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Pathway of Dephosphorylation of *myo*-Inositol Hexakisphosphate by Phytases of Legume Seeds

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Using a combination of high-performance ion chromatography analysis and kinetic studies, the pathway of dephosphorylation of *myo*-inositol hexakisphosphate by the phytases purified from faba bean and lupine seeds, respectively, was established. The data demonstrate that the legume seed phytases under investigation dephosphorylate *myo*-inositol hexakisphosphate in a stereospecific way. The phytase from faba bean seeds and the phytase LP2 from lupine seeds degrade phytate by sequential removal of phosphate groups via D-Ins(1,2,3,5,6)P_5, D-Ins(1,2,5,6)P_4, D-Ins(1,2,6)P_3, and D-Ins(1,2)P_2 to finally Ins(2)P, whereas the phytases LP11 and LP12 from lupine seeds generate the final degradation product Ins(2)P via D-Ins(1,2,4,5,6)P_5, D-Ins(1,2,5,6)P_4, D-Ins(1,2,6)P_3, and D-Ins(1,2)P_2.

KEYWORDS: Faba bean; legume phytase; lupine; *myo*-inositol phosphate isomers; phytate degradation

INTRODUCTION

Phytases [*myo*-inositol(1,2,3,4,5,6)hexakisphosphate phosphohydrolases] belong to a special group of phosphatases that are capable of sequentially hydrolyzing phytate [*myo*-inositol-(1,2,3,4,5,6)hexakisphosphate] to a series of lower *myo*-inositol phosphates and orthophosphate. In vitro it was shown that a single phytase can catalyze the sequential breakdown from *myo*-inositol hexakisphosphate to *myo*-inositol monophosphate or even *myo*-inositol (*1*−*8*). With respect to the final phytate degradation product, phytases can be divided into two groups. Enzymes exhibiting an acidic pH optimum release five or even all six phosphate groups of phytate and *myo*-inositol monophosphate and *myo*-inositol, respectively, have been detected as their final degradation products. On the other hand, the alkaline phytases (*9*−*11*) are not capable of accepting a *myo*-inositol phosphate with three or fewer phosphate residues as a substrate. Thus, *myo*-inositol trisphosphate was shown to be the end product of phytate degradation by these alkaline phytases. Depending on the phytase used, different phosphate residues of phytate may be released at different rates and in different order, resulting in the generation of different phosphate-containing intermediates.

Certain *myo*-inositol phosphates have been suggested to have beneficial health effects, such as amelioration of heart disease by controlling hypercholesterolemia and atherosclerosis (*12*, *13*), prevention of renal stone formation (*14*, *15*), and reduced risk of colon cancer (*16*, *21*). Because different structural isomers may result in different physiological functions, identification of the *myo*-inositol phosphates generated during enzymatic phytate degradation is of great importance to exploit the full potential of the naturally occurring phytases. The complete phytate degradation pathway was elucidated for only a few phytases, focusing on those of microbial (*1*−*3*, *11*, *22*) or cereal origin (*4*−*7*). There is only one paper describing the phytate degradation pathway of a legume phytase (*8*).

Because the bioactive *myo*-inositol phosphate isomers generated by legume phytases may be different from those generated by phytases from microbial or cereal origin and because it was shown recently 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 (*23*), the objective of this study was to elucidate the complete hydrolysis pathway of phytate by phytases purified from faba bean and lupine seeds, respectively.

MATERIALS AND METHODS

Chemicals. *Vicia faba* var. Alameda was obtained from CIDA of Cordoba (Spain), *Lupinus albus* var. Amiga from Südwestsaat GbR (Rastatt, Germany), and *Aspergillus niger* phytase from Novo Nordisk (Copenhagen, Denmark). Phytic acid dodecasodium salt was purchased from Aldrich (Steinheim, Germany). Ultrasep ES 100 RP18 was obtained from Bischoff (Leonberg, Germany), and a high-performance ion chromatography (HPIC) column Carbo-Pac PA-100 from Dionex

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(Sunnyvale, CA). AG1 X-4, 100−200 mesh, resin was purchased from Bio-Rad (München, Germany).

Purification of the Phytases. Purification of the phytases of *Aspergillus niger* (*3*), *Escherichia coli* (*24*), rye (*25*), faba bean seeds (*26*), and lupine seeds (*23*) was performed as described previously. All phytases were purified to apparent homogeneity according to denaturing and non-denaturing polyacrylamide gel electrophoresis.

