Genetic engineering of an *Escherichia coli* mutant phytase for thermostability does not affect the enzymatic efficacy in a diet for young pigs

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Abstract

Previous protein engineering research in this laboratory has yielded an *Escherichia coli* AppA2 mutant phytase with improved thermostability and an *Aspergillus niger* PhyA mutant phytase with improved thermostability and pH profile. The objective of this study was to determine the effectiveness of these phytase variants in improving phytate phosphorus utilization by weanling pigs. A total of 40 pigs (5-week old, Yorkshire-Landrace-Hampshire crossbred) were fed a corn-soybean meal based basal diet (BD, without supplemental inorganic phosphorus) or the BD supplemented with the wild-type of AppA2, the AppA2 mutant, or the PhyA mutant at 300 U/kg of diet for four weeks. The phytase activity was assayed in citrate buffer, pH 5.5. Pigs (n = 10/treatment) were housed individually and had *ad libitum* access to feed and water. Daily feed intake and weekly body weight change of individual pigs were recorded, and blood samples of individual pigs were taken weekly to measure plasma inorganic phosphorus concentrations and alkaline phosphatase activity. Pigs fed the wild-type and the AppA2 mutant phytases had higher ($P < 0.01$) plasma inorganic phosphorus concentrations from week 2 through week 4 and lower ($P < 0.05$) plasma alkaline phosphatase activity in week 4 than did pigs fed the BD or the PhyA mutant. The latter two groups showed similar values in these measures. The overall growth performance of pigs was not affected by the dietary treatments. In conclusion, the AppA2 mutant phytase engineered for improved thermostability was as effective as the wild-type in releasing phytate-phosphorus from the diet for weanling pigs. The feeding performance of the PhyA mutant reinforces the difficulty in predicting nutritional value of recombinant enzymes.

Acknowledgements

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Introduction

Phosphorus is an essential mineral for proper bone formation and development. Deficiencies in phosphorus can cause rickets in growing animals, bone loss in adult animals and milk fever. Much of the phosphorus stored in the plant matter that we feed our livestock is stored as phytic acid and is relatively unavailable to simple stomached animals. It is estimated that phytate phosphorus bioavailability ranges from <15% to >50% (Cromwell *et al.* 1972, 1974) with an average bioavailability near 33% (Reddy *et al*. 1982). It is neither cost effective nor environmentally sound to supplement cereal grain and oilseed meal diets with inorganic forms of phosphorus. Phytase is an enzyme that catalyzes the hydrolysis of phosphate groups from the *myo*-insitol ring of phytate. This enzyme is produced by plants, bacteria, and fungi. The phytase produced by rumen microorganisms releases phytate- phosphorus for utilization by ruminant animals. Supplementation of non-ruminant diets with phytase has been shown to be an effective means of increasing phosphorus availability to these animals. By increasing phytic acid phosphorus availability, we can supplement less inorganic phosphorus and thus reduce the phosphorus excreted by the animals. Previous protein engineering research in this laboratory has yielded an *Escherichia coli* AppA2 mutant phytase with improved thermostability (Kim *et al.* 2008) and an *Aspergillus niger* PhyA mutant phytase with improved thermostability and pH profile (Zhang and Lei 2007). The objective of this study was to determine the effectiveness of these phytase variants in improving phytate phosphorus utilization by weanling pigs.

Review of the Literature

Phosphorus in animal nutrition; The National Research Council (1998) addresses phosphorus as an essential macromineral in conjunction with calcium since the utilization and absorption of these minerals are closely associated. The major role of these two elements is in the development and maintenance of the skeletal system (Hays 1976, Kornegay 1985). Other important molecules in vertebrates that include phosphorus are adenosine triphosphate, the main energy storage molecule at the cellular level, phospholipids found in cell walls, and DNA and RNA, the genetic information storage and translation molecules. At the organism level, phosphorylation plays an important role in cellular signaling.

Under current management practices the need for mineral supplementation and meeting mineral requirements of our animals is influenced by the bioavailability of these nutrients in feed stuffs since we control all of the nutrients that the pigs receive so tightly. There must be an adequate supply of both nutrients in available forms in the animals' diet and the minerals must be in a suitable ratio. There must also be adequate vitamin D present (Peo 1991). A ratio of calcium to phosphorous that is too wide can lead to decreased phosphorus absorption and a consequent reduction in growth and bone calcification (Peo et al., 1969) whereas a narrow calcium to phosphorus ratio may result in a more efficient usage of phosphorus (NRC 1998). The council notes that the requirements of calcium and phosphorus for maximizing bone strength and bone-ash content are higher than those to maximize both rate of gain and feed efficiency measures. Feeding excess calcium and phosphorus to maximize bone mineralization does not have

an effect on health or longevity (Cromwell *et al*. 1970, Crenshaw *et al*. 1981, Kornegay *et al*. 1984).

Signs of phosphorus deficiency have been well documented in young pigs fed low phosphorus diets by researchers (Cromwell *et al*. 1995a, Lei *et al*. 1993 a, b). As dietary phosphorus decreases, there is a corresponding decrease in weight gain and feed efficiency as well as in bone breaking strength (Cromwell *et al*. 1995a). Early signs of phosphorus deficiency can be detected in young pigs fed a phosphorus deficient diet in the form of decreased plasma inorganic phosphorus concentrations and increased plasma alkaline phosphatase activity (Lei *et al*. 1993a, b). The NRC (1998) notes that signs of phosphorus deficiency are similar to those of vitamin D deficiency: decreased growth and poor bone mineralization. The results of a chronic deficiency are manifested as rickets in young animals and osteomalacia in mature animals. Sows that have a deficiency of either phosphorus or calcium can suffer from posterior paralysis in which their hind legs are paralyzed.

Phosphorus in animal feeds; Phosphorus exists mainly in the form of phytate in cereal grains and grain by-products and oilseed meals, which are currently the basis for swine diets in the United States, and is poorly available to pigs (Cromwell and Coffey 1991, Reddy *et al*. 1982, Cosgrove, 1966). The bioavailability of phosphorus in these feedstuffs is highly variable, ranging from less than 15% in corn to about 50% in wheat, according to Cromwell *et al*. (1972, 1974). Phosphorus stored in oilseed meals, like soybean meal, also has low bioavailability (Cromwell, 1992). Previous research attributes the higher availability of phosphorus in wheat to a naturally occurring phytase enzyme (McCance & Widdowson 1944, Mollgaard 1946, Pointillart *et al*. 1984).

When working with the phytase enzyme in the laboratory, it is important to consider what substrate is used. The phytate present in animal feed is a complex that involves several different cations (Augspurger *et al.* 2003). The sodium phytate used in laboratory evaluation of phytase activity has mainly sodium salt bound as a cation. The phytate found in animal feeds may also be complexed with proteins or carbohydrates (Augspurger *et al.* 2003), further complicating the difficulty in assessing activity in the laboratory.

The NRC Nutrient Requirements of Dairy Cattle (2001) state that the phytase activity from ruminal microbes makes almost all of the phosphorus bound as phytate available to ruminant animals. Since non-ruminant animals like pigs, chickens, and humans do not have this microbial population before the site of phosphorus absorption in their gut, phosphorus must be supplemented in another form. Several sources of phosphorus are supplemented into swine diets. These include animal protein sources, bone or meat meals, and inorganic phosphorus complexed with other elements.   The Animal protein sources, such as milk and blood byproducts, have high levels of available phosphorus (Cromwell *et al.* 1976, Hew *et al.* 1982, Coffey & Cromwell 1993). The availability of phosphorus from bone and meat meals has been contested; with some studies reporting about 67% (Cromwell 1992) available and others reporting up to 90% (Traylor and Cromwell 1998). Feeding animal by-products to other animals has become a source of great concern to the public and these products are quite expensive.

