Stereospecificity of *myo*-Inositol Hexakisphosphate Dephosphorylation by a Phytate-Degrading Enzyme of Baker's Yeast

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During food processing such as baking, phytate is dephosphorylated to produce degradation products, such as *myo*-inositol pentakis-, tetrakis-, tris-, bis-, and monophosphates. Certain *myo*-inositol phosphates have been proposed to have positive effects on human health. The position of the phosphate groups on the *myo*-inositol ring is thereby of great significance for their physiological functions. Using a combination of high-performance ion chromatography analysis and kinetic studies the stereospecificity of *myo*-inositol hexakisphosphate dephosphorylation by a phytate-degrading enzyme from baker's yeast (*Saccharomyces cerevisiae*) was established. The data demonstrate that the phytate-degrading enzyme from baker's yeast dephosphorylates *myo*-inositol hexakisphosphate in a stereospecific way by sequential removal of phosphate groups 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, D-Ins(1,2)P_2, to finally Ins(2)P (notation 3/4/5/6/1). Knowledge of the absolute stereochemical specificity of the baker's yeast phytase allows use of the enzyme to produce defined *myo*-inositol phosphates for kinetic and physiological studies.

Keywords: *myo-Inositol phosphate isomers; phytase; phytate degradation; Saccharomyces cerevisiae*

INTRODUCTION

Mixed salts of phytic acid [*myo*-inositol(1,2,3,4,5,6)-hexakisphosphoric acid] are common constituents of foods because phytate is a naturally occurring compound formed during the maturation of seeds and cereal grains (*1*). The main concern about the presence of phytate in the diet is its negative effect on mineral uptake (*2*). The formation of insoluble cation−phytate complexes at physiological pH values is regarded as the major reason for the poor mineral bioavailability because these complexes are essentially nonabsorbable from the gastrointestinal tract. Especially zinc and iron deficiencies were observed as a consequence of high phytate intake. Removal of dietary phytate significantly improves the bioavailability of those minerals in humans. In the past few years, however, it has been suggested from epidemiological and animal studies that phytate and degradation products of phytate may also exhibit beneficial health effects, such as reducing the risk of heart disease (*3*, *4*), renal stone formation (*5*, *6*), and certain type of cancers (*7*−*12*).

During food processing, phytate can be partially dephosphorylated to yield a large number of positional isomers of *myo*-inositol pentakis-, tetrakis-, tris-, bis-, and monophosphates. Especially with respect to the isomers, their chelating potential and their physiological function have not been carefully studied, but this is of great importance because processed foods may contain lower *myo*-inositol phosphates in great amounts and the position of the phosphate groups on the *myo*-inositol ring is of great significance for their properties. Phytate degradation during food processing is mainly due to enzymatic cleavage. Phytate-degrading enzymes (phytases), which catalyze the sequential hydrolysis of phytate, occur in a variety of organisms including plants, microorganisms, and animal tissues. With respect to the position of initial hydrolysis, different types of phytases have been recognized: 3-phytases (EC 3.1.3.8), 4-phytases, and 6-phytases (EC 3.1.3.26). In addition, different phytases may produce different isomers of the lower *myo*-inositol phosphates. To attribute observed physiological effects to certain *myo*-inositol phosphate isomers, the *myo*-inositol phosphate content and composition of a food have to be known and defined *myo*-inositol phosphates have to be available in pure form and sufficient quantity for physiological studies.

The aim of this work was to study the stereospecificity of *myo*-inositol hexakisphosphate degradation by a phytate-degrading enzyme from baker's yeast (*Saccharomyces cerevisiae*). To date, knowledge of the positional isomers formed by enzymatic degradation of *myo*-inositol hexakisphosphate is very limited. Presently, only the stereospecificity of *myo*-inositol hexakisphosphate dephosphorylation by the cereal phytases from wheat (*13*), spelt D21, rye, barley P1 and P2, and oat (*14*) as well as the bacterial phytases from *Paramecium* (*15*) and *Escherichia coli* (*16*) is established.

MATERIALS AND METHODS

Chemicals. Baker's yeast was obtained from a local supermarket and *Aspergillus niger* phytase from Novo Nordisk (Copenhagen, Denmark). Phytic acid dodecasodium salt was from Aldrich (Steinheim, Germany). High-load 16/60 Sephacryl S-300 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 high-

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performance ion chromatograph (HPIC) Omni Pac PA-100 from Dionex (Sunnyvale, CA). AG1 X-4, 100−200 mesh resin, was obtained from Bio-Rad (München, Germany).

Culture Conditions. For the growth of *S. cerevisiae* MSA medium as described by Lambrechts et al. (*17*) was used. The pH of the medium was adjusted to pH 5.4 using a 0.2 M phthalate/NaOH buffer. Media were autoclaved at 121 °C for 20 min with the exception of the phytate and vitamin solutions, which were sterilized by filtration through a 0.2 μm membrane. The cultures were grown at 30 °C in Erlenmeyer flasks (500 mL) using a rotary shaker at 150 rpm.

