Protein-Calcium-Phytic Acid Relationships in Soybean

Part II. Effects of Phytic Acid on Combination of Calcium with Soybean Meal Protein

By Kyoko SAIO, Emiko KOYAMA* and Tokuji WATANABE

*Food Research Institute, Ministry of Agriculture and Forestry, *Japan Tofu Association*

Received October 2, 1967

This paper constitutes the second in a research series undertaken to clarify the interactive relationships among soybean meal protein, calcium and phytic acid. The extent of bound calcium by cold insoluble fraction protein which is low in phosphorus content was examined by differential equilibrium dialysis, differing concentrations of calcium and phosphorus. The results indicate clearly that many molecules of calcium and phosphorus may be bound by a single protein molecule and that extent of calcium increases as phosphorous compounds in dialysis system such as phytic acid increase. The maximum number of bound calcium was calculated after statical equation by Klotz.

In the previous paper¹⁾ attention had been drawn to the remarkable effects of calcium and phosphorus on the solubility characteristics of soybean meal protein. The purpose of the present investigation was to pursue the binding of calcium ion by soybean meal protein which is effected by phytic acid. Klotz and co-workers²⁾ extensively studied the multiple equilibrium between protein and other substances. And Tamura carried out the investigation on combination of calcium ion with protein at coagulation of soybean milk, in which maximum number of bound calcium by protein,³⁾ effects of other ions than calcium on coagulation⁴⁾ and changes of electric conductivity or pH during coagulation⁵⁾ were studied. But from the view point of the marked effects of phosphorus, it appeared of interest to study further the mechanism of the combination of calcium with soybean meal protein.

EXPERIMENTAL

Material. The defatted meal of Hawkeye soybeans, 1966 crops, were used throughout this study. Phytic acid was a commercial sample of reagent grade, Tokyo Kasei Co. Ltd. Hexa-polyphosphate was supplied by Chiyoda Kagaku Company but was purified by gel-filtration on Sephadex G-25 column before using. Cold insoluble fraction protein (CIF) was prepared after Wolf's method.^{1,6)}

Differential equilibrium dialysis. Visking cellulose tubes were filled up with 5 ml of dilute protein solution, varying the amounts of added phosphorous compounds such as phytic acid. The bag was immersed in a solution of calcium chloride in different concentration and placed in cold room at 2~4°C for a period of 3 days, an interval sufficient for the attainment of equilibrium. The bag was then removed and the external solution was analyzed for calcium and phosphorus by Yanagisawa's method⁷⁾ and Nakamura's method⁸⁾ respectively. Control dialysis in which the protein was absent was set up to correct the absorption error caused by membrane. The depletion of calcium from the solution caused by

1) K. Saio, E. Koyama and T. Watanabe, *Agr. Biol. Chem.*, **30**, 1195 (1967).

2) I. M. Klotz, *The protein*, **1**, B, 727 (1953).

3) T. Tamura, *Kogyo Kagaku Zasshi*, **62**, 78 (1959).

4) T. Tamura, *ibid.*, **62**, 80 (1959).

5) T. Tamura, *ibid.*, **64**, 559 (1961).

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

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

8) M. Nakamura, *Agr. Biol. Chem.*, **3**, 244 (1950).

membrane was quite few and that of phytic acid was not so much. Phytic acid, ortho-phosphoric acid and hexa-polyphosphate were neutralized with NaOH to pH 6.7 before addition to protein solution.

Fractionation of phosphorous compounds in sample solutions. To 5 g of defatted soybean meal, 25 ml of 2% HCl containing 10% sodium sulfate anhydrate was added. The mixture was stirred, centrifuged and filtered (Extract). On the other hand CIF prepared from 25 g of defatted soybean meal was dissolved in 30 ml of above 2% HCl (Solution). Both 10 ml of Extract and Solution were added with 10 ml of 10% trichloroacetic acid respectively. Resulting precipitates were separated by centrifugation, washed twice with 5 ml of above 2% HCl and the residues were transferred to porcelain dishes and submitted to phosphorus analysis after ashing. The supernatants and washes were mixed and 20 ml of the mixture was taken in centrifuge tubes, neutralized, added with 5 ml of 1.5% ferric chloride in 1N HCl and incubated for 15 min in boiling water bath. The precipitates were centrifuged after cooling and washed with 0.6% HCl containing 10% sodium sulfate anhydrate. The precipitates were suspended in small amount of water, added with 2 ml of 1N NaOH and warmed for 15 min in boiling water bath. The precipitates were filtered while the mixture was warm and the aliquots of filtrates were submitted to phosphorus analysis after digestion. One ml of Extract was examined for labile phosphorus immediately after the extraction. Throughout this process, phosphorus was analyzed after Nakamura's method⁸ and total nitrogen was after micro-Kjeldahl method. The fractionation mainly followed the method described by Pon's *et al.*⁹ with a little modification.