Feeding inorganic phosphorus also has its disadvantages. Inorganic phosphorus is a limited, non-renewable resource and represents a major expense for animal feeding (Lei *et al.* 1993a, b, Kim 2006). There is also increasing concern about the high levels of

excreted phosphorus in areas where swine production is intensive (Lei *et al*. 1993a, b, Cromwell *et al*. 1995b). The bioavailability of phosphorus in inorganic phosphorus supplements is highly variable. Even within the same kind of inorganic phosphorus supplement, bioavailability can vary with source and processing (Kornegay and Radcliffe 1997).

Some research has explored the possibility of engineering low phytate plants and phytase transgenic plants and animals (Golovan *et al.* 2001, Li *et al.* 1997, Spencer *et al.* 2000, Ullah *et al*. 2002). Cromwell *et al.* (1998) report that phosphorus in low phytate corn can be as much as 77% available. Considering that, as previously mentioned, phosphorus in traditional corn is less than 15% available, this is a marked improvement. Despite great potential in phytase engineering of feedstuffs or animals, there has been little use of these products in the commercial industries.

Because of the cost associated with inorganic phosphorus supplementation to a plant-based diet and the poor reception of altering our plants and animals for higher dietary phosphorus availability, the next option is to explore supplementing non-ruminant diets with phytase. This enzyme releases phytate phosphorus for absorption. Major improvements in the bioavailability of phytate phosphorus have been observed when microbial phytase is supplemented into high-phytate cereal grain and oilseed meal diets. In addition, the magnitude of response to phytase supplementation is influenced by many factors, including available and total dietary phosphorus, amount of phytase supplemented, calcium to phosphorus ratio, and the level of vitamin D present (Jongbloed *et al*. 1993, Lei *et al.* 1994, Kornegay 1996).

Phytase; The NRC (1998) describes phytase as an enzyme that cleaves the ortho-phosphate groups from phytate by catalyzing the step-wise removal of phytate groups from the *myo*-inositol ring. There have been low levels of phytase activity reported in the small intestine of some mammalian species; however it is not believed to contribute significantly to the degradation of phytate and absorption of phosphorus (Lei *et al*. 1993a). Yi and Kornegay (1996) reported that there were varying levels of phytase activity in different species and Augspurger *et al*. (2003) found that different species had different responses to phytase supplementation.

Supplementing microbial phytases in low phosphorus diets has been shown to improve both bone strength and growth performance of pigs (Cromwell *et al.* 1995a, Cromwell *et al.* 1993, Simons *et al.* 1990, Young *et al.* 1993). It has also been shown that addition of phytase to a low phosphorus diet decreases fecal phosphorus excretion (Cromwell *et al.* 1995b, Lei *et al.* 1993a, b). By feeding phytase and therefore lowering the level of dietary phosphorus, phosphorus excretion can be lowered by 30% to 60% (Bridges *et al.* 1995, Carter *et al.* 1996).   Han *et al.* (1997) showed that it was feasible to feed phytase to not only young pigs but also throughout the entire growing-finishing period. They also showed that by supplementing a corn-soybean meal diet low in phosphorus with phytase decreased both fecal phosphorus and fecal nitrogen excretion, making the feeding of phytase as a supplement environmentally appealing.

It has also been shown that diets composed of cereal grains with high phytate but low phytase activity supplemented with other grains that have high phytase activity, like wheat, have improved phytate phosphorus bioavailability (Han *et al.* 1997). Since there is such a low activity to supplement mass ratio, addition of this type of phytase

supplement affects the nutrient density of the diet. Microbial phytase concentrations have been shown repeatedly to be a viable alternative to feeding plant phytases as diet supplements (Lei *et al.* 1993a, b).

One important commercial phytase is the PhyA phytase isolated from *Aspergillus niger*, currently marketed as Natuphos. Another important commercial phytase, marketed as Optiphos, is the AppA2 phytase, isolated from *E. coli*. The *E. coli* that produced AppA2 was isolated from pig colon by Rodriguez *et al.* (1999). Rodriguez *et al*. (1999) reported that the AppA2 enzyme exhibits only 19% amino acid homology with PhyA, suggesting that there are very different possibilities for engineering these phytases to be better feed additives.

Reasons for engineering the phytase enzyme; The mutant enzymes utilized for this study were engineered for improved thermostability and pH activity profile. It is important improve the thermostability of the phytase enzyme in order to allow it to withstand the temperatures necessary for feed pelleting (Gentile *et al*. 2003, Kim *et al*. 2008, Mullaney *et al*. 2002, Zhang & Lei 2008). Simons *et al*. (1990) reported that feed pelleting caused up to a 52% loss of activity in phytase enzymes. Vieille and Zeikus (1996) stated that using naturally thermostable enzymes isolated from thermophillic bacteria was not practical because physiological temperatures are too low for them to function. Additional glycosylation sites have been reported to improve thermostability (Rodriguez *et al.* 2000).

It has previously been shown that the primary site of phytase activity in pigs is the stomach where the pH is about 3.5 (Yi & Kornegay 1996). Many researchers have noticed that the low activity of *Aspergillus niger* PhyA phytase at pH 3.5 made it an

inefficient feed additive because this is the site for phytase to function in the digestive system (Han *et al*. 1999, Pagano *et al*. 2007a, Wyss *et al*. 1999). In addition, it is necessary to consider the hydrolytic effect of pepsin and other proteases on phytases. Indeed both Augspurger *et al*. (2003) and Rodriguez *et al*. (1999) commented on the ability of some phytases to resist this degradation more than others. Although this study did not focus on enzymes engineered for this purpose, it would be a logical path for research to follow should an enzyme that is ideal in other parameters (e.g. thermostability and pH profile) be found.

  Genetic engineering of mutants used in this study; Mutants of the *E. coli* AppA2 phytase were created using site directed mutagenesis (Rodriguez *et al.* 2000) and directed evolution (Kim *et al*. 2008). The individual mutations, Asp144Asn, Val227Ala, and Gly344Asp were incorporated sequentially to determine if there were additive effects from combining these mutations designed to improve thermostability (Kim *et al.* 2008). Asp144Asn introduced a hydrogen bond between side chains, which strengthened the tertiary structure of the protein and stabilized local interactions. Val227Ala is thought to decrease structural hindrance due to alanine having a smaller side chain than valine. Gly344Asp was intended to introduce another hydrogen bond between side chains but was not shown to increase the thermostability over mutants containing the previous two mutations; however it did increase the catalytic efficiency of this mutant over other mutants (Kim *et al.* 2008). Kim *et al*. (2008) hypothesized that this mutation might interact with the Asp144Asn mutation electrostatically to decrease the predicted effect. This is just one example of the difficulty in making predictions about the effects of many mutations.

The mutant containing all three of these mutations, M2 was selected for this study. It was shown to release 25% more inorganic phosphorus from soy phytate and had an overall catalytic efficiency nearly three times higher than the wild type AppA2 (Kim *et al*. 2008). After heating to 80°C for 10 minutes, M2 retained 17-19% more activity than the wild type enzyme. Kim *et al*. (2008) reported that there was no change in glycosylation or pH activity profile for M2 when compared to wild type AppA2.