Purification of the Phytases. FPLC columns were run at 25 °C and a flow rate of 1 mL min^{-1}.

A. niger. The protein powder was suspended in 20 mM Tris−acetate buffer, pH 7.0, and dialyzed against the same buffer. Any insoluble material was removed by centrifugation at 20000*g* for 30 min. The dialysate was applied onto a Mono Q HR 5/5 column equilibrated with 20 mM Tris−acetate buffer, pH 7.0. The column was washed with the same buffer for 30 min first, and then a linear gradient consisting of 0−0.5 M NaCl in 20 mM Tris−acetate buffer, pH 7.0, for 60 min was applied. Two milliliter fractions were collected. The fractions containing phytase activity were pooled, dialyzed against 20 mM sodium acetate buffer, pH 4.0, and applied onto a Mono S HR 5/5 column equilibrated with 20 mM sodium acetate buffer, pH 4.0. The column was washed with the same buffer for 30 min first, and then a linear gradient consisting of 0−0.5 M NaCl in 20 mM sodium acetate buffer, pH 5.0, for 30 min was applied. Two milliliter fractions were collected. The fractions containing phytase activity were pooled.

S. cerevisiae. The yeast cells were removed by centrifugation at 5000*g* for 15 min. The supernatant was concentrated 10-fold by using a PM 10.000 ultrafiltration membrane and dialyzed against 20 mM Tris-HCl buffer, pH 7.5. The dialysate was applied onto a Mono Q HR 5/5 column equilibrated with 20 mM Tris-HCl buffer, pH 7.5. The column was washed with the same buffer for 30 min first, and then a linear gradient consisting of 0−0.5 M NaCl in 20 mM Tris-HCl buffer, pH 7.5, for 60 min was applied. Two milliliter fractions were collected. The fractions containing phytase activity were pooled and loaded onto a 16/60 Sephacryl S-300 HR column equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 0.2 M NaCl in 2 mL portions. Two milliliter fractions were collected. The fractions containing phytase activity were pooled and rechromatographed on the 16/60 Sephacryl S-300 HR column using the same conditions. Thereafter, the phytase-active pool was dialyzed against 20 mM sodium acetate buffer, pH 5.0, and applied onto a Mono S HR 5/5 column equilibrated with 20 mM sodium acetate buffer, pH 5.0. The column was washed with the same buffer for 30 min first, and then a linear gradient consisting of 0−0.5 M NaCl in 20 mM sodium acetate buffer, pH 5.0, for 30 min was applied. Two milliliter fractions were collected. The fractions containing phytase activity were pooled.

Both phytate-degrading enzymes were purified to apparent homogeneity according to denaturing and non-denaturing polyacrylamide gel electrophoresis.

Assay of Phytase. Phytase 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 determination consisted of 350 μL 0.1 M sodium acetate, pH 4.5, containing 500 nmol of sodium phytate. After an incubation time of 30 min, the liberated inorganic phosphate was measured according to a modification of the ammonium molybdate method (*18*).

Preparation of the *myo*-Inositol Pentakisphosphates. *myo*-Inositol hexakisphosphate (50 μmol) was incubated at 35 °C in a mixture containing 50 mM ammonium acetate, pH 4.5, and 0.4 unit of the phytate-degrading enzymes in a final volume of 20 mL. After an incubation period of 60 min at 35 °C, the reactions were stopped by heat treatment (95 °C, 10 min). The incubation mixtures were lyophilized, and the dry residues were dissolved in 10 mL of 1.0 M ammonium formate, pH 2.5. The solutions were loaded onto a Q-Sepharose column (2.6 × 90 cm) equilibrated with 1.0 M ammonium formate, pH 2.5, at a flow rate of 2.5 mL min^{-1}. The column was washed with 500 mL of 1.0 M ammonium formate, pH 2.5; the bound *myo*-inositol pentakisphosphates were eluted with a linear gradient from 1.0 to 1.4 M ammonium formate, pH 2.5 (1000 mL), at 2.5 mL min^{-1}. Fractions of 10 mL were collected. From even-numbered tubes, 100 μL aliquots were lyophilized. The residues were dissolved in 3 N sulfuric acid and incubated for 90 min at 165 °C to hydrolyze the eluted *myo*-inositol phosphates completely. The liberated phosphate was quantified according to a modification of the ammonium molybdate method (*18*). The contents of the fraction tubes corresponding to the *myo*-inositol pentakisphosphates were pooled and lyophilized until only a dry residue remained. Ten milliliters of water was used to redissolve the residues. Lyophilization and redissolving were repeated twice. *myo*-Inositol pentakisphosphate concentrations were determined by HPLC ion-pair chromatography on an Ultrasep ES 100 RP18 (2 × 250 mm). The column was run at 45 °C and 0.2 mL min^{-1} with an eluant consisting of formic acid/methanol.water/tetrabutylammonium hydroxide (TBAH) (44:56:5:1.5 v/v), pH 4.25, as described by Sandberg and Ahderinne (*19*). A mixture of the individual *myo*-inositol phosphate esters ($InsP_3$−$InsP_6$) was used as a standard. The purity of the *myo*-inositol pentakisphosphate preparations was determined on an HPIC system as described by Skoglund et al. (*20*).

