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Purification and Characterization of Three Phytases from Germinated Lupine Seeds (*Lupinus albus* Var. Amiga)

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Three phytases were purified about 14200-fold (LP11), 16000-fold (LP12), and 13100-fold (LP2) from germinated 4-day-old lupine seedlings to apparent homogeneity with recoveries of 13% (LP11), 8% (LP12), and 9% (LP2) referred to the phytase activity in the crude extract. They behave as monomeric proteins of a molecular mass of about 57 kDa (LP11 and LP12) and 64 kDa (LP2), respectively. The purified proteins belong to the acid phytases. They exhibit 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 = 80 μM (LP11), 300 μM (LP12), and 130 μM (LP2) and k_{cat} = 523 s^{-1} (LP11), 589 s^{-1} (LP12), and 533 s^{-1} (LP2) at pH 5.0 and 35 °C. The phytases from lupine seeds exhibit a broad affinity for various phosphorylated compounds and hydrolyze phytate in a stepwise manner.

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

INTRODUCTION

Especially in the South American Andean region, lupine seeds have been used as a source of protein and oil from ancient times. In recent years lupine protein has been studied as an alternative protein for human consumption with growing interest as a result of its good functional properties and its high concentration in lupine seeds. In addition, the development of new applications of lupine protein and its use as a food ingredient would promote the production of this crop with a positive impact in terms of agriculture on marginal soils. A major obstacle in utilizing lupine seeds for food and feed is the presence of antinutrients such as phytate [*myo*-inositol(1,2,3,4,5,6)hexakisphosphate]. Trugo et al. have reported a phytate content of 0.4−1.2% dry matter in seeds of different wild species and cultivated varieties of lupine (*1*).

Phytases (*myo*-inositol hexakisphosphate phosphohydrolase) are widely distributed in nature (*2*, *3*), for example, in plants, microorganisms, and certain animal tissues. These enzymes catalyze the stepwise degradation of phytate, the principle storage form of phosphorus in mature seeds of cereals and legumes (*4*), to a series of lower *myo*-inositol phosphates and orthophosphate. Phytases have been studied intensively in recent years because of the interest in such enzymes for reducing the phytate content in animal feed and food for human consumption. Phytases were originally proposed as an animal feed additive to enhance the value of plant material in animal feed by liberating orthophosphate (*5*). More recently, addition of phytases has also been seen as a way to reduce the level of phosphate pollution in areas of intensive livestock management. A number of studies have already shown that addition of phytases enhances phosphate utilization from phytate and drastically reduces orthophosphate excretion (*6*, *7*). As phytate can act as an antinutrient by binding to proteins and by chelating minerals, such as zinc, iron, calcium, and magnesium (*8*), addition of phytases can improve the nutritional value of plant-based foods by enhancing protein digestibility and mineral availability through phytate hydrolysis during digestion in the stomach (*9*, *10*) or during food and feed processing (*4*). Phytases are also of interest for producing individual breakdown products of phytate for kinetic and physiological studies.

The objective of this work was to purify and characterize a phytase from lupine seeds. This enzyme may find application in improving the physiological quality of lupine protein based products. Therefore, this work is in line with the recent general trend to find alternatives to animal and soybeans as protein sources in food. There are several publications on the purification and characterization of phytases from microbial (*11*−*18*) and cereal (*19*−*24*) origins, but the knowledge on phytases from legumes is rather limited. The only phytases from legumes purified to apparent homogeneity and characterized so far are the enzymes from soybean seeds (*25*), faba bean seeds (*26*), mung bean seeds (*27*), and scallion leaves (*28*). A first report on lupine phytase was given by Silva and Trugo (*29*), but for characterization only a partly purified enzyme preparation was used.

