Research Article

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The degradation of phytate by microbial and wheat phytases is dependent on the phytate matrix and the phytase origin

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Abstract

BACKGROUND: Phytases increase utilization of phytate phosphorus in feed. Since wheat is rich in endogenous phytase activity it was examined whether wheat phytases could improve phytate degradation compared to microbial phytases. Moreover, it was investigated whether enzymatic degradation of phytate is influenced by the matrix surrounding it. Phytate degradation was defined as the decrease in the sum of $InsP_6 + InsP_5$.

RESULTS: Endogenous wheat phytase effectively degraded wheat $InsP_6 + InsP_5$ at pH 4 and pH 5, while this was not true for a recombinant wheat phytase or phytase extracted from wheat bran. Only microbial phytases were able to degrade $InsP_6 + InsP_5$ in the entire pH range from 3 to 5, which is relevant for feed applications. A microbial phytase was efficient towards $InsP_6 + InsP_5$ in different phytate samples, whereas the ability to degrade $InsP_6 + InsP_5$ in the different phytate samples ranged from 12% to 70% for the recombinant wheat phytase.

CONCLUSION: Wheat phytase appeared to have an interesting potential. However, the wheat phytases studied could not improve phytate degradation compared to microbial phytases. The ability to degrade phytate in different phytate samples varied greatly for some phytases, indicating that phytase efficacy may be affected by the phytate matrix.
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Keywords: phytate; inositol phosphate; wheat phytase; microbial phytase

INTRODUCTION

Phosphorus (P) is an essential mineral for all living organisms. In plant seeds P is stored as phytate (salts of *myo*-inositol hexaphosphate, $InsP_6$), which is deposited in single-membrane storage protein bodies, mainly as globoids.[1] Globoid crystals consist of phytate salts predominantly with potassium (K) and magnesium (Mg).[2] It is important to improve the bioavailability of phytate P in plant feed materials as this limits the need for inorganic P in feed for pigs and poultry and thus reduces the excretion of undigested P to the environment. Phytate and lower inositol phosphates ($InsP_5$ – $InsP_1$) can be hydrolyzed by the enzyme phytase (*myo*-inositol hexaphosphate phosphohydrolase, EC 3.1.3), which is found naturally in plants and other organisms. In plants the activity varies considerably, from below 100 FTU kg^{-1} in maize up to approximately 1200 FTU kg^{-1} in wheat and 5000 FTU kg^{-1} in rye.[3] The endogenous activity in plant feed materials may be inactivated during feed processing.[4] Thus for intensive pig[5] and poultry[6] farming microbial phytases are commonly used. In pigs[7–10] and broilers[11,12] the major phytate degradation is presumed to take place in the upper gastrointestinal tract. The present study focuses on the potential of wheat phytases. Wheat enzymes with phytase activity include at least two types of phytases: purple acid phosphatase phytases (PAPhys) and multiple inositol phosphate phosphatases. Based on enzyme kinetics the PAPhys, encoded by the TaPAPhy_a and TaPAPhy_b genes, is presumably the most important group.[13,14] TaPAPhy_a and TaPAPhy_b are synthesized mainly during seed development and germination, respectively.[13] In mature seeds the TaPAPhy is associated with the globoids present in protein storage vacuoles in the aleurone layer.[13] In the present study the two isoforms were represented by PAPhy_a purified from wheat bran (denoted wheat bran phytase) and recombinant PAPhy_b1 produced in *Pichia pastoris* (denoted *rec*PAPhy_b1). The concerted wheat phytase activities, referred to as endogenous wheat phytase, were represented by non-heat-treated (NHT) wheat grains. The first objective was to test the hypothesis that the ability of wheat phytases to improve phytate degradation is superior to that of microbial phytases. The respective efficacies of plant and microbial phytases have previously been compared.[15,16] However, the way in which the plant matrix surrounding the phytate may affect plant P bioavailability has received limited attention. Phytases are often evaluated using phytase activity assays, where the

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substrate is commercially available purified sodium (Na) phytate. However, if the degradation of phytate depends on the matrix surrounding it, this means that such evaluation can be misleading. Therefore, the second objective was to test the hypothesis that enzymatic degradation of phytate is influenced by 'phytate source' representing the phytate matrix. In this study phytate source refers to purified phytate samples from wheat, purified Na phytate or phytate as it is present in different feed materials.

MATERIALS AND METHODS

Phytases

Six phytases were studied; three were of plant origin (wheat) and three were of microbial origin. Recombinant PAPhy, isoform b1 (*rec*PAPhy_b1) was cloned from wheat and expressed in *Pichia pastoris* as described in Dionisio *et al*.[13] Wheat bran phytase was purified from the NHT wheat used in the current study and characterized as described below. Endogenous wheat phytase was represented by the NHT wheat. Microbial phytases included two histidine acid phosphatase phytases purified from RONOZYME® HiPhos and RONOZYME® P (DSM Nutritional Products, Basel, Switzerland). The phytases were denoted HP and RP, respectively. The third phytase was an experimental β-Propeller phytase derived from *Bacillus* (Novozymes A/S, Bagsvaerd, Denmark), denoted BP.

