	1.23	44.8
cottonseed meal control	1.85	2.07	0.51	4.43	0
phytase treated	1.42	1.76	0	3.18	28.2

[a] Two grams each of soybean meal or cottonseed meal was mixed with 30 mL of water (for control) or enzyme solution (1 unit of phytase/g of substrate) and incubated 1 h at 37 °C with constant shaking. Both solutions were adjusted to pH 5.4 with acetate buffer. [b] Sup. 1 and Sup. 2 were prepared according to Scheme I. Sup. 3 was obtained by repeating the step for Sup. 2.

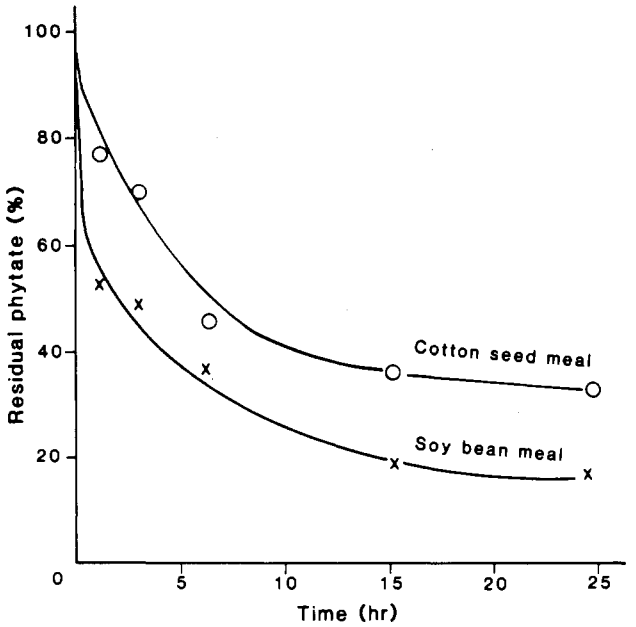


Figure 1. Time course of phytate hydrolysis in soybean and cottonseed meals by *A. ficuum* phytase.

of phytate extractable from the enzyme-treated sample after the first two extractions was almost negligible. About 45% of phytate in soybean meal and about 28% of phytate in cottonseed meal were hydrolyzed by treating with *A. ficuum* phytase.

The time course of phytate hydrolysis in soybean and cottonseed meals by *A. ficuum* phytase was studied by incubating the substrate with the enzyme for 1–24 h at 37 °C (Figure 1). *A. ficuum* phytase hydrolyzed about half of soybean phytate and about one-third of cottonseed phytate during the 1-h treatment. On prolonged incubation, the seed phytate was further hydrolyzed, and at the end of 24-h incubation, about 85% of phytate in soybean meal and about 70% of phytate in cottonseed meal were destroyed. The pattern of phytate hydrolysis in soybean and cottonseed meals was similar, but soybean phytate was more readily and extensively hydrolyzed than the cottonseed phytate. This is probably due to the fact that soybean phytate is evenly distributed in the aleurone grains whereas cottonseed phytate is segregated in small globoid deposits (Yatsu and Jacks, 1968).

Hydrolysis of phytate in soybean and cottonseed meals by the microbial enzyme increased as the temperature increased from 28 to 50 °C (Table III). The temperature

Table III. Effect of Temperature on Hydrolysis of Phytate in Soybean and Cottonseed Meals by *A. ficuum* Phytase

temp, °C	soybean meal res phytate[a]	% hydrolyzed	cottonseed meal res phytate[a]	% hydrolyzed
untreated control[b]	2.05 ± 0.28	0	4.27 ± 0.22	0
28	1.35 ± 0.09	34.1	2.91 ± 0.12	31.8
37	1.16 ± 0.04	43.4	2.92 ± 0.22	31.6
50	0.92 ± 0.16	55.1	2.59 ± 0.10	39.3

[a] Residual phytate (% of total solid) in enzyme-treated sample. Substrates were treated with microbial phytase (1 unit/g of substrate) for 1 h at different temperature. Values are means and standard deviations. [b] The values of total extractable phytate at 28 °C without enzyme treatment were used as controls.