MATERIALS AND METHODS

Chemicals. *Lupinus albus* var. Amiga was obtained from Südwestsaat GbR (Rastatt, Germany). Fractogel CMD COO^- 650(S) and most of the enzyme substrates were purchased from E. Merck (Darmstadt, Germany). Phytic acid dodecasodium salt was obtained

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from Aldrich (Steinheim, Germany), and all other phosphorylated compounds came from Fluka and Sigma. CM-Sepharose CL 6B, DEAE-Sepharose CL 6B, and high-load 16/60 Sephacryl S-200 HR were obtained from Pharmacia (Freiburg, Germany). Ultrasep ES 100 RP18 was purchased from Bischoff (Leonberg, Germany). AG1 X-4, 100−200 mesh, resin was obtained from Bio-Rad (München, Germany). All reagents were analytical grade.

Seed Germination. For surface sterilization lupine 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 extraction of the phytases, the germinated lupine 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 phytase activity in comparison to seeds extracted immediately after the germination process.

Enzyme Extraction. Freeze-dried lupine 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, containing 0.1 M phenylmethanesulfonyl fluoride 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 (*30*).

Assay of Phytase Activity. Enzyme extracts were dialyzed against 20 mM sodium acetate buffer, pH 5.0. The enzymatic reactions were started by adding 50 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 and 400 μg of bovine serum albumin. After incubation at 35 °C for 30 min, the liberated phosphate was measured by using the ammonium molybdate method (*31*) 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 measurement of 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.

To determine the substrate selectivity of the phytases from lupine seeds, several phosphorylated compounds in addition to phytate were utilized for K_M and v_{max} estimation by using the standard assay for phytase activity and the phosphorylated compound in a serial dilution of a concentrated stock solution (10 mM).

To study the pH optimum and pH stability of the purified enzymes, 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.

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 purified enzymes for 15 min at 35 °C before the standard assay for phytase activity 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 enzymes was conducted from 10 to 80 °C using the standard assay for phytase activity at the given temperature. To check thermal stability, the purified enzymes were incubated at different temperatures, cooled to 4 °C, and assayed using the standard assay for phytase activity.

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 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 (*32*). A mixture of the individual *myo*-inositol phosphate esters ($InsP_3$−$InsP_6$) was used as a standard. The lower *myo*-inositol phosphates have been generated by controlled enzymatic hydrolysis of phytate.

Purification of the Phytases. *Ammonium Sulfate Precipitation.*The crude extract was used for an ammonium sulfate precipitation at 4 °C and 30−80% saturation. The precipitate was collected by centrifugation at 12000*g* for 30 min and suspended in 20 mM sodium acetate buffer, pH 4.5, containing 0.1% Trition X-100 and dialyzed against the same buffer. Any insoluble material was removed by centrifugation at 12000*g* for 30 min.

CM-Sepharose CL 6B Chromatography. The dialyzed ammonium sulfate fraction was loaded onto a CM-Sepharose CL 6B column (3.5 × 20 cm) equilibrated with 20 mM sodium acetate buffer, pH 4.5, containing 0.1% Triton X-100. The column was run at 4 °C and a flow rate of 50 mL h^{-1}. After elution of the unbound inactive (phytate degradation) 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, containing 0.1% Triton X-100 was applied. Two peaks of phytase activity, called LP1 and LP2, were separated. Both enzyme preparations were further investigated separately. The fractions (10 mL) containing phytase activity were pooled and dialyzed against 20 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100.

DEAE-Sepharose CL 6B Chromatography. The pooled fractions from the CM-Sepharose CL 6B column were loaded onto a DEAE-Sepharose CL 6B column (4 × 15 cm) equilibrated with 20 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100. The column was run at 4 °C and a flow rate of 50 mL h^{-1}. After elution of the unbound protein from the column with equilibration buffer, a linear gradient from 0 to 0.5 M NaCl (1000 mL) in 20 mM Tris-HCl buffer, pH 8.0, containing 0.1% Triton X-100 was applied. LP1 could be separated into LP11 (unbound) and LP12 (bound). Both enzyme preparations were further investigated separately. The fractions (10 mL) containing phytase activity were pooled.

