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Performance of Seven Commercial Phytases in an in Vitro Simulation of Poultry Digestive Tract

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ABSTRACT: The aim of this study was to compare the biochemical properties of seven commercially available phytase products as well as their catalytic performance in an in vitro simulation of the digestive tract of poultry. Their enzymatic properties relevant with respect to phytate dephosphorylation in the digestive tract of birds were determined under identical assay conditions. All phytase products included in the study showed an acid pH optimum of activity and were capable of releasing the organically bound phosphate from phytate during the in vitro simulation. However, their overall biochemical properties and relative catalytic performances were remarkably different. The in vitro degradation system was considered as a simple and useful tool to evaluate the suitability of a phytase to be used as a feed supplement. Although relevant factors such as dietary P levels, intestinal phytase, and P absorption are not implemented in the system, this approach might help to reduce the number of feeding trials necessary in the search for a better suited phytase for animal feeding application.

KEYWORDS: *in vitro degradation system, phytase, phytate dephosphorylation*

INTRODUCTION

Phosphorus (P) is an essential nutrient for animals that strongly affects bone development and metabolic processes.[1] To ensure optimal growth of the animals, feeds are in general supplemented with calcium phosphate.[2] The rock phosphate used to produce the phosphate supplements are a non-renewable, limited natural resource, and recently, it has been speculated that this resource may become scarce in the near future.[3,4] P content of grain-based feeds is in general sufficient to support adequate animal growth. However, it is present predominantly as phytate[2] and thus only available to birds in low-P diets.[5,6]

Today, the use of phytases as a supplement for feeds for monogastric animals is a well-studied and widely applied technology.[2,7,8] Due to the enzymatic release of the organically bound phosphate, supplementation of feeds with calcium phosphate could be significantly reduced, resulting in a reduced total phosphorus excretion (organic and inorganic) and environmental dispersion.[2,7] Although poultry and swine feed sectors are the main phytase consumers, other potential application fields of phytases have been proposed in feed supplements for aquaculture, human nutrition and health, and plant nutrition.[2,8]

In the application of phytases as feed supplements, their functionality in animal feeds and digestive systems is of utmost importance. Because the phytases used for animal feed application differ in their enzymatic properties[7,8] such as pH profile, stability under digestive tract conditions, temperature stability, kinetic constants, and substrate specificity, their biochemical characterization is essential. However, it is virtually impossible to compare the different commercialized phytase products by the properties reported by the manufacturers, because of the different assay conditions used to determine the enzymatic properties. Therefore, the enzymatic properties of seven commercially available phytase products were determined under identical assay conditions. To our knowledge this is the first time that comparable data on the most important commercialized phytases have been reported.

Nevertheless, enzymatic properties could only be guidelines on potential functionality of phytases in animal feeds and digestive systems. There is no unique property responsible for a better performance in vivo, but a conjunction of all properties. Therefore, biological efficacy can be fully determined only by direct feeding trials. Those trials are long-lasting and cost-intensive. Therefore, an attempt was made to establish an in vitro degradation model to obtain information on the biological efficacy of phytases. This approach is aimed at ranking phytases with respect to their capability to release the organically bound phosphate from the *myo*-inositol ring and is considered as a possibility to reduce the number of feeding trials necessary in the search for a better suited phytase for animal feeding application.

MATERIALS AND METHODS

Materials. Phytase products used in this study were Quantum and Quantum Blue (AB Vista, Marlborough, Wiltshire, UK), Ronozyme NP and Ronozyme Hiphos (Novozymes/DSM, Copenhagen, Denmark), PhyzymeXP and AxtraPHY (Danisco, Northamptonshire, UK), and Natuphos (BASF, Manheim, Germany). General information about these phytase products are listed in Table 1. The liquid phytase preparations were diluted with distilled water to give a final activity of 10 U mL^{-1} prior to characterization and use in the in vitro degradation model. The solid phytase preparation was extracted with distilled water (1:10 w/v). The water extract was concentrated by acetone precipitation (0−55%). The precipitate was collected by centrifugation (10000 rpm, 10 min), resuspended, and diluted with distilled water to

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Table 1. General Information about the Phytase Products Used in the in Vitro Digestion Model

	name in this work						
	EC1	EC2	EC3	BSP	CB	PL	AN
trademark	Quantum	Quantum Blue	PhyzymeXP	AxtraPHY	Ronozyme Hiphos	Ronozyme NP	Natuphos
supplier	AB Vista	AB Vista	Danisco	Danisco	Novozymes/ DSM	Novozymes/ DSM	BASF
donor organism	*Escherichia coli*	*Escherichia coli*	*Escherichia coli*	*Buttiauxella* sp.	*Citrobacter braakii*	*Peniophora lycii*	*Aspergillus niger*
production organism	*Trichoderma reesei*	*Trichoderma reesei*	*Schizosaccharomyces pombe*	*Schizosaccharomyces pombe*	*Aspergillus oryzae*	*Aspergilus oryzae*	*Aspergillus niger*
formulation	liquid	liquid	liquid	liquid	liquid	liquid	solid

a final concentration of 10 U mL^{-1}. Phytic acid dodecasodium salt, pancreatin from porcine pancreas (4 × USP), and pepsin from porcine gastric mucosa (>250 U mg^{-1}) were obtained from Sigma (Steinheim, Germany). Ultrasep ES 100 RP18 was purchased from Bischoff (Leonberg, Germany) and AG1 X-4, 100−200 mesh, resin from Bio-Rad (München, Germany). All reagents were of analytical grade.