Assay of Phytase Activity. Phytase activity measurements were carried out at 35 °C. The enzymatic reactions were started by the addition of 10 μL of enzyme to the assay mixtures. The incubation mixture for phytase activity determination consisted of 350 μL of 0.1 M sodium acetate, pH 4.5 (*Escherichia coli* and *Aspergillus niger*), 0.1 M sodium acetate, pH 6.0 (rye), and 0.1 M sodium acetate, pH 5.0 (faba bean and lupine), respectively, containing 500 nmol of sodium phytate.

To determine the kinetic parameters for enzymatic dephosphorylation of individual *myo*-inositol phosphates by the phytase from faba bean and lupine seeds, 10 milliunits of the phytase were added to sequentially diluted solutions of the purified *myo*-inositol phosphate isomer (2.0, 1.0, 0.5, 0.25, 0.125, 0.06, 0.03, and 0.015 mM) in 400 μL of 0.1 M sodium acetate buffer, pH 5.0, at 35 °C. The following *myo*-inositol phosphate isomers were used: D-Ins(1,2,4,5,6)P_5, D-Ins(1,2,3,4,5)P_5, D-Ins(1,2,3,5,6)P_5, and the *myo*-inositol pentakisphosphate produced by the legume phytase under investigation.

After an incubation period of 30 min, the liberated phosphate was quantified by using the ammonium molybdate method (*27*) with some modification. The rate of reaction was linear for the 30 min incubation time (data not shown); 1.5 mL of a freshly prepared solution of acetone/5 N sulfuric acid/10 mM ammonium molybdate (2:1:1 v/v) and thereafter 100 μL of 1.0 M citric acid were added. Any cloudiness was removed by centrifugation prior to the measurement of absorbance at 355 nm. To quantify the released phosphate, a calibration curve was produced over the range of 5−600 nmol phosphate. Activity (units) was expressed as micromoles of phosphate liberated per minute. Blanks were run by addition of the ammonium molybdate solution prior to addition of the enzyme solution to the assay mixture. The kinetic constants (K_M and v_{max}) were calculated from Lineweaver−Burk plots of the data. For calculation of k_{cat} the following molecular masses were used: faba bean, 66 kDa (*26*); lupine LP11, 57 kDa; lupine LP12, 57 kDa; and lupine LP2, 64 kDa (*23*).

Preparation of Lower *myo*-Inositol Phosphates. The phytases from *Aspergillus niger*, *Escherichia coli*, and rye were used to generate D-Ins(1,2,4,5,6)P_5, D-Ins(1,2,3,4,5)P_5, and D-Ins(1,2,3,5,6)P_5. *myo*-Inositol hexakisphosphate (500 μmol) was incubated at 35 °C in a mixture containing 50 mM ammonium acetate, pH 4.5 (*Escherichia coli* and *Aspergillus niger*), 50 mM ammonium acetate, pH 6.0 (rye), and 50 mM ammonium acetate, pH 5.0 (faba bean and lupine), respectively, and 10 units of the phytases in a final volume of 200 mL. After an incubation period of 30 min, the reactions were stopped by heat treatment (95 °C for 10 min). The *myo*-inositol pentakisphosphates were purified as described previously (*3*).

Production of Enzymatically Formed Hydrolysis Products. The enzymatic reaction was started at 35 °C by the addition of 50 μL of the suitably diluted solution of the different legume phytases to the incubation mixtures (100 milliunits mL^{-1}). The incubation mixture consisted of 1250 μL of 0.1 M sodium acetate buffer, pH 5.0, containing 2.5 μmol of sodium phytate. From the incubation mixture, 100 μL samples were removed periodically, and the reaction was stopped by heat treatment (90 °C for 5 min).

Identification of Enzymatically Formed Hydrolysis Products ($InsP_6$—$InsP_2$). Fifty microliters of the heat-treated samples was chromatographed on an HPIC system using a Carbo Pac PA-100 (4 × 250 mm) analytical column and a gradient of 5−98% HCl (0.5 M, 0.8 mL min^{-1}) as described by Skoglund et al. (*28*). The eluants were mixed in a postcolumn reactor with 0.1% $Fe(NO_3)_3 \cdot 9H_2O$ in a 2% $HClO_4$ solution (0.4 mL min^{-1}) according to the method of Phillippy and Bland (*29*). The combined flow rate was 1.2 mL min^{-1}.

