PREPARATION OF LOW-PHYTATE SOYBEAN PROTEIN ISOLATE AND CONCENTRATE BY ULTRAFILTRATION

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ABSTRACT

A process is described for removal of phytate from defatted soybean meal and meal extracts to produce low-phytate protein concentrates and isolates. The process consists of two steps: 1) dissociation of phytate from protein; 2) removal of the dissociated phytate from the protein by ultrafiltration using a membrane permeable to phytate but impermeable to protein. Conditions for phytate dissociation were established above, below, and at the isoelectric point of soybean globulins (pI 4.9). At pH 8.5 where multivalent cations such as calcium ion appear essential for the integrity of the protein-phytate complexes, ethylenediamine tetraacetic acid (EDTA) effected dissociation. At pH 3 calcium ion resulted in phytate dissociation. At pH 5 to 5.5 and 65°C the indigenous phytase present in the meal and meal extract promoted continuing dissociation of the complex by enzymatic hydrolysis of the dissociated phytate. Subsequent removal of phytate and other low-molecular-weight impurities by ultrafiltration thus afforded low-phytate soybean isolates and concentrates of high-protein content.

Phytic acid, the hexa*ortho*monophosphate ester of *myo*-inositol, occurs at fairly high levels in grains and oilseeds as the calcium magnesium salt, phytin. In soybean meal, roughly 70% of the total phosphorus is accounted for by phytin (1). Based on a 0.6%-phosphorus content of defatted soybean meal, approximately 2% by weight of phytin is, therefore, calculated to be present. During the preparation of isolates and concentrates much of the phytate remains associated with the protein in the form of complexes. In the case of commercial soybean-protein isolates, a phosphorus content of 0.7 to 0.8% is usual, indicating that as much as 2 to 3% by weight of the isolate is phytin.

Phytate removal from isolates and concentrates is advantageous because phytin phosphorus appears to be unavailable to monogastric animals (2), and thus interferes with the adsorption of nutritionally essential multivalent cations such as calcium (3), iron (4), and zinc (5). The last-mentioned phenomenon is a consequence of the strong complexing ability of the orthophosphate esters for multivalent cations. Phytate-protein complexes, which can vary in composition, compound the problem of isolation of pure protein components by altering both the electrophoretic mobility as well as decreasing the solubility of the proteins themselves (1).

Our work, therefore, has been directed toward finding methods to promote dissociation of phytate-protein complexes over a broad range of pH, and to couple these methods with the process of ultrafiltration to separate phytate and other low-molecular-weight impurities from soybean protein. Ultrafiltration appears to be a viable industrial process as a separation technique and is especially suited for our purpose in that excellent removal of phytate can be achieved with little or no loss of protein.

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MATERIALS AND METHODS

All chemicals were reagent grade unless specified otherwise and deionized water was used throughout the work. All operations were performed at ambient temperature unless indicated otherwise.

Analytical Procedures

Dry weights were determined after drying at 110°C to constant weight at atmospheric pressure and analytical values are expressed on a dry-weight basis unless noted otherwise. Nitrogen was determined by the Kjeldahl procedure and protein calculated to be N × 6.25. During the dialysis and ultrafiltration experiments, protein was determined by the biuret reaction (6). In the presence of EDTA, extra Cu^{++} was added (equal to the equivalents of EDTA present) to prevent interference of the EDTA in formation of the biuret color. Protein concentrations were determined from the biuret color at 540 nm using a calibration curve for soybean protein determined by Kjeldahl nitrogen. Total and inorganic phosphorus were determined by the procedure of King (7). Total phosphorus content is expressed as gP/100 g protein. Phytase activity of extracts was measured by the method of Peers (8). The sodium phytate used for the phytase assay was prepared (8) from crude calcium phytate obtained from Calbiochem. The sodium phytate so prepared contained 20.76% P and less than 0.1% of the total phosphorus was inorganic.