Production of Enzymatically Formed Hydrolysis Products. The enzymatic reaction was started at 35 °C by the addition of 50 μL of the suitably diluted enzyme solution to the incubation mixtures (50 milliunits mL^{-1}). The incubation mixture consisted of 1250 μL of 0.1 M sodium acetate buffer, pH 4.5, 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, 5 min). For the identification of *myo*-inositol bis- to hexakisphosphate isomers, 50 μL of the heat-treated samples was chromatographed on an HPIC system as described by Skoglund et al. (*20*).

Identification of Enzymatically Formed Hydrolysis Products ($InsP_6$—$InsP_2$). *myo*-Inositol phosphate isomers were separated on an Omni Pac PA-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 (*21*). 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 0.5 unit of the yeast phytase with a limiting amount of *myo*-inositol hexakisphosphate (0.1 μmol) in a final volume of 500 μL of 50 mM ammonium formate. 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: 100−340 °C, rate increase of 4 °C min^{-1}. Ionization was performed by electron impact at 70 eV and 250 °C.

Kinetic Studies with the Pure *myo*-Inositol Pentakisphosphates. To determine the kinetic parameters for enzymatic *myo*-inositol pentakisphosphates dephosphorylation, 10 milliunits of the phytases was added to sequentially diluted solutions of the purified *myo*-inositol pentakisphosphate isomer (2.0, 1.0, 0.5, 0.25, 0.125, 0.06, 0.03, and 0.015 mM) produced by enzymatic phytate degradation by either the *A. niger* or the yeast phytase in 400 μL of 0.1 M sodium acetate, pH 4.5, at 35 °C. After an incubation period of 30 min, the liberated phosphate was quantified by a modification of the ammonium molybdate method (*18*). The rate of reaction was linear for the 30 min incubation time (data not shown). Activity (units) was expressed as 1 μmol of phosphate liberated/min. 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: yeast, 60 kDa; *Aspergillus*, 85 kDa (*22*).

Quantification of the Liberated Phosphate. The liberated phosphate was quantified according to a modification of the ammonium molybdate method (*18*). 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 to 400 μL of the suitably diluted hydrolysis mixtures or the mixtures of the phytase assay. 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 of phosphate. The activity (units) was expressed as 1 μmol of phosphate liberated/min.

RESULTS

HPIC Analysis of Enzymatic *myo*-Inositol Hexakisphoshate Dephosphorylation. A detailed characterization of the hydrolysis pathway of *myo*-inositol hexakisphosphate by baker's yeast phytase purified to apparent homogeneity revealed that this enzyme degrades *myo*-inositol hexakisphosphate by stepwise dephosphorylation 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) (Figure 1). All theoretically existing *myo*-inositol pentakis- and tetrakisphosphate isomers are well resolved on the HPIC system used (Figure 1a), so the identity of the *myo*-inositol pentakis- and tetrakisphosphate isomers produced by the yeast phytase as D/L-Ins(1,2,4,5,6)P_5 and D/L-Ins(1,2,5,6)P_4 is well established. A clear identification of the formed *myo*-inositol tris-, bis-, and monophosphate isomers by HPIC had not been possible until now because all theoretically existing isomers were not available.

To determine the absolute configuration of the *myo*-inositol pentakisphosphate intermediate produced by the yeast phytase, kinetic studies with the purified *myo*-inositol pentakisphosphates generated either by the *A. niger* or by the yeast phytase were performed.

Isolation of Pure *myo*-Inositol Pentakisphosphate Isomers. To isolate pure *myo*-inositol pentakisphosphates obtained by enzymatic hydrolysis, ion-exchange chromatography on Q-Sepharose was used. This method is capable of resolving the different *myo*-inositol phosphate groups ($InsP_6$−InsP), but a separation of the individual positional isomers is not possible under the conditions used. Because the *A. niger* phytase as well as the baker's yeast phytase generate predominantly only one *myo*-inositol pentakisphosphate isomer, ion-exchange chromatography on Q-Sepharose is a suitable method to obtain the pure *myo*-inositol pentakisphosphates. The purity of the *myo*-inositol pentakisphosphate preparations was determined by HPIC (data not shown).

