CHAPTER 1

INTRODUCTION

1.1 Phytic acid and phytate

Phytic acid was discovered by W. Pfeffer first in 1872 (Oberleas, 1972; Maga 1982; Reddy et al., 1982). He differentiated particles found in seeds and suggested globoid particles were a combination of phosphate and carbohydrate (Reddy et al., 1982). Posternak studied phytic acid extensively from 1900 to 1905 (Reddy et al., 1982; Wodzinski and Ullah, 1996). In 1907, Suzuki and his associates determined that inositol was a major part of phytic acid (Reddy et al., 1982). Today phytic acid is called *myo*-inositol hexaphosphoric acid or specifically as 1,2,3,4,5,6-hexakis (dihydrogen phosphate) *myo*-inositol (Maga, 1982) and exists as phytate after phytic acid chelates divalent minerals (Erdman, 1979; Lei and Stahl, 2001).

Phytic acid is a major component of cereal grains and oil seeds (Oberleas, 1972; Maga 1982; Wodzinski & Ullah, 1996). The Anderson model (Fig. 1.1) is widely accepted over the Neuberg (Fig. 1.2) model as the predominant form in plants (Erdman, 1979). Conjectural models (Fig. 1.3 and 1.4) by Weingartner and Erdman (1979) demonstrate phytic acid and phytate at neutral pH. Common animal diets consisting of oilseeds, legumes and cereals and can be assumed to have approximately 50-80% of P present in phytate form (Reddy et al., 1982; Cromwell, 1992; Eeckhout and De Paepe, 1994; Ravindran et al., 1994; Harland and Morris, 1995).

In phytate form, P has a low bioavailability to simple-stomached species including swine and human (Nelson et al, 1971; Oberleas, 1972; Cromwell, 1980) due to little phytase activity, an enzyme that hydrolyzes phytate, in their digestive tracts (Bitar and Reinhold, 1972; Wise and Gilbert, 1982; Pointillart et al., 1984; Sandberg and Andersson, 1988). Phytate reduces the bioavailability of minerals, such as zinc,

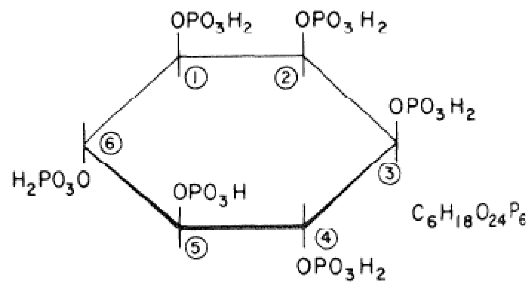

Figure 1.1 Anderson model of phytic acid.

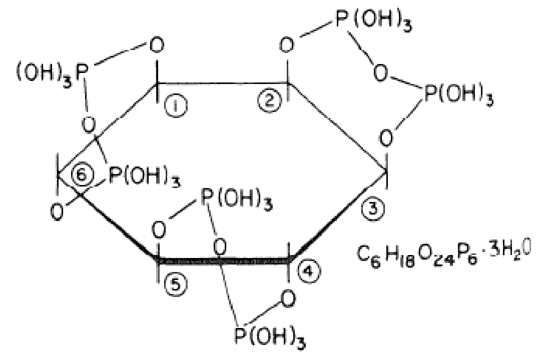

Figure 1.2 Neuberg model of phytic acid.

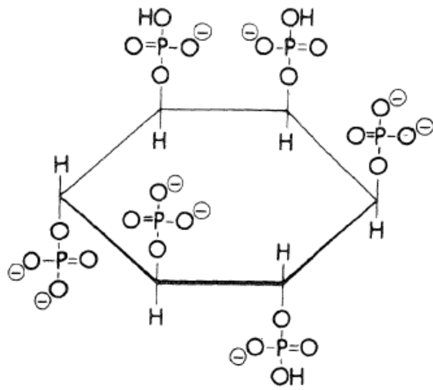

Figure 1.3 Weingartner and Erdman model of phytic acid at neutral pH.

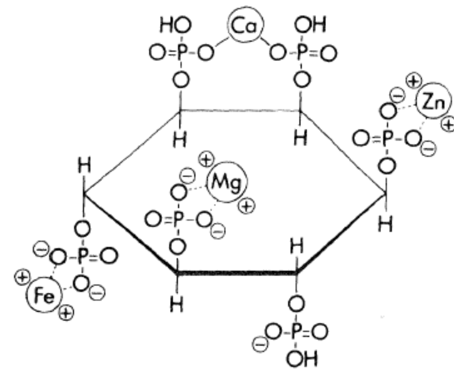

Figure 1.4 Weingartner and Erdman model of phytic acid chelate at neutral pH.

(Figures from Erdman, 1979)

magnesium,calcium, copper and iron (Reinhold, 1971; Davies and Olpin, 1978; Nolan et al., 1987; Hallberg et al., 1987). Phytic acid complexes formed with di- or trivalent cations are less soluble (Erdman, 1979). Phytic acid may also reduce protein solubility (Ritter et al., 1987; Honig and Wolf, 1991).

Ruminants sustain anaerobic microflora in the gut, such as *Selenomonas ruminatium*, *Megasphaera elsdenii*, *Prevotella* sp. and *Mitsuolkella multiacidus* in their gut (Yanke et al., 1998). These organisms produce phytases that release P from phytate.

Since conventional corn-soy diets are high in phytate-P, which is not readily available to pigs, those diets are supplemented with inorganic P to prevent P deficiency (Nelson, 1967). P is one of the most expensive ingredients of pig diets, and high amounts excreted in feces cause environmental concern (Adeola et al., 1998; Lei and Stahl, 2000). Phytic acid remaining in the feces is hydrolyzed, and the free phosphorus is carried into water supplies and causes eutrophication (Wodzinski and Ullah, 1996). As of 1992, Sweeten reported that 36% of total P excretion by production animals in the United States was attributable to swine and poultry. Supplementation by phytase may reduce or eliminate the need for inorganic supplementation of phosphorus, which could reduce feed costs and lessen eutrophication. Pigs, for example, that consumed a phytase supplemented diet excreted up to 50% less phosphorus (Lei et al., 1993a,b). Cromwell et al. (1995) showed a decrease in fecal P by 31% when inorganic P (iP) was partially replaced by supplemental phytase. These findings support the idea that phytases improve the retention of dietary phosphorus and reduce the amount lost to the environment.

1.2 Phytase

Phytases are a group of enzymes discovered by Suzuki and co-workers while researching rice and wheat bran in 1907 (Wodzinski and Ullah, 1996). Phytase is necessary for the hydrolysis of phytate into inositol and phosphate (Nelson, 1967). The International Union of Biochemistry and Molecular Biology (IUBMB) lists three phytases: a 3-phytase (microbial or fungal) which hydrolyzes the ester bond at the three position of *myo*-inositol hexakisphosphate into 1D-*myo*-inositol 1,2,4,5,6-pentakisphosphate + phosphate, a 6- phytase (plant) which hydrolyzes phytic acid to produce 1D-*myo*-inositol 1,2,3,4,5-pentakisphosphate + phosphate, and a 5-phytase (plant) that hydrolyzes phytic acid to 1L-*myo*-inositol 1,2,3,4,6-pentakisposphate + phosphate. Remaining ester bonds are hydrolyzed at different rates by phytase in a stepwise removal (Reddy et al., 1982), by alkaline phosphatases (Bitar and Rheinhold, 1972), and non-specific acid phosphatases, which are present in the digestive tract (Maenz and Classen, 1998).

Phytases are highly expressed in germinating seeds (Reddy et al., 1982; Gibson and Ullah, 1990). Eeckhout and De Paepe (1994) found cereals: rye, triticale, wheat and barley to be high in phytase in a comparison to other cereals, oil meals, beans, roots and other plant products. They also found that wheat-bran contained phytase. Since phytases are proteins, they are susceptible to digestion by other enzymes such as pepsin and trypsin and to denaturation at different temperature and pH conditions. For plant phytase, the pH optimum ranges from 4.0 to 7.5, with most plant phytases having an optimum between 5.0 and 5.6. The optimal temperature is between 45 and 60°C (Wodzinski and Ullah, 1996).

1.3 Intrinsic sources of phytase

The variation of intrinsic phytases in cereal grains and oil seeds are the reason why phytate-P bioavailability differs among them (Cromwell, 1980; Pointillart et al., 1984; Pointillart et al., 1987; Eeckhout and De Paepe, 1994). The role of cereal phytase in phytate digestion in the stomach and small intestines of humans was examined by Sandberg and Andersson (1988). They provided ileostomy subjects with diets containing 16g/day of raw or phytase-deactivated wheat bran. They found that nearly all the phytate was recovered from ileostomy contgetns of subjects fed phytase-deactivated wheat bran, but only 40% was recovered in subjects fed raw wheat bran. They concluded that human mucosal phytase did not play a significant role in the digestion of phytate but that intrinsic wheat bran phytase was the major factor in phytate hydrolysis. The use of high phytase cereals is one alternative to improving phytate-P use of simple-stomached animals, yet this is not fully desirable to the swine industry because of the high fiber contents of the cereals (Stahl, 1998).

1.4 Fungal sources of phytase

Howson and Davis (1983) and Shieh and Ware (1968) did large screenings of phytase activity from soil samples and fungi. Aspergilli had the highest phytase production among those samples. Both groups found *A. niger* produced the most active extracellular phytase. *A. niger* produces two different phytases: phy A with a pH optima at 5.0 and 2.5 and phy B with pH optimum at 2.5. Fungal phytases have an optimal pH range of 2.5-7.5 and an optimal temperature range of 35-63°C. Due to pH optimums in the range of digestive tract pH and a fairly broad temperature optimum, phy A has been the most widely used. Some other phytase-containing fungi studied include *Emericella nidulans*, *Talaromyces thermophilus*, *A. fumigatus* (Pasamontes et al., 1997), *A. terreus* and *Mycleiophthora thermophila* (Mitchell et al., 1997). More

than 200 fungal phytases have been tested for production of extracellular phytase (Liu et al., 1998).

1.5 Bacterial sources of phytase

Sources of bacterial phytase include *Aerobacter aerogenes* (Greaves et al., 1967), *Pseudomonas sp.* (Irving and Cosgrove, 1971), *Bacillus subtilis* (Powar and Jagannathan, 1982; Shimizu, 1992), *Escherichia coli* (Greiner et al., 1993), and *Klebsiella aerogenes* (Tambe et al., 1994). Bacteria were not considered a good source of phytase for a feed additive due to a low yield and a pH optimum of neutral to alkaline (Wodzinski and Ullah, 1996). However, the *E. coli* appA gene, isolated from contents of pig colon, was cloned and expressed (appA2) in *Pichia pastoris* (Rodriguez et al., 1999a). They found that appA2 had a broad pH optimum between 2.5-3.5 for sodium phytate and a temperature optimum of 55°C. AppA2 incubated in soybean meal released more P from phytate than appA or phyA (Rodriguez et al., 1999a). These properties make appA2 a viable phytase supplement.

In contrast to intrinsic plant phytases, microbial phytases are concentrated and therefore do not affect nutrient concentrations when included in swine feeds. Studies on the addition of microbial phytase to swine and poultry diets have shown an improvement in phytate-P bioavailability (Nelson et al., 1971; Simons et al., 1990; Jongbloed et al., 1992; Lei et al., 1993a,b). Microbial supplementation of phytase could potentially replace iP supplementation in swine and poultry diets (Simons et al., 1990; Lei et al., 1993a; Cromwell et al., 1993; Sebastian et al., 1996). Lei et al. (1993a) found that growth performance of weanling pigs fed a diet containing 1,200 U/kg microbial phytase was equal to that of pigs fed a diet supplemented with 0.21% of iP and actual phosphorus retention was only 7% less for pigs fed phytase. Remarkably, pigs supplemented with iP were ingesting 44% more P yet excreting

100% more P daily than pigs fed the basal diet + phytase. Similar results have been seen in young pigs (Young et al., 1993; Yi et al., 1996; Murry et al., 1997), finishing pigs (Han et al., 1997;O'Quinn et al., 1997) and lactating sows (Kemme et al., 1997). These studies indicate that replacement of iP with supplemented microbial phytase in swine diets could decrease current P pollution from industry by half.

Supplemental dietary phytase has the ability to diminish antinutrient properties of phytate. When phytate is hydrolyzed by phytase, it increases the bioavailability of minerals that are chelated by phytate. Zn bioavailability is reduced by phytate in simple-stomached animals (O'Dell and Savage, 1960; Bobilyea et al., 1991; Saha et al., 1994) and is improved when phytase is added to a diet with low available Zn (Lei et al., 1993c; Adeola et al, 1995). Fe bioavailability is also negatively affected by phytate (Saha et al., 1994) and phytase improves its bioavailability (Stahl et al., 1999). Positive effects of phytase on apparent digestibilities and retentions of Ca, Mg and Cu in simple-stomached animals have also been demonstrated (Pallauf et al., 1994; Sebastian et al., 1996; Kemme et al., 1997; Li et al, 1998).

1.6 Factors influencing phytase function

It is necessary to know optimal levels of phytase supplementation based on the P requirements of swine in different phases of production, as well as source of diet. In studies of young pigs fed similar diets, different levels of phytase have been tested to optimize phytate-P utilization. Young et al. (1993) found 500 U of phytase/kg corn-soy diet to be maximal because there was no significant difference in average daily gain or feed efficiency between pigs supplemented with 500 or 1000 U of phytase/kg of diet. However, Lei et al. (1993a) found an increase in growth performance as well as a decrease in plasma alkaline phosphatase activity when pigs were fed 1050 instead of 750 U of microbial phytase/kg corn-soy diet, suggesting an optimal

supplementation level greater than observed by Young and associates. They also showed a maximum response of 1,200 U of phytase/kg of diet on plasma P levels. Differences seen in these studies could be a result of different enzyme efficacies dependent on microbial source (Matsui et al., 2000), form of the enzyme, and the temperature and pH optima of the enzyme.

