Journal of Chromatography A, 658 (1994) 371–380
Elsevier Science B.V., Amsterdam

CHROM. 25 497

Use of polyethylene glycol and high-performance liquid chromatography for preparative separation of *Aspergillus ficuum* acid phosphatases

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ABSTRACT

Proteins of *Aspergillus ficuum* culture filtrate were sequentially fractionated with 4, 9, 15, 19, 24, 30 and 36% polyethylene glycol (PEG) into seven acid phosphatases (APases) with 93% and 52% overall recoveries of activity and protein, respectively. Crude extract was also separated into seven APase peaks on a 30 cm × 2.5 cm I.D. anion-exchange column using 0.1 *M* Tris–HCl (pH 8.0) and a 0–0.4 *M* KCl gradient as the eluent, but their resolution was incomplete. However, when individual PEG precipitates were injected on to the column, each APase was eluted in a single, large peak resulting in 85% recovery and fifteen-fold purification of APase activity over the PEG precipitates. Use of PEG prior to HPLC separations also reduced the separation time to half and allowed a tenfold increase in sample load with complete resolution. The APases in PEG fractions and their corresponding HPLC peaks varied significantly in their kinetic parameters, including substrate specificity and pH optimum. The method developed is most beneficial for the isolation of these closely related APases from microbial or other sources for further molecular biology studies.

INTRODUCTION

Acid phosphatases (EC 3.1.3.2) hydrolyze a broad spectrum of phosphate esters and occur in numerous organisms including *Aspergillus* spp. [1–6] and yeast. [7,8] An acid phosphatase (APase) with a pH optimum of 2.2 was partially purified from *Aspergillus ficuum*. [9]. Recently, an *A. ficuum* APase with an pH optimum of 2.5 was purified and characterized [10]. Another APase was also purified from the same fungal filtrate of *A. ficuum* and was reported to have a pH optimum of 6.0 [11]. Phytase, which is a phytate-specific acid phosphatase, has been partially purified from numerous bacterial [12,13] and fungal [14,15] sources and from yeast [16,17]. The phytases of *A. terreus* [18,19], *Bacillus subtilis* [20], *B. subtilis (natto)* N-77 [21] and *A. ficuum* [22] have been purified to homogeneity and characterized. Segueilha *et al.* [23] purified the phytase from *Schwanniomyces castellii* to homogeneity and studied its properties.

The use of phytases and acid phosphatases in food applications or particularly in supplementing feed is not possible economically because of the prohibitive cost of these enzymes. Fortunately, developing low-cost stable phytases and acid phosphatases with optimum properties that are suitable for the production of inositol phosphate isomers and for animal feed and food processing can be accomplished with the recombinant DNA techniques and site-directed mutagenesis. A preparative separation method for the isolation of the entire APases is needed in order to identify these closely related APases that possess appropriate parameters and thus are considered most suitable for cloning and for further molecular biology studies. Once these APases have been isolated into distinctive APases fractions, they can be easily purified further to homogeneity

SSDI 0021-9673(93)E0915-H

using chromatographic and electrophoretic techniques. Purified proteins are required for accurate probing of cDNA libraries, which is critical for successful recombinant DNA research. Large-scale methods for the extraction and purification of the acid phosphatases are needed also to improve their use in industry, which would complement our molecular cloning efforts. [24]

The objectives of this research were to isolate the entire forms of the APases and their isozymes from *A. ficuum* by large-scale preparative purification techniques and to study some kinetic and activity parameters such as pH optimum and substrate affinity to distinguish between these enzymes.

EXPERIMENTAL[a]

Fermentation of A. ficuum for production of APases

Fermentation of *A. ficuum* NRRL 3135 [4] was carried out in a liquid starch phosphate culture media at 30°C for 72 h [22]. Culture filtrates were obtained by centrifugation of the culture medium at 10 000 *g* for 20 min to remove fungal hyphae and gelatinized starch. Filtrates were freeze-dried and stored at −25°C until used.