Soybean Meals

Concentrates were prepared from Nutrisoy 7B flour (<100 mesh). Extracts of Nutrisoy 7B flakes were used to prepare isolates as well as to examine the dissociation of phytate-protein complexes. Both starting materials had been hexane-extracted as received and were obtained from the Archer Daniels Midland Co. On an "as-is" basis, the flakes contained 8.82% N and 0.77% P, and the flour 8.73% N and 0.73% P. Prior to use, the flakes and flours were extracted batchwise at 25°C three times by shaking with *n*-hexane: ethyl alcohol azeotrope (79:21, v:v) (9) using 1 hr extraction periods and 250 ml azeotrope/100 g material for each extraction. Total extracted solids, the bulk of which were lipoidal (9), were 2.4% by weight.

Extracts

Azeotrope-washed flakes were stirred with 15 parts by weight of water for 1 hr at 25°C, maintaining the pH at 8.6 throughout the extraction period with 5 N KOH. The gross insolubles, retained by passage of the slurry through a 100-mesh screen, were reextracted twice by the above procedure with 5 parts by weight of water. The three extracts were pooled and clarified by centrifugation at $1800 \times g$ for 10 min. The extracts were then adjusted to the appropriate experimental conditions and used for dialysis and ultrafiltration experiments, as well as isolate preparation.

The azeotrope-washed flakes contained 8.94% N and 0.77% P (1.38 gP/100 g protein), the extracted solids usually contained 10.42% N and 0.93% P (1.43 gP/100 g protein) and represented 72.2%, by weight, of the flakes. Therefore, 84.3% of the nitrogen and 87.0% of the phosphorus were extracted from the flakes.

Dialysis Experiments

Extracts at a 2%-protein concentration were dialyzed in Visking cellulose acetate tubing using toluene-saturated solvents at 25° C. Sample-to-bath volume was 1 to 12.5, magnetic stirring was used for equilibration, and baths were changed every 24 hr. Retentate samples were analyzed for total phosphorus and protein (biuret) after 0, 1, 2, 4, and 6 days of dialysis.

Ultrafiltration Experiments

Ultrafiltration equipment was purchased from the Amicon Corp., Lexington, Mass. A TC-1 apparatus having two laminar flow modules each containing 0.125 ft^2 of PM-30 membrane (impermeable to substances having molecular weights greater than 30,000 daltons) was used. Pressure of 10 to 40 psig nitrogen was used to drive the ultrafiltration. In some experiments, a Model 401 cell containing 0.05 ft^2 PM-30 membrane was used. In either case, the extracts were first concentrated to 4% protein by ultrafiltration, and then toluene-saturated solvents contained in a reservoir under equivalent nitrogen pressure were passed through the retentate. The retentate was thus maintained at a constant volume throughout the dialysis by ultrafiltration (diafiltration). During diafiltration, at various volume ratios of ultrafiltrate to retentate, V_D, the retentate was analyzed for total phosphorus. Upon completion of diafiltration, the retentates were recovered by freeze drying and analyzed for nitrogen and phosphorus.

Isolate Preparation

The three methods used to prepare soybean protein isolates are described below:

1) *pH 8.5, EDTA Method*—Azeotrope-treated flakes were extracted as previously described, except that 0.05 M EDTA (Na^+) at pH 8.5 instead of water was used. After concentration of the extract to 4% protein, 0.01 M $CO_3^=/HCO_3^-$ (Na^+), pH 8.5, $V_D = 4.5$, was passed through the retentate to remove the EDTA followed by water made alkaline to pH 8.5, $V_D = 5$. Temperature throughout the ultrafiltration was 65° C.

2) *pH 5, Phytase Method*—The aqueous extract of protein was lowered to pH 5 with 5 N HCl, the resulting suspension concentrated to 4% protein, and water passed through, $V_D = 5$. Temperature throughout the ultrafiltration was 65° C. Concentrate was also prepared by this procedure using a 6%, by weight, flour suspension.

3) *pH 3, Calcium Method*—The extract was lowered to pH 3 with 5 N HCl, made 0.5 M in $CaCl_2$, and concentrated to 4% protein. Calcium chloride, 0.5 M at pH 3, was passed through the retentate, $V_D = 5$, followed by water acidulated to pH 3 with HCl, $V_D = 15$, to remove the calcium. Temperature of ultrafiltration was 25° C.