Standard Phytase Activity Assay. The phytase activity assay was performed according to the method of Greiner et al.[9] Briefly, 10 μL of an appropriately diluted phytase preparation was incubated with 350 μL of phytate solution (2.5 mM of sodium phytate in 100 mM sodium acetate−acetic acid buffer, pH 5.5) at 37 °C. After an incubation time of 30 min, the liberated inorganic phosphate was measured by using a modification of the ammonium molybdate method.[10] One unit of phytase activity was defined as 1 μmol of P released in 1 min at test conditions. Blanks were performed by adding stop solution prior to enzyme addition.

Biochemical Characterization of the Phytases. To study the pH dependency of the phytase activity, the following buffers were used: pH 2.0−3.5, glycine−HCl; pH 4.0−5.5, sodium acetate−acetic acid; pH 6.0−7.0, sodium acetate−HCl; and pH 8.0−9.0, Tris−HCl.

To determine kinetic constants, the incubation mixture consisted of 350 μL of incubation buffer (100 mM glycine−HCl, pH 3.0, or 100 mM sodium acetate−acetic acid, pH 5.0) containing sodium phytate in a serial dilution of a concentrated stock solution (20 mM). Kinetic constants (K_M, k_{cat}) were calculated from Michaelis−Menten plots of the data. For the calculation of k_{cat}, the molecular masses of the nonglycosylated phytases were used: 42 kDa for the phytase from *Escherichia coli*,[9] 45 kDa for the phytases from *Peniophora lycii*[11] and *Buttiauxella*,[12] 47 kDa for the *Citrobacter braakii* phytase,[13] and 49 kDa for the *Aspergillus niger* phytase.[14]

Phytase inactivation by pepsin was determined by incubating 20 mU of the different phytase preparations in 100 mM glycine−HCl, pH 3.0, containing 3000 U of pepsin for 45 min at 37 °C. As a control the pH stability of the different phytases was determined under the same conditions. After incubation, residual phytase activity was determined using the standard phytase assay.

To study the dependency of phytase activity by ionic strength, 50 mM sodium acetate−acetic acid buffer (pH 4−5.5) was used and ionic strength was increased by adding NaCl to 600 mM.

Simulation of Poultry Digestive Tract. A simple in vitro degradation model was used to simulate the digestive tract of poultry, respectively, the crop, stomach (proventriculus and gizzard), and small intestine.[15] Because poultry diets are usually corn−soybean-based, ground wheat was used as a model feed in this study. The intrinsic wheat phytase was inactivated by microwave treatment of the ground wheat. One gram of ground wheat was incubated with 3 or 6 mL of 50 mM sodium acetate−acetic acid buffer, pH 5.0, buffer containing different amounts of phytase activity (0, 250, 500, 750, and 1000 U kg^{-1}) at 40 °C for 30 min. Subsequent stomach simulations were performed by adding 280 μL of 1 M HCl and 520 μL of 50 mM sodium acetate−acetic acid buffer, pH 3, containing 21 mg mL^{-1} pepsin to the crop digesta. The entire mixture was incubated at 40 °C for 45 min under continuous shaking at 100 rpm on a rotary shaker to guarantee a homogeneous distribution of all ingredients. Simulations of the small intestine were performed by adding 650 μL of 1 M $NaHCO_3$ and 650 μL of 14.8 mg mL^{-1} aqueous pancreatin solution to the stomach digesta. The entire mixture was incubated at 40 °C for 60 min. Inorganic P release and *myo*-inositol phosphate (IP_6−IP_3) quantification were used to follow phytate dephosphorylation throughout the simulated digestion. The samples obtained from the simulated digestion studies were freeze-dried overnight, and 1 g of the freeze-dried samples was extracted with 20 mL of 2.4% HCl for 3 h at room temperature. The resulting suspensions were centrifuged (30 min, 10000 rpm). The supernatants were collected and used for inorganic phosphate determination[10] and *myo*-inositol phosphate quantification.[16]

Quantification of the Liberated Inorganic Phosphate. The liberated phosphate was quantified by using a modification of the ammonium molybdate method.[10] One and a half milliliters of a freshly prepared solution of acetone/2.5 M sulfuric acid/10 mM ammonium molybdate (2:1:1 v/v) and thereafter 100 μL of 1.0 M citric acid were added to 400 μL of the suitably diluted supernatants or to the mixtures of the phytase activity assays. Any cloudiness was removed by centrifugation prior to the measurement of absorbance at 355 nm. To quantify the released phosphate, a calibration curve was produced over the range of 5−600 nmol of phosphate.

