Effectiveness of an experimental consensus phytase in improving dietary phytate-phosphorus utilization by weanling pigs


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ABSTRACT: Consensus phytase is a new biosynthetic, heat-stable enzyme derived from the sequences of multiple homologous phytases. Two experiments were conducted to determine its effectiveness, relative to inorganic P and a mutant enzyme of *Escherichia coli* phytase (Mutant-EP), in improving dietary phytate-P availability to pigs. In Exp. 1, 36 pigs (3 wk old, 7.00 ± 0.24 kg of BW) were fed a low-P corn-soybean meal basal diet plus consensus phytase at 0, 250, 500, 750, 1,000, or 1,250 U/kg of feed for 5 wk. Plasma inorganic P concentration, plasma alkaline phosphatase activity, bone strength, and overall ADG and gain:feed ratio of pigs were improved (*P* < 0.05) by consensus phytase in both linear (R² = 0.20 to 0.70) and quadratic (R² = 0.30 to 0.70) dose-dependent fashions. In Exp. 2, 36 pigs (4 wk old, 9.61 ± 0.52 kg BW) were fed the basal diet + inorganic P at 0.1 or 0.2%, consensus phytase at 750 or 450 U/kg of feed, Mutant-EP at 450 U/kg of feed, or 225 U consensus + 225 U Mutant-EP/kg of feed. Pigs fed 750 U of consensus phytase or 450 U of Mutant-EP/kg feed had plasma inorganic concentrations and bone strength that fell between those of pigs fed 0.1 or 0.2% inorganic P. These two measures were 16 to 29% lower (*P* < 0.05) in pigs fed 450 U of consensus phytase/kg of feed than those of pigs fed 0.2% inorganic P. Plasma inorganic P concentrations were 14 to 29% higher (*P* < 0.05) in pigs fed Mutant-EP vs. consensus phytase at 450 U/kg at wk 2 and 3. In conclusion, the experimental consensus phytase effectively releases phytate P from the corn-soy diet for weanling pigs. The inorganic P equivalent of 750 U of consensus phytase/kg of feed may fall between 0.1 and 0.2%, but this requires further determination.

Key Words: Alkaline Phosphatase, Bone Strength, Consensus, *Escherichia coli* Phytase, Phosphorus, Pigs

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Introduction

Effectiveness of supplemental microbial phytases in diets for swine in improving phytate P bioavailability and reducing manure P excretion has been demonstrated by many research groups around the world (Simons et al., 1990; Cromwell et al., 1993; Young et al., 1993). One major limitation of currently available commercial phytases in animal feed is their inability to withstand the heat denaturation (60 to 80°C) involved in feed pelleting. To overcome this limitation, Lehmann et al. (2000) have used a novel consensus approach and have developed a synthetic phytase named “experimental consensus phytase,” based on the homologous sequences of 13 known fungal phytases. Whereas the naturally occurring phytases have temperature optima between 45 and 55°C, the consensus phytase has a temperature optimum at 71°C. The unfolding temperature, at which the enzyme melts and becomes denatured, is between 56 to 63°C for all known phytases, but is 78°C for the consensus phytase (Lehmann et al., 2000). In addition, the catalytic activity and pH optimum of consensus phytase are comparable with those of *Aspergillus fumigatis* and *Emericella nidulans* phytases at 37°C (Lehmann et al., 2000). Apparently, consensus phytase has tremendous potential to become a heat-stable and catalytically efficient enzyme for use in heat-processed (e.g., pelleted) animal feed. Therefore, its effectiveness in releasing phytate P from typical diets for swine needs to be assessed. Our objectives of this study were to determine the dose-dependent effectiveness of consensus phytase in a corn-soybean meal diet for weanling pigs and its efficacy relative to that of inorganic P or a mutant enzyme of the *Escherichia coli* phytase (Mutant-EP) (Rodriguez et al., 2000) that was developed in our laboratory, as well as possible synergistic effects between these two phytases.

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Materials and Methods

Phytases

Consensus phytase was used in granular form and was provided by Roche Vitamins Ltd. (Basel, Switzerland). The Mutant-EP was produced in a yeast system from an E. coli phytase variant gene prepared in our laboratory (Rodriguez et al., 2000). The actual activities of consensus phytase and Mutant-EP phytase used for this study were 3,250 and 4,500 U/g by analysis, respectively. To make their activity comparable and practically relevant, we determined phytase activity of both enzymes by the release of inorganic P from sodium phytate in 0.2 M citrate buffer, pH 5.5, at 37°C (Han et al., 1998). One unit of phytase was defined as the amount of enzyme that releases 1 μmol of inorganic P per minute from sodium phytate under the assay conditions. The released inorganic P was measured colorimetrically at 820 nm (DU 640 Spectrophotometer, Beckman Instruments Inc., Fullerton, CA).