16/60 Sephacryl S-200 HR Chromatography. The pools containing phytase activity 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 and 0.1% Triton X-100 in 2 mL portions. The FPLC column was run with the same buffer at 25 °C and a flow rate of 1 mL min^{-1}. The fractions (2 mL) containing phytase activity were pooled and dialyzed against 20 mM sodium acetate buffer, pH 5.0, containing 0.1% Triton X-100.

Affinity Chromatography. The pools from the previous step were applied onto an affinity column (0.5 × 5 cm) [affinity resin: 1-*O*-3-aminopropyl-1-phospho-*myo*-inositol 3,4,5-trisphosphate coupled to Affi-Gel 10 (Bio-Rad), kindly provided by Dr. György Dormán, State University of New York], equilibrated with 20 mM sodium acetate buffer, pH 5.0, containing 0.1% Triton X-100. The column was run at 4 °C and a flow rate of 20 mL h^{-1}. After elution of the unbound protein from the column with equilibration buffer, a linear gradient of 0−0.5 M sodium chloride (200 mL) in 20 mM sodium acetate buffer, pH 5.0, containing 0.1% Triton X-100 was applied. The fractions (2 mL) containing phytase activity were pooled and dialyzed against 20 mM sodium acetate buffer, pH 5.0, containing 0.1% Triton X-100.

Gel Electrophoresis. Native gel electrophoresis was carried out with 5% gels at pH 4.8 (*33*). 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 (*34*). SDS electrophoresis using 10% gels was performed according to the method of Laemmli (*35*). 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

Table 1. Phytase Activity during Germination of Lupine Seeds[a]

germination time (days)	phytase activity (units g^{-1} of dry matter)	germination time (days)	phytase activity (units g^{-1} of dry matter)
0	0.017 ± 0.004	6	0.086 ± 0.005
1	0.020 ± 0.003	7	0.082 ± 0.004
2	0.025 ± 0.003	8	0.077 ± 0.005
3	0.041 ± 0.004	9	0.075 ± 0.003
4	0.097 ± 0.002	10	0.070 ± 0.003
5	0.089 ± 0.004		

[a] Temperature, 35 °C; buffer, 0.1 M sodium acetate, pH 5.0. The data are mean values ± standard deviation of three independent experiments.

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 Lupine Seeds. Only a very low phytase activity was detected in dry lupine seeds (0.017 ± 0.004 units g^{-1} of grain). During germination, maximum activity was reached after 4 days; a 5.7-fold increase in phytase activity was observed (0.097 ± 0.002 units g^{-1} of grain) (**Table 1**). Therefore, lupine seeds germinated for 4 days were used as a source of the enzymes.

Purification of the Phytases. A summary of the purification scheme is given in **Table 2**. Three phytases (LP11, LP12, and LP2) were purified from germinated lupine seeds using ammonium sulfate precipitation, ion-exchange chromatography, gel filtration, and affinity chromatography. The addition of 0.1% Triton X-100 helped stabilize phytase activity during CM-Sepharose CL 6B chromatography and subsequent steps. The phytase activity was completely bound to the cationic-exchange resin and eluted as two well-separated activity peaks (LP1 at 0.3 M NaCl and LP2 at 0.5 M NaCl) from that column. LP1 was found to consist of two phytases during the subsequent anion-exchange chromatography. LP12 and LP2 were retained on this column and could be eluted by increasing the ionic strength of the buffer, whereas LP11 was found to be in the unbound fraction. Rechromatography of LP11 under identical conditions did not result in binding of this phytase to the anion-exchange resin, indicating that LP11 and LP12 are different enzymes. The final chromatography of the three fractions containing phytase activity on a affinity resin (**Figure 1**) gave the highest purification in a single purification step and resulted in homogeneous enzyme preparations.

The phytases were purified about 14200-fold (LP11), 16000-fold (LP12), and 13100-fold (LP2) with recoveries of 13% (LP11), 8% (LP12), and 9% (LP2) referred to the phytase activity in the crude extract. The purified enzymes exhibit activities of about 539 units mg^{-1} (LP11), 607 units mg^{-1} (LP12), and 498 units mg^{-1} (LP2).

