APPLICATION OF MICROBIAL PHYTASE AND ITS INFLUENCING FACTORS *IN VIVO* AND *IN VITRO*

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(ABSTRACT)

Five experiments were conducted *in vivo* to investigate the efficacy of phytase in improving the availability of phytate P as influenced by dietary Ca:total P (**tP**) ratios for pigs, broilers, and turkey poults. In pigs and poultry, microbial phytase was effective in improving performance, P and Ca digestibility, bone mineralization , and in decreasing fecal P excretion by enhancing hydrolysis of phytate P for young pigs, broilers and turkey poults fed a corn-soybean meal diet. Maximum responses were achieved at supplemental phytase levels of 750 to 1,050 units (**U**)/kg diet for young pigs and 600 to 900 U/kg diet for poultry. Based on nonlinear and linear response equations generated from the phytase and available P (**aP**) data in young pigs and nonphytate P (**nP**) in broilers and turkeys, P-equivalency functions for phytase were developed. For pigs, the P-equivalency equation was $Y = .2622(1 - .9706e^{-.00185X})$; for broilers, the equation was $Y = .2330(1 - .9818e^{-.00074X})$; and for turkey poults, the equation was $Y = .1220(1 - 1.7721e^{-.00533X})$. For these three equations, X = added phytase (U/kg diet) and Y = P-equivalency values (%). Based on these equations, 1 g of P as inorganic deflourinated phosphate could be replaced by 300 and 208 U of phytase/kg of diet for pigs fed diets containing .07 and .16% aP, by 937 U of phytase for broilers fed with .27% nP diet, or by 340 and 511 U of phytase/kg diet for turkey poults fed diets containing .27 and .36% nP, respectively. Phosphorus-equivalency values of phytase were also obtained by generating P-equivalency functions at each P level and each Ca:tP ratio. The phytase efficacy was influenced by dietary Ca:tP

ratios, P, and vitamin D_3 levels. In pigs and poultry, a wide Ca:tP ratio decreased phytase efficacy because all measurements were decreased as the dietary Ca:tP ratio became wider. In young pigs, widening the ratios from 1.2 to 2.0:1 resulted in a decrease in phytase efficacy of 21.1 and 12.1% for .07 and .16% aP diets, respectively. In poultry, widening the ratio from 1.4 to 2.0 led to a decrease in phytase efficacy by 7.3% for broilers fed diets containing .27% nP, and by 6.3 and 4.2% for turkey poults fed diets containing .27 and .36% nP, respectively. A synergistic effect of vitamin D_3 addition and phytase supplementation was observed for broilers. Addition of vitamin D_3 indicated a potential for improving utilization of phytate P and Ca in the presence and absence of microbial phytase. Average daily gain, apparent P digestibility and bone ash content were the most sensitive measurements to assess microbial phytase efficacy for the replacement of inorganic P for pigs and poultry. These measurements were also sensitive for assessing the effects of varying Ca:tP ratios and levels of P. In summary, 1 g of P from deflourinated phosphate could be replaced by 250 to 400, 600 to 950 and 340 to 550 U of phytase/kg diet, respectively for young pigs, broilers and turkey poults when they were fed a corn-soybean meal diet. Dietary Ca:tP ratio of 1.2:1 for young pigs and 1.1 to 1.4:1 for poultry resulted in maximum phytase efficacy. An *in vitro* study was performed for the evaluation of effects of cations on the characteristics of microbial phytase from *A. niger*. A discontinuous assay was applied to assay *A. niger* phytase. The enzyme was observed to have a high affinity for sodium phytate with a K_m of 62 μM and a V_{max} of 139 U of specific activity per mg of phytase protein. Malachite green was used as the color reagent in the discontinuous assay, which increased the sensitivity 50 fold over molybdovanadate as the color reagent. All cations tested *in vitro* (Mg^{2+}, Mn^{2+}, Ca^{2+}, Cr^{3+}, Fe^{3+}, Cu^{2+} and Zn^{2+}) inhibited phytase activity, and imposed a competitive or mixed inhibition; a binding of cations with phytate also was involved in the inhibition by decreasing the effective substrate concentration. The inhibition by Ca^{2+} and Mg^{2+} caused only a partial inhibition

because the enzymatic reaction rate was never reduced to zero and replots of slopes for Ca^{2+} and Mg^{2+} were hyperbolic. Cations of Zn^{2+}, Cu^{2+}, Fe^{3+}, Cr^{3+} and Mn^{2+} gave a pure inhibition. A decreasing order of the inhibitory effect from cations was observed on the phytase activity: $Zn^{2+} > Cu^{2+} > Fe^{3+} > Cr^{3+} > Ca^{2+} > Mn^{2+} > Mg^{2+}$ based on the K_i value that increased from a low value for Zn^{2+} to a high value for Mg^{2+}. In summary, cations possess a potential for decreasing *A. niger* phytase activity by a competitive or mixed-type inhibition system; binding of cations with the phytate substrate also inhibited the activity of *A. niger* phytase.

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TABLE OF CONTENTS

LIST OF TABLES

CHAPTER II

CHAPTER III

CHAPTER IV

CHAPTER V

CHAPTER VI

CHAPTER VII

LIST OF FIGURES

CHAPTER II

CHAPTER V

CHAPTER VII

CHAPTER I

Introduction

The current strong interest in microbial phytase additives is attributed mainly to the heightened environmental awareness of P and N pollution originating from animal manure. Because of the high nutrient content of manure, and thus fertilizing value, land application has been the major means of disposal. On the other hand, however, there are limits to the amount of manure that can be applied to the land because of nutrient build-up in and on the soil. The potential environmental impact of nutrient contamination of surface run-off and ground water is perceived as a major issue facing livestock producers (Coffey, 1992). The impact of very large amounts of manure being produced in a relatively small land area has led to the development of strict legislation controlling animal manure pollution in some countries.

Of the nutrients present in manure, P is of greatest concern (Kornegay, 1995). Over 60 to 70% of P in feed ingredients is bound with *myo*-inositol phosphates as phytate. Phytate P is poorly available to pigs and poultry; and bioavailability estimates of P in corn and soybean meal range from 10 to 30% (Nelson, 1967; Calvert et al., 1978; Jongbloed and Kemme, 1990; Cromwell and Coffey, 1991). Most of the indigestible phytate P is excreted in manure (Gerritse and Zugec, 1977; Cromwell and Coffey, 1991). This low availability of phytate P poses two problems for producers: 1) the need to add inorganic P supplements to diets to meet the P requirements of pigs and poultry, and 2) the excretion of large amounts of P in the manure.

Phytase, especially from microbial origin, is very effective in the hydrolysis of phytate, and thus releasing the P and making it bioavailable. The amount of inorganic P added in diets can be reduced and the excretion of P by pigs and poultry can be decreased with the addition of phytase (Nelson et al., 1971; Han and Wilfred, 1988; Simons et al.,

1990). Although current advances in biotechnology have produced a highly active enzyme additive, the microbial product is not widely used in animal husbandry due to costs and to limited information available about its application. For practical application of phytase in pig and poultry diets, P-equivalency values of phytase for inorganic phosphorus have to be generated. Phytase efficacy in pigs and poultry is variable because many dietary factors, especially Ca, possess a potential to influence the phytase activity. Therefore, in order to improve the efficacy of application of phytase to the greatest degree, characterization of these factors is necessary. In addition, it also is important to characterize and quantify the effect of microbial phytase on the availability of Ca, N, and dry matter.

Characteristics of phytase from plant sources such as soybeans, corn, wheat and rapeseed, and from intestinal mucosa of chickens, rat, calf and man have been extensively investigated (Bitar and Reinhold, 1972; Gibson and Ullah, 1987; Yang et al., 1991). Characteristics of microbial phytase from *A. ficuum* were reported by Han (1988) and Ullah (1988ab). No reports are available regarding the characteristics of microbial phytase from *A. niger*. The results of available *in vitro* studies are not consistent; and some *in vitro* results are inconsistent with that of *in vivo* studies. Especially, the influence of cations (minerals) on the microbial phytase activity is not clear, although cations (minerals) tended to influence the enzyme activity *in vivo* and *in vitro*.

The objectives of this research were: 1) to evaluate microbial phytase as a replacement for inorganic phosphorus in pig and poultry diets; 2) to determine the effect of phytase on the utilization of Ca, N and dry matter for pigs and poultry; 3) to characterize the effect of the Ca:total P ratio on the phytase efficacy for pigs and poultry; 4) to investigate the effects of dietary P and vitamin D_3 on supplemental phytase efficacy; 5) to evaluate the sensitivity of measurements for assessing the effects of dietary supplemental phytase and P levels and Ca:tP ratios for pigs and poultry by generating second-order translog, nonlinear and linear equations; 6) to study the characteristics of

microbial phytase (*A. niger*), and investigate the effects of cations (minerals) on *in vitro* phytase activity.

CHAPTER II

Literature Review

Phosphorus

Phosphorus Nutrition

Phosphorus is an essential element for animals; it makes up about 1% of the body weight of mature pigs, and ranks second after Ca in the total mineral content in the body (Peo, 1991; Cromwell et al., 1991). Approximately 80% of the total body P is formed primarily as hydroxyapatite salts of Ca and Mg in the bones and teeth in the development and maintenance of vital skeletal structures of the hard tissues (Underwood, 1981). The remaining 20% is distributed in the fluids and soft tissues in which P serves as a component of many organic compounds such as phospholipids, nucleotides, etc. and is involved in almost all metabolic reactions in the body (Peo, 1991). Phosphorus probably is the most versatile of all of the mineral elements. In addition to taking part in the formation and maintenance of bone and teeth, which serve as a reservoir of P, phosphorus is involved in the metabolism of amino acids in the synthesis of proteins, and in the utilization of energy including the metabolism of carbohydrates and fat.

As a part of organic phosphates with high energy phosphate bonds such as acetyl phosphate, adenosine triphosphate (ATP), etc. and with low energy phosphate bonds including glucose-6-phosphate, glycerol phosphate, 3-phosphoglyceric acid and others, phosphorus is involved in most reactions catalyzed by enzymes in every cell of the body (Underwood, 1981). As a part of phospholipid, phosphorus contributes to the integrity and function of cell membranes. As a component of DNA and RNA, phosphorus serves as the basic substance of inherence and is essential for cell reproduction, protein synthesis and growth of body tissues (Pike and Brown, 1984). As an anion, phosphorus plays an

important role in maintaining the proper acid-base balance involved with other anions (Cl^- and S^{2-}) and cations (Na^+, K^+, Ca^{2+} and Mg^{2+}) in the body (Melliere and Forbes, 1966). Phosphorus serves as the major intracellular buffer in the body and is involved in the maintenance of osmotic balance in body fluids. Moreover, phosphorus is a critical element for maintaining the appetite and efficiency of feed utilization of animals.