Determination of the Absolute Configuration of the *myo*-Inositol Pentakisphosphate Isomer Produced by the Yeast Phytase. To determine the kinetic parameters for *myo*-inositol pentakisphosphate hydrolysis by the phytase from baker's yeast, the enzyme was added to sequentially diluted solutions of the purified *myo*-inositol pentakisphosphates produced either by the phytase from baker's yeast or by the phytase from *A. niger*. The kinetic parameters (K_M and V_{max}) were calculated from Lineweaver−Burk plots of the data (Table 1). K_M, V_{max}, and k_{cat} for the enzymatic hydrolysis of the *myo*-inositol pentakisphosphates generated by the yeast phytase were almost identical with the kinetic constants for the dephosphorylation of the *myo*-inositol pentakisphosphate intermediate generated by the *A. niger* phytase. Thus, the *myo*-inositol pentakisphosphate produced by the yeast phytase is identical with the *myo*-inositol pentakisphosphate formed by the *A. niger* phytase. Because it is known that the *A. niger* phytase predominantly produces the D-Ins(1,2,4,5,6)P_5 isomer (*23*) [identical with L-Ins(2,3,4,5,6)P_5], D-Ins(1,2,4,5,6)-P_5 and D-Ins(1,2,5,6)P_4 are the first two breakdown products of *myo*-inositol hexakisphosphate dephosphorylation by the yeast phytase.

Table 1. Kinetic Constants for Enzymatic *myo*-Inositol Pentakisphosphate Dephosphorylation[a]

		IP_5 generated by	
phytase	kinetic constant	yeast phytase	*Aspergillus* phytase
yeast	K_M (μmol L^{-1})	217 ± 13	210 ± 16
	V_{max} (units mg^{-1})	135 ± 11	136 ± 9
	k_{cat} (s^{-1})	135 ± 11	136 ± 9
Aspergillus	K_M (μmol L^{-1})	158 ± 8	155 ± 10
	V_{max} (units mg^{-1})	96 ± 4	98 ± 6
	k_{cat} (s^{-1})	136 ± 6	139 ± 9

[a] 10 mU of the phytases was added to varied concentrations of the purified *myo*-inositol pentakisphosphates (2.0, 1.0, 0.5, 0.25, 0.125, 0.06, 0.03, and 0.015 mM) produced by enzymatic phytate degradation by either yeast or *Aspergillus* phytase in 400 μL of 0.1 M sodium acetate buffer, pH 4.5, at 35 °C. After an incubation period of 30 min, the liberated phosphate was quantified by a modification of the ammonium molybdate method (*18*). The rate of reaction was linear for the 30 min incubation times. Activity (units) was expressed as 1 μmol of phosphate liberated/min. The kinetic parameters (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: yeast, 60 kDa; *Aspergillus*, 85 kDa (*22*). The data are mean values of five independent experiments.

Identification of the *myo*-Inositol Monophosphate Isomer. The end product of *myo*-inositol hexakisphoshate dephosphorylation was received by incubation of the yeast phytase with a limiting amount of *myo*-inositol hexakisphosphate. It was identified by gas chromatography−mass spectrometry as Ins(2)P. Ins(2)P may be produced from D-Ins(1,2,5,6)P_4 via the six different routes indicated in Figure 2. Thereby three *myo*-inositol trisphosphate [D-Ins(1,2,5)P_3, D-Ins(1,2,6)-P_3, and D-Ins(2,5,6)P_3] and three *myo*-inositol bisphosphate isomers [D-Ins(1,2)P_2, D-Ins(2,5)P_2, and D-Ins(2,6)-P_2] may occur. According to HPIC, D-Ins(2,5,6)P_3 and D-Ins(2,6)P_2 have to be excluded as intermediates because these *myo*-inositol phosphates elute well-resolved from the $InsP_3$ and $InsP_2$ peaks, respectively, generated during *myo*-inositol hexakisphosphate dephosphorylation by the yeast phytase (Figure 1a,c). It is not possible to discriminate between D-Ins(1,2)P_2 and D-Ins(2,5)P_2 because these two isomers coelute on the HPIC system used. In addition, discrimination between D-Ins(1,2,5)P_3 and D-Ins(1,2,6)P_3 is impossible because pure D-Ins(1,2,5)P_3 is not available. According to the results of Sirén (*24*) *myo*-inositol hexakisphosphate dephosphorylation by the yeast phytase results in D-Ins-(1,2,6)P_3.