Identification of the *myo*-Inositol Monophosphate Isomer. *myo*-Inositol monophosphate was produced by incubation of 1.0 unit of the legume phytase with a limiting amount of *myo*-inositol hexakisphosphate (0.1 μmol) in a final volume of 500 μL of 50 mM ammonium formate. After lyophilization, the residues were dissolved in 500 μL of a solution of pyridine/bis(trimethylsilyl)trifluoroacetamide (1:1 v/v) and incubated at room temperature for 24 h. The silylated products were injected at 270 °C into a gas chromatograph coupled with a mass spectrometer. The stationary phase was methylsilicon in a fused silica column (0.25 mm × 15 m). Helium was used as the carrier gas at a flow rate of 0.5 m s^{-1}. The following heating program was used for the column: increase from 100 to 340 °C at 4 °C min^{-1}. Ionization was performed by electron impact at 70 eV and 250 °C.

Statistical Methods. For statistical comparison Student's *t* test was used.

RESULTS

Intermediates of Enzymatic *myo*-Inositol Hexakisphosphate Dephosphorylation. The identification of the hydrolysis products of *myo*-inositol hexakisphosphate generated by the phytase purified from faba bean seeds was performed by isomer-specific HPIC analysis (**Figure 1**). The chromatographic profile of the zero-time control indicated only the *myo*-inositol hexakisphosphate peak (peak 1). After 15 min of incubation, the quantity of peak 1 (*myo*-inositol hexakisphosphate) had decreased and peak 4 [D/L-Ins(1,2,3,4,5)P_5] appeared as the major degradation product, accompanied by small amounts of peak 3 [D/L-Ins(1,2,4,5,6)P_5]. After 30 min of incubation, a further decrease in peak 1 (*myo*-inositol hexakisphosphate) was observed. Both *myo*-inositol pentakisphosphates peak 4 [D/L-Ins(1,2,3,4,5)P_5] and peak 3 [D/L-Ins(1,2,4,5,6)P_5] had also decreased, whereas peak 8 (D/L-Ins(1,2,5,6)P_4) had increased. Furthermore, small amounts of peak 12 [D/L-Ins(1,2,3,4)P_4] and peak 18 [D/L-Ins(1,2,6)P_3 and/or Ins(1,2,3)P_3] were found. After 60 min of incubation, a further decrease in peaks 1, 3, and 4 (*myo*-inositol hexakisphosphate and pentakisphosphates) and a further increase in peak 8 [D/L-Ins(1,2,5,6)P_4], peak 12 [D/L-Ins(1,2,3,4)P_4], and peak 18 [D/L-Ins(1,2,6)P_3 and/or Ins(1,2,3)P_3] was observed. In addition, small amounts of peak 22 [D/L-Ins(1,2)P_2, Ins(2,5)P_2, and/or D/L-Ins(4,5)P_2] appeared. After 90 min of incubation, *myo*-inositol hexakisphosphate (peak 1) and the *myo*-inositol pentakisphosphates (peaks 3 and 4) were completely degraded to lower *myo*-inositol phosphates, mainly peak 8 [D/L-Ins(1,2,5,6)P_4], peak 18 [D/L-Ins(1,2,6)P_3 and/or Ins(1,2,3)P_3], and peak 22 [D/L-Ins(1,2)P_2, Ins(2,5)P_2, and/or D/L-Ins(3,5)P_2]. In addition, traces of peak 12 [D/L-Ins(1,2,3,4)P_4] were still present. After 120 min of incubation, only small amounts of peak 8 [D/L-Ins(1,2,5,6)P_4] remained within the *myo*-inositol tetrakisphosphates. They were nearly completely degraded to peak 18 [D/L-Ins(1,2,6)P_3 and/or Ins(1,2,3)P_3] and peak 22 [D/L-Ins(1,2)P_2, Ins(2,5)P_2, and/or D/L-Ins(4,5)P_2]. Incubation of the faba bean phytase with a limiting amount of phytate resulted in the generation of one *myo*-inositol monophosphate, which was identified as Ins(2)P by gas chromatography−mass spectrometry (data not shown).

A detailed characterization of the hydrolysis pathway of *myo*-inositol hexakisphosphate by the phytases from lupine seeds (**Figure 2**) revealed that LP11 and LP12 degrade phytate via D/L-Ins(1,2,4,5,6)P_5 (peak 3), D/L-Ins(1,2,5,6)P_4 (peak 8), Ins(1,2,3)P_3 or D/L-Ins(1,2,6)P_3 (peak 18), and D/L-Ins(1,2)P_2 or Ins(2,5)P_2 or D/L-Ins(4,5)P_2 (peak 22) to finally Ins(2)P, whereas LP2 hydrolyzes phytate, as the phytase from faba bean seeds, via D/L-Ins(1,2,3,4,5)P_5 (peak 4), D/L-Ins(1,2,5,6)P_4 (peak 8), Ins(1,2,3)P_3 or D/L-Ins(1,2,6)P_3 (peak 18), and D/L-Ins(1,2)P_2 or Ins(2,5)P_2 or D/L-Ins(4,5)P_2 (peak 22) to finally Ins(2)P.