Dietary P insufficiency could cause animals to have various impairments of tissues and performance. Animals suffering from P deficiency grow poorly, and are accompanied by depressed appetite and low efficiency of feed utilization (Underwood, 1981). The impairment of bone growth is one of the most obvious signs of a P deficiency. Rachitic bones, osteodystrophy and osteomalacia result from the irregular growth and arrangement of cartilaginous and bone cells in cartilaginous, proliferating and hypertrophic zones, and are due to incomplete ossification or demineralization from P deprivation. The histological hallmark of a P deficiency in poultry is a widening of the hypertrophy zone that results from defective cartilage and bone differentiation. A loss of trabecular bone density along with a decrease in bone minerals and ash content also is apparent, indicating demineralization of the bone (Long et al., 1984; Qian et al., 1994bc). At the same time, bones become shorter and wider with reduced mass and mineral contents (Edwards and Veltmann, 1983; Yoshida, 1986). Inorganic P concentrations in the plasma of normal pigs is approximately 8 to 10 mg/dL. A phosphorus deficiency results in reduced-plasma P concentrations (hypophosphatemia), increased-plasma Ca (hypercalcemia), and enhanced plasma alkaline phosphatase activity (**ALP**) (Underwood, 1981; Pointillart et al., 1987; Pointillart, 1991), and is accompanied by reduced-urine P (hypophosphaturia), increased-urine Ca (hypercalciuria) and increased-urine hydroxyprolinuria (hyperhydroxyprolinuria) (Nasi, 1990; Pointillart et al., 1991). Maintenance of plasma P at normal levels ensures adequate P for the absorption of tissues, otherwise, impairment of tissues appears due to deprivation of P supply (Hayes, 1976).

Phosphorus Utilization

Phosphorus is absorbed in the form of orthophosphate (PO_4^{3-}), principally in the proximal end of the duodenum (Bartter, 1964; Irving, 1964); the absorption in the large intestine seems negligible (Jongbloed, 1987). There are two factors that should be considered for the P utilization in practice. The first one is the P requirement for growing animals, which is based on the requirement for maintenance and production, and the second is the dietary factors that affect the P availability. The NRC (1988; 1994) suggests that the P requirement for weanling pigs, broilers and turkey poults is: .32, .45 and .60% available P (**aP**) [.60, .70 and .80% total P (**tP**)], respectively. Several factors influence the rate of P absorption. These include the amount and the source of dietary P, the ratio of dietary Ca to P, adequacy of dietary vitamin D, and the presence of other minerals such as Al, Mn, Cu, Mg, Fe and Zn that are antagonistic to P absorption and utilization (Peo, 1991). Different P sources, including organic P (plant P or phytate P) and inorganic P sources, vary in P availability (NRC, 1988; Beers and Jongbloed, 1993). For example, only about 30% of P in ingredients are assumed to be used by pigs and poultry (NRC, 1988; 1994) (Table 1). In comparison, Coffey et al. (1994) reported that the bioavailability of five commercial defluorinated phosphate sources ranged from 60 to 90% for chicks and 70 to 90% for young pigs.

Because the mobilization and deposition of P share the same parathyroid hormone and thyrocalcitonin hormone control system with Ca, both Ca and P are regulated by the same hormone-regulated metabolism process (Underwood, 1981). It seems more important for a proper Ca:P ratio than the absolute amounts of Ca and P when formulating swine and poultry diets (Mahan, 1982). In swine production, diets often are mixed with more Ca relative to aP levels because of an over-estimated P availability. Phosphorus is a rather expensive additive while Ca is inexpensive (Peo, 1991). A wider ratio of Ca:P results in poor growth, reduced feed conversion and impaired bone development due to

Table 1. Bioavailability of phosphorus in feedstuffs for pigs and poultry

Feedstuff	Bioavailability of P for pigs[a]	Bioavailability of P for poultry[b]
	%	
Cereal grains		
Corn	12	28
Oats	23	33
Barley	31	36
Triticale	46	33
Wheat	50	31
Corn, high moisture	53	--
High protein meals - plant origin		
Peanut meal	12	21
Canola meal	21	26
SBM, dehulled	25	35
SBM, 44% protein	35	40

[a]Adapted from Kornegay (1995). Relative to the availability of phosphorus in monosodium phosphate, which is given a value of 100.

[b]NRC (1994) - Poultry.

the adverse effects of relatively excessive Ca on P absorption and bone mineralization. Many researchers have confirmed that the optimum ratio of dietary Ca to P ranges between 1 and 1.3:1, with outside limits of .9 and 1.6:1 (Peo, 1976). These data are in agreement with the ratio of Ca to P in the total body, which is between 1.5 and 1.6:1 (Oslage, 1964).

Also, vitamin D influences the absorption, transport, and metabolism of P as well as Ca. Vitamin D stimulates not only renal P reabsorption but also P transport through the intestinal walls (Walling, 1977). A vitamin D deficiency disturbs the absorption and metabolism of Ca and P; this results in rickets in young growing pigs and poultry, and osteomalacia in mature pigs and birds due to insufficient bone calcification (NRC, 1988; 1994). It is recommended of 200 to 220 IU of vitamin D_3 ($\mathbf{D_3}$) per kg of diet for young

piglets (NRC, 1988). In poultry, the NRC (1994) suggested requirement for D_3 is 200 IU per kg of diet. One IU of vitamin D is defined as the biological activity of .025 μg of D_3 (NRC, 1988; 1994). Recently, however, the amount of D_3 added in diets tended to be supplemented at very high levels for pigs and poultry because of its additional benefit in increasing phytate P utilization (Hancock et al., 1986; Mohammed et al., 1991).

Phosphorus utilization is also influenced by dietary energy, protein, and mineral contents. The absorption of P tends to be reduced in high fat or high energy diets, and thus causes a reduction in feed intake (Mullins and Boulware, 1967; Holler and Hill, 1968). Increasing the dietary protein level by increasing the level of soybean meal results in bone demineralization because of reduced Ca and P absorption. The high levels of phytic acid in soybean meal apparently also contribute to the detrimental effect on the absorption of Ca and P. Some minerals, such as Fe, Al, Mn, and Zn at high levels, also tend to adversely affect the utilization of P, but this seems meaningless in the practical feeding levels (Furgouri, 1972).

Response Criteria of Phosphorus

The accuracy and efficiency of the estimation of P availability are dependent on sensitivity of measurements being used. The criteria for estimation of P availability may be classified as productive performance (weight gain, feed intake and feed conversion), bone measurements (shear force, shear stress, shear energy, ash content, mineral concentrations, organic matrix and ash weight, and histologic traits), serum criteria (serum P, ALP and parathyroid hormone level), and small intestinal parameters (the activity of mucous ALP and phytase) (Koch et al., 1984; Koch and Mahan, 1985; Pointillart et al., 1985). Weight gain, feed intake, and bone shear force and ash content rank high in sensitivity for assessing dietary P availability of pigs and poultry. Serum P and ALP and bone shear energy, histologic traits of bone exhibit relatively good sensitivity. Measurements that exhibit low sensitivity include gain:feed ratio, serum parathyroid hormone level, bone

shear stress, mineral concentrations and organic matrix weight of bone, the activity of mucous ALP and phytase for the dietary P intake (Koch et al., 1984; Koch and Mahan, 1985; Pointillart et al., 1985; Peo, 1991; Qian et al., 1994bc).

Phytate Phosphorus

Chemistry and Occurrence of Phytate

Phytate is a storage form of P known as *myo*-inositol hexaphosphate or the mono to dodeca anion of phytic acid in plants while phytic acid is commonly called *myo*-inositol hexaphosphoric acid or chemically, 1, 2, 3, 4, 5, 6-hexakis (dihydrogen phosphate) *myo*-inositol (Reddy et al., 1982; Maga, 1982) (Figure 1).

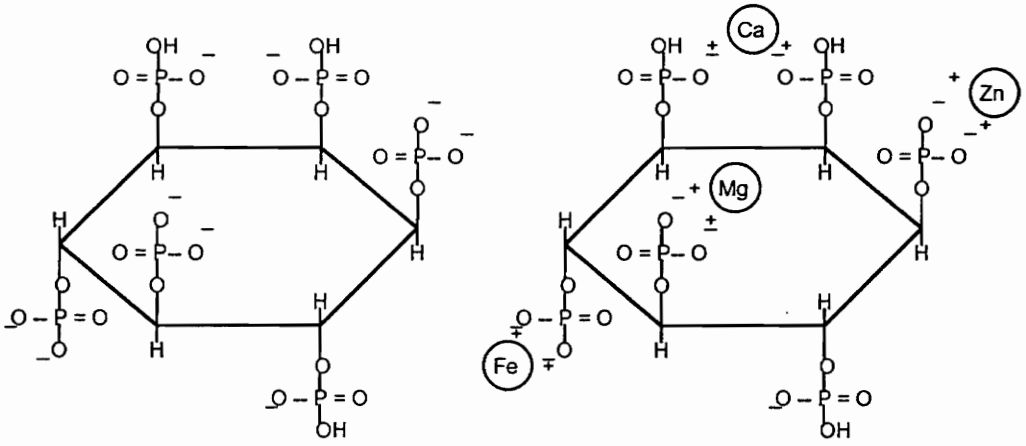

Figure 1. Structure of *myo*-inositol hexaphosphoric acid (left) and *myo*-inositol hexaphosphate (right) (Maga, 1982)

Phytate in plants often is called phytin, the Ca-Mg-K salts of phytic acids (Cosgrove, 1980). In plants, phytic acid is assumed to contain about 28.2% of P and its six phosphoric acid residues have varying affinities for cations (deBoland et al., 1975). Phytate is widely distributed in feedstuffs, and accounts for 40 to 85% of total P in

feedstuffs (Nelson et al., 1968a). Approximately two-thirds of P of ingredients is in the form of phytate while one-third is in the nonphytate form (NRC, 1988) (Table 2). Phytate

Table 2. Phytate-phosphorus content and phytase activity of some common feed ingredients

Ingredient	Phytate P, g/kg[a]	Phytate P, as % of total P[a]	Phytase activity, U/kg[b]
Cereals and by-products			
Wheat	2.4 (1.9-2.9)	68 (61-78)	1190
Maize	2.0 (1.6-2.6)	73 (61-85)	15
Sorghum	2.2 (1.9-2.9)	68 (61-76)	25
Barley	2.1 (1.9-2.4)	58 (55-62)	580
Oats	2.8 (1.6-3.5)	69 (48-78)	40
Wheat bran	8.8 (6.0-12.7)	76 (68-93)	2960
Grain Legumes			
Lupines	3.0 (2.9-3.0)	55 (54-55)	0
Peas	1.7 (1.3-2.1)	45 (36-53)	115
Chicks peas	2.1 (2.0-2.3)	51 (49-53)	--
Oilseed meals			
Soybean meal	3.7 (2.8-4.0)	57 (46-61)	40
Canola meal	6.5 (4.6-7.8)	58 (36-70)	15
Sunflower meal	4.4 (3.2-5.1)	44 (35-47)	60

[a]Data adapted from the following sources: Kornegay (1995); Eeckhout and de Paepe (1994); Kirby and Nelson (1988); Nelson et al. (1968a); Ravindran et al., (1995c). Values within parentheses refer to ranges reported in the literature.