The *Aspergillus niger* PhyA mutant phytase utilized for this study (PhyA27) included mutations designed to alter both thermostabilty and pH activity profile (Zhang & Lei 2007). This phytase exhibited a unique bimodal pH activity profile, with a dip in activity at pH 3.5. According to Kim *et al*. (2006) even though PhyA has good catalytic efficacy, its pH profile makes it a poor feed additive for animals. The thermostability mutations Ala58Glu, Pro65Ser, Gln191Arg, and Thr271Arg were incorporated using site-directed mutagenesis (Zhang *et al*. 2007). Ala58Glu and Pro65Ser introduced two new hydrogen bonds in the same loop area and had a greater effect on the thermostablity of the molecule than the other two mutations because the loop was more flexible than the alpha helix area and thus its tertiary structure was more affected by the mutations (Zhang *et al*. 2007). The Gln191Arg substitution created a new salt bridge and eliminated a repulsive ionic interaction in the alpha helix domain of the molecule. Thr271Arg also created a new salt bridge in the alpha helix region, as well as a new hydrogen bond, also in the alpha helix (Zhang *et al*. 2007). Zhang *et al*. (2007) reported a 7°C increase in melting temperature as well as higher efficiency at hydrolyzing phytate phosphorus from soybean meal over the wild type PhyA enzyme. Zhang *et al*. (2007) also report that the

increased thermostability did not compromise function of the mutant enzyme at physiological temperature.

Previous efforts to alter the pH activity profiles of other enzymes have been largely unsuccessful (Kim *et al*. 2006). Amino acid residues located in the substrate binding site were targeted for mutation due to their expected impact on the pH activity profile. Mutations were incorporated using site specific means (Kim *et al*. 2006). The Lys300Glu mutation in our mutant enzyme of interest was shown to alter the bimodal pH profile of the PhyA wild type enzyme. It eliminated the dip at pH 3.5 and improved the catalytic efficiency of the enzyme toward sodium phytate between pH 3.5 and 5.0 (Mullaney *et al*. 2002, Zhang *et al*. 2008). The specific activity of the thermostable mutant with the Lys300Glu substitution was increased 31% at pH 5.5 and >50% at pH 3.5 from that of the wild type PhyA, but the addition of Lys300Glu did not alter the thermostability of the parent enzyme (Zhang *et al*. 2007). Thus the chosen mutantPhyA27 included all of the five discussed mutations: Ala58Glu, Pro65Ser, Gln191Arg, Thr271Arg, and Lys300Glu.

Previous evaluations of phytase using pigs; There have been numerous previous studies conducted to evaluate the efficacy of phytases fed to pigs (Pagano *et al.* 2007a,b, Kim *et al.* 2006, Crowe 2003, Stahl *et al.* 2004, Han *et al.* 1997). All of these studies used weanling pigs. Han *et al.* (1997) also studied the effects of supplementing phytase through the finishing period. The overall experimental design of these studies was similar. Pigs were given *ad libitum* access to feed and water throughout the treatment period, and their growth performance data were collected on a weekly basis, as were blood samples (Pagano *et al.* 2007a,b, Kim *et al.* 2006, Stahl *et al.* 2004, Crowe 2003).

The studies also included a preliminary or adjusting period before the start of data collection to allow pigs to acclimate to diets and their environment. Studies that evaluated the effectiveness of new phytases at releasing phosphorus from phytate utilized a BD without supplemented inorganic phosphorus during this adjustment period (Kim *et al*. 2006, Pagano *et al*. 2007a, Crowe 2003). Studies that utilized treatment diets that include inorganic phosphorus fed either no adjustment diet (Pagano *et al*. 2007b) or an adjustment diet with low supplementation of inorganic phosphorus (Stahl *et al*. 2004).

In studies where pigs were housed individually and daily feed waste was recorded (Pagano *et al*. 2007a, Stahl *et al*. 2004, Crowe 2003) average daily feed intake (ADFI) and feed efficiency were also monitored. In all cases pigs were fasted before blood samples were taken. The most common length of fasting was 8 hours (Pagano *et al*. 2007a, b, Stahl *et al*. 2004, Kim *et al*. 2006). Blood samples were collected from the anterior vena cava into heparinized tubes and then fractionated by centrifugation to allow for plasma collection and assay. Plasma was used to assay for inorganic phosphorus concentration (PIP) and alkaline phosphatase activity (AKP) (Pagano *et al*. 2007a, b, Kim *et al*. 2006, Stahl *et al*. 2004, Crowe 2003, Han *et al*. 1997).

Materials and Methods

All animal protocols and housing was reviewed and approved by the Cornell University Institutional Animal Care and Use Committee.

Two mutant phytases (PhyA27, M2) produced in our laboratory were selected using information from previous studies (Kim, Weaver, & Lei, 2008; Zhang & Lei, 2007). Four treatment diets were prepared to compare the two mutant phytases to a BD without supplemental inorganic phosphorus and to a previously studied phytase to aide in

determination of the performance of the mutant enzymes. The basal diet utilized by

Pagano *et al.* (2007a) was modified to account for a change in the vitamin mineral premix

from the manufacturer (Appendix 1). The basal diet (BD) was formulated without

supplemented inorganic phosphorus. The three other diets were supplemented with

various phytase enzymes at 300 U/kg. The three enzymes used were AppA2, PhyA27,

and M2. Fifty weanling pigs (Yorkshire-Landrace-Hampshire cross, 4 weeks) were fed

BD for one week prior to the start of the study to allow for adjustment to a ground corn

based diet from commercial pelleted creep feed and to normalize the body phosphorus

status of the pigs. After that week, the pigs were weighed and blood samples taken.

Forty pigs were allotted to the four treatment diets (n=10) by weight, plasma

inorganic phosphorus (PIP) concentration, sex, and litter. The pigs were housed

individually and fed treatment diets for four weeks. Daily feed offered and orts were

recorded. Feeders were removed 8 hours prior to the weekly blood collections to

normalize blood chemistry among the pigs. Before feeders were replaced, the amount of

feed left in the feeder was recorded. Weekly feed intakes were recorded to calculate

ADFI. Using weekly weight data, average daily gain (ADG) was calculated. The feed

efficiency was calculated as the ratio of feed to gain.

Pigs were weighed before weekly blood samples were collected. The pigs were

restrained in dorsal recumbency for collecting blood samples into heparinized vacutainer

tubes. The blood was stored and transported to the laboratory on ice. Blood was

centrifuged in order to separate the plasma and cellular fractions (Pagano *et al*. 2007b)

(Appendix 2). PIP assays were completed on the same day as samples were collected

according to the method described by Pagano *et al*. (2007b) (Appendix 3). Plasma

samples were collected and stored at -20°C until they could be assayed for alkaline

phosphatase (AKP) activity, as outlined by Bowers & McComb (1966) (Appendix 4).

Phytase activity was determined using 0.2M citrate buffer, pH 5.5, with sodium

phytate as the substrate. Phosphorus released from the sodium phytate was determined

by a colorimetric reaction producing a reduced phosphomolybdate complex that is yellow

to blue in color depending on the concentration of phosphorus present. The color

reactions were evaluated using a 96-well spectrophotometer measuring absorbance at

820nm (Kim & Lei 2005). Phytase activity was determined prior to mixing the broth

from the yeast fermentation with corn meal to create a concentrate feed additive with

activity 1000U/g. The phytase activity of this concentrate feed additive was also

determined to weigh the amount needed for the final concentration to be 300U/kg in the

diets (Appendix 5). Phytase activity in the final diets was also determined to confirm that

all three enzymes were mixed at similar levels (Appendix 5).

Statistical analysis was performed using JMP7 Statistical Discovery from SAS

(SAS Inst. Inc., Cary, NC). Data means were compared using ANOVA for the main

effect and then a Tukey-Kramer Honestly Significant Difference test, alpha level 0.05 to

determine differences.

Results

Growth performance; The overall growth performance of pigs was not affected

by the dietary treatments. The average group weights did not ever differ throughout the

study (Figure 1). The ADG (Figure 2) of pigs did not differ throughout the trial. ADFI

(Figure 3) did not significantly differ for any of the groups at any of the time points. The

feed efficiency (Figure 4), calculated as feed to gain, did not differ at any of the time points.

