Research Article

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Limitations of an *in vitro* model of the poultry digestive tract on the evaluation of the catalytic performance of phytases

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Abstract

BACKGROUND: The study aims to investigate the limitation of a poultry digestive tract model developed by Menezes-Blackburn *et al.* [*J Agric Food Chem* 63: 6142–6149 (2015)] on the evaluation of the bioefficacy of phytases.

RESULTS: It was confirmed that the *in vitro* model does not mimic the *in vivo* situation in the birds sufficiently well to identify the best phytase product under real conditions, or to draw conclusion on the effect of phytate concentration, phytate source or feed composition on the bioefficacy of phytase. Addition of calcium ion (Ca^{2+}) up to a concentration of 10 g kg^{-1} to the feed substrate, for example, did not affect enzymatic phytate dephosphorylation in the *in vitro* model in contrast to the observation in poultry.

CONCLUSION: The *in vitro* approach was shown to be applicable as a complementary tool in the pre-selection of promising phytase candidates, resulting in a reduction in the number of feeding trials in the initial screening phase.

Keywords: *in vitro* model; phosphate release; phytase; phytate dephosphorylation; poultry digestive tract

INTRODUCTION

Supplementation of animal feed with phytases was seen as a strategy to utilize the organically bound phosphate for the growth and development of monogastric animals. In 1991 the first phytase product (*Aspergillus niger*) for feed application appeared on the market. Phytase products from alternative sources such as *Escherichia coli*, *Peniophora lycii*, *Penicillium funiculosum*, *Citrobacter braakii*, *Buttiauxella* sp., and *Hafnia* sp. were launched to the market thereafter.[1] In 2016 the market volume of phytases was reported to be about €350 million and their market volume is expected to reach €520 million in 2021.[2] The phytase market is very competitive and therefore, the search for and the development of phytases better suited for animal feed application is an ongoing process. Direct feeding trials are the only option to fully determine the biological efficacy of phytases. Those feeding trials however, are cost-intensive and time-consuming. As a simple means to investigate enzymatic phytate dephosphorylation under gastrointestinal conditions, *in vitro* poultry digestive tract models were applied.[3–7] The aims of the different studies and the parameters measured were different among those studies.

Morgan *et al.*[4] successfully predicted phytase efficacy in a two-step *in vitro* digestion assay by determining phosphorus solubility. The same group also studied the effect of zinc on the susceptibility of phytate to phytase using an *in vitro* digestion model by monitoring phosphate release and phytate dephosphorylation.[5] Walk *et al.*[6] evaluated the effect of limestone, dicalcium phosphate, and phytase on phosphorus solubility in a two-step *in vitro* digestion assay. An *in vitro* digestion model was also successfully applied to examine the degradation pathway in feed under gastrointestinal conditions.[7] Menezes-Blackburn *et al.*[3] used the phytase activities required to accomplish either a reduction of the phytate content by 50% or a release of 50% of the organically bound phosphate to compare seven commercial phytase products. The values obtained for the seven phytase products included in the aforementioned study might be misinterpreted to be useful in ranking the phytase products in respect to their bioefficacies. The authors however, stated that their approach did not allow the identification of the best phytase product under real conditions, due to the small differences in behaviour of the phytase products in the *in vitro* digestion model compared to the simplification of the digestion process in the model.[3] However, the *in vitro* digestion model might be applicable as a complementary tool in the pre-selection of promising

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phytase candidates, resulting in a reduction in the number of feeding trials in the initial screening phase.

Thus, the follow-up study aims to investigate the limitation of a previously developed *in vitro* model of the poultry digestive tract[3] for evaluating bioefficacy of phytases. To achieve this aim, two further commercial phytases (Optiphos® and Natuphos® E) as well as a plant phytase (rye) and two bacterial phytases (*Bacillus amyloliquefaciens*, *Pantoea agglomerans*) were included in the present study. According to the enzymatic properties available for the two bacterial phytases,[8,9] both are not expected to be good candidates for animal feed applications and a proper *in vitro* digestion model should be capable of confirming this assumption. Furthermore, the effect of more realistic feed substrates for poultry (soybean-maize mix) as well as addition of calcium ion (Ca^{2+}) to the feed substrates on the bioefficacy of the phytases were investigated.

