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Influence of Three Phytase Preparations in Broiler Diets Based on Wheat or Corn: *In vitro* Measurements of Nutrient Release

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Abstract: Using an *in vitro* poultry digestion model, potential beneficial effects from the side activities in a microbial phytase produced by solid state fermentation were examined by comparing the release of phosphorus, reducing sugars and α-amino nitrogen by two other phytase preparations in wheat- and corn-based diets. Because of the technology involved, the phytase produced by solid state fermentation is known to contain several side enzyme activities, including protease, amylase, cellulase, xylanase and β-glucanase. Phytase produced by solid state fermentation released more ($P<0.05$) phytate-bound phosphorus (11.0% and 7.8% in wheat- and corn-based diets, respectively) and α-amino nitrogen (1.7% and 6.2% in wheat- and corn-based diets, respectively) than a phytase produced by submerged liquid fermentation, which had no detectable side activities. Phytase produced by solid state fermentation also released 2.9% more reducing sugars in the wheat-based diet and 6.2% more α-amino nitrogen in the corn-based diet. The superiority of this phytase product in releasing nutrients in both types of diets is likely to be due to activities of other enzymes present, but these results need to be confirmed in future *in vivo* studies.

Key words: Microbial phytase, solid state fermentation, *In vitro* nutrient release, wheat, corn

Introduction

The usefulness of microbial phytase in releasing phytate-bound phosphorus (P) and improving P availability in diets for monogastric animals is now well documented and the inclusion of this feed enzyme in pig and poultry diets to reduce P excretion continues to meet growing acceptance (Ravindran *et al*., 2000). Several commercial microbial phytase products are currently available and two distinct fermentation technologies are used to produce these products - one involving submerged liquid fermentation and the other based on solid state fermentation. Because of the technology employed, the phytase produced by solid state fermentation also contains several side enzyme activities, including protease, amylase, cellulase, xylanase and β-glucanase. Several recent studies have shown that phytase produced by solid state fermentation is effective in enhancing the utilization of nutrients in a range of diet types for broiler chickens and these responses were, in part, attributed to the presence of side enzyme activities (Wu *et al*., 2003, 2004). The trial designs used in these studies, however, did not permit any definite conclusion about the benefits of the side activities.

In vitro simulation models have been recently developed and successfully used to predict the release of nutrients by exogenous enzymes for turkeys (Zyla *et al*., 1995) and broilers (Zyla *et al*., 1999a; 2000). The objective of the present study was to evaluate the potential beneficial effects from the other enzymes present in the phytase produced in solid state fermentation compared to two other phytase preparations by determining *in vitro* nutrient release in wheat- or corn-based diets.

Materials and Methods

Enzymes: Phytase A (Allzyme® SSF; supplied by Alltech, Inc., Nicholasville, KY, USA) was determined to contain the following enzyme activities: phytase, fungal protease, fungal amylase, cellulase, xylanase, and β-glucanase. Phytase activity in the product exceeded product guarantees of 1216 PU/g. One unit of phytase (PU) is defined as the quantity of enzyme that releases 1 μmol of inorganic phosphorus/min from 0.00015 mol/l sodium phytate at pH 5.5 at 37°C. Phytase B was formulated, using pure sources of enzymes, to contain similar enzyme profile to Phytase A. Phytase C was Natuphos® 5000 (Gist Brocades BSD V.V., Delft, the Netherlands) which was determined to contain 3645 PU/g phytase activity and had no detectable side enzyme activities.

Experimental Design: There were eight treatments (Table 1). Treatments 1 to 5 were phytase A supplying five levels of phytase (0, 250, 500, 750 and 1000 PU/kg diet). Treatments 6 and 7 were Phytase B supplying two levels of phytase (500 and 750 PU/kg). Treatment 8 was Phytase C supplemented at a level of 500 PU/kg.

The influence of the enzyme treatments was tested with two diet types. The composition of the diets are shown in Table 2.

