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Quantitative Analysis of Phytate Globoids Isolated from Wheat Bran and Characterization of Their Sequential Dephosphorylation by Wheat Phytase

LISBETH BOHN,† LONE JOSEFSEN,† ANNE S. MEYER,§ AND SØREN K. RASMUSSEN*,†

Department of Agricultural Sciences, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark, and Bioprocess Engineering, Department of Chemical Engineering, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

Wheat phytase was purified to investigate the action of the enzyme toward its pure substrate (phytic acid − *myo*-inositol hexakisphosphate) and its naturally occurring substrate (phytate globoids). Phytate globoids were purified to homogeneity from wheat bran, and their nutritionally relevant parameters were quantified by ICP-MS. The main components of the globoids were phytic acid (40% w/w), protein (46% w/w), and several minerals, in particular, K > Mg > Ca > Fe (in concentration order). Investigation of enzyme kinetics revealed that K_m and V_{max} decreased by 29 and 37%, respectively, when pure phytic acid was replaced with phytate globoids as substrate. Time course degradation of phytic acid or phytate globoids using purified wheat phytase was followed by HPIC identification of inositol phosphates appearing and disappearing as products. In both cases, enzymatic degradation initiated at both the 3- and 6-positions of phytic acid and end products were inositol and phosphate.

KEYWORDS: Inositol phosphates; wheat phytase; wheat bran; phytate globoids; minerals; kinetics; *Triticum aestivum*

INTRODUCTION

Phytic acid is the main phosphorus storage compound in most seeds and grains. The phytic acid molecule consists of a phosphorylated *myo*-inositol ring and has a very specific conformation with one axial and five equatorial phosphate groups. Phytic acid is a strong chelating agent. It readily binds metal cations from, for example, Ca, Fe, Zn, Mg, and Mn, making them insoluble and thus unavailable as nutritional factors (*1*). The salt of phytic acid and these minerals is known as phytate, and it is concentrated in electron-dense parts of the protein storage vacuoles called phytate globoids (*2*). How this interaction between the protein storage vacuole and the phytate globoid exactly is assembled is still being investigated. It could be that either the proteins form a matrix with a few large phytate globoids inside or there could actually be an inner membrane in the vacuole surrounding the globoid (*3*). The globoids are localized predominantly in the protein storage vacuoles in the aleurone layer (wheat and barley) or in the embryo (maize) (*4*). The size of the phytate globoids depends on the amount of phytic acid in the grain. In wild-type wheat, globoids up to 4 μm in diameter have been detected (*5*), whereas a low phytic acid wheat mutant (Js-12-LPA) with the same amount of phosphate but a lower content of phytic acid has smaller globoids, organized in clusters (*6*). In rice it has been shown that 11% of the globoids consist of proteins with Mg and K in large amounts (*7*). In wheat, the greatest concentrations of minerals have been found in the bran (*8*, *9*). Direct positive associations between grain phytic acid and Zn have been reported (*10*), and it has been shown that redistribution within the kernel of phosphate and Mg, from the aleurone layer to the flour, takes place in the wheat mutant Js-12-LPA (*11*).

Phytic acid can be degraded by phytase enzymes (*myo*-inositol hexakisphosphate phosphohydrolase, EC 3.1.3.26), which are defined as a class of phosphatases with the in vitro capability to release at least one phosphate group from phytic acid, thereby releasing the minerals and phosphorus. Wheat phytase was first mentioned by Posternak and Posternak in 1929 (*12*), but it was not until 1973 that Lim et al. concluded that there were two enzymes in wheat, Phy1 and Phy2, with the ability to hydrolyze phytic acid (*13*). Phy1 has a pH optimum of 6.0 and an optimal temperature of 45 °C, whereas Phy2's optimum pH lies at 5.5 with an optimal temperature of 50 °C. V_{max} for Phy1 is about half the V_{max} of Phy2, and Phy1 is more easily inhibited by P_i than Phy2 (*14*). One wheat phytase cDNA has been cloned, and the sequence revealed that the enzyme has similarity to the class of purple acid phosphatases (*15*). The hydrolysis of phytic acid by wheat phytase has been shown to be inhibited when the phytate is in complex with the minerals Al^{3+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, and Zn^{2+} (*16*). Phytic acid degradation by wheat phytase has been investigated on a number of occasions in connection

