Purification and Characterization of a Phytate-Degrading Enzyme from Germinated Faba Beans (*Vicia faba* Var. Alameda)

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A phytate-degrading enzyme was purified ~2190-fold from germinated 4-day-old faba bean seedlings to apparent homogeneity with a recovery of 6% referred to the phytase activity in the crude extract. It behaves as a monomeric protein of a molecular mass of ~65 kDa. The phytate-degrading enzyme belongs to the acidic phytases. It exhibits a single pH optimum at 5.0. Optimal temperature for the degradation of sodium phytate is 50 °C. Kinetic parameters for the hydrolysis of sodium phytate are $K_M = 148$ μmol L^{-1} and $k_{cat} = 704$ s^{-1} at 35 °C and pH 5.0. The faba bean phytase exhibits a broad affinity for various phosphorylated compounds and hydrolyzes phytate in a stepwise manner. The first hydrolysis product was identified as D/L-*myo*-inositol(1,2,3,4,5)pentakisphosphate.

Keywords: *Legume phytase; myo-inositol phosphate phosphohydrolase; phytate degradation*

INTRODUCTION

Legumes are claimed to provide health benefits, but they also contain antinutrients such as phytate. Because excessive amounts of phytate in the diet can lead to mineral deficiencies, phytate was branded as an antinutrient that should be avoided and eliminated by processing (*1*). Phytase (*myo*-inositol hexakisphosphate phosphohydrolase), a phytate-specific phosphatase, catalyzes the stepwise hydrolysis of phytate [*myo*-inositol-(1,2,3,4,5,6)hexakisphosphate], the principal storage form of phosphorus in mature seeds of cereals and legumes (*2*), to a series of lower *myo*-inositol phosphates and orthophosphate. Phytate-degrading enzymes are widely distributed in nature (*3*, *4*), for example, in plants, microorganisms, and certain animal tissues. Adding exogenous phytate-degrading enzymes or activating endogenous ones may improve phytate reduction during feed or food processing (*5*, *6*). Isolating and accurately characterizing phytate-degrading enzymes from any microorganism or plant source can facilitate obtaining effective enzymes for reducing phytate content during food processing.

Phytate-degrading enzymes are also of interest for producing defined breakdown products of phytate and may find applications in the processing of foods with improved nutritional value and health benefits and retained sensory properties (functional foods) (*7*, *8*). Some novel metabolic effects of phytate and some of its degradation products have been recognized, such as the amelioration of heart disease by controlling hypercholesterolemia and atherosclerosis (*9*, *10*), prevention of renal stone formation (*11*, *12*), and reduced risk of colon cancer (*13*−*16*). The use of different phytases may result in different positional isomers of the lower *myo*-inositol phosphates and therefore in different physiological effects. Until now, two types of phytate-degrading enzymes have been known: 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26), indicating the initial attack of the susceptible phospho−ester bond.

Because there is only limited knowledge on phytate-degrading enzymes in legumes, the objective of this work was to purify and characterize a phytate-degrading enzyme of faba beans. The only phytate-degrading enzymes from legumes that have been purified and characterized so far are the enzymes from soybean seeds (*17*), mung bean seeds (*18*), and scallion leaves (*19*).

MATERIALS AND METHODS

Chemicals. *Vicia faba* var. Alameda was obtained from CIDA of Cordoba (Spain). Fractogel CMD COO^- 650(S) and most of the enzyme substrates were purchased from E. Merck (Darmstadt, Germany). Phytic acid dodecasodium salt was obtained from Aldrich (Steinheim, Germany), and all other chemicals came from Fluka, Boehringer Mannheim, Serva, and Sigma. CM-Sepharose CL 6B, high-load 16/60 Sephacryl S-200 HR, Mono Q HR 5/5, and Mono S HR 5/5 were obtained from Pharmacia (Freiburg, Germany). Ultrasep ES 100 RP18 was purchased from Bischoff (Leonberg, Germany) and HPIC Omni Pac PAX-100 from Dionex (Sunnyvale, CA). AG1 X-4, 100−200 mesh resin was obtained from Bio-Rad (München, Germany). All reagents were of analytical grade.

Seed Germination. Faba bean seeds were soaked in the following solutions: (1) 0.1% Tween-80 for 5 min; (2) 0.5% NaOCl for 2 min; (3) 0.75% H_2O_2 for 1 min. The seeds were thoroughly rinsed with sterile water after each treatment. They were allowed to germinate on sterile boxes in the dark at 20 °C for up to 10 days. The seeds were rinsed once a day with sterile water, which was removed completely.

For phytate analysis and phytase extraction the germinated faba bean seeds were frozen at −80 °C for 24 h and thereafter dried at 0.37 mbar to complete dryness. This procedure does not result in significant differences in extractable phytate and phytase in comparison to seeds extracted immediately after the germination process.

