Isolation and Identification of the Multiple Forms of Soybean Phytases

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ABSTRACT: Sequential precipitation with polyethylene glycol (PEG) and KCl followed by preparative anion exchange high-performance liquid chromatography was used to isolate acid phosphatases (APases) from germinating soybean seeds. KCl inhibited the PEG precipitation of APases. Thus individual APases can be isolated directly from crude extracts by controlling PEG and KCl concentrations. Four of the isolated APases were phytases. APases 3 and 5 exhibited optimal activity at pH 4.5 and 3.0 and APases 4 and 6 had a dual pH of 3.0 and 5.5 and 3.5 and 5.8. Phytase activity was at its peak at 40°C for APase 4 and at 60°C for APases 3, 5, and 6. This isolation method can be used to identify endogenous and/or exogenous phytases that are suitable for phytic acid hydrolysis in many food and feed systems, especially during the processing of oilseeds and cereal-based ingredients.
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KEY WORDS: Food and feed processing, isolation, phytate hydrolysis, soy phytases.

The hydrolysis of phytic acid in foods increases the bioavailability of essential minerals, protein, and phosphorus. Phytase is an acid phosphatase (APase) that is capable of the stepwise hydrolysis of phytic acid, *myo*-inositol 1,2,3,4,5,6-hexakisphosphate, to inorganic phosphate and *myo*-inositol. Thus phytases can improve the nutritional value of plant-based foods through reduction of phytic acid during food processing such as soaking, germination, fermentation, and heat treatment in cereals, tubers, legumes, and oilseeds (1). Beleia *et al.* (7) showed a 36.1% reduction of soybean phytate during soaking in water for 4 h at 50°C. Substantial reduction in phytate content was observed during the germination of many seeds including lettuce (8), barley (9), wheat (10,11), corn (12), fava bean (13), pea (7), canola (14), and rice (6). The reduction of phytate by yeast fermentation in breadmaking also has been shown to be substantial (15–17). Other processes such as oriental food fermentation (18), preparation of soybean tempeh (19,20) and cooking (21) also affect phytate hydrolysis.

About 50–70% of the phosphorus in cereal grains and oilseed meals occurs as phytate (22,23). Ruminants readily utilize phytate phosphorus because of the abundance of phytase produced by ruminal microorganisms. However, phosphorus in this form is poorly utilized by pigs and chicks because they lack phytase. Numerous investigators used fungal phytases to improve the utilization of phosphorus, calcium, magnesium, zinc, and protein in animal feed (22,24–26). Cromwell *et al.* (26) demonstrated the effectiveness of a recombinant-derived phytase in improving the bioavailability of phosphorus in animal feed.

Conventional precipitation and chromatography methods have been used to isolate and purify phytases from many plant and microbial sources. However, due to the abundance and the multiple forms of APases, isolation of phytases from cell extracts is difficult and usually results in preparations with mixed APase activities. Nayini and Markakis (27) gave an excellent review of the purification and characterization of phytases of plant and microbial origins. Phytases with diverse kinetic properties have been isolated from many plant sources including most legumes and cereals. Singh and Mirtakharaee (28) were the first investigators to purify and characterize one form of soybean Phytase. Sutard and Buckle (29) and Gibson and Ullah (30) later carried out a slightly different purification scheme to update some of the characteristics of this soybean phytase. The pH optimum for soybean phytase was 4.2–4.8, which is typical of many APases as well as phytases in plants (27). The optimum enzyme activity was at 55–57°C and was thermally unstable at 70°C for 10 min.

The multiple forms of phytases in microorganisms have been first reported by Shieh *et al.* (31) in *Aspergillus ficuum*. Irving and Cosgrove (32) used cation-exchange dextran chromatography to obtain five phytase peaks from *A. ficuum* culture filtrate. Hamada (33) used polyethylene glycol and anion high-performance liquid chromatography (HPLC) to isolate from *A. ficuum* two phytases with a dual pH optima of 2.5 and 4.0 and 3.0 and 4.5, and three that had only one optimal activity peak at pH 2.5, 3.0, and 5.0. Recently, Ullah and Philippy (34) found the extracellular *A. ficuum* APase with pH optimum of 2.5 that they had previously reported as nonspecific APases, to be a potent phytase at pH 2.5. Lim and Tate (35) were the first to report the multiple forms of phytases from plant origin. They used diethylaminoethyl cellulose (DEAE) to separate two distinctive phytase fractions

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from wheat bran. Multiple forms of plant phytases were also found in barley (36), aleurone protoplasts of *Avena fatua* (37), rice (38,39), and soybean storage proteins (40).

Depending on ingredients and processing conditions during processing of oilseeds and cereal ingredients, exogenous phytases must be added or endogenous phytases must be isolated and concentrated to reduce the phytate content in the final products to a nutritionally acceptable level. Isolating and accurately identifying the phytases from any microbial or plant source can have an impact on obtaining effective phytases for use in the removal of phytic acid during food and/or feed processing. Further, identifying APases that are suitable for food and feed processing can help in developing low-cost stable effective phytases *via* recombinant DNA techniques and site-directed mutagenesis. The objectives of this research were to isolate all APases from germinated soybean seeds by preparative-scale precipitation and chromatography methods and to isolate from them and characterize the soybean phytases.