MATERIALS AND METHODS

Materials

Phytic acid dodecasodium salt, pepsin (> 250 U mg^{-1} from porcine gastric mucosa) and pancreatin (4 × USP from porcine pancreas) were purchased from Sigma (Steinheim, Germany). Commercially available phytase products (Quantum® Blue (EC2): AB Vista, Marlborough, UK; Phyzyme®XP (EC3): Danisco, Northampton, UK; Optiphos® (EC4): Huvepharma, Sofia, Bulgaria; Ronozyme® Hiphos (CB) and Ronozyme® NP (PL): Novozymes/DSM, Copenhagen, Denmark; Natuphos® E (NPE): BASF, Mannheim, Germany) and the purified phytases from rye,[10] *Pantoea agglomerans* (PA)[9] and *Bacillus amyloliquefaciens* (BA)[11] were used in the present study. The commercialized phytase products have been produced in genetically modified organisms using the phytase-encoding genes from *Escherichia coli* (Quantum® Blue, Phyzyme®XP, Optiphos®), *Citrobacter braakii* (Ronozyme® Hiphos) and *Peniophora lycii* (Ronozyme® NP). Natuphos® E is an engineered hybrid enzyme from the three donor organisms *Hafnia* sp., *Buttiauxella gaviniae* and *Yersinia mollaretii*, conserving the active site codon from *Hafnia* sp.[12] A phytase activity of 10 U mL^{-1} was adjusted with distilled water for all phytase preparations prior to the determination of their biochemical properties and their use in the digestive tract model.

Determination of phytase activity (standard phytase activity assay)

Briefly, 50 µL of the phytase preparations were added to 350 µL of a 0.1 mol L^{-1} sodium acetate–acetic acid buffer, pH 5.5 containing 2.5 mmol L^{-1} of sodium phytate and pre-heated to 37 °C. Incubation was performed for 30 min at 37 °C. The method described by Heinonen and Lahti[13] was applied to quantify the liberated orthophosphate with minor modifications. In brief, 1.5 mL of a freshly prepared solution consisting of 2 volumes acetone, 1 volume 2.5 mol L^{-1} sulphuric acid (H_2SO_4) and 1 volume 10 mmol L^{-1} ammonium-molybdate (AAM solution) followed by 100 µL 1.0 mol L^{-1} citric acid were added to the mixtures of the phytase activity assays. Any cloudiness was removed by centrifugation prior to measuring absorbance at 355 nm. A calibration curve over the range of 5 to 600 nmol orthophosphate was produced to quantify orthophosphate (ε = 8.7 cm^2 $nmol^{-1}$). Furthermore, 1 µmol phosphate released per minute under the conditions applied was defined as one unit of phytase activity. Blanks were performed by adding the AAM solution prior to the addition of the phytase.

Biochemical characterization of the enzymes

Glycine–hydrochloric acid buffer (pH 2.0–3.5), sodium acetate–acetic acid buffer (pH 4.0–5.5), sodium acetate–hydrochloric acid buffer (pH 6.0–7.0) and Tris-hydrochloric acid buffer (pH 8.0–9.0) were used to investigate the phytase activity at different pH environments.

Kinetic constants of enzymatic phytate dephosphorylation were determined in 350 µL 0.1 mol L^{-1} glycine–hydrochloric acid buffer (pH 3.0) or 0.1 mol L^{-1} sodium acetate–acetic acid buffer (pH 5.0) containing sodium phytate in a serial dilution of a 20 mmol L^{-1} stock solution. The apparent Michaelis–Menten constant (K_m) and catalytic constant (turnover number, k_{cat}) were obtained from Michaelis–Menten plots of the data. The following molecular masses of the non-glycosylated phytases were used for the calculation of the turnover numbers: 40 kDa for the *Baccillus amyloliquefaciens* phytase,[8] 42 kDa for the phytases from *Escherichia coli*[14] and *Pantoea agglomerans*,[9] 45 kDa for the *Peniophora lycii* phytase,[15] 47 kDa for the *Citrobacter braakii* phytase[16] and the Natuphos® E phytase,[17] 67 kDa for the phytase from rye.[10]

Pepsin tolerance of the different phytases (20 mU) was studied in 0.1 mol L^{-1} glycine–hydrochloric acid buffer, pH 3.0 at 37 °C in the presence of 3000 U pepsin. After an incubation for 45 min, residual enzyme activity was determined with the standard phytase activity assay. The same conditions were used to study pH stability of the phytases.