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Phytate Analysis. Phytate was quantified by a combination of anion-exchange chromatography (*20*) and ion-pair chromatography (*21*). Freeze-dried faba bean seeds were milled to a fine powder, and 1 g of this powder was extracted with 20 mL of 2.4% HCl for 3 h at room temperature. The slurries were centrifuged at 30000*g* for 30 min. Aliquots (1 mL) of the supernatants were diluted 1:25 with water and applied to a column (0.7 × 15 cm) containing AG1-X8, 100−200 mesh resin. The column was washed with 25 mL of water and 25 mL of 25 mM HCl. *myo*-Inositol phosphates were eluted with 25 mL of 2 M HCl. The eluate was concentrated in a vacuum evaporator to 500 μL. Aliquots (20 μL) of the samples were analyzed by HPLC ion-pair chromatography as described under Identification of the Hydrolysis Products.

Enzyme Extraction. Freeze-dried faba bean seeds were milled to a fine powder. Soluble compounds were extracted with a 6-fold amount (w/v) of 50 mM sodium acetate buffer, pH 5.0, by shaking for 2 h at 4 °C. The cell debris was removed by centrifugation at 20000*g* for 30 min.

Protein Determination. Total protein concentration was determined according to the Coomassie blue G-250 dye-binding assay using bovine serum albumin as a standard (*22*).

Assay of Phytase. Enzyme extracts were dialyzed against 20 mM sodium acetate buffer, pH 5.0. The enzymatic reactions were started by adding 10 μL of enzyme to the assay mixtures. The assay mixture consisted of 350 μL of 0.1 M sodium acetate buffer, pH 5.0, containing 250 nmol of sodium phytate. After incubation at 35 °C for 30 min, the liberated phosphate was measured according to the ammonium molybdate method (*23*) with some modifications. Added to the assay mixture were 1.5 mL of a freshly prepared solution of acetone/5 N H_2SO_4/10 mM ammonium molybdate (2:1:1 v/v) and 100 μL of 1.0 M citric acid. Any cloudiness was removed by centrifugation prior to measuring the absorbance at 355 nm. To calculate the enzyme activity, a calibration curve was produced over the range of 5−600 nmol of phosphate ($\epsilon = 8.7$ cm^2 μmol^{-1}). Activity (units) was expressed as 1 μmol of phosphate liberated per minute. Blanks were run by adding the ammonium molybdate solution prior to adding the enzyme to the assay mixture.

Enzymatic hydrolysis of other phosphorylated compounds was tested as described above, but sodium phytate was replaced by 5 μmol of the phosphorylated compounds.

To study the pH optimum and the pH stability of the faba bean phytase, the following buffers were used in the above-described standard assay: pH 1.0−3.5, 0.1 M glycine−HCl; pH 3.5−6.0, 0.1 M sodium acetate−HCl; pH 6.0−7.0, 0.1 M Tris−acidic acid; pH 7.0−9.0, 0.1 M Tris-HCl; pH 9.0−10.0, 0.1 M glycine−NaOH.

Assay of Acid Phosphatase. Acid phosphatase was determined in 200 μL of 50 mM citrate−NaOH, pH 5.0, containing 1 μmol of *p*-nitrophenyl phosphate. After 15 min at 35 °C, the reaction was stopped by adding 1.0 mL of 1 N NaOH. Acid phosphatase activity was determined by measuring the absorbance of the formed *p*-nitrophenolate at 405 nm. One unit of enzyme was defined as the amount of acid phosphatase releasing 1 μmol of *p*-nitrophenolate per minute.

Effect of Cations and Potential Inhibitors on Enzyme Activity. The effect of cations and potential inhibitors on enzyme activity was investigated by preincubating the compounds with the phytase for 15 min at 35 °C before the standard phytase assay was performed. The following cations and potential inhibitors were used in concentrations of 0.1, 0.2, 0.5, 0.8, and 1.0 mM: Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, *o*-phenanthroline, EDTA, oxalate, citrate, tartrate, cyanide, azide, 2-mercaptoethanol, iodoacetate, phenylmethanesulfonyl fluoride, phosphate, molybdate, wolframate, and vanadate. Fluoride was used in the following concentrations: 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.5, 0.6, 0.8, and 1.0 mM.

Effect of Temperature on Enzyme Activity. The temperature profile of the purified phytase was conducted from 10 to 80 °C using the standard phytase assay at the given temperature. To check thermal stability, the purified enzyme was incubated at different temperatures, cooled to 4 °C, and assayed using the standard phytase assay.

