# Towards Complete Dephosphorylation and Total Conversion of Phytates in Poultry Feeds

K. Żyła,*,[1] M. Mika,* B. Stodolak,* A. Wikiera,* J. Koreleski,† and S. Świątkiewicz†

*University of Agriculture, Department of Food Biotechnology, 29-Listopada Ave. 46, 31-425 Kraków, Poland; and †Institute of Zootechnics, Research Farm of Balice, Department of Animal Nutrition in Aleksandrowice, 32-083 Balice, Poland

**ABSTRACT** The rate of phytate P removal from feed (level of dephosphorylation, DL) and the extent to which the molecule of phytic acid is deprived of phosphate moieties (conversion degree, CD) were studied in vitro and in a feeding trial with broilers fed corn-soybean diets. In the in vitro model, phytase A asymptotically increased DL and CD. Phytase B influenced DL only at low dosages of phytase A [0 or 250 phytase activity units (FTU)/kg], but it enhanced CD irrespective of phytase A activity. In the feeding trial, 3-phytase A and 6-phytase A (at 750 FTU/kg) exerted similar effects on broiler performance and similarly influenced bone mineralization, P retention, and Ca retention. Phytase B [6,400 acid phosphatase activity units (ACPU)/kg] enhanced feed intake, BW gain (BWG), toe ash, and P retention but not the retention of Ca. *Myo*-inositol fed at 0.1% significantly increased BWG, but it reduced P retention. Under conditions of a higher CD (excess of phytase B), 3-phytase A was more effective in enhancing performance than 6-phytase A, but it reduced Ca retention. Lower phytase B activities (0 to 3,200 ACPU/kg) with added 6-phytase A were more necessary for optimal growth of chickens than for enhanced P and Ca retention (4,800 to 6,400 ACPU/kg). The efficacy of both forms of phytase A and phytase B depended on the Ca level in feed. There is enough evidence to conclude that *myo*-inositol phosphates resulting from simultaneous action of 3-phytase A and phytase B affect bird physiology differently than intermediates accumulated by the action of 6-phytase A and phytase B.

(*Key words*: broiler, conversion degree, dephosphorylation level, phytase A, phytase B)

2004 Poultry Science 83:1175–1186

## INTRODUCTION

Since the pioneering work of Nelson et al. (1968) numerous studies have been performed on phytase applications in poultry feeding. In those studies the efficacy of *Aspergillus niger* (*ficuum*) 3-phytase A (EC.3.1.3.8) has been investigated as it relates to phytate P removal from feed, increased digestibility of minerals and protein, and reduced content of P in animal manures (Sebastian et al., 1998; Wodzinski and Ullah, 1999). In contrast to 3-phytase A, which hydrolyzes first D-3 phosphate residue on the *myo*-inositol ring of phytate, 6-phytase A (EC.3.1.3.26) removes first the L-6 residue. This enzyme, although found mainly in plant seeds, is commercially available as microbial (*Peniophora lycii*) 6-phytase A engineered in an *Aspergillus oryzae* host (Ward, 2000). Phytase B, the enzyme with broad substrate specificity, more commonly known as a nonspecific acid phosphomonoesterase (EC.3.1.3.2) that removes phosphate groups from many different compounds and dephosphorylates *myo*-inositol phosphates lower than hexaphosphate, is also available, at least in experimental preparations.

The efficacy of 3- and 6-phytases A in poultry feeds is limited. Phytate occlusion with other tissue components, its limited solubility in different parts of the intestinal tract, and its short reaction time in the intestine of birds are major obstacles reducing efficacy of both forms of commercial phytase A in phytate removal from plant tissues. Different strategies have been suggested to overcome the intrinsic barriers of feed dephosphorylation by both forms of commercial phytase A (Denbow et al., 1998; Huff et al., 1998), and their effectiveness has also been compared (Żyła, 2001). It is important to realize that the pH and time limitations of a bird's gastrointestinal tract as well as the catalytic properties of microbial both forms of phytase A do not permit phytate molecules to be totally converted into free *myo*-inositol and inorganic phosphate, as both forms of phytase A are not able to liberate the C-2 axial phosphate group from phytic acid molecule. The



Received for publication December 15, 2003.

Accepted for publication February 9, 2004.

[1]To whom correspondence should be addressed: kzyla@ar.krakow.pl.

**Abbreviation Key:** ACPU = acid phosphatase activity unit; BWG = BW gain; CD = conversion degree; DL = dephosphorylation level; FCR = feed conversion ratio; FTU = phytase activity unit; IP3 = *myo*-inositol triphosphate; NC = negative control; NPP = nonphytate phosphorus.

[2]Finase P, AB Enzymes Finland Oy, Rajamäki, Finland.

[3]Ronozyme P, Hoffman la Roche, Switzerland.

C-2 phosphate residue on the *myo*-inositol ring of phytate is hydrolyzed, however, by the action of phytase B.

The theory of enzymic breakdown of phytate compounds distinguishes between liberation of phytate molecules from complexes with other tissue components and enzymic cleavage of phosphate residues on the *myo*-inositol ring. The concept of complete dephosphorylation and total conversion of feed phytates relates to these 2 distinct phenomena (Żyła, 2001). Complete dephosphorylation means that each phytate molecule of feed ingredients is extracted from plant tissues and undergoes at least limited enzymatic degradation, and consequently, the concentration of phytates measured in feed after digestion is close to zero. The extent of dephosphorylation may be expressed by dephosphorylation level (DL), defined as a percentage of total P that is removed from feeds. Phytases A and organic acids are the necessary tools to differentiate degree of feed dephosphorylation, but removal of the whole phytate contents from feeds is not possible. Total conversion, on the other hand, may be accomplished at different levels of feed dephosphorylation when phytate molecules, liberated previously from plant tissues, are hydrolyzed into *myo*-inositol and phosphate, and these 2 substances are the sole reaction end products. The extent of conversion may be expressed by conversion degree (CD), which we defined as a percentage of P freed from feeds that is dialyzable in an in vitro procedure simulating conditions of the intestinal tract. Better measure of the CD would be an increase in free *myo*-inositol concentration, provided that an appropriate method is available. The concentration of lower *myo*-inositol phosphates in the gastrointestinal tract may be altered by simultaneous catalytic action of 3- or 6-phytase A and phytase B. It seemed of interest therefore to study the nutritional role of lower *myo*-inositol phosphates and the role of free *myo*-inositol that may be produced in substantial quantities when 3- or 6-phytase A and phytase B are added to feeds. Furthermore, the 2 types of phytase A (3- and 6-phytase) with distinct substrate specificity produce different species of lower *myo*-inositol phosphates (Wyss et al., 1999; Oh et al., 2003). The phytate molecule should be perceived, therefore, not only as a chelating agent whose antinutritional properties may be reduced by catalytic action of both forms of phytase A but also as a source of *myo*-inositol and lower phosphates of *myo*-inositol supplied by the common action of phytases A and B. It remains an open question whether under time and pH limitation of the gastrointestinal tract phytate conversion may advance to such an extent that the organism is deprived of *myo*-inositol phosphate intermediates.

The objectives of the current studies were to measure the influence of tiered concentrations of 3-phytase A and phytase B on the DL and the CD using an in vitro digestion procedure as well as to distinguish effects of improved dephosphorylation from those related to enhanced phytate conversion on the performance, bone mineralization, and P and Ca retentions in growing broilers fed corn-soybean meal diets. Other objectives were to study the effects of different types of phytase A (3- and 6-phytase) and variable Ca levels on the in vitro release of P and Ca from feeds as well as on performance and P and Ca metabolisms of growing broilers.

## MATERIALS AND METHODS

### Enzymes

We used commercial preparations of microbial 3-phytase A (EC.3.1.3.8; Finase P),[2] which had a phytase activity of 5,250 units/g (declared by the producer). Finase P phytase is synthesized by *Trichoderma reesei* GMO carrying the *phyt A* gene. The preparation (Ronozyme P) of 6-phytase A[3] (EC.3.1.3.26) was donated by the manufacturer. The phytase, with declared activity of 2,583 units/g, is produced by a genetically modified *Aspergillus oryzae* strain that hosts *phyt A* gene from the fungus *Peniophora lycci* (*Basidiomycetes* class). The preparation Finase AP of phytase B (acid phosphatase; EC.3.1.3.2) was obtained from the same supplier as in the previous study (Żyła et al., 2000a).[2] It had acid phosphatase declared activity of 184,000 units/g.