MATERIALS AND METHODS

Soybean seeds of the variety "Pioneer 1667I" were from Pioneer HiBred International Inc. (Des Moines, IA). Unless noted otherwise, laboratory grade chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Soybean seeds were cleaned, treated with 0.5% NaOCl, and rinsed with 10 vol of water. Germination was carried out at 20°C in trays containing three layers of germination paper for six days. Cotyledons were extracted for 1 min at 4°C in Waring blender (New Hartford, CT) with 1:1 (wt/vol) of 0.1 M sodium acetate, pH 5.5, containing 20 mM $CaCl_2$, 1 mM dithiothreitol, and 1 mM phenylmethyl sulfonyl fluoride. After filtration and centrifugation at 5,000 × *g* and 4°C for 20 min, the extract was lyophilized.

Fractionation of soybean APases by polyethylene glycol precipitation. The design of the experiment for the PEG precipitation of the crude extract of six-day-old germinated soybean seeds was based on the PEG precipitation method developed for *A. ficuum* filtrate (33). Average molecular weight of PEG was 4,600. Initially, proteins of the soy cell extract were consecutively precipitated by PEG at increasing levels of PEG concentration (2.5–20% at 2.5% intervals) in 0.1 M acetate buffer (pH 5.5) and 0.1 M KCl. Precipitation was monitored by assaying the precipitates after dispersing in 0.1 M acetate buffer by *p*-nitrophenol phosphate (NPP) at pH 4.0 and 60°C. Individual precipitation curves for the APases were then prepared by precipitation at narrower PEG concentration range for each precipitate. These precipitation curves were prepared by assaying precipitates dispersed in 0.1 M acetate buffer (pH 5.0) at 60°C then plotting the absorbance at 410 nm against PEG concentration. Based on these curves, precipitation of soybean APase from the freeze-dried soybean extract (300 mg dispersed in 100 mL) was carried out at 3, 7.5, 10, 13, and 18% PEG concentration in 0.1 M KCl and 0.1 M extracting acetate buffer (pH 5.5). The 3% PEG precipitate was further fractionated into two PEG precipitates at 1.5 and 4.5% PEG and 0.5 M KCl concentrations, according to Figure 1.

Preparative quaternary methylamine (QM) anion-exchange HPLC. The QM anion-exchange preparative HPLC separation of crude extract and PEG precipitates was performed at 5°C on a steel column (25 mm x 30 cm) packed with Accell Plus QMA medium (Waters, Milford, MA) using a preparative chromatography unit "Delta Prep 3000" from Waters. Elution programs, detection, and computer integration of the eluted peaks, and collection of fractions were carried out as previously described (33).

Assays for phytase and APases. Cell extract, precipitates, and HPLC eluents were assayed using 1.0 mL of diluted enzyme solution or eluent and 1.0 mL of substrate solution containing 0.2 μmoles in 0.2 M of acetate or citrate buffer and HCl to adjust the pH to desired value. Reaction mixtures containing NPP or phytic acid were incubated at 60°C for 10–60 min. The P_i released from NPP or phytic acid after reaction with NaOH or molybdate reagent was determined by reading the absorbance respectively at 410 or 355 nm and using standard curves. The effect of pH (2.1–6.5) on the nonspecific and phytate-specific APases was determined in 0.1 M sodium citrate buffer, using NPP or phytate as the substrates, respectively, at 60°C. The effect of temperature on activity was determined at 30, 40, 50, and 60°C in 0.1 M acetate buffer (pH 5.0) using NPP or phytate as the substrate.

Protein content of APases was measured by the macro or micro method of Bradford (41) using "Coomassie Plus Protein Assay Reagent" from Pierce Co. (Rockford, Il).

RESULTS AND DISCUSSION

Precipitation of soybean APases by PEG. APases in six-day germinated soybean seeds were fractionated by PEG precipitation into six APases. To determine the PEG precipitation range of each APase, proteins of the soy cell extract were consecutively precipitated by PEG at increasing levels of PEG concentration. Individual precipitation curves for the APases were then prepared by precipitation at narrower PEG concentration range for each precipitate (e.g., the 2.5 and 7.5% fractions shown in Fig. 2). The plot of 2.5% PEG precipitate contained two APases with their precipitation peaked at 0.5 and 2.0% PEG when no KCl was used. Two peaks were obtained at 1 and 2.5% PEG upon using 0.1 M KCl. The precipitation of the 7.5% PEG fraction was also inhibited by KCl as its precipitation in 0.1 M KCl peaked at 7.5% PEG and its precipitation in 0.5 M KCl was at 13.5% PEG.