Animals, Diets, and Measures

Our protocols were approved by the Institutional Animal Care and Use Committee of Cornell University. A total of 72 weaning gilts (Duroc-Landrace-Yorkshire cross) was selected from the Cornell University Swine Farm, and placed into treatment groups based on litter, BW, and plasma inorganic P concentrations. In Exp. 1, 36 weaning pigs (3 wk old, 7.00 ± 0.24 kg of BW) were allotted into six treatment groups (n = 6) to determine the dose effects of dietary consensus phytase. Each group was assigned to a diet containing 0; 250; 500; 750; 1,000; or 1,250 U/kg of feed. In Exp. 2, 36 weaning pigs (4 wk old, 9.61 ± 0.52 kg of BW) were allotted into six treatment groups (n = 6) to compare relative efficacy of consensus phytase to inorganic P and Mutant-EP, and to check for a possible synergistic effect between the two phytases. Each group was assigned to a diet containing inorganic P at 0.1 or 0.2% (designated as 0.1% P and 0.2% P, respectively), consensus phytase at 450 or 750 U/kg of feed, Mutant-EP at 450 U/kg of feed or the two phytases each at 225 U/kg of feed. The duration for Exp. 1 and 2 was 5 and 4 wk, respectively. Prior to the beginning of both experiments, all pigs were fasted for 8 h, from 2200 to 0600) were collected weekly from the anterior vena cava into heparinized syringes for the assay of plasma inorganic P concentrations and alkaline phosphatase activity. At the end of both studies, the pigs were killed to collect the third and fourth metacarpals and metatarsals to determine bone strength.

Biochemical Analysis

Plasma was prepared by spinning chilled whole blood samples for 10 min at 4°C and 3,000 × g (GS-6KR Centrifuge, Beckman Instruments Inc.). After being deproteinized with 12.5% trichloroacetic acid, plasma inorganic P concentration was determined using Elon (p-methylaminophenol sulfate) solution (Gomori, 1942). Plasma alkaline phosphatase activity was assayed by the hydrolysis of p-nitrophenol phosphate to p-nitrophenol (Bowers and McComb, 1966). The enzyme unit was defined as 1 μmol of p-nitrophenol released from p-nitrophenol phosphate per minute at 30°C.

Bone Strength

The collected third and fourth metacarpals and metatarsals were prepared by removing the skin, muscle, connective tissue, tendons, and the other nearby bones and stored in closed plastic bags at 4°C (<7 d) before the

Table 1. Composition of basal diet, as-fed basis

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn</td>
<td>67.10</td>
</tr>
<tr>
<td>Soybean meal, 48% CP</td>
<td>28.00</td>
</tr>
<tr>
<td>Spray-dried plasma protein</td>
<td>1.50</td>
</tr>
<tr>
<td>Limestone</td>
<td>1.05</td>
</tr>
<tr>
<td>L-Lysine-HCl</td>
<td>0.10</td>
</tr>
<tr>
<td>Corn oil</td>
<td>1.00</td>
</tr>
<tr>
<td>Vitamin/mineral premixa</td>
<td>0.25</td>
</tr>
<tr>
<td>Salt</td>
<td>0.50</td>
</tr>
<tr>
<td>Antibioticsb</td>
<td>0.50</td>
</tr>
<tr>
<td>Total</td>
<td>100.0</td>
</tr>
</tbody>
</table>

aVitamin and mineral premix supplies (per kilogram of diet): 5,500 IU of vitamin A, 1,100 IU of vitamin D3, 24 IU of vitamin E, 0.73 mg of vitamin K, 4.4 mg of riboflavin, 17.6 mg of pantothenic acid, 26.4 mg of niacin, 66 mg of choline, 26 μg of vitamin B12, 0.27 g of Mg (MgO), 32 mg of Mn (MnO), 0.4 mg of I (C2H8 N2 I2), 0.5 mg of vitamin B6, 55 mg of penicillin/kg of diet.
bProvided 110 mg of chlortetracycline, 110 mg of sulfathiazole, and 55 mg of sulfathiazole.

<sup>a</sup>Vitamin and mineral premix supplies (per kilogram of diet): 5,500 IU of vitamin A, 1,100 IU of vitamin D3, 24 IU of vitamin E, 0.73 mg of vitamin K, 4.4 mg of riboflavin, 17.6 mg of pantothenic acid, 26.4 mg of niacin, 66 mg of choline, 26 μg of vitamin B12, 0.27 g of Mg (MgO), 32 mg of Mn (MnO), 0.4 mg of I (C2H8 N2 I2), 0.5 mg of vitamin B6, 55 mg of penicillin/kg of diet.