Efficacy of phytase can be affected by ingredients in the diet such as various minerals, pelleting and other processing methods, diet form and vitamin D level (Ravindran et al., 1995; Sandberg and Andlid, 2002). Phytase enzymes isolated from plant (Mahajan and Dua, 1997) and microbial (Greiner et al., 1993) sources are inhibited by iP. Studies in swine and poultry have shown a greater phytase efficacy at lower iP levels (Ravindran et al., 1995; Denbow et al., 1995; Kornegay et al., 1995; Qian et al., 1996). Greiner et al. (1993) also showed an inhibition of phytase activity *in vitro* by minerals such as Ca, Zn, Cu and Fe. Phytase from rapeseed was also inhibited by Zn, Fe, and Cu (Mahajan and Dua, 1997) possibly by competing with the enzyme for substrate. However, the concentration of Zn, Fe, and Cu in diets used to study phytase efficacy in pigs is generally comparable and are unlikely to cause discrepancy in maximal phytase activity as much as iP concentration or $Ca:P_{total}$ ratio in the diet. Swine and poultry studies have shown that high Ca supplementation leading to a large $Ca:P_{total}$ ratio, reduces dietary supplemental phytase activity reducing its positive effect on animals fed available P-deficient diets (Lei et al, 1994; Qian et al., 1996; Qian et al., 1997).

1.7 Digestive system

Pigs and humans are very similar in their digestive system and physiology (Pond, 1991). The digestive system of pigs includes mouth, pharynx, esophagus, stomach, small intestine and large intestine that together act as a portal for nutrients,

electrolytes and fluids to enter the body. Food is moistened and carbohydrates are hydrolyzed by salivary amylase in the mouth. Amylase action continues through the esophagus until the fundus of the stomach where the pH drops below 3.6 (Yen, 2001). The low pH also serves as a barrier to microbes. Hydrochloric acid, three forms of pepsin, rennin, and lipase make up the main digestive secretions of the stomach. The pepsins have pH optima at about 2 and 3.5 but are inactive at a pH above 6 (Cranwell, 1995). Primary functions of the stomach include mixing, storage and controlled release of digesta into the duodenum. Liquid empties from the stomach due to the pressure gradient between the stomach and the duodenum, whereas solids are released when the particles are 2mm or less. Solids that are not broken down are swept into the small intestine during fasting (Yen, 2001). Emptying of the stomach is fastest in the first hour after a meal (Yen, 2001).

In a full-grown pig, the small intestine is 16 to 21 m long, of which the first 4 to 5% is duodenum, the last 4 to 5% is the ileum and the remainder is jejunum (Schummer et al., 1979). In a newborn pig, the duodenum is also the same proportion of small intestine but the differentiation between the jejunum and ileum is not clear (Widdowson et al., 1976) although it seems likely it too is proportional to that of a full-grown pig. Digesta entering the duodenum is mixed with bile, pancreatic juice and other small intestine alkaline secretions, which raise the pH of the digesta out of the pepsins' range. Nutrients are hydrolyzed by enzymes in the small intestine and are absorbed along it. Vitamins, water and minerals of dietary and endogenous origin are also absorbed there (Yen, 2001).

The large intestine of a full grown pig is 3.5 to 6 m in length (Schummer et al., 1979). The first 7 to 8 % is the cecum. The colon of the pig is between the ileum and rectum and has three parts, the ascending, transverse and descending colons (Schummer et al., 1979). The large intestine holds 30-60% of the total digesta, which

is retained for 20-38 hours. In comparison, digesta only remains 0 to 6 hours in the stomach and 2 to 6 hours in the small intestine (Low and Zebrowska, 1989). Absorption of fluids and minerals, as well as bacterial digestion of protein and carbohydrates, are gained by the extended retention time (Low and Zebrowska, 1989).

1.8 Sites of phosphorus absorption and phytase function

Phosphorus is mainly absorbed as inorganic phosphate (Jongbloed, 1987) but may be absorbed in the organic form when part of phospholipids (Wilkinson, 1976). Moore and Tyler (1955b), using labeled P in the diet, found that phosphorus absorption took place in the proximal half of the small intestine. Some investigators have concluded there is P-absorption in the large intestine (O'Quinn et al., 1997; Liu et al. 2000; Seynaeve et al., 2000); yet others have found that exogenous P is not absorbed in the large intestine (Gueguen et al., 1968; Partridge, 1978; Ajakaiye et al., 2003). Differences in the diets used in these studies, such as the grinding of diet, or the flow of digesta back from the large to small intestine may deceptively give the impression of absorption. Therefore to ensure absorption of phytate-P it is important that it is hydrolyzed as much as possible before mid-jejunum.

Early *in vivo* studies evaluated the efficacy of dietary plant phytase (Pointillart et al., 1987; Lantzsch et al., 1992) and diets without any dietary phytase supplementation (Sandberg et al., 1993). Studies of the dietary plant phytase showed an increased hydrolysis and absorption of phytate-P with plants of higher phytase activity such as, wheat, barley and triticale, as compared to corn. Lantzsch et al. (1992) determined through postslaughter techniques that hydrolysis of phytate in the stomach and proximal intestine was about 50, 45, and 38% for wheat, barley and corn, respectively, and about 55-60% by the distal half of the small intestine for all. Sandberg et al., (1993) determined that phytate in rapeseed, a plant with virtually no

intrinsic phytase, fed to pigs degraded 35-45% in the stomach and small intestine. This was about 25% lower than a control diet composed of barley and wheat. Therefore plant phytase can contribute to the hydrolysis of phytate more than intestinal phytase or unspecific phosphatase activity of the pig. However, even with plant phytase, nearly half the phytate reaches the colon intact (Lantzsch et al., 1992); so about half the phytate-P might not be digested by the pig and therefore would be excreted into the environment.

Microbial fungal phytase from *Aspergillus niger* has also been evaluated using cannulated pigs (Jongbloed et al., 1992) and slaughter technique (Yi and Kornegay, 1996). These studies confirmed stomach as the main site of *A. niger* function. Jongbloed et al., (1992) saw a 50-64% improvement in phytate-hydrolysis in digesta at the end of the small intestine in groups fed corn-soy diet with 1,500 U/kg phytase as compared to an unsupplemented group. Yi and Kornegay, (1996) saw about a 50% reduction in phytase activity in the stomach from feed levels, that was further reduced in the upper small intestine and found negligible in the lower small intestine. The high activity in the stomach makes microbial phytase ideal for allowing great phosphorus absorption. Since AppA2 has a more acidic pH optimum and a greater resistance to pepsin digestion than *A. niger* (Rodriguez et al., 1999) it is possible that it may function in different sections in the digestive tract since more may pass from the stomach not hydrolyzed.

1.9 Pigs as models for phytase applications in humans

Swine is an excellent model for human nutritional research because of the many similarities between their digestive tracts (Dodds, 1982; Miller and Ullrey, 1987). One such benefit of phytase for humans is the aforementioned ability of the enzyme to improve the bioavailability of minerals such as Fe, Zn, Cu and Ca. High

phytase levels in the diet, for this reason, could be especially beneficial to persons in developing nations where mineral deficiencies are common.

Another, more novel, area in which pigs can serve as a model for human health is researching phytases' ability to improve bone strength. It has long been known that phytase has the ability to improve bone strength in swine by increasing the bioavailability of phytate-P from feed in low-iP diets (Harper et al., 1997; O'Quinn et al., 1997; Nunes and Guggenbuhl, 1998; Gentile et al., 2003). However, it does seem possible that bone strength is improved by other factors when pigs are supplemented with dietary phytase (Cromwell, 1991; Yi et al., 1996; Murry et al., 1997). Pigs make an excellent model of bone improvement studies through dietary means also for humans because of similar bone morphology as compared to other animal models (Aerssens et al., 1998).

Improving bone strength in humans is very important because osteoporosis is a highly prevalent disease, affecting millions of people around the world (Consensus Development Conference, 1993). Osteoporosis is defined by low bone mass and micro-architectural deterioration of bone tissue, leading to bone fragility and a consequent increase in risk of fracture (Consensus Development Conference, 1993). Fractures from osteoporosis are major causes of morbidity and disability in the elderly and sometimes lead to death. These fractures represent a large economic burden (Johnell, 1997). In developed countries, which have a high rate of fracture incidence, rates are much greater in women than men (Cooper et al., 1992; Cohen and Roe, 2000). It is believed as demographic changes occur, steep increases of fractures will be observed in Asia and Latin America making osteoporosis a truly global problem (Cooper et al., 1992). Since no magic bullet is known to prevent osteoporosis, it is necessary to look into alternate forms of treatments and preventions.

It may be more advantageous to try to prevent osteoporosis from an early age, since optimal peak bone mass accumulated in youth may delay the onset of osteoporosis (Matkovic et al., 1994; Heaney et al., 2000). Behavior modification, such as increased physical exercise and intake of Ca-rich foods, can be implemented at a young age to prevent osteoporosis. However, most pharmacological therapies (bisphosphonates, hormone replacement therapy (HRT), selective estrogen receptor modulators (SERMs), etc.) are not considered for use until a person, usually being a Caucasian or Asian post-menopausal female, is identified at risk (Lindsay and Cosman, 1999). Therefore it may be advantageous to increase peak bone mass in these populations at a young age. Since the effects of phytase on bone in pigs has been seen in weaning to finishing pigs (Cromwell, 1991; Yi et al, 1996; Nunes and Guggenbuhl, 1998, it is conceivable that phytase used as a dietary supplement for humans could be used particularly early in life to help strengthen bones and delay or prevent osteoporosis.

1.10 Objectives

It is important that most of phytate-P is hydrolyzed prior to the mid-jejunum because it has been shown that most extrinsic P absorption occurs in the proximal half of the small intestine (Moore and Tyler, 1955b). Research indicates that the stomach is the main function site of *A. niger* activity (Jongbloed et al., 1992; Yi and Kornegay, 1996). *E. coli* phytase AppA2 has a more acidic pH optimum and greater pepsin resistance than fungal phytases which could lead to a superior performance (Rodriguez, 1999b). These differences between the enzymes could potentially result in different sites of activity in the digestive tract. One objective of this thesis was to determine the fate and function of *E. coli* AppA2 in the digestive tract of pigs. An increase in bone integrity related to phytase supplementation has been seen in pigs

(Cromwell, 1991; Young et al., 1993; Yi et al., 1996; Murry et al., 1997; Gentile et al., 2003). It remains unclear whether high levels of supplemental phytase could produce additional benefit on bone strength in young pigs fed P-adequate diets. Since pigs are a good model for human nutrition (Dodds, 1982; Miller and Ullrey, 1987) and bone health (Aerssens et al., 1998), the second objective of this thesis was to determine if phytase could improve bone integrity by factors other than P-accretion in young pigs.

CHAPTER 2

FATE AND FUNCTION OF SUPPLEMENTAL *ESCHERICHIA COLI* PHYTASE APPA2 IN THE GASTROINTESTINAL TRACTS OF YOUNG PIGS

2.1 Introduction

Microbial phytase supplementation has been shown to effectively improve utilization of phytate-P in plant feeds by pigs (Sands et al., 2001; Traylor et al., 2001) and poultry (Johnston and Southern, 2000), reducing their fecal P excretion to the environment and improving bioavailability of phytate-P (Cromwell et al., 1993) and chelated minerals (Martinez et al, 2004). Jongbloed et al. (1992) and Yi and Kornegay (1996) have shown that the stomach serves as the major site for supplemental *Aspergillus niger* fungal PhyA phytase in hydrolyzing digesta phytate-P in pigs. Recently, several bacterial phytases have been expressed and characterized (Rodriguez et al., 1999). The *Escherichia coli* phytase AppA2, isolated from pig colon, has been shown to be more effective than fungal phytases in releasing phytate-P in diets for both swine and poultry (Applegate et al., 2003; Augspurger et al., 2003). The superior performance of this bacterial phytase is probably attributable to a more acidic pH optimum and a greater resistance to pepsin digestion than that of fungal PhyA phytase (Rodriguez et al., 1999). However, it is unclear if these distinct enzymatic properties of bacterial phytase lead to actions and fates in the gastrointestinal tract different from those of fungal phytases. Experimental data in this regard will help explain the nutritional role of bacterial phytases and the improved feeding efficacy. Thus, our objective of this study was to determine the effects of supplemental dietary E. *coli* AppA2 phytase on total phytase activity, soluble and total P, and total calcium in stomach, duodenum, upper jejunum, lower jejunum, ileum, and colon of young pigs.

2.2 Materials and Methods

2.2.1 Animals, Diets, and Treatments

Our protocol was approved by the Institutional Animal Care and Use Committee of Cornell University. All pigs used in the study were weanling crossbreds (Landrace-Hampshire-Duroc) selected from the Cornell University Swine Farm and allotted into treatment groups based on BW, litter and sex. Prior to experiments, pigs were weaned at 4 wk of age and fed a corn-soybean meal basal diet (BD) (**Table 2.1**) without supplemental inorganic phosphorus. In Exp. 1.1, 18 pigs (6-wk old, 8.3 ± 0.2 kg BW) were allotted to three groups (n = 6), and fed BD, BD + 500 U of phytase/kg of feed), or BD + inorganic P (0.1%) for 4 wk. In Exp. 1.2, 30 pigs (8-wk old, 14.5 ± 0.2 kg BW) were divided into 3 groups (n = 10), and fed BD, BD + 500 U of phytase/kg of feed or BD + 2,000 U of phytase/kg of feed for 2 wk. The phytase used in both experiments was an *E. coli* appA2 (40,750 units/g), produced by Phytex (Portland, ME), and added into the diets at mixing. The actual phytase activity in the experimental diets was analyzed (Kim and Lei, 2005). The BD contained adequate levels of all nutrients (NRC, 1998), but had no supplemental inorganic P and a reduced Ca concentration. The ratios of Ca:P in all diets were maintained at 1.24:1 to ensure the function of phytase (Lei et al., 1994). All pigs were penned individually in an environmentally controlled barn (21 to 26°C; 12:12 h light:dark cycle) and allowed free access to feed and water.

