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ORIGINAL ARTICLE

Dietary phytate (inositol hexaphosphate) regulates the activity of intestinal mucosa phytase

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Summary

The role of dietary phytate (inositol hexaphosphate) in the regulation of intestinal mucosa phytase was investigated in chicks. Seven-day-old chicks were grouped by weight into six blocks of three cages with six birds per cage. Three purified diets [a chemically defined casein diet, a chemically defined casein diet plus sodium phytate (20 g/kg diet) and a chemically defined casein diet plus sodium phytate (20 g/kg diet) and microbial phytase (1000 units/kg diet)] were randomly assigned to cages within each block. Chicks were fed experimental diets from 8 to 22 days of age then killed, and duodenal mucosa and left tibia removed. Phytase activity in duodenal mucosa, growth performance and bone ash content were determined. Addition of phytate to the chemically defined casein diet reduced ($p < 0.05$) the V_{max} of the duodenal brush border phytase, but the K_m of the enzyme was not affected. Addition of phytate also reduced ($p < 0.05$) weight gain, feed intake, feed efficiency and percentage ash. Addition of microbial phytase fully restored the feed efficiency ($p < 0.05$), but V_{max} and body weight gain were only partially restored ($p < 0.05$). In conclusion, it would seem that dietary phytates non-competitively inhibit intestinal mucosa phytase.

Introduction

Phytates are salts of phytic acid [inositol hexaphosphate, myo-inositol 1,2,3,4,5,6-hexakis (dihydrogen phosphate)] and they occur in many oilseeds and cereals used in human and animal diets. Phytates occur as mixed salts of magnesium, calcium and potassium (Odani et al., 1997; Selle et al., 2000), and can be hydrolysed by phytase (myo-inositol-hexakisphosphohydrolase), a specific phosphohydrolase, to yield inositol monophosphate and orthophosphate via inositol penta- to monophosphates as intermediary products (Liu et al., 1998). Presence of intestinal mucosa phytase has been demonstrated in most animal species, including human and many domestic and laboratory animals (Bitar and Reinhold, 1972; Maenz and Classen, 1998; Onyango et al., 2001). The role of intestinal mucosa phytase in the hydrolysis of phytates in human and animal diets has not been fully investigated. In addition, the regulation of its activity is not clearly understood. An understanding of the factors involved in its regulation would be an important step in understanding the role of this intestinal enzyme. Previous studies have indicated that the amount of phytate in the diet could be important in influencing intestinal phytase activity (Lopez et al., 2000). However, these studies used crude preparations and measured the enzyme at alkaline pH. Crude preparations could contain other phytate-hydrolysing enzymes, especially intracellular phosphatases. Studies using purified brush border vesicles

have demonstrated that the pH optimum for chick intestinal mucosa phytase is approximately 6.0 (Maenz and Classen, 1998). Hence, measurements at alkaline pH may have been misleading. Therefore, the main objective of this study was to determine the role of dietary phytate in the regulation of purified chicken duodenal brush border phytase measured at a pH of 6. In addition, the role of exogenous phytase in modulating this possible regulatory role of phytate was investigated.

Materials and methods

One hundred thirty day-old Ross 308 male chicks were obtained from a commercial hatchery, wing-banded and raised in electrically heated cages and provided *ad libitum* access to water and a standard chick starter diet containing 223 g crude protein, 10 g calcium and 7.7 g total phosphorus per kg for 7 days. Cages had continuous lighting and temperature was kept at 35 °C. At 8 days of age, chicks were weighed and sorted by weight to select 108 chicks with least weight variation. These were grouped by weight into six blocks of three cages with six birds each. Birds were assigned such that the average body weight was similar across diets. Three diets were randomly assigned to the three cages within each block (randomized complete block design). The diets were: chemically defined casein diet (control diet; NRC, 1994), chemically defined casein diet + phytate and chemically defined casein diet + phytate + 1000 units/kg phytase (Phyzyme XP phytase, Danisco Animal Nutrition, Marlborough, Wiltshire, UK; Table 1).