Identification of Hydrolysis Products. Enzyme and sodium phytate were incubated as described for the activity determination but omitting the phosphate assay. Samples (50 μL) were removed periodically from the incubation mixture, and the reaction was stopped by heat (90 °C, 5 min) in a PCR thermocycler to avoid evaporation of solvent. Then 20 μL of the samples was chromatographed on an Ultrasep ES 100 RP18 (2 × 250 mm). The column was run at 45 °C and 0.2 mL min^{-1} of an eluant consisting of formic acid/methanol/water/tetrabutylammonium hydroxide (44:56:5:1.5 v/v), pH 4.25, as described by Sandberg and Ahderinne (*21*). A mixture of the individual *myo*-inositol phosphate esters ($InsP_3$−$InsP_6$) was used as a standard. For the identification of the *myo*-inositol pentakisphosphate isomer 50 μL of the heat-treated samples was chromatographed on a high-pressure ion chromatography (HPIC) system as described by Skoglund et al. (*24*). *myo*-Inositol phosphate isomers were separated on an HPIC Omni Pac PAX-100 (4 × 250 mm) analytical column using a gradient of 5−98% HCl (0.5 *M*) in water/2-propanol (1:1 v/v). The eluants were mixed in a postcolumn reactor with 0.1% $Fe(NO_3)_3 \cdot 9\ H_2O$ in a 2% $HClO_4$ solution according to the method of Phillippy and Bland (*25*). The combined flow rate was 1.2 mL min^{-1}.

Purification of the Phytase. Precipitations and soft-gel chromatographies were carried out at 4 °C. Soft-gel columns were run at a flow rate of 50 mL h^{-1}; 10 mL fractions were collected. The FPLC columns were run at 25 °C and a flow rate of 1 mL min^{-1}.

*Ammonium Sulfate and Acetone Precipitation.*The crude extract was used for an ammonium sulfate precipitation at 35−80% saturation. The precipitate was collected by centrifugation at 12000*g* for 30 min and suspended in 20 mM sodium acetate buffer, pH 5.0, and dialyzed against the same buffer. Any insoluble material was removed by centrifugation at 12000*g* for 30 min. At certain intervals, cold acetone (−20 °C) was slowly added to the ammonium sulfate fraction during stirring to yield a final concentration of 55% (v/v). The acetone solution was stirred for another hour and then centrifuged at 12000*g* for 30 min. The protein material obtained was suspended in 20 mM sodium acetate buffer, pH 4.5, and dialyzed against the same buffer. Any insoluble material was removed by centrifugation.

CM-Sepharose CL 6B Chromatography. The dialyzed acetone fraction was loaded onto a CM-Sepharose CL 6B column (3.5 × 20 cm) equilibrated with 20 mM sodium acetate buffer, pH 4.5. After elution of the unbound phytase-inactive protein from the column with equilibration buffer, a linear gradient of 0−0.5 M sodium chloride (1000 mL) in 20 mM sodium acetate buffer, pH 4.5, was applied. The fractions (10 mL) containing phytase activity were pooled and dialyzed against 20 mM sodium acetate buffer, pH 6.0.

Fractogel CMD COO$^-$ 650(S) Chromatography. The pooled fractions from the CM-Sepharose CL 6B column were loaded onto a Fractogel CMD COO$^-$ 650(S) column equilibrated with 20 mM sodium acetate buffer, pH 6.0. The column was washed with equilibration buffer; then the bound proteins were eluted with a linear gradient from 0 to 0.5 M NaCl in 20 mM sodium acetate buffer, pH 6.0, within 180 min. The fractions (5 mL) containing phytase activity were pooled and dialyzed against 20 mM sodium acetate buffer, pH 4.5.

Mono S HR 5/5 Chromatography. The dialysate from the previous step were applied onto a Mono S HR 5/5 column equilibrated with 20 mM sodium acetate buffer, pH 4.5. The column was washed with the same buffer for 30 min, and then a gradient consisting of 0−0.5 M NaCl in 20 mM sodium acetate buffer, pH 4.5, for 90 min was applied. The fractions (2 mL) containing phytase activity were pooled.

16/60 Sephacryl S-200 HR Chromatography. The phytase-containing pools from the previous step were loaded onto a 16/60 Sephacryl S-200 HR column equilibrated with 50 mM sodium acetate buffer, pH 5.0, containing 0.2 M NaCl in 2 mL portions. The fractions (2 mL) containing phytase activity were pooled and dialyzed against 20 mM Tris−acetate, pH 6.5.

