

Effect of Phytate on Isoelectric Focusing of Soybean Whey Proteins

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ABSTRACT

Changes in isoelectric points of soybean whey proteins as a result of phytate elimination or addition were studied by isoelectric focusing between pH 3 and 6. Major whey proteins concentrated at pH 4.2, 4.6, and 5.5. Adjusting the pH of whey solution to pH 8.0 precipitated phytate and decreased a protein peak focused at pH 4.6 in the whey. Anion-exchange resin treatment of whey also removed phytate and lowered the protein peak focused at pH 4.2, as well as other peaks at lower pH values. When sodium phytate was added to proteins from which phytate had been eliminated by the resin method, protein peaks again focused in low pH regions. Proteins focused in the pH 4 region changed more on addition of phytate than those in pH 5 region. Protein mobilities in polyacrylamide-gel electrophoresis also were changed by added phytate. Phytate undoubtedly influenced the net charges of proteins.

Soybeans contain phytate (1). In extracting proteins from soybeans, phytate interacts with protein. The interaction products formed may vary in composition to compound the problem of evaluating homogeneity of protein. In isolation and purification of soybean protein, phytate needs to be eliminated (2). In soybean protein isolation, phytate elimination by alkali precipitation or treatment with Dowex-1 resin has been proposed (1). A shift of the isoelectric point by 0.8 unit occurs in the acid-precipitated protein on removing phytate. Electrophoretic studies indicated the presence of an interaction product of protein-phytate. Phytate is present in soybean whey, but evidence of its effect on whey proteins is lacking. Soybean whey protein has been separated by using isoelectric focusing (3). This method provides a direct way to demonstrate isoelectric point shifts of proteins. Knowing these differences in proteins is useful in obtaining purified proteins. This paper describes changes in focusing behavior of soybean whey protein as affected by elimination or addition of phytate.

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MATERIALS AND METHODS

Preparation of Soybean Whey Proteins

Kanrich soybeans were cracked, dehulled, and ground into meals (4). The full-fat meals (200 g.) were extracted with water (1:10 ratio of meal:water) for 1 hr. at room temperature. The extract was centrifuged at $10,000 \times g$ for 15 min. to separate the residue. The residue was reextracted with water (1:5 ratio of meal:water). Undissolved materials were discarded and the extracts were combined. The pH of the extract was then adjusted to 4.5 with 1N HCl and the precipitate was discarded after centrifugation. The total extract (2.7 l.) was divided into two equal parts. One part of the supernatant was titrated with 2N NaOH to pH 8.0 and kept overnight at 4°C . The solution was then centrifuged to separate phytate, and the supernatant was dialyzed for 48 hr. against water at 4°C . and freeze-dried. The other part of the original pH 4.5 supernatant was dialyzed and freeze-dried the same way but without addition of alkali. The yields of whey protein varied from 8 to 9% of meal.

Isoelectric Focusing

An LKB 8102 electrofocusing column of 440 ml. capacity and carrier ampholytes were purchased from the LKB Instruments, Inc. The principle involved and application to protein separation have been described (5,6). The density gradient was prepared with a gradient mixer, as described in the instruction sheet supplied by the company. The anode solution (0.8 ml. conc. H_3PO_4 in 2.5M sucrose) was placed at the bottom of the column and the cathode solution (14.3N NaOH) at the top. The sample was usually prepared by dissolving 200 mg. of lyophilized soybean whey protein in 30 ml. water and centrifuging at $10,000 \times g$ for 15 min. to remove insoluble materials. Centrifugation removed some lyophilized whey proteins (72% protein from the original whey and 83% from the pH 8.0 treated whey were soluble in water). The protein sample was poured first into a light solution and then mixed with a dense solution to fill up the column.

After focusing for 72 hr. with initial potential at 300 v. and final at 400 v. (20°C .), the contents of the column were drained slowly through the bottom tubing into a fraction collector. Fractions (4.5 ml.) were collected and the absorbance at 280 nm. of every other tube was determined on a Beckman DU spectrophotometer. The pH of every other fraction was measured at 25°C . with a Beckman pH meter (Zeromatic 9600).

Selected fractions were pooled according to the protein distribution curve, and the samples were dialyzed against at least five to six changes of water at 4°C . for 48 hr. to remove sucrose and ampholytes. The proteins were then lyophilized for gel electrophoresis.