Dextrose in the chemically defined casein (control) diet formulation was substituted with sodium phytate or phytase premix to make the phytate or phytate plus phytase diets. Dextrose was used as a carrier for phytase in the phytase premix. Water and the assigned experimental diets were provided *ad libitum* to the chicks from 8 to 22 days of age.

Birds were kept in cages with continuous lighting and temperatures were kept at 32 and 27 °C, for days 8–14 and 15–22 respectively. Growth performance data (body weight gain and feed intake) were recorded during the 14-day experimental period. All procedures were approved by the Purdue University Animal Care and Use Committee.

At 22 days of age, birds were killed by carbon dioxide asphyxiation and the small intestine was exteriorized. The duodenum was resected and contents gently squeezed out and flushed using 20-ml ice-cold 0.9% (physiological, 154 mM NaCl) saline. The mucosa of the resected section was dislodged out from the underlying tissue by firmly squeezing the section between a spatula and a solid surface placed on ice. The mucosa for all birds in a cage were pooled and put in a pre-weighed 50-ml centrifuge tube, weighed, placed in liquid nitrogen to allow quick freezing of the mucosa, and stored at −80 °C until further processing. The left tibia was excised from each bird, placed in a plastic bag and stored frozen at −20 °C. The tibiae were processed and percentage ash determined as described by Onyango et al. (2004). Crude brush border membrane vesicles were prepared from frozen intestinal mucosa according to the modified Mg^{2+}-precipitation procedure for chicken of Maenz and Engele-Schaan (1996). Frozen mucosa was homogenized for 2 min in buffer (50 mM D-mannitol, 50 mM HEPES, pH 7.4, 20 ml/g of mucosa) using a polytron homogenizer (Omni 2000 polytron homogenizer, Omni International, Waterbury, CT, USA). The resulting homogenate was centrifuged at 3750 *g* for 15 min at 2 °C. The supernatant was decanted into a beaker, volume measured, placed on ice on a magnetic stirrer and 1.01 M magnesium chloride added, while stirring, to achieve a concentration of 10 mM $MgCl_2$. Stirring was continued for 20 min and the homogenate was centrifuged again at 3750 *g* for 15 min at 2 °C. The resulting supernatant was centrifuged again at 29,400 *g* for 45 min at 2 °C to generate crude brush border membrane pellets. Pellets were resuspended in 1 ml buffer (300 mM D-Mannitol, 50 mM HEPES, pH 7.4), and kept in liquid nitrogen until further processing.

Purified brush border vesicles were prepared by thawing and resuspending 1-ml crude brush border vesicles in 20 ml buffer (300 mM D-Mannitol, 50 mM HEPES, pH 7.4) and homogenizing with 10 strokes in a pre-chilled glass tissue grinder (Wheaton tissue grinder, Wheaton, Miliville, NJ, USA). The preparation was centrifuged for 15 min at 591 *g* at 2 °C and the supernatant centrifuged for 45 min at 29,000 *g* at 2 °C to obtain purified brush border vesicle pellets. The supernatant was discarded and the pellet resuspended in buffer (300 mM D-Mannitol, 50 mM HEPES, pH 7.4) and kept on ice.

The protein content of the preparation was determined using a colorimetric assay kit (Pierce Biotechnology kit #23296/23596; Pierce Biotechnology, Rockford, IL, USA), based on the method of Bradford (1976). A Coomassie dye reagent (Coomassie Dye G-250, a modified Bradford reagent) coated on to wells was reacted with addition and mixing of 100 μl of sample or standard. The dye binds protein

Table 1 Composition (g/kg) and nutrient content of the experimental purified diets on as-fed basis†