[b]Data from Eeckhout and de Paepe (1994). One unit is defined ad that amount of phytase which liberates inorganic phosphorus from a 1.5 mM Na-phytate solution at a rate of 1 μmole/min at pH 5.5 and 37°C.

levels also are influenced by the degree of seed maturity. No phytate is found in the early stages of seed development while at maturity usually 60% or more of the total P is present in phytate (Saio et al., 1977). Although there are various factors (intestinal pH of animals, the type of protein, and cations associated with phytate) that influence the solubility of phytate in feedstuffs and results in a low availability of P, the source of phytate is probably

more critical for the solubility and availability of phytate. The solubility in water of phytate from corn germ, soybean meal, soybean flakes, and cottonseed meal, are 90%, 60%, 70%, and 50%, respectively, while those from sesame meal and isolated soybean protein are relatively low. At pH 6, pure inositol hexaphosphate is more stable in rice, wheat, and sesame meal than in aqueous solution where it is readily hydrolyzed (deBoland wheat, and sesame meal than in aqueous solution where it is readily hydrolyzed (deBoland et al., 1975; O'Dell and deBoland, 1976; Han, 1988).

Approximately 90% of the phytate in corn is concentrated in the germ, whereas, in wheat and rice, most of the phytate is in the outer layers, pericarp and aleurone (O'Dell et al., 1972; Cheryan, 1980). In oilseeds such as peanuts, sunflower, and cottonseed, phytate is concentrated within the protein body and the globoids that might serve as storage sites (Dieckert et al., 1962; Lui and Altschul, 1967; Saio et al., 1977). However, the location of phytate in soybean is still controversial. Martinez (1979) demonstrated that soybeans do not contain globoids and suggested that the phytate might exist in the kernel. On the other hand, Lott and Buttrose (1978), and Prattley and Stanley (1982) found globoids in soybean that were rich in phytate.

Combination of Phytate with Minerals and Proteins

Many researchers have documented that phytate can combine with protein and/or minerals such as Ca, Mg, Zn, Fe, etc. (O'Dell, 1976; Saio et al., 1968). O'Dell and deBoland (1976) demonstrated with soybean flakes extracted with water, that approximately 40% of phytate were firmly bound with protein. Similarly, this phenomenon also occurs in corn. Calcium markedly decreased the solubility of the soybean protein-phytate complex, and resulted in the protein being less subject to proteolytic digestion (O'Dell and deBoland, 1976). Phytate was able to combine with minerals such as Ca, Mg and Mn in the presence and absence of protein; the binding of proteins, especially agglutinin, appeared to be through minerals to be (Honig and Wolf,

1991). However, in some cases, phytate may be combined only with protein, and in others only with minerals to form an insoluble compound. The complexes of phytate with protein and/or minerals are low in solubility, which result in low protein and mineral availabilities for monogastric animals (Saio et al., 1967; Wise, 1983; Ritter et al., 1987). The phytate-complexes with minerals and proteins were easily formed above the isoelectric point of proteins (Saio et al., 1967). The soy protein isolate, of which the bound phytate was reduced by alkaline treatment, increased in isoelectric point, which led to proteins more soluble and functional at a wider pH range (Chen and Morr, 1985). Ritter et al. (1987) observed that phytate exerted an inhibitory effect on *in vitro* protein digestibility; the chemical treatment (i.e., alkaline treatment) of phytate increased the digestibility of protein.

At low pH (3.5 to 5.0), phytate, a polyvalent anionic compound combines with positively charged protein molecules as described in the following reaction (Figure 2):

$$P^{n+} + nA^{-} \rightarrow PA_n$$

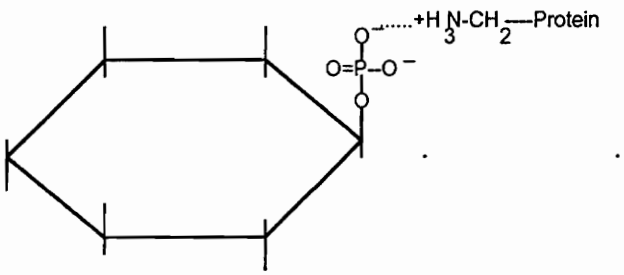

Figure 2. Possible structure of phytic acid-protein complex at low pH (Cheryan, 1980)

Based on kinetic and thermodynamic studies, phytate forms salt-like linkages with basic groups such as α-NH_2 terminal group, ε-NH_2 of lysine, and histidine and guanidyl groups

of arginine in proteins (Barre and van Huot, 1965). This causes a modification in structure brought about by close packing of protein molecules around the relatively small and highly charged phytate anion, resulting in the formation of an insoluble complex. The content of basic amino acid residues (arginyl, lysyl and histidyl) and glutamic residues in proteins are critical in phytate-binding (O'Dell and deBoland, 1976). At low pH, phytate shows greater affinity for H^+ than for mineral atoms. Thus, Mg, Ca, and Zn salts tend to be soluble and are not easily incorporated into the complex of phytate and protein at low pH (Maddaiah, 1964; Saio et al., 1968). One exception is iron that can combine strongly with phytate to form a completely insoluble compound (Yoshida, 1989; Honig and Wolf, 1991).

At an intermediate range of pH, close to the isoelectric point of plant proteins (pH 5.0 to 6.5), protein carries little net charges, hence no insoluble complexes of phytate and protein would be formed because of negligible electrostatic interaction between both phytate and protein (Prattley and Stanley, 1982). At this pH range, proteins, phytate, and minerals of diets are most soluble, and seem easy to be digested by animals (Okubo et al., 1975; Prattley and Stanley, 1982).

At an alkaline range of pH (6.0 to 10.0), the behavior of phytate appears to be strongly affected by a salt linkage or an alkaline-earth ion bridge (Cheryan, 1980). Insoluble compounds of phytate with protein and/or cations will be formed and the stability increases with increasing pH up to 10 at a proper concentration of cations:

$$\text{Cation} + \text{Phytic Acid} \rightarrow \text{Cation-Phytic Acid}$$

$$\text{Protein} + \text{Cation} + \text{Phytic acid} \rightarrow \text{Protein-Cation-Phytic Acid}$$

Phytate and protein are both negatively charged and capable of forming complexes at

alkaline pH through a cation bonding mechanism (i.e., salt bridges) (Maga, 1982; Prattley and Stanley, 1982; Chen, 1985).

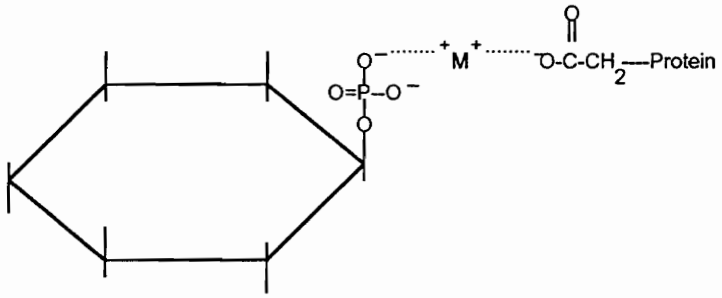

Figure 3. Possible structure of the phytate-mineral-protein complex at alkaline pH (Cheryan, 1980)

Ionized carboxyl groups as suggested in Figure 3 are not the only sites for protein-salt interaction. The imidazole group of histidine, in fact, is a possible site because chelation of metals by histidine are much stronger than that of other amino acids. At alkaline pH, the association between phytate and protein occurs only in the presence of divalent cations (Prattley and Stanley, 1982). The chelates of proteins, cations, and phytate are very labile in alkaline pH, and some complex is in the form of cation-phytate (e.g. Ca-Phytate) especially with increasing concentrations of cations (Saio et al., 1967; DeRham and Jost, 1979). Maddaiah et al. (1964) suggested a decreasing order of stability of the metal phytate complex: $Zn^{2+} > Cu^{2+} > Co^{2+} > Mn^{2+} > Ca^{2+}$,whereas, a different order was reported by Vohra et al. (1965): $Cu^{2+} > Zn^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Fe^{3+}$. The binding of phytate with minerals and proteins renders these minerals and proteins water-insoluble and unavailable for intestinal absorption.

Phytate Phosphorus Utilization

Most of the P in feedstuffs is unavailable for pigs and poultry because of the

presence of phytate P (Table 1). Pigs are able to utilize about 12% of the P in corn, 25% in rice bran, 29% in wheat bran, and 25 to 35% in soybean meal (Cromwell, 1989ab, 1990, 1992). Oilseed meals including peanut, sunflower and cottonseed meal are also low in P availability (12 to 21%). Some grains such as barley, triticale and wheat are relatively high in available P (30, 46, and 49% of P, respectively), but this is attributed to the presence of phytate occurring naturally in the seed coat, which is more soluble and digestible (Pointillart et al., 1984; Jongbloed and Kemme, 1990; Cromwell, 1992). The presence of natural phytase is another factor for the high P availability of these grains (Table 2). In other words, the P supplied by a corn-soybean meal diet for pigs is, on average, only about 20% available (Kornegay, 1995). Similar results have been found with growing chicks (NRC, 1994). In practice, about one-third of the P in feedstuffs of plant origin is assumed biologically available to pigs and poultry (NRC, 1988; 1994).