Gel Electrophoresis

Seven percent of Cyanogum 41 in 0.025N glycine buffer (pH 9.2, 0.005 ionic strength, without urea) was prepared (7). Proteins were dissolved in water (lyophilized whey dissolved readily in water or 0.025N glycine buffer; we selected water) at concentrations between 5 and 10 mg. per ml. Different amounts of protein sample (80 μl or less) were applied to the slot with a syringe. Electrophoresis was for 1.5 hr. at 300 v., 75 ma. Protein bands were stained with

1% Amido Black for 10 min. and the gel was destained with 15% acetic acid.

Resin Treatment of Soybean Whey Protein

Phytate in whey proteins was removed by an anion-exchange resin (1). Dowex-1 (X8, 200 to 400 mesh) in formate form was used to fill a column 2×20 cm. Proteins (200 mg. in one experiment and 400 mg. in another) were dissolved in 50 ml. water, and then the pH was brought to 7.0 with 1N NaOH (water was preferred because it did not affect the exchange capacity of resin and focusing later). The solution was centrifuged to remove undissolved materials and then added to the column after the resin was washed with water. Elution of proteins with 150 ml. water was carried out by gravity with the 200-mg. sample and with pressure (2 p.s.i.) for the 400-mg. sample. About 60 to 75% of the added protein was recovered as estimated by absorbance at 280 nm. The solution from the 200-mg. sample was clear, whereas the other solution was slightly turbid. The protein solutions were used directly in the isoelectric focusing experiments.

Preparation of Phytate Fractions

For comparative purposes, phytate-containing fractions were prepared from commercial defatted flakes (Archer-Daniels-Midland Co.) and Kanrich full-fat meals by precipitating phytate at pH 8.0. From the full-fat meal, a phytate-containing fraction was also prepared by barium acetate precipitation (8). Phytate fractions precipitated at pH 8.0 were not dialyzed, whereas the one precipitated with barium acetate was dialyzed against water at 4°C. for 48 hr. (more details will be described in another paper). Phosphorus was determined by Fiske and SubbaRow's method (9), nitrogen by Kjeldahl (10), and ash by burning at 300°C. until constant weight was reached.

Sodium phytate used in one experiment was purchased from K&K Laboratory. The phytate contained 20.2% phosphorus. Sodium phytate solution (25 mg. in 10 ml. water) was added to Dowex-1-treated protein solution (120 mg. protein in 130 ml. water) and stirred at 4°C. overnight before adding to the focusing column.

RESULTS

Isoelectric Focusing of Soybean Whey Protein between pH 3 and 6

The bulk of soybean whey proteins focuses in the range of pH 3 to 8 (3). Between pH 3 and 6 whey proteins concentrate mainly at pH 4.2, 4.6, and 5.5 with minor peaks in other regions (Fig. 1). The electrofocusing pattern was reproducible provided there was no abrupt change of pH gradient. Little difference was observed when the cooling temperature of the focusing column varied from 4° to 20°C. Results reported here were run in duplicate to obtain pH focusing points and are slightly different from published results (3). (Different focusing conditions and defatted meal were used by Catsimpooolas et al.) Changes of pH focusing of whey proteins were observed when treatments of whey solution varied. Here we selected phytate elimination or addition to whey proteins as one of these treatments.

Phytate Elimination

pH 8.0 Precipitation. Phytate is precipitated at pH 8.0 from whey solution recovered from acid-precipitated protein in water extract of soybean meals (1). The

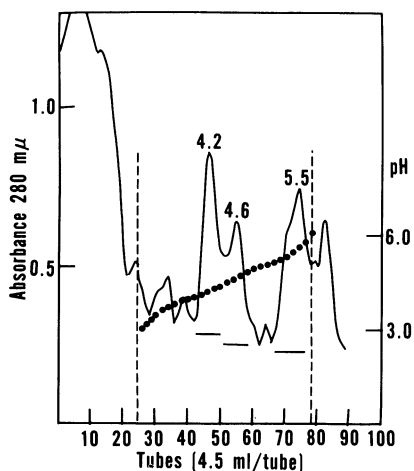


Fig. 1 (left). Isoelectric focusing of soybean whey proteins between pH 3 and 6. Solid line represents the protein concentration as measured by absorbance at 280 nm., solid circles between the dotted lines represent the pH gradient, and solid bars show the pH range of the fractions pooled.