Phytase activity determination

The phytase activity was determined by colorimetric measurement of the released phosphate according the AOAC method for purified phytases[17] and the AOAC 'in feed' method,[18] which includes a procedure to extract phytase, e.g. from feed materials. In both cases 1 FTU is defined as the amount of enzyme required to release 1 μmol of inorganic orthophosphate per minute from Na phytate (5 mmol L^{-1} Na phytate) at pH 5.5 and 37 °C. The pH profiles were determined in the pH range 2.0–7.5 using an assay buffer consisting of 50 mmol L^{-1} glycine, 50 mmol L^{-1} acetic acid and 50 mmol L^{-1} Bis-Tris.

Purification of wheat bran phytase

NHT wheat (1 kg) was milled and sieved and the bran fraction (>140 μm) was dissolved in1 L Buffer A (0.1 mol L^{-1} acetate buffer pH 5.5, 1 mmol L^{-1} $CaCl_2$, 1 mmol L^{-1} phenylmethylsulfonate fluoride, 5 mmol L^{-1} benzamidine, 50 μmol L^{-1} tosyl-lysyl chloromethylketone, 0.1% Nonidet P40). The mixture was heated to 40 °C under stirring (200 rpm) and 5000 U xylanase (Sigma X2753), 2300 U β-glucanase (Fluka 74 385) and 500 U phospholipase D (Sigma P0515) were added. The mixture was stirred for 3 h before centrifugation at 6000 × *g* for 30 min. Proteins were precipitated with $(NH_4)_2SO_4$ (60% saturation) and pellets were resuspended in 50 mL Buffer B (20 mmol L^{-1} acetate pH 4.3, 0.1 mmol L^{-1} $CaCl_2$) and dialyzed against 10 L water for 12 h using dialysis tubes (10 kDa cut-off). Dialyzed proteins were subjected to cation exchange chromatography using Buffer B and a linear gradient of Buffer B + 0.5 mol L^{-1} NaCl. Fractions with phytase activity were combined and dialyzed against Buffer C (50 mmol L^{-1} Tris-HCl, pH 7.5) and subsequently subjected to anion exchange chromatography and eluted by a linear gradient of Buffer C including 0.5 mol L^{-1} NaCl. Finally, the fractions with phytase activity were pooled and loaded on to a ConA-Sepharose column and eluted in Buffer C including 0.25 mol L^{-1} NaCl and 0.2 mol L^{-1} β-D-glucopyranoside. Pooled fractions were dialyzed and concentrated using Vivaspin cartridges (10 kDa cut-off). The purified wheat bran phytase was stored with 50% glycerol at 4 °C.

Development of antibodies against purple acid phytase

Rabbit polyclonal antibodies raised against *rec*PAPhy_b1[17] were prepared by DAKO A/S (http://www.dako.dk). The antibody specificity was verified via western blotting of wheat bran protein extracts and endoglycosidase H (New England Biolabs, Ipswich, MA, USA) treated *rec*PAPhy_b1.[13] Immunodecoration was performed with 1 : 1000 diluted affinity purified anti-PAP phytase antibodies.

Identification of wheat bran phytase by tandem mass spectrometry (MS/MS) analysis

The wheat bran phytase sample was alkylated with iodoacetamide and separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The 60 and 66 kDa protein bands were cut out of the gel and digested with trypsin. The resulting peptides were analyzed by liquid chromatography–mass spectrometry (LC-MS) on an LTQ Orbitrap XL LC-MS/MS system (Thermo Scientific, Waltham, MA, USA) coupled to an Accela LC HPLC system. Both parent peptide ions (MS data) and peptide fragments (MS/MS data) were measured in the high-precision Orbitrap. Peptide sequence fragmentation was obtained using the higher-energy C-trap dissociation (HCD) fragmentation of the system. The combined MS and MS/MS data were searched against the public databases UNIPROT and GENESEQP using the MASCOT search engine (Mascot server version: 2.2.0, Matrix Science, Boston, MA, USA), which is considered a standard software tool for protein identification from combined MS and MS/MS data.[19]

Feed materials

Feed materials included five common feedstuffs used in Denmark, Danish Agriculture & Food Council (http://www.agricultureandfood.dk). These included whole grain wheat, maize, barley, soybean and rapeseed meal and two common feed diets, including a wheat-based diet and a maize–soybean meal diet. The wheat based diet was composed of wheat (44.1%), barley (26.1%), wheat bran (16.9%) and soybean meal (13.3%). The maize–soybean meal diet was composed of maize (65%) and soybean meal (35%). As calcium (Ca) influences phytate solubility,[20–23] extra Ca (6 g Ca^{2+} kg^{-1} dry matter (DM)) was added to the feed materials to reach a normal to low range of Ca in pig[24] and broiler[25] feed. Feed materials were processed at and obtained from the Danish Technological Institute (http://www.dti.dk). Except for a subsample of wheat, referred to as non-heat-treated wheat and denoted NHT wheat, all feed materials were heat treated (95 °C, 10 min). Heat treatment was carried out in a cascade mixer to which steam was injected at a pressure of 200 kPa. Heat-treated wheat is referred to as HT wheat. All feed materials were ground to pass a 1 mm sieve. Sample DM of feed materials was determined by drying samples to a constant weight at 105 °C for 24 h. Endogenous phytase activity in HT and NHT wheat was 27 ± 8 and 396 ± 60 FTU kg^{-1} DM, respectively (n = 4).