In addition to differences in the source of phytate P, some factors such as Ca, Mg, Zn, inorganic P, vitamin D, pH, protein, citric acid, moisture, EDTA and ionic strength (the concentration of NaCl) in diets or digestive tracts influence the availability of phytate P. Increasing Ca, Mg, or Zn levels *in vitro* reduced the solubility of phytate and stabilized the metal-phytate complex in which the digestibility of phytate P was low (DeRham and Jost, 1979; Cheryan, 1980; Prattley and Stanley, 1982; Wise, 1983). The pH was found to be the most important factor influencing the solubility of phytate and its complex, thus in influencing the *in vitro* digestibility of phytate P (Okubo, 1975; Cheryan, 1980; Honig and Wolf, 1991). The digestibility of phytate P is greater in high moisture corn than in dry corn (Cornelius and Harmon, 1974; Abrams et al., 1965).

In pigs and poultry, dietary Ca had a strong adverse effect on the availability of phytate P, while vitamin D had a positive effect (Mohammed et al., 1991; Edward, 1992; Lei et al., 1994). The dietary level of Ca or the Ca:P ratio is an important factor determining the extent of phytate hydrolysis because an insoluble Ca-phytate complex is

easily formed in the digestive tracts of animals (Nelson, 1967). Harms et al. (1962) reported that widening the Ca:tP ratio in broilers from 1:1 to 2:1 lowered the availability of the P in phytic acid to a greater extent than in inorganic P supplements. For pigs fed a diet containing high levels of phytate, a wide Ca:tP ratio lowers P absorption, which results in decreased growth and bone calcification (Koch et al., 1984; Reinhart and Mahan, 1986; Pointillart et al., 1989). Detrimental effects of wide Ca:tP ratios on performance, bone characteristics and serum criteria were generally observed when the Ca:tP ratio exceeded 2.0:1; however, no significant effects were observed when the Ca:tP ratio was under 2.0:1, especially at the range of 1.0 to 1.6:1 (Koch et al., 1984; Reinhart and Mahan, 1986; Pointillart et al., 1989; Ketaren et al. 1993ab).

The active form of D_3, 1, 25-dihydroxycholecalciferol [**1,25-$(OH)_2D_3$**], has been shown to increase the utilization of phytate P (Shafey et al., 1990; Edwards, 1993). This improvement in the utilization of phytate P by vitamin D might result from an increase in the phytase activity in the small intestines of chicks (Pointillart et al., 1985). Recently, the addition of vitamin D_3 or its derivatives at very high levels has been recommended to increase phytate P utilization (Shafey et al., 1990; Mohammed et al., 1991). Mohammed et al. (1991) reported that the addition of D_3 at 1,250 μg/kg diet increased the availability of phytate Ca and P for the chick when the diet contained normal amounts of iP (.45%), Ca (1.0%) and D_3 (12.5 μg/kg diet). An addition of 1,250 μg of D_3/kg of diet further improved the availability of phytate-P and -Ca while simultaneously lowing dietary Ca contents.

Some chemical methods such as alkaline treatment have been tried for removing the phytate from plants in order to improve the availability of minerals and protein from feedstuffs, but high cost makes them impractical for the feed industry. At present, most efforts are focused on enzymatic methods that utilize phytase from plant and microorganism origins to enhance the availability of phytate P, protein, and minerals of

feedstuffs.

Considerations in Phytate P Utilization

Phytate in some feeds can reduce not only the availability of P but also the availability of protein and minerals for swine and poultry. The amounts of aP abtained from feeds of plant origins are usually insufficient to obtain good performance of pigs and poultry (Jongbloed, 1987). Therefore, it is necessary to supply inorganic phosphorus (**iP**) to meet the P requirement of pigs and chicks (NRC, 1988; 1994). This increases feed costs for pigs and poultry because P additives are quite costly and rank third next to corn and soybean meals as the major cost for swine and poultry diets (NAS, 1974).

More importantly, a large excretion of P can lead to environmental problems because most of phytate P cannot be utilized by swine and poultry and is excreted through manure (Gerritse and Zugec, 1977; Cromwell and Coffey, 1991). Phosphorus together with N from animal manure exceeds the amount needed for plant growth, and progressively accumulates in the soil (Lenis, 1989; Cromwell, 1991; Kornegay, 1995). Along with N, K and trace minerals, P is thought as a major nutrient that limits land application of manure in most intensive swine and poultry producing areas in the United States (Kornegay, 1995) (Table 3). Excess land application of P and N results in a potential contamination of fresh water resources such as streams, rivers, and lakes, which can stimulate the growth of algae and other aquatic plants in surface waters (Cromwell and Coffey, 1991). This process, also called 'eutrophication', creates a marked deterioration in the quality of fresh water by diminishing the oxygen content in the water and thereby creating an undesirable environment for fish and other wildlife (Cromwell, 1991; Swick and Ivey, 1992). More than one million tons of P are excreted annually by farm animals in the United States. Of the P excreted, approximately 200,000 and 120,000 tons of P come from swine and poultry manure, respectively. That is to say, almost one-third of total P excreted from animal waste is attributed to the swine and poultry

Table 3. Average mineral concentration of swine feed and manure (dry matter basis)[a]

Element[b]	Feed	Manure
	% of DM	
N	2.7	4.5
P	.62	2.3
Ca	.94	4.1
Na	.16	.46
K	.83	1.7
Mg	.17	.82
	mg/kg of DM	
Cu	257	1329
Zn	93	465
Fe	184	1194
Mn	55	313
B	10	21

[a]Data adapted from Kornegay (1995).

[b]Each value is the average of triplicate analyses for two samples taken annually at the same time of total fourteen years.

(Gilbertson et al., 1984; Cromwell, 1991). At present, the environmental pollution resulting from manure P and N is of great concern in the Netherlands, Belgium, Denmark, France, and in some states in the USA (Lenis, 1989; Cromwell and Coffey, 1991). Some countries including Great Britain, the Netherlands, Korea, Singapore, and some states of the United States have set some strict criteria, or even have considered applying some legislation to regulate the amount and method of waste disposal (Brown, 1992; Schwarz and Hoppe, 1992).

Phytase

Function, Occurrence and Characterization of Phytase

Phytase, *myo*-inositol hexaphosphate phosphohydrolase, consists of a family of

enzymes catalyzing the stepwise removal of inorganic orthophosphate from phytate (Ullah, 1988; Jongbloed and Kemme, 1990) (Figure 4) and some other natural and synthetic phosphorylated substrates (Gibson and Ullah, 1990).

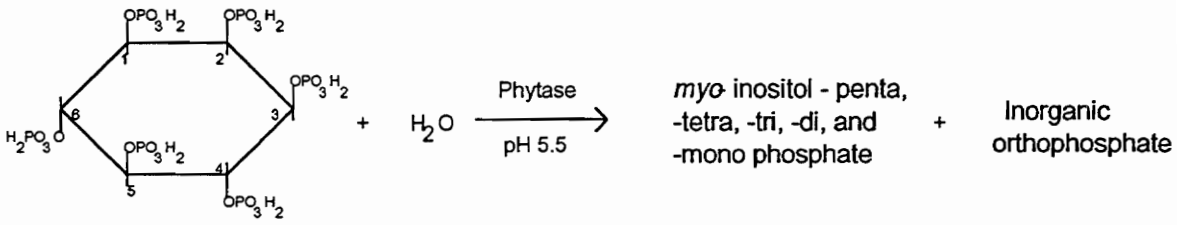

Figure 4. A step-wise removal of inorganic orthophosphate from *myo*-inosital-hexaphosphate by phytase (Gibson and Ullah, 1990)

Phytase is widely distributed in plant and animal tissues, and in numerous types of microorganisms (Bitar and Reinhold, 1972; Zhu et al., 1990; Beers and Jongbloed, 1993). Compared with nonspecific phosphatases, phytase may belong to special kinds of phosphatase that comprises two classes (Gibson and Ullah, 1990). One class of phytase is named as 3-phytase (E,C,3,1,3,8), and removes the phosphate attached to the 1- or 3-position of *myo*-inositol, whereas, the other class acts at the 6-position and is called 6-phytase (E,C,3,1,3,26). Both enzymes have the same effect in hydrolyzing phytate into inositol and inorganic phosphates. In addition to its dephosphorylation ability, phytase can improve the digestibility and utilization of Ca (Mroz et al., 1991; Schoner et al., 1994), Zn (Lei et al., 1993c), DM (Jongbloed, 1990; Kornegay et al., 1995), protein (Zyla et al., 1989; Mroz et al., 1991c; Nair et al., 1991), amino acids (Yi et al., 1995a), and organic matter (Mroz et al., 1991c) in *in vitro* and *in vivo* studies.

Phytase is widely distributed in feedstuffs such as wheat, barley and rye (Maga,

1982; Reddy et al., 1982; Beers and Jongbloed, 1993), animal tissues such as the intestinal tract, blood and liver (Bitar and Reinhold, 1972), and various microorganisms such as fungi, yeast, bacteria, and microbes in the rumen (Bitar and Reinhold, 1972; Cosgrove, 1980). Wheat, barley, rye and triticale are relatively rich in phytase, and contain a sufficient amount of phytase to release most of the orthophosphate (Table 2). These phytase-rich ingredients often are used as the sources of supplemental dietary phytase in animal diets (Zhu et al., 1990). In contrast, no more than 12% of the phytate P is released by phytase in corn and canola because they have very limited phytase activity (Ranhotra and Loewe, 1975; Pointillart et al., 1987; Pointillart, 1991).

In animal tissues, the characteristics of phytase activity are very similar to those of alkaline phosphatase (Reinhold et al., 1970; Bitar and Reinhold, 1972; Davies and Flett, 1978). Phytase degradation takes place mainly in the stomach and proximal end of the small intestine, while most of the small intestine and all of the large intestine have no phytase activity (Jongbloed et al., 1990; Jongbloed, 1991). Although phytase activity is found in the intestinal mucosa of rat, chicken, calf, and man (Bitar and Reinhold, 1972), the effectiveness of this phytase has not been demonstrated clearly, and it is generally considered that the activity, if present, is negligible in pigs and poultry (Pointillart et al., 1984). The phytase from microorganisms is of great interest at present, particularly the enzyme from the fungi of the *Aspergillus* species, such as *A. ficuum* and *A. niger*, because they are able to produce the greatest amount of microbial phytase (Ullah, 1988a; Zyla and Koreleska, 1993). Phytase from this fungi is commercially available, and is economically feasible in some situations.

Phytase from plant sources such as soybean, corn, wheat, and rapeseed and from small intestine mucosa of chicks, rat, calf and man has been extensively investigated and characterized (Bitar and Reinhold, 1972; Gibson and Ullah, 1988; Yang et al., 1991b). Recently, microbial phytase from *A. ficuum* has also been partially characterized by Han

(1988) and Ullah (1988ab).