In vitro digestion model designed for the determination of release of P, reducing sugars, and α-amino nitrogen The *in vitro* digestion model that was developed by Zyla

Table 1: Dietary treatments and dosage of enzymes tested

Treatment	Source of enzyme	Dosage (PU/kg)
1	Phytase A	0
2	Phytase A	250
3	Phytase A	500
4	Phytase A	750
5	Phytase A	1000
6	Phytase B	500
7	Phytase B	750
8	Phytase C	500

et al. (1999a) to study the release of P, reducing sugars, and α-amino nitrogen in broiler chickens was used, with minor modifications (Fig. 1). The modifications involved pH values in the crop, gizzard, duodenum and small intestine, which were obtained in a pilot trial from birds fed corn- or wheat- based diets. pH values used in the *in vitro* digestion model were adjusted to 5.7, 2.7 and 5.9 for the three incubation periods simulating the three sections (crop, proventriculus/ gizzard, and duodenum, respectively) of the digestive tract of chickens fed wheat-based diets. Corresponding pH values in corn-based diets was 5.9, 2.9 and 6.1, respectively.

Table 2: Ingredient composition and calculated analysis of the test diets

Ingredient	Wheat-based diet	Corn-based diet
	----------%	----------
Wheat	67.45	0.00
Corn	0.00	60.01
Soybean meal	25.46	33.10
Corn oil	4.00	4.00
Dicalcium phosphate	0.50	0.60
Limestone	1.28	1.20
Lysine	0.29	0.09
Methionine	0.42	0.40
Salt	0.30	0.30
Vitamin-mineral premix[1]	0.30	0.30
Calculated analysis:		
AME (MJ/kg diet)	13.52	13.51
Crude protein, %	21	21
Lysine, %	1.15	1.15
Methionine + Cysteine, %	0.94	0.94
Calcium, %	0.69	0.69
Total P, %	0.49	0.48
Phytate P, %	0.26	0.25
Non-phytate P, %	0.25	0.25

[1]Supplied per kilogram of diets: biotin, 0.22 mg; cholecalciferol, 528 IU; cyanocobalamin, 0.03 mg; folic acid, 1.1 mg; menadione, 2.8 mg; niacin, 55 mg; pantothenate, 17.6 mg; pyridoxine, 4.9 mg; *trans*-retinol, 11025 IU; riboflavin, 7.7 mg; thiamine, 2.2 mg; dl-α-tocopheryl acetate, 33 IU; choline chloride, 479 mg; Cu, 10 mg; Fe, 40 mg; I, 1.9 mg; Mn, 64 mg; Se, 300 µg; Zn, 75 mg.

Representative diet samples (corn- or wheat-based diets) were obtained and ground using a laboratory-scale grinder. The first step was to simulate the digestion in the crop. One gram (1 ± 0.0001) of the sample (wheat- or corn-based diet) was weighed (in triplicate) into a 10 ml plastic syringe without Luer-locks. The samples were hydrated with 1.5 ml distilled water or enzyme dilution solution.

Contents of the syringe were vortexed and incubated in a water bath at 40°C for 30 min. After 30 min incubation, 1.5M HCl (0.46 and 0.49 ml for the wheat- and corn-based diet, respectively) was added and vortexed. Half a ml of pepsin solution (6000 Units/ml; P-7012, Sigma Chemical Co, St. Louis, MO, USA) was then added and the syringe sealed with parafilm and incubated for a further 45 min at 40°C. This step simulated digestion in the proventriculus and gizzard.

The third step was to simulate the digestion in the duodenum of chickens. 0.45 to 0.50 ml of 1M $NaHCO_3$ containing 3.7 mg pancreatin per ml solution (P-3292, Sigma Chemical Co, St. Louis, MO, USA) was added to each syringe under constant stirring. The digesta slurry was then transferred to segments of dialysis tubing (molecular weight cutoff, 12000-14000 Da diameter 18.0 mm; Sigma Chemical Co., St Louis, MO, USA). The dialysis tubings were placed in a 250 ml flask containing 100 ml of 0.1M NaCl in a 0.05 M sodium succinate buffer (pH 5.90 and 6.10 for wheat- and corn-based diets, respectively) and incubated in a shaker within an incubator. The temperature of incubator was maintained at 40°C. Six milliliters of the dialysate samples were taken at second and fourth hour of the incubation. The dialysate was analyzed for inorganic phosphate (Shieh *et al.*, 1969), reducing sugars (Miller, 1960) and α-amino nitrogen using the ninhydrin method (Moore and Stein, 1954).