### Enzyme Activity Measurements and Units

Activities of phytases A and acid phosphatase were determined using procedures described previously (Żyła et al., 2000a), and units of enzymic activity used in the current study were the same as in the previous work. Measured activities of the preparations were 4,132 phytase activity units (FTU)/g for Finase P, 3,221 FTU/kg for Ronozyme P, and 12,680 acid phosphatase activity units (ACPU)/g for Finase AP.

### In Vitro Digestions and Measurements

Determinations of P in the dialyzates of feed samples ($P_{dial}$) subjected to a procedure of multiple digestions were performed using methods described previously (Żyła et al., 2000a). Briefly, in this model, pepsin and pancreatin digestion periods are preceded by preincubation at pH 5.80 to simulate digestion in broiler crops. Triplicate samples (1 ± 0.001 g) of a diet, ground through a 1-mm screen, were weighed into 5-mL plastic syringes without Luer-lock tips. The feed samples were hydrated with a double distilled water and HCl solution so that a pH value of 5.80 was obtained. When enzyme solution was applied, water was partly (or completely) substituted for by the investigated enzyme fractions. The contents of each tube were vortexed, and then the tubes were sealed with parafilm and incubated in a water bath at 40°C for 30 min. Next, 0.5 mL of 1.5 *M* HCl and 3,000 units of pepsin were added to each tube, mixed well, vortexed, sealed with parafilm, and reincubated for 45 min at the same temperature. At the end of this period, 0.455 mL of 1

[2]Finase P, AB Enzymes Finland Oy, Rajamäki, Finland.
[3]Ronozyme P, Hoffman la Roche, Switzerland.

*M* $NaHCO_3$ containing 5.6 mg/mL pancreatin was added dropwise into each tube with constant stirring. The slurry was transferred quantitatively to segments of dialysis tubing by means of the syringe piston. Segments were placed in 250-mL Erlenmeyer flasks containing 100 mL of 0.1 *M* NaCl in 0.05 *M* succinate buffer (pH 6.10) and incubated in a shaking water bath at 41.1°C (temperature of dialysate was 40°C). Samples of the dialysate were withdrawn after 240 min for determining inorganic phosphate. Additionally, samples of digested feed that remained in the dialysis tubing at the end of the multistep procedure were assayed for residual P ($P_{res}$). The contents of the dialysis tubing were dried and mineralized as described for total P ($P_{tot}$) determination. The concentrations of Ca in the dialysates of feed samples were assayed colorimetrically using chlorophosphonazo reagent and a procedure similar to that described by Ma (1999). The concentration of Ca in samples of digested feed subjected to the wet mineralization procedure was determined colorimetrically by the calmagite method (Marczenko and Balcerzak, 1998) and validated by flame atomic absorption spectrophotometry assay. The DL was calculated using the following formula:

$$DL = (P_{tot} - P_{res})/P_{tot} \times 100\ (\%),$$

whereas CD was calculated as follows:

$$CD = P_{dial}/(P_{tot} - P_{res}) \times 100\ (\%).$$

## Experimental Design, Birds, Diet Composition, and Preparation

In the in vitro part of the study, the influence of tiered concentrations of 3-phytase A and phytase B on DL and CD was determined in a multilevel factorial completely randomized design (2 factors, 5 levels). The activity of phytase A varied from 0 to 1,000 FTU/kg of feed in increments of 250 FTU/kg, whereas the activity of phytase B was changed from 0 to 6,400 ACPU/kg of feed in increments of 1,600 ACPU/kg.

In the feeding experiment, only one level (750 FTU/kg) of 3- or 6-phytase A was used, whereas the activity of phytase B in dietary treatments was 0, 1,600, 3,200, 4,800, or 6,400 ACPU/kg of feed (Table 1). One-day-old Ross broiler chickens (n = 800) were purchased from a commercial hatchery[4] and allotted randomly to stainless steel battery brooders with wire-mesh floors. The temperature was maintained at 32 ± 1°C during the first week. Every 7 d the temperature was decreased by 2°C. Lighting was continuous, and feed and water were provided ad libitum. The experimental design consisted of 20 dietary treatments with 5 replicates of 8 birds allotted randomly to each dietary treatment from d 1 to 21.

Broilers were fed the following 20 diets. A low-P, low-Ca basal diet (negative control 1, NC1, diet 1) was formulated to contain 0.12% nonphytate P (NPP) and 0.65% Ca (Table 2). Diet 2 was the NC1 diet supplemented with phytase B at 6,400 ACPU/kg of diet. Diets 3 and 4 were the NC1 diets with 3- and 6-phytase A, respectively, fed at 750 FTU/kg of feed. Diet 5 was the NC1 diet supplemented with 3-phytase A and phytase B, and diets 6 to 9 contained 6-phytase A and tiered levels of phytase B (1,600, 3,200, 4,800, and 6,400 ACPU/kg in diets 6, 7, 8, and 9, respectively). Diet 10 (NC2) had the same composition as diet 1, but the Ca content was raised to 0.8%. Diet 11 was the NC2 diet supplemented with phytase B as in diet 2, and diets 12, 13, 14, and 15 were the NC2 diets supplemented as diets 3, 4, 5, and 9, respectively. Other control diets included 0.27% NPP and 0.65% Ca (diet 16), 0.47% NPP and 0.80% Ca (diet 18), and 0.47% NPP and 0.98% Ca (diet 20, NRC). Diets 17 and 19 had the same composition as the diets 16 and 18, respectively, but were supplemented with 0.1% of *myo*-inositol.[5] In the control diets, levels of NPP and Ca were adjusted by changing amounts of dicalcium phosphate and limestone added to the feed. Diets 3 to 9 and 12 to 15 were also supplemented with 3% of citric acid. A detailed description of dietary treatments is given in Table 1. Powdered enzymes were premixed with a small quantity of feed and added to the remaining part of a diet during final mixing. All the diets were formulated to be isonitrogenous and isocaloric. The protein and energy contribution from enzyme addition was considered insignificant. In the course of the experiment all diets were stored in a cooler at 4°C.

An experimental design used in the feeding experiment was chosen so that multiple comparisons among treatments that differed in DL and in CD were possible. Comparison of treatments 1, 3, 4, 10, 12, and 13 allowed to assess effects of different DL produced by 2 types of phytase A and citric acid in feeds that contained different Ca levels at a low CD. In treatments 2, 5, 9, 11, 14, and 15, the excess of phytase B provided conditions for a higher CD as a background to learn the effects described above. In treatments 4, 6, 7, 8, and 9 the CD was increased gradually by tiered activities of phytase B from 0 to 6,400 ACPU/kg in feeds that were supplemented with a constant activity (750 FTU/kg) of 6-phytase A and citric acid. Supplementation of diets 1 and 10 with phytase B (6,400 ACPU/kg; diets 2 and 11) revealed effects of a substantial increase in CD at 2 Ca levels with low dephosphorylation. Finally, comparison of treatments 16, 17, 18, and 19 allowed estimation of impacts of pure *myo*-inositol supplemented at 0.1%, the concentration that reflected total conversion of phytate, in diets with higher NPP levels that differed in Ca/P ratios.

## Sample Collection and Assays

At the end of wk 3 of the experiment, chicks were weighed individually, and feed consumption was determined for each pen. During the second and third weeks (d 11 to 16) total collection of excreta from each pen was

[4]Commercial Hatchery "Zielonki", Krakow, Poland.
[5]Sigma Chemical Inc., St. Louis, MO.