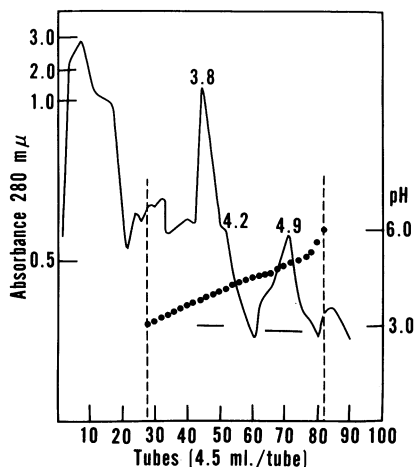


Fig. 2 (right). Isoelectric focusing of soybean whey proteins after phytate was eliminated by pH 8.0 precipitation. For other conditions see Fig. 1.

precipitate contains 13.8% phosphorus and 1.7% nitrogen. We found these percentages were true only when defatted flakes were used (see Results). We further found that the protein peak focused at pH 4.6 (Fig. 1) was absent in the electrofocusing pattern of such treated soybean whey (Fig. 2). Other changes were that more protein concentrated at pH 3.8 than at pH 4.2, and proteins focused at pH 5.5 in Fig. 1 shifted to pH 4.9 in Fig. 2. Although there was no proof that these latter proteins were similar, they possessed the same mobility on gel electrophoresis (see Results).

In Fig. 2 the protein, which should focus at pH 4.6, was not lost by fractionation on alkali treatment. Although proteins (at pH 4.6) were unlikely to be denatured selectively by such mild treatment, still a shift of pH focusing occurred. Lyophilized proteins used in Fig. 2, when dissolved in water, gave a pH 6.0, whereas those in Fig. 1 gave a pH 4.5. Whey proteins were more soluble in water at pH 6.0 than at pH 4.5. When pH of the two solutions was reversed and when the solutions were focused, both patterns showed peaks at pH 3.8 and 4.2 but no peak at pH 4.6.

Whey solution produced precipitates in the pH 4 region during electrofocusing when the sample was more than 200 mg. However, when the same amount of whey protein (treated at pH 8.0) was focused, exceptionally large amounts of white precipitate were found in the pH 4.2 peak. This appearance of excessive white precipitate suggests that an alkaline treatment may change the charges of the protein complex.

Resin Treatment. Dialyzed water-extractable protein solution (pH 7.0) loses 78% phosphorus and 10% nitrogen on treatment with Dowex-1 resins (1). When whey proteins are treated in this manner, the protein focusing pattern shifts (Fig.

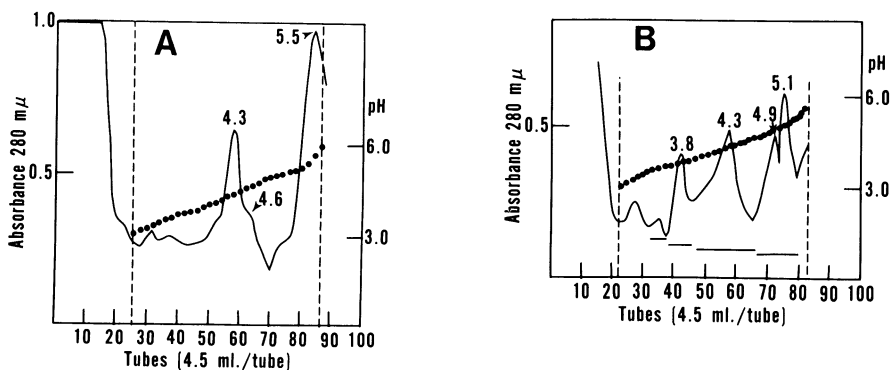


Fig. 3. Soybean whey proteins were passed through a Dowex-1 formate column 2×20 cm. and then focused as in Fig. 1 (A) (left) 200-mg. sample, eluted with water by gravity and (B) (right) 400-mg. sample, eluted with water under 2 lb. p.s.i. pressure.

3). Fig. 3, A shows two protein peaks (pH 4.3 and 5.5). Proteins treated as in Fig. 3, B peaked at four different pH values (pH 3.8, 4.3, 4.9, and 5.1).