Mono Q HR 5/5 Chromatography. The pool from the previous step was applied onto a Mono Q HR 5/5 column

Table 1. Purification Scheme of the Phytate-Degrading Enzyme of Faba Beans

step	total protein (mg)	total activity (units)	specific activity (units mg^{-1})	purification (fold)	recovery (%)
crude extract	2320	674	0.3	1	100
$(NH_4)_2SO_4$ precipitation	1179	704	0.6	2	104
acetone precipitation	712	636	0.9	3	94
CM-Sepharose CL 6B	143	420	3	10	62
Fractogel ion-exchange	10.5	251	24	82	37
Mono S HR 5/5	1.98	197	100	342	29
16/60 Sephacryl S200 HR	0.54	122	226	778	18
Mono Q HR 5/5	0.066	42	636	2190	6

equilibrated with 20 mM Tris−acetate buffer, pH 6.5. The column was washed with the same buffer for 30 min, and then a gradient consisting of 0−0.5 M NaCl in 20 mM Tris−acetate buffer, pH 6.5, for 60 min was applied. The fractions (2 mL) containing phytase activity were pooled and dialyzed against 20 mM sodium acetate buffer, pH 5.0.

Gel Electrophoresis. Native gel electrophoresis was carried out with 5% gels at pH 4.8 (*26*). Enzymatic staining of the protein was performed with 1-naphthyl phosphate coupled with Fast Blue B in 0.1 M sodium acetate buffer, pH 5.0, in the dark (*27*).

The SDS electrophoresis using 10% gels was performed according to the method of Laemmli (*28*). Gels were stained by Coomassie brilliant blue R-250.

Gel Filtration. To assess the molecular mass of the native phytases, the purified proteins were gel-filtered on 16/60 Sephacryl S-200 HR equilibrated with 50 mM sodium acetate buffer, pH 5.0, containing 0.2 M NaCl. The column was calibrated with glucose-6-phosphate dehydrogenase (M_r = 120000), creatine kinase (M_r = 81000), bovine serum albumin (M_r = 68000), β-lactoglobulin (M_r = 40000), and myoglobin (M_r = 17000).

RESULTS

Germination of Faba Beans. In germinated as well as nongerminated faba beans only one phytate-degrading enzyme was identified. Only a low phytase activity was detected in dry faba bean seeds (0.06 ± 0.01 unit g^{-1} of grain). During germination, maximum activity was reached after 4 days; a 22-fold increase in phytase activity was observed (1.35 ± 0.02 units g^{-1} of grain). Therefore, faba beans germinated for 4 days were used as a source of the enzyme.

Purification of the Phytate-Degrading Enzyme. A summary of the purification scheme is given in Table 1. The phytase activity eluted as a single sharp activity peak from each column. On each ion-exchange column phytase activity was retained and could be eluted by increasing the ionic strength of the buffer. The phytate-degrading enzyme was purified ~2190-fold with a recovery of 6% referred to the phytase activity in the crude extract. The purified enzyme exhibits an activity of ~636 units mg^{-1}.

Molecular Properties. The molecular mass and homogeneity of the enzyme preparation were estimated by PAGE and gel filtration. Polyacrylamide gel electrophoresis under denaturing (Figure 1) and non-denaturing conditions (data not shown) revealed only a single protein band after the gels had been stained with Coomassie. These results indicate that the phytate-degrading enzyme was purified to apparent homogeneity.

The apparent subunit molecular mass of the purified phytate-degrading enzyme was ~66 ± 2 kDa, as estimated by SDS−PAGE (Figure 1). The molecular mass of the native enzyme was determined to be 67 ± 1 kDa on a calibrated 16/60 Sephacryl S-200 HR column with elution position being measured by determination of

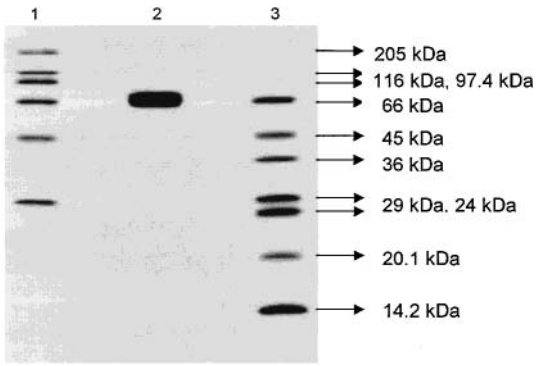

Figure 1. Electrophoretic analysis of purified faba bean phytase (10% SDS-PAGE of a preparation of the purified faba bean phytase stained Coomassie Blue): lane 1, standard SDS-6; lane 2, purified faba bean phytase (30 μg of protein); lane 3, standard SDS-7.

enzyme activity (Figure 2). Consequently, the phytase is a monomeric protein.

Enzymatic Properties. *pH Optimum and pH Stability.* The phytate-degrading enzyme of faba beans has a single pH optimum at pH 5.0. A rapid decline of the enzyme activity was observed on both sides of the pH optimum. The enzyme is virtually inactive below pH 3.0 and above pH 8.0 (data not shown).