Molecular Properties. Molecular mass and homogeneity of the enzyme preparations were estimated by PAGE and gel filtration. Polyacrylamide gel electrophoresis under denaturing (**Figure 2**) and non-denaturing conditions (data not shown) revealed only a single protein band after the gels had been stained with Coomassie blue. These results indicate that the phytases were purified to apparent homogeneity.

The apparent subunit molecular masses of the purified phytase enzymes were approximately 57 ± 2 kDa (LP11 and LP12) and 64 ± 2.5 kDa (LP2) as estimated by SDS−polyacrylamide gel electrophoresis (**Figure 2**). The molecular masses of the native enzyme were determined to be 56 ± 1.5 kDa (LP11 and LP12) and 65 ± 2 kDa (LP2) on a calibrated 16/60 Sephacryl S-200 HR column with elution position being measured by determination of enzyme activity (data not shown). Consequently, the phytases are monomeric proteins.

Enzymatic Properties. *pH Optimum and pH Stability.* All of the phytases purified from lupine seeds have 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 enzymes are virtually inactive below pH 3.0 and above pH 8.0 (data not shown).

The purified enzymes were stable over pH 3.5−7.5, whereas below pH 3.0 and above pH 7.5 activities declined rapidly. Over 90% residual activity was measured within 10 days at pH 5.0 and 4 °C. At pH 2.5 the enzymes lost about 63% (LP11), 82% (LP12), and 71% (LP2) of their initial activities during 24 h, whereas at pH 8.0 the enzymes were found to retain about 52% (LP11), 38% (LP12), and 44% (LP2) of their initial activities.

Temperature Optimum and Thermal Stability. The temperature optimum of all three phytases was found to be 50 °C. The Arrhenius activation energies for the hydrolysis of sodium phytate were calculated to be 20.3 kJ mol^{-1} (LP11), 25.1 kJ mol^{-1} (LP12), and 32.4 kJ mol^{-1} (LP11). The enzymes did not significantly lose activity during 90 min at temperatures up to 45 °C. When exposed for 90 min at 50 °C, they retained over 85% and at 60 °C 21% (LP11), 8% (LP12), and 15% (LP2) of their initial activities.

Substrate Selectivity and Kinetic Parameters. To determine the substrate selectivity of the phytases from lupine seeds, several phosphorylated compounds in addition to phytate were utilized for K_M and v_{max} estimation by detecting the appearance of the phosphate ion during hydrolysis using formation of a soluble phosphomolybdate complex in an acidic water/acetone mixture. The results are summarized in **Table 3**. The kinetic parameters for the hydrolysis of sodium phytate were determined to be $K_M = 80\ \mu M$ (LP11), 300 μM (LP12), and 130 μM (LP2) and $k_{cat} = 523\ s^{-1}$ (LP11), 589 s^{-1} (LP12), and 533 s^{-1} (LP2) at pH 5.0 and 35 °C. It is evident that in terms of the kinetic parameters, the highly negative charged phosphorylated compounds, pyrophosphate and ATP, are hydrolyzed much more quickly than phytate by all purified enzymes. A comparison of the enzymatic hydrolysis of the 5′-adenosine phosphates ATP, ADP, and AMP is interesting in that k_{cat} decreases and K_M increases with decreasing negative charge (decreasing number of phosphate residues). ADP and phytate are fair substrates of all phytases studied, whereas pyridoxal phosphate, *o*-phospho-L-serine, 2-naphthyl phosphate, and 2-glycerophosphate seem to be poor substrates. Sugar phosphates in general are fair substrates of the phytase LP12 only, whereas GTP seems to be a fair substrate of LP11 and LP12.

Like other phytases, the phytases of lupine seeds showed a substrate inhibition. The activity of the purified enzymes was inhibited at phytate concentrations >8 mmol L^{-1} (data not shown). Silva and Trugo reported an inhibition of a partly purified lupine phytase by phytate levels >0.33 mmol L^{-1} (*29*).