Analysis of inositol phosphate

Sample supernatants (HCl extracts of purified phytate samples and feed materials, and of samples from phytate degradation studies) were filtrated through Microcon filters (cut-off 30 kDa) and

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centrifuged (14 000 × *g*, 90 min at 0 °C). Filtrates were analyzed using high-performance ion chromatography (HPIC) and post-column reaction with 1 g L^{-1} $Fe(NO_3)_3.9H_2O$ in a 20 mL L^{-1} solution of $HClO_4$. InsP–Fe complexes were formed and detected by UV as described by Pontoppidan *et al.*[26] according to the method of Skoglund *et al.*[27] Inositol phosphate (InsP), which included phytate ($InsP_6$), inositol pentaphosphate ($InsP_5$), inositol tetraphosphate ($InsP_4$) and inositol triphosphate ($InsP_3$), were calculated as either InsP or InsP-P.

Substrate preparations for phytate degradation studies

Preparation of wheat slurries

Wheat slurries were prepared in the following way. To each of four beakers was added 62.4 mL diluted HCl with molarities sufficient to reach pH 3.0 ± 0.05, pH 4.0 ± 0.05, pH 5.0 ± 0.05 or pH 5.5 ± 0.05. Each beaker contained HT wheat (8 g) with additional Ca added as 22 g $CaCl_2.2H_2O$ kg^{-1} DM, corresponding to 6 g Ca^{2+} kg^{-1} DM. The slurries were left on a magnetic stirrer (350 rpm) for 30 min at 22 °C and the pH was verified using a glass pH meter (Metrohm, Herisau, Switzerland). Similarly, four wheat slurries with additional Ca were prepared with NHT wheat for degradation with the endogenous wheat phytase.

Preparation of purified phytate samples

The preparations of purified phytate samples included InsP as present in HT wheat (wheat), InsP extracted from HT wheat (extracted wheat InsP), InsP as present in globoids extracted from HT wheat (globoids), InsP extracted from wheat globoids (extracted globoid InsP) and commercial InsP from Sigma (Na-phytate) (P-3168, Sigma-Aldrich, St Louis, MO, USA). Samples are referred to by the names given in parentheses. The phytate sample, wheat, was prepared as a wheat slurry (pH 4.0 ± 0.05) described under 'Preparation of wheat slurries' with the exception that no additional Ca was added. Extracted wheat InsP was prepared according to a modified version of the method by Carlsson *et al.*[28] In brief, InsP was extracted from feed material (1 g) with 10 mL of 0.5 mol L^{-1} HCl for 3 h with magnetic stirring (500 rpm) at 22 °C, interrupted by a freezing step (minimum 24 h). Subsequently, the sample was centrifuged (14 000 × *g*) for 10 min at 0 °C and the supernatant recovered. Globoids were isolated from bran according to Tanaka *et al.*[29] with the modification that acetone was used instead of CCl_4. Globoids were wetted with 96% ethanol and suspended in 25 mL water before use. Extracted globoid InsP was prepared by dissolving globoids (50 g) in 25 mL of 0.5 mol L^{-1} HCl and shaking (300 rpm) at 25 °C for 2 h. Na phytate was prepared by dissolving 117.5 mg commercial Na phytate in 100 mL water. All samples were adjusted to pH 4 ± 0.05 with NaOH or HCl. Concentrations of InsP in the phytate samples were determined to ensure a final concentration in each sample of 0.84 ± 0.16 mmol $InsP_6$ L^{-1}. In addition to $InsP_6$, all samples except Na phytate contained $InsP_5$ (6–23%). The wheat globoids furthermore contained $InsP_4$ (14%). Mineral content in the phytate samples was determined by inductively coupled plasma optical emission spectrometry (Table 1).

Preparation of phytate in feed materials

InsP was extracted from all the feed materials, as described for extracted wheat InsP under 'Preparation of purified phytate samples', and the InsP contents were quantified and found to conform well with previously published results (data are not shown).[6,31] These results were used to calculate appropriate amounts of feed material to ensure the same concentration of $InsP_6$ in all feed slurries used in the degradation studies (1.05 ± 0.15 mmol $InsP_6$ L^{-1}). Feed slurries were prepared by mixing feed material with additional Ca (added as 22 g $CaCl_2 \cdot 2H_2O$ kg^{-1} DM, corresponding to 6 g Ca^{2+} kg^{-1} DM) and suspending samples in diluted HCl solutions to obtain pH 4 ± 0.05.

Table 1. Mineral content (ppm) in different phytate samples measured by inductively coupled plasma optical emission spectrometry (ICP-OES). Wheat and wheat extracts were prepared from heat-treated wheat. Na phytate was obtained from Sigma. Results are presented as means

Phytate samples	*n*	Ca	Fe	Mg	Mn	P	K	Na
Wheat	2	28a	2.1a	47a	1.7a	140a	185b	41c
Extracted wheat InsP	2	22ab	1.4b	34b	1.1b	84c	205b	4050a
Wheat globoids	2	18ab	1.4b	32b	1.1b	96b	85c	72c
Extracted globoid InsP	2	15ab	<1c	29c	1b	98b	260a	3600b
Na-phytate	2	10b	<1c	<10d	<1b	90bc	<10d	150c

Different letters within each column indicate a significant difference ($P < 0.05$), tested by Tukey HSD. The concentrations of Al (<4 ppm), B (<10 ppm), Cu (<1 ppm), Ni (<1 ppm) and Zn (<4 ppm) were also measured but the values were all below the detection limit. *n* indicates number of replicates.