The phytate-degrading enzyme of faba beans was stable over pH 4.0−7.5, whereas below pH 3.0 and above pH 7.5 activity declined rapidly. Over 85% residual activity was measured within 10 days at pH 4.0, but at pH 2.5 78% and at pH 8.0 60% of the original activity were lost during 24 h.

Temperature Optimum and Thermal Stability. The temperature optimum was found to be 50 °C. The Arrhenius activation energy for the hydrolysis of sodium phytate was calculated to be 25.7 kJ mol^{-1}. The enzyme did not significantly lose activity during 90 min at temperatures up to 45 °C. When exposed for 90 min at 50 and 60 °C, it retained 86 and 16%, respectively, of the original activity.

Substrate Selectivity and Kinetic Parameters. The action of the purified phytate-degrading enzyme of faba beans in 0.1 M sodium acetate buffer on several phosphorylated compounds was tested. The relative rates of hydrolysis are summarized in Table 2. The enzyme showed a broad substrate specificity, and phytate was not the compound with the highest relative rate of hydrolysis. The highest turnover numbers were found with pyrophosphate, ATP, and *p*-nitrophenyl phosphate. The kinetic parameters for the hydrolysis of sodium phytate were determined to be K_M = 148 μmol L^{-1} and k_{cat} = 704 s^{-1} at pH 5.0 and 35 °C.

Like other phytate-degrading enzymes, the phytate-degrading enzyme of faba beans showed a substrate inhibition (*29*−*38*). The activity of the purified enzyme