* Author to whom correspondence should be addressed [e-mail skr@life.ku.dk; telephone (+ 45) 35333436; fax (+45) 35283460].
† University of Copenhagen.
§ Technical University of Denmark.

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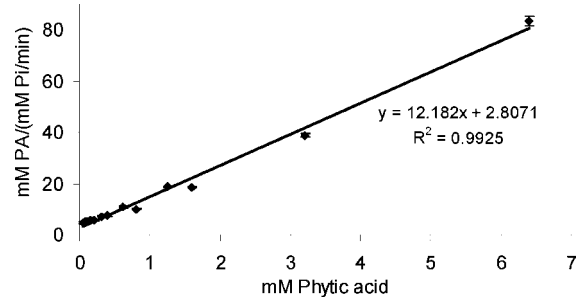

Figure 1. Hanes plot of wheat phytase kinetics using phytic acid as substrate at pH 6.0 and 45 °C. Error bars are indicated with lines. From the plot, K_m is calculated to 0.83 mM phytic acid and V_{max} to 0.230 mmol of P_i/min·mg.

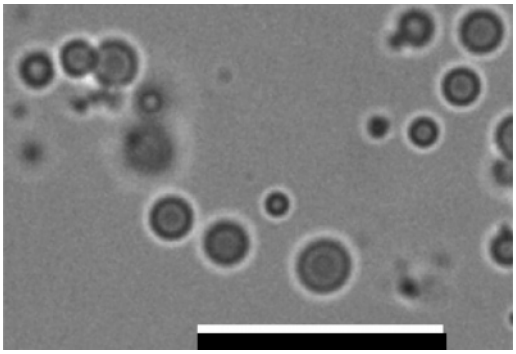

Figure 2. Phytate globoids isolated from wheat bran. Globoids are the spheres visualized by light microscopy (×100). Scale bar = 20 μm.

with characterizations of the enzyme (*17*, *18*), and it has been determined that it acts through attack on phosphate in the 6-position to yield *myo*-inositol as the final product.

The degradation patterns documented so far were, however, based on experiments in which soluble phytic acid was present for degradation. It has been proved that soluble phytic acid can be completely degraded under these conditions, but not whether—and how—phytase degrades the naturally occurring form of phytic acid—the insoluble phytate globoids. The importance of a proper degradation of phytate to improve mineral bioavailability from food has recently been extensively reviewed (*19*). The inositol phosphates must be reduced to very low levels to prevent inhibition of mineral bioavailability, and the understanding of the natural action of endogenous plant phytases on phytate globoids is of economic importance to the food-processing industry.

This study was undertaken to investigate the relationships between wheat phytase, phytic acid, and phytate globoids. The isolated phytate globoids are characterized with respect to mineral composition as compared with the bran fraction. Furthermore, the degradation patterns by wheat phytase of pure phytic acid and phytate globoids isolated from wheat are established and the kinetics of phytase using either phytic acid or phytate globoids are compared.

MATERIALS AND METHODS

Plant Material, Chemicals, and Enzymes. Wheat bran was kindly provided by Ringsted Dampmølle, Ringsted, Denmark. Phytic acid isolated from corn (P8810), crude extract of wheat phytase (P1255), bovine serum albumin (BSA) (A7906), cotton seed oil (C7767), *p*-nitrophenyl phosphate (104-0), and the Bradford reagent (B6916) were all purchased from Sigma-Aldrich (St. Louis, MO). Anhydrous carbon tetrachloride (CCl_4) was purchased from Prolabo (Fontenay sous Bois, France), and 1-naphthylphosphate sodium salt monohydrate (6815) was purchased from Merck (Darmstadt, Germany).