**TABLE 1. Characteristics of dietary treatments fed to growing broiler chicken, d 1 to 21 posthatching[1]**

| Treatment | Phytase A (750 FTU/kg) | Phytase B (ACPU/kg) | *Myo*-inositol (%) | NPP (%) | Ca (%) | Citric acid (%) |
|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0.12 | 0.65 | 0 |
| 2 | 0 | 6,400 | 0 | 0.12 | 0.65 | 0 |
| 3 | 3-phytase | 0 | 0 | 0.12 | 0.65 | 3 |
| 4 | 6-phytase | 0 | 0 | 0.12 | 0.65 | 3 |
| 5 | 3-phytase | 6,400 | 0 | 0.12 | 0.65 | 3 |
| 6 | 6-phytase | 1,600 | 0 | 0.12 | 0.65 | 3 |
| 7 | 6-phytase | 3,200 | 0 | 0.12 | 0.65 | 3 |
| 8 | 6-phytase | 4,800 | 0 | 0.12 | 0.65 | 3 |
| 9 | 6-phytase | 6,400 | 0 | 0.12 | 0.65 | 3 |
| 10 | 0 | 0 | 0 | 0.12 | 0.80 | 0 |
| 11 | 0 | 6,400 | 0 | 0.12 | 0.80 | 0 |
| 12 | 3-phytase | 0 | 0 | 0.12 | 0.80 | 3 |
| 13 | 6-phytase | 0 | 0 | 0.12 | 0.80 | 3 |
| 14 | 3-phytase | 6,400 | 0 | 0.12 | 0.80 | 3 |
| 15 | 6-phytase | 6,400 | 0 | 0.12 | 0.80 | 3 |
| 16 | 0 | 0 | 0 | 0.27 | 0.65 | 0 |
| 17 | 0 | 0 | 0.1 | 0.27 | 0.65 | 0 |
| 18 | 0 | 0 | 0 | 0.47 | 0.80 | 0 |
| 19 | 0 | 0 | 0.1 | 0.47 | 0.80 | 0 |
| 20 | 0 | 0 | 0 | 0.47 | 0.98 | 0 |

[1]One unit of phytase activity (FTU) is the amount of enzyme required to liberate 1 $\mu M$ of inorganic phosphorus from 2 m*M* sodium phytate in 1 min at 40°C, pH 4.5. One unit of acid phosphatase activity (ACPU) is equal to 1 $\mu M$/min of *p*-nitrophenol liberated from 5.5 m*M* disodium *p*-nitrophenylphosphate at 40°C and pH 4.5.

carried out. Excreta were stored in plastic bags at –20°C. After being thawed, the excreta were dried in an oven at 50°C to constant weight, weighed, and ground to pass a 1-mm sieve. On d 21, the chicks were killed by cervical dislocation. Toe samples were obtained by carefully and uniformly severing the middle toe from each foot between the second and third tarsal bones. The samples were dried at 100°C for 24 h, weighed, and dry-ashed at 600°C overnight for determination of toe ash. Selected organs (liver, spleen, and bursa of Fabricius) were excised and weighed.

**TABLE 2. Composition and nutrient content of the low-P, basal diet (negative control 1, diet 1)**

| Ingredient | (%) |
|---|---|
| Corn meal | 56.38 |
| Soybean meal | 38.00 |
| Vegetable oil | 3.60 |
| Limestone | 0.97 |
| Salt | 0.30 |
| Lysine | 0.10 |
| DL-Methionine | 0.15 |
| Vitamins and minerals[1] | 0.50 |
| Composition | |
| Metabolizable energy (kcal/kg) | 2,980 |
| Crude protein | 21.60 |
| Nonphytate P | 0.12 |
| Total P | 0.41 |
| Total analyzed P | 0.44 |
| Ca | 0.65 |
| Analyzed Ca | 0.70 |
| Lysine | 1.16 |
| Methionine | 0.47 |

[1]Supplied per kilogram of feed: retinyl acetate, 12,000 IU; cholecalciferol, 3,000 IU; DL-$\alpha$-tocopheryl acetate, 20 mg; menadione sodium bisulfite, 3 mg; thiamin mononitrate, 2 mg; riboflavin, 6 mg; pyridoxine, 2 mg; cyanocobalamin, 15 $\mu$g; nicotinic acid, 20 mg; Ca pantothenate, 12 mg; folic acid, 1 mg; biotin, 50 $\mu$g; choline-HCl, 200 mg; Mn, 65 mg; Zn, 50 mg; Fe, 20 mg; Cu, 6 mg; I, 0.5 mg; Se, 0.1 mg; Co, 0.2 mg; ethoxyquin, 125 mg; virginiamycin, 15 mg; diclazuril, 1 mg.

Duplicate samples of feed were digested by a wet-ash procedure. Phosphorus concentration was determined colorimetrically by the molybdo-vanadate method (AOAC, 1995). Calcium concentration in feed and manure samples was analyzed by flame atomic absorption spectrophotometry.

## Statistical Analysis

The influence of tiered concentrations of phytase A and phytase B on DL and on CD was determined in a multilevel factorial completely randomized design (2 factors, 5 levels) using the Experimental Design Module of Statgraphics Plus for Windows (1996). The layout was balanced, and 2 replicate observations were performed at each of the treatment combinations. Results were subjected to regression analysis that followed the model given by Wu and Hamada (2000), and nonsignificant components were removed from the regression models. The regression equations were evaluated by computing determination coefficients ($R^2$) and probabilities of the lack of fit. Data from broiler experiment were analyzed by the GLM procedure of Statgraphics Plus for Windows (1996). ANOVA was performed following models of one-way ANOVA: $X_{ij} = \mu + \alpha_i + e_{ij}$, where $\mu$ = overall mean, $\alpha_i$ = *i*th treatment effect, and $e_{ij}$ = error contribution with average 0 and variance $\sigma^2$ and two-way ANOVA: $X_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + e_{ij}$, where $\mu$ = overall mean, $\alpha_i$ = first factor effect, $\beta_j$ = second factor effect, $(\alpha\beta)_{ij}$ = interaction between factors, $e_{ij}$ = error contribution with average 0 and variance $\sigma^2$; and i = 1…a, j = 1…b (Wu and Hamada, 2000). Mean differences were determined using Fisher's least significant difference test. Statistical significance was accepted at $P < 0.05$. Organ weights were adjusted for final body weight by covariance analysis.

**TABLE 3. Effects of tiered concentrations of 3-phytase A (x1) and phytase B (x2) on dialyzable P ($P_{dial}$), dephosphorylation level (DL), and phytate conversion degree (CD) of a corn-soybean meal feed (diet 1)[1]**

| | x1 | | x2 | | x1x2 | | $x1^2$ | | $x2^2$ | | Model | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Parameter | *F* | *P* | *F* | *P* | *F* | *P* | *F* | *P* | *F* | *P* | R2 (%) | Lack of fit (*P*) |
| | ANOVA | | | | | | | | | | | |
| $P_{dial}$ | 2,518 | 0.0001 | 3,999 | 0.0003 | 9 | 0.0559 | 448 | 0.0002 | 58 | 0.047 | 95 | 0.1106 |
| DL | 523 | 0.0002 | 19 | 0.0217 | 22 | 0.0184 | 122 | 0.0016 | 0.1 | 0.836 | 97 | 0.8152 |
| CD | 1,084 | 0.0001 | 354 | 0.0003 | 6 | 0.0954 | 237 | 0.0006 | 75 | 0.033 | 92 | 0.1192 |
| | Main effects estimates | | | | | | | | | | Average | |
| $P_{dial}$ (mg/g) | 1.9949 | | 0.7943 | | –0.1643 | | –1,4374 | | –0.5175 | | 3.0861 | |
| DL/100 (%) | 0.3179 | | 0.0613 | | –0.0885 | | –0.2622 | | –0.0053 | | 0.8212 | |
| CD/100 (%) | 0.2878 | | 0.1645 | | –0.0286 | | –0.2301 | | –0.1290 | | 0.7221 | |

[1]Multilevel factorial design (2 factors, 5 levels), error df = 67, 2 blocks, 75 runs.

# RESULTS

## Preliminary Estimation

***Effects of Phytases.*** A multilevel factorial completely randomized design (2 factors, 5 levels) was used to assess the influence of tiered activities of 3-phytase A and phytase B on the P release from feeds on DL and phytate CD. All the parameters studied were influenced mainly by the activity of phytase A whose effects had significant linear and quadratic components (Table 3). Phytase B significantly enhanced all the studied parameters with the most pronounced effect on CD and only minor effect on DL. More detailed analysis of the experimental data revealed that phytase B affected DL only in absence or in the presence of a low dosage (250 FTU/kg) of phytase A (Figure 1A). To the contrary, irrespective of phytase A activity in the feed, phytase B significantly increased phytate CD (Figure 1B). Whereas CD produced by phytase A added as a sole supplemental enzyme did not exceeded 60%, simultaneous application of phytase A and phytase B raised the parameter to 75%. The highest values of CD were achieved by feed supplementation either with phytase A minimally at 500 FTU/kg and a low activity of phytase B (1,600 ACPU/kg) or with a low activity of phytase A (250 FTU/kg) and a high activity of phytase B. When the activity of phytase A was kept at 250 FTU/kg, tiered activities of phytase B caused a linear increase in CD. At higher activities of phytase A, on the other hand, the lowest dosage of phytase B was sufficient to bring CD to highest levels.