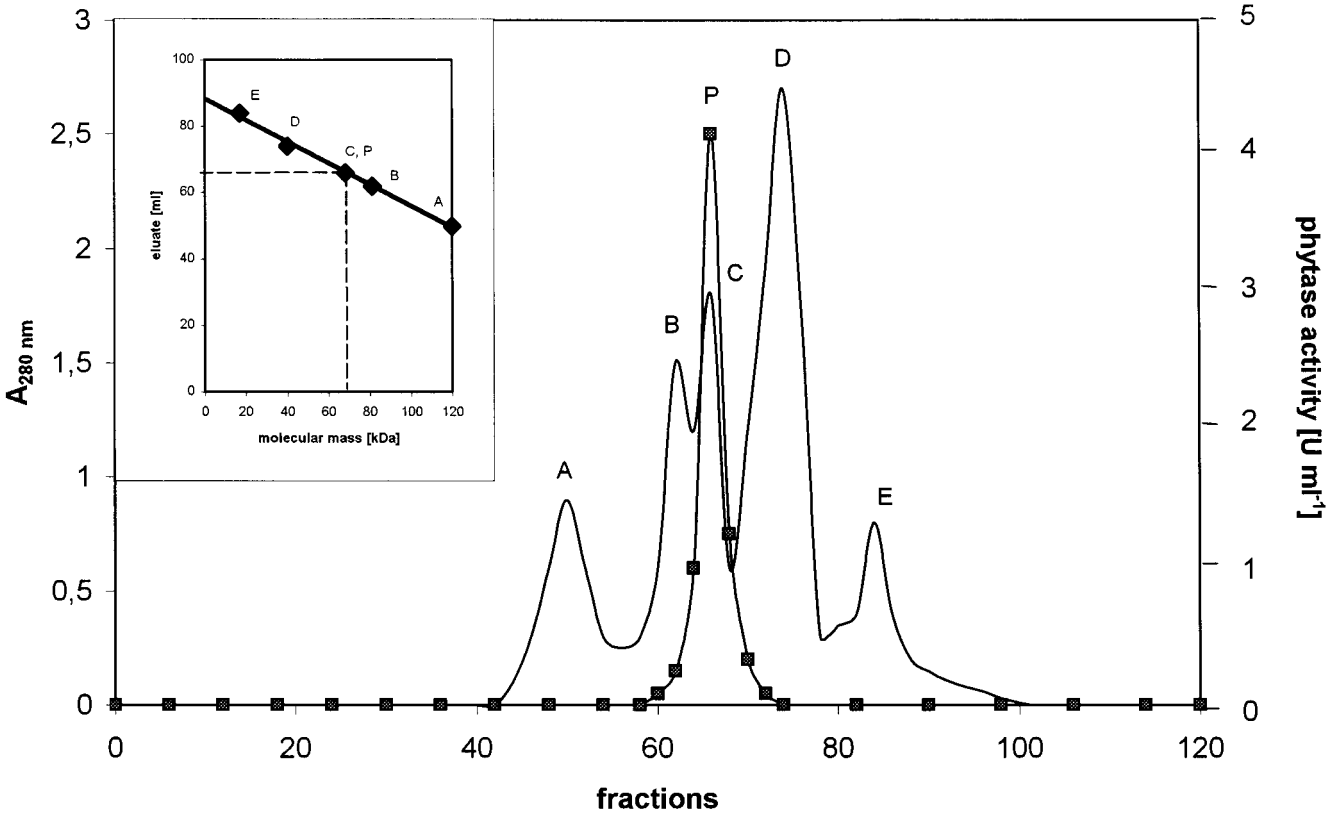

Figure 2. Estimation of the molecular mass of the faba bean phytase by gel filtration. The Sephacryl S-200 HR column was calibrated with (A) glucose-6-phosphate dehydrogenase (M_r = 120000), (B) creatine kinase (M_r = 81000), (C) bovine serum albumin (M_r = 68000), (D) β-lactoglobulin (M_r = 40000), and (E) myoglobin (M_r = 17000). (P) Faba bean (M_r estimated to be ~67000); phytase activity (■); optical density at 280 nm (—).

Table 2. Substrate Specificity and Kinetic Constants of the Faba Bean Phytase[a]

substrate	relative activity (%)	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($10^4\ s^{-1}\ M^{-1}$)
phytate	100	148	704	475
p-nitrophenyl phosphate	68	415	479	115
1-naphthyl phosphate	49	512	338	66
2-naphthyl phosphate	20	nd[b]	nd	nd
2-glycerophosphate	24	nd	nd	nd
fructose-1,6-diphosphate	98	398	690	173
fructose-6-phosphate	5	nd	nd	nd
glucose-6-phosphate	30	nd	nd	nd
AMP	11	nd	nd	nd
ADP	87	410	612	149
ATP	221	697	1556	223
GTP	10	nd	nd	nd
Na_2H_2-pyrophosphate	290	821	2042	249
pyridoxalphosphate	14	nd	nd	nd
o-phospho-L-serine	12	nd	nd	nd

[a] Hydrolysis rate of sodium phytate was taken as 100%; temperature, 35 °C; buffer, 0.1 M sodium acetate, pH 5.0; enzyme concentration, 50 milliunits mL^{-1}. [b] nd, not determined.

was inhibited at substrate concentrations >5 mmol L^{-1} (data not shown).

Enzymatic Hydrolysis of Phytate. To examine the action of the phytate-degrading enzyme of faba beans on sodium phytate, the hydrolysis products were separated by HPLC ion-pair chromatography and ion-exchange chromatography. Phytate was hydrolyzed in a stepwise manner by the phytate-degrading enzyme of faba beans. During degradation, the hydrolysis rate decreased markedly. This might be due to product inhibition (phosphate, see below) or a lower hydrolysis rate of the lower phosphate esters of *myo*-inositol. Probably both factors are responsible for the decrease in the hydrolysis rate. From the analysis of the individual stereoisomers, the pentakisphosphate was identified as D/L-*myo*-inositol(1,2,3,4,5)pentakisphsophate (Figure 3).

Effect of Cations on Enzyme Activity. The study of the effect of metal ions on enzyme activity reveals that none of them had an activating effect when concentrations between 10^{-4} and 10^{-3} M were used. Mg^{2+} and Ca^{2+} had no significant effect, whereas Mn^{2+} and Co^{2+} were slightly inhibitory. Fe^{2+}, Fe^{3+}, Cu^{2+}, and Zn^{2+} showed strong inhibitory effects. The reduced phytase activity in the presence of Fe^{2+} and Fe^{3+} is attributed to a lower phytate concentration because of the appearance of an iron phytate precipitate.

Inhibition Studies. When compounds that tend to chelate metal ions, such as *o*-phenanthroline, EDTA, oxalate, citrate, or tartrate, were tested for their effect on enzyme activity, it was noted that none of them was inhibiting at concentrations from 10^{-4} to 10^{-3} M. Therefore, the enzyme, like many other phytate-degrading enzymes, is not a metalloenzyme. Moreover, cyanide and azide did not show any effect. The absence of an effect of the sulfhydryl inhibitors 2-mercaptoethanol and iodoacetate indicates that sulfhydryl groups do not participate in the active site of the enzyme. The enzyme is also insensitive to the presence of the serine-specific reagent phenylmethanesulfonyl fluoride. The strongest inhibitors were fluoride, phosphate, molybdate, wolframate, and vanadate. Fluoride, a known inhibitor of different phytate-degrading enzymes and other phosphatases (*3*, *4*, *29*−*33*, *35*), inhibits the hydrolysis of phytate competitively with a K_i value of 0.2 mM.