Quantification of *myo*-Inositol Phosphates. Two milliliter aliquots of the supernatants were diluted with water to give a final volume of 60 mL. The entire solution was applied to a column (0.7 × 15 cm) containing 0.5 g of AG 1-X4 200−400-mesh resin. The column was washed with 25 mL of water and 25 mL of 25 mM HCl. Then *myo*-inositol phosphates were eluted with 25 mL of 2 M HCl. The eluates obtained were concentrated in a vacuum evaporator and dissolved in 1 mL of water. Then 20 μL of the samples was chromatographed on Ultrasep ES 100 RP18 (2 × 250 mm). The column was run at 45 °C and 0.2 mL min^{-1} of an eluant consisting of formic acid/methanol/water/tetrabutylammonium hydroxide (44:56:5:1.5 v/v), pH 4.25, as described by Sandberg and Ahderinne.[17] A mixture of the individual *myo*-inositol phosphate esters (IP_3−IP_6) was used as a standard.

RESULTS AND DISCUSSION

Biochemical Properties of the Phytases Used in the in Vitro Degradation Model. Some enzymatic properties of seven commercially available phytase products were determined under identical assay conditions. All phytases have been produced in genetically modified microorganisms. Therefore, it is assumed that the major constituent of the phytase products analyzed is the respective phytase, and the enzymatic properties determined reflect those of the enzyme in question. Phytases from five different donor organisms have been used in this study (Table 1). Two are of fungal (*A. niger*, *P. lycii*) and three of bacterial origin (*E. coli*, *C. braakii*, *Buttiauxella* sp.). On the basis of the carbon in the *myo*-inositol ring of phytate at which hydrolysis is initiated, the commercial phytase products consist either of a 3-phytase (*A. niger*) or a 6-phytase (*E. coli*, *P. lycii*, *C. braakii*, *Buttiauxella* sp.). Furthermore, variants of the above-mentioned phytases with significant improvements over the

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Table 2. Enzymatic Properties of the Phytase Products Used in the in Vitro Digestion Model

	EC1	EC2	EC3	BSP	CB	PL	AN
pH range (80% of the optimal activity)	4.0−5.0	3.5−5.0	3.0−5.0	3.0	3.0−4.5	4.5−5.5	4.5−5.5
phytase activity at pH 3.0[a] (%)	92.5	101.3	82.8	235.1	145.7	12.5	64.2
phytase activity at pH 7.0[a] (%)	0.8	2.2	1.7	0.5	0.6	7.8	7.0
K_M (μM) for phytate at pH 5.0 and 37 °C	228	142	285	272	364	75	35
k_{cat} (s^{-1}) for phytate at pH 5.0 and 37 °C	1545	1821	1327	1054	1478	1532	318
K_M (μM) at pH 3.0 and 37 °C	257	178	302	311	427	98	142
k_{cat} (s^{-1}) at pH 3.0 and 37 °C	1012	1274	984	768	1061	824	170
residual activity (%) (pH 3.0, 37 °C, 45 min)							
without pepsin	95	98	92	87	93	58	81
with 3000 U pepsin	93	98	92	85	92	34	47
optimal ionic strength (mM NaCl)	50−100	50−200	100−200	50−200	50−200	50−200	50−600

[a]Phytase activity at pH 5.5 was taken as 100%.

parent enzymes through selective molecular modifications have found their way to the marketplace.[2] In addition, the expression system used for phytase production has an effect on the enzymatic character of the phytase. Last but not least, postfermentation activities such as product formulation and optimization can influence the properties of the final phytase preparation.[2] Therefore, phytases of the same origin might exhibit differences in their enzymatic properties as also shown for the *E. coli* phytases in this study (Table 2; Figure 1).

With respect to phytate dephosphorylation in the gastrointestinal tract of poultry, the low pH (pH 4−5) in the forestomach (crop) as well as in the proventriculus and gizzard (pH 2−5) has to be considered.[2,15] The small intestine of poultry, however, presents a neutral pH environment (pH 6.5−7.5).[15] Thus, pH optima and pH activity profile of supplementary phytases generally determine their ability to develop catalytic activity in the aforementioned gastrointestinal compartments.[18] All phytase preparations studied showed an acid pH optimum for phytate dephosphorylation and were shown to be virtually inactive above pH 7.0 (Figure 1A,B; Table 2). Thus, the phytase preparations included in the study are expected to act most efficiently under the conditions present in the forestomach, proventriculus, and gizzard of poultry. Under intestinal pH conditions the phytases exhibit almost no phytate-degrading capability. However, considerable differences in their pH activity profiles have been observed. Even if the site of phytase action in the gastrointestinal tract of poultry has received little attention so far, the crop was reported to be very likely the primary site of phytate dephosphorylation by supplementary phytase.[19] With regard to the bioefficacy of supplementary phytase, it has also to be considered that phytases are in general supplemented according to their activity determined at standard conditions (pH 5.5, 37 °C, 5 mmol/L sodium phytate).[20] Therefore, their phytate-degrading activity will differ at other pH conditions. In Table 2, the relative phytate-degrading activities of the phytase preparations included in the studies at pH 3.0 (represents the pH conditions in the proventriculus and gizzard of the birds) and pH 7.0 (represents the pH conditions in the small intestine of the birds) are displayed. Compared to their phytate-degrading activities at pH 5.5, some of the phytase preparation studied exhibited a similar (EC1, EC2, EC3), a significantly higher (BSP, CB), or a significantly lower (PL, AN) phytate-degrading capability at pH 3.0. The lowest relative activity was determined for PL (12.5%) and the highest for BSP (235.1%). At pH 7.0 all phytase preparations included in the study showed a very low phytate-degrading capability compared to that at pH 5.5. The phytate-degrading activity at pH 7.0 of all enzymes has been found to be below 8% compared to the activity at pH 5.5.