Assays for phytase and APases

Reaction mixtures contained 0.1 or 1.0 ml of enzyme preparation, 100 m*M* sodium citrate buffer (pH 2.0–7.0), 0.03–0.30 ml of 0.01 *M* *p*-nitrophenol phosphate (*p*-NPP) or phytic acid and water to a 2.0-ml total volume. Incubations of enzyme preparations and *p*-NPP were carried out at 60°C for 10–15 min for all samples except eluents (1.0 ml each), which were incubated for 45–60 min. The reaction of *p*-NPP was terminated by adding 0.2 ml of NaOH (0.5–1.5 *M*, depending on the pH of the reaction mixture) to each tube and the absorbance was read at 410 nm. The P_i released from phytic acid was determined by the molybdate method as modified by Heinonen and Lahti [25] after 15–30 min of incubation at 60°C. To the phytate-containing reaction mixture, 2.0 ml of ammonium molybdate solution was added and the absorbance was read at 355 nm. The original crude extract was diluted twentyfold times before assaying.

PEG precipitation curves of A. ficuum acid phosphatases

The design of the experiment for the polyethylene glycol (PEG) precipitation of *A. ficuum* filtrate was based on the method of Miekka and Ingham [26]. PEG with a nominal average molecular mass of 4600 was purchased from Aldrich (Milwaukee, WI, USA). Freeze-dried crude extract (0.125 g) was suspended in 60 ml of deionized water and then stirred in an ice-bath at 400 rpm for 15 min. The pH was raised from 2.3 to 5.5 using 1.0 *M* NaOH. The sample was centrifuged at 8000 *g* at 4°C for 20 min. Fractionation of a 2.0-ml aliquot of the supernatant liquid was carried out in 0.05 *M* KCl and 0.10 *M* acetate buffers (pH 5.5) with increasing concentrations of PEG-4600 up to a 50% PEG final concentration at 2.5% PEG intervals. The PEG mixtures were kept at 4°C for 30 min, then centrifuged at 5000 *g* at 4°C for 20 min. The precipitates were dissolved in 2.0 ml of water and assayed for non-specific APase activity using *p*-NPP at pH 2.0, 3.5 and 5.0.

Isolation of APases from A. ficuum by precipitation at 4–36% PEG

Freeze-dried preparation (1.5 g) and 30 ml of water were stirred in an ice-bath at 400 rpm for 15 min, then the pH was raised from 2.3 to 5.5 using 1.0 *M* NaOH. A 5-ml volume of 1.0 *M* sodium acetate buffer (pH 5.5), 2.5 ml of 1.0 *M* KCl and 5 ml of 40% PEG solution were added and the total volume was adjusted to 50 ml. Mixtures were incubated at 4°C for 30 min then centrifuged at 5000 *g* at 4°C for 20 min. The 4% PEG precipitate was saved for later analysis. The supernatant was used for later PEG precipitation at 9, 15, 19, 24, 30 and 36% PEG using the same method. KCl and sodium acetate buffer (pH 5.5) were maintained at 0.05 and 0.1 *M*, respectively,

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throughout the precipitation steps. The precipitates were stored at −25°C until used. PEG precipitates were dissolved in 3–5 ml of water and each fraction was assayed for non-specific APase and phytase.

Effect of pH on activity of APases and phytases

The effect of pH (mixtures adjusted to pH 2, 2.25, 2.5, 3, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 7.0) on the non-specific and phytate-specific APases was determined in 0.1 *M* citrate buffer using *p*-NPP or phytate as the substrate, respectively.

Preparation of A. ficuum crude extract and PEG precipitates for chromatography

Freeze-dried filtrate (1.0 g) and 20 ml of water were mixed and the pH of the solution was raised from 2.3 to 8.0 with 0.5 *M* sodium hydroxide, then Tris–HCl buffer (pH 8.0) was added to a 0.1 *M* concentration. The solution was dialyzed in a tank containing 20 l of 0.02 *M* Tris–HCl buffer (pH 8.0) at 5°C for 3 days. Fungal debris was removed by centrifugation. Each of the seven PEG precipitates was dissolved in 0.1–0.2 ml of 1.0 *M* Tris–HCl (pH 8.0) and water to a total volume of 2.0–4.0 ml. Aliquots were filtered through a 0.45-μm Millex-HV Filter (Millipore, Bedford, MA, USA) before injection.