Kinetic Studies. To determine the kinetic parameters for the hydrolysis of some *myo*-inositol phosphate isomers at pH 6.0 and 35 °C by the legume phytase under investigation, the

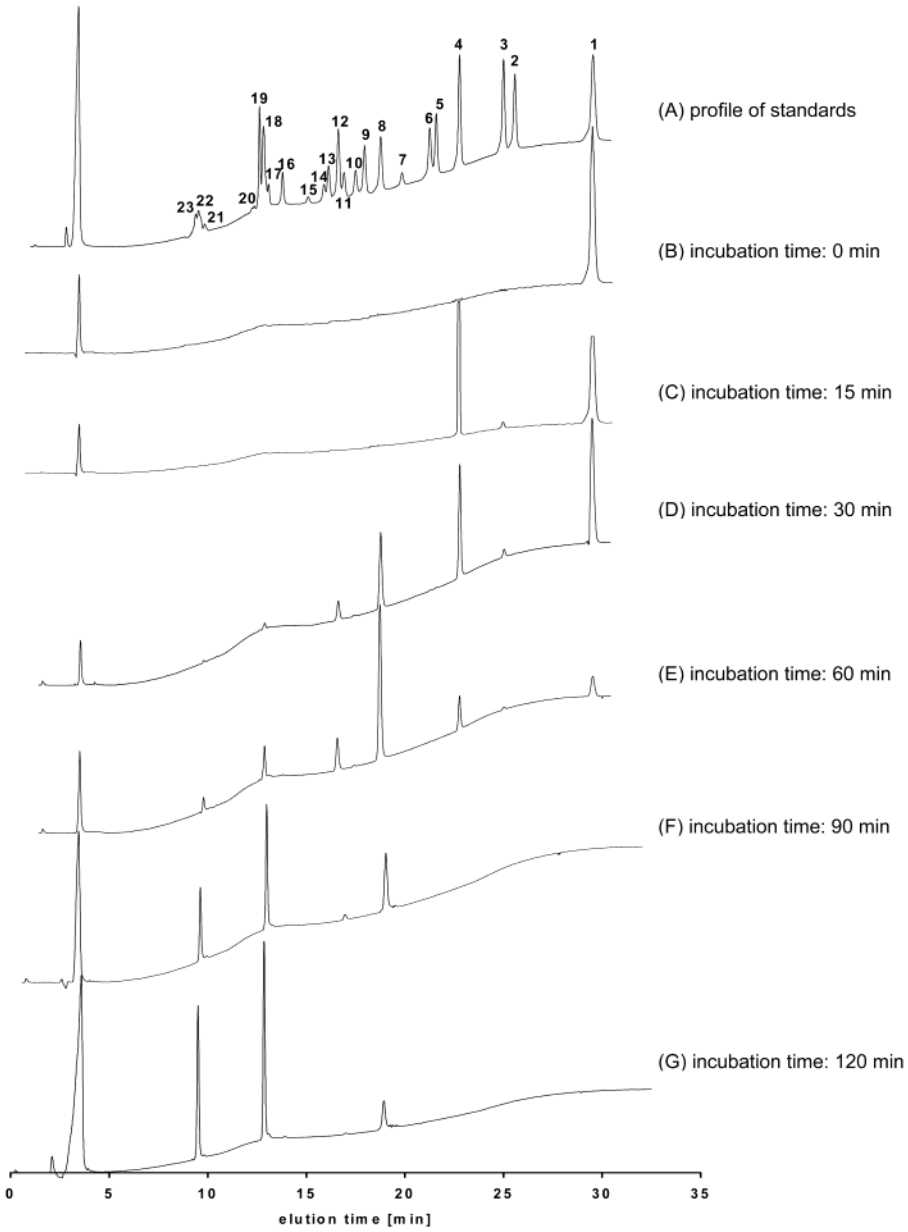

Figure 1. HPIC analysis of the enzymatically formed hydrolysis products of *myo*-inositol hexakisphosphate ($InsP_6$–$InsP_2$) by the phytase purified from faba bean seeds reference sample (A). The source of the reference *myo*-inositol phosphates is as indicated in Skoglund et al. (*28*), phytase from faba bean seeds. Peaks: (1) Ins(1,2,3,4,5,6)P_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; (6) D/L-Ins(1,4,5,6)P_4; (7) Ins(2,4,5,6)P_4; (8) D/L-Ins(1,2,5,6)P_4; (9) D/L-Ins(1,3,4,5)P_4; (10) D/L-Ins(1,2,4,5)P_4; (11) Ins(1,3,4,6)P_4; (12) D/L-Ins(1,2,3,4)P_4; (13) D/L-Ins(1,2,4,6)P_4; (14) Ins(1,2,3,5)P_4; (15) Ins(4,5,6)P_3; (16) D/L-Ins(1,5,6)P_3; (17) D/L-Ins(1,4,5)P_3; (18) D/L-Ins(1,2,6)P_3, Ins(1,2,3)P_3; (19) D/L-Ins(1,3,4)P_3; (20) D/L-Ins(1,2,4)P_3, D/L-Ins(2,4,5)P_3; (21) D/L-Ins(2,4)P_2; (22) D/L-Ins(1,2)P_2, Ins(2,5)P_2, D/L-Ins(4,5)P_2; (23) D/L-Ins(1,4)P_2, D/L-Ins(1,6)P_2.

enzymes were added to sequentially diluted solutions of Ins(1,2,3,4,5,6)P_6, D-Ins(1,2,4,5,6)P_5, D-Ins(1,2,3,5,6)P_5, D-Ins(1,2,3,4,5)P_5, and the major *myo*-inositol pentakisphosphate generated by the legume phytase under investigation. The kinetic parameters (K_M and k_{cat}) were calculated