Furthermore, it must be considered that bioefficacy is not only determined by the pH activity profile of the phytase but, among others, by its kinetic constants for phytate hydrolysis, its stability under the pH conditions of the crop or stomach, and its susceptibility to pepsin degradation and the electrostatic environment in the crop or stomach.[18] Even if so far not proven by animal studies, a high affinity for phytate and *myo*-inositol pentakisphosphate, high turnover numbers with both compounds, and narrow substrate specificity are concluded to be desirable properties for phytases used as feed additives.[18] In Table 2, the kinetic constants (K_M, k_{cat}) of the phytase preparations included in the studies for phytate dephosphorylation at pH 5.0 (represents the pH conditions in the crop of the birds) and pH 3.0 (represents the pH conditions in the proventriculus and gizzard of the birds) are given. For all phytase products the turnover numbers (k_{cat}) were lower and the Michaelis−Menten constants (K_M) higher at pH 3.0 than at pH 5.0. Thus, all phytases acted more rapidly on and exhibited a higher affinity toward phytate at pH 5.0 compared to pH 3.0. Relatively low K_M values have been found for AN and PL at pH 5.0 and for PL at pH 3.0. However, AN also exhibited relatively low turnover number at both pH values. A higher affinity toward sodium phytate of *A. niger*[14] and *P. lycii*[11,14] phytases compared to the phytases from *E. coli*,[9] *C. braakii*,[13] and *Buttiauxella*[12] has already been reported.

With the exception of PL all phytase products included in the study showed a remarkable stability at pH values representing the conditions of the proventriculus and gizzard of the birds (Table 2). PL exhibited 58% residual phytase activity after exposure at pH 3.0 and 37 °C for 45 min, whereas with all other phytase products residual activities >80% have been observed. These data are in good agreement with already published data on the pH stability of the phytases studied.[9,12,13,21] The effectiveness and limitations of feed supplementation with phytases may also depend on their susceptibility to proteolytic cleavage. By incubating the phytase products with pepsin at pH 3.0, differences in their ability to withstand degradation by this digestive protease were observed (Table 2). The bacterial phytases seem to be pepsin tolerant (residual activity >85%), whereas the fungal phytases retained only 34% (PL) and 47% (AN) of their initial activity after pepsin digestion. These data are in good agreement with already published data on the susceptibility of phytases to proteolytic cleavage by pepsin.[13,22−25]

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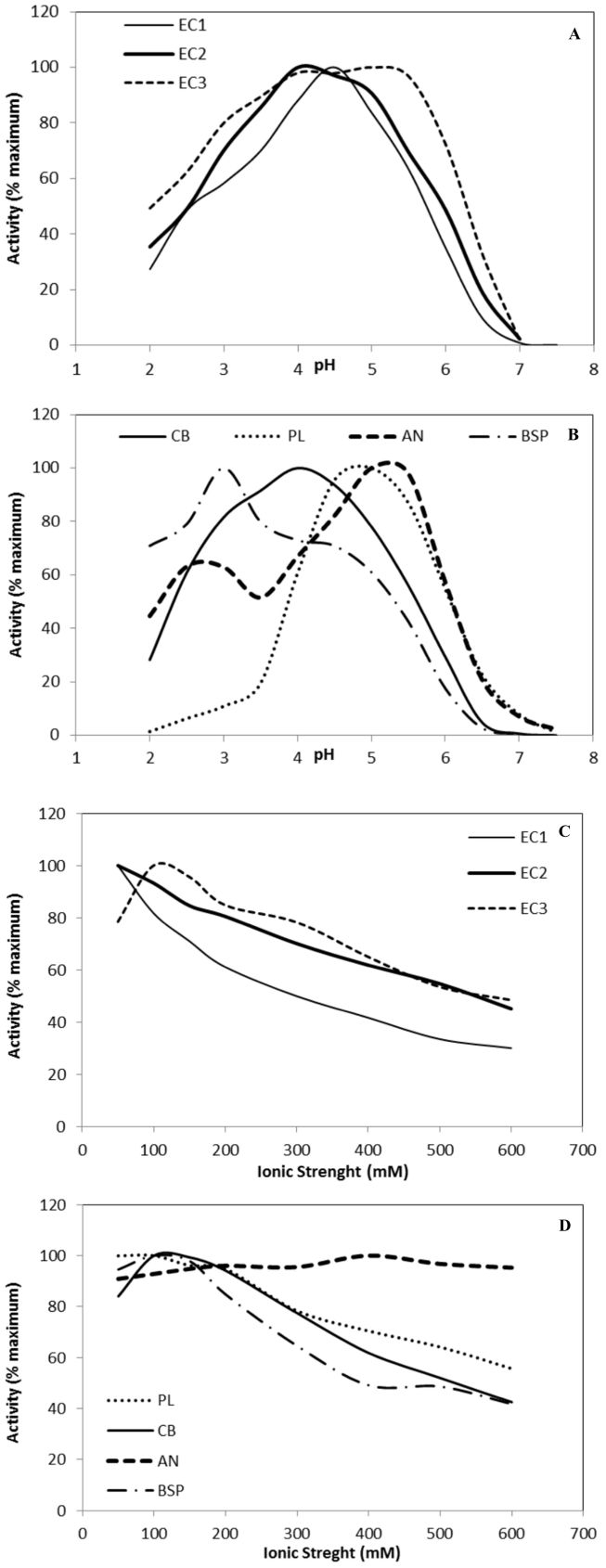

