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Optimization of Phytase Concentration from *Aspergillus ficuum* for Phytate-bound Phosphorus Release in Cereal Meals

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ABSTRACT

In order to determine the optimum phytase dose needed to release phytate-phosphorus in *Zea mays*, *Triticum aestivum* and *Sorghum bicolor* meals, six phytase concentrations (0, 100, 200, 300, 500 & 1 000 µg/kg) prepared from a commercial phytase (Natuphos®) derived from *Aspergillus ficuum*, were investigated. A dose-dependent increase in phytate-phosphorus release with an increase in phytase concentration was noted. The optimum phytase dose for phytate-phosphorus release from *Z. mays*, *T. aestivum* and *S. bicolor* meals was 700, 567 and 667 µg/kg, respectively. Non-phytate phosphorus concentration at the optima phytase doses were 1.4868, 5.742 and 2.136 g/kg for *Z. mays*, *T. aestivum* and *S. bicolor* meals, respectively; translating into incremental phosphorus release of 166.8, 31.0 and 161.4%, respectively. The optimum dose of *A. ficuum* derived phytase required for release of phytate-phosphorus is dependent on cereal type. © 2010 Friends Science Publishers

Key Words: Cereals; Exogenous phytase; Phytate-bound phosphorus; Nutrient-chelating

INTRODUCTION

Phosphorus (P), a mineral essential for growth and development, is found in substantial amounts in cereal, legume and oil seeds where up to 80% of it is present as unavailable phytic acid bound form (Heindl, 2006). Cereal grains, legumes and oil seeds constitute the principal components of animal feeds (Berka *et al*., 1998). Monogastric animals, whose diets are largely cereal, legume and oilseed based do not produce sufficient amounts of intrinsic phytases necessary to hydrolyze the phosphorus-binding phytic acid molecule (Smith *et al*., 2004). The six phosphate groups in the phytate molecule make it highly charged (Lehninger, 1982). The phytate molecule thus chelates many inorganic and organic entities, forming insoluble complexes that include phytate-proteins, phytate-digestive enzymes and phytate-minerals complexes (Reyden & Selvendran, 1993; Ravindran *et al*., 1995). Phytic acid binds calcium, magnesium and zinc very tightly at the multiple phosphate groups preventing their absorption (Lehninger, 1982). Diets for monogastric animals are mainly comprised of feedstuffs that have low P availabilities (cereal grains), making P supplementation from inorganic sources necessary to obtain maximum animal performance (Heindl, 2006).

In areas with intensive livestock production, the phytate-phosphorus output is often high (Gill, 2003). Most of the phytate-phosphorus present in monogastric animal feeds rendered unavailable to the animals is largely excreted in their dung (Abelson, 1999). The excreted phytate-phosphorus causes eutrophication that negatively affects aquatic ecosystems (Berka *et al*., 1998). Feed enzymes such as phytases have become a key weapon in the battle to improve feed utilization efficiency and reduce P excretion (Sharpley & Beegle, 2001). Phytases in animal feed ingredients release not only bound phytate-phosphorus; but, also phytate bound amino acids in cereal and legume grains and oil seeds by breaking down the phytate structure. By releasing bound phosphorus in feed ingredients, phytases make more phosphorus available for improved productive and reproductive animal performance. Additionally, phytases reduce the amount of phosphorus excreted into the environment thus help in the conservation of natural resources (Novo Nordisk, 1995; Berka *et al*., 1998). Phytases also release phytate-bound minerals such as calcium and magnesium, amino acids and proteins for utilization by the animal (Lehninger, 1982).

This study sought to determine the maximum concentration of *Aspergillus ficuum* derived phytase for optimal phytate-phosphorus release in *Zea mays*, *Triticum aestivum* and *Sorghum bicolor* meals sampled from National Foods Livestock Division, Zimbabwe and establish

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percent non-phytate phosphorus increase at optimum phytase dose for each meal with a view to better advice the livestock feed industry.

MATERIALS AND METHODS

Research site: The laboratory assays were done at the Department of Applied Biology and Biochemistry of the National University of Science and Technology, Zimbabwe located at 20° 10' S and 28° 35' E at an altitude of 1050 m above sea level, with a mean annual rainfall ranging from 350-650 mm (Philpott & Collins, 1961).

Research materials: *Zea mays* (white maize meal), *Triticum aestivum* (wheat feed meal) and *Sorghum bicolor* (red sorghum meal) were used. The cereal grain samples were randomly collected from National Feeds Limited Stockfeeds Division at Aspindale depot, Harare, Zimbabwe. Two subsamples per cereal were collected over a 6-months period. The subsamples of each cereal constituted a composite sample from which assays were done. The grain samples were separately milled through a 1 mm screen and then stored in sample bottles pending laboratory assays.

Treatments and experimental design: Six phytase concentrations had their effect tested on each cereal grain meal: 0, 100, 200, 300, 500 and 1 000 µg/kg. For each cereal meal three replicates were subjected to each test phytase concentration in a completely randomized design.