Phytase preparations for phytate degradation studies

The intention was to dose the phytases at a level equivalent to the concentration of endogenous phytase determined in the NHT wheat. Thus, in wheat slurries, phytases were dosed at 400 FTU kg^{-1} DM, equivalent to 50 FTU $mmol^{-1}$ $InsP_6$. Therefore, in purified phytate samples and in feed materials, phytases were dosed at 50 ± 8 FTU $mmol^{-1}$ $InsP_6$. Appropriate phytase activities were reached by diluting purified phytases in enzyme buffer (100 mmol L^{-1} acetate buffer, pH 6.0, including 5 mmol L^{-1} Ca, 0.01% bovine serum albumin and 0.01% Tween 20). *rec*PAPhy_b1 was activated by adding $FeSO_4 \cdot 7H_2O$ (2 mmol L^{-1} final concentration in assay) and vitamin C (2 mmol L^{-1} final concentration in assay) to the phytase stock solution as recommended in Dionosio *et al.*[17]

Phytate degradation studies

Aliquots of 0.780 mL of each substrate preparation, i.e. wheat slurries, purified phytate samples or phytate in feed materials were placed in 2 mL Eppendorf tubes. The reaction was started by adding 0.020 mL enzyme solution or enzyme buffer (blank samples) to each aliquot and incubations were carried out for 30 min at 40 °C in a thermomixer (1000 rpm) at the relevant pH (3.0 ± 0.05, 4.0 ± 0.05, 5.0 ± 0.05 or 5.5 ± 0.05).The reaction was stopped by adding 0.800 mL of 1 mol L^{-1} HCl and InsP was extracted using a slight modification of the method by Carlsson *et al.*[28] as described under 'Preparation of purified phytate samples', except that the extractions were carried out in the thermomixer (1000 rpm) at 40 °C instead of by magnetic stirring at 22 °C. The supernatants were recovered and InsP contents were analyzed.

Statistics

Statistical analysis of InsP contents was performed using the software JMP (Statistical Analysis System, SAS Institute Inc., Cary,

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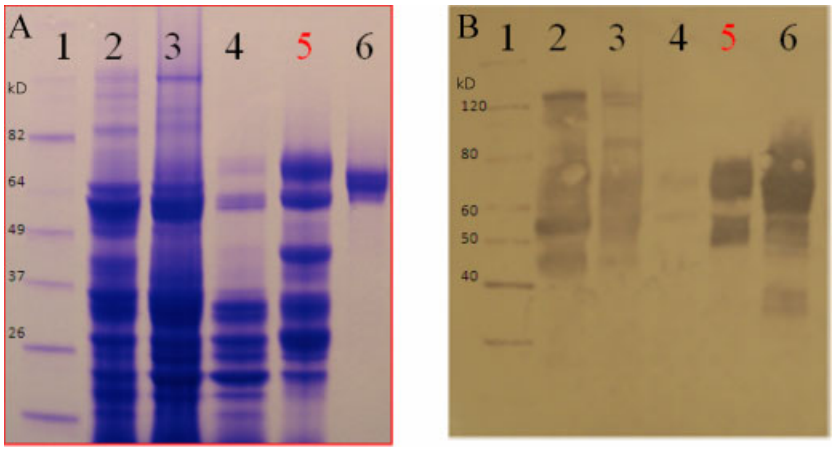

Figure 1. SDS-PAGE (A) and western blotting (B) following the purification of wheat bran phytase. Lane 1, standards; lane 2, crude extract; lane 3, SP-Sepharose phytase peak; lane 4, Q-Sepharose phytase peak; lane 5, ConA-Sepharose phytase peak, representing the wheat bran phytase; lane 6, *rec*PAPhy_b1.

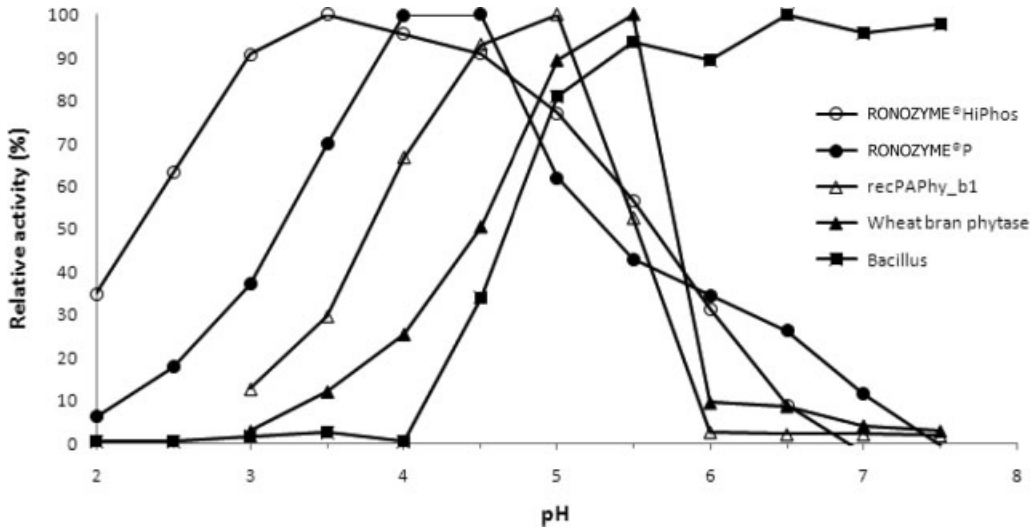

Figure 2. Relative activity (%) of five phytases in the pH range 2.0–7.5 (5 mmol L^{-1} Na phytate, 37 °C, 15 min). Maximum activity for each phytase was set to 100%. RONOZYME HiPhos (HP); RONOZYME P (RP); Bacillus (BP).