The difference in Figs. 3, A and B is due to the completeness of Dowex-1 treatment since the amount of protein used in 3, A was half of that in 3, B. The treated protein solution was clear in 3, A but turbid in 3, B. Focusing gave no peak at pH 4.6 in both 3, A and 3, B, and only 3, A peaks at lower pH range were eliminated. This sequence of peak elimination seems to agree with the supposition that phytate is involved. However, factors other than just phytate elimination may be involved since whey protein is heterogeneous.

Dowex-1 treatment removed phytate and up to 40% of proteins in the sample. The treatment separated anionic proteins from those unreacted cationic proteins. If phytate interacts with the proteins to form complexes of different net charges, proteins with higher negative charge will migrate toward the anode. Proteins at pH 4.3 will contain less phytate than those at pH 3.8 and 4.2 (Figs. 3, A and B). Indeed, when phytate was added to Dowex-1-treated proteins, the relation is evident between proteins focused at higher pH and lower pH.

Addition of Sodium Phytate to Whey Protein

Results so far indicate that treatment of whey with pH 8.0 precipitation or Dowex-1 resins influences the pH focusing of proteins. Since both treatments eliminate phytate and since phytate complexes with protein in water-extractable proteins of varying composition (2), the phytate-protein complex may be found in whey. To elucidate this point, sodium phytate was added to Dowex-1-treated whey solution and the solution was focused isoelectrically in the usual manner. The protein solution focused differently (Fig. 4). Proteins peaking originally at pH 4.3 (Fig. 3, A) were split into peaks at pH 3.8, 4.1, 4.2, and others. This evidence supports phytate-protein interaction to form various products. Proteins focused in pH 4 regions changed more than those in pH 5 regions. Proteins peaking at pH 5.5 shifted slightly to a lower pH (5.2) with a shoulder (pH 4.9).

Crystalline Soybean Trypsin Inhibitor Focused at pH 3 and 6

Kunitz' soybean trypsin inhibitor (SBTI, two times crystallized, purchased from

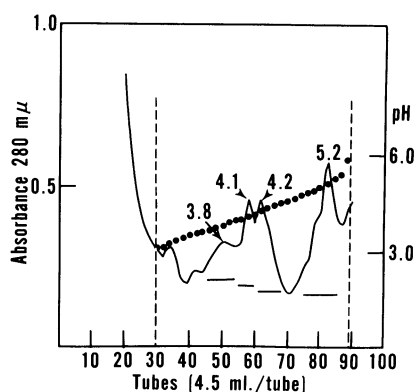


Fig. 4 (left). Soybean whey protein treated as in Fig. 3, A and then 25 mg. of sodium phytate was added. Focusing was done the same way as in Fig. 1.

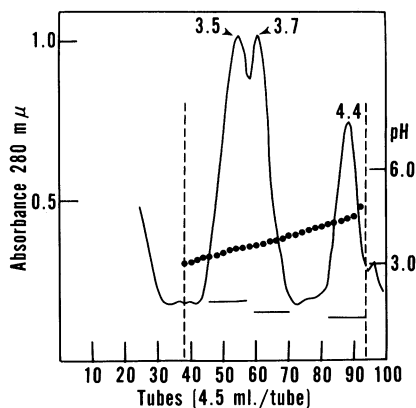


Fig. 5 (right). Commercial soybean trypsin inhibitor (100 mg.) focused the same way as Fig. 1.

Sigma Chemical Co.) has an isoelectric point at pH 4.5 (11) and its heterogeneity has been shown (7). Isoelectric focusing separated the proteins into three peaks in the range of pH 3 and 6 (Fig. 5). Proteins with isoelectric pH 4.4 were separated clearly from those at pH 3.5 and 3.7. Proteins in all three peaks are active in inhibiting hydrolysis of *n*-benzoyl-L-arginine ethyl ester by trypsin, and undoubtedly contain different net charges. Studies are under way to investigate these three types of proteins.

Since protein focuses at different pH, the net charge of protein varies and the different net charge affects the mobility of protein in gel electrophoresis. Therefore, the following experiment on gel electrophoresis was carried out.