From the data of precipitation curves at various KCl concentrations, the phosphatases of the cell extract were precipitated into five fractions using 3, 7.5, 10, and 18% PEG, and 0.1 M KCl. The 3% PEG precipitate was further separated into two precipitates using 0.5 M KCl at 1.5 and 4.5% PEG. The precipitation curves of the 1.5% and the 10% frac-

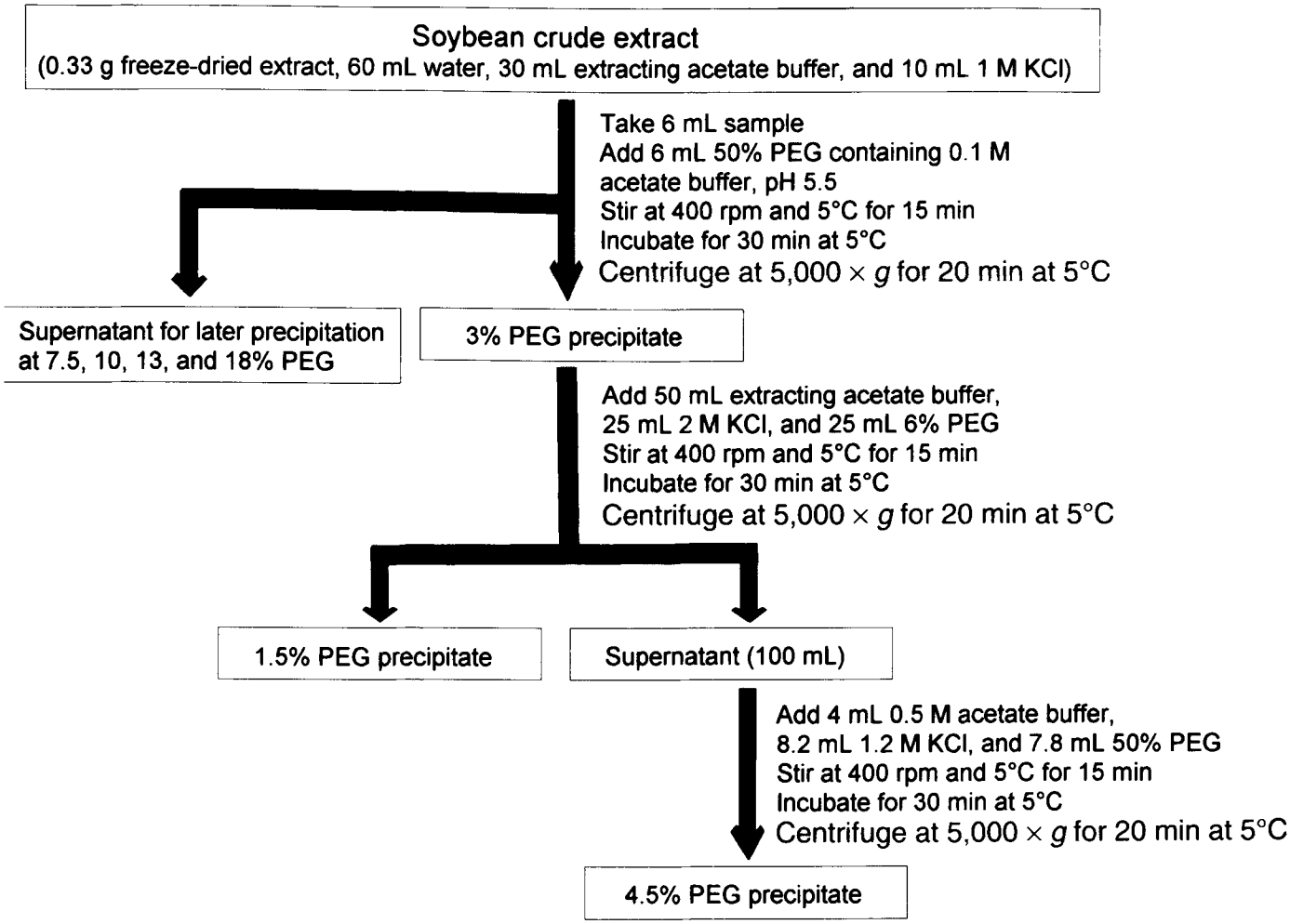

FIG. 1. Flow chart for the precipitation of two acid phosphatases (APases) from cell extract of germinating soybean by polyethylene glycol (PEG).

tions prepared by PEG reprecipitation at corresponding PEG levels and 0.5 and 0.1 M KCl concentration, respectively, are presented in Figure 2. Accordingly, KCl inhibited the PEG precipitation of APases, and thus individual APases can be initially isolated and concentrated directly from crude extracts by controlling PEG and KCl concentrations. One-half the PEG concentration was needed to effect the precipitation of all the soybean APases as compared to fungal APases where up to 40% PEG was used (33). Furthermore, the precipitation of *A. ficuum* phosphatases using PEG occurred more readily than with soy APases as evidenced by the lack of overlap between the phosphatases in precipitation curves.

The lyophilized cell extract had specific activities of 2.1 nmol/s/mg using NPP and 0.22 nmol/s/mg using phytic acid as the substrate at pH 5.0 and 60°C. From 300 mg of freeze-dried cell extract, which contained 90 mg protein, 1.7, 4.5, 19.1, 5.8, 4.9, and 4.2 mg protein were recovered respectively in the consecutive precipitates. Thus, PEG purification resulted in six APase fractions and removed nearly half of the non-APase proteins from the cell extract. The amounts of phosphate that were released after incubation of consecutive precipitates with NPP or phytate in sodium acetate buffer (pH 5.0) at 60°C are shown in Table 1. Degree of purification was calculated based on the actual specific activities of PEG precipitate and the protein content recovered from the cell extract for each APase.