     Biochemical analysis; Plasma inorganic phosphorus levels and alkaline phosphatase activity was affected by dietary treatments. Pigs fed BD+AppA2 and BD+M2 had higher ($P < 0.01$) plasma inorganic phosphorus concentrations than did pigs fed BD from week 2 through week 4 (Figure 5). Pigs fed BD+AppA2 also had higher PIP concentrations than did pigs fed BD in week 1. Pigs fed BD+PhyA27 did not differ from those fed BD. Pigs fed BD had higher AKP than did pigs fed BD+M2 in week 4 (Figure 6).

     Other observed results included general discomfort of pigs fed BD and BD+PhyA27 in the later part of the study. There were several pigs in both groups whose health records indicate that they were lame and reluctant to stand in the final week of the study as recorded by both the researcher and the manager of the facility where the pigs were housed.
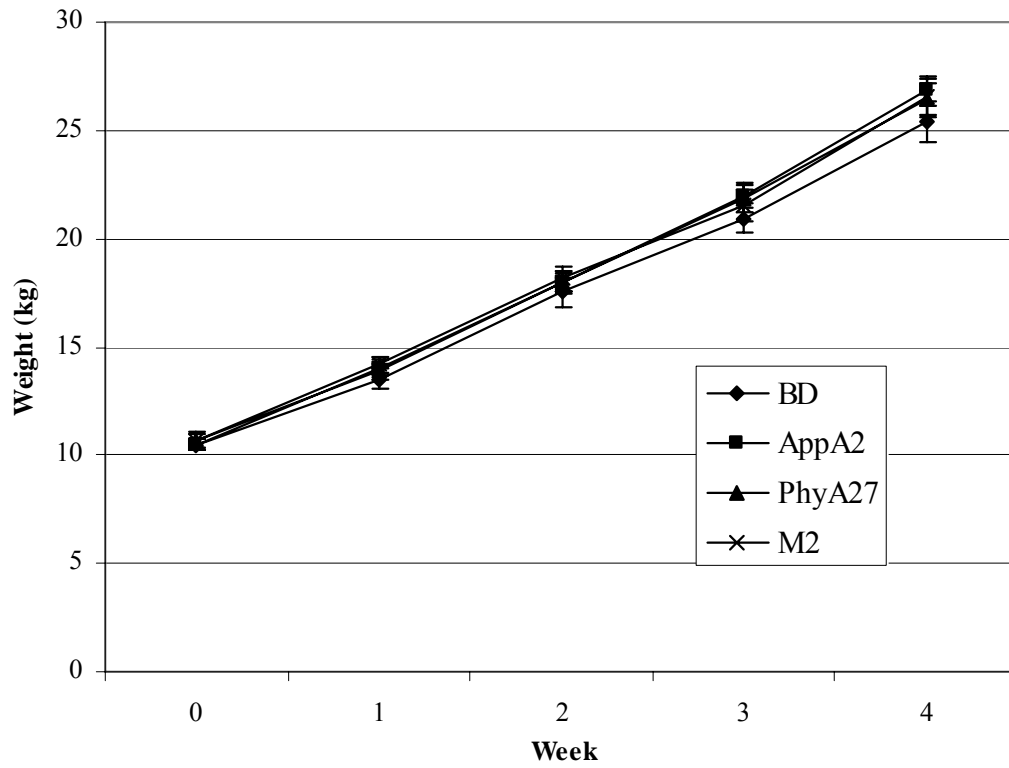
Figure 1. Body weights of pigs fed different treatment diets by week. No significant treatment differences observed.