Mobility of Proteins on Gel Electrophoresis

Polyacrylamide-gel electrophoresis provides a simple method to assay soybean whey protein (7). In glycine buffer (pH 9.2) without urea, proteins recovered from the electrofocusing experiment show distinctive bands (Fig. 6, A to E). In dialyzed whey (Fig. 1) the protein that focused at pH 4.2 had a mobility equal to that of commercial soybean SBTI (Fig. 6, A). All proteins from crystalline SBTI (Fig. 5) had the same R_f . The peak at pH 4.6 (Fig. 1) shows a slower moving band, about half R_f of SBTI. The R_f difference may indicate that these proteins are not the same as SBTI. The peak at the pH 5.5 region gives the slowest moving bands. Protein in the pH 5 region is believed to be lipoxygenase (EC 1.13.1.13) owing to its focusing, pH 5.5 (12), and certain bands in gel electrophoresis correspond to commercial lipoxygenase (purchased from Sigma Chemical Co.). Since lipoxygenase changes less during electrofocusing and gel electrophoresis, we shall concentrate on the protein focused at pH 4 regions.

When whey was treated by pH 8.0 precipitation (Fig. 2), proteins concentrated at pH 3.8 and showed a fast-moving SBTI band (Fig. 6, B). Upon Dowex-1 resin treatment as in Fig. 3, B, the proteins focused at pH 4.3 showed a mixture of a small amount of fast-moving SBTI and a large quantity of half R_f of SBTI; proteins

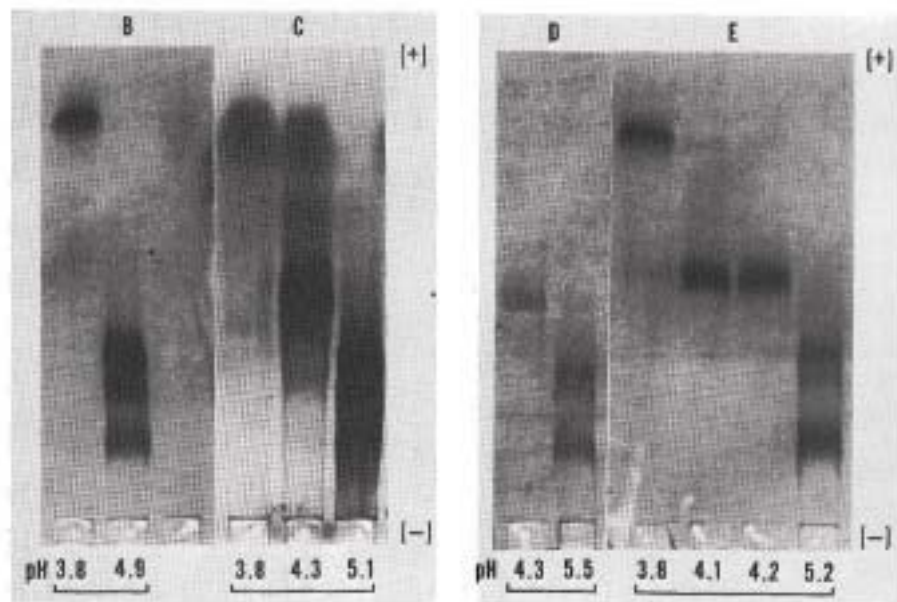
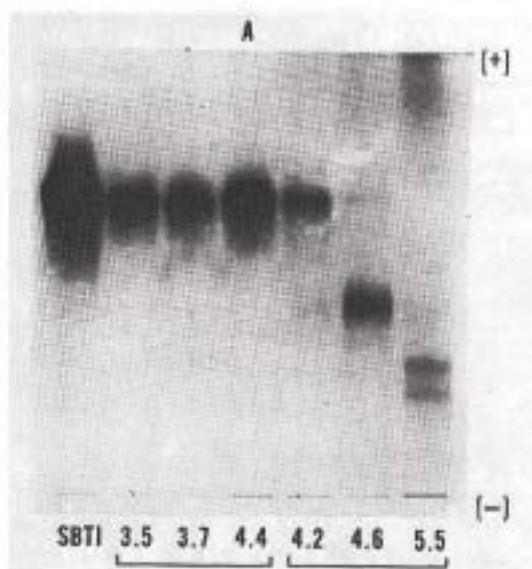


Fig. 6 Polyacrylamide-gel electrophoresis patterns. (A) Proteins pooled at pH range 4.2, 4.6, and 5.5 from Fig. 1 and commercial soybean trypsin inhibitor from Fig. 5; (B) proteins from Fig. 2; (C) proteins from Fig. 3, B; (D) proteins from Fig. 3, A; and (E) proteins from Fig. 4.