Enzymatic Hydrolysis of Phytate. To examine the action of the phytases of lupine seeds on sodium phytate, the hydrolysis products were separated by ion-pair reverse phase chromatography. All lupine phytases investigated exhibited a very similar behavior. Therefore, only the results of the action of LP2 on phytate are given in **Figure 3**. All three enzymes hydrolyzed phytate in a stepwise manner. The hydrolysis rate decreased markedly during degradation. This might be due to product

Table 2. Purification Scheme of the Phytases of Lupine Seeds

step	total protein (mg)	total activity (units)	specific activity (units mg^{-1})	purification (-fold)	recovery (%)
crude extract	573.0	21.57	0.038	1	100
$(NH_4)_2SO_4$ precipitation	219.0	19.31	0.088	2	89
CM-Sepahrose CL 6B					
LP1	4.9	10.85	2.21	58	50
LP2	1.7	4.93	2.90	76	23
DEAE-Sepharose CL 6B					
LP11	0.31	4.96	16.0	421	23
LP12	0.18	3.17	17.6	474	15
LP2	0.20	3.45	17.3	447	16
16/60 Sephacryl S200 HR					
LP11	0.061	3.37	55.2	1447	16
LP12	0.036	2.25	62.5	1658	10
LP2	0.048	2.47	51.5	1342	11
affinity chromatography					
LP11	0.0054	2.91	539.0	14184	13
LP12	0.0030	1.82	607.0	15973	8
LP2	0.0041	2.04	498.0	13105	9

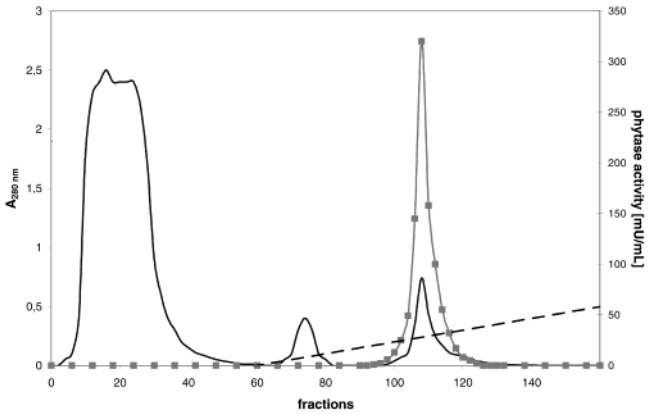

Figure 1. Chromatography of the phytase active fraction (LP2) on an affinity resin. The phytase active fractions from the gel filtration column were chromatographed on an affinity column (0.5 × 5 cm) [1-*O*-(3-aminopropyl-1-phospho)-*myo*-inositol 3,4,5-trisphosphate coupled to Affi-Gel 10 (Bio-Rad)], collecting 2 mL fractions. Fractions were analyzed for phytase activity (■): (—) optical density at 280 nm; (- - -) salt gradient.

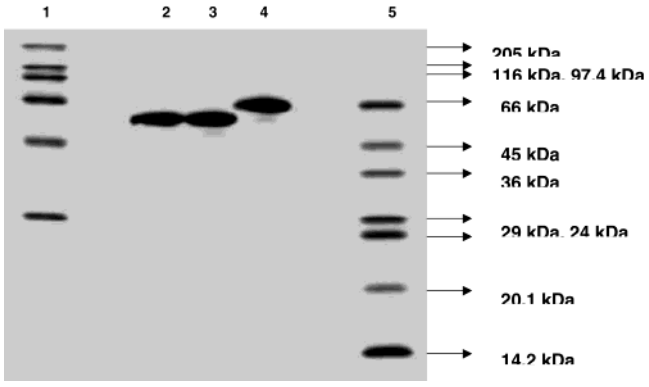

Figure 2. Electrophoretic analysis of purified lupine phytases. Coomassie blue staining of purified lupine phytases on a 10% SDS-PAGE: (lane 1) standard SDS-6; (lane 2) purified lupine phytase LP11 (30 μg of protein); (lane 3) purified lupine phytase LP12 (30 μg of protein); (lane 4) purified lupine phytase LP2 (30 μg of protein); (lane 5) standard SDS-7.