From published data, kinetic properties of phytase are apparently influenced by various sources of the enzyme. The purified phytase from soybean seeds exhibited two subunits with molecular weights (**MW**) of 59 and 60 KDa and a K_m of 48 μM for phytate (Gibson and Ullah, 1988). The K_m of phytase purified from rat intestinal mucosa was 210 μM (Yang et al., 1991a). Bitar and Reinhold (1972) extracted the mucosal phytase from rat, chicken, calf and man and found that phytases from the four species were markedly different from each other in the activity profiles and kinetic parameters. Microbial phytase of *A. ficuum* was reported to be a glycoprotein with a MW of 85 KDa and a K_m of 40 μM (Ullah, 1988a).

Although the optimal pH, temperature and cation concentrations for the enzyme action are apparently variable, and differ with phytase sources, phytase from any source seems to have a high affinity for the phytate, and have high concentrations of glutamic and aspartic acid residues (Ullah, 1988a; Gibson and Ullah, 1988). The published data on kinetic parameters for phytase were obtained by various laboratory methods which may contribute to variation among the reported results. Phytase activity has been determined mostly by using a 'one-time stop' assay, in which incubation of phytate with phytase was stopped by a 'stop-color' reagent at an assigned time period and the inorganic orthophosphate released was then photometerically determined at 415 nm. Molybdovanadate often is used as color reagent (Simons et al., 1990; Engelen et al., 1994). One unit (**U**) of phytase activity is defined as nmole of released iP per sec. or as μmole of released iP per min. under the designed test condition (Ullah, 1988ab; Engelen et al., 1994).

Effectiveness of Phytase

In Vitro

Because of recent concern about environmental pollution by P and N, much effort

is being focused on *in vitro* or *in vivo* effectiveness of microbial phytase, since commercial phytase additives are available. Results of an *in vitro* study (Han and Wilfred, 1988) showed that at 37°C and at pH 3.5 to 5.7, about 50% of soybean phytate and 30% of cottonseed phytate were hydrolyzed by the *A. ficcum* phytase after 12 h of incubation, and 85% of phytate in soybean meal and 70% in cottonseed meal were dephosphorylated at the end of a 24 h incubation. Later, Han (1989) reported that 63% of soybean phytate and 42% of cottonseed phytate were hydrolyzed at 37°C and at pH 5.4 during a 5 h incubation (the residence time of feed in the chicken's digestive tract), which suggests that one U of the *A. ficuum* phytase was able to release about 2.4 mg of P (63% of the 0.38% P in soybean meal) per g of soybean meal within 5 h. In another *in vitro* study, Simons and coworkers (1990) demonstrated that at 40°C and pH 5.5, *A. ficuum* phytase hydrolyzed 85% of the P from soybean meal phytate and almost 100% of the P from phytate in corn during a 1 h incubation.

In addition, microbial phytase is effective in releasing Ca from phytate-bound Ca. The *in vitro* constituted *myo*-inositol tris-phosphate-phytase complex was found to be highly effective in releasing Ca from plant (Samanta et al., 1993). This complex is a result of *myo*-inositol tris-phosphate, one of the products of phytate hydrolyzed by phytase, transitionally bound to phytase.

In Poultry

Nelson et al. (1968b) were the first to show that an *A. ficuum* phytase preparation improved the utilization of phytate P and bone mineralization when it was added to broiler diets. Recently, many researchers have shown that dietary supplementation of microbial phytase to broiler and laying hen diets improved the availability of phytate P and Ca (Simons et al., 1990; Swick and Ivey, 1990; Schwarz, 1992; Simons et al., 1992;). In 1990, Simons and coworkers conducted two broiler studies in which the phytase from *A. ficuum* was added to the corn-soybean-sorghum-sunflower diet. Results indicated that the

retention of P and Ca, growth rate, and feed conversion ratio were improved significantly by adding different levels of microbial phytase. Also, the maximum release of P was reached when 800 U of phytase per kg of diet were added. The BW gain of chicks fed diets supplemented with 750 U of phytase per kg of diet was equal to that of birds fed diets containing 1 g of inorganic P (a mixture of monoammonium and dicalcium phosphate). In another report, Simons et al. (1992) pointed out that 250 U of phytase was equivalent to .5 g monocalcium phosphate P (MCP-P) per kg of feed for broilers using P absorption as the measurement; in laying hens, 250 U of phytase per kg of feed resulted in an equivalency of .7 g MCP-P/kg feed. In agreement, Schwarz (1992) observed that the availability of P was increased by 20 to 60% and the excretion of P was decreased by 30 to 50% when phytase was added to a low P broiler diet. Saylor (1991) reported that more than 1,000 U of phytase/kg diet must be supplemented to a corn soybean meal diet fed to broilers to hydrolyze all of the phytate.

Kornegay et al. (1995) showed that microbial phytase was also effective for improving Ca and DM utilization when added to broilers diets. Schoner et al. (1993, 1994) reported that improved Ca retention of broilers was obtained when phytase was added to corn-soybean meal diets. In a broiler study designed to measure the effect of phytase on Ca availability, Schoner et al. (1994) reported that 500 U of microbial phytase was equivalent to .35 g Ca as measured by BW gain and .56 g Ca as measured by phalanx ash. In a turkey study, within a Ca range of 6 to 9.6 g/kg and a phytase level up to 500 U/kg diet, Kornegay and Denbow (1995) estimated, based on BW gain, that 500 U of phytase was equivalent to about 2 g of Ca. Within a Ca range of 6 to 12 g/kg and a phytase range of 0 to 625 U, toe ash as a percentage of dried toes was not influenced by dietary treatments; all toe ash values appeared normal (11 to 12%). Body weight gain appeared to be depressed above 500 U of phytase and above 9.6 g of Ca/kg.

Supplemental microbial phytase also is effective for improving the availability of

Zn in a corn-soybean meal diet. Thiel and Weigand (1992) reported that the addition of phytase (800 U/kg diet) to a diet containing 27 mg of Zn/kg of diet increased the retention of Zn and decreased Zn excretion. Thiel et al. (1993) reported that the femur Zn content of chicks fed a diet containing 30 mg of Zn/kg plus 700 U of phytase/kg of diet was equal to that of chicks fed a diet containing 39 mg of Zn/kg without added Zn. Biehl et al. (1995) fed chicks a glucose-soy concentrate diet containing 13 mg of Zn/kg diet with 0, 5 and 10 mg of added Zn/kg diet and with 1,200 U of phytase or 10 mg of 1, 25-$(OH)_2D_3$/kg for 12 d. The addition of 10 mg of Zn increased BW gain by 40% and total tibia Zn by 108%. Phytase or 1, 25-$(OH)_2D_3$ supplementation increased BW gain and tibia Zn to a similar extent. The combination of phytase and 1, 25-$(OH)_2D_3$ increased BW gain by 43% and tibia Zn by 159%. Supplementation of phytase increased Zn as well as Ca, P, and Mg tibial retention, which resulted in an improvement of tibial histological structure and bone density of chicks and turkey poults (Qian et al., 1994bc).

The effect of phytase on protein digestibility was observed for turkey poults fed low P and low protein levels in a corn soybean meal diet with 750 U of phytase added (Yi et al., 1995a): phytase improved apparent and true ileal digestibility of most essential amino acids by about two percentage units. Improving protein digestibility may cause the enhancement of the metabolic energy (ME) value of the cottonseed meal for chicks by the supplementation of phytase (Rojas and Scott, 1969; Miles and Nelson, 1974).

In Pigs

Microbial phytase is effective for improving phytate P utilization of diets fed to pigs. Studies using pigs fed corn soybean meal diets supplemented with microbial phytase from *A. ficuum* have reported similar conclusions to those reported for poultry (Simons et al., 1990; Nasi, 1990; Cromwell, 1991; Beers and Jongbloed, 1993): 1) 800 to 1,000 U of phytase/kg diet resulted in a significant increase in the apparent digestibility of P ranging from 30 to 60%, an increase in the retention of P by about 40 to 50%, and a decrease in

the excretion of P by 35 to 50%; 2) supplementation with 500 U of phytase was equivalent with .7 to .8 g of P from MCP (ranged between .5 to 1.0 g); 3) supplementation with 1,000 to 1,200 U of phytase/kg diet resulted in a maximum release of the phytate P, almost meeting the P requirement for weaning pigs; 4) phytase supplemented in the diets for pigs improved ADG, ADFI, feed conversion ratio (**FCR**) and bone ash content; and 5) degradation of phytate by phytase mainly took place in the stomach and the proximal end of the small intestine (duodenum) and there was no phytase activity at the end of the small intestine (ileum) of the pig.

Phytase effectively increased the utilization of Ca, and decreased the excretion of Ca for pigs fed a corn soybean meal diet (Simons et al., 1990; Nasi, 1990; Lei et al., 1993ab). Radcliffe et al. (1995) suggested that 500 U of phytase was equivalent to 1.1 and .65 g of Ca, respectively, based on ADG and digested Ca. Mroz et al. (1993) reported enhanced Ca and P digestibility of pigs when 300 and 600 U of phytase was added to a diet containing a suboptimal level of Ca (.43 %) and tP (.43 %). They also observed that hypophosphaturia and hypercalcuria appeared in pigs fed a basal diet, although ADG and FCR were similar among treatments; adding either .5 g P from KH_2PO_4 or 300 U of phytase were equally effective in preventing a P deficiency. Pointillart (1993) indicated, primarily with cereal phytase, that improved P utilization was generally accompanied by improved Ca retention.

The effects of supplemental microbial phytase on the utilization of DM, OM, protein, amino acids, and minerals such as Zn, Fe, Cu and Mn by pigs are not as clear as with Ca and P, and some results are contradictory. Research is limited in this area. Although no effects of phytase supplementation were reported for the digestibility of DM for pigs fed corn-soybean meal diets (Simons et al., 1990; Beers and Jongbloed, 1992), Mroz et al. (1991a), Jongbloed et al. (1992), and Simons et al. (1992) showed DM digestibility was improved when phytase was added to the diet. Based on the research

reported by Mroz et al. (1991a), OM digestibility was increased by supplementary phytase; but, Nasi (1990) reported that OM digestibility was not significantly different between control and a phytase supplemented group.