Ingredient	Chemically defined casein diet (g/kg)	Chemically defined casein diet + phytate (g/kg)	Chemically defined casein diet + phytate + phytase (g/kg)
Dextrose	678.8	658.8	642.8
Casein	200	200	200
DL-methionine	5	5	5
L-arginine	10	10	10
Glycine	20	20	20
Corn oil	30	30	30
Vitamin A	0.008	0.008	0.008
Vitamin D_3	0.001	0.001	0.001
Vitamin E	0.04	0.04	0.04
Vitamin K	0.002	0.002	0.002
Ascorbic acid	0.25	0.25	0.25
Vitamin B_{12}	0.004	0.004	0.004
Biotin	0.03	0.03	0.03
Choline chloride	2	2	2
Folacin	0.005	0.005	0.005
Inositol	0.1	0.1	0.1
Niacin	0.05	0.05	0.05
Calcium panthothenate	0.03	0.03	0.03
Para-aminobenzoic acid	0.002	0.002	0.002
Pyridoxine HCl	0.006	0.006	0.006
Riboflavin	0.013	0.013	0.013
Thiamin HCl	0.022	0.022	0.022
$CaCO_3$	3	3	3
$Ca_3(PO_4)_2$	28	28	28
$MgSO_4 \cdot 7H_2O$	3.5	3.5	3.5
KH_2PO_4	9	9	9
NaCl	8.8	8.8	8.8
H_3BO_3	0.009	0.009	0.009
$CoSO_4 \cdot 7H_2O$	0.001	0.001	0.001
$CuSO_4 \cdot 5H_2O$	0.02	0.02	0.02
Ferric citrate	0.5	0.5	0.5
$MnSO_4 \cdot H_2O$	0.65	0.65	0.65
KI	0.04	0.04	0.04
$Na_2MoO_4 \cdot 2H_2O$	0.009	0.009	0.009
Na_2SeO_3	0.0002	0.0002	0.0002
$ZnCO_3$	0.1	0.1	0.1
Na phytate	0	20	20
Phytase premix‡	0	0	16
Calculated nutritive value			
ME_n (kJ/kg)	12,697	12,463	12,459
CP (%)	17	17	17

†Appropriate amount of dextrose in the basal (control) diet was replaced with phytate or phytase premix to make the phytate or phytate–phytase diet.
‡Phyzyme XP phytase enzyme premix contained 62.5 units of phytase per gram.

and changes colour from reddish-brown to blue, corresponding to an absorbance shift from 465 to 595 nm. Colour response is proportional to the amount of protein in the sample. Protein was determined colorimetrically with a spectrophotometer (SLT Spectra Shell Reader; Tecan US, Durham, NC, USA) at 595 nm. Protein contents of the samples were then diluted to contain 16 μg protein/μl.

Purified brush border vesicles have a 21-fold enrichment in the specific activity of alkaline phosphatase relative to the initial homogenate (Maenz and Engele-Schaan, 1996; Maenz and Classen, 1998). Specific activity of phytase [nanomoles of inorganic phosphate (Pi)/mg protein/min] in the diluted purified brush border vesicles was determined at a pH 6 and 25 mM $MgCl_2$, following the

method described by Maenz and Classen (1998). Purified brush border vesicle samples were incubated in triplicate wells with various concentrations of sodium phytate for a period of 10 min and at 37 °C. Each well contained 80 μg of vesicle protein. Phytase hydrolyses phytate to release inorganic phosphate. Released Pi was reacted with ammonium molybdate in an acid solution to form phosphomolybdate. A mixture of sodium bisulphite, sodium sulphite and 1-amino-2-naphthol-4-sulphonic acid reduces the phosphomolybdate to form a phosphomolybdenum blue complex. The intensity of the colour being proportional to the phosphate concentration and was measured at 660 nm. In order to describe the kinetics of the enzyme reaction, eight concentrations of the substrate, sodium phytate, was used (0, 0.025, 0.05, 0.075, 0.1, 0.2, 0.4 and 0.6 nM).

Enzyme kinetics data were analysed using nonlinear regression curve-fitting software (GraphPad® Prism, GraphPad Software, San Diego, CA, USA). Kinetics, growth performance and bone ash data were analysed using GLM procedures of SAS (SAS, 2002) appropriate for randomized complete block design using cage as the experimental unit. The level of statistical significance was set at $p < 0.05$. Means were separated using the possible differences option in SAS (SAS, 2002).