<sup>b</sup>Provided 110 mg of chlortetracycline, 110 mg of sulfathiazole, and 55 mg of penicillin/kg of diet.

<sup>c</sup>Calculated (NRC, 1998).
Table 2. Effects of dietary supplemental consensus phytase on overall growth performance of pigs in Experiment 1

<table>
<thead>
<tr>
<th>Item</th>
<th>0</th>
<th>250</th>
<th>500</th>
<th>750</th>
<th>1,000</th>
<th>1,250</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplemental dietary consensus phytase, U/kg feed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daily body weight gain, g</td>
<td>308</td>
<td>389</td>
<td>437</td>
<td>465</td>
<td>443</td>
<td>440</td>
<td>25</td>
</tr>
<tr>
<td>Daily feed intake, g (as-fed)</td>
<td>642</td>
<td>717</td>
<td>769</td>
<td>792</td>
<td>792</td>
<td>747</td>
<td>49</td>
</tr>
<tr>
<td>Gain:feed, g/kg</td>
<td>483</td>
<td>545</td>
<td>572</td>
<td>590</td>
<td>562</td>
<td>588</td>
<td>18</td>
</tr>
</tbody>
</table>

*Values are means of six individually penned pigs during the 5-wk study.

*Linear (*P* < 0.001, R² = 0.29) and quadratic (*P* < 0.001, R² = 0.45) responses.

*Linear (*P* = 0.06, R² = 0.10) and quadratic (*P* < 0.03, R² = 0.20) responses.

*Linear (*P* < 0.001, R² = 0.29) and quadratic (*P* < 0.001, R² = 0.40) responses.

strength analysis. The bone strength was determined at room temperature (23°C) by subjecting each bone to a three-point bending test (Turner and Burr, 1993) using the Instron machine (model 4502, Instron, Canton, MA). During the testing, the crosshead speed was set at 50.0 mm/min, and the two supports were spaced 2 cm apart. From each test, a load-displacement curve was generated, which indicated the maximum load at the breaking point of each bone. Actual values were expressed as the means of the third and fourth metacarpals or metatarsals or the overall averages of all four bones.

Statistical Analyses

All data were processed using SAS (SAS Inst., Inc., Cary, NC). In Exp. 1, dose effects of dietary consensus phytase activity on weekly and/or overall growth performance, plasma inorganic P concentration, and alkaline phosphatase activity, and bone strength were evaluated by polynomial evaluations. In Exp. 2, growth performance and plasma data were analyzed using ANOVA with time-repeated measurements (Gill, 1986). The bone data were analyzed by ANOVA. The individually penned pig was used as the experimental unit. The Bonferroni t-test was used to compare treatment means. The significance level was set at *P* < 0.05.

Results

Experiment 1

The overall ADG of pigs responded to dietary consensus phytase doses in both linear (*P* < 0.001, R² = 0.29) and quadratic fashions (*P* < 0.001, R² = 0.49; Table 2). Likewise, effects of the phytase doses on the overall gain:feed ratio of pigs fit linear (*P* < 0.001, R² = 0.29) and quadratic (*P* < 0.001, R² = 0.40) polynomial equations. However, the linear response of the overall ADFI of pigs to the dietary phytase doses was fairly weak (*P* = 0.06, R² = 0.10), and the quadratic response was moderate (*P* < 0.03, R² = 0.20). Based on the quadratic polynomial equations, the predicted dietary consensus phytase activities for the maximal ADG, ADFI, and gain/feed ratio were 875, 868, and 913 U/kg of feed, respectively. On the weekly basis, the linear or quadratic effects of the consensus phytase doses on these three growth performance measures were similar to those with the aforementioned overall averages (data not shown).

Initial plasma inorganic P concentrations were similar among the six treatment groups (Figure 1). At the end of the trial (wk 5), plasma inorganic P concentrations were a linear (*P* < 0.001, R² = 0.69) function of dietary consensus phytase doses. Their relationship also fit a quadratic polynomial (*P* < 0.001, R² = 0.70). The linear and quadratic effects (*P* < 0.05) of the phytase doses were relatively stronger at wk 3 and 4 (R² = 0.50 to 0.70) than at wk 1 and 2 (R² = 0.3 to 0.4) (data not shown). Based on the weekly quadratic polynomial equations, the average dietary consensus phytase activity predicted for the maximal plasma inorganic P concentration was 1,497 U/kg of feed. In contrast, plasma alkaline phosphatase activity was inversely correlated with dietary phytase doses, and the relationship (*P* < 0.001, R² = 0.70) quadratic response was moderate (*P* < 0.03, R² = 0.20). Based on the quadratic polynomial equations, the predicted dietary consensus phytase activities for the maximal ADG, ADFI, and gain/feed ratio were 875, 868, and 913 U/kg of feed, respectively. On the weekly basis, the linear or quadratic effects of the consensus phytase doses on these three growth performance measures were similar to those with the aforementioned overall averages (data not shown).