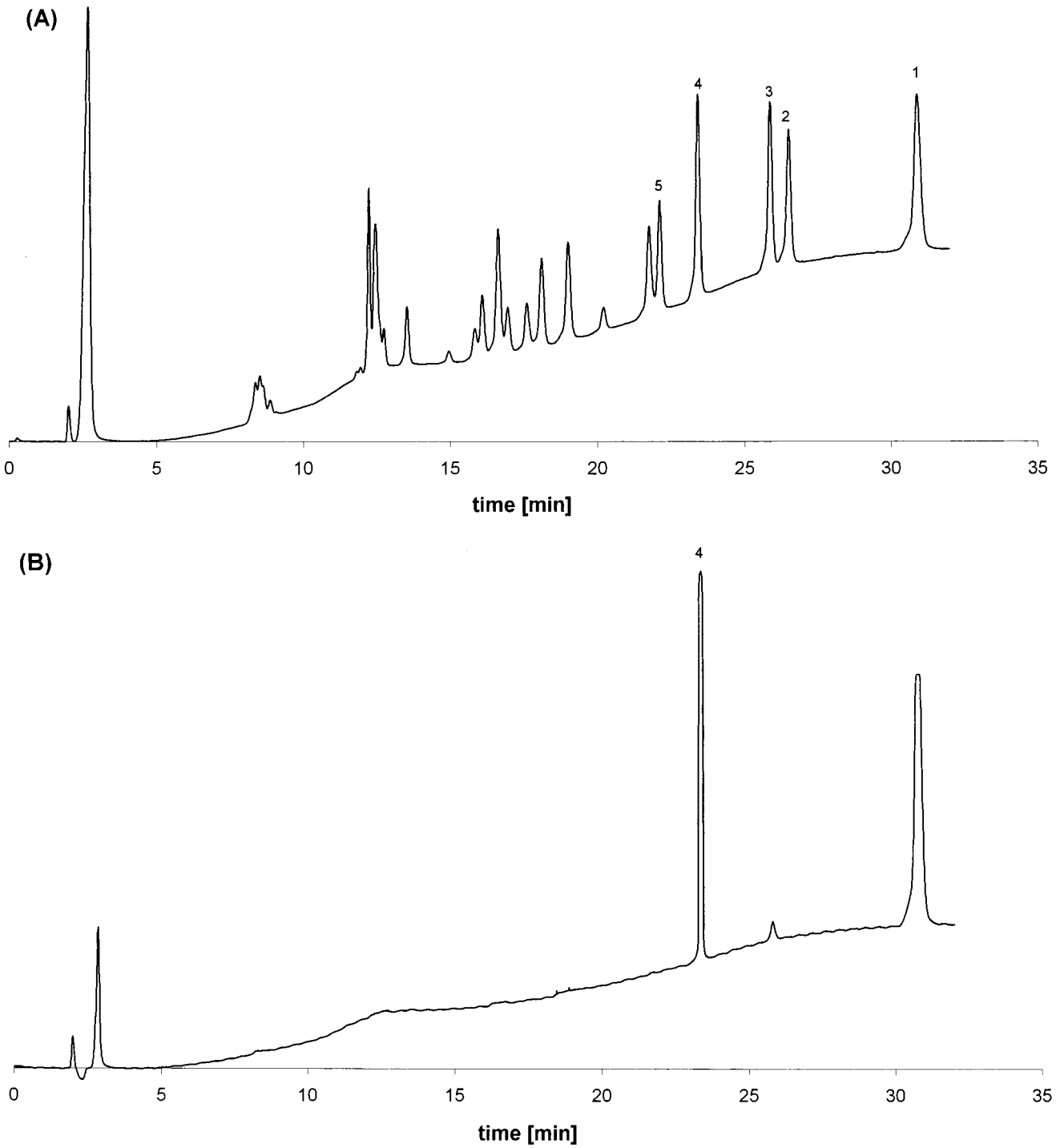

Figure 3. HPIC analysis of the $InsP_5$ isomer formed by the degradation of sodium phytate by the faba bean phytase. The enzymatic reaction products were separated by HPIC with postcolumn derivatization. (A) Reference sample. Peaks: (1) $InsP_6$; (2) Ins(1,3,4,5,6)P_5; (3)D/L-Ins(1,2,4,5,6)P_5; (4) D/L-Ins(1,2,3,4,5)P_5; (5) Ins(1,2,3,4,6)P_5. The source of the reference *myo*-inositol pentaphosphates was as indicated in ref *21*. (B) Hydrolysis mixture of sodium phytate incubated with the purified faba bean phytase.