In the study of proteins and amino acids, some researchers (Mroz et al., 1991ab; Simons et al., 1992) agreed that the addition of phytase to diets for pigs enhanced the apparent digestibility of protein and some amino acids. Mroz et al. (1991b) explained that the phytate-protein complex could be degraded by phytase. Ketaren et al. (1993b) found that the addition of phytase increased protein deposition and retention, but had no effect on protein digestibility. Mroz et al., (1994) reported that supplemental microbial phytase improved the apparent digestibility of protein and amino acids in pigs. Kemme et al. (1995) observed in pigs that the addition of phytase at a level of 900 U/kg to a diet with no extrinsic phytase exerted a positive effect on the apparent ileal digestibility of protein as well as lysine, tryptophan, isoleucine and threonine. In growing pigs, Mroz et al. (1991b) found that phytase significantly increased the digestibility of amino acids such as methionine, cysteine, arginine, isoleucine, and phenylalanine. However, the study of Nasi (1990) indicated that supplementation of phytase did not cause any improvement in protein digestibility.

Microbial phytase (*A. niger*) significantly improved the absorption of Zn, Mg, Fe, and Cu, but not Mn in piglets (Pallauf et al., 1992). Based on enhanced growth, increased plasma Zn concentration and alkaline phosphatase activity, the bioavailability of Zn for pigs was improved when phytase (1,350 U/kg) was added to a low P and Zn corn soybean meal diet with 0, 30 or 60 mg of added Zn/kg (Lei et al., 1993c); however, neither supplemental phytase nor Zn affected Zn retention. In another study, Lei et al. (1993b) reported that phytase (1,350 U/kg) from *A. niger* improved Zn bioavailability in a corn soybean meal diet containing 30 mg of Zn per kg of diet, but there was no effect of phytase on the concentrations of plasma Mg, Cu, Fe, and Zn. Adding microbial phytase to

the diets of young pigs significantly improved apparent absorption of Zn and Mg (Pallauf et al., 1992; Nasi and Helander, 1994). Using a low Zn diet (23 mg/kg) containing 8 g of Ca/kg and 6.2 g of P/kg, Adeola et al. (1995) reported that ADG, and the retention of Zn, Cu, Ca and P of pigs were increased when 1,500 U of phytase/kg of diet was fed. However, Nasi (1990) found that growing pigs fed supplemental phytase (1,200 U/kg) from *A. niger* did not show any improvement in the digestibility of Fe, Cu, and Zn for a corn soybean meal diet.

Factors Influencing Phytase Activity

In Vitro

To date, studies evaluating factors that might influence the activity of phytase are limited, but some factors such as Ca, P, Zn, Mg, vitamin D, pH, moisture and temperature have a potential to affect the activity of phytase. Of these factors, pH is most critical to the activity of microbial phytase; the optimum values are at pH 2.5 and 5.5 (Simons et al., 1990). Temperature is another important factor that can directly affect the activity of the enzyme. Results of an *in vitro* study indicated that hydrolysis of phytate from soybean meal by microbial phytase increased as the temperature increased from 28 to 55°C (Han, 1989).

Bitar and Reinhold (1972) first found that the addition of Zn^{2+} to phytase from the intestinal mucosa of rat caused the phytase activity to increase by about 40% as the Zn^{2+} concentration increased from .02 to .08 mM. However, they observed that at Zn^{2+} concentrations exceeding .625 mM, the activity of phytase from the intestinal mucosa of rat decreased because of formation of a zinc-phytate precipitate. They did not observe similar results for phytase from the intestinal mucosa of chicken, calf and man; and no effects of Ca^{2+}, Cu^{2+} and Mn^{2+} on phytase activity were observed for the intestinal phytase for any of the species examined. In contrast, soybean phytase activity increased from 30 to 80% as Ca^{2+}, Mg^{2+}, Mn^{2+} and Fe^{3+} concentrations were increased from .2 to

1.0 mM, whereas, the activity of phytase decreased by 25% as the Zn^{2+} concentration was increased from .2 to 1.0 mM (Gibson and Ullah, 1988). However, the activity of phytase was inhibited as the concentrations of these five cations were increased to 5.0 mM in the *in vitro* phytase assay system. Davies and Flett (1978) showed that the activity of phytase and alkaline phosphatase from the intestinal mucosa of rats required both Zn^{2+} and Mg^{2+}, and that the concentrations, especially of Zn^{2+}, were critical for maximal activity. Although the evidence for a correlation between the activity of the intestinal phytase and dietary Zn^{2+} for rats is less conclusive, the correlation is significant, i.e., the phytate-splitting activity of phytase is Zn^{2+}-dependent (Reinhold et al., 1970). Ullah (1988a) also observed the inhibitory effect of Cu^{2+}, Zn^{2+}, Fe^{3+} and Cu^{+} and the stimulatory effect of Co^{2+} and Mn^{2+} on *A. ficuum* phytase *in vitro*; furthermore, Cu^{2+} and Zn^{2+} were shown to be non-competitive inhibitors and Fe^{3+} and Cu^{+} to be competitive inhibitors. However, Ullah (1988a) did not observe the Ca^{2+} and Fe^{2+} effect. In another *in vitro* phytase assay system, Zn^{2+} was shown to be a strong inhibitor of intestinal phytase activity of rats; but no effects on the phytase activity were observed with Ca^{2+}, Mn^{2+} and monovalent cations such as Li^{+}, Na^{+}, K^{+} and NH_4^{+} (Yang et al., 1991b). In addition, the concentration of phytate, the substrate, is critical for measuring phytase activity. Phytate concentration in excess of 1.25 mM tended to possess an inhibitory effect; at phytate concentrations of more than 4 mM the phytase activity was totally inhibited (Ullah 1988b).

In Vivo

It is well known that P utilization by animals is influenced by dietary Ca and vitamin D. An excess of dietary Ca will interfere with the utilization of P as well as Mg, Mn and Zn (NRC, 1988; 1994). Furthermore, the effectiveness of phytase in pig and poultry diets also has been shown to be influenced by dietary Ca and P levels (Schoner et al., 1993; Lei et al., 1994). Simons and coworkers (1990) reported that high levels of Ca had a strong adverse effect on the efficacy of phytase by making phytate less available for

degradation by phytase. Wise (1983), however, thought that only excessive dietary Ca could directly reduce the activity of microbial phytase. Supplementation of phytase is thought to be effective only in a low P diet for pigs, and that maximum efficacy is achieved when diets contain no supplemental P. Also, the efficacy of phytase decreases as the inorganic P in diets is increased (Cromwell et al., 1992). In disagreement, Nelson and Kirby (1992) observed that inorganic P seemed to have a potential for improving the hydrolysis of phytate P. The key to this controversy may be due to dietary Ca:P ratio that seems more important than the absolute amount of Ca and P in pig and poultry diets supplemented with phytase. Using a 14-d and a 40-d broiler study, Schoner et al. (1993) reported that high levels of Ca with a constant level of P (3.5 g/kg) reduced increases in BW gain, feed intake and P and Ca retention that were observed when phytase was added. From their lowest (6 g/kg) to highest (9 g/kg) levels of Ca, the Ca:tP ratio varied from 1.70:1 to 2.57:1. Jongbloed et al. (1993) investigated three levels of dietary Ca (4, 6 and 8 g/kg) using a basal diet (tapioca, corn, hominy feed, barley, soybean meal and sunflower meal) containing 4.3 g of P/kg but without added iP. Apparent Ca and P absorption generally decreased as the level of Ca increased, but increased as the level of phytase increased. The magnitude of the increase due to phytase was reduced as dietary Ca increased.

The decrease in the efficacy of phytase as the Ca:tP ratio became wider could be due to: 1) a wide Ca:P ratio that may reduce growth and bone calcification by lowering P absorption, especially when the diet is marginal in P (NRC, 1988; 1994); 2) the high level of Ca that may decrease the phytate P availability by forming a complex with phytic acid which is less available for degradation by phytase (Wise, 1983; Fisher, 1992); and 3) the high level of Ca that may directly reduce the activity of phytase and thus limit the efficacy of phytase (Bhandari, 1980; Jongbloed et al., 1993; Lei et al., 1994).

Lei et al. (1994) demonstrated that a high concentration of the dietary vitamin D

improved the utilization of phytate P for pigs and offset the adverse effect of the dietary Ca on the activity of phytase. They reported that the ability of phytase to improve phytate P availability was greatly reduced at a normal level (9.2 g/kg) of dietary Ca (Ca:tP ratio was 3:1) when pigs were fed a corn soybean meal diet with no added iP (3.1 g/kg tP). Raising the level of D_3 in the diet (16.5 vs 166.5 μg/kg) partially offset this adverse effect but did not produce further improvement when the Ca level was lower. Edwards (1993) conducted two experiments in which the phytate P utilization for chicks was greatly enhanced by the addition of 5 to 10 μg of 1,25-$(OH)_2D_3$/kg diet in the presence or absence of supplemental phytase. Addition of 1,25-$(OH)_2D_3$ apparently improved the efficacy of phytase , which resulted in an improvement in BW gain, feed intake, toe ash content, and P and Ca retention of birds.

Very few studies have investigated the influence of dietary Zn on phytase efficacy for pigs and poultry. Lei et al. (1993c) reported that there was a significant interaction between supplemental phytase and dietary Zn; the highest level of phytase efficacy was achieved as weanling pigs were fed diets containing the proper level of dietary Zn. They fed diets containing 0, 30 and 60 mg of added Zn/kg diet combined with 1,350 U of phytase/kg diet. Maximum responses for performance and serum measurements were achieved at 30 mg of added Zn/kg diet. This study suggests that a high dietary Zn level does not possess a positive effect for improving phytase efficacy.

Considerations in Application of Phytase

Supplementation of microbial phytase in pig and poultry diets is the main approach for improving phytate P availability and decreasing P excretion of animals and poultry in the near future. Although the use of microbial phytase has recently been studied, the efficacy of phytase for pigs and poultry is not completely understood, especially, the effect of dietary Ca:tP ratios on the phytase efficacy. The optimum Ca:tP ratios have to be determined for pigs and poultry because this dietary factor has a great influence on the

phytase activity, phytate P utilization and P metabolism. The application of phytase in animal husbandry is determined by the cost, product stability, consistency of results, and ease of application (Swick and Ivey, 1992). Although current advances in biotechnology have produced a highly active enzyme additive, the microbial product cannot be widely used in animal husbandry due to its relatively high cost. Based on the estimation made by Kornegay (1995), the cost for microbial phytase is 1.8 to 2.3 times higher than that for feed grade phosphate additives. Thus, there are two ways to get over the obstacle. One way is to reduce the cost for producing the enzyme by means of advanced biotechnology. The other way is to improve the efficacy of application of phytase by nutritional technology, i.e., by limiting the actions of inhibitors and increasing the actions of activators.

