Protein-Calcium-Phytic Acid Relationships in Soybean

Part I. Effects of Calcium and Phosphorus on Solubility Characteristics of Soybean Meal Protein

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This paper constitutes the first report in a research series undertaken in order to clarify the interactive relationships among soybean meal protein, calcium and phytic acid in solution and in precipitate.

Data presented are mainly concerned with solubility characteristics of soybean meal proteins as a function of pH. The results support the suggestion as follows; phosphorus and calcium give the remarkable effects on solubility characteristics of protein, the complex containing protein, calcium and phosphorus is formed above isoelectric point of protein and the combinations are labile above at pH 8, especially by heating.

Soybean meals contain 500~600 mg of phosphorus on dry basis, 70~80% of which account for phosphorus of phytic acid.

It has been reported that phytic acid influences the solubility characteristics of the meal protein by the combination with basic radicals in protein molecule below its isoelectric point.¹⁻³⁾ In the course of the investigation, it was found that fairly large quantity of phosphorus precipitates with protein at about pH 5.7 by calcium salt, and practically, 70~80% of water extractable phosphorus transfer into curd (tofu) in the process of coagulation.⁴⁾ Wolf and Briggs reported that cold-insoluble fraction of soybean meal protein precipitated by cooling after addition of calcium chloride contained 1.40% of phosphorus, compared to a 0.17% of phos-

phorus content in cold-insoluble fraction precipitated in the absence of calcium ion.⁵⁾ These data indicate that protein easily combines with phytic acid in the presence of calcium even above its isoelectric point. But little attention has been paid to the relationship between protein and phytic acid above isoelectric point, since previous investigations dealt with almost exclusively with the relationship below isoelectric point.

This work was started in order to clarify interactive relationships among soybean meal protein, calcium and phytic acid in solution and also in precipitate by acid or calcium salt.

MATERIAL AND METHODS

Material. Hawkeye soybeans, 1965 crops, were used throughout this study. The defatted soybean meal was prepared by grinding, removing the hulls and defatting with a continuous flow of *n*-hexane.

Preparation of samples. Whole water extract; to 18~20 g of defatted soybean meal, 150 ml of water was added and the mixture was stirred for 30 min,

1) T. D. Fontaine, W. A. Pons, Jr. and G. W. Irving, Jr., *J. Biol. Chem.*, **164**, 487 (1946).

2) A. K. Smith and J. J. Rackis, *J. Am. Chem. Soc.*, **79**, 633 (1957).

3) J. E. Courtois and P. Delrieu, *Biochem. appl.*, **5**, 65 (1958).

4) O. Nakayama, C. Fukamachi, S. Kubo, S. Suruga, S. Miyanaga, S. Horike and T. Watanabe, *Report of the Food Research Institute*, **14B**, 52 (1960).

5) W. J. Wolf and D. R. Briggs, *Arch. Biochem. Biophys.*, **186**, 85 (1959).

centrifuged* and filtered through several layers of gauze.

Acid-precipitated protein; whole water extract was acidified to pH 4.25 with HCl and the precipitate was centrifuged, washed twice with a small amount of water and dissolved in dilute alkaline solution until about pH 8 with NaOH.

Calcium precipitated protein; calcium chloride was added to whole water extract to make it 0.02 N with respect to added calcium ion and the precipitate was centrifuged, washed twice with a small amount of water and dissolved in dilute alkaline solution.

Cold-insoluble fraction; it was prepared after Wolf's method.⁶⁾ To 40 g of defatted soybean meal, 200 ml of water containing 0.01 M mercaptoethanol was added, and the mixture was stirred for 30 min, centrifuged and filtered through gauze. After standing in ice bath for 3~4 hr, the precipitate resulted was separated by centrifugation at $10,000 \times g$ for 30 min.

Phytate-free protein; it was prepared mainly after Graine's method. To the solution containing 5 g of fresh acid-precipitated protein, 25 g of Dowex-I-X10 resin (acetate form) was gradually added, the mixture being kept at pH 2.5 and stirred for 15 min. The solution was filtered through glass wool to remove the resin and treated a second time with 25 g of resin.