**Figure 1.** Effects of supplemental dietary consensus phytase activity on plasma inorganic P concentrations of pigs in Exp. 1. Values are means of six individually penned pigs. The overall dose response at wk 5 fit linear (*P* < 0.001, R² = 0.70) and quadratic (*P* < 0.001, R² = 0.70) polynomial equations.
Figure 2. Effects of supplemental dietary consensus phytase activity on plasma alkaline phosphatase activity of pigs in Exp. 1. Values are means of six individually penned pigs. The overall dose response at wk 5 fit linear (P < 0.001, R² = 0.41) and quadratic (P < 0.001, R² = 0.46) polynomial equations.

0.001) fit linear (R² = 0.40) and quadratic (R² = 0.50) polynomial equations at wk 5 (Figure 2). The dietary treatment had no (P = 0.24 to 0.46) linear or quadratic (R² = 0.02 to 0.08) effect on plasma alkaline phosphatase activity at wk 1 or 2, but showed weak or moderate (P < 0.03, R² = 0.20 to 0.41) effects at wk 3 and 4 (data not shown). The estimated average dietary consensus phytase activity for the lowest plasma alkaline phosphatase activity was 1,094 U/kg of feed. The average breaking strength of the third and the fourth metacarpals or metatarsals as well as the mean strength of both bones responded to dietary phytase virtually in a nearly identical linear fashion (P < 0.001, R² = 0.53 to 0.57, Figure 3). The responses could also be described by quadratic polynomial equations (P < 0.001, R² = 0.55 to 0.59), and the maximal mean strength of both bones was predicated to occur at 1,559 U/kg of feed.

Experiment 2

There was no significant main effect of dietary treatments on ADG (P = 0.92), ADFI (P = 0.91), or gain:feed ratio (P = 0.44) (Table 3). Pigs fed 0.1% P and 450 U of consensus phytase/kg feed had 25 and 26% lower (P < 0.05) average bone strength of the third and the fourth metacarpals and metatarsals than that of pig fed 0.2% P, respectively. The other three groups of pigs had intermediate bone strengths that were not different (P = 0.10 to 0.84) from those of pigs fed 0.1 or 0.2% P. Separate analyses of metacarpals or metatarsals gave results similar to the means of both bones (data not shown).

At initial, plasma inorganic P concentrations of all six groups of pigs were similar (Figure 4). Throughout the experiment, pigs fed 450 U of consensus phytase/kg feed had 16 to 29% lower (P < 0.05) plasma inorganic P concentrations than those of pigs fed 0.2% P. The concentrations in pigs fed 750 U consensus phytase or 450 U of Mutant-EP/kg feed were not different from those fed 0.1 or 0.2% P (P = 0.10 to 0.99). At wk 2 and 3, plasma inorganic P concentrations were 14 to 29% higher (P < 0.05) in pigs fed 450 U of Mutant-EP/kg feed than those fed 450 U of consensus phytase/kg of feed. The latter also had lower (P < 0.05) plasma inorganic P concentrations than those of pigs fed 0.1% P at wk 2. Plasma inorganic P concentrations in pigs fed the two phytases in combination did not differ (P = 0.16 to 0.70) from those of pigs fed each enzyme alone. There was no main effect of dietary treatments (P = 0.13) on plasma alkaline phosphatase activity (data not shown).