NC, USA). Data were analyzed by a one-way analysis of variance (ANOVA) and comparison of means was done using the Tukey HSD test ($\alpha = 0.05$). Data were tested in a significant model ($P < 0.0001$). All data were comprised of means of triplicates from two individual days, where days are included in the model as fixed effects. Multivariate data analysis of mineral effect on the efficacy of phytases was performed by partial least squares (PLS)[31,32] using the software The Unscrambler® (CAMO AS, Oslo, Norway). A model for each phytase was prepared and significant effects were selected by running an uncertainty test by jackknifing as described in Martens and Martens.[33] The mineral impact on phytase potential was remodeled for each phytase with the significant minerals. All five substrates were represented by two samples prepared on two individual days.

RESULTS AND DISCUSSION

Purification and characterization of wheat bran phytase

The presence of PAPhy in the wheat bran preparation was confirmed by SDS-PAGE and western blotting (Fig. 1) as two immunoreactive bands of 60 and 66 kDa (Fig. 1(B), lane 5). Judged from SDS-PAGE the phytase bands accounted for 30–40% of the protein content. Tandem mass spectroscopy (MS/MS) confirmed that PAPhys were the only phytases present in the wheat bran preparation. The major components of the 60 and 66 kDa bands were xylosidases and the PAP isoform PAPhy_a1 (UNIPROT : C4PKK7), with three unique peptides.

Phytase pH activity profiles

The HP had a very broad pH activity profile, with optimum at pH 3–4.5, whereas the profiles for RP and the two wheat phytases – *rec*PAPhy_b1 and wheat bran phytase – were more narrow and showed optima at pH 4–4.5, pH 5 and pH 5.5, respectively (Fig. 2). The BP had maximum activity at pH above 6. The difference between the pH profiles for the two wheat phytases is interesting and indicates that it is necessary to have more emphasis on the specific isoform used when referring to wheat phytase. The profile of the wheat bran phytase is similar to data published by Tang *et al*.[34] It should be noted that the activity values are relative

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Table 2. Residual inositol hexaphosphate phosphorus ($InsP_6$-P) + inositol pentaphosphate phosphorus ($InsP_5$-P) after incubation with phytases (400 FTU g^{-1} DM) in heat-treated wheat with added calcium (6 g kg^{-1} DM). The endogenous wheat phytase is represented by non-heat-treated wheat with added calcium (6 g kg^{-1} DM). Incubation was carried out at different pH values for 30 min at 40 °C. Values are presented as means of $InsP_6$-P + $InsP_5$-P (g kg^{-1} DM) and values relative to blank are given as a percentage (%)

	pH 3			pH 4			pH 5			pH 5.5		
	n	Mean ± SE (g kg^{-1}) DM	%	*n*	Mean ± SE (g kg^{-1}) DM	%	*n*	Mean ± SE (g kg^{-1}) DM	%	*n*	Mean ± SE (g kg^{-1}) DM	%
Blank	6	1.91 ± 0.03b		6	2.05 ± 0.06a		5	1.85 ± 0.04a		6	2.05 ± 0.06a	
*rec*PAPhy_b1	6	1.97 ± 0.03b	103	6	0.99 ± 0.06b	48	5	1.43 ± 0.04bc	77	6	2.08 ± 0.06a	101
Wheat bran phytase	5	2.17 ± 0.03a	114	5	1.76 ± 0.07a	86	6	1.35 ± 0.03bc	73	6	2.12 ± 0.06a	103
Endogenous wheat phytase	6	1.95 ± 0.03b	102	5	0.33 ± 0.07c	16	5	0.95 ± 0.04d	51	5	2.01 ± 0.07a	98
RONOZYME® HiPhos (HP)	6	0.38 ± 0.03d	20	6	0.18 ± 0.06c	9	6	1.45 ± 0.03b	78	6	2.02 ± 0.06a	99
RONOZYME® P (RP)	6	1.52 ± 0.03c	80	6	0.85 ± 0.06b	41	4	1.27 ± 0.04c	69	5	2.20 ± 0.07a	107
Bacillus (BP)	6	2.16 ± 0.03a	113	6	1.82 ± 0.06a	89	6	1.30 ± 0.03bc	70	6	1.95 ± 0.06a	95

Means with different letters in the same column are significantly different ($P < 0.05$), tested by Tukey HSD. *n* indicates number of replicates.

so that for each phytase the activity is set to 100% at the pH where the activity is highest. This makes it difficult to compare actual activities at a certain pH value between different phytases.