2.2.2 Growth Performance and Sample Collection

Individual pig feed waste was collected daily, and body weight (BW) was measured weekly to calculate average daily gain (ADG), average daily feed intake (ADFI) and gain:feed ratio. Blood samples of individual pigs were collected at

Table 2.1. Composition, in percent, of basal and control diets as fed basis

Ingredients	*Basal*	*Control*
Corn, grain	67.10	66.55
Soybean meal, 48% CP	28.00	28.00
Spray-dried plasma protein	1.50	1.50
Limestone	1.05	1.05
L-Lysine·HCl	0.10	0.10
Corn oil	1.00	1.00
Vitamin/mineral premix[a]	0.25	0.25
Dicalcium phosphate	0.00	0.55
Salt	0.50	0.50
Tylan® 10	0.50	0.50
Total	100.00	100.00
Nutritive Values[b]		
ME (kcal/kg)	3325.27	3306.46
Crude protein	20.04%	19.99%
Ca, total	0.51%	0.63%
P, total	0.41%	0.51%
P, available	0.09%	0.19%
Ca:P, total	1.24	1.24

[a]Vitamin and mineral premix supplies (per kilogram of diet): 5,500 IU of vitamin A, 1,100 IU of vitamin D_3, 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 niacin, 66 mg choline, 26 μg of vitamin B_{12}, 0.27 g of Mg (MgO), 32 mg of Mn (MnO), 0.4 mg of I ($C_2H_8N \cdot 2HI$,ethylenediamine dihydroiodide), 10 mg Cu ($CuSO_4 \cdot 5H_2O$), 0.3 mg of Se (Na_2SeO_3), 90 mg Zn (ZnO), and 80 mg Fe ($FeSO_4 \cdot 7H_2O$).

[b]Calculated (NRC, 1998).

the beginning of the experiments and then weekly from the anterior vena cava into heparinized BD Vacutainer™ (Preanalytical Solutions, NJ) tubes after an overnight fast (8 h). Plasma samples were prepared at 4°C to assay for plasma inorganic P concentrations and alkaline phosphatase activity. To collect digesta samples, all pigs in Exp. 1.1 and 5 pigs from each treatment group in Exp. 1.2 were killed at the ends of the trials by electrical stunning and exsanguination. In both studies, pigs were fasted at 20h prior and resumed feeding at 12 h prior to slaughter for normalizing the presence of digesta throughout the tract. Digesta samples were collected from the stomach, duodenum (12 cm aborally from pylorus), upper jejunum (2 m aborally from duodenal sample), lower jejunum (2.12 m from the ileocaecal junction), ileum (last 12 cm before the ileocaecal junction) and colon (midpoint) for determination of phytase activity, soluble P concentration, total P concentration and total Ca concentration. Stomach contents were blended with saline, as needed, before sampling. Samples were frozen immediately in liquid nitrogen, and stored at –20°C until lyopholized.

2.2.3 Laboratory Analyses

Plasma was prepared by centrifuging whole blood samples chilled on ice at 3,000 x *g* (GS-6KR centrifuge, Beckman Instruments Inc., Palo Alto, CA) for 10 min at 4°C. Plasma inorganic P concentration was determined using Elon (*p*-methylaminophenol sulfate) solution after deproteinating with 12.5% trichloroacetic acid (Gomori, 1942). Plasma alkaline phosphatase activity was measured 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 per minute at 30°C. Phytase activity in the digesta and feed was determined by the release of inorganic P from sodium phytate in 0.2 *M* citrate buffer, pH 5.5, at 37°C (Kim and Lei, 2005). To measure soluble P, digesta samples were extracted in 0.2 *M* citrate buffer, pH 5.5 at

room temperature for 30 min. Thereafter, 200 μL of extract (50 μL for colon extract) was diluted with 1.80 mL (or 1.95 mL for colon extract) deionized water, and mixed with 2 mL of coloring reagent (1 *M* sulfuric acid, 2.5% ammonium molybdate, and 10% ascorbic acid; 3:1:1). The mixture was incubated in a 50°C water bath for 20 min and the absorbency was measured at 820 nm against a blank. Total P and Ca concentrations in stomach and colon digesta samples were analyzed (Eppard et al., 1985). Freeze-dried stomach digesta samples were vortexed in deionized water (0.1 g into 4 mL) for measuring pH using a pH meter (Accumet Model 630, Fischer Scientific, Pittsburgh, PA).

2.2.4 Statistical Analyses

All data were analyzed using SAS (SAS Inst., Inc., Cary, NC). Individual pen was used as the experimental unit. The main effects of dietary treatments on growth performance, plasma inorganic P concentrations, and plasma alkaline phosphatase activities were analyzed using one-way ANOVA with time-repeated measurements (Gill, 1986). The main effects of dietary treatments on digesta phytase activity, soluble P concentrations, and total Ca or P concentrations were analyzed using one-way ANOVA. Because of heterogeneity, digesta phytase activity data were normalized by log transformation (log activity + 1) for statistical analysis, but were presented as the original values in the results. The Bonferroni *t*-test was used to compare treatment means, with a significance level set at $P < 0.05$. Correlations between digesta phytase activity and soluble P, between digesta phytase activity and pH, or between total digesta P and total digesta calcium in Exp. 2 were also analyzed using PROC CORR of SAS.

2.3 Results

2.3.1 Experiment 1.1

Growth Performance and Plasma Measures

Compared with pigs fed BD, pigs fed the BD + 500 U/kg and BD + 0.1% iP had 22 and 25% greater ($P < 0.05$) overall ADG, respectively (**Table 2.2**). These two groups of pigs also had 12 to 18% higher ($P < 0.05$) gain:feed ratios than the pigs fed BD only. There was no significant difference in overall ADFI among the three groups. Initial plasma inorganic P concentrations or plasma alkaline phosphatase activities were similar between any two treatment groups. At wk 4, pigs fed BD had 20 to 22% lower ($P < 0.05$) plasma inorganic P concentration, but 40 to 54% higher ($P < 0.05$) plasma alkaline phosphatase activity than those fed BD + 500 U/kg or BD + 0.1% iP, respectively.

Phytase Activity in Digesta from Various Segments

Pigs fed BD + 500 U/kg had greater ($P < 0.05$) phytase activity in digesta of stomach and upper jejunum than that of the pigs fed BD or BD + 0.1% iP (Figure 2.1). These two phytase-unsupplemented groups had little (0 to 25 U/kg) phytase activity in the digesta from the two segments. Pigs fed BD + 500 U/kg maintained similar phytase activity in digesta from stomach to upper jejunum, with approximately 12 to 15% of the supplemented activity on dry matter basis. No phytase activity was detected in digesta samples from lower jejunum or ileum from any of the treatment groups. In contrast, colon digesta samples exhibited the highest phytase activity among all segments of the gastrointestinal tracts in all treatment groups.

Table 2.2. Effects of dietary treatments on growth performance and plasma biochemical measures of pigs in Experiment 1.1[a]

		Treatment			
Item	**Time**	**Basal**	**500U**	**iP 0.1%**	**SEM**
Average Daily Gain (ADG), g					
	Overall	380 [y]	485 [x]	508 [x]	40
Average Daily Feed Intake, g (as-fed)					
	Overall	810	913	893	31
Gain:Feed					
	Overall	0.47[z]	0.53[y]	0.57[x]	0.03
Plasma inorganic P concentrations, mg/dL					
	Wk 0	5.97	5.26	5.34	0.23
	Wk 4	5.20[y]	7.95[x]	8.35[x]	0.99
Plasma alkaline phosphatase activity, mU/mL					
	Wk 0	244	285	267	12
	Wk 4	452[x]	271[y]	210[y]	73

[a]Values are means of six individually penned pigs during the 4-wk study.

[x,y,z] In each row, values not sharing a common letter are different ($P < 0.05$).

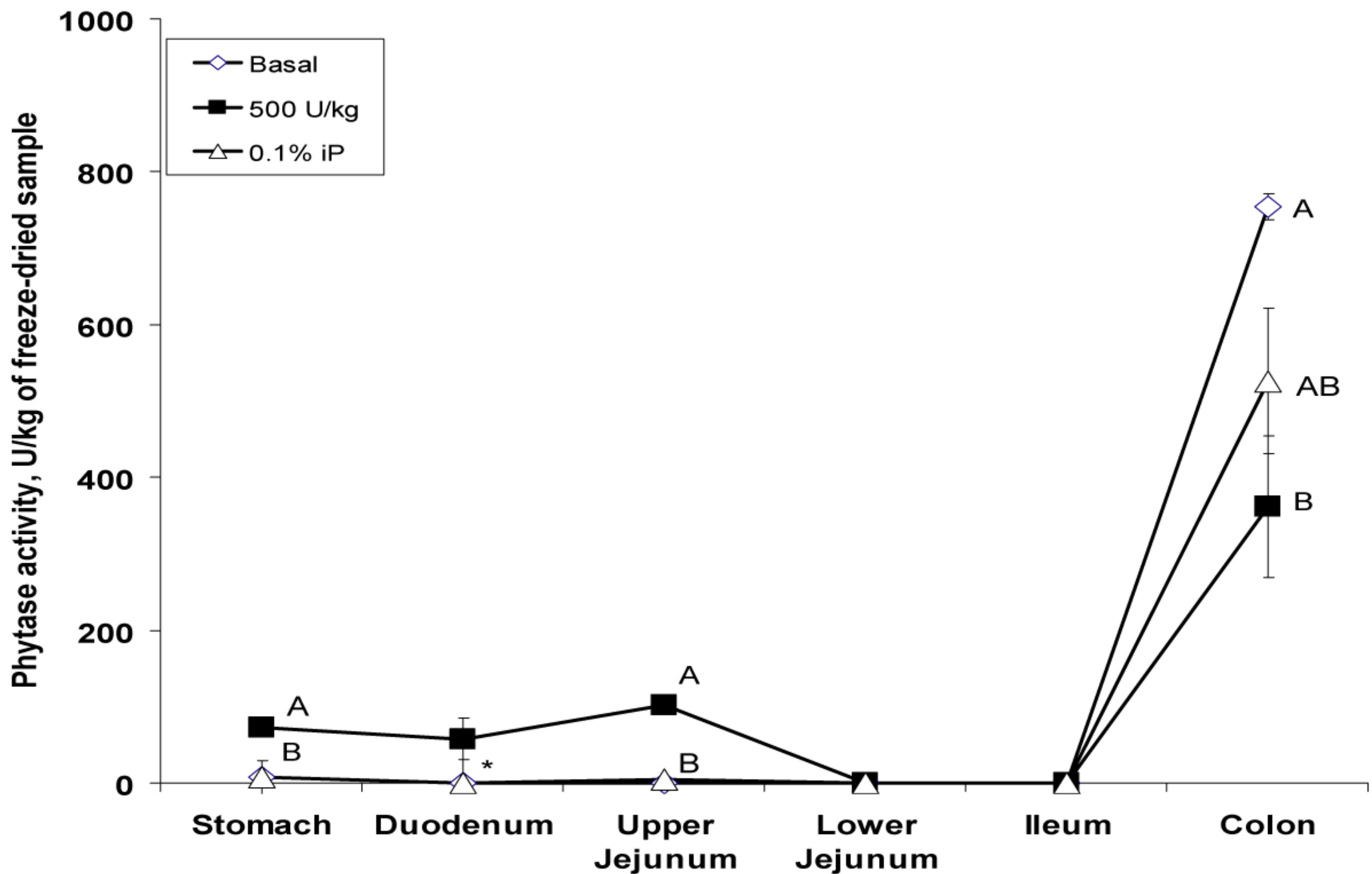

Figure 2.1 Phytase activity of freeze-dried stomach, duodenum, upper jejunum, lower jejunum, ileum and colon digesta of pigs fed BD, BD + 500 U/kg phytase, or inorganic P (0.1%) in Exp. 1. Values are the means of individually penned pigs (n = 5 to 6). At each segment, means not sharing a common letter differ ($P < 0.05$). *Only one duodenum digesta sample was available for analysis from BD group.

Comparatively, pigs fed BD had the highest, whereas pigs fed BD + 500 U/kg had the lowest phytase activity in colon digesta. The activity in pigs fed BD + 0.1% iP was not different from either group.

Soluble P Concentrations in Digesta from Various Segments

Soluble P concentration in stomach digesta of pigs fed BD was 56% ($P < 0.05$) and 51% ($P = 0.07$) lower than that of pigs fed BD +0.1% iP and BD + 500 U/kg, respectively (Figure 2.2). However, digesta from upper jejunum of pigs fed BD displayed an 80% increase in soluble P concentration over that from duodenum, and the concentration was 69% higher ($P < 0.04$) than that of the other two treatment groups. Digesta soluble P concentrations did not differ between treatment groups in lower jejunum or ileum. As in the case of phytase activity, colon digesta showed the highest soluble P concentrations among all segments assayed. The concentration in pigs fed BD was 31% higher ($P < 0.05$) than that of pigs fed BD + 500 U/kg.

Total P and Ca Concentrations in Stomach and Colon Digesta

Total P concentration in stomach digesta was 25 to 31% higher ($P < 0.02$) in pigs fed BD + 0.1% iP than that in the other two treatment groups (Figure 2.3). Total P concentrations in colon digesta from pigs fed BD and BD + 0.1% iP were similar, but 24% higher ($P < 0.05$) than that in pigs fed BD + 500 U/kg. Total calcium concentration in stomach digesta of pigs fed BD + 0.1% iP was greater ($P < 0.05$) and marginally greater ($P = 0.09$) than that in pigs fed BD and BD + 500 U/kg, respectively. Total calcium concentration in colon digesta was 53% higher ($P < 0.05$) in pigs fed BD than that in pigs fed BD + 500 U/kg.