Operating the HPLC system

A Delta Prep 3000 preparative chromatographic system (Waters, Division of Millipore, Milford, MA, USA) was used for this investigation. The Delta Prep 3000 consisted of a solvent-delivery system operated by a system controller and sample injection by a Rheodyne Model 7012 injector with a 5-ml loop. Several different elution programs consisting of Tris–HCl buffer (pH 8.0), 1.0 *M* KCl and deionized water were utilized. Elution was monitored at 280 nm with a Model 481 Lambda-Max spectrophotometic detector connected to a Baseline 810 chromatography workstation (Waters) for the integration of the proportions of various eluted peaks. Fractions were collected every 1 min using a Foxy Fractionator (ISCO, Lincoln, NE, USA).

Preparative quaternary methylamine anion exchange

The ion-exchange preparative separation of crude extract and PEG precipitates was performed on a steel column (30 cm × 25 mm I.D.) packed with Accell Plus QMA medium (Waters). The protein load was 1–10 mg in 2–4 ml of 0.05 *M* Tris–HCl buffer (pH 8.0). The buffer and 1.0 *M* KCl were used in this gradient separation at a flow-rate of 6.0 ml/min. In one experiment involving the separation of the APases of *A. ficuum* crude extract, the flow-rate was reduced to 3.0 ml/min. The pH of the fractions was lowered to 3.0 with citric acid and then the eluates were assayed for APase activity at pH 3.0. The eluates were pooled, dialyzed and then either assayed or freeze-dried and stored at −25°C until used. APase and phytase activities of samples injected into the HPLC system and also pooled peaks was determined at pH 2.0, 3.5 and 5.0.

Protein analysis

The protein content of APases was measured by the macro or micro method of Bradford [27] using Coomassie Plus Protein Assay Reagent from Pierce (Rockford, IL, USA).

Statistical analysis of variance

Multi-factor analysis of variance (ANOVA) of the total enzyme activity, determined in duplicate, was performed using the software package of Statgraphics (Rockville, MD, USA) and the procedures described by Walpole and Myers [28]. Tukey's multi-range test was used to compare the means of the different levels of each factor and to group the levels of a factor together if the different levels were not significantly different, *i.e.*, homogeneous.

RESULTS

PEG precipitation of acid phosphatases from A. ficuum crude extract

The PEG precipitation curves for each of the *A. ficuum* APases were obtained by first determining the order of precipitation and the amount of PEG required to precipitate each individual APase from the crude extract. An

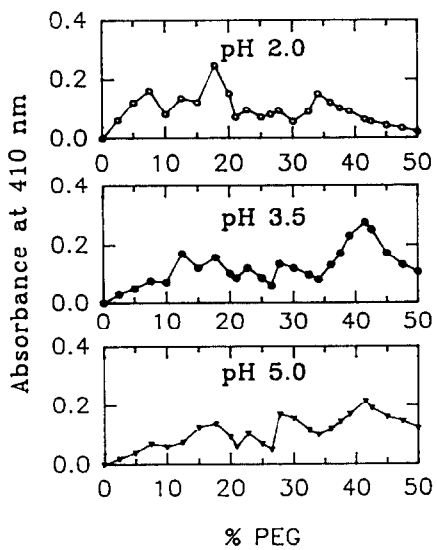

Fig. 1. Effect of PEG concentration on the precipitation of the *A. ficuum* phosphatases APases in the crude extract in 0.1 *M* acetate buffer (pH 5.5) and 0.05 *M* KCl. APase activity in precipitates was determined in 0.1 *M* citrate buffer (pH 2.0, 3.5 and 5) using *p*-NPP.

initial precipitation curve was prepared by plotting the effect of PEG concentration on the activity of APases precipitated from the crude extract using PEG-4600 in 0.1 *M* acetate buffer (pH 5.5) and 0.05 *M* KCl (Fig. 1). Seven APase peaks were apparently detected at 8, 13, 18, 23, 28, 35 and 43% PEG concentrations. These fractions were then reprecipitated at a narrower PEG concentration range pertaining to each APase peak. The precipitation conditions, especially protein concentration, *i.e.*, dilution factor, were optimized to define precisely the minimum PEG concentration required to bring the APase out of solution. Based on the precipitation curves mentioned above, PEG precipitation of the acid phosphatases in the crude extract was repeated with 4, 9, 15, 19, 24, 30 and 36% PEG concentrations, sequentially in 0.1 *M* acetate buffer (pH 5.5) and 0.05 *M* KCl. These PEG concentrations corresponded to the mid-points of the slopes of the seven APase peaks indicated in Fig. 1.