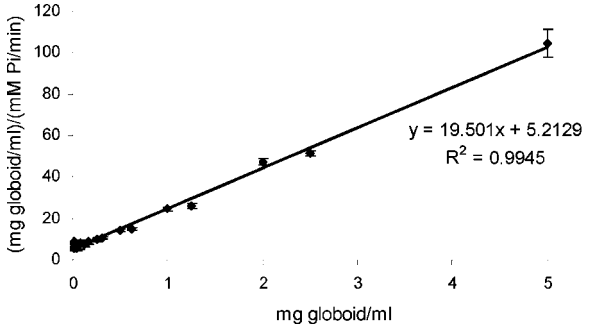

Figure 3. Hanes plot of wheat phytase kinetics using phytate globoids as substrate at pH 6.0 and 45 °C. Error bars are indicated with lines. From the plot, K_m is calculated to 0.96 mg of globoids/mL and V_{max} to 0.185 mmol of P_i/min·mg.

Phytase Purification. Chromatography was done on a fast protein liquid chromatography system (ÄktaExplorer10S) from Amersham Biosciences (Uppsala, Sweden). Wheat phytase was purified by gel filtration on a HiPrep 26/60 Sephacryl S-200 column (Amersham Biosciences). NaAc (0.1 M) containing 0.15 M NaCl, pH 5.5, was used as running buffer. Crude phytase extract (250 mg) was dissolved in 2 mL of running buffer and filtered through a 0.22 μm filter before injection. Fractions (1.8 mL) were tested for phytase activity as described below, and positive samples were pooled and concentrated using Millipore's Amicon Ultra-4 10000 MWCO filter device (Carrigtwahill, Ireland). Purity was verified by SDS-PAGE and Coomassie blue staining (Pierce GelCode 24590). The Bradford reagent was used for protein quantification throughout the purification steps using BSA as reference.

Enzyme Assay. Phytase activity was measured according to the method given in ref *20* (adapted for Eppendorf tubes) at 42 °C in a buffer containing 200 mM sodium acetate, pH 5.5, and 1 mM $CaCl_2$. The reaction was started by adding 200 μL of preheated substrate (7.5 mM phytic acid, 7.5 mM *p*-nitrophenyl phosphate, or 7.5 mM 1-naphthyl-phosphate), pH 5.5, to the enzyme (100 μL) and incubated for 60 min. The reaction was terminated by adding 200 μL of color stop mix (2.5% ammonium heptamolybdate, 5 mM ammonium vanadate, and 10.7% nitric acid). Released phosphate was recorded by measuring absorption spectrophotometrically at 415 nm using FLUOstar galaxy (BMG labtech, Offenburg, Germany) and quantified using a standard curve in the range of 0−500 nmol of NaH_2PO_4 dissolved in the assay buffer. The activity of phytase was calculated as release of inorganic phosphate (P_i) per minute.

Enzyme Characterization. Activity response to pH was examined at 42 °C using a 0.1 M glycine buffer in the pH ranges of 2−4 and 9−10, 0.1 M sodium acetate buffer in the pH range of 4−6.5, and a 0.1 M Tris buffer in the pH range of 6−9. Measurements were done in triplicate using the enzyme assay described above. The thermostability experiments were performed on a heating block (Grant QBTB, Grant Instrument). The optimum temperature was determined in 200 mM sodium acetate, 1 mM $CaCl_2$ at pH 5.5 and 7.5 mM phytic acid as substrate in the range from 25 to 60 °C with 5 °C increments. Samples were equilibrated at target temperature for 15 min before the enzyme was added and then incubated for an additional 60 min. The reaction was stopped by adding color stop mix. K_m and V_{max} measurements were performed under optimal conditions (45 °C and pH 6.0) using various concentrations of either phytate globoids or phytic acid as substrate to 0.28 mg enzyme and incubated for 30 minutes.