Anion exchange separation of APases of cell extract and its PEG precipitates. Anion-exchange HPLC for the separation of soybean cell extract and its PEG precipitates is shown in Figure 3. The APases obtained from the cell extract by PEG separation were further fractionated on the anion-exchange HPLC column at 6 mL/min using 0.1 M Tris-HCl buffer (pH 8.0) and the KCl gradient scheme illustrated in Figure 3. Each PEG precipitate contained either one large APase peak or one major APase peak and one minor peak as can be seen for the 1.5% PEG precipitate (Fig. 3). The pooled peaks contained 0.3, 0.4, 1.6, 0.5, 0.4, and 0.3 mg protein. The activities of the nonspecific APases and phytases per peak using, respectively, NPP and phytate in sodium acetate buffer (pH 5.0) at 60°C are given

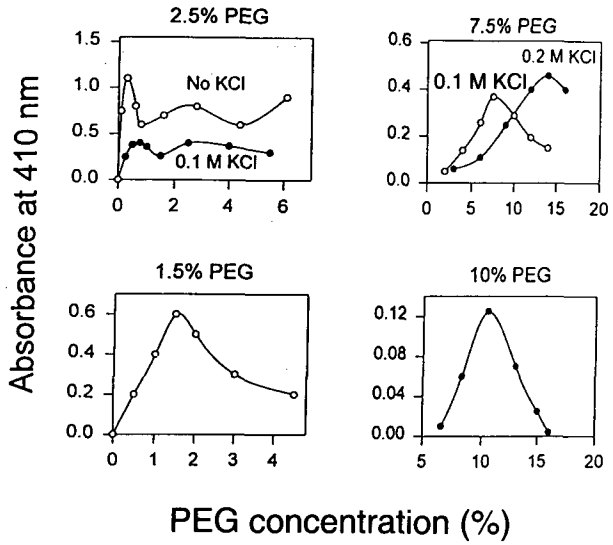

FIG. 2. PEG precipitation curves of APases isolated from cell extract of germinating soybean. The first two APases were respectively prepared by precipitation at 2.5 and 7.5% PEG concentrations in the presence of 0.1 M KCl. The 1.5 and 10% PEG fractions were reprecipitated from 3 and 10% PEG precipitates in the presence of 0.5 and 0.1M KCl, respectively. Precipitated fractions were assayed in 0.1 M sodium acetate buffer, pH 5.0 for 15 min at 60°C using *p*-nitrophenol phosphate. See Figure 1 for other abbreviations.

in Table 1. Comparing the nonspecific APase and phytase specific activities of the peaks (column 5) and PEG precipitates (column 3), it appears that the anion-exchange HPLC resulted in 4.5, 8.5, 11.2, 10.9, 11.0, and 10.5-fold purification of these enzymes. The total activity recovered in all peaks was 92.8% of the total activity in the six individual injections of the PEG protein precipitates.

The cell extract (12 mg containing 3.6 mg protein) was also fractionated by anion HPLC onto six overlapping peaks (Fig. 3). Nonspecific APase activities were 0.9, 1.1, 1.8, 0.9, 0.8, and 0.9 nmol/s, and the phytase activity was 0.04, 0.04, 0.13, 0.15, 0.28, and 0.16 nmol/s, respectively, for the isolated peaks in their order of elution. These peaks contained 13, 14, 16, 8, 6, and 8% of the total protein, respectively. The total nonspecific APase or phytase activity of all peaks was 96% of the activity in the injected extract. HPLC of cell extract resulted in 18, 9, 3, 6, 7, and 7-fold purification of the soybean APases. Considering the protein content of cell extract as the basis for calculating the fold-purification, the degree of purification of APases of crude extract, averaging 8-fold, was much less than the rates obtained by PEG fractionation followed by HPLC.

Singh and Mirtakharaee (28) isolated and purified a soybean phytase to 4-fold by ammonium sulfate precipitation and DEAE-cellulose chromatography. Gibson and Ullah (30) used ammonium sulfate (30–60% saturation) to precipitate part of the APases of 10-day-old germinating soybean seeds. The precipitate was fractionated by cation-exchange chromatography into a nonspecific APase and a phytase fraction. Gel chromatography and chromatofocusing were used to increase the purification of both enzymes from 9- to 22-fold. Sutard and Buckle (29) partially purified one phytase from soybean cell extract 100-fold using 35–80% ammonium sulfate and DEAE-cellulose chromatography. Comparing the separation data here with the methods used to purify the phytase in these three papers is difficult because their preparations were restricted to the APases that could be precipitated with ammonium sulfate at high saturation levels. These preparations could be mixtures of phytase activities. However, it is likely that their phytase preparation might have consisted mainly of the same proteins of APases 5 and 6 precipitated here at relatively higher PEG concentration.