Degradation of wheat phytate by wheat phytases and microbial phytases

The hypothesis that the ability of wheat phytases to improve the degradation of wheat phytate is superior to that of microbial phytases was tested at different pH values. The values chosen for this experiment (pH 3–5.5) are in line with the range of pH in digesta of pigs[35–39] and broilers.[40–43] It is sometimes argued that phytases need to have high optimal activity at low pH (2–3) as they are assumed to act in the upper gastrointestinal tract of pigs[7–10] and broilers.[11,12] However, it has been shown that pH in the stomach of pigs increases after feed ingestion[35,36,39] and thus feed enzymes are probably emptied out of the stomach before pH returns to very low pre-prandial values. Stomach pH ranged from 3.8 to 4.6 when pigs had free access to feed.[37,38] When feed was offered as a meal, the reported peak stomach pH values range from 4.7 to 5.6.[35,36,39] and, 2 h after feeding, stomach pH ranged from 2.6 to 4.8.[35,36,39] Similarly, pH in digesta samples from the upper gastrointestinal tract of broilers range from 4.4 to 5.6 in the crop[40,41] and from 3.6 to 4.8 in the proventriculus,[41,42] whereas it is lower in the gizzard (pH 2.6–4.1).[41–43]

It has been shown that in pigs the limiting step for high phytate P bioavailability is the hydrolysis of the first one to two phosphate groups of phytate.[7,8,44–47] Therefore phytate degradation was quantified and interpreted as the hydrolysis of the sum of $InsP_6$ and $InsP_5$ ($InsP_6$ + $InsP_5$). Residual wheat $InsP_6$-P + $InsP_5$-P levels after incubation with the six phytases are shown in Table 2. None of the phytases could degrade any wheat $InsP_6$ + $InsP_5$ at pH 5.5, which may be explained by the lack of soluble substrate.[21–23] At pH 5 approximately half of the $InsP_6$ + $InsP_5$ was degraded by the endogenous wheat phytase, whereas the degradation was between 22% and 31% for the other phytases. Overall the best degradation was obtained at pH 4, where HP, endogenous wheat phytase, RP and *rec*PAPhy_b1 degraded 91%, 84%, 59% and 52%, respectively. Low effects of wheat bran phytase and the BP were seen at all pH levels. Both of these enzymes are disfavored at the pH level between 3 and 5, because they have a pH optimum at pH 5.5, which is the pH level where the phytase determinations are made in order to dose all phytases at the same level. This may to some extent explain their low effect. The higher efficiency of the endogenous wheat phytase compared to *rec*PAPhy_b1 may be explained by the endogenous wheat phytase being located close to the substrate, since phytase as well as phytate is located in protein storage vacuoles in the wheat aleurone.[2,48,49] It is also possible that the efficacy of the endogenous wheat phytases is somewhat overestimated as it may have been dosed higher than the other candidates. Typical levels of endogenous phytase in wheat range from 915 to 1581 FTU kg^{-1} DM.[3] The phytase activity determined in the NHT wheat was 396 ± 60 FTU kg^{-1} DM and, in theory, it is possible that not all activity was extracted.[50] Finally, the capacity to degrade phytate may also have been confounded by a difference in substrate structure, as the efficacy of the endogenous phytase was determined using NHT wheat, whereas the other phytases were incubated with HT wheat and the heat treatment may have affected phytate accessibility. Nevertheless, in a previous study neither pelleting nor extrusion affected the concentration of InsP in wheat.[4] At pH 3 wheat $InsP_6$ + $InsP_5$ was degraded by HP (80%) and RP (20%). None of the wheat phytases could degrade wheat $InsP_6$ + $InsP_5$ at pH 3, which is possibly a result of both poor phytase stability[51,52] and low activity (Fig. 2). As mentioned above, the increase in stomach pH when feed is ingested by pigs and the high pH values of digesta in the crop and proventriculus of broilers do not suggest that feed enzymes should necessarily have optimal activity at pH 3. However, as the pH in local regions of the upper gastrointestinal tract may be low, it is generally assumed that feed enzymes should ideally be stable at pH 3.[36]

Assuming that all phytases are dosed similarly and that the heat treatment has not rendered the HT wheat phytate more accessible, it is concluded that the endogenous wheat phytase appears to have an interesting potential. However, it was not possible to improve wheat $InsP_6$ + $InsP_5$ degradation compared to $InsP_6$ + $InsP_5$ degradation induced by microbial phytases in a pH range that is relevant for the upper gastrointestinal tract of broiler and pigs. Moreover, the efficacy of the endogenous phytase could not be achieved either by expressing the TaPAPhy_b gene microbially or by isolating the phytase from wheat bran.

The influence of phytate source on phytate degradation

The importance of phytate source – and thus the phytate matrix – on the bioavailability of plant P has received little

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Table 3. Residual inositol hexaphosphate ($InsP_6$) + inositol pentaphosphate ($InsP_5$) after incubation with phytases (50 ± 8 FTU μmol^{-1} $InsP_6$) in phytate samples at pH 4.00 ± 0.05 for 30 min at 40 °C. Wheat and wheat extracts were prepared from heat-treated wheat. Na phytate was obtained from Sigma. Values are presented as means of $InsP_6$ + $InsP_5$ in blank samples (mmol L^{-1}) and means of the relative $InsP_6$ + $InsP_5$ contents in the phytase-treated samples compared to the blank samples are given as a percentage (%)