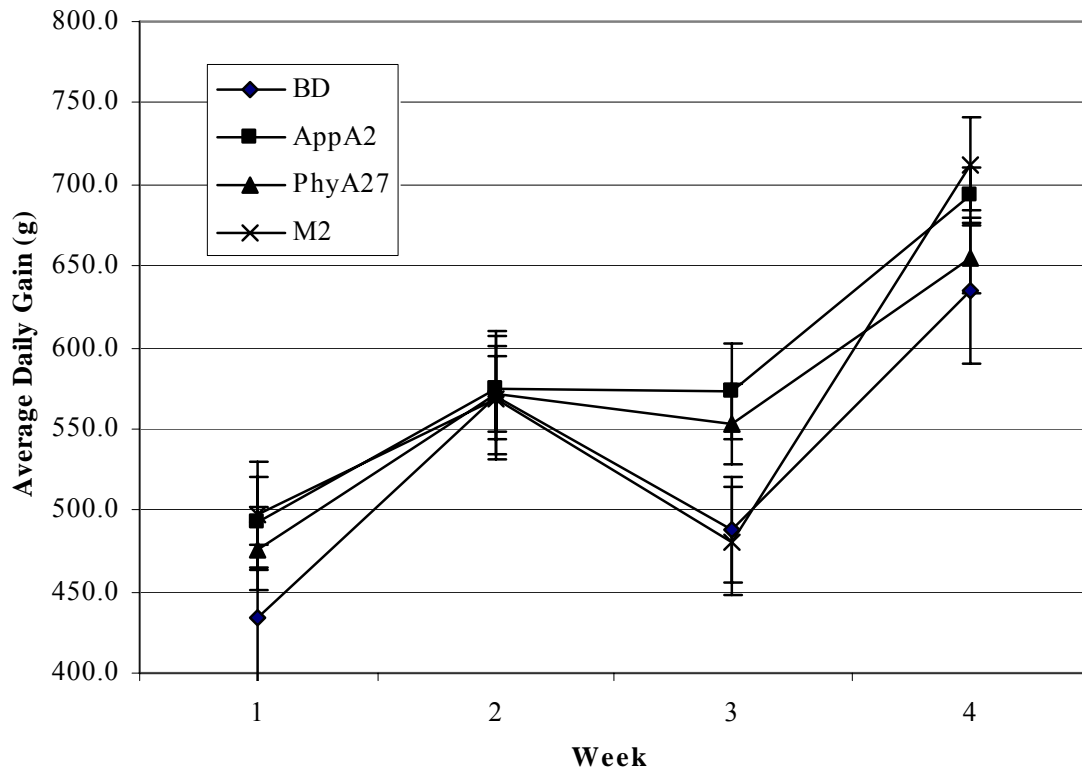
Figure 2. Average daily gain of pigs fed different treatment diets by week. Values were calculated from individual average daily gains for each pig. No significant treatment differences observed.
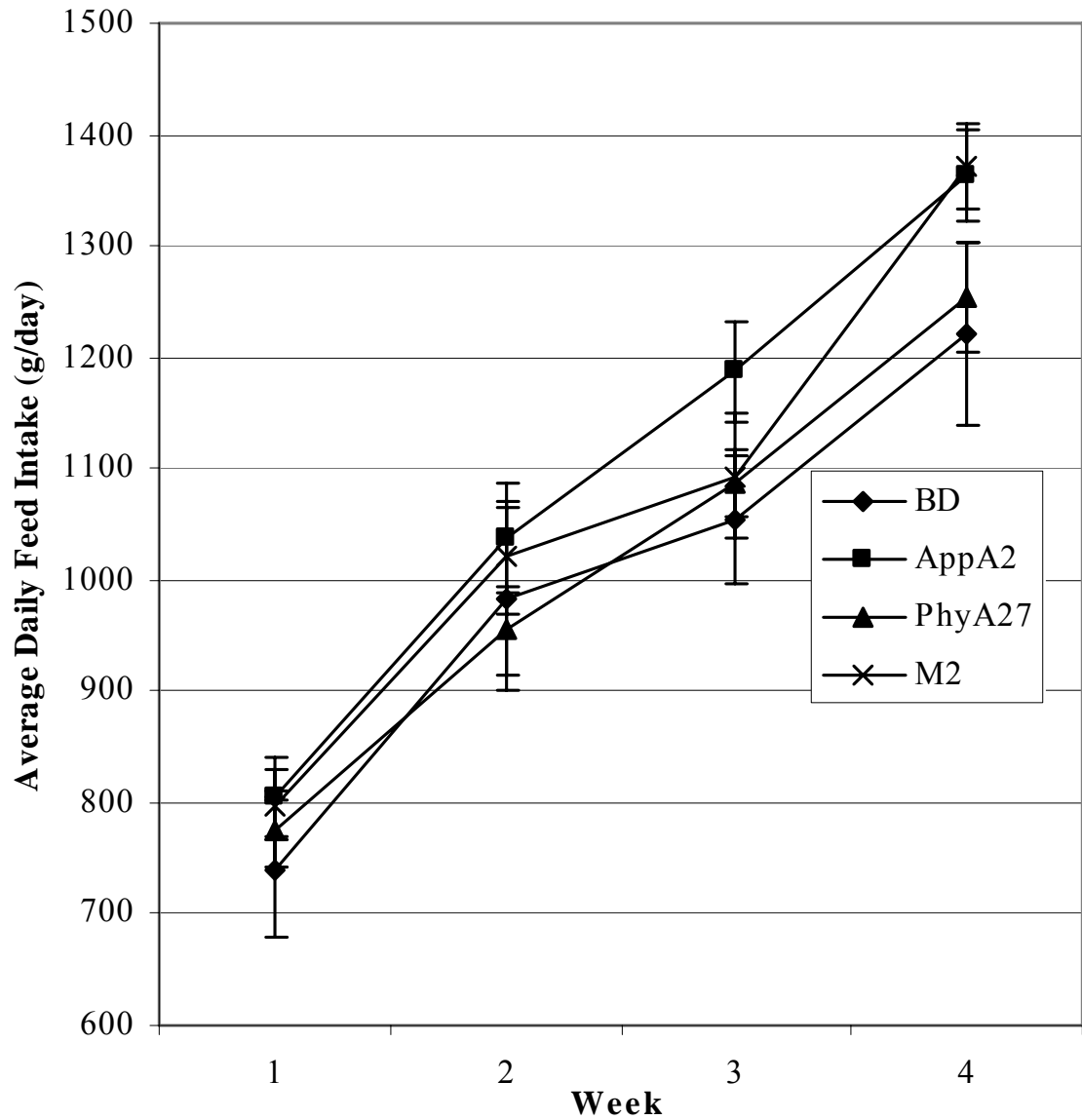
Figure 3. Average daily feed intake of pigs fed different treatment diets by week. Values were calculated using individuals averages. No significant treatment differences observed.
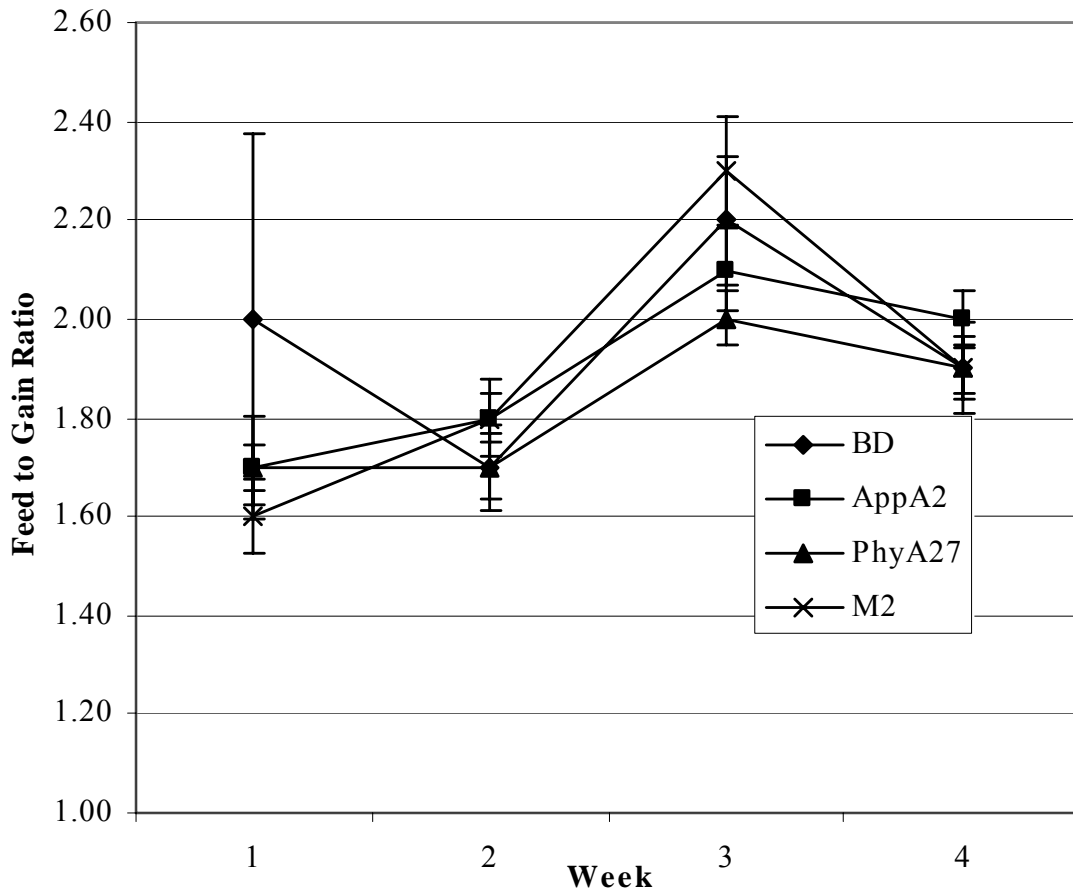
Figure 4. Average weekly feed to gain ratio for pigs fed different treatment diets by week. Values were calculated using individual feed to gain ratios. No significant treatment differences observed.
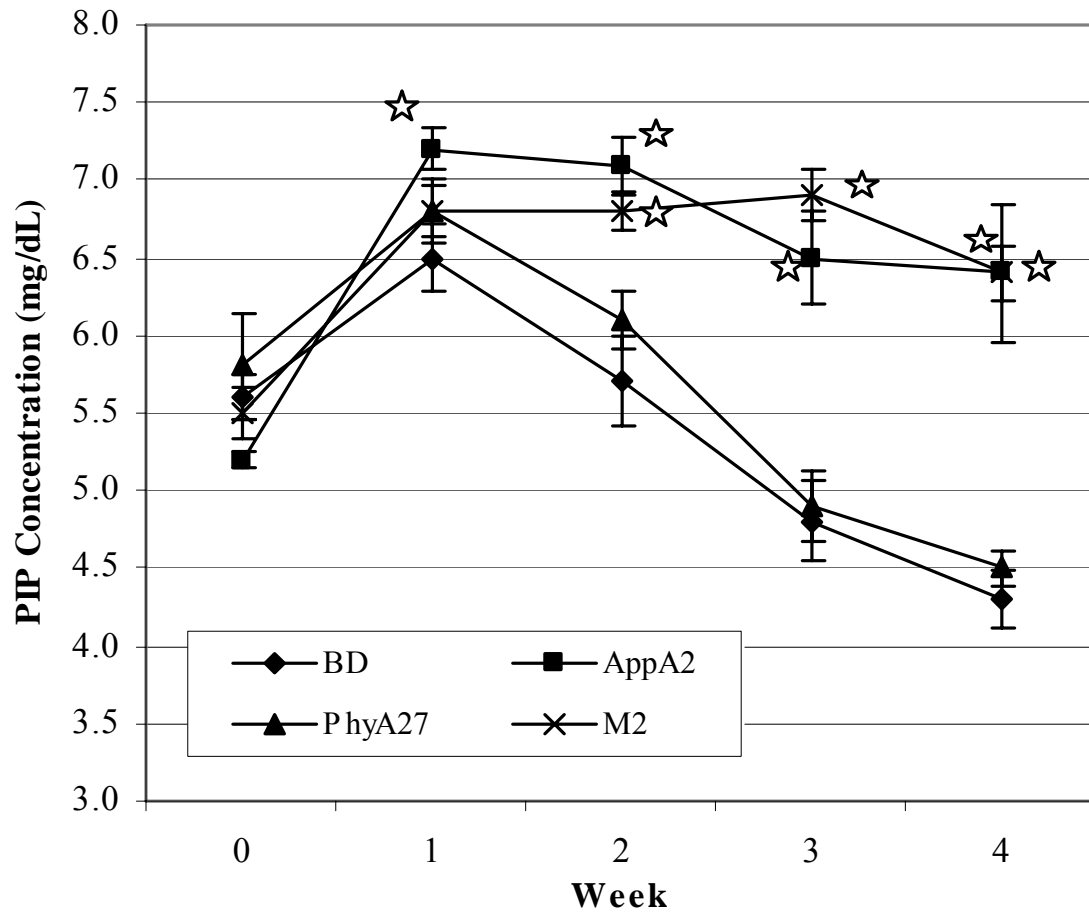
Figure 5. Plasma inorganic phosphorus concentration of pigs fed different treatment diets by week. ☆ Indicate significant treatment difference from BD in a given week (p<0.05).
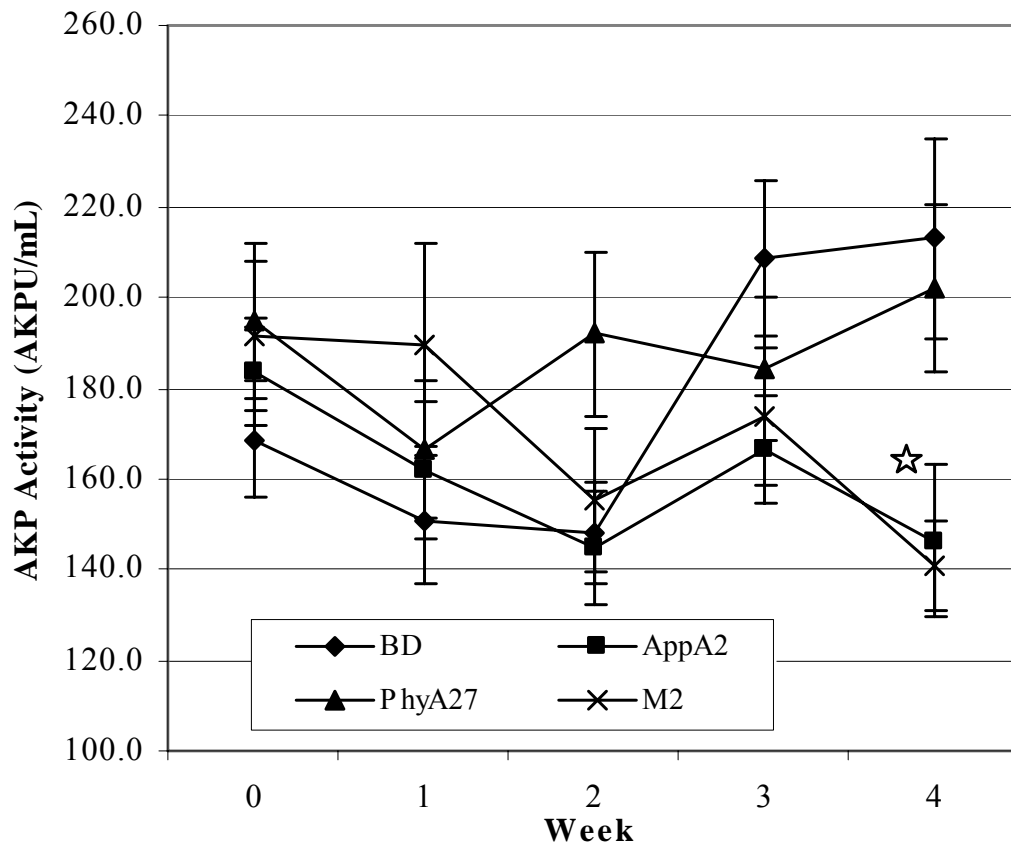
Figure 6. Alkaline phosphatase activity of pigs fed different treatment diets by week.