TABLE 1
Purification of Soybean Acid Phosphatases by PEG Precipitation and Anion HPLC[a]

Enzyme and substrate	PEG precipitates		HPLC recovered peaks		
	Total activity (nmol/s)	Specific activity (nm/s/mg)	Total activity (nmol/s)	Specific activity (nm/s/mg)	Fold purification
NPP					
APase-1	35	21	24	80	4
APase-2	30	7	28	70	11
APase-3	20	1	18	11	11
APase-4	25	4	22	44	10
APase-5	25	5	25	63	12
APase-6	40	10	30	100	11
Phytate					
APase-1	1	0.6	1	3	5
APase-2	2	0.4	1	3	6
APase-3	3	0.2	3	2	11
APase-4	4	0.7	4	8	12
APase-5	8	1.7	7	16	10
APase-6	8	1.9	6	20	11

[a]Starting cell extract contained 90 mg protein and activity was determined in *p*-nitrophenolphosphate (NPP) or phytic acid in sodium acetate buffer, pH 5 at 60°C to assay nonspecific acid phosphatases and phytases, respectively. PEG, polyethylene glycol; HPLC, high-performance liquid chromatography; APase, acid phosphatase.

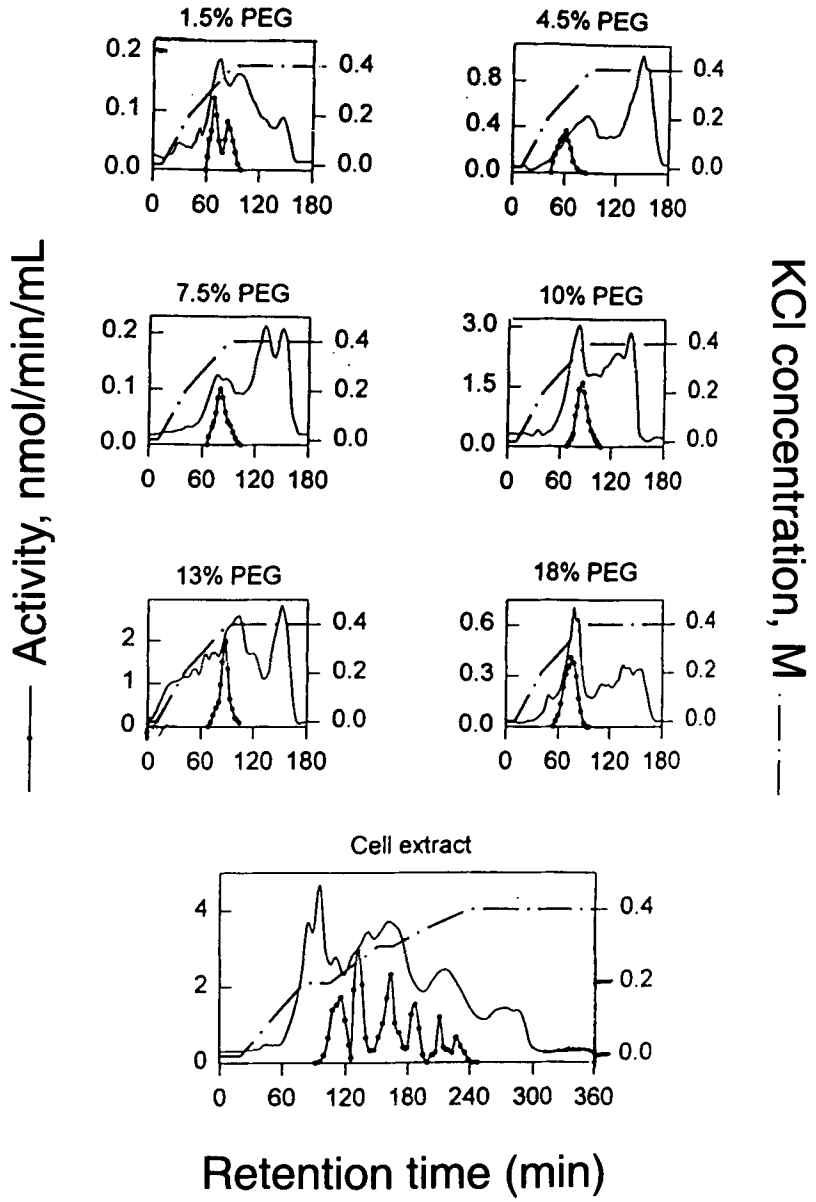

FIG. 3. Anion exchange separation of APases of cell extract and its PEG precipitates. Conditions: Waters Delta Prep 3000 using a steel column (25 mm x 30 cm) packed with Accell Plus QMA medium (Waters, Milford, MA) at 6 mL/min with protein detection at 280 nm (——) and a gradient from 0 to 80% 0.5 M potassium chloride in 0.1 M tris-HCl buffer, pH 8.0 (——). Protein loads were 2, 5, 19, 6, 5, and 4 mg for the 1.5, 4.5, 7.5, 10, 13, and 18% PEG fractions, respectively, and 4 mg for the cell extract. Activity of enzyme fractions (—o—) were assayed for one hour in 0.2 M sodium acetate buffer, pH 5.0 at 60°C. See Figure 1 for abbreviations.