RESULTS AND DISCUSSION

In order to see the effects of phosphorus compounds on the binding of calcium by soybean meal protein, it is necessary to remove phosphorus from the protein without denaturation. CIF was used throughout this study because of rather low content of phosphorus and easiness for preparation. The common procedures for the elimination of phosphorus from the protein, for instance, treatment with ion exchange resin or long period dialysis

against water, may cause the denaturation of protein and are time-consuming. It should be noted that characteristics of CIF in binding of calcium might be different from those of whole soybean protein for their different component. But the trend of binding of calcium and phosphorus by whole soybean protein is almost identical with those by CIF, as compared in Fig. 1 with Figs. 2 and 3.

Figure 2 shows the extent of bound calcium and phosphorus after equilibrium, illustrating the total amounts of calcium and phosphorus added to dialysis system. In Fig. 2, bound calcium increases as added calcium increases (1, 2, 3, 4, 5 and 6) and increases greatly as added phytic acid increases (I, II and III). Bound phosphorus slightly decreases in higher calcium concentration, whereas bound calcium increases. Fig. 3 was prepared from Fig. 2 and supplemental data, showing the binding of calcium by CIF as the function of free calcium. It indicates that a single molecule of CIF combines with a large number of calcium ions by many binding sites and that the co-existence of phytic acid with CIF gives the changes in the binding behavior of CIF with calcium. Bound calcium increases as added phytic acid increases as far as the range of phytic acid in the experiment shown in Fig. 3 concerns. Fig. 4 shows the relation

Fig. 1. Binding Affinity of Calcium by Whole Soybean Protein.

Experimental conditions; 21.3 mg of total N (20.6 mg of protein N) and 1.78 mg of total P presented in dialysis system. Soybean protein was dialyzed for a week in cold against water before using. —●— Ca —○— P

⁹ W. A. Pons, Jr., M. F. Stansbury and C. L. Hoffpauir, *J.A.O.A.C.*, **36**, 492 (1953).

FIG. 2. Total Amounts of Calcium and Phosphorus in Dialysis System and Bound Amounts of Them after Equilibrium.

Inside compartment: 5 ml of CIF solution (N 16.96 mg), varying phytic acid concentration; Outside compartment: 10 ml of calcium chloride solution in different concentration.

FIG. 3. Changes of Affinity for Calcium of CIF in the Presence of Phytic Acid.

- P 0.447 mg, N 16.96 mg
- P 0.925 mg, N 16.96 mg
- ×— P 1.423 mg, N 16.96 mg
- ▲— P 2.923 mg, N 11.92 mg
- P 3.990 mg, N 20.07 mg

P and N mg described above show total amounts of P and N presented in dialysis system.

FIG. 4. Change in Extent of Bound Calcium by CIF with Added Phytic Acid.

Experimental condition: CaCl_2 concentration in dialysis system was 0.02N.

between added phytic acid and bound calcium by CIF when the concentration of calcium chloride in dialysis system is 0.02N. The rate of increases of bound calcium is fast in lower phytic acid concentration and slow in higher concentration. It needs further consideration whether absolutely phytate-free protein can combine with calcium or not, although its possibility may be suggested by Fig. 4. Evidently phytic acid first combines calcium ion in the solution but immediately combines with CIF in company with calcium. The relationships is interesting when compared with the case in the presence of oxalic acid experimented by Tamura.⁴⁾

He reported that added oxalic acid to soybean milk immediately reacts with calcium, and coagulation caused by the reaction between calcium and soybean protein starts later only after all oxalic acid finishes to react. It means that both reactions are independent unlike the relationships among soybean protein, calcium and phytic acid.

According to Figs. 3 and 4 the highest number of bound calcium to 1 mg nitrogen of CIF is about 4.5 μmoles . Under the assumption of molecular weight of 350,000 to CIF, about 260 moles of calcium are calculated to bound to a CIF protein molecule in maximum.

Ortho-phosphoric acid and hexa-polyphosphate were also used instead of phytic acid for differential equilibrium dialysis. Figs. 5

FIG. 5. Changes of Affinity for Calcium of CIF in the Presence of ortho-Phosphate.

—●— P 0.508 mg, N 11.92 mg
 —○— P 1.518 mg, N 11.92 mg
 —×— P 2.528 mg, N 11.92 mg
 P and N mg described above show total amounts of P and N presented in dialysis system.

FIG. 6. Changes of Affinity for Calcium of CIF in the Presence of hexa-Polyphosphate.