Experiments on solubility characteristics. For preliminary experiment, whole water extract was made different pH values or different calcium concentrations with HCl or calcium chloride, and precipitates resulted were centrifuged and analyzed for the amounts of nitrogen and phosphorus. Nitrogen was determined by micro-Kjeldahl method and phosphorus by Nakamura's method.⁸⁾

Solubilities of nitrogen, calcium and phosphorus of whole water extract, solutions of acid-precipitated protein, calcium-precipitated protein, cold-insoluble fraction and phytate-free protein were determined at different pH values in the range of pH 2~11. The protein solutions were respectively pipetted in 8 ml and were adjusted to the various pH values by HCl or NaOH. Each solution was filled up to 10 ml

with water and then centrifuged at $1,000 \times g$ for 1 min or in some case ultracentrifuged at $80,000 \times g$ for 30 min. Each supernatant was submitted to the determination of total nitrogen, protein nitrogen phosphorus and calcium. Total nitrogen was determined by micro-Kjeldahl method and protein nitrogen by micro-Kjeldahl method after precipitation with 10% of trichloroacetic acid. Phosphorus was determined by Nakamura's method and calcium by Kubo and Tsutsumi's method⁹⁾ after ashing aliquots of each supernatant and removing silicic acid. The values on Y axis in Figs. illustrating solubility characteristics indicate mg of nitrogen, calcium and phosphorus per ml of protein solutions. The values were recalculated as those in solutions containing 5 mg nitrogen per ml because of the small variation of nitrogen content of sample solutions.

Five ml of sample was set on the column of Sephadex G-75, 1.5 cm $\times$ 30 cm, eluted with $10^{-5} N$ NaOH and fractionated in 3 ml. Each tube was examined on the absorbance at 280 m μ , protein by Lowry's method,¹⁰⁾ phosphorus by Nakamura's method and calcium by Yanagisawa's method.¹¹⁾ For the gel-filtration of calcium-precipitated protein solution as well as calcium chloride solution shown in the top and middle in Fig. 4, their pH values were adjusted to 9.0. In the case of acid-precipitated protein solution shown in the bottom in Fig. 4, the pH was also adjusted to 9.0 after calcium chloride was added to make the final concentration 0.02 N.

Heat treatment. To discuss the effects of heating on solubility, whole water extract was heated on boiling water bath for 10 min and was submitted to the determination of solubility characteristics. Because of the difficulty of homogeneous pipetting for precipitates resulted by heating, acid-precipitated protein, calcium-precipitated protein solutions and also whole water extract were first pipetted, adjusted the pH values and then heated on boiling water bath for 10 min.

RESULTS AND DISCUSSION

The amounts of the nitrogen and phosphorus precipitated from whole water extract at dif-

* Centrifugation was carried out at $1,000 \times g$ for 15 min, unless otherwise stated.

6) D. R. Briggs and W. J. Wolf, *Arch. Biochem. Biophys.*, **72**, 127 (1957).

7) E. M. Graine and K. E. Fahrenholtz, *Cereal Chem.*, **245**, 35 (1958).

8) M. Nakamura, *J. Agr. Chem. Soc. Japan*, **3**, 244 (1950).

9) S. Kubo and C. Tsutsumi, *Report of the Food Research Institute*, **5**, 171 (1951).

10) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

11) F. Yanagisawa, *Analysis and Instrument, Japan*, **4**, 59 (1966).

FIG. 1. Percentage of N and P Precipitated from Whole Water Extract at Different pH Values

●—● total N
○—○ phosphorus

FIG. 2. Percentage of N and P Precipitated from Whole Water Extract at Different Calcium Concentrations.