DISCUSSION

Because the investigation of *myo*-inositol hexakisphosphate dephosphorylation by the wheat phytase deduced using an NMR technique (*13*) and by the cereal phytases

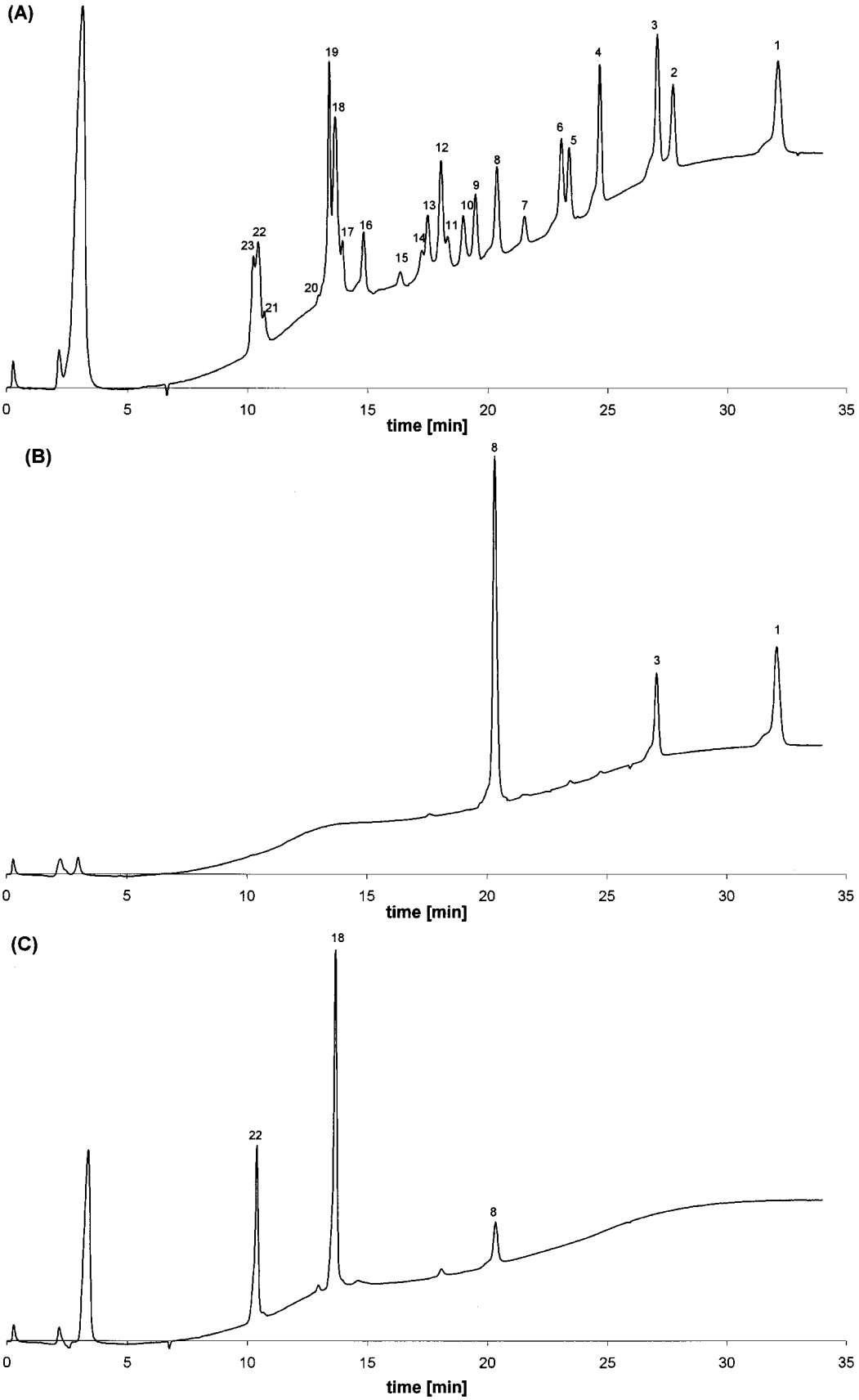

Figure 1. HPIC analysis of enzymatically formed hydrolysis products of *myo*-inositol hexakisphosphate (A) reference sample (source of reference *myo*-inositol phosphates indicated in ref *20*); (B) yeast, incubation time of 30 min; (C) yeast, incubation time of 240 min. Peaks: (1) Ins(1,2,3,4,5,6)P_6; (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.