Amino Acid Analysis

A Beckman Model 120C Analyzer equipped with a Beckman Model 125 Integrator was used. Samples were hydrolyzed at 110° C. *in vacuo* for 24 hr in 6 N HCl. Half-cystine and methionine were determined as cysteic acid and the sulfone, respectively (10).

TABLE I
Dissociation of Phytin-Protein Complex Measured by Dialysis

Conditions		Days of Dialysis				
		1	2	4	6	
pH	Solvent	% P Removed				gP/100 g Protein[a] at 6 Days
8.5	0.026M Borate (Na^+)					
	+ EDTA	33	-	62	80	0.24
	−EDTA	25	-	42	65	0.42
8.5	0.05 M *tris* (Cl^-)					
	+ EDTA	23	41	55	72	0.33
	−EDTA	26	47	47	50	0.60
7.2	0.05 M *tris* (Cl^-)					
	+EDTA	44	54	67	85	0.18
	−EDTA	47	57	72	82	0.21
5.5	Water					
	+ EDTA	62	85	88	95	0.06
	−EDTA	70	88	93	95	0.06
2.0	~0.01 N HCl	17	28	22	31	0.82

[a]Original extract used in experiments contained 1.20 gP/100 g protein.

RESULTS AND DISCUSSION

Dialysis Studies

Shown in Table I are the results of the dialysis studies which are an indication of the amount of phytate not bound to the protein. As can be seen, and as was initially observed by Fontaine et al. (11), the amount of phosphorus removed by dialysis is a function of pH. At pH 5.5, near the isoelectric point of soybean globulins, 95% of the phosphorus can be removed to result in an essentially phytate-free isolate. Below the isoelectric point at pH 2.0, only 30% of the phosphorus is permeable, the remainder being firmly bound to the protein. Since 70% of the total phosphorus in the meal is phytate (1) and this value corresponds closely to the measured amount of nonpermeable phosphorus, it is concluded that the majority of the extracted phytate is firmly bound to the protein at pH 2.0. Besides phytate, an additional source of nondialyzable phosphorus is a ribonucleoprotein fraction, representing about 3%, by weight, of the total soybean globular protein (12). Phytate binding below the isoelectric point of proteins is known to be a result of the strong electrostatic interaction between the cationic residues of the protein (lysyl, histidyl, arginyl, and amino terminal groups) and the anionic phosphate esters of phytate (13). In the absence of EDTA, phytate dissociation above the isoelectric point of soybean globulins is seen to decrease as the pH at which dialysis is performed increases. At pH 8.5 in the *tris* and borate buffers, phosphorus permeability is less than that measured at pH 7.2 in *tris*-buffer. Studies on phytate binding to soybean globulins (14) have indicated that calcium ion mediates binding above the isoelectric point of the

proteins. In support of this observation is the fact that the permeability of phosphorus is greater in both buffers at pH 8.5 when 0.01 M EDTA is present than when it is absent. Control experiments were performed at pH 7.2 and 5.5, conditions wherein calcium chelation by EDTA is minimal and nonexistent, respectively. At these pH values, dissociation of phytate, as judged by phosphorus permeability, was found to be the same in the presence and absence of 0.01M EDTA. Thus, chelation of calcium and other multivalent cations, such as magnesium, above the isoelectric point of the protein, facilitates the dissociation of phytate-protein complexes.

Effect of Phytase on Phytate Removal

The pH and temperature optimum of plant phytases is usually in the vicinity of pH 5.5 and 55°C (15). Therefore, at pH 5 and 65°C, our conditions of diafiltration, sufficient phytase activity is present to effect phytate removal. Table II illustrates the effect of phytase on promoting phytate removal from soybean meal extracts by diafiltration. The efficiency of phytate removal of two extracts is compared. One extract contains the indigenous enzyme; in the other extract the enzyme has been heat inactivated. A 90% removal of phosphorus was achieved in the extract having active phytase, whereas 74% was removed in the extract having no phytase activity. Note the preponderance of inorganic phosphate (P_i) in both the retentate and diafiltrate of the phytase-containing extract over those of the phytase-inactivated extract. Thus, phytase facilitates phytin removal from soybean globulins around pH 5, a pH coincidental with the isoelectric point, as observed initially by Fontaine (11). Presumably, phytase acts upon free phytate, thus causing a further dissociation of phytate from the

TABLE II
Effect of Phytase Activity upon Phosphorus Removal during Diafiltration with Water, pH 5.0, 65°C.