Results

The kinetics of duodenal mucosa phytase activity in chicks fed diets with different levels of phytate and microbial phytase from 8 to 22 days of age are presented in Fig. 1. Chicks fed chemically defined casein control diet with added phytate had reduced V_{max} of the intestinal mucosa phytase when compared with those fed the control diet without added phytate ($p < 0.05$; Table 2). The V_{max} of the intestinal mucosa phytase in birds fed the phytate diet with added microbial phytase was not different from that observed in chicks fed either the control diet or phytate diet. The K_m of the intestinal mucosa phytase was not different among the three diets. Mucosa yield in chicks fed the phytate diet showed a downward trend ($p = 0.058$) compared with those fed either the control diet or phytate diet with added microbial phytase.

A pattern similar to that noted for the kinetics of duodenal mucosa phytase was also seen in the effects of phytate and exogenous microbial phytase on growth performance criteria. Chicks fed a control diet with added phytate had less body weight gain, feed intake, gain-to-feed ratio and percentage tibia ash when compared with those fed the control diet without added phytate ($p < 0.05$; Table 3). Feed efficiency in chicks fed the phytate diet with added microbial phytase was higher ($p < 0.05$) than those fed the phytate diet but was not different from those fed the control diet. Although the body weight gain of chicks fed the phytate diet with added microbial phytase was higher ($p < 0.05$) than those fed the phytate diet it was, nevertheless, less ($p < 0.05$) than that of chicks fed the control diet. Feed intake and percentage tibia ash in chicks fed phytate diet with added microbial phytase were similar to those fed phytate diet, but lower ($p < 0.05$) than those chicks fed the control diet.

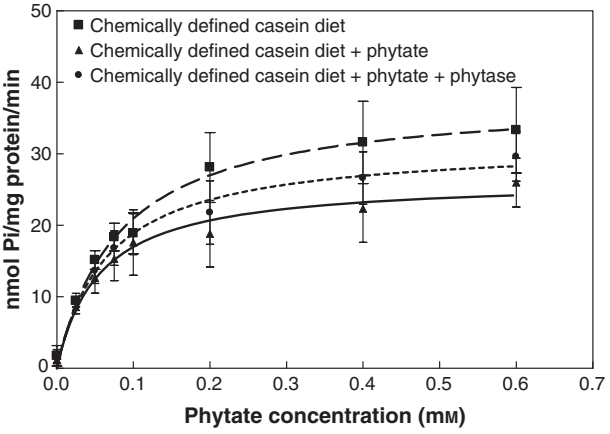

Fig. 1 Kinetics of duodenal mucosa phytase activity in chicks fed purified diets with different levels of phytate and microbial phytase from 8 to 22 days of age. Phytase activity in duodenal mucosa brushborder vesicles was determined by incubation with Na phytate for 10 min at 37 °C and pH 6. Error bars represent standard deviation. Values represent the mean of six cages with six birds per cage ($n = 6$).

Discussion

Phytates occur widely in plants (Reddy et al., 1982) and it is difficult to exclude them in diets, which use plant ingredients. The current study set out to determine the influence of a known form and amount of phytate on activity of intestinal mucosa phytase. A chemically defined casein diet was formulated in order to exclude phytates in the basal control diet. This ensured that the only source of phytate in the diet was that added into the formulation and hence the influence of added phytate could be delineated with certainty.

A number of enzymes, especially in the gastrointestinal tract, are regulated by their substrates or products (Ferraris and Diamond, 1989; Kutchai,

Table 2 Duodenal mucosa yield and phytase activity in chicks fed purified diets with different levels of phytate and microbial phytase from 8 to 22 days of age†

Item	Mucosa yield (g/bird)	V_{max} (nmol P released/mg protein/min)	K_m (mm phytate)
Chemically defined casein diet	22.5	38.57[a]	0.084
Chemically defined casein + phytate diet	19.0	26.51[b]	0.057
Chemically defined casein + phytate + phytase diet	22.8	31.52[ab]	0.068
SD‡	2.7	7.17	0.024

[ab]Means within a column not sharing a common superscript letter differ ($p < 0.05$).
†Values represent the mean of six cages with six birds per cage ($n = 6$).
‡SD = Pooled standard deviation.