***In vitro* viscosity measurement:** Samples for viscosity measurements in wheat-based diets were obtained using the *in vitro* procedures described above, with the only difference being that centrifuge tubes were used, instead of plastic syringes. Simulation of the digestion in the small intestine was performed with addition of 0.46 ml of 1M $NaHCO_3$ containing 3.7 mg per ml pancreatin solution (Fig. 1). After 120 min of digestion, the samples were centrifuged at 12500 x g and supernatant viscosity was determined at 40°C using a capillary viscometer (Brookfield Engineer Ltd, Middleboro, MA, USA) according to the method described by Almirall *et al.* (1995).

Data Analysis: The experimental data were collected in three replicates for the all response variables and analyzed by the General Linear Model (GLM) procedure (SAS, 1997). Linear and quadratic effects on dialyzable phosphorus, reducing sugars, and α-amino nitrogen were tested with five levels of phytase for Phytase A

Table 3: Effects of source (Phytase A, B and C) and concentration of phytase on *in vitro* dialyzable phosphorus (g/kg), reducing sugars (g/kg), α-amino nitrogen (g/kg) and *in vitro* viscosity (mPa.s) in wheat- and corn-based diets[1]

Treatment	Enzyme	Dose (PU/kg diet)	Phosphorus[2]		Reducing sugars[2]		α-amino nitrogen[2]		Viscosity[2]
			WB	CB	WB	CB	WB	CB	WB
1	Phytase A	0	1.325	1.065	43.42	116.23	1.194	0.625	1.93
2	Phytase A	250	1.699	1.424	43.90	114.62	1.195	0.647	1.85
3	Phytase A	500	2.041	1.786	44.73	117.10	1.224	0.665	1.84
4	Phytase A	750	2.257	2.007	45.53	121.91	1.217	0.682	1.81
5	Phytase A	1000	2.457	2.305	46.23	113.20	1.218	0.683	1.77
6	Phytase B	500	1.832	1.699	44.65	117.17	1.199	0.655	1.44
7	Phytase B	750	2.050	1.899	46.05	121.17	1.212	0.668	1.36
8	Phytase C	500	1.839	1.656	43.45	117.75	1.204	0.626	1.80
Pooled SEM			0.0285	0.0253	0.348	2.492	0.0094	0.0063	0.0195
Probabilities[3]									
Phytase A effect									
Linear			***	***	***	NS	NS	***	***
Quadratic			***	*	NS	NS	NS	NS	NS
Contrasts									
Treatment 3 vs 8			***	**	*	NS	NS	*	NS
Treatment 3 vs 6			***	*	NS	NS	NS	NS	*
Treatment 4 vs 7			***	**	NS	NS	NS	NS	*

WB, wheat-based diet; CB, corn-based diet. [1]For details of *in vitro* procedures, see Materials and Methods.
[2]Mean value from three replicate samples. [3]NS, not significant; *, P<0.05; **, P<0.01; ***, P<0.001

using the contrast statement. The linear and quadratic equations for P, reducing sugars and α-amino nitrogen against five levels of phytase for Phytase A were generated by ProcReg statement using the SAS package. Comparison between treatment 3 and 6 (or 8), and between treatment 4 and 7, were made using non-orthogonal contrasts. Significant differences were considered at P<0.05.

Results

Although dialyzable samples were taken at both second and fourth hours, only the data taken on the fourth hour are reported (Table 3). Data from the 2-hour sampling followed a similar trend.

Dialyzable P levels were quadratically increased with increasing levels of phytase A in wheat-based diets (R^2 = 0.99, P<0.0001) (Fig. 2) and corn-based diets (R^2 = 0.99, P<0.0001) (Fig. 3).