The total activities of the seven PEG precipitates (Table I) varied significantly among the fractions when NPP was used as the substrate to assay the non-specific APases. ANOVA categorized these seven APases into four statistically homogeneous groups: the 4%, 9% and 19% PEG precipitates were in group 1, the 15% and the 30% PEG precipitates in group 2 and the 24 and 36% PEG precipitates in groups 3 and 4, respectively. The activity of these PEG precipitates using *p*-NPP at pH 2.0 and 5.0 was significantly different from that at pH 3.5. On the other hand, two precipitates had no phytase activity and there was no significant difference in the total phytase activity among the remaining precipitates. When the phytase activities were compared at pH 2.0, 3.5 and 5.0, three different

TABLE I

TOTAL ACTIVITY OF *A. ficuum* ACID PHOSPHATASES PRECIPITATED WITH POLYETHYLENE GLYCOL

Protein fraction	Protein (mg)	Total activity (nmol/s per fraction)					
		p-NPP			Phytic acid		
		pH 2.0	pH 3.5	pH 5.0	pH 2.0	pH 3.5	pH 5.0
Crude Extract	67.1	623	1586	346	195	180	126
4% PEG	2.1	76	118	24	0	0	0
9% PEG	2.7	54	23	20	0	0	0
15% PEG	3.2	44	209	54	31	29	8
19% PEG	5.7	55	102	27	46	29	10
24% PEG	6.7	142	365	62	28	31	21
30% PEG	9.6	81	141	29	14	44	49
36% PEG	3.7	119	445	95	53	27	16

homogeneous groups were found. Over 50% of the original protein was precipitated by PEG: about 3.1, 4.0 4.8, 8.5, 10.0, 14.3 and 5.5% of the crude extract proteins were recovered in the seven fractions, respectively. These precipitates contained an average of 93% of the total non-specific and phytate-specific APase activities. Precipitation resulted in 2.9, 1.4, 2.6, 1.0, 2.2, 0.7 and 4.7-fold purifications, respectively, with an average increase in specific activity of 1.9.

Effect of pH on activity

The pH optimum for the APases and the phytases at 60°C in 0.1 *M* sodium citrate buffer (Fig. 2) varied significantly among these seven enzymes. The pH optimum for the APase activity of the 4, 9, 15, 19, 24, 30 and 36% PEG precipitates at 60°C was found to be 3.0, 2.0 (and 3.0), 3.0, 4.0, 4.0, 4.5 and 3.5, respectively (Fig. 2). The *p*-NPP activity of the crude extract was exhibited in one peak with activity diminishing gradually on either side of an optimum pH value of 3.5. On the other hand, the study of the pH effect on the phytase activity in the crude extract yielded two peaks at pH 3.0 and 5.0. The pH optima for the phytase activity of these APases were 3 for the 15% PEG precipitate, 2.5 and 4.0 for the 19% PEG precipitate, 3.0 and 4.5 for the 24% PEG precipitate, 5.0 for the 30% PEG precipitate and 2.5 for the 36% PEG precipitate.