Phytase activity dependency on ionic strength was determined in 50 m mol L^{-1} sodium acetate–acetic acid buffer, pH 5.5. An increase in ionic strength was achieved by adding sodium chloride (NaCl) up to 600 mmol L^{-1}.

In vitro simulation of the poultry digestive tract

The *in vitro* poultry digestive tract model applied was already described by Menezes-Blackburn *et al.*[3] All incubations were performed at 40 °C under continuous shaking using a rotary shaker at 100 rpm to ensure a homogenous distribution of all ingredients. To simulate the crop, 1 g of ground wheat (phytase inactivated) or a ground soybean–maize mix (1:2 *w/w*) was incubated for 30 min with 6 mL of 50 mmol L^{-1} sodium acetate–acetic acid buffer pH 5.0 in the presence of 0, 250, 500, 750 and 1000 U kg^{-1} phytase activity. The effect of Ca^{2+} on phytate dephosphorylation was considered by adding calcium chloride ($CaCl_2$) to the soybean–maize mix to give a final concentration of 5, 10 and 50 g Ca^{2+} kg^{-1} feed substrate. Subsequent simulation of the stomach was achieved by adding 280 µL 1 mol L^{-1} hydrochloric acid and 520 µL of 50 mmol L^{-1} sodium acetate–acetic acid buffer pH 3 containing 21 mg mL^{-1} pepsin to the crop digesta. The mixtures were incubated for 45 min. To simulate the small intestine, 650 µL 1 mol L^{-1} sodium hydrogen carbonate ($NaHCO_3$) and 650 µL of an aqueous pancreatin solution (14.8 mg mL^{-1}) were added to the stomach digesta. The mixtures were incubated for 60 min. Quantification of *myo*-inositol phosphates (IP_6–IP_3) and the liberated orthophosphate were used to study enzymatic *myo*-inositol phosphate dephosphorylation throughout *in vitro* digestion. After freeze drying the samples obtained from *in vitro* digestion, 20 mL of 2.4% hydrochloric acid per gram of the resulting dry material was used for extraction (3 h at 22 °C). Thereafter, centrifugation (30 min, 12 000 × *g*)

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was applied to precipitate the solids. The collected supernatants were used to quantify *myo*-inositol phosphates[18] and the liberated orthophosphate.[13]

Orthophosphate quantification

Briefly, 1.5 mL of a freshly prepared solution consisting of 2 volumes acetone, 1 volume 2.5 mol L^{-1} H_2SO_4 and 1 volume 10 mmol L^{-1} AAM solution followed by 100 µL 1.0 mol L^{-1} citric acid were added to 400 µL of the supernatants of the digesta of the small intestine. Any cloudiness was removed by centrifugation prior to measuring absorbance at 355 nm. A calibration curve over the range of 5 to 600 nmol orthophosphate was produced to quantify orthophosphate ($\varepsilon = 8.7\ cm^2\ nmol^{-1}$). If needed, the supernatants were diluted prior to the addition of the AAM solution to guarantee absorbance values within the calibration range.

myo-Inositol phosphate quantification

Aliquots (2 mL) of the supernatants were diluted 1:30 (*v/v*) with water. Sample preparation was performed by ion-exchange chromatography on AG 1-X4 200-400-mesh (0.5 g) (Bio-Rad, München, Germany). Prior to elution of the *myo*-inositol phosphates with 25 mL 25 mmol L^{-1} hydrochloric acid, the column (0.7 cm × 15 cm) was washed with 25 mL of water and the same volume of 25 mmol L^{-1} hydrochloric acid. A vacuum evaporator was used to concentrate the samples obtained from ion-exchange chromatography. Then, 1 mL of water was used to dissolve the samples. Ion-pair chromatography at 45 °C on Ultrasep ES 100 RP18 (2 mm × 250 mm) (Bischoff, Leonberg, Germany) was applied to separate and quantify the different *myo*-inositol phosphates.[19] An injection volume of 20 µL and a flow rate of 0.2 mL min^{-1} were used. The column was run with a mobile phase consisting of formic acid–methanol–water–tetrabutylammonium hydroxide (44:56:5:1.5 *v/v*), pH 4.25. For calibration, a mixture of the individual *myo*-inositol phosphate esters (IP_3–IP_6) was used.