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.

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. Like many other phytases, the lupine phytases studied are not metalloenzymes, because compounds that tend to chelate metal ions, such as *o*-phenanthroline, EDTA, oxalate, citrate, or tartrate, were not observed to be inhibiting at concentrations from 10^{-4} to 10^{-3} M. Moreover, cyanide and azide did not show any effect. The absence of an effect of the sulfhydryl inhibitors 2-mercaptoethanol and iodoacetate points to a missing participation of sulfhydryl groups in the active site of the enzymes. The enzymes are also insensitive to the presence of the serine-specific reagent phenylmethanesulfonyl fluoride. The strongest inhibitors were found in fluoride, phosphate, molybdate, wolframate, and vanadate.

DISCUSSION

It was shown that phytases exist in multiple chromatographic forms in germinated lupine seeds. Multiple forms of phytases have already been identified in other plant species, such as barley (*21*), wheat (*23*, *36*, *37*), spelt (*22*), maize (*38*, *39*), rice (*40*), soybean (*41*), rapeseed (*42*), pumpkin (*43*), and lily (*44*).

The phytase from lupine seeds appeared to be homogeneous by polyacrylamide gel electrophoresis under non-denaturing conditions at pH 4.8 and gave a single band upon SDS gel electrophoresis. Like the phytases from scallion leaves (*28*), faba bean seeds (*26*), soybean seeds (*25*), rice bran (*40*), mung bean seeds (*27*), spelt (*22*), rye (*19*), oat (*20*), barley (*21*), and wheat bran (*23*), the corresponding enzymes from lupine seeds are single-chain proteins. Their molecular masses (LP11, 57 kDa; LP12, 57 kDa; LP2, 64 kDa) are lower than those from phytases purified from other plant sources, that is, 160 kDa for mung bean seeds (*27*); 72 kDa for scallion leaves (*28*); 68 kDa for rice bran (*40*), rye (*19*), spelt (*22*), and oat (*20*); 66 kDa for barley (*21*) and faba bean seeds (*26*); and 76 kDa for maize seedling (dimer) (*24*). Silva and Trugo identified two phytases in lupine seeds (*29*). Lupine phytase F_1 was identified as a single-chain protein with a molecular mass of 26 kDa, whereas it was impossible to determine the molecular mass of lupine phytase F_2 due to the limiting amount obtained during purifica-

Table 3. Kinetic Constants for the Hydrolysis of Phosphorylated Compounds by Lupine Phytases LP11, LP12, and LP2 at pH 5