CHAPTER III

Replacement of Inorganic Phosphorus by Microbial Phytase for Young Pigs fed a Corn Soybean Meal Diet

ABSTRACT Ninety six young crossbred pigs (average initial age = 30 d, BW = 7.8 kg) were used in a 5-wk trial to determine the effectiveness of microbial phytase in improving the bioavailabilities of P and other nutrients in corn soybean meal diets. A 2 x 5 factorial arrangement of treatments was employed with two available P (**aP**) levels (.07 and .16%) and five phytase levels [0, 350, 700, 1,050, 1,400 unit (**U**)/kg of diet]. In addition, two extra diets were formulated to supply the NRC recommended level of aP (.32%) with 0 or 1,400 U of phytase. The addition of graded levels of phytase resulted in linear increases in ADG, ADFI and gain:feed for pigs fed diets containing .07 and .16% aP ($P < .04$). Also, the addition of phytase linearly increased apparent digestibilities of P, Ca and N ($P < .01$, .01 and .06, respectively); whereas, fecal P excretion was linearly decreased ($P < .01$). Linear increases in shear force, shear energy and ash content of both metacarpal and tenth rib, and shear stress of metacarpal were found to respond to added phytase ($P < .01$). These improvements in performance, apparent P absorption and bone measurements by phytase also were observed by increasing dietary aP levels. Adding 1,400 U of phytase to the .32% aP diet further increased ADG, ADFI, apparent absorption of P, Ca and N, and metatarsal shear force and ash content ($P < .01$ to .07). Generally, maximum responses occurred at a phytase level of 1,050 U/kg diet for the .07% aP diets, and 700 U for the .16% aP diets. Based on nonlinear and linear response equations generated for the phytase and aP levels, the average function of the equivalency of P (Y, %) by microbial phytase (X, U/kg) was developed across aP levels of .07 and .16%: $Y = .2622 - .2559e^{-.00185X}$. The replacement of 1 g of inorganic P as defluorinated phosphate would require about 246 U of microbial phytase. This represents 41% of

phytate P hydrolyzed from dietary plant sources.

Key Words: Piglets, Phosphorus, Phytase, Corn-Soybean Meal, Bioavailability

Introduction

Interest in the addition of phytase to pig and poultry diets has occurred because of a general need to reduce the amount of fecal P excretion. This is important due to the potential for environmental pollution, particularly in areas where large numbers of pigs and poultry are produced. Phosphorus is a key element in swine nutrition, and adequate amounts in diets are necessary for optimal growth, reproduction and bone development of animals (NRC, 1988). However, much of the P in swine diets (composed primarily of corn and soybean meal in the United States) cannot be utilized because it occurs bound as phytate P (Cromwell, 1992). The poor availability of this bound P leads to large amounts of P present in pig manure (Cromwell and Coffey, 1991) because inorganic P must be added to meet the P requirement. Microbial phytase supplementation has been shown to be effective in releasing a significant portion of the bound P and thus, improves the availability of P (Simons et al., 1990; Cromwell et al., 1993b; Hoppe et al., 1993). Phosphorus excretion can be reduced, with estimates ranging from 25 to 50%, by the addition of phytase to the diet (Simons et al., 1990; Jongbloed et al., 1992; Cromwell et al., 1993a; Lei et al., 1993ab). An estimated equivalency value of phytase for inorganic P was reported by Hoppe et al. (1993), but only a single level of P was fed. The objective of this study was to determine the effectiveness of Natuphos® phytase for increasing the absorption of Ca, P and N, increasing bone mineralization, and improving performance of young pigs fed a corn-soybean meal basal diet with varying levels of phytase in combination with two deficient levels of available P. Response equations were generated and evaluated; the most sensitive indicators were used to calculate equivalency values of phytase for inorganic P.

Materials and Methods

Animals and feeding management. A total of 96 crossbred pigs (equal number of males and females) were utilized. The pigs were weaned between 28 and 35 d of age and given a 7-d adjustment before diet treatments were started. During the adjustment, they were fed a pre-starter formula containing 22.0% crude protein (**CP**) for 4 d (Maximum Wean 10-15, Southern States Cooperative, Richmond, VA) and then fed a 20.0% CP corn-soybean meal diet containing 10.0% dried whey for the remaining 3 d. After the adjustment period, pigs were weighed (average weight, 7.8 ± .13 kg) and randomly placed on treatments from outcome groups based on gender and weight. Littermates were balanced across treatments as far as possible.

The pigs were housed two per pen (.6 m x .9 m) in environmentally controlled rooms with expanded metal floors. Each pen had a plastic-coated welded wire floor and was equipped with a nipple waterer and a stainless steel feeder. Room termperatures were initially set at 29°C and were lowered about 2°C per week after the second week. A continuous lighting regime and recommended air ventilation rates were maintained. Feed and water were available *ad libitum* at all times. Pigs were weighed individually at weekly intervals during the 5-wk study period. Pen feed intakes were recorded. The care and treatment of pigs followed published guidelines (Consortium, 1988).

Treatments and diets. A 2 x 5 factorial arrangement of treatments was employed to evaluate the response of weanling pigs to graded levels of phytase added to diets containing two levels of available phosphorus (**aP**) that were below the pig estimated requirement (NRC, 1988). Dietary aP levels were formulated at .07 and .16% aP, or .36 and .45% total P (**tP**), respectively, and each level of aP was supplemented with 0, 350, 700, 1,050 and 1,400 unit (**U**) of phytase/kg of diet. A U is defined as the quantity of enzyme that liberates 1 μmole of inorganic P per min from 1.5 mM sodium phytate at pH 5.5 and 37°C. These P levels were formulated below the current NRC (1988)

recommendations to ensure maximum response to phytase additions. In addition to the ten diets described above, two additional diets were formulated to supply the recommended level of P (.32% aP or .61% tP); one diet was fed without phytase and one was fed with 1,400 U of phytase/kg of diet. The diet without the addition of phytase served as the positive control. Each of the 12 dietary treatments were fed to four replicate pens of two pigs (one barrow and one female) each.

The basal diets were based on corn and soybean meal as the protein sources (Table 1). Corn and soybean meal supplied all the P (.07% aP or .36% tP) contained in the lowest P basal diet. The desired levels of aP in the other basal diets were achieved by the addition of defluorinated phosphate (CDP®, Consolidated Minerals Inc., Feed Supplement Division, Plant City, FL). The Ca:tP ratio was maintained at 2:1 in all basal diets. Defluorinated phosphate and limestone were added to the diets at the expense of corn starch. Since phytate was supplied from corn and soybean meal, the dietary content of phytate P (.244%) was similar in all diets. A chromic oxide mixture was included at a level of .4% in diets as an indigestible indicator for digestiblity measurements. To obtain a homogenous distribution of the indicator in the diet, chromic oxide was first mixed in a small mixer with corn starch at a ratio of 1:3 (w/w) and then ground in a laboratory mill to pass through 1 mm-sieve (Dellaert et al., 1990).

Sampling and analysis. Grab fecal samples of approximately the same amount were collected from each pen during the 4th and 5th wk. During each of these weeks, feces were collected twice daily (morning and evening) on three alternate days. Collections from each of the 3 d within a wk were pooled and frozen at -20°C in airtight plastic bags for subsequent analysis. After thawing, fecal samples were dried in an oven at 70°C. The dried samples, along with representative samples of diets, were ground to pass through a 1 mm-sieve. Dry matter was determined according to standard AOAC (1990) methods. After a nitric-perchloric acid wet digestion of samples, tP concentrations were assayed

photometrically (AOAC, 1990), and Ca and Cr contents were determined with an atomic absorption spectrophotometer (Perkin Elmer 5100, Norwalk, CT). Nitrogen

Table 1. Percentage of composition and calculated analysis of the basal diets

Item	aP, %		
	.07[a]	.16[a]	.32[b]
Ingredients, %			
Ground yellow corn	72.62	72.07	72.45
Soybean meal (48.5% CP)	24.50	24.50	24.50
Limestone	1.68	1.73	.47
Defluorinated phosphate[c]	---	.50	1.38
Salt	.30	.30	.30
Vitamin premix[d]	.25	.25	.25
Trace mineral premix[e]	.10	.10	.10
Selenium premix[f]	.05	.05	.05
Chromic oxide - starch mixture	.40	.40	.40
Lysine	.10	.10	.10
Calculated analysis, %			
CP	18.30	18.25	18.29
Lys	1.05	1.05	1.05
Meth. & Cys.	.61	.61	.61
Ca	.72	.90	.70
tP	.36	.45	.61
aP	.07	.16	.32

[a]0, 350, 700, 1,050 and 1,400 FTU phytase per kilogram of diet were added. Phytase (Natuphos-5000 U/g) was supplied by BASF Corp., 3000 Continental Drive North, Mount Olive, NJ 07828-1234.

[b]0 and 1,400 FTU phytase per kilogram of diet were added.

[c]Fine CDP, Southern Bag Corp., Valdosta, GA 31803.

[d]Supplied per kilogram of diet: retinyl acetate, 1514 µg; cholecalciferol, 110 µg; dl-α-topherol acetate, 22 mg; riboflavin, 4.4 mg; niacin, 22 mg; choline, 440 mg; d-pantothenic acid, 22 mg; d-biotin, 0.44 mg; cyanocobalamin, 22 µg; vitamin K (as menadione dimethylprimidinol bisulfite), 2.2 mg.

[e]Supplied per kilogram of diet: Zn, 150 mg; Fe, 175 mg; Mn, 60 mg; Cu, 17.5 mg; I, 2 mg.

[f]Supplied 0.3 mg Se per kilogram of diet.

content was determined using the Kjeldahl method. The apparent absorption coefficients of Ca, P and N over the total tract were calculated (Dellaert et al., 1990).

At the end of wk 5, the barrow in each replicate pen (four per treatment) was slaughtered for collection of bone samples. The front foot and the 10th rib on the left side were removed and frozen in airtight plastic bags. The foot samples were later thawed, extraneous tissue was removed and the fourth metacarpal was retained. The rib samples also were defleshed. The width of the bones at the narrow and wide dimensions of the bone shaft were measured. Bones then were refrozen in airtight plastic bags until shear force determination as described by Combs et al. (1991).