Table 3 Growth performance and tibia ash in chicks fed purified diets with different levels of phytate and microbial phytase from 8 to 22 days of age†

Item	Weight gain‡ (g/bird)	Feed intake‡ (g/bird)	Gain/feed‡ (g/g)	Tibia ash‡ (%)
Chemically defined casein diet	485[a]	622[a]	0.77[a]	52.2[a]
Chemically defined casein + phytate diet	348[c]	490[b]	0.71[b]	50.8[b]
Chemically defined casein + phytate + phytase diet	411[b]	541[b]	0.76[a]	50.6[b]
SD§	44	47	0.02	1.0

[abc]Means within a column not sharing a common superscript letter differ ($p < 0.05$).
†Average initial body weight of the chicks was 150 g.
‡Values represent the mean of six cages with six birds per cage ($n = 6$).
§SD = Pooled standard deviation.

1998). For example, pancreatic amylase and chymotrypsin are regulated by dietary carbohydrate and protein (Ferraris and Diamond, 1989). In addition, presence of high levels of inorganic phosphates has been shown to repress synthesis of some phosphatases (Shieh et al., 1969). Hence, presence of phytate, a substrate for phytase, in the digestive tract may be expected to regulate the intestinal phytase activity. In the current study, phytate depressed activity of the mucosal phytase activity. This finding is in agreement with that of Roberts and Yudkin (1961), who reported that addition of bran or sodium phytate (at 50 g/kg) to diets fed to rats for 4 weeks decreased intestinal phytase activity of the rats. In contrast to their findings, chicks fed, for 3–4 weeks, diets supplemented with calcium or sodium phytate did not show a change in activity of intestinal phytase (Davies et al., 1970). Yang et al. (1991), on the contrary, fed rats diets more similar to ones fed in the current study and observed increased phytase activity and amount of 90K subunit in rats fed 16 g sodium phytate/kg purified diet for 2 days. Lopez et al. (2000) reported that rats fed for 10–20 days on diets with added sodium phytate (at a rate of 5.5 g/kg diet) or wheat bran (containing 5.4 g phytic acid/kg diet) showed increased intestinal phytase activity. There seems to be contrasting findings on the effect of phytate inclusion in diets. Differences in findings reported in the different studies could be as a result of the animal species used, different levels of inclusion of phytates, diet formulations (purified vs. non-purified diets), pH at which phytase activity was measured and purity of samples (crude vs. purified preparations). For example, rats have been shown to have higher intestinal phytase activity in relation to chicks (Bitar and Reinhold, 1972). In another example, in the chick study by Davies et al. (1970), phytase activity was measured using crude preparations and at alkaline pH of 7.4, whereas on the contrary, phytase activity in the current study was measured in purified brush border vesicles at a pH of 6.0. This could contribute to the different results observed in the two studies.

In the current study, addition of sodium phytate to the control diet depressed the maximal activity of the mucosa phytase without affecting the enzyme's affinity for its substrate. When exogenous microbial phytase was added to the phytate-supplemented diet, the maximal enzyme activity increased to a level similar to that observed in chicks fed the control diet with no added phytate. This suggests that the depression in enzyme activity was indeed