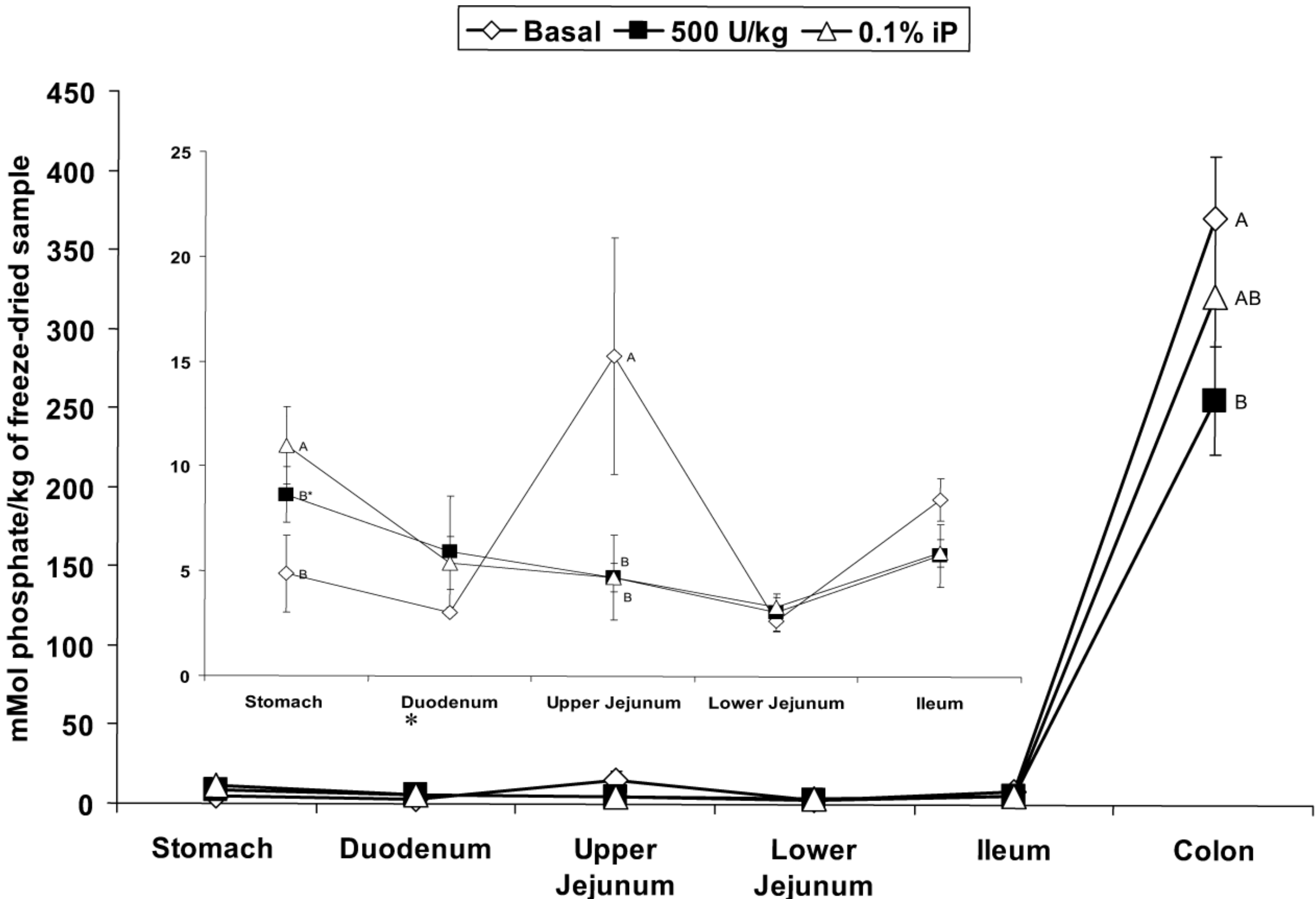

Figure 2.2 Soluble P concentrations of freeze-dried stomach, duodenum, upper jejunum, lower jejunum, ileum and colon digesta of pigs fed BD, BD + 500 U/kg phytase, or inorganic P (0.1%) in Exp. 1. Values are the means of individually penned pigs (n = 3 to 6). At each segment, means not sharing a common letter differ ($P < 0.05$). B*, marginal significance ($P = 0.065$). * Only one duodenum digesta sample was available for analysis from the BD group.

2.3.2 Experiment 1.2

Growth Performance and Plasma Measures

Pigs fed **s**upplemental phytase at 500 or 2,000 U/kg had greater (P < 0.05) overall ADG (by 13 to 27%), ADFI (12-15%), and gain:feed ratio (by 7 to 10%) than pigs fed BD (**Table 2.3**). At the beginning of experiment, pigs in all treatment groups had similar plasma inorganic P concentrations and plasma alkaline phosphatase activities. At wk 2, pigs fed BD had 40 to 47% lower (P < 0.05) plasma inorganic P concentrations than those fed BD + 500 or 2,000 U/kg. At the same time, plasma alkaline phosphatase activity was approximately 50% higher (P < 0.05) in pigs fed BD than that in the phytase-supplemented groups.

Phytase Activity in Digesta from Various Segments

Pigs fed BD + 2,000 U/kg had greater (P < 0.05) phytase activity in stomach digesta than pigs fed BD or BD + 500 U/kg (Figure 2.4). Pigs fed BD + 2,000 U/kg had greater (P < 0.05) phytase activity in the upper jejunum than pigs fed BD and marginally greater (P = 0.16) activity than those fed BD + 500 U/kg. Pigs fed BD + 500 or 2,000 U/kg maintained similar phytase activity in digesta from stomach to upper jejunum, with approximately 22 to 32% of the supplemented activity in the diets on a dry matter basis. No activity was detected in lower jejunum or ileum samples from any treatment group. Colon digesta samples had highest phytase activity among all segments of the gastrointestinal tract for all treatment groups except those fed BD + 2000 U/kg. Colon phytase activity was 81 to 99% greater (P <0.05) in pigs fed BD than those supplemented with phytase at 500 or 2,000 U/kg.

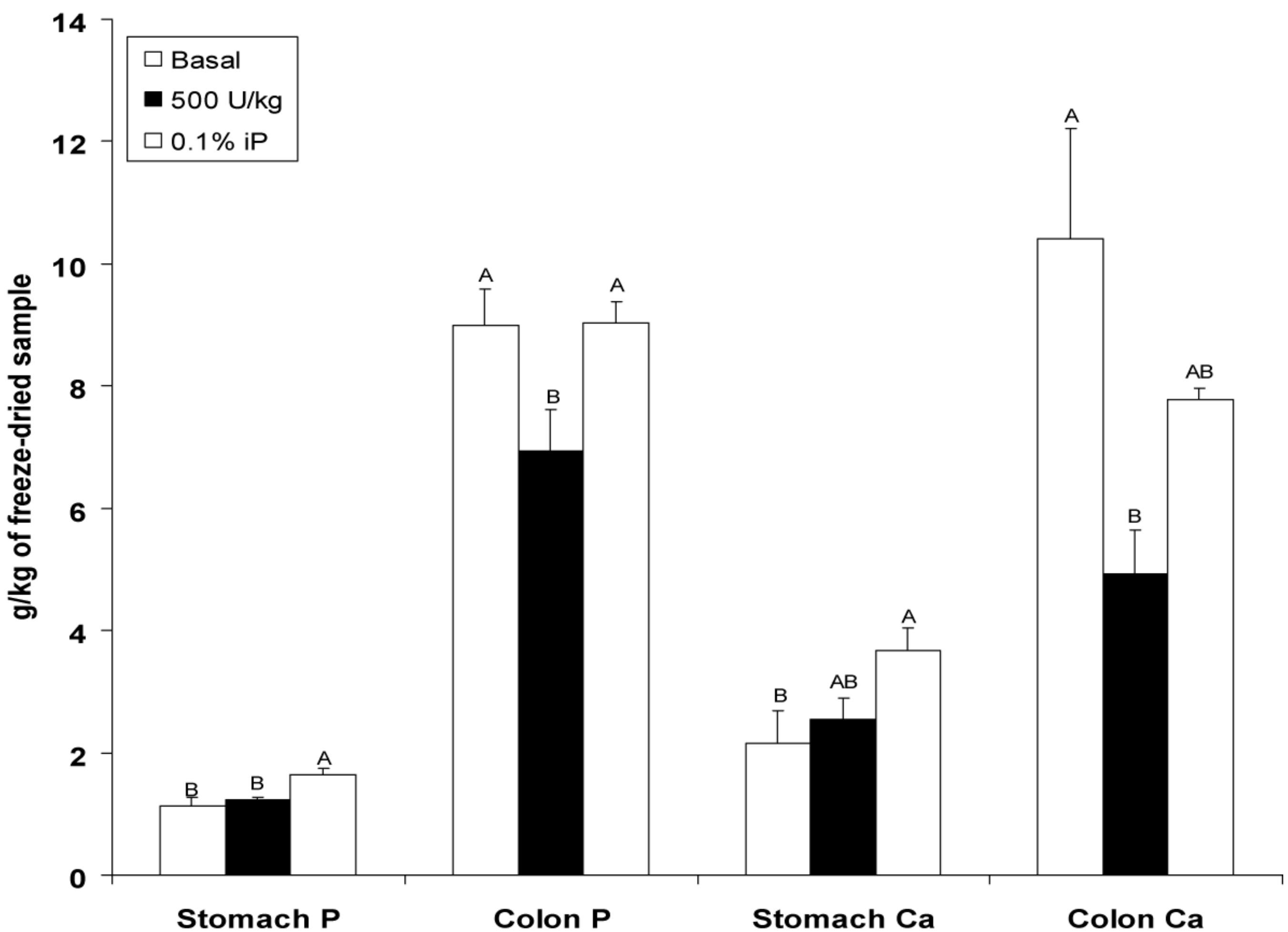

Figure 2.3 Total P and Ca concentrations of freeze-dried stomach and colon digesta samples for pigs fed BD, BD + 500 U/kg phytase, or inorganic P (0.1%) in Exp. 1. Values are means of individually penned pigs (n = 6). Means not sharing a common letter for each segment and mineral are different ($P < 0.05$).

Table 2.3. Effects of dietary treatments on growth performance and plasma biochemical measures of pigs in Experiment 1.2[a]

Item	**Time**	Treatment **Basal**	 **500U**	 **2000U**	 **SEM**
Average Daily Gain (ADG), g					
	Overall	541[y]	634[x]	706[x]	48
Average Daily Feed Intake, g (as-fed)					
	Overall	916[y]	1038[x]	1072[x]	47
Gain:Feed					
	Overall	0.59[y]	0.63[x]	0.65[x]	0.02
Plasma inorganic P concentration, mg/dL					
	Wk 0	6.06	5.81	5.79	0.09
	Wk 2	4.25[z]	7.07[y]	8.06[x]	1.14
Plasma alkaline phosphatase activity, U/L					
	Wk 0	334	297	349	15
	Wk 2	329[y]	162[x]	138[x]	60

[a]Values are means of ten individually penned pigs during the 2-wk study.

[x,y,z] In each row, values not sharing a common letter are different ($P < 0.05$).

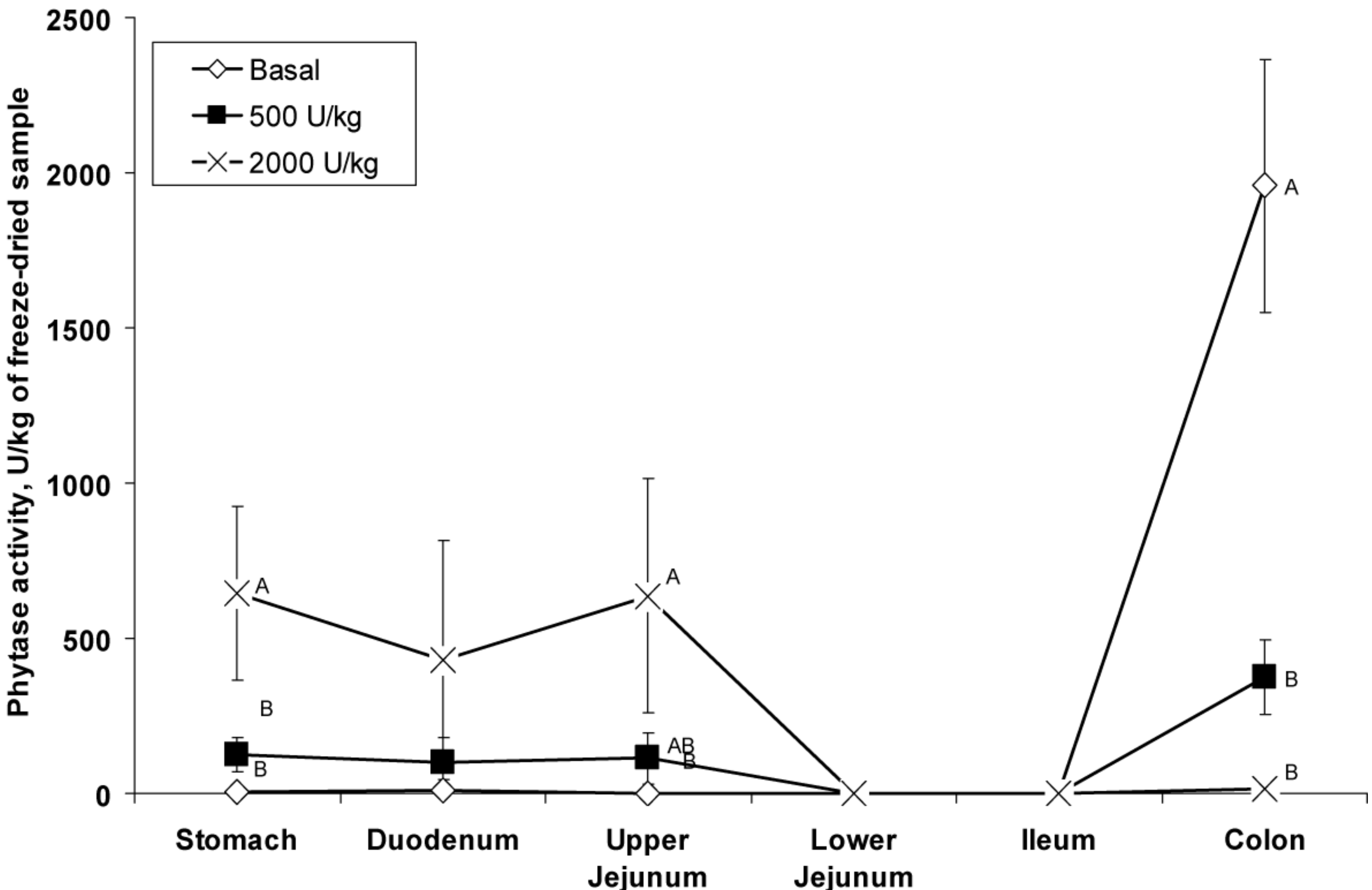

Figure 2.4 Phytase activity of freeze-dried stomach, duodenum, upper jejunum, lower jejunum, ileum and colon digesta of pigs fed BD or supplemented with 2 levels of microbial phytase (500 or 2000 U/kg) in Exp. 2. Values are the means of individually penned pigs (n = 4-5/group). At each segment, means not sharing a common letter differ ($P < 0.05$).