☆ Indicate significant treatment difference from BD in a given week (p<0.05).

Discussion

The data from the PIP is a good reflection of body status with respect to phosphorus. The PIP concentrations show that pigs fed BD+PhyA27 did not perform any better than pigs fed BD alone, in contrast the higher ($p<0.05$) PIP concentrations of pigs fed BD+AppA2 and BD+M2 indicate that these phytases were effective to the same degree. Previous studies have shown AppA2 to be an effective enzyme and it is commercially available as Optiphos, thus the new mutant, M2 has comparable activity to a proven product.

Since there are many sources for AKP in the body, and the assay used in the present study was not specific for testing bone AKP, the greater variation in the data and the longer time needed to elicit a response are not totally unexpected. Phytase at these low levels is expected to be a replacement for iP in the diet and not to alter the bone remodeling process. Thus it is expected that pigs fed BD had higher AKP activity than those fed a phosphorus adequate diet (BD+AppA2). Even though AKP in pigs fed BD+AppA2 did not statistically differ from AKP in pigs fed BD, there is still a 68% difference in their AKP activity. Therefore the difference can still be considered biologically meaningful. The rather high AKP activity in pigs fed BD+PhyA27 suggests that this phytase was unable to release enough orthophosphate for nutrient requirement by these growing pigs The lower AKP activity ($p<0.05$) of the pigs fed BD+M2 than the negative control (BD) pigs indicates the effectiveness of M2 in releasing sufficient phosphorus for the growth of these pigs