Isolation of Phytate Globoids. Aleurone particles were purified from wheat bran following the nonaqueous protocol of Tanaka et al. (*7*) through six centrifugation steps. Sixty grams of bran was homogenized with 100 mL of cottonseed oil in a Braun blender (MX32, Frankfurt, Germany) at maximum speed for 10 min at room temperature. After the homogenate had been filtered through gauze, the slurry was centrifuged at 2600*g* for 15 min and the supernatant was discharged. The pellet was suspended in cottonseed oil/CCl_4 mixtures, which were carried through the subsequent steps as outlined by Tanaka et al. (*7*).

Quantifications of Phytate Globoid Components. Two hundred milligrams of material was treated as described in ref *21* before the

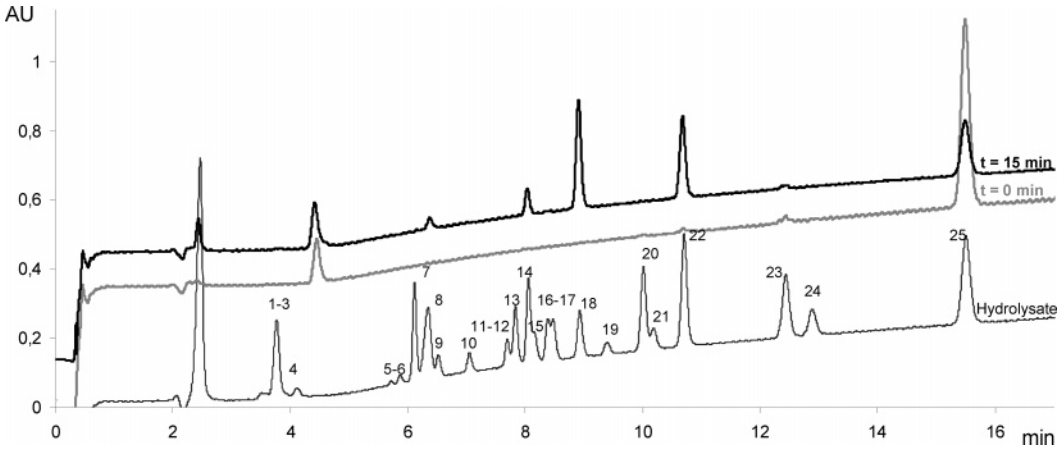

Figure 4. Chromatographic profile of (bottom) hydrolyzed sodium phytate and partly degraded phytate globoids after 0 (center) and 15 min incubation (top). Peaks: 1–3, $InsP_2$; 4, $InsP_2$; 5 and 6, unidentified; 7, DL-Ins(1,2,4)P_3, DL-Ins(1,3,4)P_3, and Ins(1,2,3)P_3; 8, DL-Ins(1,2,6)P_3 and Ins(1,2,3)P_3; 9, DL-Ins(1,4,5)P_3; 10, DL-Ins(1,5,6)P_3; 11, DL-Ins(4,5,6)P_3; 12, Ins(1,2,3,5)P_4; 13, DL-Ins(1,2,4,6)P_4; 14, DL-Ins(1,2,3,4)P_4; 15, Ins(1,3,4,6)P_4; 16, DL-Ins-(1,2,4,5)P_4; 17, DL-Ins(1,3,4,5)P_4; 18, DL-Ins(1,2,5,6)P_4; 19, Ins-(2,4,5,6)P_4; 20, DL-Ins(1,4,5,6)P_4; 21, Ins(1,2,3,4,6)P_5; 22, DL-Ins(1,2,3,4,5)P_5; 23, DL-Ins-(1,2,4,5,6)P_5; 24, Ins(1,3,4,5,6)P_5; 25, $InsP_6$.