because of the presence of phytates. Depression in maximal activity may indicate a reduction in the amount of active enzyme because of inhibition or simply because of downregulation of enzyme production. The exact mechanism seems unclear. However, the findings of Yang et al. (1991), in which they reported increased phytase activity and amount of 90K subunit when suckling rats were fed a purified diet with added sodium phytate, may suggest a possible genomic mechanism for the effects of phytate on intestinal mucosa phytase. Nevertheless, depression of the V_{max} with no effect on K_m is characteristic of pure non-competitive inhibition, in which the inhibitor interacts both with the free and bound enzyme and even with the free substrate, without affecting the binding sites for substrate (Garrett and Grisham, 1999; Jemiolo and Theg, 1999). In contrast to pure non-competitive inhibition, mixed non-competitive inhibition binding of the inhibitor affects the binding sites for substrate, altering both the V_{max} and K_m. It would, therefore, appear that phytate caused pure non-competitive inhibition of intestinal phytase in the current study. Indeed phytates have been reported to inhibit a variety of digestive enzymes such as carboxypeptidase A (Martin and Evans, 1989), lipase (Knuckles, 1988), chymotrypsin (Sathe and Sze-tao, 1997), pepsin (Kanaya et al., 1976; Inagawa et al., 1987; Vaintraub and Bulmaga, 1991), α-amylase (Sharma et al., 1978; Knuckles and Betschart, 1987) and alkaline phosphatase (Martin and Evans, 1991).

Phytic acid, a moderately strong acid, has 12 ionizable protons (Martin and Evans, 1986). Of the 12 protons, six (one from each phosphate) have pKa values of 1.1–2.1, three others are weakly acidic with pKa values of 5.7–7.6 and the pKa values for the remaining three is 10–12 (Costello et al., 1976). Therefore, the phytate ion, for a wide pH range has the capacity to chelate various cations between two phosphate groups or weakly within a phosphate group (Erdman, 1979). Consequently, the phytate ion is able to form complexes with proteins/peptides through basic and acidic elements in the amino acid residues or directly with amino acids (Sharma et al., 1978; Singh and Krikorian, 1982). Formation of such complexes is thought to be the cause of its antinutritional effect in reducing digestibility of nutrients such as minerals (Hallberg et al., 1987; Torre et al., 1991; Pallauf et al., 1992), proteins (Ravindran et al., 1999) and possibly starch (Thompson, 1986). Because enzymes are proteins, they can form phytate–protein complexes that have been shown to reduce the solubility and activity of proteins (Maga, 1982; Grynspan and Cheryan, 1989). Reduced solubility of proteins as a result of phytate–protein complexes can adversely affect certain functional properties of proteins that are dependent upon their hydration and solubility (Reddy et al., 1982). Therefore, formation of phytate–enzymic protein complexes may reduce the effectiveness of complexed enzymes in the digestive process (Jenab and Thompson, 2002). This could be one of the mechanisms by which phytate reduced the duodenal brush border phytase activity in the present study. Addition of exogenous phytase to diets containing phytates would therefore hydrolyse phytates and so interfere with phytate–enzymic protein complexes. Such action would be expected to relieve the inhibition of the intestinal phytase and consequently there would be an increase in its activity. This could explain findings in the present study where higher brush border phytase activity was observed in chicks fed phytate diet with added microbial phytase in relation to those fed phytate diet alone without added microbial phytase.

Addition of phytate to the diet depressed the weight gain, feed intake and efficiency, and percentage tibia ash of chicks in the current study. These antinutritional effects of phytates have been documented by many workers (Maga, 1982; Ravindran et al., 2000). Perhaps the observed growth depression in chicks that received phytate-supplemented chemically defined casein diet resulted from the capacity of phytate to complex and chelate nutrients with a consequence of making these nutrients unavailable to the bird. The tendency for a lower mucosa yield noted in the present study when phytate was added to the diet may have been part of the generalized growth depression noted. Alternatively, it may have been an adaptive response, by the bird, to try to reduce use of scarce nutrients to maintain the intestine. Either way, the decrease in mucosa could mean that the animal may have become less efficient in absorption of nutrients with the consequence of further growth depression. Hydrolysis of phytates using added exogenous microbial phytase mitigated some of the depression in growth performance.

In summary, the presence of phytates in a chemically defined casein diet reduced maximal activity of duodenal brush border phytase, body weight gain, feed intake and efficiency, and tibia ash of chicks. Addition of phytase reduced some of the effects of phytate. Because V_{max} was altered, whereas K_m was not; it may, therefore, be concluded that phytate

non-competitively inhibited intestinal mucosa phytase.

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