Statistical methods

The Student's *t* test was used for statistical comparison. A confidence level of 95% was used.

RESULTS AND DISCUSSION

Enzymatic properties of the phytases used in the poultry digestive tract model

According to their enzymatic properties (Table 1), Optiphos® and Natuphos® E are promising candidates for an application as feed additives to make phytate phosphorus available to the animal. Their pH optima and pH profiles suggest a good phytate dephosphorylation in the fore-stomach, proventriculus and gizzard of poultry. Both phytase products showed an acidic pH optimum and exhibited at pH 3.0 (represented the pH conditions in the proventriculus and gizzard of the birds) more than 50% of the enzymatic activity observed at pH 5.5. At pH 7.0 (represented the pH conditions in the small intestine of the birds) both enzyme products displayed less than 5% of their enzymatic activity at pH 5.5. Thus, in an intestinal environment both enzymes are expected to exhibit almost no phytate dephosphorylating activity. Besides the pH activity profile of the enzymes, other enzymatic properties need to be considered in respect to bioefficacy. Among others, bioefficacy is affected by the kinetic constants for phytate dephosphorylation, phytase stability under the pH conditions and electrostatic environments of the different compartments of the gastrointestinal tract of the birds, and the tolerance of the phytase to pepsin digestion.[20]

For Optiphos® and Natuphos® E relatively high turnover numbers (k_{cat}) were observed at the pH value representing the crop (pH 5.0) and at the pH value representing the proventriculus and gizzard of the birds (pH 3.0). Both enzymes exhibited a lower affinity towards and acted slower on phytate at pH 3.0 compared to pH 5.0. Optiphos® and Natuphos® E showed a high pH stability under acidic conditions. More than 90% of their original activity remained after exposure at pH 3.0 for 45 min at 37 °C. Both enzymes were also found to be pepsin tolerant. Their residual enzymatic activities were found to be higher than 90%. An increase in ionic strength by adding NaCl up to 200 mmol L^{-1} was shown to have no effect on the phytate dephosphorylating activity of Optiphos® and Natuphos® E. The presence of NaCl in concentrations of 600 m mol L^{-1}, however, had a strong inhibitory effect on the phytase products. Thus, the biochemical properties of Optiphos® and Natuphos® E were found to be very comparable to those obtained for the five commercially available bacterial phytase products characterized by Menezes-Blackburn *et al.*[3] using identical assay conditions.

Table 1. Enzymatic properties of the phosphatases used in the *in vitro* digestion model

	NPE	EC4	Rye	BA	PA
pH range (80% of the optimal activity)	4.0–5.5	3.5–5.5	5.5–6.8	6.0–7.5	3.5–5.5
Phytase activity at pH 3.0 (%)[a]	79.3	55.8	0.4	0.2	72.3
Phytase activity at pH 7.0 (%)[a]	2.9	3.5	48.3	92.4	4.1
K_M (µmol L^{-1}) for phytate at pH 5.0 and 37 °C	165	197	342	622	351
k_{cat} (s^{-1}) for phytate at pH 5.0 and 37 °C	1121	1365	304	12	20
K_M (µmol L^{-1}) at pH 3.0 and 37 °C	193	231	n.d.	n.d.	378
k_{cat} (s^{-1}) at pH 3.0 and 37 °C	754	998	n.d.	n.d.	18
Residual activity (%) (pH 3.0, 37 °C, 45 min)	94	99	36	76	98
Without pepsin	92	97	28	51	93
With 3000 U pepsin optimal ionic strength (mmol L^{-1} NaCl)	50–200	50–200	50–200	50–200	50–200

BA, *Bacillus amyloliquefaciens* phytase; EC4, Optiphos®; NPE, Natuphos® E; PA, *Pantoea agglomerans* phytase; K_m, apparent Michaelis–Menten constant; k_{cat}, catalytic constant (turnover number).
[a] Phytase activity at pH 5.5 was taken as 100%.