Compared to the Phytase C, Phytase A at 500 PU /kg diet released (P<0.05) more phytate-bound P (11.0 and 7.8% for wheat- and corn-based diets, respectively). Compared to the Phytase B, Phytase A released (P<0.05) more P at both 500 and 750 PU/kg diet in both types of diets.

Dialyzable reducing sugar levels were linearly (R^2 = 0.85, P<0.0001) increased with increasing levels of Phytase A in wheat-based diets (Fig. 4). Phytase A supplemented at 500 PU/kg diet released significantly (P<0.05) more reducing sugars (2.9%) than Phytase C supplemented at the similar activity. There were no differences (P>0.05) between Phytase A and B at 500 PU/kg diet. Phytase B released more reducing sugars than Phytase A at 750 PU/kg diet, but the differences were not statistically significant (P>0.05).

In the corn-based diet, increasing levels of Phytase A had no effect (P>0.05) on reducing sugar levels and no differences (P>0.05) were observed between the three phytases at 500 PU/kg diet (Fig. 5).

Increasing levels of Phytase A had no effect (P>0.05) on α-amino nitrogen levels (expressed as glycine per kg diet) in the dialysate of the wheat-based diet (Fig. 6), but quadratically (R^2 = 0.71; P<0.001; Fig. 7) increased α-amino nitrogen levels in the dialysate of the corn-based diet. Compared to Phytase C, Phytase A at 500 PU/kg diet released 1.7% more α-amino nitrogen in the wheat-based diet, but the differences were not significant (P>0.05). In the corn-based diet, Phytase A at 500 PU/kg diet released 6.2% more (P<0.05) α-amino nitrogen than Phytase C. Phytase A released more α-amino nitrogen than Phytase B at 500 and 750 PU kg diet in both types of diets (Table 3), but the differences were not significant (P>0.05).

In vitro viscosity value was highest in the unsupplemented wheat-based diet (Table 3). The viscosity value linearly decreased (P<0.001) with increasing levels of Phytase A. There were no differences between Phytases A and C at 500 PU/kg diet. Interestingly, the viscosity value for the diet supplemented with Phytase B was lower (P<0.05) than those supplemented with Phytases A and C.

Discussion

The objective of the present study was to examine whether or not there were beneficial effects from the side