As expected, the inverse relationship between PIP and AKP (Figure 7) is described by a logarithmic function ($R^2=0.52$). Since AKP should be increased in pigs
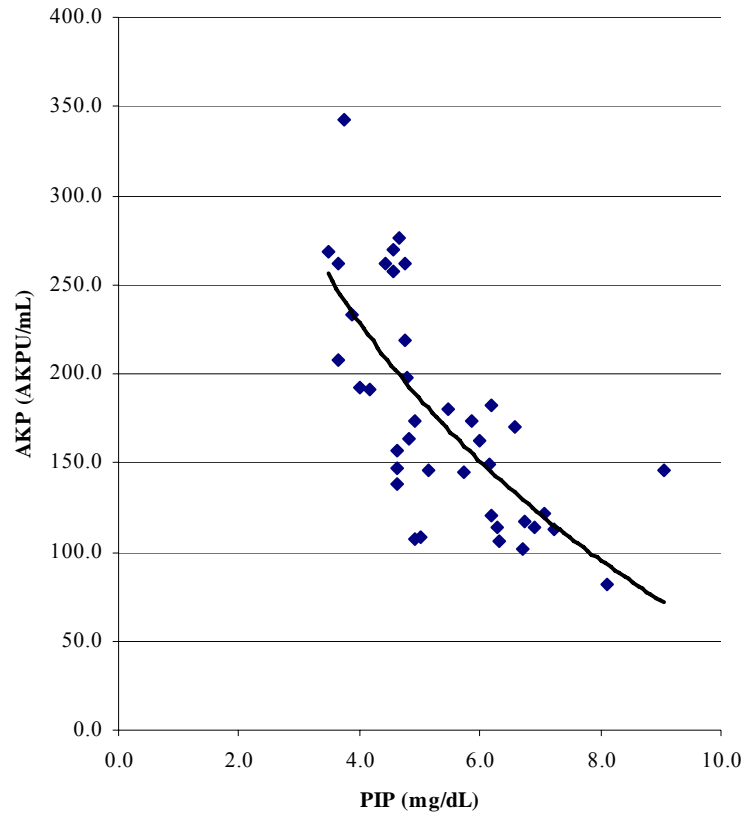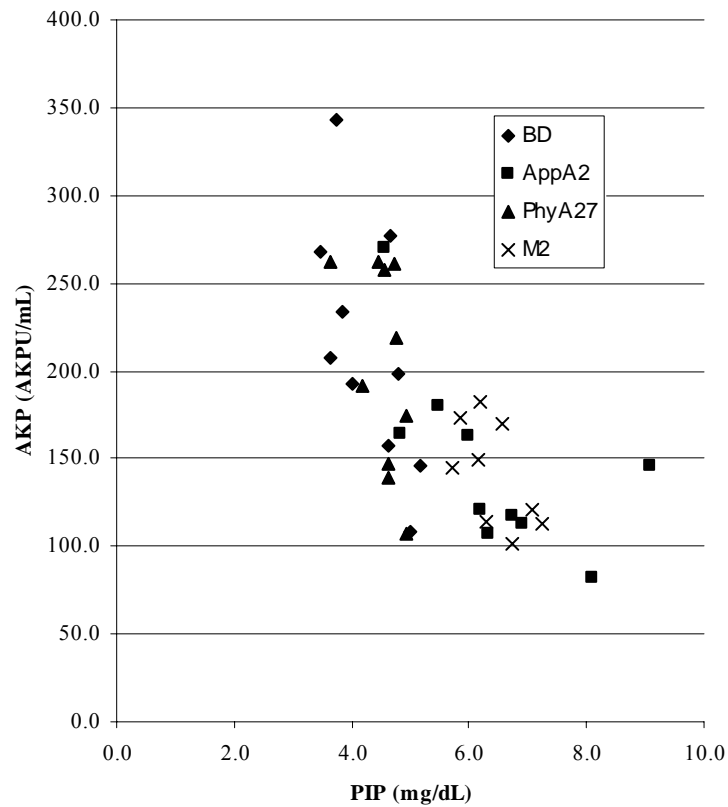
A.

Figure 7. (a) Correlation of AKP activity with PIP concentration. The trend line is a logarithmic function with equation $y = -192.5Ln(x) + 495.74$ and $R^2 = 0.5175$. Data used is from all groups in week 4. (b) Correlation data coded by treatment groups.

B.

that are phosphorus deficient, this response would be expected to be correlated with a lower PIP concentration. This relationship was also noted by Gentile *et al*. (2003).

The observed discomfort and lameness in pigs fed BD were expected since phosphorus deficiency has been shown to affect bone health. The observed discomfort in pigs fed BD+PhyA27 is explained by the lack of effectiveness of this phytase enzyme, as indicated by the PIP and AKP data as well.

The lack of a sustained difference in growth performance of pigs in different treatment groups is expected to be due to the strong genetic selection of pigs for fast growth. Indeed, in a study published sixteen years ago, there was no difference in feed efficiency due to dietary phytase levels (Lei *et al*. 1993). Gentile *et al*. (2003) found no effects of diet on growth performance. Augspurger *et al*. (2003) found that there was a poor fit between weight gain and increasing levels of phytase supplementation in pigs.

In conclusion, the AppA2 mutant phytase engineered for improved thermostability (M2) was as effective as the parent (wild-type) enzyme in releasing phytate-phosphorus from the diet for weanling pigs. The improved thermostability likely makes M2 a better feed supplement than the wild-type AppA2 because animal trials indicate that it partially overcomes problems associated with transportation, storage, and processing of the enzyme. The animal feeding results also encourage us to continue the mutation studies in order to develop an ideal phytase as the feed additive in swine diets.

The feeding performance of the PhyA mutant reinforces the difficulty in predicting nutritional value of recombinant enzymes. One reason that the PhyA27 may not have performed as well as the other two phytase enzymes is that it has lower activity at pH 3.5 than it does at pH 5.5 (Zhang & Lei 2008), meaning that the mix rate of 300

U/kg of diet is actually higher than the functional activity in the pigs' stomachs. In contrast, the *E. coli* phytases have almost twice the activity of pH 5.5 at pH 3.5 (Kim *et al.* 2008). This difficulty in comparing different phytase enzymes with different pH optima has been described before (Gentile *et al.* 2003, Yi & Kornegy 1996, Augspurger *et al.* 2003). Other factors that may have affected the activity of PhyA27 are increased rigidity or changes in active site conformation caused by the additional mutations. Previous research has shown that consensus phytases suffered from reduced specific activity while still having high thermostability (Lehmann *et al.* 2000). The sodium phytate substrate used for *in vitro* assays in the lab is slightly different from the phytate that is found in feeds, as described in the literature review of this thesis.

Future research concerning these mutants should be a mixed endeavor. Additional mutations to further improve the M2 enzyme should be explored and tested as long as progress is being made. New mutations in the PhyA27 variant may be considered to make it an effective feed additive. Other directions for research concerning *Aspergillus niger* phytases would benefit from exploring other mutants that have been created and have shown promising *in vitro* results, as described by Zhang and Lei (2008).

Future animal trials shall be conducted to determine effects of phytase on health. Current projects along this idea include studies concerning bone formation and composition, iron bioavailability, and effects on other systems that utilize P and Ca, such as the vitamin D modification pathway. An *in vitro* assay that is more reliably predictive of animal performance would also be helpful. As addressed earlier, there are important differences between the phytase substrate employed in most laboratory evaluations of phytase and the phytate found in biological systems (Augspurger *et al.* 2003). Other

researchers in this laboratory have assayed the enzymes using soybean meal as the substrate (Crowe 2003, Kim *et al.* 2008) but further research in this area is necessary to make it a reliable method.

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Appendix 1. Composition of Diets

Ingredient	% of Diet			
	BD	WT	PhyA27	M2
Corn	67.10	67.10	67.10	67.10
Soybean Meal, 48% CP	28.00	28.00	28.00	28.00
Spray-dried plasma protein	1.50	1.50	1.50	1.50
Limestone	1.05	1.05	1.05	1.05
L-Lysine HCl	0.10	0.10	0.10	0.10
Corn Oil	1.00	1.00	1.00	1.00
Swine Amicro 4 (Vitamin/Mineral Premix)	0.20	0.20	0.20	0.20
MgO_4	0.05	0.05	0.05	0.05
Dical Phosphate	0.00	0.00	0.00	0.00
Salt	0.50	0.50	0.50	0.50
Tylan 10 (Antibiotic)	0.50	0.50	0.50	0.50
Total	100.00	100.00	100.00	100.00

	Mix Rate	g/kg		
Phytase WT AppA2	0	0.3310	0	0
Phytase PhyA 27	0	0	0.3311	0
Phytase M2	0	0	0	0.3330

Appendix 2. Blood sample processing protocol: centrifugation and storage

Whole blood samples arrive from farm on ice.

Centrifugation

Turn on centrifuge and allow it to cool to 4°C.

Load samples into centrifuge tube holders, leaving one space in each holder empty for balance tubes.

Use water and empty tubes to balance pairs of holders. Be sure that rubber stoppers are on balance while adding or removing water.

Centrifuge at 3000 x g for 10 minutes. 2000 rpm on the GS-6KR Beckman Instruments centrifuge.

When complete, remove vials from holders and place back on ice. Take care not to disturb the samples.