Effect of pH on the nonspecific APase and phytase activity. The effect of pH on the activity of the cell extract and the HPLC fractions of PEG precipitates using NPP and phytate as the substrates is shown in Figures 4 and 5. In Figure 4 (A), the NPP activity of the cell extract reached the peak at pH 4.5 with the hydrolysis of 182 nmol/s of the phosphates per 100 mg protein. In Figure 4 (B), showing the changes in the phytase activity at various pH values, there were two peak pH points (4.0 and 5.5) where the activity was 49 and 32 nm/s/100 mg protein. The effect of pH on the APase and phytase activity of the anion-exchange peaks of PEG precipitates is shown in Figure 5 (A and B, respectively). As with cell extract, the highest nonspecific APase activity for all peaks (i.e., total activity) was at pH 4.5. The total phytase activity of all chromatographic peaks reached the maximum at pH 3.5 and at pH 5.5. Individually, the optimal APase activity was 5.0 (and 5.8); 4.0; 5.5; 4.5 (and 5.8); 4.0; and 2.5 (and 5.3) for the HPLC peaks of the 1.5, 4.5, 7.5. 10, 13, and 18% PEG precipitates, respectively. Optimal phytase activity of the HPLC peaks was at pH values of 4.5, 4.0 (and 2.5), 4.5, 3.0 (and

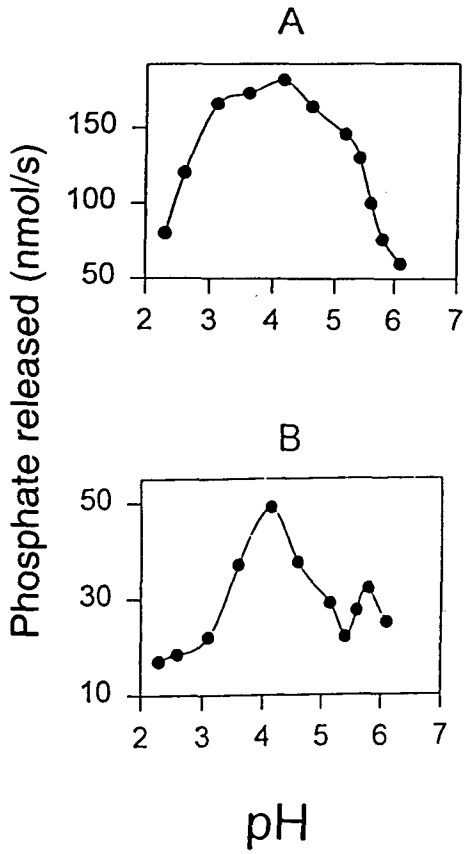

FIG. 4. Effect of pH on the activity of cell extract (100 mg protein) in 0.1 M sodium citrate buffer, pH 2.1–6.5 at 60°C using *p*-nitrophenol phosphate (A) or phytic acid (B) as the substrate.

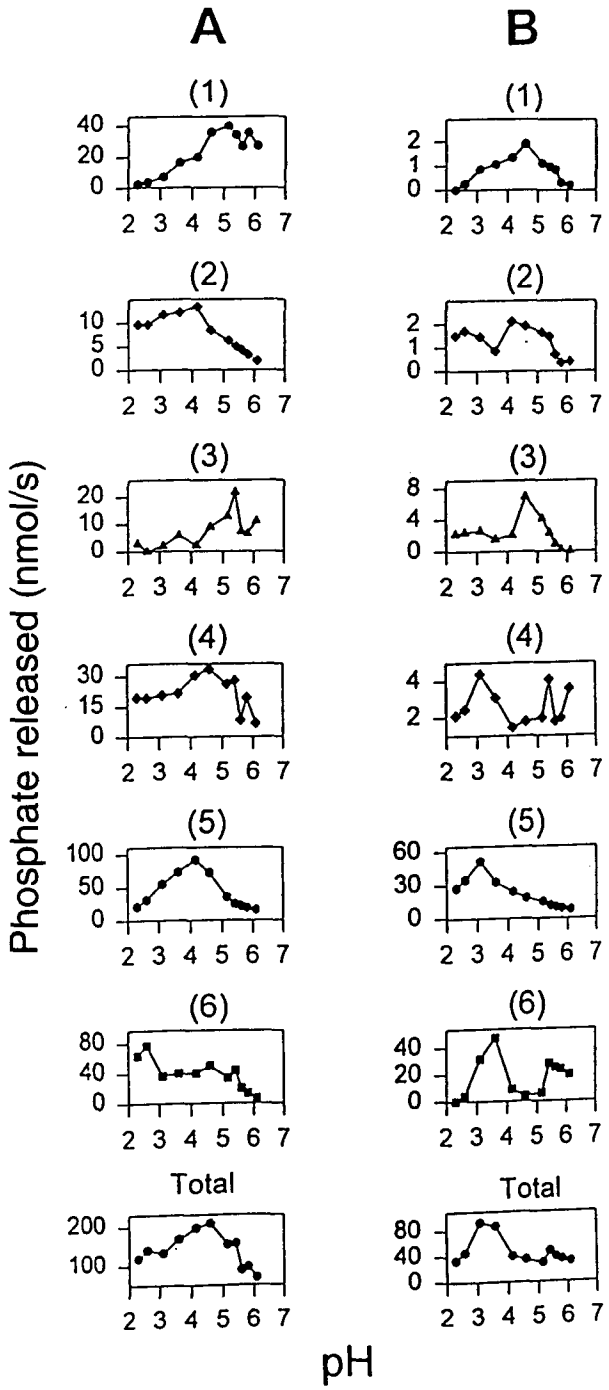

FIG. 5. Effect of pH on the activity of PEG precipitates that were further separated individually by anion-exchange in 0.1 M sodium citrate buffer, pH 2.1–6.5 at 60°C using *p*-nitrophenol phosphate (A) or phytic acid (B) as the substrate. Graphs 1–6 represent the activities of high-performance liquid chromatography peaks of the six consecutive PEG precipitates in increasing order of PEG concentration. See Figure 1 for abbreviation.