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The characteristics of the phytases from rye, *Pantoea agglomerans* and *Bacillus amyloliquefaciens* obtained in the present study are in good agreement to the enzymatic properties already published for these enzymes. [8–10,20] According to their enzymatic properties, the phytase from rye and especially the phytases from *Pantoea agglomerans* and *Bacillus amyloliquefaciens* seem to be less suited for an application as feed additives compared to the commercially available bacterial and fungal phytase products included in the present and the previous study.[3] In respect to the pH profile, only the *Pantoea agglomerans* phytase was found to be comparable to the characterized commercialized phytase products (Table 1). The rye and *Bacillus amyloliquefaciens* phytases, however, showed almost no phytate dephosphorylating activity at pH 3.0, but a reasonable high enzymatic activity at pH 7.0. Thus, both phytases were expected to dephosphorylate phytate in the small intestine rather than in the fore-stomach, proventriculus and gizzard of the birds.

The turnover number for phytate dephosphorylation (k_{cat}) of the enzymes from rye, *Bacillus amyloliquefaciens* and *Pantoea agglomerans* at pH 3.0 and pH 5.0 were significantly lower than those obtained for the commercialized phytase products. Due to the low enzymatic activity of the rye and *Bacillus amyloliquefaciens* phytases at pH 3.0, turnover numbers could not be determined. In respect to pH stability at pH 3.0 and pepsin tolerance, the phytase from *Pantoea agglomerans* was found to be comparable to the commercially available phytase products included in the present and the previous study.[3] The phytase from *Bacillus amyloliquefaciens* showed an intermediate pH stability at pH 3.0 and an intermediate pepsin tolerance, whereas the phytase from rye exhibited both a low pH stability at pH 3.0 and a low pepsin tolerance. The dependence of enzymatic activity from ionic strength was comparable to that obtained for the commercialized phytase products for all three phytases. Besides significant differences in the pH profile, the rye phytase is therefore comparable in its enzymatic properties to the phytases of fungal origin included in the previous study.[3]

Performance of the phytases in the poultry digestive tract model

The *in vitro* approach already described by Menezes-Blackburn *et al.*[3] was used to investigate the effect of two commercial phytase products (Optiphos® and Natuphos® E) and three purified phytases of different origin (rye, *Bacillus amyloliquefaciens* and *Pantoea agglomerans*) on phytate dephosphorylation. Even if the viscosity of the systems when using the lower buffer volume (3 mL per gram feed substrate) was reported to be closer to the viscosity in the digestive tract of the birds,[3] only the higher buffer volume (6 mL per gram of feed substrate) was used in the present study to avoid the difficulties to achieve a homogeneous distribution especially of the phytase activity throughout the entire crop content.

In order to accomplish a 50% reduction in phytate, between 128 U (Optiphos®) and 4637 U (*Bacillus amyloliquefaciens*) of phytase activity per kilogram of ground wheat was required (Table 2). The phytase activities required to accomplish a release of 50% of the organically bound phosphate were between 688 U (Optiphos®) and 35 714 U (*Pantoea agglomerans*). As expected from their biochemical properties (Table 1), a very similar behaviour of Optiphos® and Natuphos® E in the digestive tract model was obtained as observed for the commercially available bacterial phytase products included in the previous study.[3] Thus, both phytases were identified as promising candidates for an animal feed application by the *in vitro* digestion model applied.

The behaviour of the rye phytase in the *in vitro* digestion model was found to be comparable to the fungal phytases included in the previous study,[3] as already concluded from its biochemical properties (Table 1). To obtain information on the capability of the *in vitro* model to identify phytase candidates less or not suited for animal feed applications, two phytases (*Pantoea agglomerans* and *Bacillus amyloliquefaciens*) with less favourable enzymatic properties for phytate dephosphorylation in the stomach of the birds were included in this follow-up study (Table 1).