	Unboiled Extract (Phytase)[a]			Boiled Extract (No Phytase)[b]		
	Starting Extract	Final Retentate	Ultra-filtrate	Starting Extract	Final Rentenate	Ultra-filtrate
gP/100 g Protein	1.48	0.15	-	1.30	0.34	-
Total P (mg)	335	34	309	334	87	247
(P_i/Total P) × 100[c]	13%	34%	46%	10%	0.4%	16%
Total P Removed from Extract		90%			74%	
V_D		10.2			14.2	
Protein Retained During UF (biuret)		100%			100%	

[a]The unboiled extract prior to ultrafiltration liberated 2.7 γ P_i/hr/ml extract (in the presence of sodium phytate) when assayed by the method of Peers (8).
[b]The extract was heated in a boiling water bath 15 min at pH 8.5, after which no phytase activity could be detected by assay.
[c]P_i denotes inorganic phosphate.

TABLE III
Analytical Values of Soybean Protein Isolates Prepared by Diafiltration[a]

	% N	% P	gP/100 g Protein	% Recovery from Flakes		
				Wt	N	P
EDTA, pH 8.5	16.0	0.58	0.58	43	77	32.3
Phytase, pH 5	15.2	0.22	0.23	46	79	13.2
Ca^{++}, pH 3	15.3	0.19	0.20	46	78	7.0

[a]Expressed on a dry-weight basis.

protein-phytate complex in addition to that resulting from removal by diafiltration. No loss of protein during diafiltration occurred in either the boiled or unboiled extract as measured by the biuret reaction. Generally, a 10%-protein loss occurred during diafiltrations (see Table III) as measured by Kjeldahl nitrogen. This disparity between biuret and Kjeldahl measurements may be due to the relative insensitivity of the former and reflect the error inherent in the biuret analysis. On the other hand, defatted soybean meal contains about 8.5% nonprotein nitrogen (16); therefore, the biuret measurements may, in this situation, be a more accurate determination of protein content than the Kjeldahl nitrogen.

Phytate Removal by Diafiltration

Shown in Fig. 1 are the results of diafiltration experiments in which the logarithm of the per cent total phosphorus residing in the retentate is plotted against V_D. Little or no removal of phosphorus occurs either at pH 8.5 in the absence of EDTA or at pH 3.0 in the absence of calcium ion. These results are in accord with the dialysis experiments presented previously. Of the three possible methods of phytate removal, the use of EDTA at pH 8.5 and 65°C appears the least effective in comparison to the phytase method at pH 5 and the calcium ion/pH 3 method. In the last-mentioned method, the phytate, presumably, is displaced from the cationic groups of the protein by the high concentration of calcium ion; both species compete for the phytate through the law of mass action. Although the calcium ion/pH 3 method, because of the low pH, can be used at ambient temperature with little danger of microbial growth, soybean globulins are known to dissociate into subunits and to be denatured at this pH (17). Therefore, the three methods of phytate removal described in this paper may not be applicable to isolation of native globulins due to the harsh conditions of temperature or pH employed. The possibility of significant protein hydrolysis occurring at pH 8.5 and 65°C during diafiltration appears remote because of the high retention of protein measured by both the Kjeldahl (90%) and the biuret reaction (100%). The high temperature, 65°C, employed at pH 5, where the protein already is insoluble at ambient temperature, may cause a reduction of solubility on either side of the isoelectric point. However, these methods are applicable in principle to the production of protein isolates for food use. Shown in Table III are analytical values of isolates prepared by the three methods of phytate removal. The isolates all contain more than 90% protein, but the phosphorus content of the isolate prepared by the pH 8.5/EDTA method is