5.5), 3.0 and 3.5 (and 5.8), respectively for the consecutive precipitates.

Phytases have a broad range of their pH optima. The pH of optimal activity can be as low as 2.2 in Pichia farinosa (27) or 2.5 in *A. ficuum* (33) or can be in the slightly alkaline range such as 7–8 for most phytases of the animal intestinal mucosa (27). Phytase from barley, navy bean, mung bean, rice, soybean, wheat bran (fraction F_1), and wheat bran (fraction F_2) had pH optimum of 5.2, 5.2, 7.5, 4.2, 4.9, 5.6, and 7.2, respectively (27). The separation method used here was derived from a previous isolation scheme developed for the *A. ficuum* APases (33). The optimum pH for phytate-specific and nonspecific activity of the seven fungal APases was more acidic than that of the six soybean APases using phytate and NPP as the substrates, respectively. APases 3–7 were phytate-specific APases. Table 2 summarizes the pH optimum of the soybean and fungal phytases.

Effect of temperature on the activity of phosphatases. The effect of temperature on the activity of cell extract and the HPLC peaks of PEG precipitates using NPP (A) or phytate (B) as a substrate is shown in Figures 6 and 7, respectively. Nonspecific activity increased linearly with increasing temperature. The cell extract activity at 60°C was 194 nm/s per 100 mg protein (A of Fig. 6). The effect of temperature on the activity of the phytases differed from that of the nonspecific APases. Phytase activity increased linearly with temperature but declined at 50°C. Of the four temperatures examined, phytase was highest at 60°C (27 nmol/s per 100 mg protein)

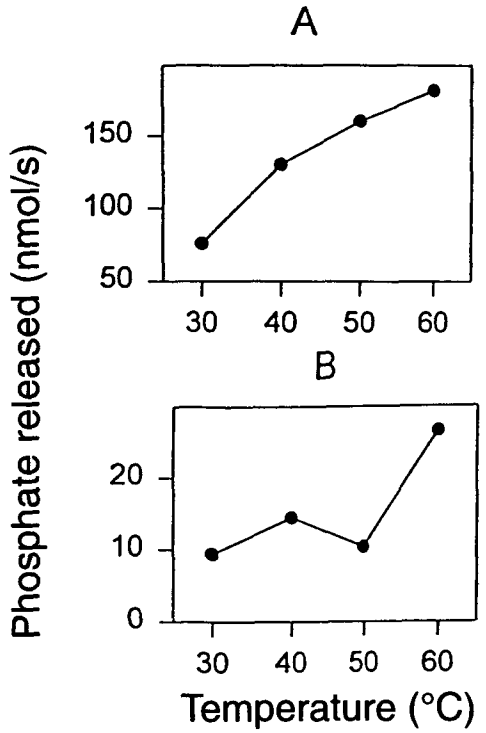

FIG. 6. Effect of temperature on the activity of cell extract (100 mg protein) in 0.1 M sodium acetate buffer (pH 5.0) at 30–60°C using *p*-nitrophenol phosphate (A) or phytic acid (B) as the substrate.

and lowest at 30°C (9 nmol/s per 100 mg protein) (B in Fig. 6).

The activity of individual nonspecific APases obtained from the anion-exchange peaks of PEG precipitates and their total increased with temperature. As with the effect of temperature on the activity of the cell extract, total phytase activity increased with temperature and was the highest at both 40 and 60°C (14 and 27 nmol/s, respectively) (Fig. 7). The phytases of 10, 13, and 18% PEG peaks each had almost equal phytase activities at 30–50°C. The optimum temperature for the nonspecific APases and the phytase (30) that were isolated from 10-day germinated soybeans was 60 and 55°C, respectively. Although the optimal temperature for the activity of plant phytases varies among sources, it has a narrow range of 45 to 57°C (27). For instance, the phytases from soy and mung beans belong to the upper range of 57°C, and corn, rice, and yeast had optimal temperature of 45°C.