In an experiment that was carried out using the in vitro multidigestion procedure, P and Ca release from corn-soybean meal feed by 3-phytase A and 6-phytase A added at 750 FTU/kg alone, as well as in combination with phytase B (6,400 ACPU/kg), were studied. Supplemental enzymes and the level of Ca in feed affected amounts of both nutrients released during in vitro digestions (Table 4). Phytase B applied in combination with phytases A enhanced both P and Ca release irrespective of the type of phytase A used. The 3-phytase A appeared to be more effective than 6-phytase A in feed dephosphorylation and in Ca release under conditions that simulated digestion in the intestinal tract of broilers. The higher level of Ca in feed lowered amounts of P released from the control diet and suppressed feed dephosphorylation by all kinds of phytases, but amounts of Ca released from feed samples were positively related to its content in feed.

## Broiler Feeding Experiment

Significant main effects of dietary additions and Ca level and a significant Ca level by dietary additions interactions were observed for feed intakes and 21-d BWG of chicken from treatments 1, 3, 4, 10, 12, and 13 (Table 5). Increasing DL by addition of 3- or 6-phytase A also influenced feed conversion ratio (FCR), percentage of ash in the toes, and retention of P but had no effect on the retention of Ca. The 2 forms of phytase A exerted similar effects on all the parameters studied.

Phytase B fed as a sole supplemental enzyme at 6,400 ACPU/kg produced conditions of increased CD at low DL in feeds containing 0.6 or 0.8% Ca. There were significant effects of phytase B and Ca levels on feed intake, BWG, and P retention as well as a significant phytase B × Ca level interaction for BWG of chickens, but no effects were observed in relation to Ca retention in broilers. The percentage of ash in the toes was significantly increased by phytase B but was not affected by different Ca levels (Table 6). Feed supplementation with phytase B resulted in substantial increases in feed intake, BWG, toe ash, and P retention. Similar to both forms of phytase A, the higher Ca level in feeds decreased phytase B efficacy and, with the exception of FCR, bone mineralization and Ca retention, suppressed all the parameters studied. The pure *myo*-inositol added at 0.1%, the concentration that reflected total phytate conversion, to feeds that differed in Ca/P ratio significantly increased BWG (537 vs. 579 g) but reduced P retention (56 vs. 50%, Table 7). One-way ANOVA on treatments 16 to 19 revealed that this unusual phenomenon was more pronounced in feeds containing 0.27% NPP and 0.65% Ca.

Under conditions of a higher CD, however, when the excess of phytase B was added to feeds with 3- or 6-phytase A, there was a significant effect of the type of phytase A on the performance of broilers (Table 8). 3-

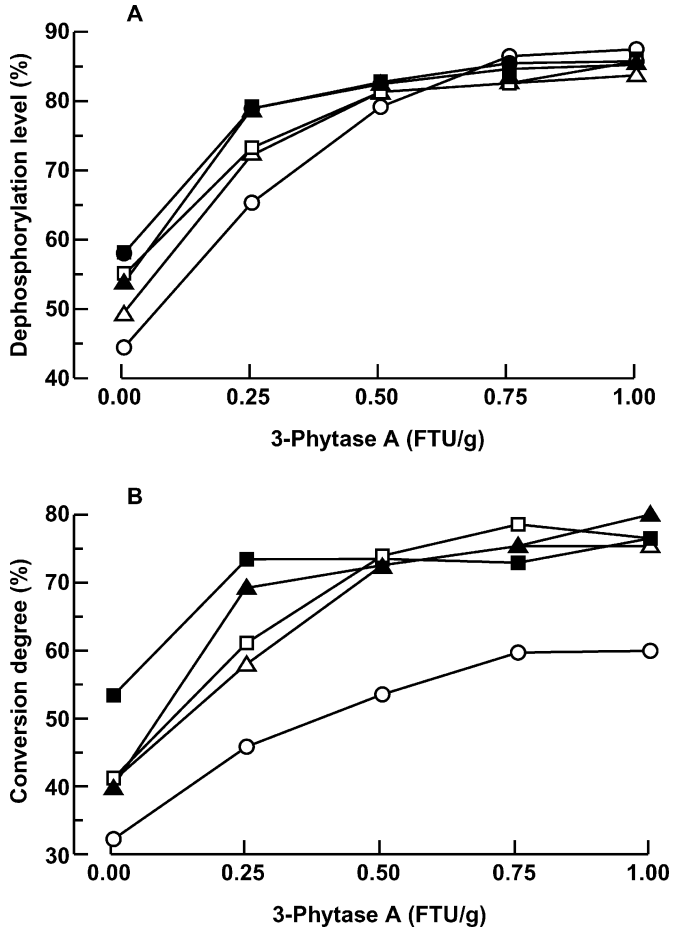


**FIGURE 1.** Panel A: The influence of tiered concentrations of 3-phytase A and phytase B (multilevel factorial design: 2 factors, 5 levels; error df = 67; 2 blocks; 75 runs) on dephosphorylation level. Phytase B activities studied were control, 0; △, 1,600; □, 3,200; ▲, 4,800; and ■, 6,400 acid phosphatase activity units (ACPU)/kg. Dephosphorylation level was calculated as $(P_{tot} - P_{res})/P_{tot} \times 100(\%)$, where $P_{tot}$ = total P; $P_{res}$ = residual P. $P_{res}$ contents were determined by the wet-ash mineralization of feed sample before and after multidigestion in vitro procedure, respectively. One unit of phytase activity (FTU) was defined as the amount of enzyme required to liberate 1 $\mu M$ of inorganic P from 2 m*M* sodium phytate in 1 min at 40°C and pH 4.5. One ACPU is equal to 1 $\mu M$/min of *p*-nitrophenol liberated from 5.5 m*M* disodium *p*-nitrophenylphosphate at 40°C and pH 4.5.

Panel B: The influence of tiered concentrations of 3-phytase A and phytase B (multilevel factorial design: 2 factors, 5 levels; error df = 67; 2 blocks; 75 runs) on conversion degree. Phytase B activities studied were control, 0; △, 1,600; □, 3,200; ▲, 4,800; and ■, 6,400 ACPU/kg. Conversion degree was calculated as: $P_{dial}/(P_{tot} - P_{res}) \times 100(\%)$, where $P_{dial}$ = dialyzable P. $P_{dial}$ was determined by the in vitro multidigestion procedure. whereas $P_{tot}$ and $P_{res}$ contents were determined by the wet-ash mineralization of feed sample before and after multidigestion in vitro procedure, respectively.

Phytase A was more effective than 6-phytase A in enhancing feed intake, BWG, and FCR. In contrast, the retention of Ca was higher in chickens consuming 6-phytase A than in birds fed 3-phytase A. Both forms of phytase A exerted similar effects on bone mineralization and P retention in broilers. Under these conditions, different levels of Ca affected BWG, FCR, and P retention. In treatments 4, 6, 7, 8, and 9, a high DL was produced by the addition of 6-phytase A (750 FTU/kg) and citric acid to feeds containing 0.12% NPP and 0.60% Ca. Conversion degree was increased gradually by the addition of tiered activities of phytase B (0 to 6,400 ACPU/kg). Tiered levels of phytase B influenced feed intake, BWG, and P and Ca retentions in broilers but did not affect FCR and bone mineralization (Table 9). Up to 3,200 ACPU/kg, phytase B did not influence performance but enhanced Ca retention. Higher levels of phytase B (4,800 to 6,400 ACPU/kg) decreased feed intake and BWG but improved P and Ca retentions.

More detailed analysis of the influence of the highest CD (phytase B at 6,400 ACPU/kg) and the high DL (750 FTU/kg of either 3- or 6-phytase A) on the performance, bone mineralization, and P and Ca metabolisms is listed in Table 10 (one-way ANOVA on treatments 3, 4, 5, 9, 12, 13, 14, and 15). At 0.65% Ca, feed intakes and BWG of chickens fed 3-phytase A were greatly enhanced by phytase B, whereas in broilers fed 6-phytase A, phytase B exerted detrimental effects on the performance but improved P and Ca retentions. The deterimental effect on performance produced by 6-phytase A at the highest CD and low Ca level was overcome by increasing Ca to 0.8%.