Table IV. Effect of Heating on Phytate Hydrolysis in Soybean and Cottonseed Meals by *A. ficuum* Phytase

treatment[a]	soybean meal res phytate[b]	% hydrolyzed[c]	cottonseed meal res phytate[b]	% hydrolyzed[c]
unheated, control	1.99		4.41	
unheated phytase	1.33	33.2	2.84	35.6
heated, control	1.86		2.42	
heated phytase	1.02	44.6	1.29	46.7

[a] One part of substrate was mixed with one part of water and heated 1 h at 121 °C. [b] Residual phytate (% of total solid) after the enzyme treatment. [c] Percent hydrolyzed was calculated from the difference between the amount of phytate extracted from the control (no enzyme treated) and the enzyme-treated sample. Substrate was hydrolyzed for 1 h under the conditions described in the Materials and Methods.

effect was more apparent in soybean meal than in cottonseed meal. The amount of phytate hydrolyzed in soybean meal by *A. ficuum* phytase during the 1-h period at 28 °C was 34.1% while that at 50 °C was 55.1%, an increase of 21%, while only 7.5% increase was observed in cottonseed meal by the same rise in temperature. Plant seeds contain endogeneous phytase activated at around 60 °C and catalyzing autolysis of their own seed phytate (Chang et al., 1977). Therefore, the increased hydrolysis at 50 °C could be due to the combined result of autolysis and hydrolysis by the microbial phytase. Thus, proper selection of treatment temperature is important in practical removal of phytate from seed meals by enzyme treatments. The optimum pH for phytate hydrolysis in soybean and cottonseed meals was between 4.5 and 5.7, which is similar to that found for hydrolysis of pure phytic acid by *A. ficuum* phytase (Han and Gallagher, 1986). The rate of hydrolysis was significantly reduced at pH values outside of this range.

In plant seeds, phytate exists in aleurone grains (protein bodies) in association with cellular protein. Also found in this body are acid proteinase and acid phosphatase activities (Yatsu and Jacks, 1968). Inactivation of these enzymes, denaturation of protein, and destruction of phytate by heat may have some effect on enzymatic hydrolysis of phytate. To see the effect of heat treatment on enzymatic hydrolysis of phytate, soybean meal and cottonseed meal were heated (1 h at 121 °C) prior to enzyme treatment, and the extent of phytate hydrolysis was determined (Table IV). In both substrates, the extent of phytate hydrolysis increased by about 11% due to the heat treatment. However, the heat treatment also reduced the amount of total extractable phytate. Almost half of the cottonseed phytate was destroyed while only 6.5% of soybean phytate was destroyed by the heat treatment. Boland et al. (1975) reported that heat destruction of

phytic acid in cereal grains was species specific. Dry heat (autoclaving without water in the substrate) destroyed little phytate but increased the rate of enzymatic hydrolysis in the heated sample.

Seed phytate can be hydrolyzed by an application of microbial enzyme. Improving the treatment conditions, such as elevating temperature (50 °C), adjusting to pH 4–5.5, and heating the substrate prior to enzyme treatment, facilitates the hydrolysis of phytate. Because large portions of seed phytate exist as water-soluble forms that are easily extracted by water and because they contain endogenous phytase, a practical way of phytate removal from seeds may be accomplished by utilizing a combination of these factors—water solubility of seed phytate, inherent phytase, and added microbial phytase.

Registry No. Phytate, 83-86-3; phytase, 9001-89-2.

LITERATURE CITED

Boland, A. R.; Garner, G. B.; O'Dell, B. L. "Identification and Properties of Phytate in Cereal Grains and Oilseed Products". *J. Agric. Food Chem.* **1975**, *23*, 1186–1189.

Chang, R.; Schwimmer, S.; Burr, H. K. "Phytate Removal from Whole Dry Beans by Enzymatic Hydrolysis and Diffusion". *J. Food Sci.* **1977**, *42*, 1098–1101.

Cosgrove, D. J.; Irving, G. C. J.; Bromfield, S. M. "Inositol Phosphate Phosphatases of Microbiological Origin. The Isolation of Soil Bacteria Having Inositol Phosphate Phosphatase Activity". *J. Biol. Sci.* **1970**, *23*, 339–343.

Greaves, M. P.; Webley, D. M. "The Hydrolysis of Myoinositol Hexaphosphate by Soil Microorganisms". *Soil. Biol. Biochem.* **1969**, *1*, 37–43.

Greaves, M. P.; Anderson, G.; Webley, D. M. "Hydrolysis of Inositol Phosphates by *Aerobacter aerogenes*". *Biochim. Biophys. Acta* **1967**, *132*, 412–418.