●—● total N
○—○ phosphorus

rent acidic pH values are shown in Fig. 1 the percentage to those of whole water extract. The maximum precipitation in total phosphorus occurs at about pH 3.5, whereas the maximum precipitation range for the nitrogen extends from pH 3.5 to 5. The ratio phosphorus to nitrogen increases on acidic side as far as this experiment concerns. Fig. 2 shows the amounts of the nitrogen and phosphorus precipitated at different calcium concentrations. It is apparent that calcium-pre-

cipitated protein contains large quantity of phosphorus and the trend of solubilities of phosphorus corresponds to that of nitrogen. The ratio of phosphorus to nitrogen is higher than that of acid-precipitated protein, because the percentage of nitrogen precipitated by calcium salt is lower than that by acid.

The solubilities characteristics expressed as the amounts of total nitrogen, protein nitrogen, phosphorus and calcium in whole water extract, acid-precipitated protein and calcium-precipitated protein, as a function of pH, are shown in Fig. 3-A, B and C. Fontaine et al. investigated protein-phytic acid relationship in peanut, cotton seed and soybean.¹¹ That relationship in rice was reported by one of the authors.¹² The solubility curve of whole water extract shown in Fig. 3-A is quite similar to that shown by Fontaine et al. on soybean and there is very little decrease in the solubility of phosphorus above pH 7 in contrast to those in peanut, cotton seeds and rice as they also pointed out. In the solubility curve of acid-precipitated protein solution shown in Fig. 3-B, the minimum solubility range for nitrogen is narrower than

FIG. 3. Solubility Characteristics of Soybean Proteins at Different pH Values.

●—● total N ●—● protein N ○—○ phosphorus ×—× calcium

12) K. Saio and S. Kubo, *J., Agr. Chem. Soc. Japan*, **19**, 217 (1965).

that of whole water extract, extending between pH 4.1 to 5.1 with sharp increase at both sides. In Fig. 3-C showing the solubility curve of calcium-precipitated protein, the minimum solubility range of nitrogen lies broadly from pH 3 to 6. A very sharp solubility of calcium at pH 5.7 and two of phosphorus at pH 3.8 and 5.7 are observed. It is interesting to note that phosphorus shows minimum point both at pH 3.8 not coinciding with that of calcium and at pH 5.7 coinciding with that of calcium, and that phosphorus and calcium still keep solubilities above pH 8, where both should be expected to react each other and to form the precipitate of Ca-phytate (Fig. 5-B). In order to discuss possible combination among protein, calcium and phosphorus at alkaline range, calcium-precipitated protein solution was gel-filtered on Sephadex G-75. As the control, the gel-filtration of both reagent

calcium chloride solution and the solution mixture of acid-precipitated protein and calcium chloride was also carried out. In Fig. 4-A, F and C, great majority of calcium in calcium-precipitated protein was eluted in the same tubes with protein, where the peaks of absorbance values at 280 m μ and phosphorus are identically indicated, whereas calcium in reagent calcium chloride was eluted later. In the case of acid-precipitated protein solution to which calcium chloride is added, calcium seems to be eluted in two groups, one is eluted with protein and the other is eluted in later stage, which might perhaps be calcium added excessively for protein. The data herein could lead the suggestion that there is something relate closely among protein, calcium and phosphorus (phytic acid) in alkaline solution as well as in precipitate.

But during the course of conducting the experiments, white flocculent precipitate was sometimes observed within several days, when calcium-precipitated protein solution was kept

FIG. 4. Chromatogram on Sephadex G-75 Column for Separation of Bound Calcium.

● — ● O. D. at 280 m μ ● — ● protein N
○ - - - ○ phosphorus × - - - × calcium

FIG. 5. Solubility Characteristics of Calcium-Precipitated Protein and Calcium Phytate.

Values in calcium-precipitated protein were analyzed after ultracentrifugation.