Phytate samples	*n*	Blank Mean ± SE (mmol L^{-1})	RONOZYME® HiPhos (HP) Mean ± SE (%)	*rec*PAPhy_b1 Mean ± SE (%)	Wheat bran phytase Mean ± SE (%)
Wheat	6	1.19 ± 0.01	11.3 ± 1.1b	67.1 ± 1.2b	86.8 ± 0.85b
Extracted wheat InsP	6	0.81 ± 0.02	23.6 ± 1.1c	86.4 ± 1.2cd	78.0 ± 1.04a
Globoid	4	0.94 ± 0.06	9.1 ± 1.1b	81.5 ± 1.2c	96.6 ± 0.94c
Extracted globoid InsP	6	0.93 ± 0.01	22.4 ± 1.2c	87.9 ± 1.2d	80.5 ± 0.85a
Na-phytate	6	1.01 ± 0.01	0 ± 1.1a	32.2 ± 1.2a	100 ± 0.85c

Different letters within each column indicate a significant difference ($P < 0.05$). Tested by Tukey HSD. *n* indicates number of replicates.

attention. In this study, phytate source refers to purified phytate samples from wheat, purified Na phytate or phytate as it is present in different feed materials. The two isolated wheat phytases (*rec*PAPhy_b1 and wheat bran phytase) and the most effective microbial phytase (HP) were selected for degradation studies with different phytate sources. The endogenous phytase was the most effective among the wheat phytases, but it was not possible to apply this phytase to the purified phytate substrates without adding wheat phytate. For practical reasons only one pH level was chosen. The incubation pH was fixed at pH 4, since it favors phytate solubility[21–23] and activity of all phytases. Furthermore, it is within the range of relevant pH values for digesta from the upper gastrointestinal tract of pigs[35–39] and broilers.[40–43]

HP degraded more $InsP_6$ + $InsP_5$ in the different wheat phytate samples and the Na phytate sample than *rec*PAPhy_b1 and wheat bran phytase did (Table 3). *rec*PAPhy_b1 was more efficient than wheat bran phytase, except when degradation of extracted wheat InsP and extracted globoid InsP were quantified. While the purified Na phytate was extensively degraded by HP and *rec*PAPhy_b1, the wheat bran phytase did not degrade Na phytate at all under these conditions. However, it should be considered that the data are based on degradation of the $InsP_6$ + $InsP_5$. The wheat bran phytase degraded 10% of $InsP_6$ to $InsP_5$, but the $InsP_5$ was not degraded further. Interestingly, the largest variation of efficacy among the phytate samples was seen for *rec*PAPhy_b, which degraded 68% $InsP_6$ + $InsP_5$ in Na phytate and only 12% in extracted globoid InsP. It was notable that *rec*PAPhy_b1 exerted much less activity on the wheat-based substrates than on pure Na phytate. Previous studies have suggested that phytate in globoids might be embedded in proteins and therefore less accessible to dephosphorylation than pure Na phytate.[53] This assumption seems plausible but does not explain why $InsP_6$ + $InsP_5$ in the wheat sample is more easily degraded than it is in extracted wheat InsP and extracted globoid InsP. Whether this is due to a change in the phytate matrix when the phytate is extracted compared to when it is located in the wheat remains to be investigated in depth. In the current study, only the mineral content of these different phytate samples was studied further. The impact of mineral content on phytase efficacy was analyzed by multivariate data analysis. For all phytases Mg, K and Na have a significant impact on phytase efficacy and models were made based on these minerals, which could explain 0.69–0.93 of the variance (R^2) (Table 4). It seemed that there was a tendency towards HP and *rec*PAPhy_b1 being negatively and wheat bran phytase positively influenced by minerals. However, it must be noted that the dataset was very small and consequently the model validity is weak.

Table 4. The impact of mineral content on phytase efficacy analyzed by partial least squares (PLS). Minerals were magnesium (Mg), potassium (K) and sodium (Na). Explained variance (R^2) and regression coefficients for the three phytase models

Enzyme	Explained variance, R^2	Regression coefficients Mg	K	Na
RONOZYME® HiPhos (HP)	0.93	−0.25	−0.46	−0.45
*rec*PAPhy_b1	0.69	−0.32	−0.39	−0.31
Wheat bran phytase	0.89	+0.27	+0.47	+0.39

The ability of HP, *rec*PAPhy_b1 and wheat bran phytase to degrade $InsP_6$ + $InsP_5$ as it is present in different feed materials also differed (Table 5). HP degraded around 80% of $InsP_6$ + $InsP_5$ in all materials except for soybean meal, where it degraded 52%. However, this lower degradation of $InsP_6$ + $InsP_5$ in soybean meal was not evident when maize and soybean meal were mixed in a 65 : 35 ratio, where HP degraded 83% of $InsP_6$ + $InsP_5$. *rec*PAPhy_b1 degraded approximately 30% of $InsP_6$ + $InsP_5$ in most feed materials and up to 65% and 70% of $InsP_6$ + $InsP_5$ in maize and rapeseed meal, respectively. Surprisingly, it was least efficient at degrading wheat phytate, where only 25% $InsP_6$ + $InsP_5$ was degraded. With regard to maize meal the higher activity may be explained by phytate being located in the maize embryo, whereas in barley and wheat phytate is predominantly located in the aleurone layer.[2] Results from a previous study on the ability of an *E. coli* phytase to hydrolyze phytate in corn, soybean meal and a corn–soybean meal also showed differences in phytate degradation between different feed materials.[54] Wheat bran phytase only degraded between 0% and 12% $InsP_6$ + $InsP_5$ in all feed materials.