Weights of spleen were not influenced by dietary additions (data not shown), but weights of liver and bursa of Fabricius significantly differed in certain treatments comparisons (Table 11). Generally, there was a significant effect of phytases A on weights of livers and bursas. Both types of phytase A decreased liver and bursal weights, but 6-phytase reduced bursal weights more effectively than 3-phytase (1,382 vs. 1,205 g). Chickens fed diets with 0.47% NPP and 0.80% Ca had significantly higher liver weights (21.03 vs. 18.79 g) than birds that consumed feeds with 0.27% NPP and 0.65% Ca. The gradual increase in CD produced by tiered activities of phytase B (from 0 to 4,800 ACPU/kg with the background of phytase A at 750 FTU/kg) did not affect liver weights with the exception of 1,600 ACPU/kg, with which liver weights were significantly lower. It is interesting to note that phytase B alone did not produce any significant changes in the organ weights of broilers (data not shown).

## DISCUSSION

Availability of a commercial phytase B and the possibility to apply phytase A and phytase B to broiler feeds create new aspects of phytase enzymology in nutrition sciences. In this work we introduced 2 parameters that quantitatively describe phenomena of phytate removal from feed and the hydrolysis of phosphate moieties on the *myo*-inositol ring of phytate. Dephosphorylation level was the measure of a relative increase in P freed from feed samples during digestion, whereas the extent to which phytic acid molecules are deprived of phosphate moieties was assessed as a ratio of dialyzable P to the P freed from feed samples. It had been assumed that phytase A would affect mainly DL, whereas phytase B added in addition to phytase A would enhance phosphate hydrolysis on the *myo*-inositol rings and consequently influence the CD. The rationale behind that assumption was tested preliminary using an in vitro multidigestion proce-

**TABLE 4. Effects of different forms of phytase A alone and in combination with phytase B on the in vitro P and Ca release from feeds containing different Ca levels[1,2,3]**

| Item | Dialyzable P (mg/g) | | Mean | SEM | Dialyzable Ca (mg/g) | | Mean | SEM |
|---|---|---|---|---|---|---|---|---|
| Ca content in feed | 0.60 | 0.80 | | | 0.60 | 0.80 | | |
| Enzyme | | | | | | | | |
| Control (none) | 0.755 | 0.703 | 0.729[A] | | 3.50 | 4.69 | 4.10[A] | |
| 3-phytase A | 2.720 | 2.559 | 2.639[C] | | 4.35 | 5.58 | 4.97[C] | |
| 6-phytase A | 1.971 | 1.887 | 1.929[B] | 0.029 | 4.33 | 5.15 | 4.74[B] | 0.0067 |
| 3-phytase A + phytase B | 3.302 | 2.981 | 3.141[E] | | 4.65 | 5.73 | 5.19[D] | |
| 6-phytase A + phytase B | 2.955 | 2.780 | 2.868[D] | | 4.59 | 5.74 | 5.17[D] | |
| Mean | 2.341 | 2.182 | 2.261 | | 4.28 | 5.38 | 4.83 | |
| SEM | 0.0184 | | | | 0.0044 | | | |

[a–e]Means within columns with no common superscript differ significantly ($P < 0.05$).

[1]Probabilities of main effects were 0.0001 for enzyme, 0.0001 for Ca, 0.0194 for enzyme × Ca and 0.0001 for enzyme, 0.0001 for Ca, 0.2305 for enzyme × Ca, for dialyzable phosphorus and Ca, respectively.

[2]Data represent means of 6 determinations.

[3]Phytases A was added at 750 phytase activity units (FTU)/kg of feed; phytase B was added at 6,400 acid phosphatase activity units (ACPU)/kg. One FTU is the amount of enzyme required to liberate 1 $\mu M$ of inorganic P from 2 m*M* sodium phytate in 1 min at 40°C and pH 4.5. One ACPU is 1 $\mu M$/min of *p*-nitrophenol liberated from 5.5 m*M* disodium *p*-nitrophenylphosphate at 40°C and pH 4.5.

dure. Although DL and CD were influenced mainly by the level of phytase A (as evidenced in Table 3), the data obtained provide new insight into cooperation phenomena of phytases A and B during hydrolysis of phytates in feed (Żyła, 1993). Phytase B, a nonspecific phosphatase, proved not to be necessary in the process of phytate removal from feed ingredients. Dephosphorylation level was constant no matter what activity of phytase B was accompanied by phytase A at 500 FTU/kg or higher. It is interesting to note, however, that in the absence of phytase A, or when the enzyme was added at a suboptimal level (250 FTU/kg), phytase B did influence DL significantly (Figure 1A). For the purpose of phytate removal from feed ingredients phytase A could, therefore, be partly substituted for by phytase B, but at the present time this does not seem economically feasible. Phytase B, however, was found to be an irreplaceable tool in enhancing CD at each level of phytase A, as evidenced in Figure 1B. It is worth mentioning that in absence of supplemental phytase A, phytase B increased both DL and CD in a dose-dependent manner, but these effects may partly be attributed to the cooperation of phytase B with the activity of endogenous phytase A in soybeans and corn.

In the in vitro model, 3-phytase A released significantly more P than 6-phytase A at both levels of Ca and also significantly more Ca from a diet that contained 0.80% Ca. Ullah and Sethumadhavan (2003) provided evidence that *Phy A* gene of *Aspergillus ficuum* and *Peniophora lycii* encodes different enzymes. The 2 phytases differ in structure, physicochemical, and catalytical properties, in spite of belonging to the same family of histidine acid phosphatases. The enzyme from *P. lycii* has a narrower pH vs.

**TABLE 5. Effects of different dietary Ca levels and types of phytase A (diets 1, 3, 4, 10, 12, and 13) on the performance, bone mineralization, and P and Ca retentions in broilers[1]**

| Main effects | Feed intake (g) | BWG[2] (g) | FCR | Toe ash (%) | P retention (%) | Ca retention (%) |
|---|---|---|---|---|---|---|
| Phytase A | | | | | | |
| None | 588[A] | 352[A] | 1.67[B] | 7.75[A] | 58[A] | 57 |
| 3-phytase | 738[B] | 519[B] | 1.44[A] | 11.4[B] | 67[B] | 52 |
| 6-phytase | 777[B] | 532[B] | 1.48[A] | 10.94[B] | 63[B] | 49 |
| nCa level | | | | | | |
| 0.65% | 738 | 486 | 1.53 | 10.43 | 63 | 53 |
| 0.80% | 664 | 424 | 1.57 | 9.65 | 63 | 57 |
| Pooled SEM | 19 | 14 | 0.06 | 0.61 | 2.1 | 4.2 |
| | Probability | | | | | |
| ANOVA | | | | | | |
| Calcium level | 0.0039 | 0.0012 | NS | NS | NS | NS |
| Phytase A | 0.0001 | 0.0001 | 0.02510 | 0.0001 | 0.0321 | NS |
| Interaction | 0.0025 | 0.0117 | NS | NS | NS | NS |

[A,B]Means within columns with no common superscript differ significantly ($P < 0.05$).

[1]Phytases A fed at 750 phytase activity units (FTU)/kg of feed containing 0.12% nonphytate P (NPP). One unit of phytase activity (FTU) was defined as the amount of enzyme required to liberate 1 $\mu M$ of inorganic P from 2 m*M* sodium phytate in 1 min at 40°C and pH 4.5.

[2]Body weight gain.

**TABLE 6. Effects of different dietary Ca levels and phytase B supplementation on the performance, bone mineralization, phosphorus and Ca retention in broilers (diets 1, 2, 10, and 11; 0.12% nonphytate P)[1]**

| Main effects means | Feed intake (g) | Body weight gain (g) | FCR | Toe ash (%) | P retention (%) | Ca retention (%) |
|---|---|---|---|---|---|---|
| Phytase B | | | | | | |
| None | 588 | 352 | 1.67 | 7.75 | 58 | 57 |
| 6,400 ACPU/kg | 707 | 458 | 1.55 | 9.49 | 62 | 57 |
| Ca level (%) | | | | | | |
| 0.65 | 700 | 452 | 1.55 | 8.95 | 61 | 58 |
| 0.85 | 595 | 358 | 1.68 | 8.29 | 57 | 57 |
| Pooled SEM | 23 | 8 | 0.07 | 0.53 | 1.13 | 2.3 |
| | Probability | | | | | |
| ANOVA | | | | | | |
| Phytase B | 0.0012 | 0.0001 | 0.094 | 0.0396 | 0.0301 | NS |
| Ca level | 0.0030 | 0.0001 | 0.08526 | NS | 0.0306 | NS |
| Interaction | NS | 0.0013 | NS | NS | NS | NS |

[1]One unit of acid phosphatase activity (ACPU) was equal to 1 $\mu M/min^{-1}$ of *p*-nitrophenol liberated from 5.5 m*M* disodium *p*-nitrophenylphosphate at 40°C and pH 4.5.