Assay procedure: Each cereal meal was incubated with a commercial phytase (Natuphos® 5000 FTU/g) derived from *A. ficuum*. One phytase unit was defined as the activity that releases 1 µmol of inorganic phosphorus from phytate per min at pH 5.5 and 37°C. One gram sample of each cereal meal was placed in three test tubes followed by the addition of 4 mL of distilled water. The respective test phytase (enzyme) levels were applied by the use of a micropipette. This was followed by addition of 0.5 mL of 0.2 M sodium hydroxide in citrate buffer (pH 5.5). The contents were thoroughly mixed and then incubated in a water bath at 37°C for 30 min. Thereafter, 0.5 mL TCA was added to stop the reaction. The contents of the test tubes were centrifuged at 13 000 rpm for 10 min using a micro-centrifuge. Supernatants were collected (1 mL) into a 100 mL-measuring cylinder to which 1 mL of 0.1 M molybdate and 1 mL of 0.1 M ascorbic acid reagents were added. The contents were then made up to 60 mL by addition of distilled water. The solutions were placed into beakers and were allowed to stand for 5 min. A total of 5 test tubes were setup with known concentrations of phosphate to act as the standards. Extinctions were read using a Milton Roy® spectrophotometer set at a wavelength of 660 nm against the blank filled with TCA only. Phosphorus was tested using the Ascorbic Acid-Molybdate Method, based on the Standard Methods for Examination of Water and Waste Water method for analysis of P (SMEWW, 1985). Extinctions from the standards were used to plot a graph (calibration curve) that showed the relationship between absorbance and phosphate concentration.

Data analysis: The calibration curve was regressed using a statistical package from Genstat® 7.1 Edition (GenStat, 2003). The regression model was used to generate phosphate concentrations in the cereals. The first order differentiation derivative was equated to zero to determine the point of inflexion in each cereal, where enzyme concentration ceased to be the limiting factor in making phytate phosphorus available. An analysis of variance on the generated phosphorus (phosphate) levels under different phytase concentration was done using Genstat® 7.1 Edition with mean separation through use of the Least Significant Difference (LSD) procedure.

RESULTS

A dose-dependent phytate-phosphorus release was noted with phytase concentration in all the cereal grain meals (Table I). The differences in phytate-phosphorus release between any two levels of phytase activity in the maize meal were significant ($P<0.001$, Table I). Maize meal initially had a non-phytate phosphorus concentration of 0.557 g/kg (Table I), which increased to 1. 486 g/kg at the maximum phytase level (Table II). Total incremental amounts of phosphorus released from the maize meal in 30 min were 0.112, 0.483, 0.669, 0.966 and 1.226 g/kg for 100, 200, 300, 500 and 1000 µg/kg phytase levels, respectively (Table I). The hydrolysis of phytate phosphorus increased very sharply up to 700 µg/kg phytase concentration as evidenced by the computed maximum enzyme (phytase) addition level for maize (Table II).

The differences in phytate-phosphorus release between any two levels of phytase activity in the wheat fed meal were significant ($P<0.001$, Table I). Wheat feed with an initial non-phytate phosphorus concentration of 4.384 g/kg increased to 5.742 g/kg at the maximum phytase level (Table I & II) and had 0.372, 0.818, 1.041, 1.189 and 1.784 g/kg incremental release of phytate-phosphorus in 30 min at 100, 200, 300, 500 and 1000 µg/kg phytase level in the wheat feed, respectively. The hydrolysis of phytate-phosphorus in the wheat feed reached a plateau at 567 µg/kg maximum phytase level (Table II).

Sorghum had an initial non-phytate phosphorus concentration of 0.817 g/kg (Table I), which increased to 2.136 g/kg at the maximum phytase level (Table II). Total amounts of incremental phytate-phosphorus released in 30 min were 0.372, 0.595, 1.041, 1.338 and 1.412 g/kg of sorghum meal at 100, 200, 300, 500 and 1000 µg/kg phytase in the meal, respectively. The hydrolysis of phytate-phosphorus in the sorghum meal reached its optima at 667 µg/kg (Table II).

The first order differentiation derivative showed a very strong relationship between the phosphate (phosphorus) concentration and the absorbance as evidenced by the high R^2 values (Table II). Beyond the optimum phytase level,

Table I: Mean inorganic phosphorus release (g/kg) at different phytase concentrations by cereal

Phytase conc. (µg /kg)	Maize	Sorghum	Wheat feed
0	0.557±0.08^{c}	0.817±0.11^{d}	4.384±0.12^{c}
100	0.669±0.10^{bc}	1.189±0.25^{c}	4.756±0.16^{bc}
200	1.040±0.12^{b}	1.412±0.14^{c}	5.202±0.23^{bc}
300	1.226±0.09^{b}	1.858±0.11^{b}	5.425±0.20^{b}
500	1.523±0.15^{a}	2.155±0.42^{ab}	5.573±0.19^{ab}
1000	1.783±0.25^{a}	2.229±0.28^{a}	6.168±0.38^{a}
Grand Mean	1.133	1.610	5.251
SE	0.0841	0.1012	0.2253
LSD	0.2650	0.3190	0.7098
CV (%)	12.9	10.9	7.4
P-Value	0.001	0.003	0.001