Figure 1. Effect of pH (A, B) and ionic strength (C, D) on phytase activity of the phytase products used in the in vitro degradation model.

In addition, it was already shown that the electrostatic environment could have an effect on phytase activity. The pH profiles of the phytases from *A. niger* and *E. coli*, for example, have been modified by both the buffer and the introduction of salt (NaCl, $CaCl_2$).[26] Addition of NaCl up to 600 mM to increase the ionic strength of the phytase environment resulted in a strong inhibition of all phytase products with the exception of AN (Figure 1C,D).

No clear conclusion about the bioefficacy of the phytase products could be drawn from the enzymatic properties determined. Some enzymatic properties would point to a certain phytase product as better suited for application as an animal feed supplement; some other enzymatic properties would give preference to a different phytase product (Table 2). Furthermore, there is no evidence that a single property is more important with respect to bioefficacy. A better performance in vivo is a conjunction of all enzymatic properties as well as the interaction with phosphatase activity occurring in the gastrointestinal tract.[18] Thus, enzymatic properties could only be guidelines on potential functionality of phytases in animal feeds and digestive systems.

Phytase Performance in an in Vitro Simulation of Poultry Digestive Tract. The effect of seven commercial phytase products on phytate dephosphorylation was studied in an in vitro simulation of the digestive tract of poultry based on that of Zyla and co-workers.[15] The simulation included some key conditions of the crop, stomach (proventriculus and gizzard), and small intestine of the birds. In general, supplementation of animal feed with 250−1000 U kg^{-1} is recommended by the phytase suppliers.[27−29] Therefore, 250, 500, 750, and 1000 U kg^{-1} were used in this simulation study. Whole wheat flour (ground wheat grains) was used as a feed matrix. The intrinsic wheat phytase activity was reduced to 27.6 ± 2.1 U kg^{-1} dry matter by microwave treatment. Thus, the residual activity of the intrinsic wheat phytase corresponds to 11% of the phytase activity added to the simulation at the lowest dose. A complete inactivation of the intrinsic wheat phytase could not be achieved by microwave treatment, and structural changes of the wheat by microwave treatment could not be avoided. To simulate the crop two different volumes of buffer were used. The lower volume (3 mL per gram of wheat flour) represented much better the conditions in the digestive track of the birds with respect to viscosity. However, it was extremely difficult to homogeneously distribute the phytase added throughout the entire crop content by mixing, because the mixture had a pasty consistency. To overcome this problem, 6 mL of buffer per gram of microwave-treated wheat flour was used in a second setup. Phytate dephosphorylation was followed by quantification of phytate (IP_6) and partly phosphorylated *myo*-inositol phosphates (IP_5−IP_3) as well as the inorganic phosphate (P_i) released. The variation of the analytical methods applied was determined using 10 replicates of the artificial crop, stomach, and intestine digesta without phytase addition. The standard deviation of P_i, IP_6, and IP_{total} (IP_6 + IP_5 + IP_4 + IP_3) represented 5−15% of the average (coefficient of variation; CV), whereas the standard deviation of the partly phosphorylated *myo*-inositol phosphates represented 30−100% of average. The high CVs for IP_3, IP_4, and IP_5 can be explained by the fact that these are transient forms in the phytate degradation pathway and occur in a significantly lower concentration compared to IP_6 and P_i. Conclusions drawn from the simulated digestion studies were therefore based only on IP_6, IP_{total}, and P_i.

The following trends in the simulated gastrointestinal degradation studies have been observed: IP_6 as well as IP_{total}