Multiple forms of phytases and their significance. This study classified the APases of soybean into two groups containing nonspecific APases and phytases. Since the phytase activity of the APases -1 and -2 was negligible, they must belong to the first group in which APases are capable of hydrolyzing a number of phosphoesters but not phytic acid. The rest of soybean APases are considered phytate-specific APases or phytases since they readily hydrolyzed phytic acid as well as NPP as a general phosphoester substrate. Many investigators have separated multiple forms of phytases from plant and microbial origins. These endogenous phytases can play a major role in the reduction of phytic acid during processing of cereals, tubers, and legumes. Such processes contribute variably to reduction of phytate levels in the final products. For instance, Knorr *et al.* (17) used commercial phytase and nonspecific phosphatase to reduce phytic acid in whole wheat flour-yeast doughs up to 92% reduction of its initial value. The effectiveness of the exogenous nonspecific APase (as assayed at pH 5.0 and 60°C) was explained by the similarity of its optimal pH to the pH of the dough. In the event the level of endogenous phytases at certain processing conditions is relatively small, such as in rye (2), it may be necessary to add pure exogenous phytases or phytase-rich fractions from other cereals for the effective removal of phytate. The activity of soybean phytase is considerable and compares well with the activity of many other oilseeds and cereal sources such as rice bran (38,39). This can have an impact on the effective removal of phytic acid during food processing. Also, the benefits of microbial phytases in animal feeding is substantial.

Therefore, isolating and accurately identifying the phytases from any microbial or plant source can be of great value to the molecular biologist and food or feed processor alike. Identification of all the forms of phytase from any microbial or plant source will help in increasing the effectiveness of the APases during processing. This is accomplished by the selection of the proper phytase that can be tailored to the process. Therefore, identification of the phytases is important in optimizing phytate reduction during processing. Also, identifying the phytases with the desired kinetic parameters can be help-

TABLE 2
Optimal pH for the Activities of APases in Germinating Soybean and *Aspergillus ficuum*

	Soybean			*A. ficuum*[a]		
	%PEG	Substrate—pH		%PEG	Substrate—pH	
APase	(KCl, M)	NPP	Phytate	(0.05 M KCl)	NPP	Phytate
1	1.5 (0.5)	5.0 and 5.8	—	4	3.0	—
2	4.5 (0.5)	4.0	—	9	2.0 and 3.0	—
3	7.5 (0.1)	5.5	4.5	15	3.0	3.0
4	10 (0.1)	4.5 and 5.8	3.0 and 5.5	19	4.0	2.5 and 4.0
5	13 (0.1)	4.0	3.0	24	4.0	3.0 and 4.5
6	18 (0.1)	2.5 and 5.3	3.5 and 5.8	30	4.5	5.0
7	—	—	—	36	3.5	2.5

[a]From Reference 33. See Table 1 for abbreviations.

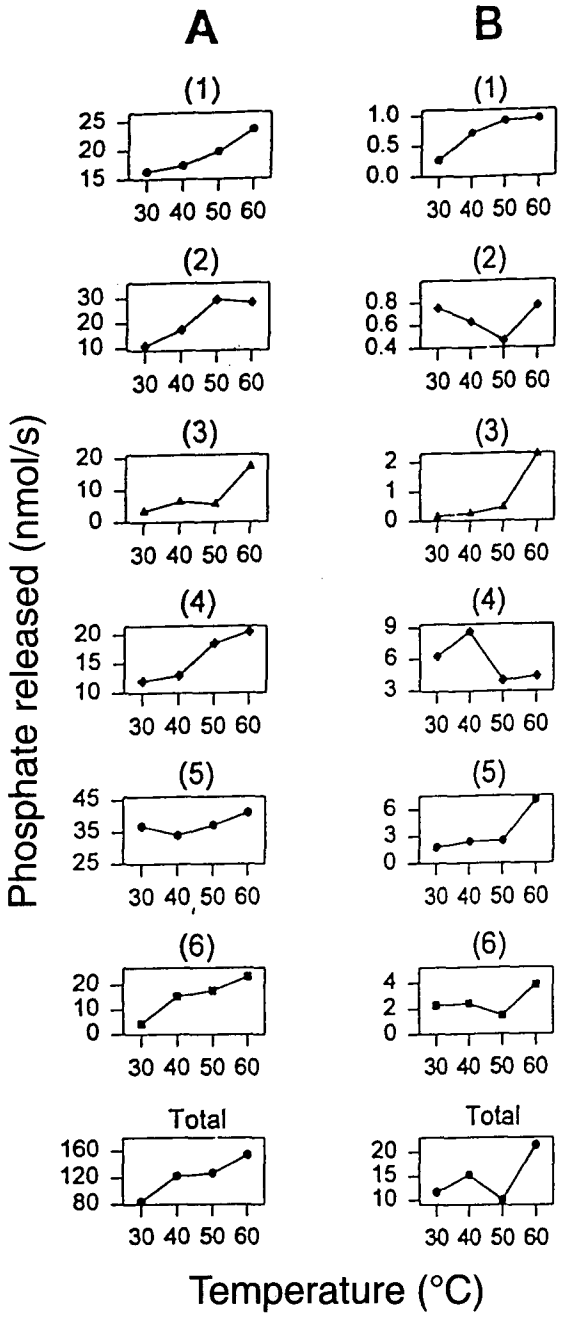

FIG. 7. Effect of temperature on the activity of anion-exchange peaks of PEG precipitates using *p*-nitrophenol phosphate (A) or phytic acid (B) as the substrate in 0.1 M sodium acetate buffer, pH 5.0 at 30–60°C. Graphs 1–6 represent the activities of high-performance liquid chromatography peaks of the six consecutive PEG precipitates in increasing order of PEG concentration. See Figure 1 for abbreviation.

ful in breeding and site-directed mutagenesis research to yield low-cost stable phytases and acid phosphatases.

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