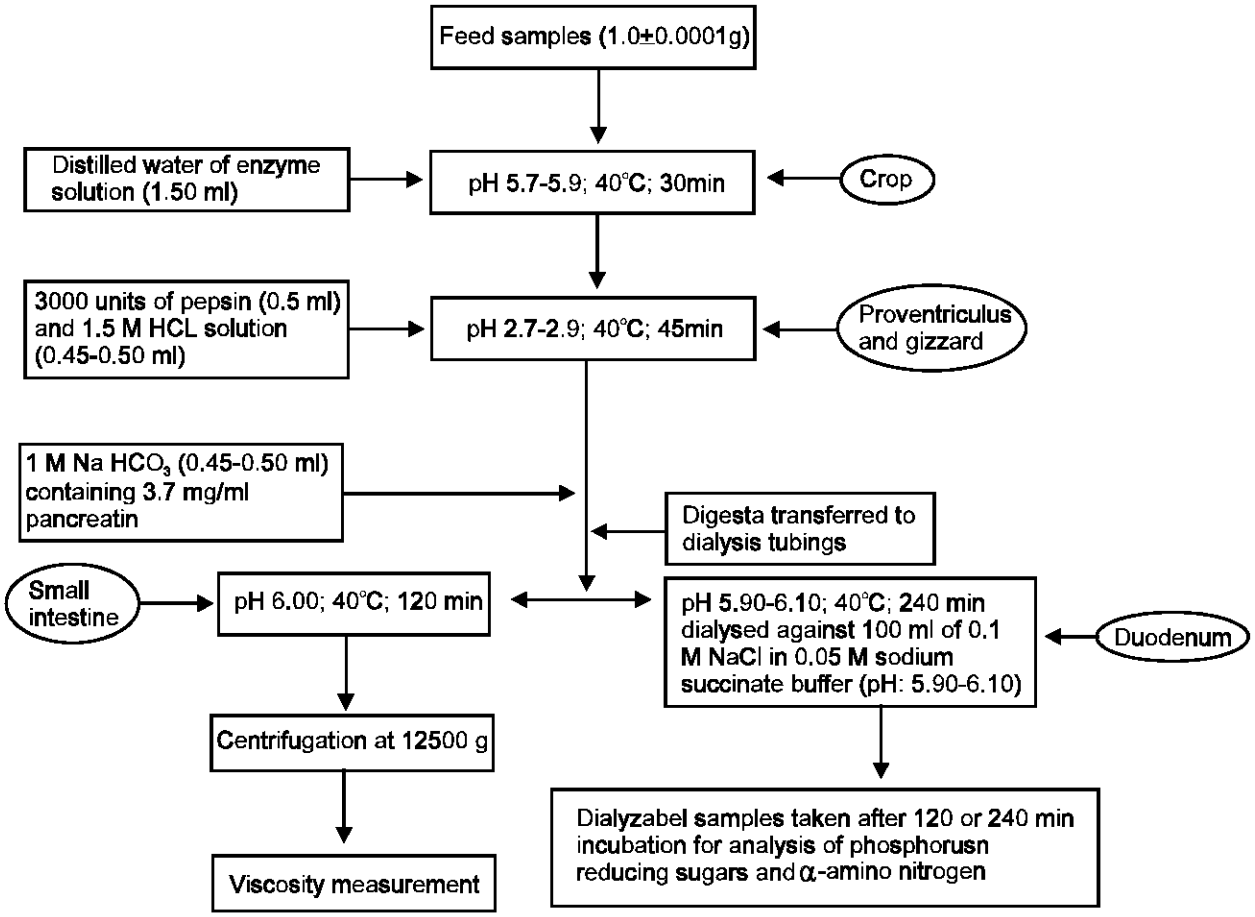

Fig. 1: Flow chart of the *in vitro* procedure used to study the release of phosphorus, reducing sugars, α-amino nitrogen and *in vitro* viscosity (modified from Zyla *et al.*, 1999a)

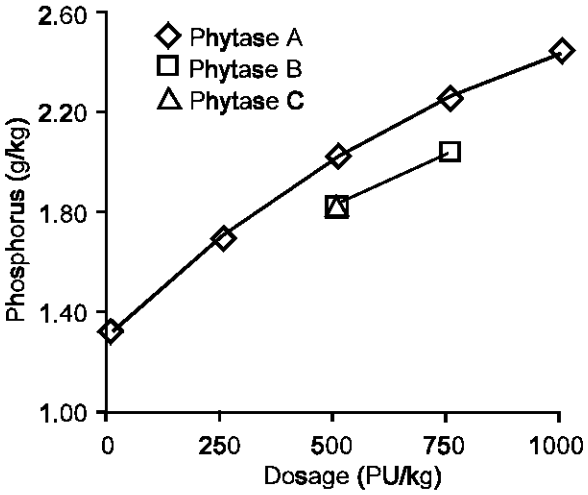

Fig. 2: Effect of source and concentration of phytase on dialyzable phosphorus in the wheat-soy diet

activities present in Phytase A, which was produced by solid state fermentation. In order to ensure P release responses, the diets were formulated to contain 0.25% non-phytate P, which is 0.20% lower than the NRC (1994) recommendation for broiler starters, as the *in vivo* responses are known to be greater in low-P diets (Kornegay *et al.*, 1996).

Dialyzable P levels for phytase were increased with increasing levels of phytase in both diet types. These results are consistent with previous reports (Zyla *et al.*, 1999a,b; Zyla *et al.*, 2000) with several commercial phytase products, including Phytase C, in wheat-based diets. Compared to Phytase C produced by submerged liquid fermentation with no detectable side activities, Phytase A significantly ($P<0.05$) released more phytate-bound P (11.0 and 7.8% in wheat-based and corn-based diets, respectively).