	lupine phytase LP11			lupine phytase LP12			lupine phytase LP2		
substrate	K_M (μM)	k_{cat} (10^{-3} s^{-1})	k_{cat}/K_M (s^{-1} M^{-1})	K_M (μM)	k_{cat} (10^{-3} s^{-1})	k_{cat}/K_M (s^{-1} M^{-1})	K_M (μM)	k_{cat} (10^{-3} s^{-1})	k_{cat}/K_M (s^{-1} M^{-1})
phytate	80 ± 5	523 ± 21	6538	300 ± 17	589 ± 26	1963	130 ± 7	533 ± 18	4100
p-nitrophenyl phosphate	152 ± 7	431 ± 34	2835	123 ± 9	319 ± 18	2593	176 ± 10	254 ± 11	1443
1-naphthyl phosphate	512 ± 21	161 ± 12	314	718 ± 56	67 ± 10	93	492 ± 37	112 ± 6	228
2-naphthyl phosphate	653 ± 37	97 ± 11	149	634 ± 34	49 ± 7	77	516 ± 26	49 ± 2	95
2-glycerophosphate	667 ± 43	51 ± 4	76	881 ± 75	84 ± 9	95	704 ± 67	67 ± 3	95
Na_2H_2-pyrophosphate	445 ± 29	1824 ± 93	4098	691 ± 31	2312 ± 126	3342	812 ± 51	1686 ± 32	2076
AMP	365 ± 21	91 ± 10	249	315 ± 19	136 ± 15	432	319 ± 32	31 ± 2	97
ADP	403 ± 19	367 ± 23	911	398 ± 12	789 ± 63	1982	451 ± 39	435 ± 14	965
ATP	531 ± 36	1512 ± 76	2847	412 ± 21	1819 ± 91	4415	596 ± 42	1207 ± 27	2025
GTP	398 ± 27	313 ± 34	786	423 ± 15	612 ± 44	1447	217 ± 12	24 ± 2	111
fructose 1,6-diphosphate	229 ± 14	114 ± 17	498	731 ± 36	467 ± 37	639	359 ± 21	498 ± 21	1387
fructose 6-phosphate	478 ± 33	99 ± 7	207	676 ± 38	312 ± 21	462	531 ± 23	11 ± 1	21
glucose 6-phosphate	401 ± 36	217 ± 19	541	634 ± 46	398 ± 36	628	302 ± 20	52 ± 3	172
pyridoxal phosphate	776 ± 59	56 ± 3	72	876 ± 56	32 ± 5	36	915 ± 72	17 ± 1	19
o-phospho-L-serine	891 ± 71	19 ± 1	21	740 ± 49	21 ± 2	28	811 ± 69	10 ± 1	12

[a] Temperature, 35 °C; buffer, 0.1 M sodium acetate, pH 5.0; enzyme concentration, 50 milliunits mL^{-1}. Data are mean values ± standard deviation of three independent experiments.

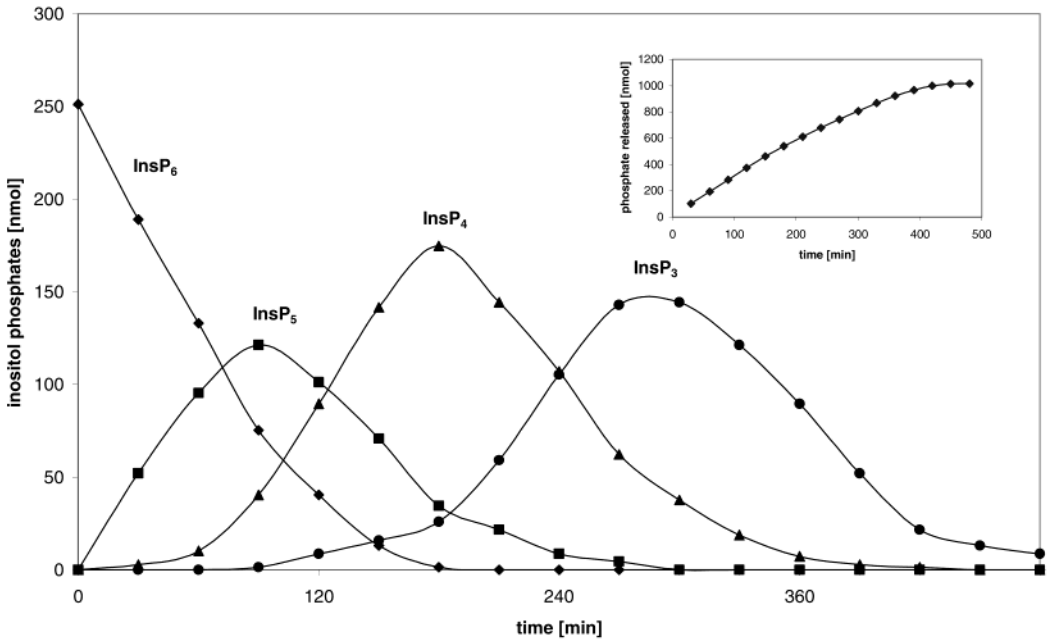

Figure 3. Time course of the action of lupine phytase LP2 on sodium phytate. Temperature, 35 °C; buffer, 0.1 M sodium acetate, pH 5.0; enzyme concentration, 8.5 milliunits mL^{-1}; substrate concentration, 0.625 mM. Enzymatic reaction products were separated by ion-pair chromatography. $InsP_6$, $InsP_3$; *myo*-inositol hexakisphosphate to -trisphosphate.

tion. The difference in the observed molecular masses is not easy to explain, but it has to be kept in mind that only a partly purified phytase preparation was characterized by Silva and Trugo (*29*).