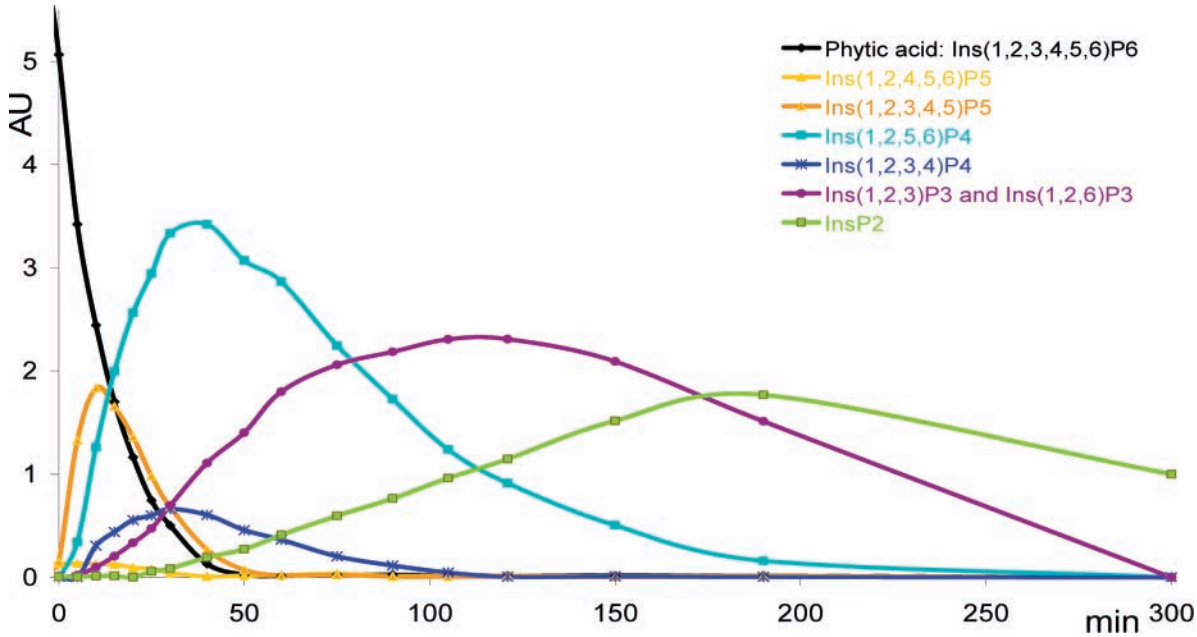

Figure 5. Wheat phytase degradation of phytate globoids from wheat. The peaks are not quantified, but the time of the maximum height tells us in which order the products are produced. A similar experiment was performed using pure phytic acid as substrate, and the pattern was the same, although the reaction was faster (data not shown).

Table 1. Quantification of the Composition of Major Elements and Minerals in Wheat Phytate Globoids Compared to Wheat Bran (Average Coefficient of Variation <4 %)

	mg/g			μg/g										
	phytic acid	protein	moisture	B	Ca	Cu	Fe	K	Mg	Mn	Na	P	S	Zn
globoid	402	462	103	1.3	4290.1	58.0	589.0	76443.9	32351.4	122.2	318.1	81739.8	1724.1	223.1
bran	129	13	73	2.0	832.9	11.3	82.6	17157.6	4377.5	71.7	79.9	11616.4	1227.8	47.4

composition of the minerals Al, B, Ca, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, P, S, and Zn was measured by inductively coupled plasma−mass spectrometry (ICP-MS) (Agilent 7500ce, Agilent Technologies, Manchester, U.K.). Proteins were extracted in Milli-Q water overnight at 8 °C and quantified using the Bradford reagent. Phytic acid was extracted in 0.1 M HCl overnight at 8 °C and quantified on HPIC as described below. Moisture was removed by freeze-drying samples overnight (Alpha 1-4, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The potential presence of starch in the globoids was assessed by quantitative measurement of glucose by high-performance anionic chromatography (HPAEC) of globoid hydrolysates resulting after acid hydrolysis (2 M HCl, 100 °C, 1 h). ICP-MS quantifications were done in triplicates. Protein, phytic acid, and moisture quantifications were done 5-fold.