**TABLE 7. Effects of *myo*-inositol supplementation and different Ca/P ratios on the performance, bone mineralization, phosphorus and Ca retention in broilers (diets 16, 17, 18, and 19)[1]**

| Main effects means | Feed intake (g) | Body weight gain (g) | FCR | Toe ash (%) | P retention (%) | Ca retention (%) |
|---|---|---|---|---|---|---|
| *Myo*-inositol | | | | | | |
| None | 838 | 537 | 1.57 | 11.9 | 56 | 61 |
| 0.1% | 861 | 579 | 1.47 | 12.2 | 50 | 59 |
| Ca/P ratio | | | | | | |
| 1.143 | 828 | 535 | 1.55 | 11.1 | 54 | 61 |
| 1.182 | 861 | 581 | 1.49 | 13.1 | 52 | 59 |
| Pooled SEM | 21 | 11 | 0.04 | 0.17 | 1.4 | 2.3 |
| | Probability | | | | | |
| ANOVA | | | | | | |
| *Myo*-inositol | NS | 0.0199 | 0.0573 | NS | 0.0015 | NS |
| Ca/P ratio | NS | 0.0128 | NS | 0.0001 | NS | NS |
| Interaction | NS | NS | NS | 0.0001 | NS | NS |

[1]Diets 16 and 17 had 0.27% nonphytate P (NPP); diets 18 and 19 had 0.47% NPP.

**TABLE 8. Effects of different dietary Ca levels and types of phytase A fed excess phytase B on performance, bone mineralization, and P and Ca retentions in broilers (diets 2, 5, 9, 11, 14, and 15)[1]**

| Main effects means | Feed intake (g) | Body weight gain (g) | FCR | Toe ash (%) | P retention (%) | Ca retention (%) |
|---|---|---|---|---|---|---|
| Phytase A | | | | | | |
| None | $707^{A}$ | $458^{A}$ | $1.55^{B}$ | $9.49^{A}$ | $62^{A}$ | $57^{B}$ |
| 3-phytase | $834^{C}$ | 601 [$sm^{C}$ | $1.39^{A}$ | $11.77^{B}$ | $70^{B}$ | $49^{A}$ |
| 6-phytase | $775^{B}$ | $510^{B}$ | $1.52^{B}$ | $11.68^{B}$ | $70^{B}$ | $61^{B}$ |
| Calcium level | | | | | | |
| 0.65% | 770 | 535 | 1.45 | 10.74 | 68 | 57 |
| 0.80% | 774 | 511 | 1.52 | 11.22 | 64 | 59 |
| Pooled SEM | 14 | 10.5 | 0.03 | 0.37 | 1.86 | 2.12 |
| | Probability | | | | | |
| ANOVA | | | | | | |
| Calcium level | NS | 0.0033 | 0.01428 | NS | 0.0400 | NS |
| Phytase A | 0.0001 | 0.0001 | 0.0003 | 0.0005 | 0.0469 | 0.0415 |
| Interaction | 0.0316 | 0.0007 | NS | NS | 0.0350 | NS |

[A–C]Means within columns with no common superscript differ significantly ($P < 0.05$).

[1]Both forms of phytase A were provided at 750 phytase activity units (FTU)/kg; phytase B was provided at 6,400 acid phosphatase activity units (ACPU)/kg of feeds containing 0.12% nonphytate P. One FTU is the amount of enzyme required to liberate 1 $\mu M$ of inorganic P from 2 m*M* sodium phytate in 1 min at 40°C and pH 4.5. One ACPU is equal to 1 $\mu M$/min of *p*-nitrophenol liberated from 5.5 m*M* disodium *p*-nitrophenylphosphate at 40°C and pH 4.5.

**TABLE 9. Effects of tiered concentrations of phytase B added with 750 phytase activity units (FTU)/kg of 6-phytase A on the performance, bone mineralization, and P and Ca retentions in broilers (diets 4, 6, 7, 8, and 9 containing 0.12% nonphytate P and 0.65% Ca)[1]**

| Item | Feed intake (g) | Body weight gain (g) | FCR | Toe ash (%) | P retention (%) | Ca retention (%) |
|---|---|---|---|---|---|---|
| Main effect means | | | | | | |
| Phytase B level | | | | | | |
| 0 | 831$^{A}$ | 581$^{A}$ | 1.49 | 11.10 | 64$^{A}$ | 48$^{A}$ |
| 1,600 ACPU[2]/kg | 852$^{A}$ | 563$^{AB}$ | 1.52 | 10.38 | 68$^{B}$ | 57$^{B}$ |
| 3,200 ACPU/kg | 847$^{A}$ | 583$^{A}$ | 1.45 | 10.83 | 67$^{B}$ | 57$^{B}$ |
| 4,800 ACPU/kg | 759$^{B}$ | 516$^{BC}$ | 1.48 | 11.25 | 75$^{C}$ | 61$^{C}$ |
| 6,400 ACPU/kg | 741$^{B}$ | 488$^{C}$ | 1.52 | 11.35 | 74$^{C}$ | 59$^{BC}$ |
| Pooled SEM | 13 | 15 | 0.04 | 0.49 | 1.21 | 1.86 |
| Probability | 0.0001 | 0.0036 | NS | NS | 0.0011 | 0.0256 |

$^{A–C}$Means within columns with no common superscript differ significantly ($P < 0.05$).

[1]One unit of phytase activity (FTU) was defined as the amount of enzyme required to liberate 1 *M* of inorganic phosphorus from 2 m*M* sodium phytate in 1 min at 40°C and pH 4.5. One unit of acid phosphatase activity (ACPU) was equal to 1 $\mu M$/min of *p*-nitrophenol liberated from 5.5 m*M* disodium *p*-nitrophenylphosphate at 40°C and pH 4.5.

[2]Acid phosphatase activity units.

**TABLE 10. Effects of a type of phytase A (3- or 6-phytase) with and without phytase B on the performance, bone mineralization, and P and Ca retentions in broilers (diets 3, 4, 5, 9, 12, 13, 14, and 15 containing 0.12% nonphytate P)[1]**

| Phytase type | Calcium (%) | Feed intake (g) | Body weight gain (g) | FCR | Toe ash (%) | P retention (%) | Ca retention (%) |
|---|---|---|---|---|---|---|---|
| 3-A | 0.65 | 712$^{A}$ | 536$^{AB}$ | 1.40$^{AB}$ | 11.76 | 65$^{AC}$ | 42$^{A}$ |
| 3-A + B | 0.65 | 842$^{C}$ | 631$^{C}$ | 1.30$^{A}$ | 11.33 | 70$^{CD}$ | 43$^{AB}$ |
| 6-A | 0.65 | 831$^{C}$ | 581$^{BC}$ | 1.49$^{B}$ | 11.14 | 64$^{AB}$ | 48$^{AB}$ |
| 6-A + B | 0.65 | 741$^{A}$ | 488$^{A}$ | 1.48$^{B}$ | 11.34 | 74$^{D}$ | 59$^{CD}$ |
| 3-A | 0.80 | 764$^{AB}$ | 502$^{A}$ | 1.49$^{B}$ | 11.07 | 68$^{AD}$ | 61$^{CD}$ |
| 3-A + B | 0.80 | 826$^{BC}$ | 572$^{B}$ | 1.41$^{A}$ | 12.21 | 70$^{BD}$ | 55$^{C}$ |
| 6-A | 0.80 | 723$^{A}$ | 483$^{A}$ | 1.46$^{B}$ | 10.74 | 62$^{A}$ | 49$^{B}$ |
| 6-A + B | 0.80 | 810$^{BC}$ | 533$^{AB}$ | 1.48$^{B}$ | 12.00 | 65$^{AC}$ | 63$^{D}$ |
| Pooled SEM | | 22 | 20 | 0.038 | 0.44 | 2.15 | 2.07 |
| Probability | | 0.0005 | 0.0001 | 0.016 | 0.3103 | 0.0131 | 0.0001 |

$^{A–D}$Means within columns with no common superscript differ significantly ($P < 0.05$).