—●— P 0.508 mg, N 11.92 mg
 —○— P 1.422 mg, N 11.92 mg
 —×— P 2.336 mg, N 11.92 mg
 —□— P 3.130 mg, N 20.07 mg
 P and N mg described above show total amounts of P and N presented in dialysis system.

and 6 show the binding of calcium by CIF in the presence of ortho-phosphoric acid and hexa-polyphosphate respectively. In the case of ortho-phosphoric acid increase of phosphorus resulted in increase of bound calcium but the extent reaches soon at the maximum value, being 1.78 μ moles, almost the same as phosphorus. The addition of hexa-polyphosphate increases bound calcium more remarkably than that of ortho-phosphoric acid. As com-

pared with the case of phytic acid, the extent of bound calcium is higher in low concentration of free calcium but lower in high concentration.

The law of mass action has been applied by Klotz²¹ to the case of multiple combination under condition that all the binding sites have the same intrinsic association constant. The derivations finally led to statical equation as below;

$$1/r = K/n(I) + 1/n,$$

where, r represents bound molecules of cation or anion by protein, (I) is concentration of free cation or anion and K depends on the nature of the protein and n represents the average maximum number of binding sites per protein. According to the equation a plot of $1/r$ against $1/(I)$ will give a straight line and n , the maximum binding sites, is obtained by extrapolation to $1/r$ axis.

The curves in Fig. 7 were calculated from the data of differential equilibrium dialysis in

FIG. 7. Calculation of Maximum Binding Extent of Calcium by CIF.

—●— CIF (0.508 mg)
 —○— ortho-phosphate (2.528 mg)
 —×— hexa-polyphosphate (2.336 mg)
 —□— phytic acid (2.923 mg)

The numbers in the brackets show total P mg presented in dialysis system.

TABLE I. CALCULATION OF MAXIMUM NUMBER OF BOUND CALCIUM BY CIF PROTEIN

	P mg	Intersept of Y axis	Ca μ moles/ N 1 mg	P μ moles/ N 1 mg
CIF	0.508	0.6	1.65	1.27
Ortho-P	2.528	0.5 ~0.55	1.8~2.0	1.8
Hexa-PP	2.336	0.2 ~0.25	4 ~5	4.5~5
Phytic acid	2.923	0.15~0.2	5 ~6.7	4.5~5

the presence of about 2000 mg of phytic acid, ortho-phosphoric acid or hexa-polyphosphate. The relations do not show the strict straight lines, in accordance with Klotz's result on the combination of azosulfathiazole with bovin serum albumin.¹⁰⁾ The data mean that all binding sites are not independent and added calcium exerts an additional electrostatic repulsion toward further encoming calcium ion. The values in Table I are approximately calculated from the intercepts of curves with $1/r$ axis. The maximum number of bound calcium of initial CIF which is less than 2μ moles increases by addition of phosphorus compounds and the extent of bound phosphorus increases at the same time, reaching to the maximum values described on Table I. It makes clear that n , maximum number of bound calcium, is changeable depending on the kinds and amounts of phosphorous compounds presented with CIF protein.

It may suggestable that phytic acid presented with soybean protein in natural gives some influences on binding affinity for calcium; consequently, on the characteristics of soybean protein in coagulation by calcium.

Table II shows total phosphorus, phosphorus of phytic acid, in labile form and precipitated with protein by trichloroacetic acid per nitrogen in whole water extract of CIF. A great majority of total phosphorus is occupied by phytic acid, especially in whole water extract. In

TABLE II. FRACTIONATION OF PHOSPHORUS COMPOUNDS IN SAMPLE PROTEINS

	P mg/N 10 mg (%)			
	Total P	P in phytic a.	labile P	P in ppt.
Whole water extract	1.56	1.4 (91)	0.085 (5.4)	0.056 (4)
Cold insoluble fraction	0.36	0.27 (75)	0.011 (3)	0.073 (20)

CIF lower ratio of phytic acid to total phosphorus was obtained, since phosphorous compounds eliminated by precipitation in cold were mainly phosphorus of phytic acid and in labile form. It should be noted that phosphorus in CIF still consists of phytic acid in higher rate and therefore calcium bound by CIF even in the presence of ortho-phosphoric acid or hexa-polyphosphate might be partly bound by phytic acid.

From the behavior of phytic acid affecting the binding of calcium by CIF, phytic acid is almost fit to the low molecular substances indicated by Tamura,³⁾ having high binding affinity for calcium, and being precipitable by acid and dializable against water. But comparing with Tamura's discussion that 3 parts of bound calcium contribute to the combination with protein itself and 1 part to the combination with the low molecular substances, we rather emphasize that phytic acid co-operatively reacts with calcium and protein and contributes to binding of calcium in higher quantity.

The data in this paper may prove the interactive relationships among soybean meal protein, calcium and phytic acid, which was suggested in the previous paper.¹⁾ And the structural mechanism of binding will be investigated in the progressing work.

This research has been financed in part by a grant to Japan Tofu Association from the U.S. Dept. of Agriculture under P.L. 480.

10) I. H. Klotz, F. H. Walker and R. B. Pivan, *J. Amer. Chem. Soc.*, **68**, 1486 (1946).