Overall, HP was most efficient at degrading $InsP_6$ + $InsP_5$ in the purified wheat samples, Na phytate as well as in the different feed materials. *rec*PAPhy_b1 was moderately efficient and wheat bran phytase degraded almost nothing. The ability to degrade $InsP_6$ + $InsP_5$ in the different phytate samples differed particularly for the *rec*PAPhy_b1, which degraded 65–70% in Na phytate, maize and rapeseed meal, around 30% in most other feed materials and only 12% in extracted globoid InsP. This indicates that *rec*PAPhy_b1 is more sensitive to the matrix surrounding the phytate samples than HP is. It was speculated that this may be due to the mineral content of the different phytate samples, but the current dataset was not

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Table 5. Residual inositol hexaphosphate ($InsP_6$) + inositol pentaphosphate ($InsP_5$) after incubation with phytases (50 ± 8 FTU $mmol^{-1}$ $InsP_6$) in feed materials at pH 4.00 ± 0.05 for 30 min at 40 °C. All feed materials were heat treated and additional calcium (6 g Ca^{2+} kg^{-1} DM) was added. Values are presented as means of $InsP_6$ + $InsP_5$ in blank samples (mmol L^{-1}) and means of the relative $InsP_6$ + $InsP_5$ contents in the phytase-treated samples compared to the blank samples are given as a percentage (%)

Feed material	*n*	Blank Mean ± SE (mmol L^{-1})	*n*	RONOZYME® HiPhos (HP) Mean ± SE (%)	*n*	*rec*PAPhy_b1 Mean ± SE (%)	*n*	Wheat bran phytase Mean ± SE (%)
Wheat	6	1.35 ± 0.01	6	21.5 ± 1.3ab	5	75.1 ± 1.7d	6	98.9 ± 1.2c
Maize	6	1.34 ± 0.01	6	24.5 ± 1.3b	6	36.8 ± 1.9a	6	99.7 ± 1.2c
Barley	6	1.25 ± 0.02	6	21.3 ± 1.3ab	6	70 ± 1.7cd	6	91.7 ± 1.2a
Soybean meal	6	1.39 ± 0.01	6	47.9 ± 1.3c	6	61.9 ± 1.7b	6	89.8 ± 1.2a
Rapeseed meal	6	1.34 ± 0.01	5	21.3 ± 1.3ab	6	29.7 ± 1.7a	6	92.7 ± 1.2ab
Wheat based blend	6	1.30 ± 0.01	6	21.3 ± 1.3ab	6	65.3 ± 1.7bc	6	97.8 ± 1.2bc
Soybean meal-maize blend	6	1.11 ± 0.01	4	17.2 ± 1.5a	6	73.1 ± 1.9d	4	87.8 ± 1.3a

Different letters within each column indicate a significant difference ($P < 0.05$), tested by Tukey HSD. *n* indicates number of replicates.

sufficient to show a stronger dependence of the *rec*PAPhy_b on the mineral content than of the HP, which appears much less influenced by phytate sample. It is especially interesting to note the difference between degradation of highly purified Na phytate and phytate as it is present in the feed material, since Na phytate is the substrate for the standardized determination of phytase activity, e.g. in the AOAC method.[17] For some phytases, e.g. *rec*PAPhy_b1, the ability to degrade Na phytate will not reflect the ability to degrade phytate in some feed materials. Obviously, the only way to fully determine and compare the potential of feed phytases is to perform dose–response feeding trials in the animal species of interest. However, it was shown in the current study that more information may be obtained from laboratory evaluations if application-relevant phytate substrates are included in the evaluation of phytase efficacy.

CONCLUSION

The degradation of wheat $InsP_6$ + $InsP_5$ was dependent on both pH and type of phytase. The highest degradation rates were obtained at pH 4, possibly due to a combination of high substrate solubility and optimal activity of the phytases. Assuming that all phytases are dosed similarly and that the heat treatment has not rendered the HT wheat phytate more accessible, it is concluded that the endogenous wheat phytase has an interesting potential to degrade phytate in wheat, where it is located close to the substrate. However, it was not possible to improve degradation of wheat $InsP_6$ + $InsP_5$ compared to the degradation induced by microbial phytases in the pH range from 3 to 5.5, which is relevant for the upper gastrointestinal tract of broilers and pigs. Moreover, the attempts to isolate the wheat phytase activity, either by expressing the TaPAPhy_b gene microbially or by isolating phytase from wheat bran, did not result in the same level of degradation as for the endogenous wheat, RP or HP phytases. Of the three selected phytases (HP, *rec*PAPhy_b1 and wheat bran phytase), HP was most efficient at degrading $InsP_6$ + $InsP_5$ in the purified wheat phytate samples, Na phytate and the different feed materials at pH 4.0. *rec*PAPhy_b1 was moderately efficient and wheat bran phytase degraded almost nothing. The ability to degrade $InsP_6$ + $InsP_5$ in the different phytate samples differed considerably for the *rec*PAPhy_b1. This indicates that phytase efficacy may be affected by the matrix surrounding the phytate. The current data thus show that more information may be derived from laboratory studies if application-relevant phytate substrates are included in the evaluation of phytase efficacy.

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