[1]Phytases A fed at 750 phytase activity units (FTU)/kg, phytase B at 6,400 acid phosphatase activity unit (ACPU)/kg. One FTU is the amount of enzyme required to liberate 1 $\mu M$ of inorganic P from 2 m*M* sodium phytate in 1 min at 40°C and pH 4.5. One unit of acid phosphatase activity (ACPU) was equal to 1 $\mu M$/min of *p*-nitrophenol liberated from 5.5 m*M* disodium *p*-nitrophenylphosphate at 40°C and pH 4.5.

**TABLE 11. Significant effects of dietary additions on organ weights[1,3]**

| Organ | Phytases A | Ca (%) | Inositol | Ca/P ratio | Tiered phytase B effects |
|---|---|---|---|---|---|
| | | | | | 0–19.53B |
| Liver (g) | 0–18.64B | 0.65–17.54 | 0–19.54 | 1.143–18.79 | 1,600–16.68A |
| | 3–16.26A | 0.80–16.93 | 0.1%–20.28 | 1.182–21.03 | 3,200–18.63B |
| | 6–16.80A | | | | 4,800–20.85B |
| | | | | | 6,400–19.27B |
| Probability | 0.0157 | NS | NS | 0.0025 | 0.0205 |
| Bursa of Fabricius (g) | 0–1.555C | 0.65–1.477 | | | |
| | 3-1.383B | 0.80–1.285 | | | |
| | 6–1.205A | | | | |
| Probability | 0.0452 | 0.0443 | | | |

[1]Absolute organ weights were adjusted for final body weight by analysis of covariance.

[2]Data represent means of quadruplicate groups of three chicken aged 21 d posthatching.

[3]Phytases A fed at 750 phytase activity units (FTU)/kg, phytase B was at 6,400 acid phosphatase activity units (ACPU)/kg of feeds containing 0.12% of nonphytate P. One FTU is the amount of enzyme required to liberate 1 $\mu M$ of inorganic P from 2 m*M* sodium phytate in 1 min at 40°C and pH 4.5. One ACPU is equal to 1 $\mu M$/min of *p*-nitrophenol liberated from 5.5 m*M* disodium *p*-nitrophenylphosphate at 40°C and pH 4.5.

activity profile and is less acid-stable than *A. ficuum* phytase. Furthermore, Igbasan et al. (2000) reported *P. lycii* phytase to be less thermostable with lower resistance to pepsin and pancreatin digestions than its counterpart from *A. niger*. Differences in the efficacies between 3- and 6-phytase A in releasing P and Ca from the experimental feed found by the in vitro multidigestion procedure were not reflected in results from the feeding experiment. Performance, bone mineralization, and P retention in broilers fed low P diets were improved by 3- or 6-phytase A to a comparable extent. In the literature, similar to our findings, there are no data from animal experiments that show lower efficacy of 6-phytase A than 3-phytase A in enhancing performance or bone mineralization in broilers. Klünter and Steimle (2001) reported comparable BWG and tibia ash in chicken fed *P. lycci* or *A. niger* phytase at 500 FTU/kg. The authors observed, however, higher concentrations of inorganic phosphate in the plasma of broilers fed *A. niger* phytase than in birds receiving enzyme from *P. lycii* and attributed this phenomenon to differences in kinetics of P liberation from phytate by 2 phytases. Differences in the mode of action of the 2 enzymes might be a further explanation of an inferior ability of 6-phytase A to liberate inorganic P from feed in the in vitro part of our study. A general lack of differences between the efficacies of 3- and 6-phytase A has also been reported by Payne and Southern (2003) who studied growth and tibia ash of 3,360 broilers for 41 d.

To our knowledge, this report is the first that demonstrates significant effects of phytase B added to low-P diets as a sole supplemental enzyme on the performance, toe ash, and P retention in growing broilers. Furthermore, the well-known detrimental effect of higher Ca levels on the efficacy of phytase A was observed for phytase B in relation to BWG. Näsi et al. (1999a,b) reported a lack of acid phosphatase (phytase B) influence on P retention and excretion in growing swine, but the level of feed supplementation in phytase B used in those studies was approximately 10 times lower than 6,400 ACPU/kg used in our experiment.

With the exception of Pearce (1975), no literature data are available on the effects of *myo*-inositol supplementation on broilers performance or metabolism. In the current study, pure *myo*-inositol was added to feeds with 0.27 and 0.47% NPP so as to mimic diversified DL and the highest phytate CD. The improvement in BWG as well as the decrease in P retention resulting from *myo*-inositol supplementation were significant in birds fed diets with 0.27% NPP and 0.65% Ca but not in those receiving 0.47% NPP and 0.80% Ca. As a result of *myo*-inositol addition to feeds that contained 0.27% NPP and 0.65% Ca, amounts of ingested P increased, but, surprisingly, amounts of retained P decreased, and consequently the percentage of P retained decreased. No simple explanation for that phenomenon can be offered. Waagbo et al. (1998) reported an enhanced growth of Atlantic salmon fry fed fish meal based diets supplemented with 0.08% or more of *myo*-inositol in the first 4 wk, but the main effect of *myo*-inositol supplementation observed in that study was a significantly lower content of triacylglicerols in blood plasma. However, in studies on broilers fed wheat-based diets Pearce (1975) found no effects of 0.5% supplementary *myo*-inositol on hepatic lipid metabolism or on the fatty liver and kidney syndrome. In our studies, although liver weights were affected by the Ca/P ratio, no strict relationship was found between *myo*-inositol level in the diets and weights of chicken livers. Studies in the field of human nutrition showed an important role of dietary *myo*-inositol in the formation of surfactant phospholipids in lung, brain, and reproductive organs (Beemster et al., 2002; Fisher et al., 2002). Increased phospholipids synthesis, however, would rather promote than decrease P retention as observed in our studies.

Under conditions of a higher CD produced by the excess of phytase B, 3-phytase A was more effective in enhancing chicken performance than 6-phytase A, but in contrast to 3-phytase A, 6-phytase A enhanced Ca retention. Increased Ca retention by 6-phytase A might reflect a discovery of Padmanabhan et al. (2001), who, in the molecule of a plant 6-phytase, found an allosteric cocatalytic binding site of *myo*-inositol triphosphate ($IP_3$) that mobilizes Ca reserves during germination of seeds. Some of the intermediates of phytic acid degradation have also been suspected to play a role in the Ca entry from the external environment into cells through the plasma membrane (Phillippy, 1999). Taking into account that saturation of the high affinity $IP_3$ binding site in the molecule of 6-phytase led to increasd affinity of the enzyme for phytic acid (Padmanabhan et al., 2001) and, consequently, to a higher DL and P retention, 2 main factors might be influencing performance and metabolism of chickens fed a mixture of different *myo*-inositol phosphates—products of enzymic phytic acid degradation. *Myo*-inositol enhances performance but reduces P retention, and $IP_3$ enhances P retention, exerting an unknown effect on the performance.

In our experiment, when tiered concentrations (0 to 6,400 ACPU/kg) of phytase B were added with 750 FTU/kg of 6-phytase A, the highest BWG were found in birds fed phytase B at 0 to 3,200 ACPU/kg, but the highest values of P retention were for those receiving 4,800 to 6,400 ACPU/kg. It seems, therefore, that the range of phytase B applied in the experiment resulted in an increased concentration of $IP_3$ rather than in accumulation of substantial quantities of *myo*-inositol. It is not clear, however, why performance of broilers was reduced in birds fed the highest activities of 6-phytase A and phytase B at 0.65% Ca. Furthermore, it should be noted that under the highest CD (phytase B added at 6,400 ACPU/kg), chickens fed 6-phytase A and 0.6% Ca performed worse, but when fed 0.8% Ca they performed better than birds 6-phytase A as the sole supplemental enzyme. In case of 3-phytase A, addition of phytase B (6,400 ACPU/kg) with phytase A resulted in enhanced feed intake and BWG, regardless of Ca level in feed. Furthermore, increased CD, with *myo*-inositol phosphate intermediates produced by 6-phytase action resulted in significantly higher P and Ca retention, whereas with 3-phytase A, a higher CD was

reflected exclusively in performance. Indeed, the one-way ANOVA on all treatments clearly indicated that the highest BWG and the lowest FCR was in birds fed low-P, low-Ca diets supplemented with 3-phytase A and phytase B at 6,400 ACPU/kg, but the highest P and Ca retention was observed in broilers consuming 6-phytase A and phytase B at the highest level (data not shown). The content of ash in the toes was sensitive to changes in DL but not in CD, irrespective of the type of phytase A used.