Soluble P Concentrations in Digesta from Various Segments

Soluble P concentrations in stomach digesta of pigs fed BD was 81 to 85% lower ($P < 0.05$) that that of pigs fed BD + 500 U/kg and BD + 2,000 U/kg. Pigs fed 2,000 U/kg had 43 to 47% greater ($P < 0.05$) soluble P concentrations in the duodenum than pigs fed BD + 500 U/kg and BD. Pigs fed BD displayed a 41% increase in soluble P from the duodenum to upper jejunum, however there was no difference in soluble P concentrations of the upper jejunum among treatment groups. Soluble P concentrations in ileum digesta of pigs fed BD was 52 to 62% lower ($P < 0.05$) than that of pigs fed BD + 500 U/kg and BD + 2,000 U/kg. Consistent with phytase activity, colon digesta had the highest soluble P concentrations among all segments. The concentration in pigs fed BD was 52 to 78% higher ($P < 0.05$) than that of pigs supplemented with 500 and 2,000 U/kg phytase (Figure 2.5). There was a positive linear correlation for pH and soluble P in the stomach ($r = 0.75$, $P < 0.05$) and between pH and remaining phytase activity percentage ($r = 0.83$, $P < 0.05$).

Total P and Ca Concentrations in Stomach and Colon Digesta

There was no dietary treatment effect on total P or Ca concentrations in stomach digesta (Figure 7). Total P concentrations in colon digesta were different ($P < 0.05$) between any two treatment groups, and the concentration was inversely ($r = -0.8$, $P < 0.0002$) related to dietary phytase activity. Total calcium concentrations in colon digesta showed the same pattern as that of total P concentration, but the difference between the two phytase-supplemented groups was only marginal ($P = 0.06$). There was a positive correlation ($r = 0.9$, $P < 0.001$) between total P and Ca concentrations in colon digesta.

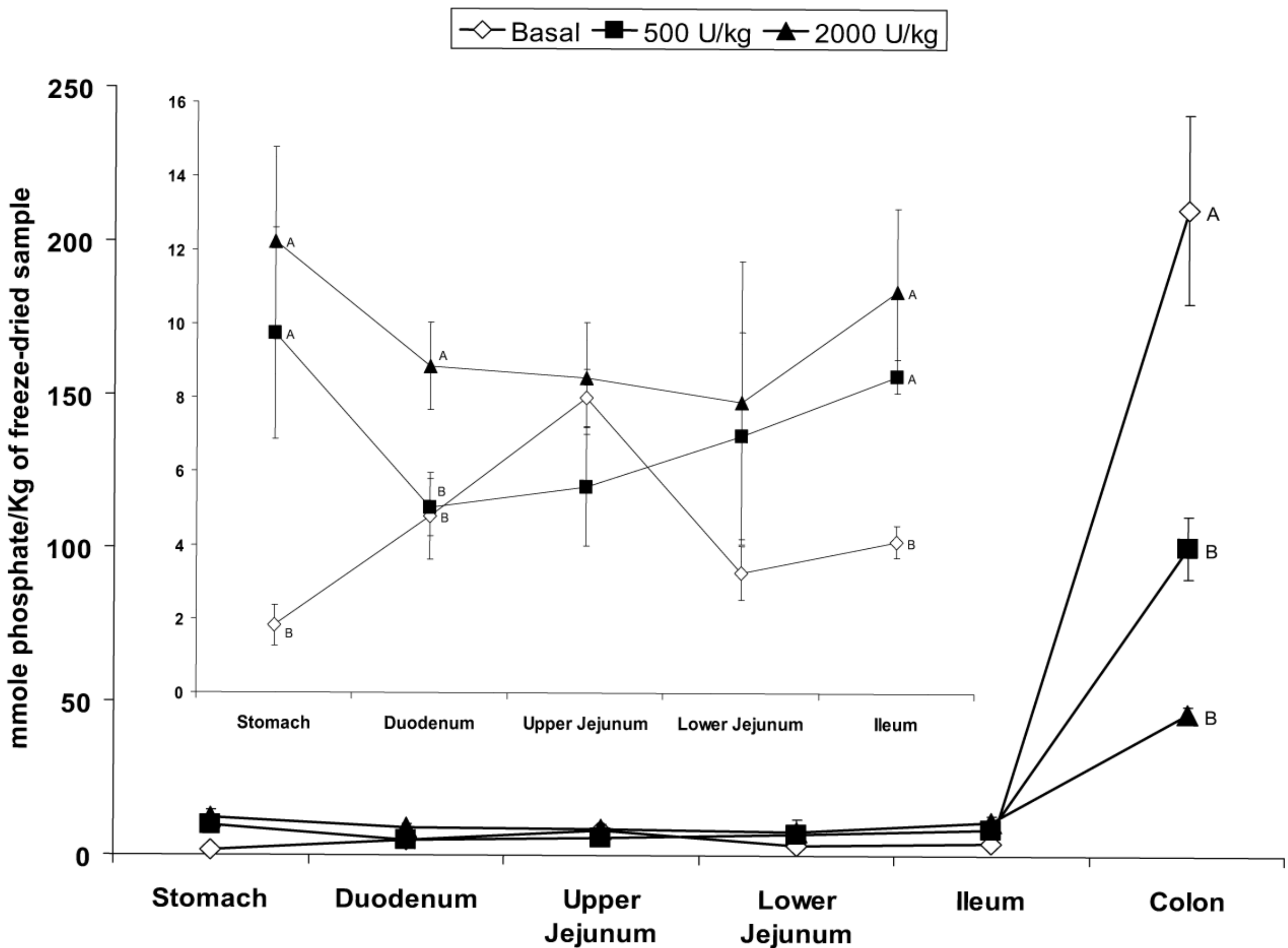

Figure 2.5 Soluble P concentrations of freeze-dried stomach, duodenum, upper jejunum, lower jejunum, ileum and colon digesta of pigs fed BD or supplemented with 2 levels of microbial phytase (500 or 2000 U/kg) in Exp. 2. Values are the means of individually penned pigs (n = 3 to 5/group). At each segment, means not sharing a common letter differ ($P < 0.05$).

2.4 Discussion

Our results demonstrate that stomach is the main site of *E. coli* appA2 phytase function, based on changes in digesta phytase activity and soluble P concentrations among different segments of the gastrointestinal tract. Because the growth performance and the plasma biochemical measures in pigs fed BD + appA2 phytase our experiments were comparable to those fed BD + iP, responses of these phytase-supplemented pigs represented an adequate P nutritional status or normal dietary P digestion and metabolism (Lei et al., 1993). Earlier studies have shown stomach as the function site for PhyA fungal phytase, the activity decreased from stomach to upper jejunum (Jongbloed et al, 1992; Yi and Kornegay, 1996). In contrast, *E. coli* AppA2 phytase activity remained fairly constant throughout stomach and upper intestine. This difference was associated with the greater resistance of *E. coli* phytase to proteolysis of pepsin than PhyA phytase (Rodriguez et al., 1999b), and helps explain the efficacy difference between these enzymes in swine and poultry feeding (Applegate et al., 2003; Augspurger et al., 2003). Stomach digesta pH analysis in Exp. 1.2 showed a positive correlation ($r = 0.9$, $P = 0.01$) to phytase activity in the stomach and upper jejunum. Similarly, Yi and Kornegay (1996) reported a reduced phytase activity in the stomach when pH was reduced by citric acid. Since pepsin is most active at pH 2 to 3.8, (Kotts and Jenness, 1976), pepsin might cause greater phytase degradation in pigs with lower stomach pH. However, digesta pH was positively correlated ($r = 0.86$, $P < 0.002$) to stomach digesta soluble P released by phytase, which might act as a buffer. Therefore, dietary phytase activity itself might create poor pepsin conditions and prolong its activity. Because of the susceptibility to trypsin denaturation (Rodriguez et al., 1999b), supplemental AppA2 phytase activity was not detectable in the lower intestine.

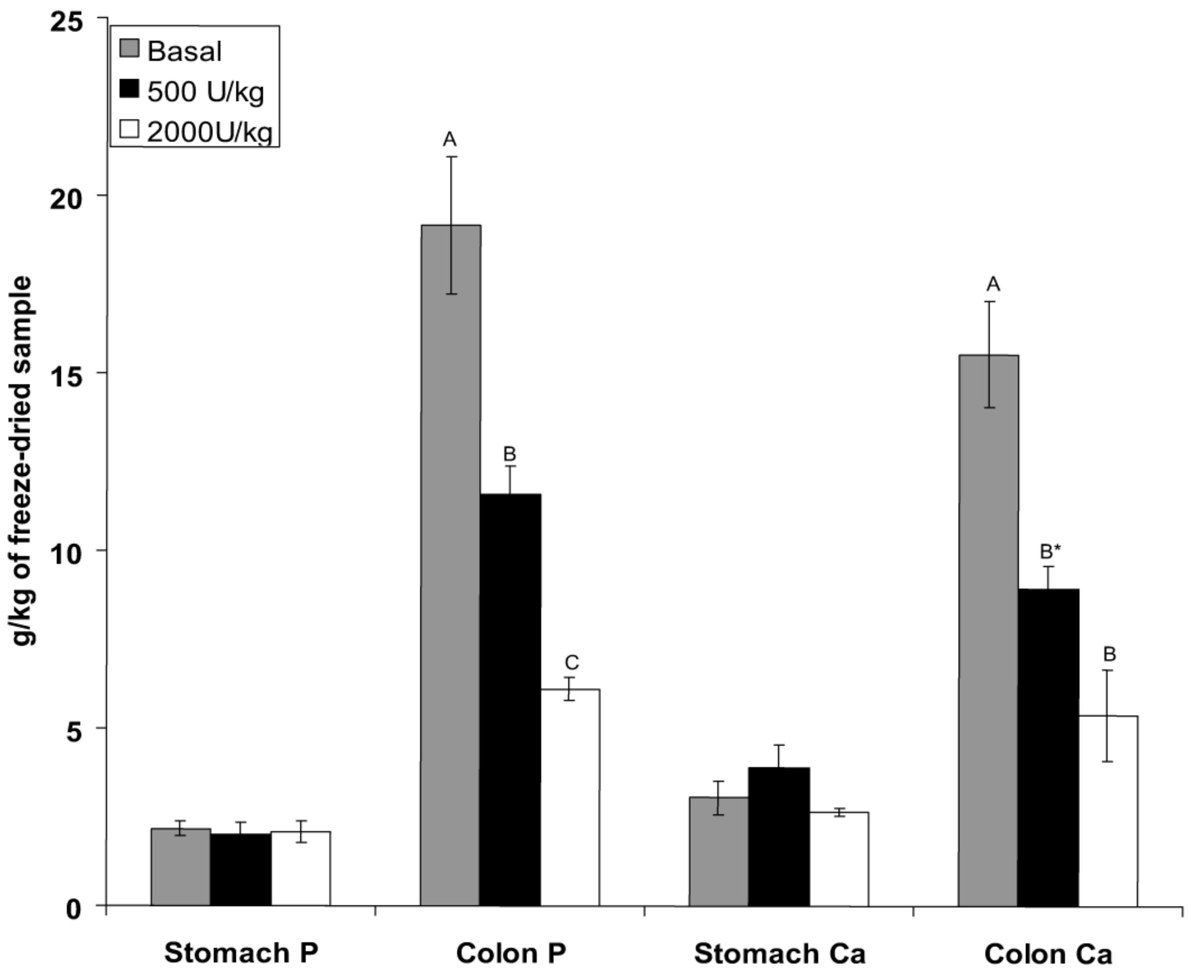

Figure 2.6 Total P and Ca concentrations of freeze-dried stomach and colon digesta samples for pigs fed BD, BD + 500 U/kg phytase or 2000 U/kg in Exp. 2. Values are means of individually penned pigs (n = 5). Means not sharing a common letter for each digesta type and mineral are different (P < 0.05).

Soluble P in the colon was proportional to colon phytase activity, but inversely related to supplemental dietary phytase levels. In both experiments, pigs fed 500 U/kg phytase had lower colon phytase activity than pigs fed BD; and in Exp. 1.2, pigs fed BD + 2,000 U/kg had lower colon phytase activity than those fed BD + 500 U/kg. Colon soluble P concentrations followed the same trends. The inverse relationship between dietary phytase activity and colonic phytase activity is explained by greater phytate hydrolysis in the upper gastrointestinal tract leaving less available substrate for the colon microbial phytase (Porres et al., 1999). Likewise, soluble P concentrations in colon digesta were inversely related to dietary phytase activity because enhanced phytate-hydrolysis upstream by dietary phytase led to reduced dietary-P to reach colon for the degradation by colonic phytase (Schlemmer et al., 2001; Leytem et al., 2004). Although there was no significant difference in colon digesta phytase activity and soluble P concentrations between pigs fed BD + iP and BD + 500 U/kg, the total P concentration in colon digesta was higher in pigs fed BD + iP. Therefore, those pigs had more phytate-P and greater potential for soluble P to accumulate further along the colon. Supplemented phytase hydrolyzed phytate in the upper digestive tract and rendered more soluble P absorbed in the upper intestine (Liu et al., 2000). In all, greater levels of supplemental phytase allow for more absorption of phytate-P in the stomach and upper jejunum leading to a decreased release of soluble P into the environment from phytate-P degradation in colon, as the colon does not seem to be a major site for absorption of digesta P (Civitelli and Avioli, 1994).

A sharp decrease in digesta soluble P concentrations occurred between the stomach and the duodenum, indicating these segments may be the main sites of P absorption. In Exp. 1.1, that decrease in pigs fed BD + 500 U/kg and BD + 0.1% iP was 30 and 50%, respectively. In Exp. 1.2, that decrease in pigs fed BD + 500 or 2,000 U/kg was approximately 45%. Compared with the initial total P concentrations

in the diets, stomach digesta of pigs from all treatment groups showed more than 50% reduction. This reduction could also be evidence that a large portion of dietary P was absorbed in the stomach. However, there was only negligible difference in soluble P concentrations between duodenum and upper jejunum digesta of pigs fed BD + phytase or iP, indicating no major P absorption (extraction) post duodenum. In contrast, digesta soluble P increased from the stomach to the upper jejunum (6 to 14%) in pigs fed BD, and that change was similar to intestinal P secretion observed by Moore and Tyler (1955) in the first 270 cm of pig small intestines. Other studies based on analysis of ileal digesta of pigs fed soybean meal have also shown an endogenous P release into the upper intestine (Fan et al, 2001; Ajakaiye et al., 2003). Our results indicate that the upper jejunum was the location of the endogenous P secretion. Endogenous P was only observable in pigs fed BD. This observation may be because endogenous excretion decreases exponentially as dietary P increases (Civitelli and Avioli, 1994).