Hydrolysis of Phytic Acid by Phytase. Six milligrams of phytate or phytate globoids was incubated in 2.3 mL of 200 mM sodium acetate, pH 5.5, and 1 mM $CaCl_2$ buffer. The assay was started by adding 3.3 units of preheated phytase in 0.2 mL of buffer to the reaction mixture.

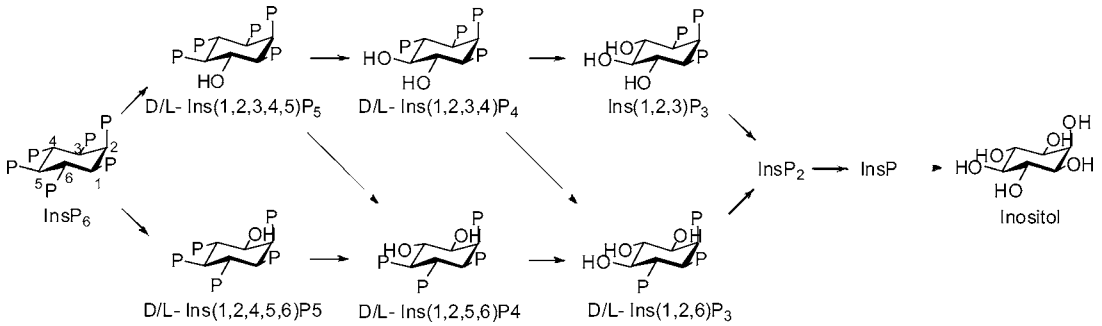

Figure 6. Degradation pattern of phytic acid in globoids by purified Sigma wheat phytase as suggested from the data of **Figure 5**. In this figure only, P represents inorganic phosphate.

At appropriate times from −2 min to 1260 min 100 μL was transferred to 200 μL of 95 °C Milli-Q water, and the enzyme was inactivated by incubation at 95 °C for 15 min. Samples were spun at full speed on an Eppendorf Centrifuge 5417R (Radiometer, Brønshøj, Denmark) for 2 min before the degradation products were extracted with 0.4 M HCl, filtered, and analyzed at HPIC.

Quantification of Inositol Phosphates. Phytic acid and other inositol phosphates were quantified on a HPLC (chemically inert HPLC system 10Avp series, Shimadzu, Kyoto, Japan) equipped with a guard column (Dionex CarboFac PA-100, 4 × 50 mm) and a HPIC CarboPac PA-100 (4 × 250 mm) analytical column (Dionex Corp., Sunnyvale, CA) at 40 °C in a column oven (CTO-10A). The protocol was performed according to that of Carlsson et al. (*22*). Samples (100 μL) were loaded with an autoinjector (SIL-10Ai). The eluants (0.8 mL/min) were mixed with 0.1% $Fe(NO_3)_3 \cdot 9H_2O$ (Sigma-Aldrich) in a 2% solution of $HClO_4$ in a postcolumn reactor using a Shimadzu HPLC pump LC-10Ai. The combined flow rate was 1.2 mL/min. A mixing tee and a 4 m PTFE knitted open tubular reactor coil $^1/_{16}$ in. o.d., 0.25 mm i.d. (from a local supplier) was used to ensure sufficient reaction time and mixing. Phytate was detected after postcolumn reaction by monitoring the absorbance at 290 nm using a Shimadzu SPD-M10Avp diode array detector. A hydrolysate of phytic acid was prepared by boiling pure phytic acid for 12 h in 6 M HCl, vaporizing the acid, and redissolving the sample in Milli-Q water. The peaks were then compared with the hydrolysate of refs *22* and *23*. For data analysis Class-VP, release 6.12 SP (Shimadzu) was used.