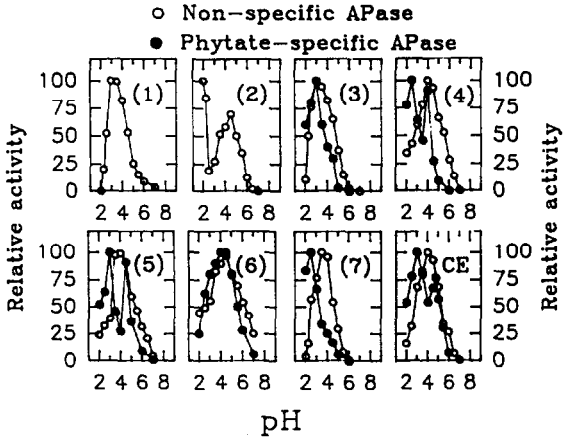

Fig. 2. Effect of pH on the activity of the *A. ficuum* (○) non-specific and (●) phytate-specific APases in crude extract (CE) and its proteins precipitated at 4, 9, 15, 19, 24, 30 and 36% PEG concentrations, numbered 1–7, respectively. Enzymes were incubated with *p*-NPP or phytate in 0.1 *M* sodium citrate buffer for 15 min at 60°C.

Quaternary methylamine anion-exchange chromatography of crude extract

Proteins secreted from *A. ficuum* (2.0-mg load) were applied to a 15 cm × 19 mm I.D. column containing Accell QMC and eluted at 6.0 ml/min with the run gradient presented in Fig. 3. Proteins were separated into eleven major peaks containing more than 2% of the total protein, of which seven peaks contained the APase activity, but there was overlap between the peaks. The proteins in the APase peaks accounted for 75% of the total protein. The Accell separation was also carried out with a 1-mg load at a 3.0 ml/min flow-rate, which gave 24 major peaks with 89% of the APase units recovered in seven peaks (Fig. 4). The overlapping areas between the seven peaks were much smaller than observed for the separation of the 2.0-mg load at a 6.0 ml/min flow-rate (Fig. 3).

The total activities of the seven pooled peaks using *p*-NPP as the substrate at pH 2.0, 3.5 and 5.0 (Table II) varied significantly. ANOVA categorized these seven APases into four homogeneous groups based on whether there was a significant difference or not in their activity means. Peaks 1, 2 and 4 were in group 1, peaks 3

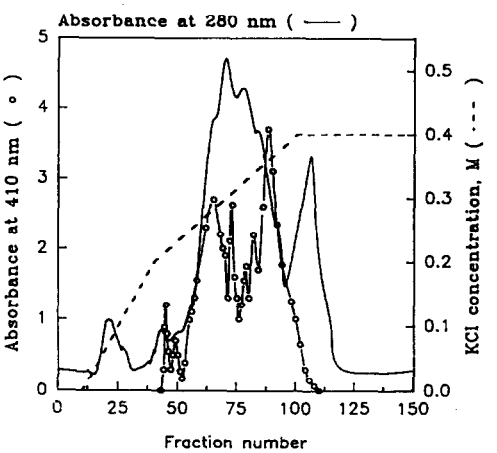

Fig. 3. Separation of *A. ficuum* proteins (2.0 mg) in crude extract using QMA anion-exchange medium at a flow-rate of 6.0 ml/min. Fractions were collected every 1 min. Activity in each fraction was determined using *p*-NPP in 0.1 *M* citrate buffer (pH 3.0).

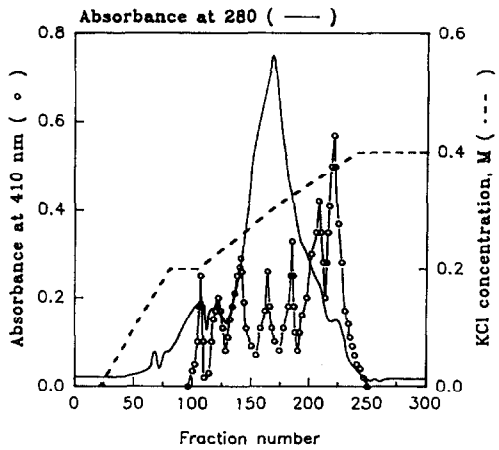

Fig. 4. Separation of 1.0 mg of protein of *A. ficuum* crude extract using QMA anion-exchange medium at a flow-rate of 3.0 ml/min. Volume of each fraction was 3.0 ml. Activity in each fraction was determined using *p*-NPP in 0.1 *M* citrate buffer (pH 3.0).