^{abcd}Means within a column with different superscripts are significantly at P<0.05

Table II: Maximum phytase level by cereal as determined by differentiation derivative and corresponding inorganic phosphorus levels

Cereal	Differential equation	R^2 Value	Maximum phytase dose	P concentration at maximum phytase dose (g/kg)
Maize	$Y = -2E - 06X^2 + 0.0028X + 0.5061$	0.9873	700 µg/kg	1.486 (*166.79)
Sorghum	$Y = -3E - 06X^2 + 0.0040X + 0.8022$	0.9902	667µg/kg	2.136 (*161.44)
Wheat	$Y = -2E - 06X^2 + 0.0034X + 4.4569$	0.9701	567 µg/kg	5.742 (*30.98)

*Figures in parentheses are % increase in non-phytate phosphorus release at the respective maxima phytase dose for each cereal grain meal

release of phytate-phosphorus increased at a decreasing rate, an indicator that the enzyme was not the limiting factor past the point of inflexion.

DISCUSSION

The method used in this study to determine phosphate concentration had a high precision but was limited in the range of phosphate that could be determined. The spectrophotometer used had an absorbance that ranged from 0 to 1.5 therefore, the more concentrated cereal grain samples had to be diluted by a factor of 60. However, results of the standard curve agreed with (Strickland & Parsons, 1972) who stated a direct proportional relationship between the absorbance and the concentration of phosphate (Beer's Law) as evidenced by the high R^2 value of the regression analysis curve.

In cereal grains and other plant seeds, most of the phosphate is in the form of phytic acid, which is largely indigestible by monogastric animals and is the single most important factor hindering the uptake of a range of minerals (Brinch-Pedersen *et al*., 2002). Phytic acid is reported to be one of the critical antinutritional factors for the availability of divalent and trivalent minerals (e.g., zinc & calcium & possibly also iron) in monogastric farm animals and humans as it readily forms insoluble complexes with Zn^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, Ca^{2+} and Fe^{2+} (Cheryan, 1980; Ravindran *et al*., 1995; Sebastain *et al*., 1998). In the gastrointestinal tract (GIT) phytic acid complexes with both dietary proteins and digestive enzymes resulting in dietary proteins refracting hydrolysis by digestive enzymes and also effecting a reduction in the activity of digestive enzymes (Sebastain *et al*., 1998). This leads to reduced digestion especially of protein thus exacerbating protein malnutrition in cereal-dependent communities of the developing world. About 2 billion people (women & children) from the developing world suffer from phytic acid induced iron and zinc deficiency since phytic acid inhibits the uptake of the minerals from the staple diets (Bouis, 2000). Cereals constitute the large proportion of the diets of people in the developing third world. This scenario explains key mineral deficiencies since results from this study amply prove that a large proportion of phosphorus in cereals is locked up (together with other minerals) with the phytic acid as evidenced by incremental release of phosphorus from the three cereal grains when subjected to an exogenous phytase.

The antinutritional properties of phytic acid have been supported by several feeding experiments. Dietary supplementation with microbial phytase improved utilization of Zn^{2+} in rats and broiler chickens, while in pigs, phytase supplementation increased the apparent absorption of Mg^{2+}, Zn^{2+}, Cu^{2+} and Fe^{2+} (Rimbach & Pallauf, 1993; Sebastian *et al*., 1996). Results from the current study indicated an increase in phosphorus availability with phytase use, were in agreement with the observed and reported increased mineral availability and improved monogastric animal performance. The noted increase in the release and hence availability to animals of phytate-phosphorus with addition of exogenous of phytase proves the efficacy of the enzyme in releasing phytate-bound phosphorus and in the process other minerals and possibly phytate-bound dietary proteins. It has been observed that simultaneous use of the exogenous enzyme (phytase) with crop engineering improved phosphate bioavailability and reduced phytic acid excretion into the environment (Brinch-Pedersen *et al*., 2002). The improved phosphate bioavailability reduces the phosphate load on agricultural ecosystems and thereby, alleviates eutrophication of the aquatic environment (Brinch-Pedersen *et al*., 2002).

Furthermore, improved phosphate availability, from cereals use in diets of monogastric animals, reduces the need to add inorganic phosphate, a non-renewable resource, to monogastric animal feeds, thus positively impacting on the cost of monogastric animal protein production.

CONCLUSION

A determination of the optimum exogenous phytase dose by cereal is necessary in the formulation of least cost monogastric animal feeds. Optimum dose of *A. ficuum* derived phytase for maximum phytate-phosphorus release in *Zea mays*, *Triticum aestivum* and *Sorghum bicolor* meals used in the experiment were 700, 567 and 667 µg/kg, respectively. The optimum dose of *A. ficuum* derived phytase required for release of phytate-phosphorus is dependent on cereal type.

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