TABLE I. VARIATION OF PHYTATE FRACTION PRECIPITATED FROM SOYBEAN WHEY

Soybean Meal	Methods of Preparation	Yield from 100 g. Meal g.	Percent of		
			Phosphorus	Nitrogen	Ash
Defatted	Precipitated at pH 8.0	0.94	11.34	1.33	48.93
Full-fat	Precipitated at pH 8.0	1.88	6.93	2.58	25.61
Full-fat	Barium acetate ^a precipitation	4.52	3.73	5.97	28.29

^aMethod described by Cardini and Leloir (8).

focused at pH 3.8 were mostly the fast-moving SBTI (Fig. 6, C). When the resin-to-protein ratio is increased as in Fig. 3, A, proteins mainly focused at 4.3 and showed a slow-moving SBTI band (Fig. 6, D).

When sodium phytate is added to Dowex-1-treated soybean whey, proteins focus again at three different positions (Fig. 4; pH 3.8, 4.1, and 4.2); their gel patterns are shown in Fig. 6, E. Proteins with higher pH focusing (pH 4.2) move slower on the gel while those with lower pH focusing (pH 3.8) migrate faster. Proteins focused at pH 4.1 give a mixture of both fast- (very faint) and slow-moving bands and probably are not well separated with proteins focused at pH 3.8 and 4.2. These results demonstrate the heterogeneity of protein caused by addition of phytate to form phytate-protein complexes of varying composition. Commercial SBTI is probably different from crude whey proteins (pH 4.6).

Comparison of Phytate-Containing Fractions from Defatted Flakes and Full-Fat Meals

Previous studies of phytate fractions from soybean whey by adjusting the pH of the whey to pH 8.0 were on defatted flakes (1). The variation between the fraction from full-fat meals and that prepared by barium acetate precipitation is presented in Table I. A sample from defatted flakes was high in phosphorus (11.34%) and ash (48.93%), and low in nitrogen (1.33%); yield was 0.94%. These figures agree with published results (1). But when full-fat meal is used, a decrease of phosphorus (6.93%) and ash (25.61%) coincides with an increase of nitrogen (2.58%) and yield (1.88%). The changes are more noticeable in the barium acetate-precipitated phytate fraction from full-fat meals. The last method recovers the highest amount of total phosphorus (0.17 g. phosphorus as compared to 0.13 g. from full-fat meal and 0.11 g. from defatted meal by pH 8.0). More significantly, the last preparation has a distinct taste and turns rancid on storage (no formal taste panel test). On water extraction it yields large amounts of water-insoluble white precipitate. After separation of the white precipitate from water-soluble protein, the separated fractions lose their original taste. Formation of insoluble reaction products between phytate and whey components and instability of whey solution were the main reasons for eliminating phytate from whey (2). Phytate interactions between proteins and the white precipitates and their significance in the taste of protein are under investigation.

DISCUSSION AND SUMMARY

Two facts are noted in this study: (a) phytate elimination or addition changes the pH focusing of whey protein and mobility of protein in gel electrophoresis, and (b) phytate-containing fractions prepared from defatted flakes differ from those from full-fat meals.

Isoelectrofocusing between pH 3 and 6 separates soybean whey protein into peaks at pH 4.2, 4.6, and 5.5 (Fig. 1). Phytate seems to interact nonspecifically with soybean proteins. Between pH 4 and 5 proteins show more effect of phytate interaction than between pH 5 and 6 (Figs. 2 to 4). The pH 6 boundary may affect protein separation more at pH 5 region. Nevertheless, the ampholyte chosen (pH gradient of pH 3 to 6) provides a good range for studying interaction of phytate with soybean proteins.

Multiple forms of SBTI in soybeans (13,14,15) and various methods to prepare a sizable quantity of the inhibitor from soybeans (14,15,16,17) have been reported. Evidence presented here on the focusing pattern of commercial SBTI supports the view of heterogeneity of the protein. Presence of phytate in the inhibitor is a rare possibility, since the Kunitz crystalline sample contains only 0.01% or less phosphorus (11). However, we did observe the presence of white precipitates in soybean whey protein as well as in the Kunitz crystalline SBTI. At present the white precipitates were not identified, but their association with phytate or protein fractions seemed clear. This association may be the cause of some discrepancies in isoelectric points and R_f in gel electrophoresis. Attempts to remove these white precipitates from protein caused a shift of isoelectric point of proteins. These results will be reported in a later paper.

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