and 6 were in group 2, and peaks 5 and 7 were each in the single groups 3 and 4, respectively. Based on the multiple range test, the activity at pH 2.0 was not statistically different from that at pH 5.0. The activity of the enzymes at these two sets of pH values was significantly different from that at pH 3.5. The total phytase activity profile was different from the non-specific APase activities. There was no significant difference between the total activities of the phytate-specific APase peaks 3–7. However, the multiple range test classified the activity at the three pH levels (2.0, 3.5 and 5.0) into three groups. Peaks 1 and 2 exhibited no phytase activity but all the remaining APase peaks contained all the phytase activity. Peaks 3 and 4 were most active in the acidic pH range of 2–4 but their activities were relatively low at pH 5.0. The phytase activity of peak 5 was highest at pH 3.5 and contained only 75% of this activity at both pH 2.0 and 5.0. In contrast with peak 6, which was most active at pH 5.0, peak 7 was most active at pH 2.0 with relatively very high activity at pH 2–3.5. Peaks contained 2.4, 4.0, 5.6, 7.3, 1.3, 9.3 and 2.0% of the injected protein. This represents about one third of the total protein. The specific activity of non-specific APases and phytases increased in these peaks by 23, 14, 10, 7, 38, 10 and 59, respectively. An average purification of 23-fold was found for these samples compared with the crude extract.

Quaternary methylamine anion-exchange chromatography of PEG precipitates

The seven APases of the crude extract previously fractionated by PEG precipitation were each further purified individually by anion-exchange HPLC on the 30 × 2.5 cm I.D. column. The sample load varied as it was dependent on the amount of protein recovered for each APase in the replicates of PEG precipitation, which yielded 3–10 mg of protein (Table I). Fig. 5 shows the separation of the 15% PEG precipitate on the Accell column as a representative chromatogram for the separations obtained for

TABLE II

TOTAL ACTIVITY OF THE PEAKS OF *A. ficuum* CRUDE EXTRACT SEPARATED BY ANION-EXCHANGE CHROMATOGRAPHY

Peak No.	Retention time (min)	Collected fractions (No.)	Recovered protein (mg)	APase activity (nmol/s per peak)			Phytase activity (nmol/s per peak)		
				pH 2.0	pH 3.5	pH 5.0	pH 2.0	pH 3.5	pH 5.0
1	102	98–106	0.02	1.0	1.3	0.5	0	0	0
2	122	118–126	0.04	1.2	0.2	0.6	0	0	0
3	141	137–145	0.06	1.1	3.1	0.6	0.36	0.37	0.11
4	170	167–176	0.07	1.1	1.5	0.4	0.62	0.39	0.14
5	188	186–195	0.01	1.8	4.9	0.6	0.38	0.45	0.31
6	212	207–216	0.09	1.5	1.7	0.6	0.21	0.60	0.64
7	225	220–228	0.02	1.6	6.4	1.6	0.75	0.36	0.18

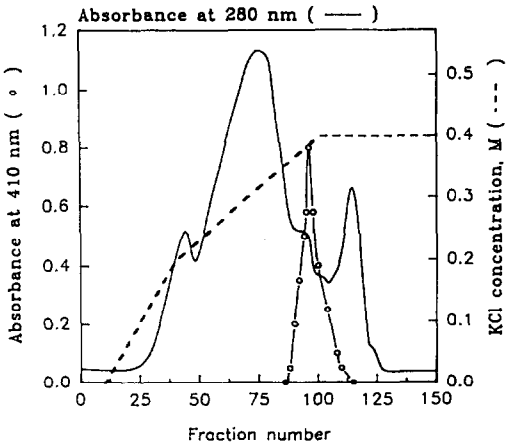

Fig. 5. QMA anion-exchange chromatography of the *A. ficuum* proteins precipitated at 15% PEG concentration. A 3-mg amount of protein was injected; the flow-rate was 6.0 ml/min. Activity in each fraction (6.0 ml) was determined using *p*-NPP in 0.1 *M* citrate buffer (pH 3.0).