Han, Y. W.; Gallagher, D. J. "Phytase Production by *Aspergillus ficuum*". *J. Ind. Microbiol.* **1986**, *1*, 295–301.

Han, Y. W.; Gallagher, D. J.; Wilfred, A. G. "Phytase Production by *Aspergillus ficuum* on Solid Substrate". *J. Ind. Microbiol.* **1987**, *2*, 195–200.

Heinonen, J. K.; Lahti, R. J. "A New and Convenient Colorimetric Determination of Inorganic Orthophosphate and its Application to the Assay of Inorganic Pyrophosphate". *Anal. Biochem.* **1981**, *113*, 313–317.

Knudsen, D.; Clark, R. B.; Denning, J. L.; Pier, P. A. "Plant Analysis of Trace Elements by X-Ray". *J. Plant Nutr.* **1981**, *3*, 61–75.

Latta, M.; Eskin, M. J. "A Simple and Rapid Colorimetric Method for Phytate Determination". *J. Agric. Food Chem.* **1980**, *28*, 1313–1315.

Liener, I. E. "Removal of Naturally Occurring Toxicants through Enzymatic Processing". *Adv. Chem. Ser.* **1977**, *No. 160*, 283–300.

Nelson, T. S.; Shieh, T. R.; Wodzinski, R. J.; Ware, J. H. "The Availability of Phytate Phosphorus in Soybean Meal before and after Treatment with Mold Phytase". *Poultry Sci.* **1968**, *47*, 1842–1848.

Shieh, T. R.; Wodzinski, R. J.; Ware, J. H. "Regulation of the Formation of Acid Phosphatases by Inorganic Phosphate in *Aspergillus ficuum*". *J. Bacteriol.* **1969**, *100*, 1161–1165.

Whitaker, F.; Brunnert, H. "Enzymatic Modification of Protein Applicable to Foods". *Adv. Chem. Ser.* **1977**, *No. 160*, 95–155.

Yamada, K.; Minoda, Y.; Yamamoto, S. "Phytase from *Aspergillus terreus*. Part 1. Production, Purification and Some General Properties of the Enzyme". *Agric. Biol. Chem.* **1968**, *32*, 1275–1282.

Yatsu, L. Y.; Jacks, T. J. "Association of Lysosomal Activity with Aleurone Grains in Plant Seeds". *Arch. Biochem. Biophys.* **1968**, *124*, 466–471.

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Refolding of Thermally Unfolded Soy Proteins during the Cooling Regime of the Gelation Process: Effect on Gelation

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The thermal denaturation and gelation of soy proteins were studied. Evidence suggests that soy 11S globulin undergoes partial refolding during the cooling regime of the thermal gelation process. At 8% protein concentration, in the absence of any additive, soy 11S refolded considerably during the cooling regime and failed to form gel. However, addition of 0.5 M $NaClO_4$ inhibited the extent of refolding and caused gelation of 11S even at 8% concentration, whereas addition of 0.5 M NaCl promoted refolding and prevented gelation. On the basis of these findings a general mechanism for thermal gelation of globular proteins is proposed. It is suggested that by controlling the extent of refolding of the protein during the cooling regime, it is possible to improve the gelation of globular proteins even at suboptimum protein concentrations. The thermal denaturation of soy proteins under various solution conditions is also discussed.

A considerable amount of research has been done on the heat-induced gelation of soy globulins and other proteins in order to understand the general mechanism of protein gelation (Catsimpoolas and Meyer, 1970, 1971; Ishino and Kudo, 1977; Babajimopoulos et al., 1983; Bello et al., 1962). Studies have shown that several factors, such as protein concentration, pH, ionic strength, reducing agents, urea, etc., affect the gelation of soy globulins and other globular proteins (Catsimpoolas and Meyer, 1970, 1971; Hermansson, 1982; Schmidt et al., 1979; Furukawa et al., 1979). The results of those studies have led to the general conclusion that the three-dimensional network in gel structures is formed via hydrogen bonds, hydrophobic bonds, and electrostatic interactions between polypeptides. However, no attempts have been made to understand the relationship between the extent of formation of gel network and the state of protein structure in the gel.

The important initial step in heat-induced reversible gelation of globular proteins is the heating of the protein solution above the denaturation temperature of the pro-

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