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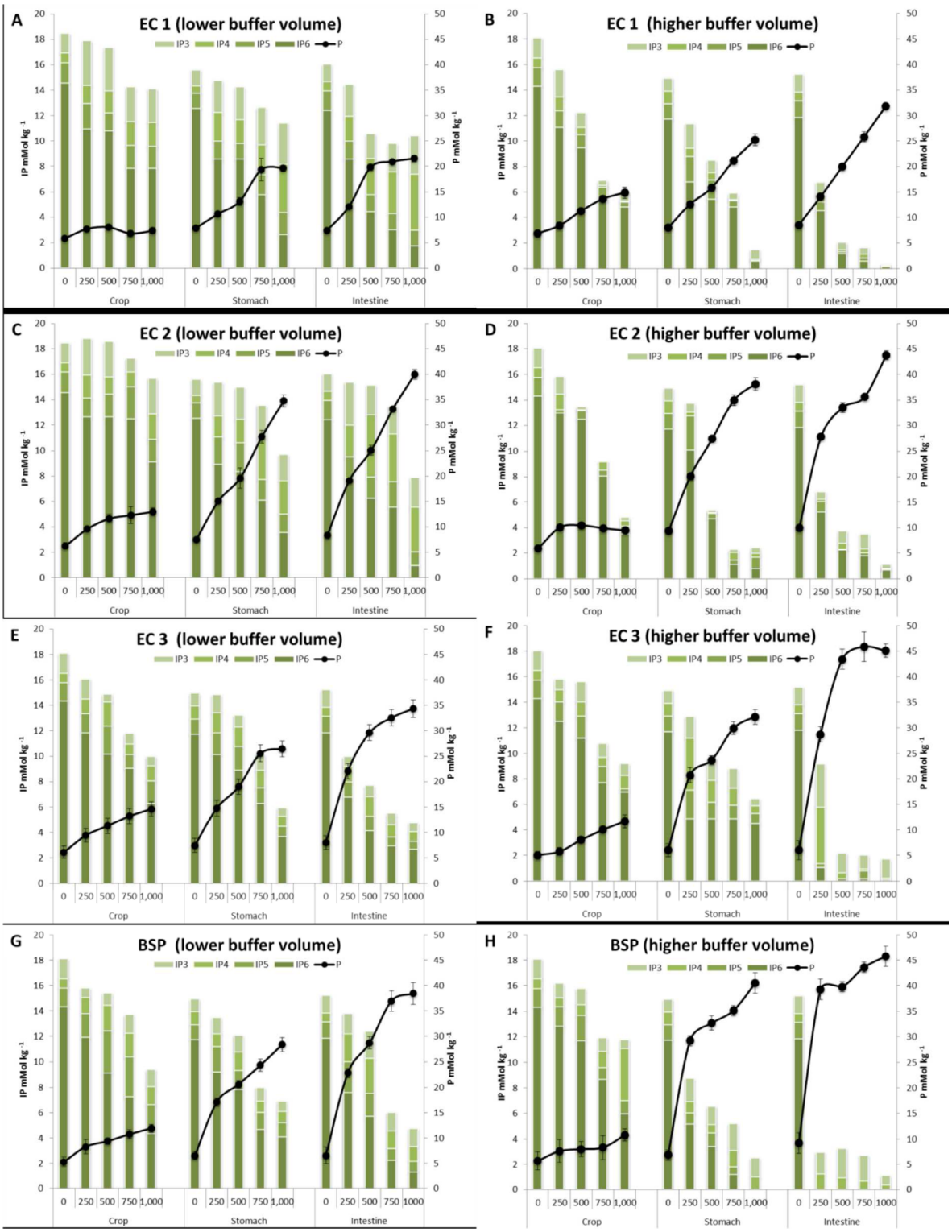

Figure 2. continued

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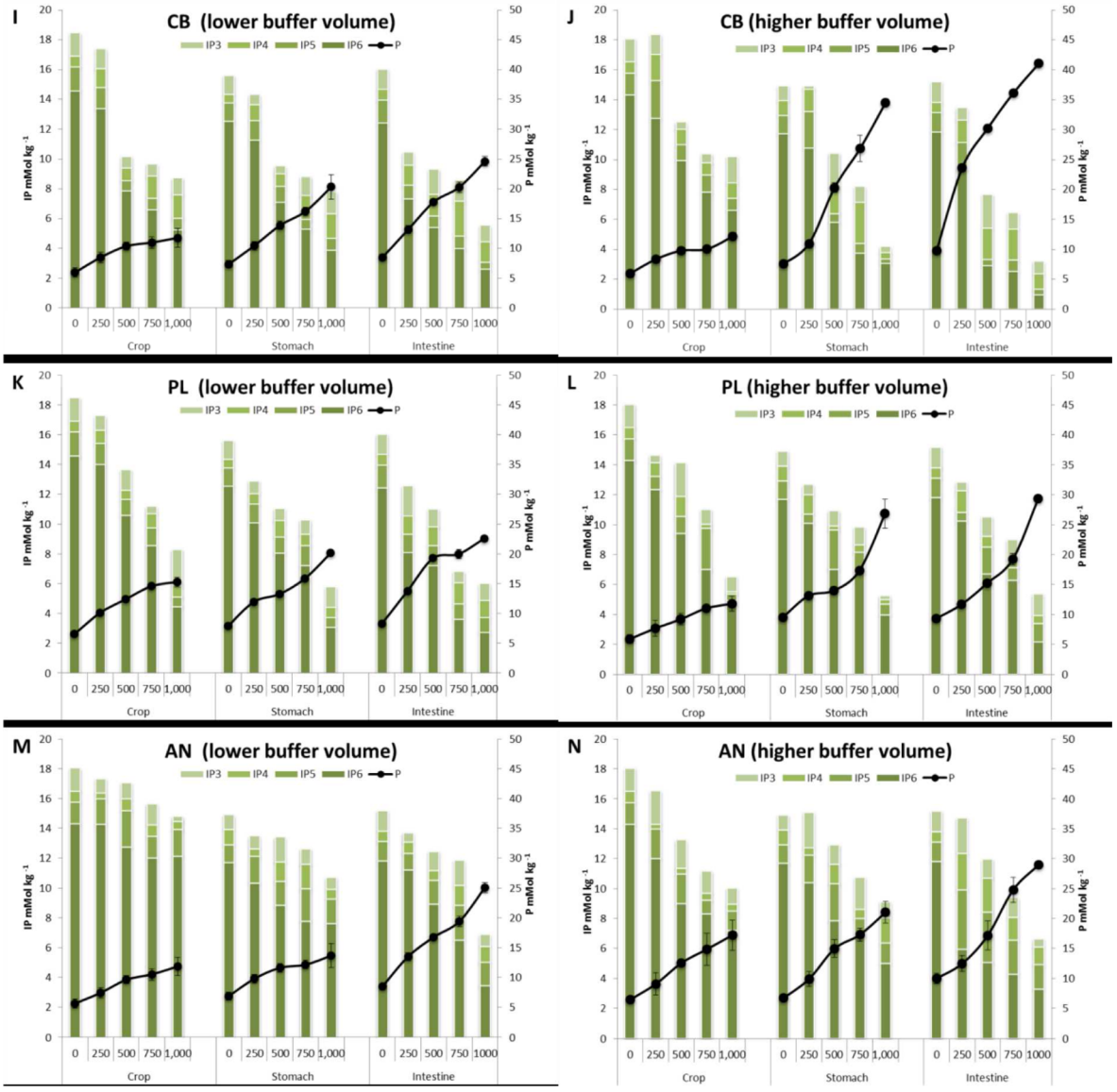