from Lineweaver−Burk plots of the data (**Table 1**). K_M and k_{cat} for the enzymatic hydrolysis of Ins(1,2,3,4,5,6)P_6 were determined to be $K_M = 148$ μmol L^{-1} (faba bean), 80 μmol L^{-1} (lupine LP11), 300 μmol L^{-1} (lupine LP12), and 130 μmol L^{-1} (lupine LP2) and $k_{cat} = 704$ s^{-1} (faba bean), 523 s^{-1} (lupine LP11), 589 s^{-1} (lupine LP12), and 533 s^{-1} (lupine LP2), respectively. In comparison to *myo*-inositol hexakisphosphate, the affinity of the *myo*-inositol pentakisphosphates included in the study for the legume phytases under investigation and their maximal rates of hydrolysis were higher. Comparing the hydrolysis of the *myo*-inositol pentakisphosphates included in the study for every single enzyme, it is obvious that the kinetic parameters for the *myo*-inositol pentakisphosphate generated by the enzyme under investigation are not statistically significant different ($P < 0.05$) from the kinetic parameters for the hydrolysis of D-Ins(1,2,3,5,6)P_5 (faba bean and lupine LP2) and D-Ins(1,2,4,5,6)P_5 (lupine LP11 and LP12), respectively.

DISCUSSION

Several studies have been performed to elucidate the hydrolysis pathway of phytate by phytases from bacterial (*1*, *2*, *11*), fungal (*3*, *22*), and cereal (*4*−*7*) origins, but only one study on the phytate hydrolysis pathway of a legume phytase has been reported (*8*). The phytase from mung beans seeds was reported