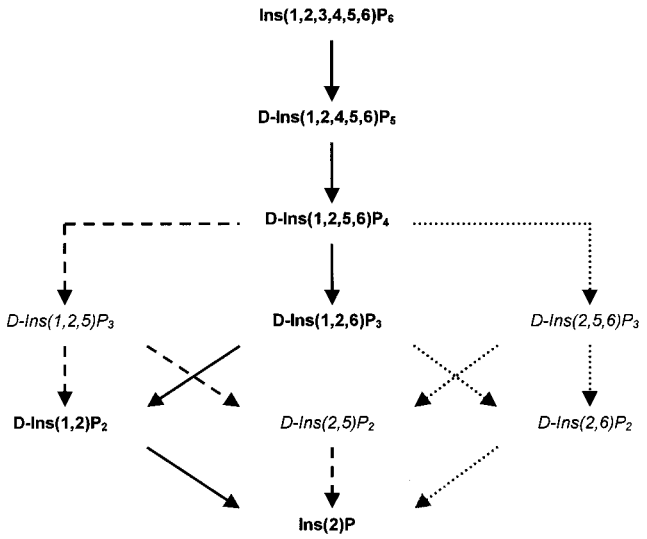

Figure 2. Theoretically possible degradation pathways from D-Ins(1,2,5,6)P_4 to Ins(2)P: (dotted arrows) to be excluded according to the results of HPIC (Figure 1a,c); (dashed arrows) to be excluded according to the findings of Ullah and Phillippy (*23*) and Sirén (*24*); (solid arrows) major pathway of *myo*-inositol hexakisphosphate dephosphorylation by the phytase from baker's yeast.

from spelt D21, rye, barley P1 and P2, and oat (*14*) using a combination of HPIC analysis and kinetic studies resulted in identical results, the latter can be considered as a suitable method to reveal the stereospecificity of enzymatic *myo*-inositol hexakisphosphate degradation. In summary, the combined data indicate that *myo*-inositol hexakisphosphate is dephosphorylated by the yeast phytase in a stereospecific way by sequential removal of phosphate groups 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 (notation 3/4/5/6/1). With the exception of the *myo*-inositol pentakisphosphate isomer, the phytate degradation pathway by the baker's yeast phytase is identical with that of the cereal phytases from wheat (*13*), spelt D21, rye, barley P1 and P2, and oat (*14*). In contrast to these cereal phytases, which are identified as 4-phytases (EC 3.1.3.26), the phytase from baker's yeast has to be considered a 3-phytase because the phytate dephosphorylation is preferentially initiated at the 3-position of the *myo*-inositol ring. Thus, the baker's yeast phytase fits into the general consideration that 3-phytases (EC 3.1.3.8) are characteristic for microorganisms and 4-phytases as well as 6-phytases for the seeds of plants. However, this is not a general rule, as exemplified by indications of 3-phytase activity in soybean seeds (*25*) and 6-phytae activity in *Paramecium* (*15*) and *E. coli* (*16*). Especially in plant seeds phytate-degrading enzymes are found in multiple forms (*26*−*29*), and there are hints that those multiple forms may even exhibit different stereospecificities of *myo*-inositol hexakisphosphate dephosphorylation (R. Greiner, unpublished work).

To date, two main types of phytases have been identified: acidic phytases with a pH optimum between pH 2.5 and 6.0 and alkaline phytases with a pH optimum between pH 7.0 and 8.0. Independent of their bacterial (*16*), fungal (*30*), or plant origin (*14*), the so far characterized acidic phytases release five of the six phosphate groups of phytate and the final degradation product of phytate was identified as Ins(2)P. The final phytate degradation product generated by the acidic phytase from baker's yeast was also identified as Ins(2)P. Thus, acidic phytases seem to produce Ins(2)P as a common final degradation product of phytate. On the other hand, alkaline phytases, which have been identified in the pollen of *Lilium longiflorum* (*31*, *32*), in *Typha latifolia* (*33*), and in *Bacillus* species (*34*−*36*) do not accept *myo*-inositol phosphates with three or fewer phosphate groups as substrates (*32*, *37*). Thus, an InsP_3 isomer is the final phytate degradation product after the action of these alkaline phytases.

The physiological role of different *myo*-inositol phosphates is presently undergoing extensive research. The effects observed with the different *myo*-inositol phosphate isomers seem to be very specific for the certain isomer. For example, D-Ins(1,2,6)P_3, an isomer generated by the action of cereal phytases as well as the phytase from baker's yeast, has been studied with respect to the prevention of diabetes complications (*38*, *39*) and treatment of chronic inflammations as well as cardiovascular diseases (*40*, *41*). Although much research has been done to discover the exact role of all *myo*-inositol phosphates, the investigation is still far from finished. Knowledge of the absolute configuration of the phytate degradation products generated by the baker's yeast phytase allows us to use and monitor the enzyme in a fermentation process for the production of defined *myo*-inositol phosphate isomers for pharmacological and biochemical studies. If the phytate degradation products induce physiological effects, the yeast phytase may find application in food processing to produce foods with improved nutritional value and health benefits and maintained sensory properties (functional foods).

LITERATURE CITED

(1) Cosgrove, D. J. Nutritional aspects of inositol phosphates. In *Inositol Phosphates. Their Chemistry, Biochemistry and Physiology*; Cosgrove, D. J., Ed.; Elsevier Scientific Publishing: Amsterdam, The Netherlands, 1980; pp 158−172.

(2) Zhou, J. R.; Erdman, Jr., J. W. Phytic acid in health and disease. *Crit. Rev. Food Sci. Nutr.* **1995**, *35* (6), 495−508.