Prepare Microcentrifuge Tubes

Prepare labels to print using the Avery 5167 template. Use Avery 5667 labels.

Pig identification number, date collected, week of study, type of sample (ex. plasma), and your initials should be on the label. 2-3 tubes are needed for each sample

Be careful not to fill the label all the way across as they overlap some on the tubes.

Print labels.

Write pig identification number on the top of each microcentrifuge tube and stick a label to it, being careful not to winkle the label.

Collect Samples

Pipette supernatant into the microcentrifuge tubes.

Place tubes back on ice until ready to store.

<u>Storage</u>

Place samples in labeled sample storage box.

Box label should included date, week of study, name of study, description of sample (ex. plasma), and your name.

Put box in -20°C or -80°C freezer.

<u>Clean Up</u>

Dispose of remaining fractions of blood in sink and rinse glass collection tubes.

Discard glass tubes in glass collection bin. Rubber stoppers can be disposed of in the regular trash.

Appendix 3. Plasma Inorganic Phosphorus Assay (Fiske and Subbarow 1925, Gomorri 1942)

<u>Reagents</u>

*Label all solutions that you make with the name of the solution, concentration, pH (if appropriate), date made, and your initials.

Sodium Molybdate (MS) Solution

- Dissolve 5.0 g of Sodium Molybdate Dihydrate, $Na_2MoO_4 \cdot 2H_2O$ (Sigma, 331058-100G), in 500 mL ddH_2O in a 1 L beaker.

- Add 14 mL concentrated Sulfuric Acid, H_2SO_4 (Fischer, A300-212), while stirring constantly.

- Transfer to 1 L volumetric flask.

- Add ddH_2O until volume reaches 1 L.

- Store at room temperature.

Metol Solution (3% sodium bisulfate and 1% metol)

- Dissolve 3.45 g Sodium Bisulfate Monohydrate, $NaSO_4 \cdot H_2O$ (Sigma, S-9631), to 100 mL ddH_2O

- Add 1.0 g Metol, 4-methylamino phenol hemisuflate (Fluka, 69748).

- Mix well.

- Store in brown bottle wrapped in aluminum foil at 4°C.

12.5% Tricholracetic Acid (TCA) Solution

- Dissolve 125 g TCA, CCl_3COOH (Fischer, A322), in 800 mL ddH_2O.

- Pour into 1L volumetric flask.

- Add ddH_2O until volume reaches 1L.

- Store at room temperature.

Phosphorous Standard Stock Solution (1000 ppm)

- Dry 5.0-10.0 g of Ammonium Phosphate Dibasic, $(NH_4)_2HPO_4$ (Sigma, A-1167), in the oven at 80°C for 2 hours.

- Cool in desicator for 30 minutes.

- Dissolve exactly 4.3533 g in 1 L of ddH_2O.

- Store at room temperature.

Standard Preparation

Prepare a 1:5 dilution of the 1000 ppm P stock solution in a 15 mL tube (1 mL 1000 ppm P + 4 mL ddH_2O).

Vortex.

Prepare standard solutions according to following table in 15 mL tubes.

Store at room temperature

Standard	1	2	3	4	5	6	7	8	9	10
P 200ppm (mL)	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	1.0
ddH_2O (mL)	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.0
12.5% TCA (mL)	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Final Volume (mL)	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
P (mg/dL)	0.0	0.4	0.8	1.2	1.6	2.0	2.4	2.8	3.2	4.0

Sample Preparation

- Pipette 800 µL 12.5% TCA into microcentrifuge tubes.

- Add 200 µL plasma to each tube.

- Vortex.

- Incubate at RT for 15 minutes.

- Centrifuge samples at 2500 RPM for 3 minutes at 4°C.

Assay

- Pipette 40 μL of ddH$_2$O in duplicate wells for a blank.

- Pipette 40 μL of each standard into a 96-well plate in duplicates. Be careful not to touch the bottom of the plate when handling.

- Pipette 40 μL of each sample's supernatant into the 96-well plate in duplicates, recording the identification number of each sample.

- Add 200 μL of MS solution to each well.

- Add 20 μL of Metol to each well. Use the multipipettor for last two steps, do these in order.

- Cover plate with a plastic cover slip and agitate gently being careful not to splash any of the solution onto the cover slip.

- Incubate plate at RT for one hour.

Measurement of Phosphorus

- Start up reader and computer according to directions.

- Use the "Plasma Phosphorus" protocol. Settings: End point, λ=700 nm, correct for path length.

- Adjust the layout to match your plate. Check for correct P concentration in standards and label samples with a dilution factor of 5.

- Read the plate.

Appendix 4. Alkaline Phosphatase (AKP) Activity Assay (Bowers and McComb 1966)

Reagents

Buffer – 0.84 M 2-Amino-2-Methyl-1-Propoanol (2A2M1P), pH 10.17

- Dissolve 52.752 g 2-Amino-2-Methyl-1-Propoanol (Sigma, A5888-500G) in 300 mL ddH$_2$O.

- Adjust pH to 10.17 using 5M MaOH.

- Transfer to 500 mL volumetric flask.

- Add ddH$_2$O until volume reaches 500 mL

Substrate – 60 mM Phoshpatase Substrate in 1.5 mM MgCl$_2$

*Make fresh each time.

- Dissolve 20 mg of Phosphatase Substrate, *p*-nitrophenol phosphatae hexahydrate (Sigma, P4744-10G), in 1.0 mL 1.5 mM MgCl$_2$ (Sigma M4880-100G)

- Vortex.

Assay

- Allow plasma samples to thaw on ice.

- Prepare appropriate amount of phosphatase substrate in 15 mL tube.

- Place substrate in water bath or incubator at 30°C.

- Record sample identification numbers on a 96-well plate template, use 2 or 3 duplicates per sample.

- Place 270 µL 2A2M1P buffer into each sample well, use multipipettor.

- Place 280 µL 2A2M1P buffer into two wells for blanks.

- Add 10 µL of plasma to each well.

Microplate Reader

- Turn on the plate reader and computer according to the start up protocol located next to machine.

- Use the "AKPhosphatase" protocol. Settings: kinetic, $3\lambda=405$, 900, 977, heat to 30°C, 2 minute reading at 33 second intervals.

- Adjust the layout to match your plate.

- Select read.

- The reader will begin to heat up, allow it to reach 30°C and then incubate for 5 minutes.

- At about 4:30 in the incubation, retrieve the warmed substrate and put it into a trough.

- After the 5 minute incubation, remove the plate and place 20 μL of substrate into each well. Use multipipettor.

- Place plate back in reader and select read plate.

- After reading the plate, export the 900 and 977 readings and the Mean V 405 (Mean V#1) for further calculations.

Calculations

Import data into Excel using tab-delimited protocol.

Correction for path length (Held 2004)

- Subtract the 977 values from the 900 values.

- Divide by 0.18

Other calculations

- Subtract the average blank Mean V #1 value from the rest of the Mean V#1 values.

- Using the corrected mean V#1 values, divide by the path correction for each well.

- Multiply by 1.6.

- Calculate the average value of each sample across your duplicates.

- Calculate the standard deviation between your duplicates.

Appendix 5. Results of Phytase Activity Assay

Phytase activity in cornmeal concentrate feed additive.

Enzyme	Activity (U/g)	% Recovery [a]
AppA2	917	92
PhyA27	920	92
M2	913	91

[a] Based on 1000 U/g

Phytase activity in final research diets.

Diet	Activity (U/kg)[b]	% Recovery [c]
BD	22	(endogenous activity in grains)
AppA2	175	58
PhyA27	189	61
M2	200	67

[b] Endogenous (BD) activity has been subtracted from all other diet phytase activities.
[c] Based on 300 U/kg