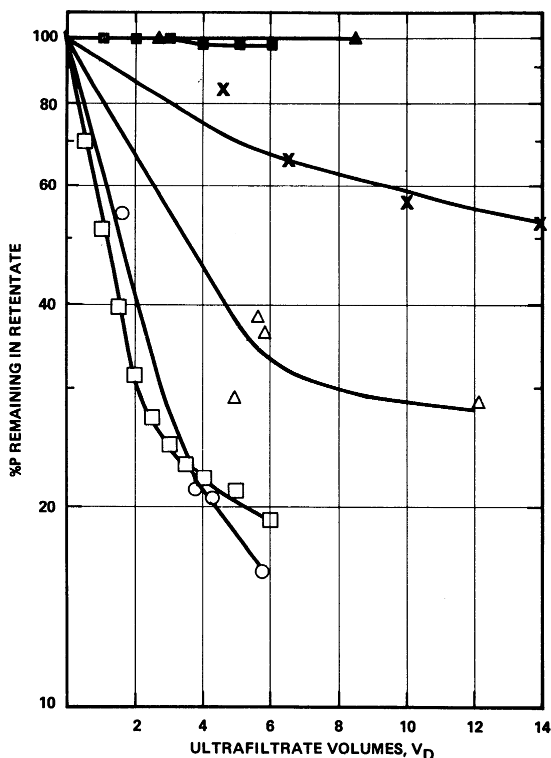

Fig. 1. Removal of phytate as measured by phosphorus remaining in retentate during diafiltration: (▲) pH 8.5, 0.015 M Na borate, 65°C; (△) pH 8.5, 0.01 Na carbonate/bicarbonate, 65° C − 0.05 M EDTA initially present in extract; (×) pH 7.1, Na carbonate/bicarbonate, 65° C; (O) pH 5.0, water, 65° C; (■) pH 3.0, water, 25° C; (□) pH 3.0, 0.5 M $CaCl_2$, 25° C.

TABLE IV
Essential Amino Acid Composition of Soybean Protein Isolate Prepared by Diafiltration at pH 5, 65° C.

	g aa/16 g N	
	Isolate	Promine D[c]
Lysine	6.9	6.0
Cystine/2	1.4[a]	0.9
Valine	5.2	5.0
Methionine	1.4[b]	1.0
Isoleucine	5.3	4.9
Leucine	8.1	8.1
Phenylalanine	5.5	5.6
Tyrosine	4.1	3.6

[a]Determined as cysteic acid.
[b]Determined as sulfone.
[c]Taken from the Product Information Sheet issued by Central Soya Co., Chicago, Ill.

TABLE V
Preparation of Soybean Protein Concentrates[a]

Process	% Retained from Flour		Concentrate		
	N	P	% Protein	% P	gP / 100 g Protein
Diafiltration	90	17	79	0.24	0.30
Acid leaching	89	52	78	0.47	0.60

[a]Analytical values expressed on a dry-weight basis.

significantly higher than that of the other isolates. For all methods, about 78% of the flake nitrogen is recovered in the corresponding isolates. The pH 3/calcium-ion method is apparently the most effective method of phytate removal. Table IV indicates that the essential amino acid composition of the isolate prepared by the pH 5/phytase procedure compares favorably with that of Promine D, especially in terms of lysine and the sulfur-containing amino acids.

Soybean-Protein Concentrates by Diafiltration

Diafiltration is an efficient way to prepare protein concentrates low in phytate from defatted soybean meals and flours. This efficiency is evident when phytate removal under conditions of high nitrogen retention is comparatively examined on Nutrisoy 7B flour by using diafiltration vs. the conventional process of leaching with water acidified to pH 4.5 with HCl. The data in Table V illustrate the differences observed during preparation of concentrate by both procedures. In the case of the conventional process, acid leaching was repeated five times using an overall amount of water in five equal portions equal to the total filtrate collected by diafiltration of the other sample. Prior to acid leaching, the flour was incubated at pH 5, 65°C for the same length of time used for the diafiltration of the other sample in order to subject both samples, equally, to the action of phytase. The results indicate that the total phosphorus content of the concentrate prepared by diafiltration is half that of the concentrate prepared by acid leaching, whereas the protein content of both concentrates is identical.

Ultrafiltration is becoming an economically feasible operation in the food-processing industry (18). Hence, the methods described in this work appear promising for the manufacture of low-phytate soybean-protein isolates and concentrates of high-nutritional quality. Since the experiments described here have not been performed on a pilot plant scale, it would, therefore, be difficult to make a cost estimate and comparison with the conventional process of isolate and concentrate preparation.

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