● — ● total N ○ - - - ○ phosphorus
× - - - × calcium

alkaline pH, and also similar precipitate as observed within several hours, when the calcium-precipitated protein prepared with excessively added phytic acid was dissolved in dilute alkaline solution. Although Fig. 5-A shows the solubility curve of calcium-precipitated protein, it is different from Fig. 3-C at the point that the values in Fig. 5-A were determined after ultracentrifugation at $80,000\times g$ instead of low-speed centrifugation at $1,000\times g$. At alkaline pH above 8, the solubilities of calcium and phosphorus decrease after ultracentrifugation, whereas that of protein does not change. On the other hand, Fig. 5-B shows the solubilities of calcium and phosphorus in the mixture of reagents calcium chloride and sodium phytate. Calcium and phytic acid ions immediately react each other and resulted in the precipitate with limited solubility above pH 7, which sediments easily even at $1,000\times g$.

On the basis of available data, it can be assumed that the combinations among protein, calcium and phytic acid are very labile in alkaline range and some part of complex is in the form of calcium phytate (phytic acid is not necessarily saturated with calcium).

FIG. 6. Solubility Characteristics of CIF Protein and Calcium-Precipitated Protein from CIF.

● — ● total N ○ --- ○ phosphorus
× --- × calcium

Cold-insoluble fraction contained about 30% of phosphorus in that of original soybean meal extract and phytate-free protein about 10%. Fig. 6-A indicates the solubility curves of cold-insoluble protein and Fig. 6-B shows those of calcium-precipitated protein from cold-insoluble fraction. The solubility characteristics of nitrogen, calcium and phosphorus in Fig. 6-B, comparing with the values in Fig. 3-C, are quite similar as a function of pH but calcium content per total nitrogen decreased.

FIG. 7. Solubility Characteristics of Phytate-free Proteins.

● — ● total N ○ --- ○ phosphorus
× --- × calcium

Fig. 7-A shows the solubility curve of phytate-free protein without addition of calcium ion, and Fig. 7-B shows that with addition of calcium ion. The minimum solubility range of total nitrogen in Fig. 7-A and B is shifted to pH 4.5~5.8 from 3.5~5 observed in Fig. 3-A and B, and the solubility at acidic side below pH 4 remarkably increases, whereas that at alkaline side increases gradually. As precipitate of phytate-free protein at pH 5.7 contains little calcium, there is a small difference between Fig. 7-A and Fig. 7-B in the solubility of total nitrogen, leaving calcium in supernatant in Fig. 7-B.

These evidences suggest that calcium content

in protein precipitates is effected by phosphorus content and that solubility characteristics are influenced by phosphorus content. However, the reasonable conclusion will be sent to the further work in progress, because there leave some doubts in the points that denaturation might occur on phytate-free protein during the treatment by ion-exchange resin and that cold-insoluble protein is different from initial soybean protein on the protein components, which consist of 89~90% of 11S.

Fig. 8-A and B indicate the solubility characteristics of whole water extract after heating. Fig. 8-A shows those heated after pH adjustment and Fig. 8-B those heated before pH adjustment. But there seems little difference between Fig. 8-A and Fig. 8-B in insolubility characteristics of total nitrogen, calcium and phosphorus. Fig. 9-A and B show the solubility curves of acid-precipitated protein and calcium-precipitated protein after heating, respectively. In Figs. illustrating

Fig. 8. Solubility Characteristics of Whole Water Extracts after Heating.

A; heated before pH adjustment, B; heated after pH adjustment.

● — ● total N ● — — ● protein N
○ — — — ○ phosphorus × — — × calcium

Fig. 9. Solubility Characteristics of Acid-Precipitated Protein and Calcium-Precipitated Protein after Heating.

● — ● total N ● — — ● protein N
○ — — — ○ phosphorus × — — × calcium

solubility characteristics after heating, the marked change by heating is observed above pH 7 in the presence of calcium (Figs. 8 and Fig. 9-B). Solubilities of calcium and phosphorus decrease sharply, because heat treatment perhaps makes calcium and phosphorus more labile. But the minimum solubility range of phosphorus and calcium at pH 5.7~6.0 suggests the existence of combinations among protein, calcium and phytic acid at this range even after heating.

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