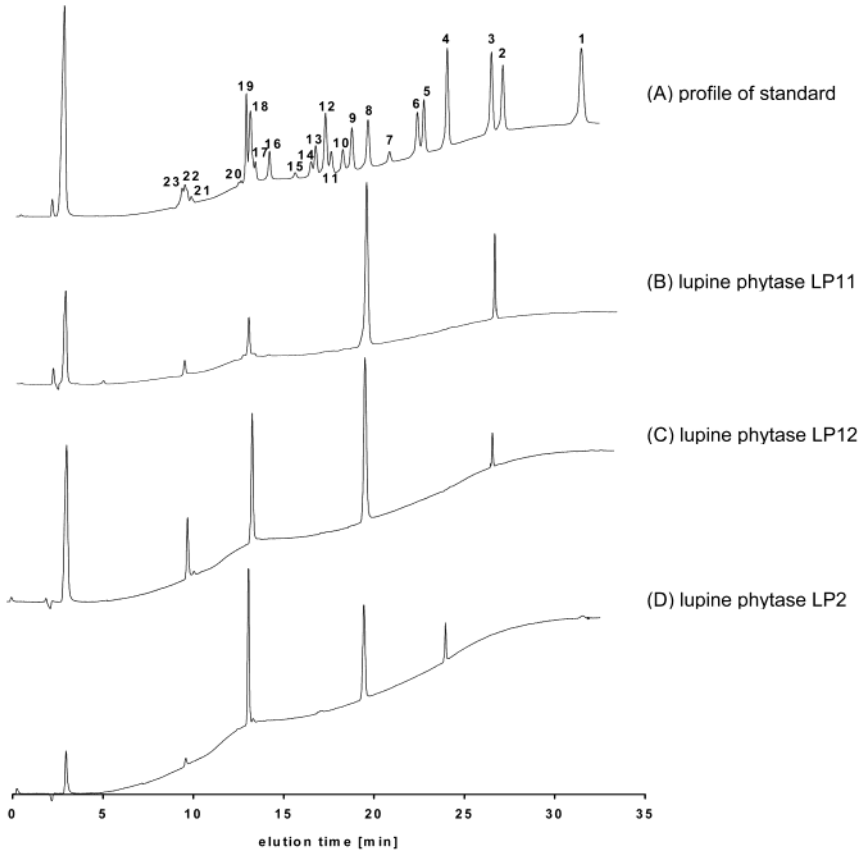

Figure 2. HPIC analysis of the enzymatically formed hydrolysis products of *myo*-inositol hexakisphosphate ($InsP_6$–$InsP_2$) by the phytase purified from lupine seeds reference sample (A). The source of the reference *myo*-inositol phosphates is as indicated in Skoglund et al. (*28*). Reference sample: see **Figure 1**, incubation time = 60 min. (B) Phytase LP11; (C) phytase LP12; (D) phytase LP2. Peaks: (1) Ins(1,2,3,4,5,6)P_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; (6) D/L-Ins(1,4,5,6)P_4; (7) Ins(2,4,5,6)P_4; (8) D/L-Ins(1,2,5,6)P_4; (9) D/L-Ins(1,3,4,5)P_4; (10) D/L-Ins(1,2,4,5)P_4; (11) Ins(1,3,4,6)P_4; (12) D/L-Ins(1,2,3,4)P_4; (13) D/L-Ins(1,2,4,6)P_4; (14) Ins(1,2,3,5)P_4; (15) Ins(4,5,6)P_3; (16) D/L-Ins(1,5,6)P_3; (17) D/L-Ins(1,4,5)P_3; (18) D/L-Ins(1,2,6)P_3, Ins(1,2,3)P_3; (19) D/L-Ins(1,3,4)P_3; (20) D/L-Ins(1,2,4)P_3, D/L-Ins(2,4,5)P_3; (21) D/L-Ins(2,4)P_2; (22) D/L-Ins(1,2)P_2, Ins(2,5)P_2, D/L-Ins(4,5)P_2; (23) D/L-Ins(1,4)P_2, D/L-Ins(1,6)P_2.

to degrade phytate via two independent routes: The routes proceed via D-Ins(1,2,3,5,6)P_5, D-Ins(1,2,3,6)P_4, D-Ins(1,2,6)P_3, and D-Ins(2,6)P_2 to finally Ins(2)P and via D-Ins(1,2,3,5,6)P_5, D-Ins(1,2,3,6)P_4, D-Ins(1,2,3)P_3, and D-Ins(1,2)P_2 to finally Ins(2)P.

It was concluded that the faba bean phytase and the phytase LP2 from lupine seeds generate D-Ins(1,2,3,5,6)P_5 as the major *myo*-inositol pentakisphosphate intermediate, whereas the major *myo*-inositol pentakisphosphate intermediate generated by the phytases LP11 and LP12 from lupine seeds is D-Ins(1,2,4,5,6)P_5, because the kinetic parameters for the *myo*-inositol pentakisphosphate generated by the individual phytase under investigation are not statistically significantly different ($P < 0.05$) from the kinetic parameters for the hydrolysis of D-Ins(1,2,3,5,6)P_5 (faba bean and lupine LP2) and D-Ins(1,2,4,5,6)P_5 (lupine LP11 and LP12), respectively. Because the phytases under investigation generate D-Ins(1,2,4,5,6)P_5 (lupine LP11 and LP12) and D-Ins(1,2,3,5,6)P_5 (faba bean and lupine LP2), respectively, as the major *myo*-inositol pentakisphosphate intermediate, the lupine phytases LP11 and LP12 are 3-phytases (EC 3.1.3.8), whereas the phytase purified from faba bean seeds and the phytase LP2 from lupine seeds are 6-phytases (EC 3.1.3.26). Phytases from microorganisms are considered to be 3-phytases (EC 3.1.3.8), whereas seeds of higher plants are said to contain 6-phytases (EC 3.1.3.26). Thus, the phytases from faba bean seeds and the phytase LP2 from lupine seeds as well as the phytases from mung bean, rye, barley, spelt, oat, wheat bran, rice, *Saccharomyces cerevisiae*, *Pseudomonas*, *Klebsiella terrigena*, and *Aspergillus niger* (*1*, *2*, *4*−*8*, *30*, *31*) fit into this general consideration. However, this is not a general rule, as exemplified by indications of 3-phytase activity in lupine (LP11 and LP12) and soybean seeds and 6-phytase activity in *Paramecium* (*22*) and *Escherichia coli* (*2*).