Total P and Ca concentrations in the stomach digesta of pigs fed BD or BD + 500 or 2000 U/kg did not differ, but were less (25 and 35%) than pigs fed BD + iP. In the colon digesta, there was a positive correlation between total P and Ca concentrations. Pigs fed BD had higher total P and Ca in colon digesta than pigs fed BD + 500 U/kg ($P < 0.05$). The latter had greater levels than those fed BD + 2,000 U/kg. Much of this correlation was probably because phytate chelated Ca (Vohra et al., 1965) and therefore when phytate was not degraded in the upper gastrointestinal tract, Ca remained chelated in the colon. Apparently, phytase supplementation improved absorption of P and Ca in the stomach and duodenum (Liu et al., 1997), resulting in less passage of digesta P and Ca to colon and eventually in the environment.

2.5 Implications

A supplemental bacterial phytase improves digestion of feed phytate-phosphorus in growing pigs fed corn-soy diets. The supplemental enzyme mainly functions in the stomach, but remains fairly active in the upper small intestine due to its resistance to pepsin hydrolysis. Essentially no phytase activity is detectable in digesta of lower jejunum and ileum, indicating the susceptibility of the supplemental phytase to trypsin hydrolysis. The enhanced dietary phytate-phosphorus release in the stomach and upper small intestine by the supplemental bacterial phytase leads to a substantial reduction in microbial phytase activity in colonic digesta. Consequently, the phytase-supplemented pigs have low concentrations of phosphorus and calcium in the colon digesta and low fecal excretion of these elements into environment.

CHAPTER 3

SUPPLEMENTAL *ESCHERICHIA COLI* PHYTASE IMPROVES BONE STRENGTH OF YOUNG PIGS FED A PHOSPHORUS-ADEQUATE DIET

3.1 Introduction

Bone strength is one of the primary concerns for human health. The occurrences of bone fractures are great at puberty, likely due to poor bone mineral density during growth spurts, and post-menopause for women (Alffram and Bauer, 1962). Elderly Caucasian women are at high risk of osteoporosis (Cohen and Roe, 2000), a metabolic bone disease characterized by low bone mass and deterioration of architecture that decreases bone strength and thus increases risk of fracture (Consensus development conference, 1993). Hip fractures occur mostly in people over the age of 70 (Wasnich, 1999). These fractures are estimated to exceed 6 million cases a year by 2050 (Cooper et al., 1992), It is believed that achieving a high peak bone mass early in life will postpone or prevent osteoporosis (Matkovic et al., 1994; Heaney et al., 2000). The FDA guidelines recommend using small and large animal models to test potential agents for osteoporosis treatment (Thompson et al., 1995). Since only surrogates for bone strength can be measured *in vivo* for humans (Heaney et al., 2000), it is advantageous to use animals in which actual bone strength and composition can be measured. Canine and porcine bones, in comparison with those of cow, sheep, chicken and rat, most resemble human samples in bone density and stress fracture properties (Aerssens et al., 1998). Estrogen depletion due to menopause is a major factor in the occurrence of osteoporosis for women (Masse et al., 2004). Since pigs have an estrus cycle similar to the human menstrual cycle, they are a better model for osteoporosis

research than dogs that reach estrus only every 1 or 2 years (Miller et al., 1995).

Phytate-P in plant foods is unavailable to simple-stomached animals (Reddy et al., 1982). Many studies have shown that microbial phytase supplementation in swine improves bone strength in low-P diets (Murry et al., 1997; Gentile et al., 2003) because of phytase's ability to release P from phytate, making it available for incorporation into bone (Young et al., 1993). Phytate also has the ability to chelate cations, such as Ca, Fe, Zn, Mn and Cu to form insoluble salts (Vohra et al., 1965) decreasing these minerals' bioavailability. Supplementing dietary phytase breaks down phytate-P and releases the chelated minerals for absorption in the gastrointestinal tract and for possible incorporation into bone. A couple of experiments with phytase added to P-adequate diets (Cromwell, 1991; Yi et al., 1996) have shown potential benefit of phytase to bone metabolism in addition to P-accretion.. However, a definitive conclusion could not be drawn from these studies due to confounding effects of enhanced P-accretion and suboptimal Ca:P ratios. Since the primary goal of those studies was to improve P nutrition for animal production, rather than bone metabolism, the results from those experiments have limited implications in human health. In the present study, we chose young female pigs as a model. The objective of our study was to use young female pigs as a model for young girls (Aerssens et al., 1998; Pond and Houpt, 1978) to maximize peak bone strength for reducing risk of osteoporosis later in life (Matkovic et al., 1994; Heaney et al., 2000). Our objectives were to determine: 1) whether high levels of phytase supplementation in P-adequate diets offered extra improvement in bone strength over the adequate dietary P supplementation; 2) whether the improvement was mediated by factors other than P-accretion.

3.2 Materials and Methods

3.2.1 Animals, Diets, and Treatments

Our protocol was approved by the Institutional Animal Care and Use Committee of Cornell University. All pigs used in the study were weanling crossbreds (Landrace-Hampshire-Duroc) selected from the Cornell University Swine Farm and allotted into treatment groups based on body weight, litter and sex. Prior to experiments, pigs were weaned at 4 wk of age and fed a corn-soybean meal basal diet (BD, **Table 3.1**) without supplemental inorganic phosphorus (iP). In Exp. 2.1, 24 female pigs (5-wk old, 10.1 ± 0.3 kg body weight (BW)) were allotted to 4 groups (n = 6), and fed BD, BD + phytase (1,000 U/kg), BD + 0.2% iP or BD + 0.2% iP + phytase (1000 U/kg) for 5 wk. In Exp. 2.2, 32 pigs (5-wk old, 9.9 ± 0.1 kg BW) were divided into 4 groups (n = 8), and fed BD, BD + iP (0.25%), BD + iP (0.25%) + phytase (1,000 U/kg) or BD + iP (0.25%) + phytase (2,000 U/kg) for 4 wk. In Exp. 2.3, 24 female pigs (4-wk old, 8.6 ± 0.1 kg BW) were divided into 2 groups (n = 12), and fed BD + iP (0.25%) or BD + iP (0.25%) + phytase (2,000 U/kg) for 6 wk. The phytase used in all experiments was *Escherichia coli* AppA2 (Rodriguez et al., 1999) (provided by Phytex, Portland, ME) and was added to the diets at feed mixing. The actual phytase activity in the experimental diets was analyzed (Kim and Lei, 2005). The BD contained adequate levels of all nutrients (NRC, 1998), but had no supplemental inorganic P and an adjusted Ca concentration to maintain a Ca:P of 1.2:1 (Lei et al., 1994). Pigs were penned individually in Exp. 2.1 and 2.2 and group-penned in Exp. 2.3, in an environmentally controlled barn (19 to 27°C; 12-light:12-dark cycle). All pigs were allowed free access to feed and water.

Table 3.1. Composition of basal diet of Experiment 2.

Ingredients	*%*
Corn, grain	67.10
Soybean meal, 48% CP	28.00
Spray-dried plasma protein dried	1.50
Limestone	1.05
L-Lysine·HCl	0.10
Corn oil	1.00
Vitamin/mineral premix[a]	0.25
Dicalcium phosphate	0.00
Salt	0.50
Tylan® 10	0.50
Total	100.00
Nutritive Values[b]	
ME (kcal/kg)	3325.3
Crude protein	20.04%
Ca, total	0.51%
P, total	0.41%
P, available	0.09%
Ca:P, total	1.24

[a]Vitamin and mineral premix supplies (per kilogram of diet): 5,500 IU of vitamin A, 1,100 IU of Vitamin D_3, 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 niacin, 66 mg choline, 26 μg of vitamin B_{12}, 0.27 g of Mg (MgO), 32 mg of Mn (MnO), 0.4 mg of I ($C_2H_8N\cdot 2HI$,ethylenediamine dihydroiodide), 10 mg Cu ($CuSO_4\cdot 5H_2O$), 0.3 mg of Se (Na_2SeO_3), 90 mg Zn (ZnO), and 80 mg Fe ($FeSO_4\cdot 7H_2O$).

[b]Calculated (NRC, 1998).

3.2.2 Growth Performance and Sample Collection

In all experiments, individual body weight was measured weekly. Daily feed consumption was recorded individually in Exp. 2.1 and 2.2, and by group in Exp. 2.3. Blood samples were collected from all pigs, initially (day 0) and then weekly, from the anterior vena cava into heparinized BD Vacutainer™ (Preanalytical Solutions, NJ) tubes after an overnight fast (8 h). Chilled whole-blood samples were centrifuged at 3,000 x *g* (GS-6KR centrifuge, Beckman Instruments Inc.) for 10 min. at 4°C to prepare plasma for determination of inorganic P concentrations and alkaline phosphatase activity. At the end of experiments, 4 pigs from each treatment group in Exp. 2.1 and 2.2, and 8 pigs from each treatment group in Exp. 2.3 were killed by electrical stunning and exsanguination. Both front legs were amputated and stored on ice at 4°C until the 3rd and 4th metacarpals were isolated for strength test (see below). Following the strength test in Exp. 2.3, bones were stored at –20°C for mineral composition analysis.

3.2.3 Biochemical Analysis

After being deproteinated with 12.5% tricholoacetic acid, plasma samples were assayed for inorganic P concentrations using Elon (*p*-methylaminophenol sulfate) solution (Gomori, 1942). The hydrolysis of *p*-nitrophenol phosphate to *p*-nitrophenol was used to measure plasma alkaline phosphatase activity (Bowers and McComb, 1966). The enzyme unit was defined as 1 μmol of *p*-nitrophenol released per minute at 30°C. Phytase activity in diets was determined by the release of inorganic P from sodium phytate in 0.2 M citrate buffer, pH 5.5, at 37 ^{o}C using filtration and spin column steps prior to hydrolysis (Kim and Lei, 2005).

3.2.4 Bone Strength

Third and fourth metacarpals from both legs were prepared by manually removing surrounding skin, muscle and other tissues. Bones were stored in closed plastic bags at $4^{o}C$ until analysis. Bone strength of the metacarpals was measured in kilonewtons using an Instron 4500 Machine (Canton, MA) at room temperature ($23^{o}C$) by subjecting each bone to a three-point bending test (Turner and Burr, 1993). During testing, force was applied to the center of the bone held by supports 2.0 cm apart. The crosshead speed was set at 50 mm/min and the sample rate was 10 points/sec. Final strength was recorded as the average strength of four bones per pig determined from load-displacement curves indicating the maximum loads. In Exp. 2.3, samples (~100-200 mg) of right metacarpal were taken for mineral content analysis. Pieces were isolated by cracking the bone with pliers wrapped in plastic and a paper towel (to avoid possible contamination), removing excess connective tissue with a stainless steel scalpel and removing individual shards with needle-nosed pliers with plastic tubing covering pincers. The samples were placed on filter paper on watch glasses and heated for 8 h at $105^{o}C$ to measure bone dry weight. Total P and Ca content were measured using inductively coupled argon plasma spectroscopy, (ICAP 61E Trace Analyzer, Thermo Jarell Ash corporation, Franklin, MA) (Eppard et al., 1985).

3.2.5 Statistical Analyses

All data was analyzed by SAS (SAS Inst., Inc., Cary, NC). The main effects of dietary treatments on all measures were analyzed using one-way ANOVA with or without time-repeated measurements (Gill, 1986). The Bonferroni *t*-test was used to compare treatment means, with a significance level set at $P < 0.05$.

3.3 Results

3.3.1 Experiment 2.1

Compared to pigs fed BD, pigs fed BD + 1,000 U/kg, BD + 0.20% iP, and BD + both had bone strength improved (P < 0.05) by 39, 53, and 60%, respectively (**Figure 3.1**). Bone strength was not significantly different between pigs fed BD + 1,000 U/kg or BD + 0.20% iP diets, but was improved (36%, P < 0.05) when phytase and iP were supplemented in comparison to phytase alone. A trend towards improvement in bone strength (16%, P = 0.16) was shown by feeding pigs BD + 1,000 U/kg + 0.20% iP over pigs fed BD + 0.20% iP. There was no interaction between phytase and iP on bone strength. The calculated overall, improvement by phytase and iP in bone strength (P < 0.05) was 25 and 44% respectively.

Initial plasma iP concentrations and alkaline phosphatase activities were similar among all treatment groups (**Table 3.2**). At the end of the experiment, pigs fed BD had lower (P < 0.05) plasma iP concentrations than pigs fed BD + 1,000 U/kg and(or) 0.2% iP. Plasma iP concentrations of pigs fed BD + 0.2% iP were nearly identical to those of pigs fed BD + 0.2% iP + 1,000 U/kg. Alkaline phosphatase activity was 45% higher (P < 0.05) in pigs fed BD than in the other three treatment groups that had similar activity. Overall growth performance, including daily gain, daily feed intake, and feed efficiency was improved (P < 0.05) by supplementing phytase and(or) iP. However, growth performance was similar between pigs fed BD + 0.2% iP and BD + 0.20% iP + 1,000 U/kg.