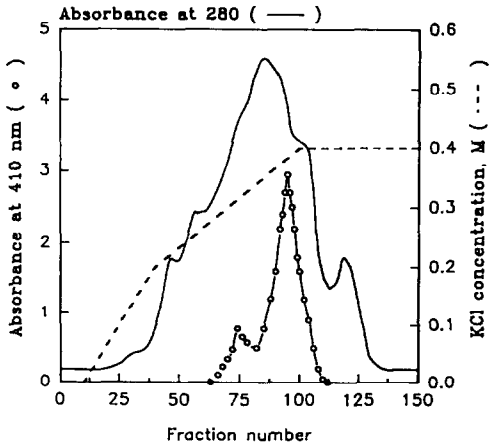

Fig. 6. QMA anion-exchange chromatography of the *A. ficuum* proteins precipitated with PEG at 27% concentration (6 mg load) at a flow-rate of 6.0 ml/min. The APase activity in each fraction (6.0 ml) was determined using *p*-NPP in 0.1 *M* citrate buffer (pH 3.0).

the other PEG precipitates. Each precipitate was eluted as a single peak in 26 min from 81 to 107 min retention with an average retention time of 93 min (Table III). The total NPP activities of the seven pooled peaks of PEG precipitates varied significantly. According to the multiple range test, the seven APases were grouped into four homogeneous groups. Peaks of the 4%, 9% and 19% PEG precipitates were in group 1, peaks of the 15 and 30% PEG precipitates in group 2 and peaks of the PEG fractions 24 and 36% PEG precipitates in groups 4 and 5, respectively. The total activities of these peaks at pH 3.5 was significantly different from that at pH 2.0 and 5.0. There was no significant difference between the activities of the last five peaks containing the phytase activity, but their activities were significantly different when measured at pH 2.0, 3.5 and 5.0. There was an average fifteenfold increase in the specific activities of APases in the seven runs. Individual APases were purified by 8, 14, 7, 24, 21, 20 and 11 for the 4%, 9%, 15%, 19%, 24% 30% and 36% PEG precipitates, respectively. Fig. 6 shows

TABLE III

ACTIVITY OF *A. ficuum* POLYETHYLENE GLYCOL PRECIPITATES SEPARATED BY ANION-EXCHANGE CHROMATOGRAPHY

Precipitate	Retention time (min)	Collected fractions (No.)	Protein Recovered (mg)	Activity (nmol/s per peak)					
				Using *p*-NPP			Using phytic acid		
				pH 2.0	pH 3.5	pH 5.0	pH 2.0	pH 3.5	pH 5.0
4% PEG	95	82–103	0.23	62	102	16	0	0	0
9% PEG	93	82–108	0.18	49	22	17	0	0	0
15% PEG	96	83–110	0.46	37	187	47	27	22	6
19% PEG	94	82–106	0.23	48	93	19	39	23	9
24% PEG	94	83–107	0.23	117	350	57	26	27	17
30% PEG	95	82–108	0.48	58	123	24	13	36	44
36% PEG	98	84–108	0.32	92	440	87	48	21	10

the anion-exchange HPLC separation of an APase precipitated at 27% PEG concentration, a mixture of the 24% and 30% PEG proteins (see Fig. 1). Two peaks were eluted, containing 17 and 82% of the APase activity at pH 3.0 and 60°C.

DISCUSSION

Analytical PEG precipitation curves for each of the APases of *A. ficuum* determined the maximum concentration of PEG that could be added without precipitating the APase of interest. They also determined the minimum concentration required to bring the APase out of solution. Based on these curves, the proteins of *A. ficuum* crude extract were fractionated into seven separate individual APases. The fractions varied significantly in their total activity and an average of 1.9-fold purification was possible with this isolation method. PEG precipitation is markedly dependent on the size of PEG up to M_r 6000. Precipitation also increases with increasing size of the protein, suggesting a steric exclusion mechanism [29]. In the absence of specific interactions, the sequence of precipitation of several proteins will depend primarily on the ratios of their initial concentrations relative to their respective solubility in the absence of PEG [29]. As a preparative tool for protein separation, PEG has the unique advantage over other precipitating agents such as acetone, ethanol or ammonium sulfate of the shorter time required for the precipitated proteins to equilibrate and achieve a physical state suitable for large-scale centrifugation.