(3) Jariwalla, R. J.; Sabin, R.; Lawson, S.; Herman, Z. S. Lowering of serum cholesterol and triglycerides and modulation of divalent cations by dietary phytate. *J. Appl. Nutr.* **1990**, *42* (1), 18−28.

(4) Potter, S. M. Overview of proposed mechanisms for the hypocholesterolemic effect of soy. *J. Nutr.* **1995**, *125*, 606S−611S.

(5) Modlin, M. Urinary phosphorylated inositols and renal stone. *Lancet* **1980**, *2*, 1113−1114.

(6) Ohkawa, T.; Ebisuno, S.; Kitagawa, M.; Morimoto, S.; Miyazaki, Y.; Yasukawa, S. Rice bran treatment for patients with hypercalciuric stones: Experimental and clinical studies. *J. Urol.* **1984**, *132*, 1140−1145.

(7) Baten, A.; Ullah, A.; Tomazic, V. J.; Shamsuddin, A. M. Inositol-phosphate-induced enhancement of natural killer cell activity correlates with tumor suppression. *Carcinogenesis* **1989**, *10* (9), 1595−1598.

(8) Graf, E.; Eaton, J. W. Suppression of colonic cancer by dietary phytic acid. *Nutr. Cancer* **1993**, *19*, 11−19.

(9) Shamsuddin, A. M.; Vucenik, I.; Cole, K. E. IP-6: A novel anti-cancer agent. *Life Sci.* **1997**, *61* (4), 343−354.

(10) Ullah, A.; Shamsuddin, A. M. Dose-dependent inhibition of large intestinal cancer by inositol hexaphosphate in F344 rats. *Carcinogenesis* **1990**, *11*, 2219−2222.

(11) Vucenik, I.; Sakamoto, K.; Bansal, M.; Shamsuddin, A. M. Mammary carcinogenesis inhibition by inositol compounds. *Cancer Lett.* **1993**, *75*, 95−102.