Optimal conditions (pH 5.0; $T = 50$ °C) for the enzymatic phytate degradation by the purified lupine phytases are identical to those reported by Silva and Trugo (*29*) for a partly purified lupine phytase. Acidic pH and moderate temperature optimum are properties typical for plant acid phytases. The K_M values of the phytases from lupine seeds for phytate (LP11, 80 μmol L^{-1}; LP12, 300 μmol L^{-1}; LP2, 130 μmol L^{-1}) are similar to those found for other phytases from plant sources, that is, 72 μmol L^{-1} for barley (*21*), 117 μmol L^{-1} for maize (*24*), 148 μmol L^{-1} for faba beans (*26*), 300 μmol L^{-1} for rye (*19*), and 130 μmol L^{-1} for lupine phytase F_1 (*29*). The turnover numbers of phytate were determined to be 523 s^{-1} (LP11), 589 s^{-1} (LP12), and 533 s^{-1} (LP2). This is in the same magnitude as the molecular activity for the phytase of scallion leaves (600 s^{-1}) (*28*).

As reported for many other plant acid phytases, the purified lupine enzymes exhibit a broad substrate specificity and the highest turnover numbers are found with ATP and pyrophosphate and not with phytate. The natural substrate for these enzymes is not known, but their natural role is presumably related to some aspects of seed germination. In nongerminated seeds, the bulk of total phosphate appears as phytate and phytase activity increases during germination. Therefore, some of these enzymes might have a role in phosphate mobilization from phytate. Phytate degradation in vivo is not necessarily due to the action of one single enzyme, but could be realized by the concerted action of several phosphatases. Thus, the lupine phytases could conceivably be active in phytate breakdown in a cooperative role.

Like the phytases purified from faba bean (*26*), soybean cotyledon (*25*), wheat bran (*23*), rice bran (*40*), barley (*21*), rye (*19*), spelt (*22*), and oat (*20*), the lupine phytases are not metalloenzymes. The first metalloacid phytase has recently been isolated from soybean (*45*). A violet color attributed to the presence of binuclear Fe(III)−Me(II) centers where Me is Fe, Mn, or Zn is characteristic for this enzyme. However, no violet coloration is exhibited by the lupine seed phytases after the extensive purification that was carried out in the present study.

Common with many other acid phytases purified from plant sources, the phytases purified from lupine seed were inhibited by Cu^{2+}, Zn^{2+}, fluoride, phosphate, molybdate, wolframate, and vanadate, but depending on the plant source, their affinities to fluoride and phosphate have been shown to differ.

Due to the physicochemical and enzymatic properties, it can be concluded that legume phytases 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. The endogenous phytase activity in lupine seeds may find application especially for the production of lupine protein with improved nutritional and physiological qualities. Those protein preparations can be used as a beneficial alternative to soybean protein in food applications.

In addition, certain *myo*-inositol phosphates have been suggested to have beneficial health effects (*46*). The position of the phosphate groups on the *myo*-inositol ring is thereby of great significance for their physiological function. Depending on the source of the phytase, different phosphate-containing intermediates have been detected (*23*, *29*, *47*−*52*). Thus, the elucidation of the complete hydrolysis pathway of phytate by the phytase purified from lupine seeds, including the identification of the absolute configuration of the individual phytate degradation products, is currently under way. Because purification of large amounts of the phytases from lupine seeds remains a problem, cloning and overexpressing the corresponding genes may provide the best approach to make sufficient enzyme available for a biotechnological application and further studies.

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