Phytase A was determined to release more phytate-bound P than the Phytase B at 500 and 750 PU/kg diet in both wheat-based and corn-based diets. This is an interesting finding, since Phytase B was blended, using pure sources of enzymes, to contain similar enzyme activities as Phytase A. This may be suggestive of the presence of an unidentified factor that promotes P release when Phytase A is used.

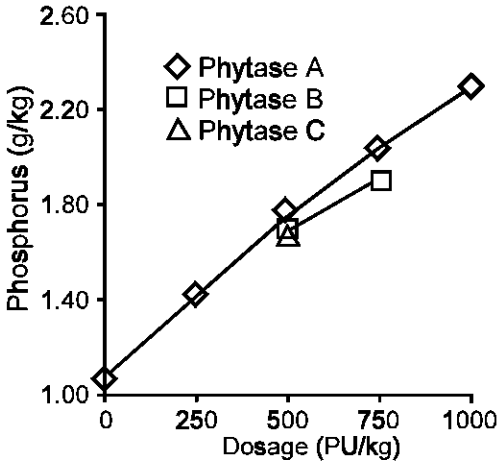

Fig. 3: Effect of source and concentration of phytase on dialyzable phosphorus in the corn-soy diet

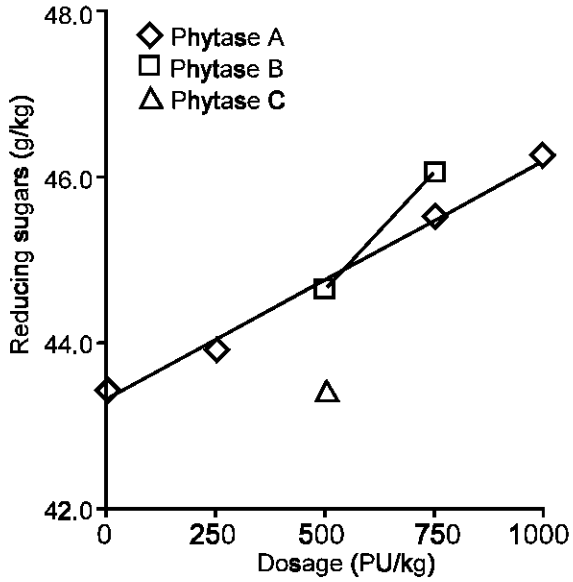

Fig. 4: Effect of source and concentration of phytase on dialyzable reducing sugars in the wheat-based diet

Phytase A at 500 PU/kg diet released ($P<0.05$) more reducing sugars (2.9%) than Phytase C in wheat-based diet. But there were no responses in corn-based diet, which is in general agreement with the observation from the *in vivo* studies by Wu *et al.* (2003). It appears that the magnitude of responses to added phytase is dependent on the dietary cereal base, with corn generally responding to a lesser degree than wheat (Ravindran *et al.*, 1999; Rosen, 2002).

Increasing levels of Phytase A had no effect ($P>0.05$) on the α-amino nitrogen levels in the dialysate in the wheat-based diet, but quadratically increased the α-amino nitrogen levels in the corn-based diet. The lack of response in the wheat-based diet is in disagreement with previous research where reported increases in the

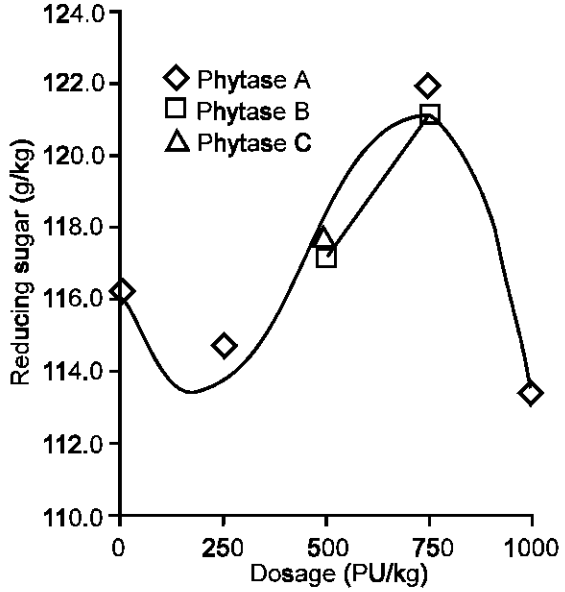

Fig. 5: Effect of source and concentration of phytase on dialyzable reducing sugars in the corn-based diet