DISCUSSION

This paper describes for the first time the purification and characterization of a phytate-degrading enzyme of faba beans. The purified enzyme shares many enzymatic properties in common with other phytate-degrading enzymes, especially with those of plants. As the phytate-degrading enzyme of scallion leaves (M_r = 72 kDa) (*19*), soybean seeds (M_r = 60 kDa) (*17*), mung bean seeds (M_r = 160 kDa) (*18*), spelt (M_r = 68 kDa) (*35*), rye (M_r = 67 kDa) (*31*), oat (M_r = 67 kDa) (*32*), and barley (M_r = 67 kDa) (*33*), the phytate-degrading enzyme of faba beans (M_r = 66 kDa) is a monomeric enzyme, whereas the phytate-degrading enzyme of maize is a dimer consisting of two subunits with a molecular mass of 38 kDa (*34*, *39*).

In plants, two main types of phytate-degrading enzymes have been identified: acidic phytate-degrading enzymes with optimum activity around pH 5.5 and alkaline phytate-degrading enzymes with a pH optimum around pH 8.0. The phytate-degrading enzyme of faba

beans belongs, as do most of the so far characterized phytate-degrading enzymes of plants, to the acidic ones. As expected for acidic phytate-degrading enzymes, the faba bean enzyme exhibits a broad affinity for various phosphorylated compounds. Like the phytate-degrading enzymes of wheat (*36*), spelt (*35*), rye (*31*), oat (*32*), and barley (*33*), the highest relative rates of hydrolysis were with pyrophosphate and ATP. Of all phosphate esters and anhydrides tested, phytate gave the highest k_{cat}/K_M value (Table 2). Thus, under physiological conditions the likely substrate for the phytate-degrading enzyme of faba beans is phytate, and therefore this enzyme should be a phytase. The turnover number of phytate was determined to be 693 s^{-1}. This is of the same magnitude as the molecular activity for the phytate-degrading enzyme of scallion leaves (600 s^{-1}) (*19*) but considerably higher those of the corresponding plant enzymes reported so far [spelt (368 s^{-1}) (*35*), rye (358 s^{-1}) (*31*), oat (356 s^{-1}) (*32*), tomato (350 s^{-1}) (*40*), barley P1 (136 s^{-1}) (*33*), barley P2 (43 s^{-1}) (*33*), and soybean (<10 s^{-1}) (*17*)].

Germinated faba bean seeds contain phytase activity comparable to that in germinated seeds of cereal such as wheat, spelt, and barley (*41*). The response to temperature and pH of the phytate-degrading enzyme of faba beans is similar to that of cereals (*31*−*33*, *35*, *39*) and soybean seeds (*17*). Although phytate-degrading enzymes of microorganisms are generally more stable to temperature and pH and exhibit a higher activity between pH 2 and 4, phytate-degrading enzymes of cereal seeds reduce phytate content in foods to an extent similar to that shown by their microbial counterparts (*5*). Thus, it can be concluded that legume phytate-degrading enzymes may be an alternative to the corresponding enzymes of microorganisms and cereal seeds for a biotechnological application to reduce phytate content during food and feed processing. In this context it is of practical interest that legume (*42*) as well as cereal (*43*) phytate-degrading enzymes exhibit a higher thermostability in the plant tissue compared to the isolated enzyme.

Phytate-degrading enzymes with different hydrolysis pathways from phytate have been identified in various organisms (*7*, *8*, *44*, *45*). The faba bean enzyme was identified as a 4-phytase or a 6-phytase (EC 3.1.3.26), because from the analysis of the individual stereoisomers the pentakisphosphate was identified as D/L-*myo*-inositol(1,2,3,4,5)pentakisphosphate. Cereal phytate-degrading enzymes are known to form D-*myo*-inositol1,2,3,5,6)pentakisphosphate (*8*), and it is likely that the phytate-degrading enzyme of faba beans does as well. The elucidation of the complete hydrolysis pathway of phytate by the phytate-degrading enzyme of faba beans, including the identification of the absolute configuration of the individual phytate degradation products, is currently under way. This is of interest because some of the breakdown products probably have useful chemical and biological activities.

In accordance with many cereals and legumes, germination is a suitable method to increase phytase activity in faba beans. Although large increases in phytase activities have been extensively reported in germinating seeds as well as in germinating pollen, the biochemical mechanism leading to this rise in phytase activity is not well understood. It was suggested that phytate-degrading enzymes induced during germination may be synthesized from long-lived, pre-existing mRNA or by de novo synthesis. Furthermore, the rapid increase of activity in the very beginning of germination may be due to activation of an inactive form of the phytate-degrading enzyme.

To study the control of expression of the phytate-degrading enzyme in faba beans and to produce sufficient amounts of this enzyme for a biochemical application to improve the nutritional value of plant-based foods, the corresponding encoding gene will have to be isolated and characterized.

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