In order to accomplish a 50% reduction in phytate, a 6- to 23-times higher phytase activity (*Pantoea agglomerans*) and a 9- to 36-times higher phytase activity (*Bacillus amyloliquefaciens*) were required (Table 2) compared to the commercialized phytase products studied. The corresponding phytase activities to accomplish a release of 50% of the organically bound phosphate were 5- to 15-times (*Pantoea agglomerans*) and 20- to 65-times (*Bacillus amyloliquefaciens*) higher than those obtained for the commercialized phytase products included in the studies. Thus, the *in vitro* approach was shown to be capable of identifying phytases less suited for animal feed applications and could therefore be used as a complementary tool in the pre-selection of promising phytase candidates.

The feed substrate used in the *in vitro* digestion model might also have an effect on the efficacy of the different phytases to dephosphorylate phytate. This has already been shown comparing soybean and rapeseed meal[4] and soybean and corn,[6] respectively, as feed substrates in *in vitro* models. The amount of phytate present in the feed substrates used was assumed to be responsible for the observed differences in phytase efficacy.[1] In the present study, all phytases showed a better efficacy with ground wheat as a substrate (Table 2) in the digestive tract model than with the ground soybean–maize mix (Table 3). The phytate contents of the two feed substrates (ground wheat: 7.92 $\pm$ 0.46 g kg^{-1}, ground soybean-maize mix: 8.35 $\pm$ 0.53 g kg^{-1}) however, were not significantly different ($P < 0.05$). Both feed substrates exhibited significant differences in their intrinsic

Table 2. Enzyme activity needed to achieve a 50% reduction in myo-inositolhexakisphosphate, phytate (IP_6) and an increase in orthophosphate, inorganic phosphate (P_i) of 50% of the maximum reachable value (ground wheat)

	NPE	EC4	Rye	BA	PA
50% IP_6	147 (0.93)	128 (0.89)	516 (0.88)	4637 (0.86)	2959 (0.94)
50% P_i	712 (0.87)	688 (0.82)	2653 (0.86)	9471 (0.83)	35 714 (0.87)

Values (U kg^{-1}) obtained by non-linear fit of the observed data; coefficient of determination in parentheses.
BA, *Bacillus amyloliquefaciens* phytase; EC4, Optiphos®; NPE, Natuphos® E; PA, *Pantoea agglomerans* phytase.

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Table 3. Enzyme activity needed to achieve a 50% reduction in IP_6 and an increase in P_i of 50% of the maximum reachable value (ground soybean–maize mix (1:2 *w/w*)

	Ca^{2+}-level	NPE	EC4	CB	PL	EC2	EC3	Rye
50% IP_6	0 g kg^{-1}	252 (0.94)[a]	196 (0.92)[a]	376 (0.92)[a]	595 (0.94)[a]	179 (0.92)[a]	225 (0.94)[a]	534 (0.91)[a]
50% P_i	5 g kg^{-1}	261 (0.89)[a]	192 (0.94)[a]	395 (0.91)[a]	602 (0.91)[a]	180 (0.90)[a]	248 (0.91)[a]	512 (0.86)[a]
	10 g kg^{-1}	258 (0.87)[a]	213 (0.91)[a]	382 (0.94)[a]	619 (0.87)[a]	185 (0.91)[a]	250 (0.87)[a]	547 (0.92)[a]
	50 g kg^{-1}	512 (0.87)[b]	432 (0.89)[b]	565 (0.90)[b]	682 (0.91)[b]	272 (0.88)[b]	346 (0.90)[b]	723 (0.88)[b]
	0 g kg^{-1}	698 (0.89)[a]	789 (0.83)[a]	911 (0.93)[a]	1958 (0.87)[a]	702 (0.88)[a]	827 (0.85)[a]	3017 (0.84)[a]
	5 g kg^{-1}	677 (0.85)[a]	802 (0.85)[a]	883 (0.87)[a]	1904 (0.81)[a]	723 (0.81)[a]	840 (0.87)[a]	3119 (0.86)[a]
	10 g kg^{-1}	704 (0.87)[a]	823 (0.84)[a]	926 (0.88)[a]	1943 (0.84)[a]	739 (0.82)[a]	839 (0.82)[a]	3373 (0.89)[a]
	50 g kg^{-1}	1163 (0.86)[b]	1325 (0.82)[b]	1377 (0.83)[b]	2435 (0.87)[a]	1203 (0.80)[b]	1361 (0.81)[b]	5231 (0.88)[b]