Crude extract was also fractionated by ion-exchange chromatography (IEC) into seven APases. The results of the multiple range test suggested that the seven APases of the crude extract fractionated by ion exchange (Table II) were eluted in the same order of consecutive PEG precipitation (Table I). These peaks and the seven APases in PEG precipitates (Table I) were grouped into the same statistically homogeneous groups using *p*-NPP or phytate as the substrate. Also, the effect of pH on both the activities of non-specific APase and phytase was the same in the two experiments. The seven APases of the crude extract previously fractionated by PEG precipitation were each further purified individually by IEC on a 30×2.5 cm I.D. preparative column. Each PEG precipitate was eluted as a single peak with an average increase in the specific activities of fifteen-fold. Considering the purification values observed for the PEG precipitation, these enzymes were purified 23, 19, 17, 22, 52, 13 and 49 times, respectively, over the crude extract. This represents an average of 28-fold purification.

Two APases with pH optima of 2.2 [9] and 2.5 [10] were purified from *A. ficuum*. As these two enzymes had no phytase activity, they could be the same as the APases precipitated here at 9% PEG concentration. However, among the seven APases isolated, none matched a non-specific APase with a pH optimum of 6.0 previously isolated from *A. ficuum*. [11] As the pH 6.0 optimum APase was not evaluated for phytase activity at pH 5.0, it is possible that this enzyme corresponds to the 30% PEG precipitate with pH optima pH of 4.5 and 5.0 using *p*-NPP and phytate in citrate buffer at 60°C, respectively. The disagreement in the optimum pH value for this particular enzyme could be due to the use of two different buffers. Data for the effect of pH on the phytase activity in crude extract are in general agreement with those reported for *A. ficuum* phytase [22] in that both had two peaks representing two types of activities. However, the *A. ficuum* phytases were fractionated here into five phytases, of which only two had a dual pH optima, the other three having only one peak at pH 2.5, 3.0 and 5.0. It is possible that these phytases are isozymes of two different phytases. This should be followed up in future work that includes the purification of these enzymes to homogeneity and then characterizing their proteins.

Electrophoresis and IEC are the techniques usually used for the successful fractionation of complex mixtures of closely related proteins or enzymes such as isozymes. IEC, however, has a larger resolving power as it depends on both the charge density and the distribution of charges on the protein surfaces, *i.e.*, charge anisotropy [30]. Therefore, it was not surprising that IEC was

effective in the separation of the closely related acid phosphatases of *A. ficuum*. Further, IEC can be of considerable value in early stages of enzyme purification when a large portion of the polyelectolytes are associated with the matrix [31]. An average of 67% and 93% of the non-APase proteins in the crude extract and the PEG precipitates, respectively, were immobilized in the column and eluted later in the chromatographic run in separate peaks. Despite peak overlap, individual APases could be separated directly from the *A. ficuum* extract in one chromatographic run using recycle LC and peak shaving. Peak shaving is practised to obtain pure compounds when the apparent resolution is incomplete by collecting the portion of the eluting material before the cut point of the overlap between two peaks. Peak shaving is also a technique for making recycle LC an even more effective tool [32].

However, the use of PEG prior to IEC seems more attractive than either recycle or peak shaving as it provides many benefits: comparing the chromatogram of the 27% PEG precipitate (Fig. 6), a mixture of the 24 and 30% PEG precipitates (see Fig. 1) with the other chromatograms for PEG precipitates led us to believe that the preparation of samples by PEG precipitation before application to HPLC was most helpful in obtaining only one peak of the 24% PEG acid phosphatase. Therefore, the use of PEG precipitation to prepare the samples for chromatography ended peak overlapping. In addition, when individual PEG precipitates were injected on to the column, each APase was eluted in a single large peak, resulting in an average of 28-fold purification of APase activity over crude extract. Use of PEG before HPLC separations also reduced the separation time by half and allowed a tenfold increase in sample load from 1.0 to 10.0 mg with complete resolution and good recovery of activity, up to 79% of that of starting crude extract. Prior PEG fractionation increased the concentration of APase protein relative to non-APase proteins, which might have had a dramatic effect on the resolution by maximizing the separation factor (α) between the APase and other protein components. Maximizing α between components is the single most significant step that can be taken to optimize a preparative LC separation [32].

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