The *myo*-inositol phosphate intermediates generated by the legume phytases under investigation are consistent with the degradation pathways shown in **Figure 3**. All enzymes remove phosphate stepwise from the phytate molecule, whereby each *myo*-inositol intermediate is released from the enzyme and may become a substrate for further hydrolysis. The major phytate degradation pathway of the phytases LP11 and LP12 from lupine seeds proceeds via D-Ins(1,2,4,5,6)P_5, D-Ins(1,2,5,6)P_4, D-Ins(1,2,6)P_3, and D-Ins(1,2)P_2 to finally Ins(2)P, whereas the faba bean phytase and the phytase LP2 from lupine seeds degrade phytate via D-Ins(1,2,3,5,6)P_5, D-Ins(1,2,5,6)P_4, D-Ins(1,2,6)P_3, and D-Ins(1,2)P_2 to finally Ins(2)P. The phytase purified from faba bean seeds exhibited to a small extent also 3-phytase activity. Thereby, D/L-Ins(1,2,4,5,6)P_5 is generated, which may be linked to the major degradation pathway by dephosphorylation to D/L-Ins(1,2,5,6)P_4. A further minor pathway seems to proceed from D-Ins(1,2,3,5,6)P_5 via D-Ins(1,2,3,6)P_4, Ins(1,2,3)P_3, and/or D-Ins(1,2,6)P_3 and D-Ins(1,2)P_2 to finally Ins(2)P.

All theoretically existing *myo*-inositol pentakis- and tetrakisphosphate isomers are well resolved on the HPIC system

Table 1. Kinetic Constants for Enzymatic *myo*-Inositol Phosphate Dephosphorylation

substrate	enzyme	K_M[a] (μmol L^{-1})	k_{cat}[a] (s^{-1})
$InsP_6$	faba bean	148 ± 9	704 ± 31
	lupine LP11	80 ± 5	523 ± 21
	lupine LP12	300 ± 17	589 ± 26
	lupine LP2	130 ± 7	533 ± 18
$InsP_5$[b]	faba bean	99 ± 5a	921 ± 54a
	lupine LP11	65 ± 3b	671 ± 19b
	lupine LP12	152 ± 12c	684 ± 42b
	lupine LP2	84 ± 4d	691 ± 38b
D-Ins(1,2,4,5,6)P_5	faba bean	nd	nd
	lupine LP11	68 ± 5b	664 ± 23b
	lupine LP12	146 ± 9c	696 ± 31b
	lupine LP2	nd	nd
D-Ins(1,2,3,5,6)P_5	faba bean	104 ± 6a	936 ± 42a
	lupine LP11	nd	nd
	lupine LP12	nd	nd
	lupine LP2	87 ± 3d	679 ± 19b
D-Ins(1,2,3,4,5)P_5	faba bean	411 ± 27e	271 ± 18c
	lupine LP11	nd	nd
	lupine LP12	nd	nd
	lupine LP2	521 ± 35f	196 ± 13d

[a] Entries followed by different letters in a column differ ($P < 0.05$). nd, not determined. The data are mean values ± standard deviation of five independent experiments. [b] Generated by the legume phytase under investigation.

used (**Figure 1**), so the identities of the *myo*-inositol pentakis- and tetrakisphosphate isomers generated by the phytases under investigation is well established. A clear identification of the formed *myo*-inositol trisphosphate isomers by HPIC was not possible until now, because not all theoretically existing isomers are available. Theoretically D-Ins(1,2,5)P_3, D-Ins(1,2,6)P_3, and D-Ins(2,5,6)P_3 as well as D-Ins(1,2)P_2, D-Ins(2,5)P_2, and D-Ins(2,6)P_2 may occur during degradation of D-Ins(1,2,5,6)P_4 to Ins(2)P. According to HPIC, D-Ins(2,6)P_2 (peak 21) has to be excluded as an intermediate, because this *myo*-inositol bisphosphate elutes well resolved from the $InsP_2$ peak (peak 22) generated during *myo*-inositol hexakisphosphate dephosphorylation by the phytases under investigation (**Figures 1** and **2**). In addition, only a *myo*-inositol trisphosphate intermediate coeluting with D-Ins(1,2,6)P_3 (peak 18) could serve as a precursor of Ins(2)P. Thus, Ins(2)P is generated from D-Ins(1,2,5,6)P_4 via D-Ins(1,2,6)P_3, and D-Ins(1,2)P_2.