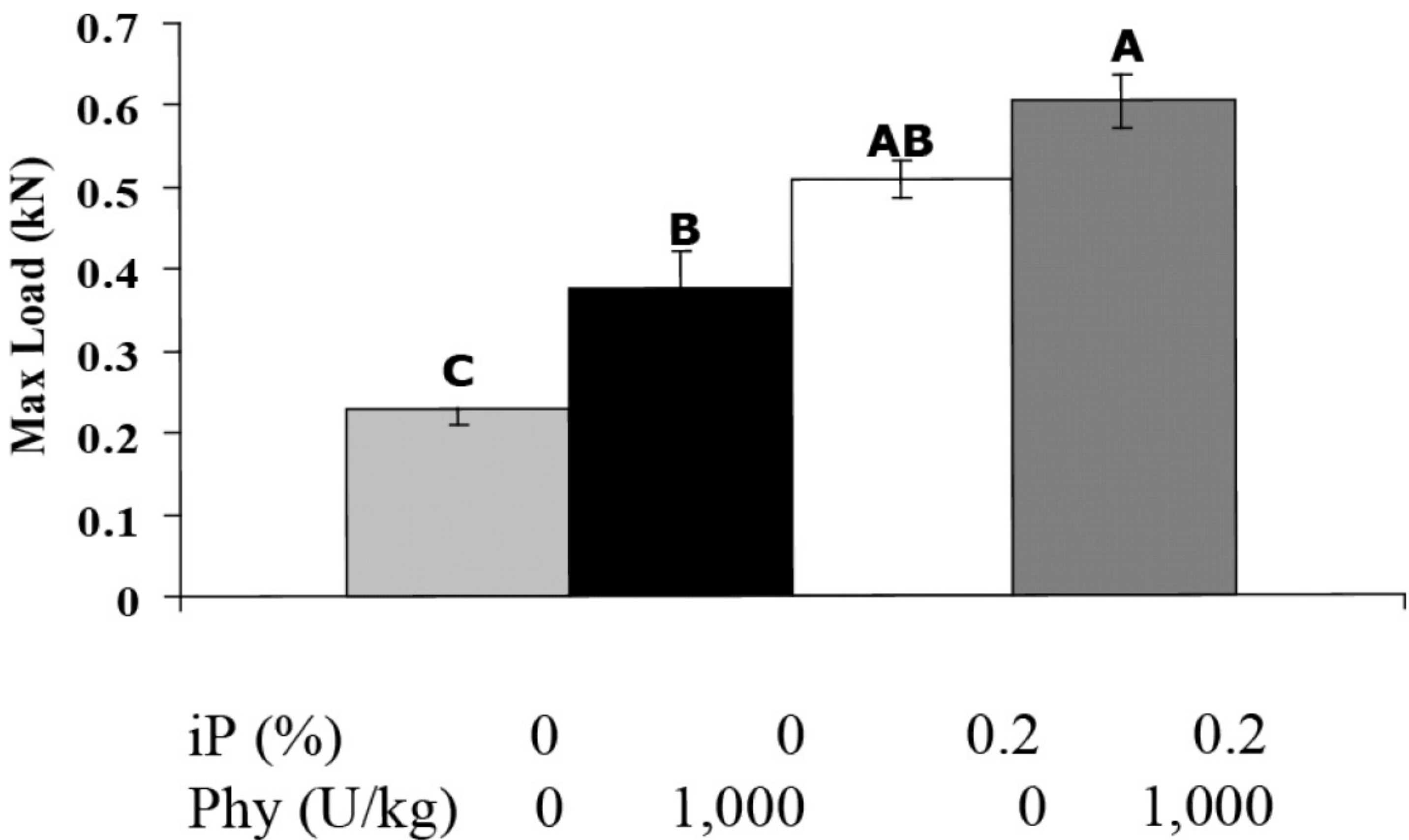

Figure 3.1 The bone breaking strength of pigs fed BD, BD + inorganic P (iP, 0.2%), BD + 1000 U phytase/kg or BD + iP (0.2%) + 1000 U phytase/kg. Values are the means of individual pigs (n = 4). Means not sharing a common letter differ ($P < 0.05$).

Table 3.2. Effects of supplemental dietary phytase and inorganic P on growth performance and plasma measures of pigs in Experiment 2.1[a]

	Treatment				
iP (%)	**0**	**0**	**0.2**	**0.2**	
Phytase (U/kg)	**0**	**1000**	**0**	**1000**	**SEM**
Overall daily gain, g	384 [z]	544 [y]	625 [x,y]	660[x]	17
Overall daily feed intake, g (as-fed)	774[y]	1041[x]	1050[x]	1085[x]	25
Overall gain:feed	0.50[z]	0.55[xy]	0.60[xy]	0.61[x]	0.01
Plasma inorganic P, mg/dL					
Week 0	6.04	5.91	6.50	6.14	0.13
Week 5	5.60[z]	7.52[y]	8.79[x]	8.73[x]	0.19
Plasma alkaline phosphatase activity, mU/ml					
Week 0	294	281	305	271	59
Week 5	357[x]	165[y]	151[y]	168[y]	66

[a]Values are means of five or six individually penned pigs during the 5-wk study.

[x, y,z] At each sampling time point, values not sharing a letter are different ($P < 0.05$).

3.3.2 Experiment 2.2

Bone strength was improved 22% ($P = 0.06$) in pigs fed BD + 0.25% iP + 2,000 U/kg over pigs fed BD + 0.25% iP and/or 1,000 U/kg phytase (**Figure 3.2**). Bone strength was 57% greater ($P < 0.002$) in pigs fed the latter two diets than pigs fed BD. Initially, pigs in all treatment groups had similar plasma iP concentrations and alkaline phosphatase activity (**Table 3.3**). At Wk 4, pigs fed BD had 35-36% lower ($P < 0.05$) plasma iP concentrations and greater ($P < 0.01$) plasma alkaline phosphatase activity than those fed BD + 0.25% iP and/or either level of dietary phytase. Pigs fed BD had lower ($P < 0.05$) average daily gain (34-42%) and average daily feed intake (26-33%) than pigs fed iP (0.25%) and/or phytase (1,000 or 2,000 U/kg). Addition of 1,000 U/kg phytase did not improve average daily gain over pigs fed 0.25% iP, but average daily gain did improve ($P < 0.03$) with the addition of 2,000 U/kg (13%). Pigs fed BD had a lower ($P < 0.05$) gain:feed ratio (by 20%) than pigs fed BD + 0.25% iP + 1,000 or 2,000 U/kg.

3.3.3 Experiment 2.3

Bone strength was 12% greater ($P = 0.02$) in pigs fed BD + both 0.25% iP and 2,000 U/kg than those fed diet supplemented with BD + only 0.25% iP (**Figure 3.3**). The former had 7% ($P < 0.05$) higher bone Sr concentrations than the latter (63 ± 1.1 vs. 58 ± 1.7 ppm). However, these two groups of pigs showed no significant difference in bone total Ca and P concentrations (**Figure 3.4**) or in total K, Na, S, Mg, Fe, Mn, Zn, B or Cr levels (data not shown). Plasma iP concentrations, plasma

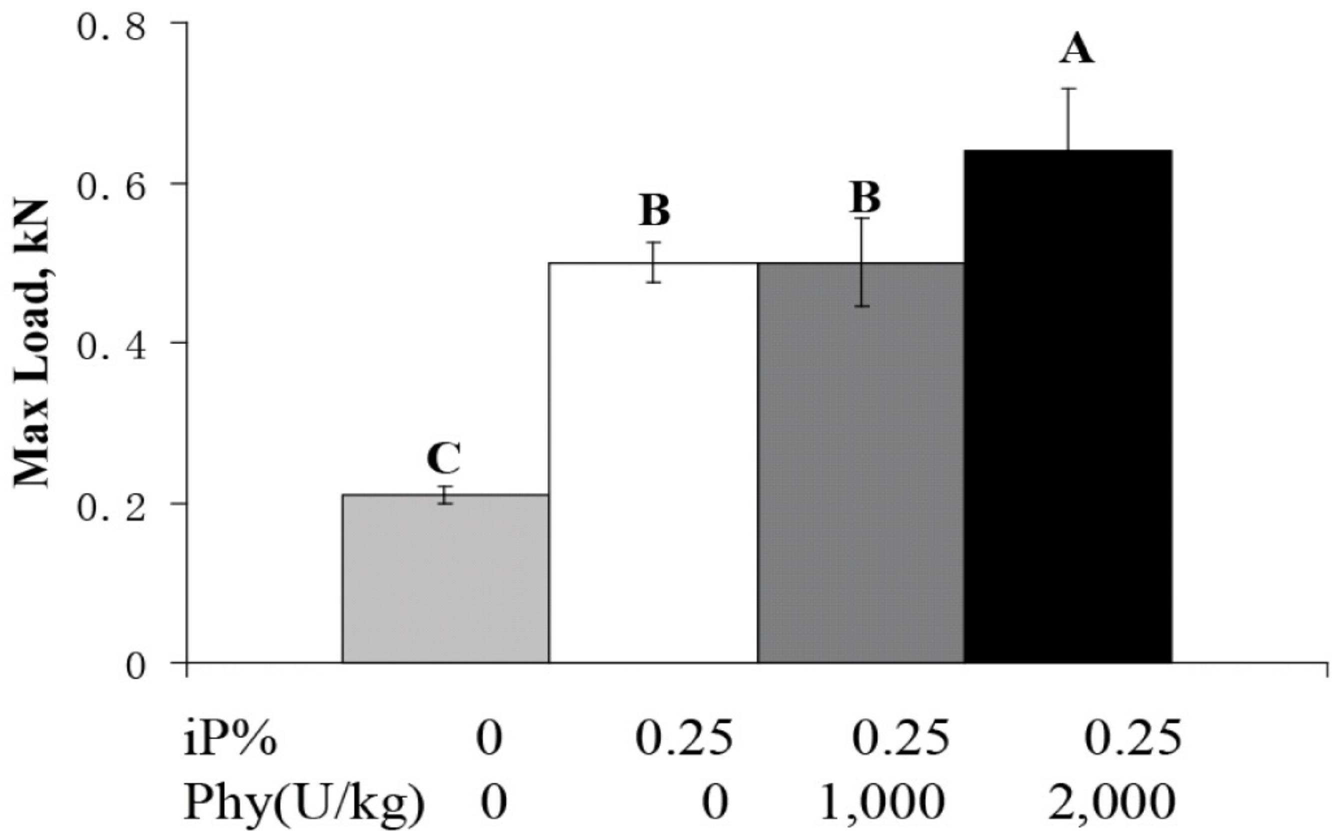

Figure 3.2 The bone breaking strength of pigs fed BD, BD + iP (0.25%), BD + iP (0.25%) + 1000 U phytase/kg and BD + iP (0.25%) + 2000 U phytase/kg. Values are the means of individual pigs (n = 4). Means not sharing a common letter differ ($P < 0.05$).

Table 3.3. Effects of supplemental dietary phytase and inorganic P on growth performance and plasma measures of pigs in Experiment 2.2[a]

	Treatment				
iP (%)	0	0.25	0.25	0.25	
Phytase (U/kg)	0	0	1000	2000	SEM
Overall daily gain, g	323^{z}	489^{y}	486^{y}	557^{x}	11
Overall daily feed intake, g (as-fed)	695^{y}	983^{x}	936^{x}	1032^{x}	24
Overall gain:feed	0.53^{y}	0.59^{x}	0.60^{x}	0.60^{x}	0.01
Plasma inorganic P, mg/dL					
Week 0	5.72	6.09	5.78	6.11	0.09
Week 4	4.39^{y}	8.67^{x}	8.25^{x}	8.14^{x}	0.11
Alkaline phosphatase activity, mU/ml					
Week 0	191	150	173	190	51
Week 4	188^{x}	122^{y}	102^{y}	127^{y}	46

[a]Values are means of seven or eight individually penned pigs during the 4-wk study.

^{x,y,z} At each sampling time point, values not sharing a letter are different ($P < 0.05$).

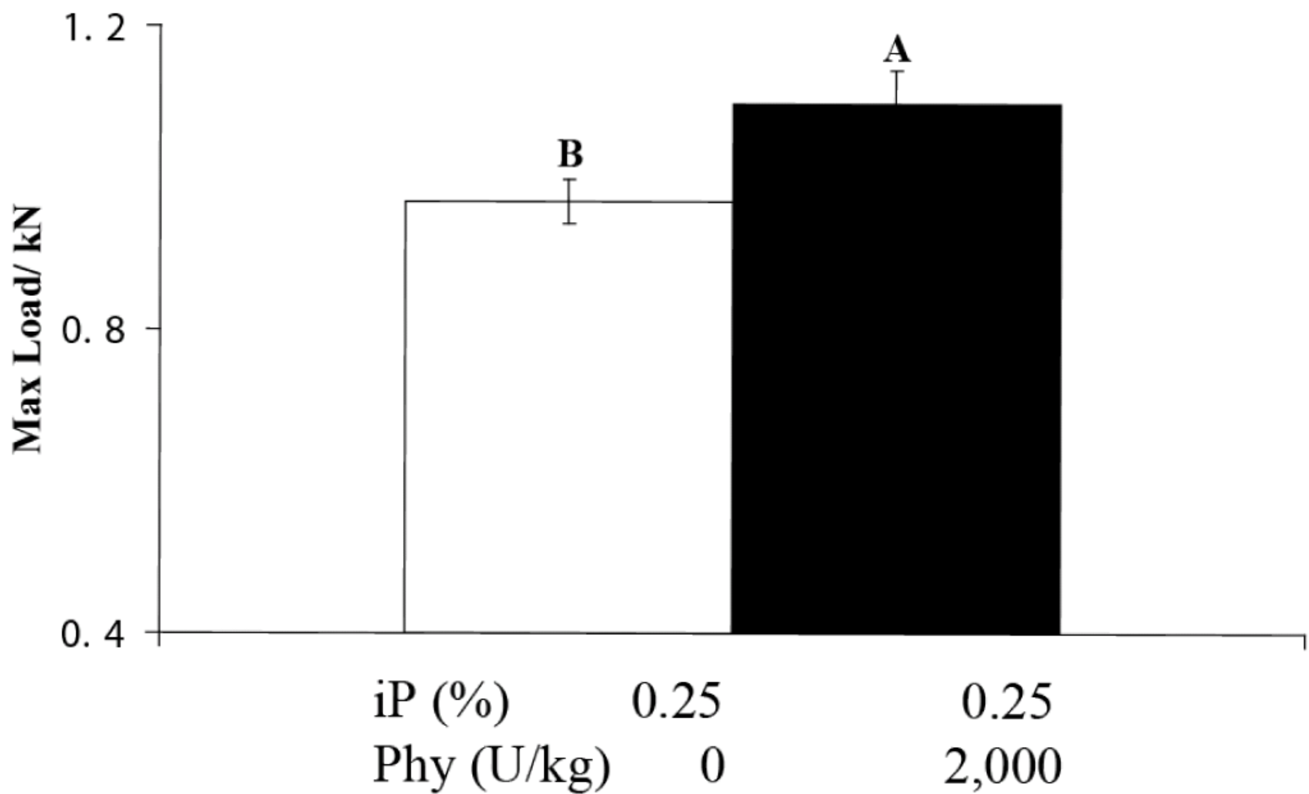

Figure 3.3 The bone breaking strength of pigs fed BD + iP (0.25%) and BD + iP (0.25%) + 2000 U phytase/kg. Values are the means of individual pigs (n = 8). Means not sharing a common letter differ ($P < 0.05$).