Values (U kg^{-1}) obtained by non-linear fit of the observed data; coefficient of determination in parentheses. Different letters in a column indicate a significant difference of data in a column at $P < 0.05$.
CB, Ronozyme® Hiphos; EC2, Quantum® Blue; EC3, Phyzyme®XP; EC4, Optiphos®; NPE, Natuphos® E; PL, Ronozyme® NP.

phytase activities (ground wheat:[3] 27.6 ± 2.1 U kg^{-1}, ground soybean–maize mix: (73.2 ± 2.7 U kg^{-1}). In both cases, the intrinsic phytase activity is low compared to the exogeneous phytase activity added and therefore it may only add significantly to the enzymatic phytate dephosphorylation during *in vitro* digestion in the presence of no or low (250 U kg^{-1} feed substrate) exogeneous phytase activity. Thus, differences in the accessibility of the phytate to the phytases might explain the observed results. In order to be dephosphorylated by the phytase added to the *in vitro* digestion model, phytate must be released from the plant matrix into the surrounding medium. It is well known, that the distribution of phytate within the plant matrix differs among grains[21] and therefore, its release might differ among feed substrates. Furthermore, the microwave treatment applied to inactivate the intrinsic wheat phytase[9] might facilitate the release of phytate. More feed substrates need to be studied in the *in vitro* digestion model in order to evaluate its suitability to draw conclusion on the effect of phytate concentration or phytate source on phytase efficacy.

To check if the *in vitro* approach allows to draw conclusions about the effect of feed constituents on the performance of phytases, Ca^{2+} was chosen as the model compound. In order to support the complex process of eggshell formation, commercial laying hens require about 4 to 5 g of Ca^{2+} per day.[22] Diets high in Ca^{2+} have been linked to poor growth performance in broilers,[23] inefficient utilization of phosphorus or calcium,[24] and a reduction in phytate dephosphorylation in the digestive tract of poultry. For example, addition of 1.2 to 5.2 g kg^{-1} diet reduced phytate dephosphorylation from 55.0% to 5.6%.[25] Tamim and Angel[24] reported a reduction in phytate dephosphorylation from 68.6 to 21.3% after adding Ca^{2+} in a concentration of 5 g kg^{-1} poultry diet. Therefore, 5, 10 and 50 g Ca^{2+} (as $CaCl_2$) were added per kilogram of soybean–maize mix to study the effect of Ca^{2+} addition on phytase performance in the poultry digestive tract model (Table 3). Up to a concentration of 10 g kg^{-1} no significant effect on phytate dephosphorylation respectively dephosphorylation of the partially phosphorylated *myo*-inositol phosphates was observed for all phytases included in the present study. Only, the addition of 50 g Ca^{2+} per kilogram of feed substrate resulted in a significant reduction in phytate dephosphorylation respectively phosphate release. The differences between the *in vivo* and *in vitro* results might be due to a better accessibility of phytate to the exogeneous phytase added *in vitro*. In order to be enzymatically dephosphorylated, phytate needs to be released from the feed matrix into the surrounding medium and stay in solution. It can be assumed that grinding of the feed substrates facilitates such a release. The phytate concentration in the liquid phase of the digestion mixture might therefore be higher in the *in vitro* compared to the *in vivo* situation. Thus, higher Ca^{2+} concentrations are needed to precipitate phytate and to reduce enzymatic phytate dephosphorylation.

CONCLUSION

The *in vitro* approach used in the present study was shown to be capable of identifying promising phytase candidates and distinguishing them from phytates less suited for animal feed applications and could therefore be used as a complementary tool in the pre-selection of promising phytase candidates. The *in vitro* system of the poultry digestive tract, however, does not mimic the *in vivo* situation in the birds sufficiently well to be used for questions such as which phytase performs best under real conditions, the effect of phytate concentration, phytate source or feed composition on phytase efficacy.

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