Statistical Analysis. Data are presented as means ± standard deviation ($n = 3$). In **Figures 1** and **3** linear regression is used and linearity (R^2) is calculated using Microsoft Excel 2003.

RESULTS AND DISCUSSION

Phytase Purification and Characterization. Size exclusion chromatography of the crude extract of wheat phytase resulted in one peak of activity, and the fractions containing the activity were pooled and concentrated; subsequently, a 4-fold increase in activity was shown as compared to the activity of the crude phytase extract. Purity was tested by SDS-PAGE of the fractions collected after chromatography. It revealed copurification of two proteins of almost identical size, of which only one had phytase activity. The phytase displayed highest activity at pH 6.0 and an optimum temperature of 45 °C (data not shown), which is in agreement with previously published results for wheat Phy1 (*14*). The phytase was also inhibited by 0.3 mM sodium phosphate added to the solution as expected from previous descriptions of Phy1 and its sensitivity to product inhibition (*13*, *14*). When pure sodium salt of phytate was used as substrate (**Figure 1**), K_m (0.83 mM phytic acid) was higher than previously reported values for Phy1, which range from 0.02 mM (*13*) to 0.48 μM (*14*). V_{max} (0.230 mmol of P_i/min·mg of protein) corresponded well with the V_{max} value of 127 μmol/min·mg of protein for purified wheat phytase previously reported by Nakano et al., considering differences in buffer systems and assay conditions (*14*). More comparable than these values were the differences observed using the pure phytic acid or phytate globoids as substrate for the measurements under exactly the same conditions, as we describe in the following sections.

Phytate Globoid Purification and Characterization. Particles from the aleurone layer were isolated from wheat bran by a six-step differential centrifugation procedure using nonaqueous media. After the final purification step, the preparation had an ivory white powder appearance and was only partially soluble in aqueous solutions. The particles were spherical with diameters ranging from ∼1.5 to 5 μm (**Figure 2**). This is comparable to the average size globoid of 4 μm for aleurone grains in wheat bran (*5*). The yield of globoids was in the range of 0.4−0.7% (w/w) of the initial weight of the bran. These phytate globoids contained 402 mg of phytic acid/g of globoid, 462 mg of protein/g of globoid, 10.3% moisture, high contents of P (82 mg/g) and the minerals Mg and K (32−76 mg/g), and relatively high contents of Fe and Ca (0.6−4.3 mg/kg) (**Table 1**). Besides the listed minerals Al, Mo, and Ni were also quantified, but with too high variation to make the values significant. No starch granules were observed visually by light microscope (**Figure 2**), nor was any glucose detected in the particles by HPAEC after acid hydrolysis. We will therefore from now on refer to them as phytate globoids. In general, an increase in the concentration of several cations during the purification of the globoids was observed (**Table 1**). The concentrations of Fe, Mg, and P were all 7-fold higher in the purified globoids as compared with wheat bran. This copurification of Fe and Mg with phytate-P allows us to suggest an association between these compounds and confirms a connection between phytic acid and Mg, verifying what has been documented in low-phytate wheat [Js-12-LPA (*11*)] and rice [lpa1−1 (*24*)]. Other minerals such as Ca, Zn, and K increased 5-fold in concentration from wheat bran to purified globoids (**Table 1**). This indicates that they are more loosely bound to the phytic acid, and it can be inferred that they also are stored in other parts of the seed or bran. This theory is supported by the fact that compared with whole wheat grain (*25*, *26*), the concentrations of Ca and Fe in bran are both about twice as high in bran compared to the whole grain, but in the enrichment step from bran to globoids, the relative increase in Fe is much higher than what is detected for Ca.