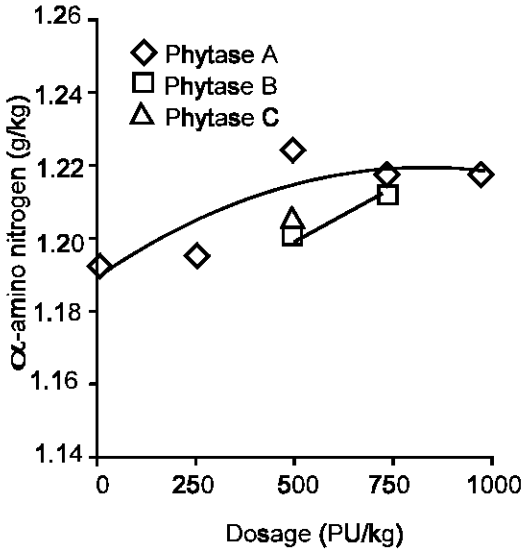

Fig. 6: Effect of source and concentration of phytase on dialyzable α-amino nitrogen in the wheat-soy diet

in vivo amino acid digestibility were greater in wheat compared to corn (Ravindran *et al.*, 1999). Compared to Phytase C, Phytase A at 500 PU/kg diet released similar amounts of α-amino nitrogen in the wheat-based diet. In the corn-based diet, Phytase A at 500 PU/kg diet released 6.2% more α-amino nitrogen than Phytase C. As expected, *in vitro* viscosity value was highest in the unsupplemented wheat-based diet. The viscosity values in the diet supplemented with the Phytase B (500 and 750 PU/kg) were significantly lower than those in Phytase A. These results are difficult to explain since

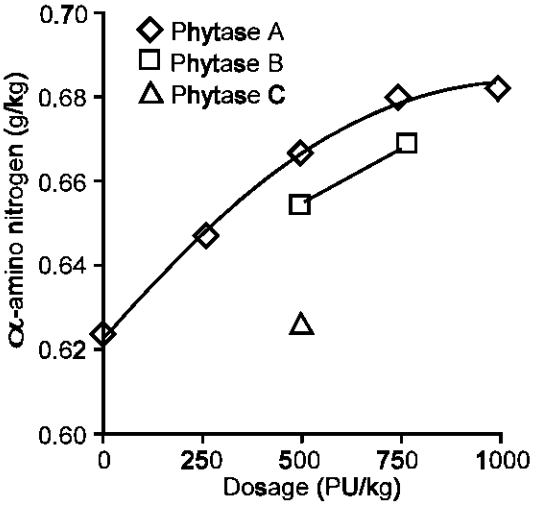

Fig. 7: Effect of source and concentration of phytase on dialyzable α-amino nitrogen in the corn-soy diet

Phytase B was blended to contain similar enzyme activities as Phytase A. It must be also noted that the viscosity measurements determined in this study (1.36 to 1.93 mPa.s) are too low to be of practical significance under *in vivo* situations. Digesta viscosity becomes a significant issue with regard to the performance responses of birds fed wheat-based diets, only in situations where the digesta viscosity is more than 10 mPa.s (Bedford and Schulze, 1998).

In summary, the results from this *in vitro* study showed that Phytase A, a product with side enzyme activities, produced better response in terms of nutrient release than Phytase C, a source of pure phytase. It should be, however, noted that these *in vitro* results may not be directly applicable to *in vivo* situations and therefore should be considered only as crude indicators of the relative efficacy of the enzymes evaluated. It will be necessary that these preliminary results be examined and confirmed in well-designed *in vivo* studies.

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