Discussion

Our results from Exp. 1 have clearly demonstrated the effectiveness of this experimental consensus phytase in improving dietary phytate P utilization by weanling pigs. When it was supplemented up to 1,250 U/kg of feed into the corn-soybean meal diet without inorganic P added, the enzyme resulted in linear and quadratic improvements in growth performance, bone strength, and plasma inorganic P concentrations in pigs. The phytase dose-dependent (linear and quadratic at wk 3, 4, and 5) decrease in plasma activity of alkaline phosphatase, an enzyme that catalyzes phosphorus release from bone, indicates that consensus phytase in-
Table 3. Effects of dietary supplemental inorganic P, consensus phytase, and Mutant-EP alone or in combination on overall growth performance and bone strength of pigs in Experiment 2\textsuperscript{a,b}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ADG, g</th>
<th>ADFI, g</th>
<th>Gain:feed, g/kg</th>
<th>Bone strength, kg(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1% P</td>
<td>563</td>
<td>1,002</td>
<td>561</td>
<td>28.7\textsuperscript{y}</td>
</tr>
<tr>
<td>0.2% P</td>
<td>603</td>
<td>1,028</td>
<td>591</td>
<td>38.3\textsuperscript{z}</td>
</tr>
<tr>
<td>750 U Consensus</td>
<td>604</td>
<td>1,030</td>
<td>586</td>
<td>30.6\textsuperscript{y}z</td>
</tr>
<tr>
<td>450 U Consensus</td>
<td>538</td>
<td>966</td>
<td>559</td>
<td>28.5\textsuperscript{y}</td>
</tr>
<tr>
<td>450 U Mutant-EP</td>
<td>583</td>
<td>1,065</td>
<td>552</td>
<td>34.4\textsuperscript{z}</td>
</tr>
<tr>
<td>Consensus + Mutant-EP (225 U each)</td>
<td>579</td>
<td>1,045</td>
<td>554</td>
<td>37.1\textsuperscript{y}</td>
</tr>
<tr>
<td>SEM</td>
<td>38</td>
<td>68</td>
<td>17</td>
<td>2.6</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Phytases were added on a per kilogram of feed basis.

\textsuperscript{b}Values of growth performance are means of six individually penned pigs during the 4-wk study.

\textsuperscript{c}Average breaking strength of the third and the fourth metacarpals and metatarsals bones of four individually penned pigs.

\textsuperscript{y,z}Means within the same column not sharing a common superscript letter differ \((P < 0.05)\).
pigs fed 0.2% P. More directly, 450 U of Mutant-EP phytase/kg of feed yielded 14 to 29% higher (P < 0.05) plasma inorganic P concentrations at wk 2 and 3 than 450 U of consensus phytase/kg feed. Seemingly, Mutant-EP phytase was more efficacious than consensus phytase in releasing dietary phytate P for maintaining plasma inorganic P concentrations in weanling pigs. Despite this, caution should be given in generalizing an overall efficacy comparison between these two enzymes, due to the lack of statistically significant differences in the final plasma inorganic P concentrations or bone strength between the two groups of pigs in Exp. 2. However, efficacy of E. coli phytases has been determined in chicks by Leeson et al. (2000) and in pigs by Stahl et al. (2000). An improved effectiveness of this Mutant-EP phytase over that of other two fungal phytases in both swine and poultry diets has been reported (Applegate et al., 2003; Augspurger et al., 2003). Any efficacy difference between Mutant-EP phytase and consensus phytase or other fungal phytases might be partially related to their pH optima. Although the optimal pH for consensus phytase is 6.5 (Lehmann et al., 2000), the Mutant-EP phytase is most active at a more acidic pH (Rodriguez et al., 2000). It is understandable that 450 U of Mutant-EP phytase, measured at pH 5.5, bears more functioning activity at the stomach pH of pigs (Yi and Kornegy, 1996) than that of consensus phytase. In addition, E. coli phytase has a much greater turnover rate than the fungal enzyme (Greiner et al., 1993), and is resistant to proteolysis (Rodriguez et al., 1999).

A combination of these two rather different phytases resulted in no apparent synergistic effect on the measures in Exp. 2. Although pigs fed 225 U of consensus phytase + 225 U of Mutant-EP phytase/kg had numerically higher plasma inorganic P concentrations and bone strength than the calculated averages of pigs fed each enzyme alone at 450 U/kg, there was no significant difference among these three groups. Future research should examine if there is any synergistic effect of these two enzymes under dietary conditions different from those in the present study. Because properties of phytases vary, it is possible to maximize dietary phytate P bioavailability to pigs by using multiple phytases in combination, along with low-phytate grain (Spencer et al., 2000; Sands et al., 2001), organic acids (Han et al., 1998; Radcliffe et al., 1998), intrinsic phytases in feeds (Han et al., 1997), and an effective feeding regimen (Mroz et al., 1994).

Implications

A newly developed experimental heat-stable consensus phytase was effective in improving phytate phosphorus bioavailability in corn-soybean meal diets for weanling pigs. Linear and quadratic responses in growth performance and body nutritional status of phosphorus were observed in these pigs fed this enzyme at 0 to 1,250 U/kg of corn-soybean meal diet, without supplemental inorganic phosphorus. Future research will be needed to determine the exact inorganic phosphorus equivalents of consensus phytase and any possible synergistic effects between the enzyme and other phytases.

Literature Cited