Thus, the phytate degradation pathways elucidated for the legume phytases under investigation are identical either to the phytate degradation pathway determined for the phytases from *Saccharomyces cerevisiae* (*3*) and *Pseudomonas* (*1*) (lupine LP11 and LP12) or to the phytate degradation pathway obtained for the phytases from rye, barley, spelt, oat (*4*), wheat bran (*7*), and rice (*5*) (faba bean and lupine LP2). Only the minor phytate degradation pathway of the phytase from faba bean seeds shows some similarities to that of the phytase from mung bean (*8*). The most striking difference is the appearance of only D-Ins(1,2,3,6)P_4 as the *myo*-inositol tetrakisphosphate intermediate during phytate hydrolysis by the phytase from mung bean seeds, whereas the phytase from faba bean seeds generates mainly D-Ins(1,2,5,6)P_4. The difference may be due to not only the different specificities of the two enzymes but also the different analytical approaches to elucidate the enzymatic phytate degradation pathways. However, because *myo*-inositol hexakisphosphate dephosphorylation by the wheat phytase deduced using an NMR technique (*7*) and by the phytase D21 from spelt, an old wheat variety, using a combination of HPIC analysis and kinetic studies (*4*), gave identical results, the suitability of the latter to reveal the stereospecificity of enzymatic *myo*-inositol hexakisphosphate degradation has already been demonstrated.

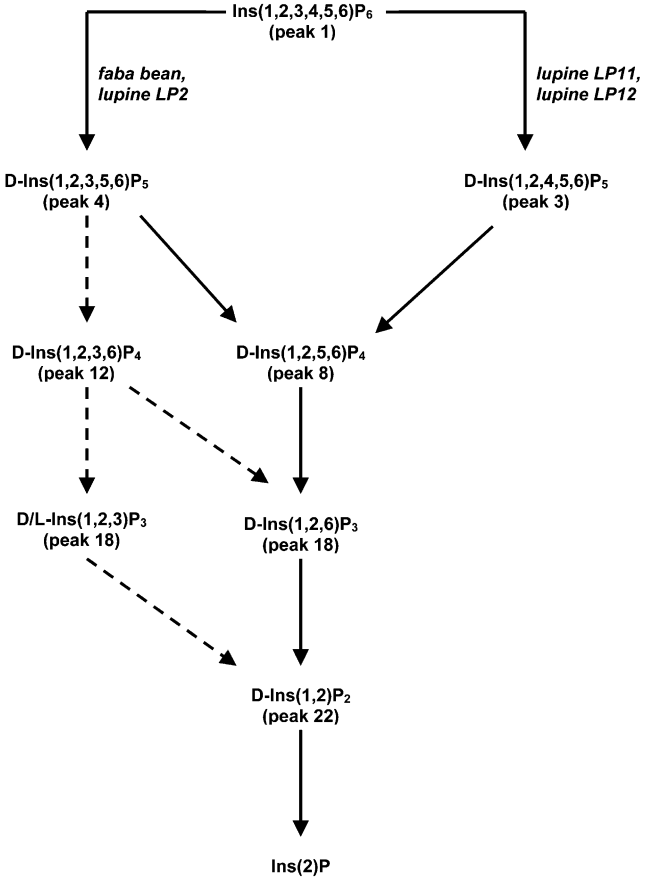

Figure 3. Suggested degradation pathways of phytate by the phytase under investigation: (solid arrow) Major pathway of *myo*-inositol hexakisphosphate dephosphorylation by the legume phytase under investigation; (broken arrow) minor pathway of *myo*-inositol hexakisphosphate dephosphorylation by the faba bean phytase. The faba bean enzyme generates also small amounts of D/L-Ins(1,2,4,5,6)P_5.

Inositol phosphates containing the 1,2,3-trisphosphate cluster are iron-binding antioxidants (*32*, *33*), and D-Ins(1,2,3,6)P_4 has a structure that is moderately effective in opening calcium channels (*34*, *35*). In addition, D-Ins(1,2,6)P_3 has anti-inflammatory and antisecretory properties (*36*). Whether *myo*-inositol phosphate isomers have nutritional significance in food is for the most part speculative, even if an in vivo antioxidative effect of dietary *myo*-inositol phosphates has been reported (*37*, *38*). Until now, the diversity and practical unavailability of the individual *myo*-inositol phosphate intermediates precluded their being tested as bioactive. Studies are now under way to make individual *myo*-inositol phosphates available in pure form and in sufficient quantities for physiological studies.

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