Figure 2. Enzymatic phytate dephosphorylation during in vitro simulation of poultry digestive tract quantified by reversed-phase high-performance liquid chromatography of *myo*-inositol phosphates (columns: IP_6 to IP_3) and P_i (dots and black lines). In the individual graphs the data obtained for the phytase products EC1, EC2, EC3, BSP, CB, PL, and AN are shown. A, C, E, G, I, K, and M represent the lower buffer volume, whereas B, D, F, H, J, L, and N the higher buffer volume. For every enzyme and buffer system samples were analyzed after simulation of the crop, crop and stomach, as well as crop, stomach, and small intestine at increasing phytase doses (0−1000 U kg^{-1}).

decreased, whereas P_i increased with increasing phytase supplementation (Figure 2). This result was expected, because phytases were added to release the organically bound phosphate in the phytate molecule. Histidine acid phytases (HAP) as used in this study are capable of releasing up to five phosphate moieties from the phytate molecule.[18] However, P_i release determined experimentally and P_i release calculated from the HPLC data of *myo*-inositol phosphate turnover did not match. The calculated values were always higher. The impossibility to quantify IP_2 and IP_1 with the HPLC method applied might explain this observation at least in part, because the difference in IP_{total} during in vitro digestion compared to IP_{total} in the diets was taken as IP_1 for P_i release calculations. The experimentally determined P_i release in the in vitro degradation system was comparable to values already available in the literature. In poultry, P availability was reported to increase up to 1 g P kg^{-1} of feed when feeds are supplemented with 766 U kg^{-1} of HAP phytase.[30,31] P_i release in the in vitro degradation system at 750 U kg^{-1} phytase was determined to be 0.83 ± 0.24 g P kg^{-1} (lower buffer volume) and 0.93 ± 0.31 g P kg^{-1} (higher buffer volume), respectively. Selle et al.[31] reported that 42% of the organically bound phosphate from phytate was released under the conditions mentioned above, whereas the corresponding values in the in vitro degradation system were 72.7 ± 6.8% (lower buffer volume) and 85.6 ± 9.7% (higher buffer volume), respectively. However, this study was performed with microwave-treated ground wheat, whereas Selle et al.[31] used a complete feed. A higher buffer volume and, respectively, a lower viscosity in the in vitro digestion studies resulted in general in improved IP_6 dephosphorylation and in lower IP_{total} and higher P_i amounts (Figure 2). The extent of the improvement in the release of the organically bound phosphate dephosphorylation was dependent on the phytase used. EC1 and EC2 were most sensitive to modification of the

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Table 3. Phytase Activity Needed To Achieve a 50% Reduction in IP_6 and an Increase in P_i of 50% of the Maximum Reachable Value[a]

	EC1	EC2	EC3	BSP	CB	PL	AN
lower buffer volume							
50% IP_6	326 (0.95)	319 (0.86)	395 (0.92)	323 (0.92)	445 (0.87)	418 (0.80)	586 (0.89)
50% P_i	2194 (0.84)	955 (0.94)	1159 (0.93)	952 (0.94)	2200 (0.97)	2606 (0.89)	2398 (0.93)
higher buffer volume							
50% IP_6	148 (0.94)	211 (0.82)	140 (0.76)	129 (0.88)	269 (0.96)	480 (0.75)	503 (0.90)
50% P_i	1459 (0.97)	780 (0.86)	636 (0.86)	654 (0.77)	841 (0.94)	1719 (0.82)	1773 (0.93)