(12) Yang, G.; Shamsuddin, A. M. P-6-induced growth inhibition and differentiation of HT-29 human colon cancer cells: Involvement of intracellular inositol phosphates. *Anticancer Res.* **1995**, *15*, 2479−2488.
(13) Nakano, T.; Joh, T.; Narita, K.; Hayakawa, T. The pathway of dephosphorylation of myo-inositol hexakisphosphate by phytases from wheat bran of *Triticum aestivum* L. cv. Nourin #61. *Biosci., Biotechnol., Biochem.* **2000**, *64*, 995−1003.
(14) Greiner, R.; Larsson Alminger, M. Stereospecificity of myo-inositol hexakisphosphate dephosphorylation by phytate-degrading enzymes of cereals. *J. Food Biochem.* **2001**, in press.
(15) van der Kaay, J.; van Haastert, J. M. Stereospecificity of inositol hexaphosphate dephosphorylation by *Paramecium* phytase. *Biochem. J.* **1995**, *312*, 907−910.
(16) Greiner, R.; Carlsson, N.-G.; Larsson Alminger, M. Stereospecificity of *myo*-inositol hexakisphosphate dephosphorylation by a phytate-degrading enzyme of *Escherichia coli*. *J. Biotechnol.* **2000**, *84*, 53−62.
(17) Lambrechts, C.; Boze, H.; Moulin, G.; Galzy, P. Utilization of phytate by some yeasts. *Biotechnol. Lett.* **1992**, *14*, 61−66.
(18) Heinonen, J. K.; Lahti, R. J. A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay of inorganic phosphatase. *Anal. Biochem.* **1981**, *113*, 313−317.
(19) Sandberg, A.-S.; Ahderinne, R. HPLC method for determination of inositol tri-, tetra-, penta-, and hexaphosphate in foods and intestinal contents. *J. Food Sci.* **1986**, *51*, 547−550.
(20) Skoglund, E.; Carlsson, N.-G.; Sandberg, A.-S. Determination of isomers of inositol mono- to hexaphosphates in selected foods and intestinal contents using high-performance ion chromatography. *J. Agric. Food Chem.* **1997**, *45*, 431−436.
(21) Phillippy, B. Q.; Bland, J. M. Gradient ion chromatography of inositol phosphates. *Anal. Biochem.* **1988**, *175*, 162−166.
(22) Ullah, A. H. J.; Gibson, D. M. Extracellular phytase (E.C. 3.1.3.8) from *Aspergillus ficuum NRRL 3135*: Purification and characterization. *Prep. Biochem.* **1987**, *17* (1), 63−91.
(23) Ullah, A. H. J.; Phillippy, B. Q. Imobilization of *Aspergillus ficuum* phytase: Product characterization of the bioreactor. *Prep. Biochem. Biotechnol.* **1998**, *18*, 483−489.
(24) Sirén, M. J. U.S. Patent 4735939, 1984.
(25) Phillippy, B. Q.; Johnston, M. R.; Tao, S.-H.; Fox, M. R. S. Inositol phosphates in processed foods. *J. Food Sci.* **1988**, *53*, 496−499.
(26) Greiner, R.; Jany, K.-D.; Larsson Alminger, M. Identification and properties of *myo*-inositol hexakisphosphate phosphohydrolase (phytase) from barley (*Hordeum vulgare*). *J. Cereal Sci.* **2000**, *31*, 127−139.
(27) Konietzny, U.; Greiner, R.; Jany, K.-D. Purification and characterization of a phytases from spelt. *J. Food Biochem.* **1995**, *18*, 165−183.
(28) Lim, P. E.; Tate, M. E. The phytases: II. Properties of phytase fraction F_1 and F_2 from wheat bran and the *myo*-inositol phosphates produced by fraction F_2. *Biochim. Biophys. Acta* **1973**, *302*, 326−328.
(29) Nagai, Y.; Funahashi, S. Phytase (*myo*-inositol hexaphosphate phosphohydrolase) from wheat bran. *Agric. Biol. Chem.* **1962**, *26*, 794−803.
(30) Wyss, M.; Brugger, R.; Kronenberger, A.; Remy, R.; Fimbel, R.; Oesterhelt, G.; Lehmann, M.; van Loon, A. P. G. M. Biochemical characterization of fungal phytases (*myo*-inositol hexakisphosphate phosphohydrolase): Catalytic properties. *Appl. Environm. Microbiol.* **1999**, *65* (2), 367−373.
(31) Lin, J.-J.; Dickinson, D. B.; Ho, T.-H. D. Phytic acid metabolism in Lily (*Lilium longiflorum* Thunb.) pollen. *Plant Physiol.* **1987**, *83*, 408−413.
(32) Baldi, B. G.; Scott, J. J.; Everard, J. D.; Loewus, F. A. Localization of constitutive phytases in lily pollen and properties of the pH 8 form. *Plant Sci.* **1988**, *56*, 137−147.
(33) Hara, A.; Ebina, S.; Kondo, A.; Funaguma, T. A new type of phytase from pollen of *Typha latifolia* L. *Agric. Biol. Chem.* **1995**, *49*, 3539−3544.
(34) Choi, Y. M.; Noh, D. O.; Cho, S. H.; Lee, H. K.; Suh, H.-J.; Chung, S.-H. Isolation of a phytase-producing *Bacillus* sp. KHU-10 and its phytase production. *J. Microbiol. Biotechnol.* **1999**, *9* (2), 223−226.
(35) Kerovuo, J.; Lauraeus, M.; Nurminen, P.; Kalkinnen, N.; Apajalahti, J. Isolation, characterization, molecular gene cloning and sequencing of a novel phytase from *Bacillus subtilis*. *Appl. Environ. Microbiol.* **1998**, *64*, 2079−2085.
(36) Kim, Y.-O.; Kim, H.-K.; Bae, K.-S.; Yu, J.-H.; Oh, T.-K. Purification and properties of a thermostable phytase from *Bacillus* sp. DS11. *Enzyme Microb. Technol.* **1998**, *22*, 2−7.
(37) Kerovuo, J.; Rouvinen, J.; Hatzack, F. Hydrolysis of phytic acid by *Bacillus* phytase. *Biochem. J.* **2000**, *352*, 623−628.
(38) Carrington, A. L.; Calcutt, N. A.; Ettlinger, C. B.; Gustafsson, T.; Tomlinson, D. R. Effects of treatment with *myo*-inositol or its 1,2,6-trisphosphate (PP56) on nerve conduction in streptozotocin-diabetis. *Eur. J. Pharmacol.* **1993**, *237*, 257−263.
(39) Ruf, J. C.; Ciavatti, M.; Gustafsson, T.; Renaud, S. Effects of PP-56 and vitamin E on platelet hyperaggregability, fatty acid abnormalities, and clinical manifestations in streptozotocin-induced diabetis rats. *Diabetis* **1991**, *40*, 233−239.
(40) Claxon, A.; Morris, C.; Blake, D.; Sirén, M.; Halliwell, B.; Gustafsson, T.; Löfkvist, B.; Bergelin, I. The anti-inflammatory effects of D-*myo*-inositol-1.2,6-trisphosphate (PP56) on animal models of inflammation. *Agents Actions* **1990**, *29* (1/2), 68−70.
(41) Sirén, M.; Linne, L.; Persson, L. Pharmacological effects of D-*myo*-inositol-1,2,6-trisphosphate. In *Inositol Phosphates and Derivatives. Synthesis, Biochemistry and Therapeutic Potential*; Reitz, A. B., Ed.; American Chemical Society: Washington, DC, 1991; pp 103−110.

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