Degradation of Phytate Globoids by Phytase. Because phytate in globoids may be embedded in proteins (*2*), it is not expected to be as readily accessible to dephosphorylation as chemically pure phytic acid. We have tested the way that phytase behaves toward its natural substrate (**Figure 3**). When phytate globoids were used as substrate, K_m and V_{max} were calculated to 0.96 mg of globoids/mL [equivalent to 0.59 mM phytic acid/mg of protein based on the levels of phytate determined in the globoids (**Table 1**)] and 0.185 mmol of P_i/min·mg of protein,

respectively. Both of these values are significantly lower than the values computed for wheat phytase when phytic acid was the substrate (**Figure 1**). K_m using phytate globoids decreased by 29% as compared to the value determined using chemically pure phytate as substrate, and V_{max} decreased 37% under similar conditions. This result could be explained as a simple question of space: The proteins in the globoid may retard the action of the enzyme simply by blocking access to phytic acid. If phytate crystals are embedded in a protein matrix or membrane (*2*), the enzymatic activity may be hindered by the structure of the complex. A similar experiment using the smaller globoids from a low phytic acid mutant (*24*) could investigate this theory, because a small membrane-covered phytate crystal would be expected to have the same K_m and V_{max} values as larger globoids, if the proteins covered that globoid with a membrane. An alternative explanation could be that the minerals in complex with the phytic acid inhibit dephosphorylation by forming insoluble salts as studied by Tang et al. (*16*). However, in this investigation HPIC analysis of phytic acid and its lower inositol phosphate products (**Figures 4**−**6**) showed that wheat phytase was able to attack phytic acid in globoids and that all inositol phosphates were completely degraded within 20 h (data not shown). In addition, the speed of degradation slowed, especially in the last dephosphorylation steps from inositol di-kisphosphate to inositol (**Figure 5**). This could be a consequence of product inhibition, as the phosphate concentration slowly increased during the reaction.

The analysis of intermediate InsPx products indicated that wheat phytase was capable of attacking both the 3- and the 6-phosphate of the inositol hexakisphosphate ring. **Figure 4** shows the HPIC analysis of an undegraded sample of phytate globoids (center chromatogram) and a partly degraded sample (top chromatogram) compared to a standard hydrolysate (bottom chromatogram). It shows how phytase catalyzed the conversion of phytic acid in multiple pathways. **Figure 5** recaptures the sequential dephosphorylation of the phytic acid in the samples over time as illustrated in **Figure 6**. The quantifications in **Figure 5** are based on the absorbance of the different inositol phosphates. They document how phytic acid was hydrolyzed within 50 min and replaced by lower inositol phosphates during the enzyme-catalyzed degradation. After 10 min, two $InsP_5$s peaked, and in time all of the phosphates groups were removed from the inositol in a very distinct pattern, ultimately ending in inositol and phosphate (**Figure 6**). The same degradation pattern was observed when pure phytic acid was used as substrate instead of phytate globoids (results not shown). These current findings thus illustrate that wheat phytase is much less predictable in its actions than previously reported. In their study on wheat phytase Nakano et al. (*17*) identified the P6 pathway as the only route for dephosphorylation. However, recently other phytases have been shown to share a similar tendency toward multiple degradation pathways (*27*), suggesting that the active site of phytase in general is less specific than previously anticipated.

In conclusion, we have established a connection between several minerals and phytate globoids. Furthermore, data here shown indicated that wheat phytase apparently was able to release phosphate directly from attack on the globoids. Despite inhibition from other substances in the globoids, the enzyme was able to degrade phytic acid through attack of either the 6- or 3-positioned C in the inositol hexakisphosphate ring. Finally, use of the naturally occurring phytate globoids as substrate for phytase slowed the action of the enzyme compared to using commercially available phytic acid as substrate. Manufacturers of food or feed with phytase added should consequently expect a lower activity level and a lower yield of phosphate and free minerals than the values calculated in advance.

ABBREVIATIONS USED

HPIC, high-performance ion chromatography; InsP*x*, inositol mono-hexakisphosphate; WT, wild type; PA, phytic acid; P_i, inorganic phosphate.

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