[a]Values (U kg^{-1}) obtained by nonlinear fit of the observed data; coefficient of determination in parentheses.

buffer volume, whereas BSP, CB, and EC3 were moderately sensitive and PL as well as AN showed similar behaviors at both buffer volumes. Apparently, bacterial phytases were more affected by the modification in buffer volume during in vitro digestion, which may suggest that viscosity is of more concern regarding bioefficacy of bacterial compared to fungal phytases. In contrast to the enzymatic dephosphorylation of phytate in a buffered system, no real accumulation of partially phosphorylated *myo*-inositol phosphates such as IP_4 or IP_3 has been observed in the in vitro digestion system (Figure 2). A reduced mobility of the phytase and the *myo*-inositol phosphate molecules due to the higher viscosity in the in vitro digestion compared to the buffered system seems to result in the preference of the phytase to act upon the just released *myo*-inositol phosphate instead of binding a different phytate molecule into its active site. As expected from their pH profiles (Table 2; Figure 1), the major site of action of the different phytase products used was determined to be the stomach, but a significant *myo*-inositol phosphate dephosphorylation was also observed in the crop (Figure 2). About 80−90% of the enzymatic *myo*-inositol phosphates dephosphorylation occurred in the stomach and crop.

All phytase products included in the study were capable of releasing the organically bound phosphate from *myo*-inositol phosphate moiety. Their relative performances, however, were remarkably different (Figure 2). When the enzymatic properties of the different phytases (Table 2) as well as incubation time in the different parts of the intestinal tract and the phytate concentration in the microwave-treated ground wheat are taken into consideration, all phytases should be able to dephosphorylate all phytate accessible in the wheat to *myo*-inositol monophosphate, the final phytate dephosphorylation product of histidine acid phytases, even at the lowest dose (250 U kg^{-1}) used. Because IP_6, IP_5, IP_4, and IP_3 were still present in the intestinal samples of the in vitro degradation system, further parameters need to affect phytase performance. An attempt was made to explain enzyme performance in the in vitro degradation system by considering incubation time and substrate concentrations in the different parts of the digestive tract as well as pH dependence of enzymatic activity individually and in combination. Neither correlation tables nor component analysis or regression plots were able to explain the differences in phytase performance (data not shown). Therefore, further parameters such as lower catalytic activity toward precipitated forms of phytate, product inhibition (P_i) of enzymatic activity, reduction in enzymatic activity due to adsorption processes, and limited access of the phytase to phytate because of mobility and physical constraints may affect phytase performance.

Due to the vast number of parameters affecting phytase performance in the in vitro degradation system used as well as in animal studies, an in vitro system will hardly be able to fully mimic the in vivo situation. Besides determination of the enzymatic properties of a phytase, however, in vitro simulation is considered as a good complementary tool to preselect promising phytase candidates as a feed supplement. To compare the different enzyme products used in the study, the phytase activity needed to achieve a 50% reduction in IP_6 and a 50% release of the P_i bound to the *myo*-inositol ring was estimated using nonlinear fit of a negative exponential model of the observed data (Table 3). According to the model, between 319 and 581 U phytase per kilogram of microwave-treated ground wheat was needed to achieve a 50% reduction in IP_6, whereas between 952 and 2606 U phytase per kilogram of ground wheat was required to release 50% of the P_i bound to the *myo*-inositol ring when the lower buffer volume was used. With the higher buffer volume, the corresponding values were estimated to be 129−503 and 636−1791 U per kilogram of microwave-treated ground wheat, respectively. Thus, significantly lower phytase activity values were calculated to achieve a 50% reduction in IP_6 compared to a 50% release of the organically bound P_i. This result was expected because in the former case more or less 50% of the IP_6 present in the microwave-treated ground wheat was converted to IP_5, and in the latter case all IP_6 was enzymatically dephosphorylated to IP_3. In addition, it must be considered that partially phosphorylated *myo*-inositol phosphates are dephosphorylated with a different velocity compared to IP_6 and that the rate of phosphate release decreases progressively as the enzymatic reaction proceeds.

The differences in the behavior of the different commercially available phytase products in the in vitro degradation model system do not precisely reflect their performance in the animal. Therefore, the model cannot be used to rank phytases with respect to their bioefficacy, but it is considered as a simple and useful tool to evaluate the suitability of a phytase to be used as a feed supplement. Although relevant factors such as dietary P levels, intestinal phytase, and P absorption are not implemented in the system, this approach might help to reduce the number of feeding trials when searching for a better suited phytase for animal feed application in the initial screening phase. Based on of the phytase activity needed to achieve either a 50% reduction in IP_6 or a 50% release of the organically bound P_i, studies are currently carried out to establish a threshold value for the need to conduct animal feeding trials with the phytase in question. Furthermore, these studies should answer the question of whether the in vitro model will prove satisfactory when only the higher buffer volume is used and only P_i is quantified. In that case, the model system is more easily handled; the in vitro studies are less time-consuming and need less analytical effort. Further research work is in progress to study the value of in

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vitro models with more realistic feed formulations for poultry and swine.

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Notes

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