Because in our previous study (Żyła et al., 2000b) certain levels of 3-phytase A and phytase B had significant influence on the bursa and proventriculus weights of broilers, we wanted to verify whether different DL and CD values affect organ weights of chickens. The only significant effect on the weights of chicken bursas resulted from feeding 3- or 6-phytase A as the sole supplemental enzyme, which suggests an influence of phytic acid degradation products with a low CD on the immune system of birds. Products of phytate degradation by 6-phytase A seem to be more efficacious in lowering bursal weights than those resulted from 3-phytase A action. More advanced hydrolysis of *myo*-inositol phosphate intermediates (a higher CD) removed the effect.

In conclusion it can be stated that phytase B, when added to feeds that have already been supplemented with adequate activities of phytase A, does not influence DL. Phytase B, however, does influence CD, which is reflected in increased accumulation of *myo*-inositol phosphates intermediates—products of phytate degradation by 3- or 6-phytase A. The efficacy of both forms of phytase A and phytase B depends on the Ca level in feed. Further research is necessary to explain in greater details changes in the physiology of birds produced by lower *myo*-inositol phosphates resulting from simultaneous action of 3-phytase A and phytase B, in comparison to those accumulated by the action of 6-phytase A and phytase B, but the current study provides sufficient evidence that they change physiology of broilers in a different manner.

## ACKNOWLEDGMENTS

This work was supported by a grant from the Polish Research Council (Nb. 6P06E 052 20). Authors acknowledge Hoffman la Roche (Switzerland) for financial support and for providing Ronozyme P preparation and AB Enzyme Finland Oy (Rajamäki, Finland) for supplying the Finase P and Finase AP preparations.

## REFERENCES

Association of Official Analytical Chemists. 1995. 4. Animal feeds. Phosphorus. 965.17 Photometric method. Page 27 in Official Methods of Analysis. Association of Official Analytical Chemists, Washington, DC.

Beemster, P., P. Groenen, and R. Steegers-Theunissen. 2002. Involvement of inositol in reproduction. Nutr. Rev. 60:80–87.

Denbow, D. M., E. A. Grabau, G. H. Lacy, E. T. Kornegay, D. R. Russell, and P. F. Umbeck. 1998. Soybeans transformed with a fungal phytase gene improve phosphorus availability for broilers. Poult. Sci. 77:878–881.

Fisher, S. K., J. E. Novak, and B. W. Agranoff. 2002. Inositol and higher inositol phosphaes in neural tissues: homeoastasis, metabolism and functional significance. J. Neurochem. 82:736–754.

Huff, W. E., P. A. Moore, P. W. Waldroup, J. M. Balog, G. R. Huff, N. C. Rath, T. C. Daniel, and V. Raboy. 1998. Effect of dietary phytase and high available phosphorus corn on broiler chicken performance. Poult. Sci. 77:1899–1904.

Igbasan, F. A., K. Männer, G. Miksch, R. Borriss, A. Farouk, and O. Simon. 2000. Comparative studies on the in vitro properties of phytases from various microbial origins. Arch. Anim. Nutr. 53:353–373.

Klünter, A. M., and C. Steimle. 2001. Effects of *Peniophora lycii* phytase in broiler chickens fed a low-phosphorus diet. Pages 188–189 in Proceedings of the 13th European Symposium on Poultry Nutrition, Blankenberge, Belgium. WPSA—Belgium Branch.

Ma, H.-M. 1999. Direct spectrophotometric determination of calcium in human serum and cerebrospinal fluids with aminoG acid chlorophosphonazo. Anal. Lett. 32:799–809.

Marczenko, Z., and M. Balcerzak. 1998. Spectrophotometric methods in inorganic analysis. WNT, Warsaw. (in Polish).

Näsi, M., K. Partanen, and J. Piironen. 1999. a. Comparison of Aspergillus niger phytase and Trichoderma reesei phytase and acid phosphatase on phytate phosphorus availability in pigs fed on maize-soybean meal or barley-soybean meal diets. Arch. Anim. Nutr. 52:15–27.

Näsi, M., J. Piironen, and K. Partanen. 1999b. Efficacy of Trichoderma reesei phytase and acid phosphatase activity ratios in phytate phosphorus degradation *in vitro* and in pigs fed maize-soybean meal or barley-soybean meal diets. Anim. Feed Sci. Technol. 77:125–137.

Nelson, T. S., T. R. Shieh, R. J. Wodzinski, and J. H. Ware. 1968. The availability of phytate phosphorus in soybean meal before and after treatment with a mold phytase. Poult. Sci. 47:1842–1848.

Oh, B.-C., W.-C. Choi, S. Park, Y.-O. Kim, and T.-K. Oh. 2003. Biochemical properties and substrate specificities of alkaline and histidine acid phytases. Appl. Microbiol. Biotechnol. 63:55–62.

Padmanabhan, U., S. Dasgupta, B. B. Biswas, and D. Dasgupta. 2001. High affinity association of *myo*-inositol trisphosphates with phytase and its effect upon catalytic potential of the enzyme. J. Biol. Chem. 276:43635–43644.

Payne, R. L., and L. L. Southern. 2003. A comparison of two sources of phytase on growth performance and bone ash in commercial broilers. Pages 25–26 in Proceedings of the 14th European Symposium on Poultry Nutrition, Lillehammer. WPSA-Norwegian Branch.

Pearce, J. 1975. The effects of choline and inositol on hepatic lipid metabolism and the incidence of the fatty liver and kidney syndrome in broilers. Br. Poult. Sci. 16:565–570.

Phillippy, B. Q. 1999. Susceptibility of wheat and *Aspergillus* niger phytases to inactivation by gastrointestinal enzymes. J. Agric. Food Chem. 47:1385–1388.

Sebastian, S., S. P. Touchburn, E. R. Chavez, and P. C. Lague. 1998. Implications of phytic acid and supplemental microbial phytase in poultry nutrition: A review. Worlds Poult. Sci. J. 54:27–47.

Statgraphics Plus for Windows. 1996. Advanced Regression. Manugistics, Inc., Rockville, MD.

Ullah, A. H. J., and K. Sethumadhavan. 2003. Phy A gene product of Aspergillus ficuum and Peniophora lycii produces dissimilar phytases. Biochem. Biophys. Res. Com. 303:463–468.

Waagbo, R., K. Sandnes, and Ø. Lie. 1998. Effects of inositol supplementaton on growth, chemical composition and blood chemistry in Atlantic salmon, *Salmo salar* L., fry. Aquacult. Nutr. 4:53–59.

Ward, N. E. 2000. Phytase in commercial poultry diets: an update. Pages 8–21 in Proceedings of the 62nd Cornell Nutrition Conference, Ithaca, NY.

Wodzinski, R. J., and A. H. J. Ullah. 1999. Phytase. Adv. Appl. Microbiol. 42:263–303.

Wu, C. F. J., M. Hamada. 2000. Experiments. Planning, Analysis, and Parameter Design Optimization. J. Wiley, Inc., New York.

Wyss, M., R. Brugger, A. Kronenberger, R. R. Fimbel, G. Oesterhelt, M. Lehmann, and A. P. G. M. van Loon. 1999. Biochemical characterization of fungal phytases (myo-inositol heksakisphosphate phosphohydrolases): Catalytic properties. Appl. Environ. Microbiol. 65:367–373.

Żyła, K. 1993. The role of acid phosphatase activity during enzymic dephosphorylation of phytates by *Aspergillus niger* phytase. World J. Microbiol. Biotechnol. 9:117–119.

Żyła, K. 2001. Phytase applications in poultry feeding. Selected issues. J. Anim. Feed. Sci. 10:247–258.

Żyła, K., J. Koreleski, S. Świątkiewicz, A. Wikiera, M. Kujawski, J. Piironen, and D. R. Ledoux. 2000a. Effects of phosphorolytic and cell wall-degrading enzymes on the performance of growing broilers fed wheat-based diets containing different calcium levels. Poult. Sci. 78:66–76.

Żyła, K., A. Wikiera, J. Koreleski, S. Świątkiewicz, J. Piironen, and D. R. Ledoux. 2000b. Comparison of the efficacies of a novel *Aspergillus niger* mycelium with separate and combined effectiveness of phytase, acid phosphatase, and pectinase in dephosphorylation of wheat-based feeds fed to growing broilers. Poult. Sci. 79:1434–1443.