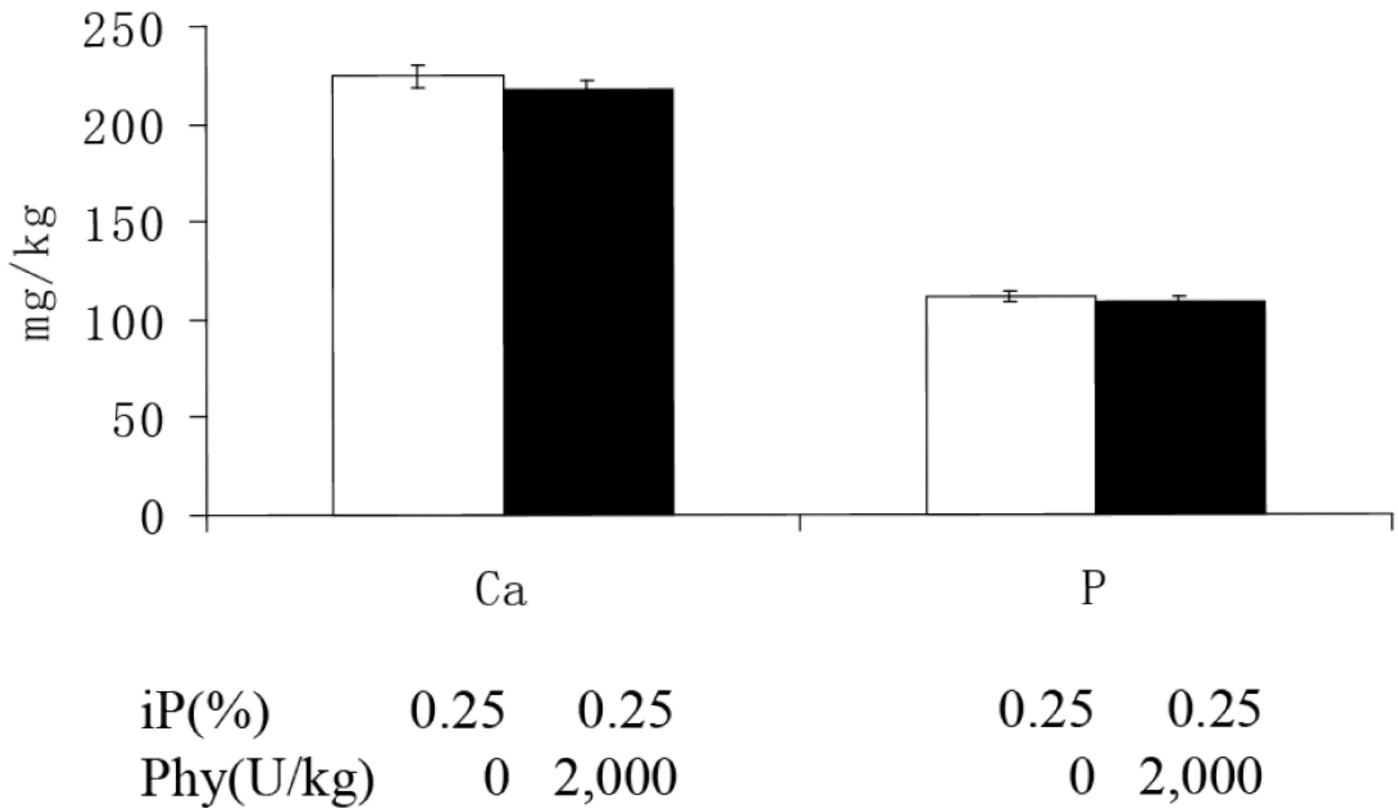

Figure 3.4 Bone calcium and phosphorus concentrations of pigs fed BD + iP (0.25%) and BD + iP (0.25%) + 2000 U phytase/kg. Values are the means of individual pigs (n = 8).

Table 3.4. Effects of supplemental dietary phytase and inorganic P on growth performance and plasma measures of pigs in Experiment 2.3

		Treatment			
		iP (%)	**0.25**	**0.25**	
Measures	**Time**	**Phytase (U/kg)**	**0**	**2000**	**SEM**
Overall daily gain, g			527	500	9
Overall daily feed intake[b], g (as-fed)			1089	1010	-
Overall gain:feed[b]			0.53	0.56	-
Plasma inorganic P[a], mg/dL					
	Week 0		5.27	5.27	0.08
	Week 6		8.36	8.18	0.11
Alkaline phosphatase activity, mU/ml					
	Week 0		263	273	87
	Week 6		173	162	35

[a]Values are means of eleven individual pigs during the 6-wk study.

[b]Values are means of individual pens holding 11-12 pigs during the 6-wk study.

[x,y,z] At each sampling time point, values not sharing a letter are different ($P < 0.05$).

alkaline phosphatase activity and growth performance were not significantly different between these two dietary treatment groups (**Table 3.4**).

3.4 Discussion

The central finding of this study is that supplemented *Escherichia coli* AppA2 phytase at 2,000 U/kg of diet produced significant improvement of bone strength of young female pigs fed P-adequate diets. Results of the three consecutive experiments indicate a strong dependence of such improvement on the supplemental phytase dose and the sample size of treatment groups. While in Exp. 2.1 supplementing 1,000 U of phytase/ kg in a low-P diet resulted in a 39% ($P < 0.05$) increase in bone strength, the same supplementation of phytase in a P-adequate diet (0.2% iP) produced only a marginal improvement (16%, $P = 0.16$), if any. A phytase dose response became evident in Exp. 2.2. Compared with those fed P-adequate diets, pigs fed additional phytase at 1,000 U/kg, as in Exp. 2.1, did not show significant enhancement in bone strength. In contrast, pigs fed additional phytase at 2,000 U/kg showed 22% improvement in bone strength over those fed P-adequate diet even with an increase in inorganic P supplementation from 0.2 to 0.25%. However, this biological improvement ($P = 0.06$) just missed statistical significance ($P < 0.05$), possibly because of the limited sample size of bone strength analysis ($n = 4$). When the sample size was 8 in Exp. 2.3, the difference in bone strength between the same two treatments became significant ($P = 0.02$).

Since bone strength is a sensitive and reliable indicator of bone integrity (Koch and Mahan, 1985; Combs et al., 1991a; Combs et al., 1991b; Ketaran et al., 1993), our above-described findings demonstrate a novel role and potential for dietary phytase in improving bone health of mammals. Similar findings have been implied, but not

proven, in previous studies (Murry et al., 1997; Cromwell, 1991; Yi et al., 1996). Yi et al. (1996) showed that supplementing a fungal phytase at 1,400 U/kg in a diet containing 0.32% available-P increased bone ash by 10% and shear force by 19%. Although the reported dietary available P was relatively high, these researchers still observed an elevated absorption of P associated with the addition of phytase, probably due to a suboptimal total Ca: total P ratio (1.46:1), instead of the recommended 1.2:1(Lei et al., 1994). Thus, the bone improvement by supplemental phytase in their study might still be attributed to enhanced P accretion. In contrast, we believe that the bone strength improvement by 2,000 U of phytase/kg over the P-adequate diet in the present study was due to factors other than P. Three results of our research are evidence to support this notion.

First of all, plasma iP concentrations and plasma alkaline phosphatase activity were nearly identical between pigs fed diet supplemented with 0.25% iP and 2,000 U of phytase/kg and those fed the diet supplemented with only 0.25% iP throughout all experiments and timepoints. As both measures are very responsive to body P deficiency (Boyd et al., 1983; Koch and Mahan, 1985; Lei et al., 1993a), the lack of differences in the two measures demonstrates that the P-adequate diet provided sufficient available P levels to maintain normal body P status. Conversely, the high level of phytase (2,000 U/kg) did not further improve body P status to exert its effect on bone strength. Secondly, the bone strength improvement produced by supplementing 2,000 U of phytase/kg in the P-adequate diet was not attributed to increased body mass of tested animals (van Langendonck et al., 2004). Animal growth is very sensitive to dietary P levels ranging from the deficient to adequate levels (Koch and Mahan, 1985; Traylor et al., 2005), and bone strength is positively correlated to body weight. However, no growth performance was altered by the high level of phytase in the P-adequate diets except in Exp. 2.2, in which 2,000 U of phytase/kg

resulted in 13% improvement ($P < 0.03$) in overall ADG. Even so, the final body weights were not significantly different between the two groups and the heights and widths of the metacarpals were very similar between treatment groups (data not shown). Thirdly, the lack of differences in bone Ca and P concentrations between pigs fed the diet supplemented with both iP (0.25%) and phytase (2,000 U/kg) and those fed the diet supplemented with only iP (0.25%) in Exp. 2.3 further excludes the possibility that the bone strength improvement by the high level of phytase was due to an increased P or Ca deposition in bone. A previous study (Murry et al., 1997) has shown no change in bone mineral concentration, but a significant increase in global mineral density of pigs by supplementing 700 or 1000 U of phytase/kg in a P-adequate diet (0.93% tP). Therefore it is possible for the bone strength or bone mineral density to increase without a concurrent increase in the mineral concentration.

As the primary objective of this research was to determine if high levels of dietary phytase exerted non-P effect on bone strength, our experiments do not unveil the specific mechanisms for the observed benefit of phytase. However, it is fascinating to see the small, but significant increase in bone deposition of Sr by supplementing 2,000 U of phytase/kg in the P-adequate diet. Many cell culture and animal studies have shown the positive effect of Sr on bone formation while negatively impacting resorption (Boivin et al., 1996; Grynpas et al., 1996; Dahl et al., 2001; Ammann, 2005). Supplemental Sr has been suggested and used to prevent and treat osteoporosis (Meunier et al., 2004; Ammann, 2005). Apparently, it warrants future research to find out whether the non-P effect of high levels of phytase on bone strength is mediated by improving bone Sr deposition. In addition, possible impacts of high levels of phytase may be on the metabolism of organic components of bone (Ketaran et al., 1993; Burstein et al., 1977; Leichter et al., 1982; Currey et al., 1996) and the development of bone morphology (Judex et al., 2003) should also be investigated.

In summary, our research has demonstrated the additional benefit to bone strength by supplementing a high level of bacterial phytase in diets containing adequate inorganic P in young female pigs. Although the biochemical mechanism of this non-P effect of phytase on bone strength still remains to be elucidated, this benefit illustrates a novel function of phytase. Since pigs are excellent models for humans (Pond and Houpt, 1978), our findings offer a new strategy for improving human bone health and preventing bone fractures. Current approaches in that regard include calcium supplementation, increased physical activity and sun exposure and application of bone resorption reducers such as estrogen, calcitonin, bisphosphonates and selective estrogen-receptor modulators (SERMs), and bone formation enhancers like fluoride (Dahl et al., 2001). Supplementing high levels of dietary phytase, starting early in life, may be a more effective, convenient, and economical intervention than those approaches. However, possibilities of mineral toxicities and possible links to colon cancer due to high levels of iron would need to be investigated to ensure safe consumption by humans.

CHAPTER 4

CONCLUSION

Two studies have been conducted to examine the location of dietary *E. coli* AppA2 microbial phytase function in the gastrointestinal tract of pigs and to evaluate the ability of microbial phytase to improve bone strength by factors other than P-accretion.

In the first study, two experiments with a total of 33 weanling pigs were conducted to examine dietary phytase activity disappearance along various segments of the digestive tract. Activity of supplemented AppA2 phytase was compared to a low-P corn-soy basal diet and/or BD + iP (0.1%) at 500 and 2,000 U of phytase/kg. The study demonstrated that AppA2 mainly functioned in the stomach with activity remaining through the upper jejunum, but that the phytase was undetectable by the time the digesta reached the lower jejunum. This is similar to PhyA fungal phytase in which the stomach also serves as the main site of activity (Jongbloed et al, 1992; Yi and Kornegay, 1996), whereas the AppA2 activity remained fairly constant from stomach to upper jejunum, the activity decreased for PhyA. The difference along this section of tract was most likely due to AppA2's lower pH optima and greater pepsin-resistance in comparison to PhyA (Rodriguez et al., 1999b).

The study showed that supplementing different levels of AppA2 phytase in the diet diminished the amount of colon phytase activity and therefore also decreased the amount of iP excreted in feces. The higher levels of phytase release more phytate-P in the upper digestive tract, where it is readily absorbed (Moore and Tyler, 1985; Lantsch et al., 1992) and resulted in less substrate for the colon microflora. Overall, there was a reduction in total P and Ca that was detectable in colon digesta, indicating greater

absorption in the small intestine of both minerals due to phytase supplementation. The minimal colon phytase activity associated with a 2,000 U AppA2 phytase/kg indicate that this is approximately the optimal level to add to a corn-soy diet to release most phytate-P prior to reaching the large intestine. Labeled-P could be used in the future to determine true P-absorption of phytate-P versus supplemental iP in a similar study. If the released P is truly absorbed in the digestive tract prior to the colon, then high levels of dietary AppA2 phytase is a highly effective weapon against P pollution in the environment.

In the second study, three experiments were conducted with a total of 80 pigs fed a low-P (0.4%) corn-soy BD, or BD with 0.2% or 0.25% iP and(or) 1,000 or 2,000 U *E. coli* AppA2 phytase/kg for 4-6 wk. The study demonstrated that dietary phytase supplementation increased bone strength of pigs fed a P-adequate diet. The experiments showed strong dependence of such improvement on the supplemental phytase dose. In conclusion, phytase improved bone parameters when supplemented at 2,000 U/kg but not at 1,000 U/kg, showing that high levels of phytase are needed for this function. No interaction between the supplemented iP and the phytase was detected in the study. Bone composition analysis also showed that improvement by 2,000 U/kg phytase was not due to increased bone mineral concentrations of P, Ca, Mg, K, Na, S, Fe, Mn, Zn, B and Cr. The lack of increase in bone P concentrations, as well as lack of difference in growth or plasma P parameters strongly supports that the improved bone strength in the study was not due to P-accretion. However, the increase in bone Sr concentrations might be attributable to the increase in strength as many recent studies have shown supplementation of Sr positively affects bone strength (Boivin et al., 1996; Grynpas et al., 1996; Dahl et al., 2001; Ammann, 2005). Further research needs to be carried out to find the exact mechanism. Bone density and

mineral composition analysis of other tissues may help elucidate the role of phytase in bone strength.

Since the bone improvement study was carried out in young pigs with normal P status, it is warranted that phytase be supplemented to human youth in a study as a preventative of osteoporosis. Phytase supplementation could offer a viable way of controlling osteoporosis worldwide if found an effective treatment for humans.

Both studies support levels of 2,000 U/kg AppA2 phytase as a beneficial dietary level for weanling pigs fed corn-soy diet. At 2,000 U/kg AppA2 phytase was able to release most phytate-P, as well as improve bone strength, which was not observed when supplemented at half that amount. The improvement in bone strength by phytase may be related to Sr released and incorporated into bones.

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