The shear forces of the 4th metacarpal and the 10th rib were determined using an Instron Universal Testing Machine (Model 1123, Instron Corp., Canton, MA). Bones were thawed in airtight plastic bags immediately prior to testing to prevent desiccation. After the shear failure test, wall thickness was measured using dial calipers. Shear stress values were calculated according to the formula of Combs et al. (1991) for metacarpals and of Wilson (1991) for ribs. After the shear test, the bones were oven-dried at 100°C for 24 h and ashed in a muffle furnace at 600°C for 24 h. Bone ash was expressed as percentage of dry weight.

Statistical analysis and calculation of P-equivalency values. The data were analyzed by the GLM procedure of the SAS (1990). Performance and absorption data was analyzed using the pen as the experimental unit, whereas bone measurements were analyzed using individual pig values as the experimental unit. The digestibility of the DM was used as a covariate for the digestibility of Ca, P and N as suggested by Dellaert et al. (1990), but only very small differences were observed and unadjusted coefficients are reported. Linear and quadratic effects of supplemental phytase within each aP level, and within dietary aP levels without phytase were tested using orthogonal polynomials. A comparison between the positive control diet with and without phytase was made using

nonorthongonal contrasts. Second order translog functions were derived for the 2 x 5 factorial with the following model: $LnY=\alpha_0 + \alpha_1 D_1 + \alpha_2 LnX + \alpha_3 (LnX)^2 + \alpha_4 D_1 LnX$. Where, Y = response measurement such as ADG (g) or P absorption (%); X = phytase added (U/kg of diet); D_1= available P in the diet - when aP = .07%, D_1 = 0; when aP = .16%, D_1 = 1.

Nonlinear functions were derived using treatment means for phytase levels at each P level and linear functions for the three aP levels without added phytase. The nonlinear regression model used was $Y = a(1 - be^{-kX})$ and the linear regression model was Y = a + bX, where Y = response measurements; X = phytase levels added (U/kg of diet) or aP (%) levels of diet. The sensitivity of various measurements was determined by examining the r^2 values of the second order translog equations and the nonlinear and linear equations for all of the measurements. The functions generated from all measurements with the higher r^2 values for aP levels and phytase levels were used to generate P-equivalency equations of phytase. The equation for aP and the equation of added phytase at each of the two levels of aP were set equal. For example, the equation for ADG at .07% aP was as follows (Table 7):

$$212.4 + 345.0Y = 376.7 - 133.4e^{-0.00095X}$$

$$Y = .4762 - .3867e^{-0.00095X}$$

where, Y = available P (%); X = phytase added (U/kg of diet). The resulting equations were used to calculate the equivalent aP (%) at 250, 500, 750 and 1,000 U of phytase/kg of diet. Values for ADG and apparent digestibility of P then were used to determine the amount of P released and the released P was expressed as a percentage of phytate P. The amount of P released per 100 U of phytase also was calculated.

Results

Growth performance. Cumulative average daily gain, ADFI, and gain:feed (**G:F**) of young pigs linearly increased ($P < .01$, .01 and .08, respectively) as the level of aP increased (only diets without phytase) (Tables 2 and 3). The addition of graded levels of phytase resulted in linear increases of ADG ($P < .01$), ADFI ($P < .04$) and G:F ($P < .03$) for pigs fed diets containing .07 and .16% aP ($P < .04$). This linear increase in ADG reached a plateau at 1,050 U of phytase/kg of diet for the .07% aP diets, and at 700 U of phytase for the .16% aP diets. Average daily feed intake continued to increase to the highest level of phytase (1,400 U) for the .07% aP diets, but appeared to reach a plateau at 700 U of phytase for the .16% aP diets. Gain:feed appeared to reach a plateau at 700 U phytase for the .07% aP diets, and continued to increase linearly ($P < .03$) to 1,400 U phytase for .16% aP diets. The addition of 1,400 U phytase to the diet containing a NRC (1988) recommended level of .32% aP (.61% tP) increased ($P < .07$) ADG and ADFI, with no effect on G:F.

Apparent digestibility coefficients. For diets without added phytase, increasing the aP level linearly increased ($P < .01$) apparent digestibility of P, but linearly decreased the digestibility of DM and N ($P < .05$ and .01, respectively) (Tables 2 and 3). The effect of aP level on Ca absorption was not significant. The apparent absorption of P continued to increase to the highest level of phytase (1,400 U/kg) for pigs fed the .07% aP diets with both linear ($P < .01$) and quadratic ($P < .02$) effects. For pigs fed the .16% aP diets, P absorption reached a plateau at 1,050 U phytase with both linear ($P < .01$) and quadratic ($P < .01$) effects. Dry matter digestibility was only decreased ($P < .05$) by phytase addition for pigs fed .16% aP diets. The apparent absorption of Ca linearly increased ($P < .01$) as phytase was added, with the magnitude of the increase greater for the .07% aP diets than for the .16% aP diets ($P < .03$). A quadratic decrease ($P < .06$) in N digestibility was observed for pigs fed phytase addition at both aP levels. Adding 1,400 U

Table 2. Performance, apparent digestibility of DM, P, Ca and N, fecal P excretion of and bone characteristics of young weanling pigs fed a corn-soybean meal based diet containing varying levels of aP and supplemental phytase (PY)[a]

Diets	1	2	3	4	5	6	7	8	9	10	11	12	
aP, %			.07					.16			.32		
Added phytase (U/kg diet)	0	350	700	1,050	1,400	0	350	700	1,050	1,400	0	1,400	SEM
Measurements[b]													
Performance, 1-5 wk													
ADG, g	244	282	300	338	337	256	328	387	357	386	327	368	24.1
ADFI, g	528	569	621	645	675	554	628	706	633	621	596	658	51.6
G:F, g/kg	463	501	491	529	517	469	523	549	580	625	550	563	50.3
Apparent digestibility coefficients[c]													
DM, %	87.9	86.8	87.1	87.2	87.4	87.6	86.7	86.5	86.8	86.2	86.1	87.1	.43
P, %	27.6	40.2	42.1	47.7	52.5	35.3	47.6	53.9	56.2	54.4	57.2	68.3	2.35
Ca, %	72.1	77.3	77.5	79.6	81.0	72.9	74.8	78.1	76.9	77.8	74.6	76.8	1.68
N, %	85.4	83.2	83.4	83.4	83.9	83.4	82.8	81.8	83.1	83.4	81.0	83.0	.73
Fecal excretion of													
P, g/d	1.38	1.22	1.29	1.21	1.15	1.61	1.48	1.46	1.25	1.27	1.45	1.23	.08
Bone characteristics[d]													
Metacarpal													
Shear force	0.65	0.67	0.75	0.82	0.87	.71	.86	.89	.91	.97	.77	1.01	.06
Shear stress	1.15	1.10	1.17	1.30	1.42	1.15	1.27	1.31	1.45	1.43	1.30	1.45	.11
Shear energy	1.33	1.61	2.32	2.31	2.67	1.96	2.30	2.28	2.61	2.79	2.25	2.42	.21
Ash percent	29.5	31.9	32.9	32.8	33.8	30.7	33.7	35.4	34.2	37.7	33.2	36.1	.70
Tenth rib													
Shear force	.65	.74	.83	.75	.83	.67	.79	1.12	.85	1.09	.74	.85	.05
Shear stress	3.30	3.39	3.24	3.05	3.55	2.92	3.32	3.59	3.00	3.73	3.86	3.37	.37

Shear energy	.60	.68	.81	.78	.88	.65	.84	1.14	.85	1.19	.72	1.09	.06
Ash percent	37.0	44.2	44.3	45.7	46.2	40.7	45.9	49.7	48.0	48.3	48.0	48.2	.90

[a]Probability values are given in Table 3.
[b]Four pens (two pigs per pen) per treatment mean. Average initial weight, 7.8 kg.
[c]Eight pens (two pigs per pen for week 4 and 5) per treatment mean.
[d]Four pigs per treatment mean, units are N, N/cm^2 and N-mm, respectively for shear force, shear stress and energy.

of phytase/kg to the diet containing .32% aP increased the absorption of P ($P < .01$), Ca ($P < .05$) and N ($P < .05$), but did not change the absorption of DM. Differences for P and Ca absorption were slightly greater when adjusted for DM digestibility, whereas, the effect on N digestibility was very limited.

Fecal P excretion was increased (linear, $P < .01$; quadratic, $P < .07$) as the level of aP (without phytase) was increased. Addition of graded levels of phytase decreased fecal P excretion; both linear ($P < .01$) and quadratic ($P < .01$) effects were observed. Supplementing 1,400 U of phytase to the .32% aP diet decreased ($P < .01$) fecal P excretion.

Bone characteristics. Bone measurements, shear force, shear energy, and ash percentage of metacarpal and tenth rib, for pigs fed diets without added phytase, increased linearly as the level of aP increased ($P < .05$ to .01, respectively; Tables 2 and 3). The addition of graded levels of phytase resulted in linear increases ($P < .01$) in shear force, shear energy and ash percent for both metacarpals and tenth ribs at both aP levels. Also, shear stress linearly increased ($P < .01$) for metacarpals as phytase was added, but the effect of phytase on shear stress of tenth ribs was not significant. A quadratic response of added phytase on shear force and ash percent of tenth rib was also obtained ($P < .02$ and .01, respectively). The aP x phytase interactions generally were not significant, except for shear force ($P < .01$) and energy ($P < .05$) of tenth ribs. When pigs were fed .32% aP, the addition of 1,400 U of phytase increased shear force and ash percent of metacarpals ($P < .02$), and shear force and energy of tenth ribs ($P < .07$ and .01, respectively).

Response curves. Second order translog functions were generated from data for the five phytase and two aP levels on performance, apparent digestibility, fecal P excretion, and bone characteristics of young pigs fed corn-soybean meal diets (Table 4). Response equations of apparent digestibility coefficients of P and Ca, and fecal P excretion indicated high r^2 (> .9) and significant P-values ($P < .01$). Equations for ADG, apparent

Table 3. Probability values of main effects, interactive effects, linear and quadratic effects, and contrast of pigs fed a corn-soybean meal diet containing varying amounts of phosphorus and supplemental phytase

Items	PY[a]	aP	PYxaP	Lin[b]	Lin 1[b]	Lin 2[b]	Quad[c]	Quad 1[c]	Quad 2[c]	Con 1[d]	Con 2[d]	Con 3[d]
Performance												
ADG	.01	.01	.58	.01	.01	.01	.08	.42	.02	.05	.01	.46
ADFI	.29	.21	.68	.04	.03	.42	.24	.83	.16	.07	.08	.44
G:F	.09	.28	.87	.03	.40	.03	.13	.36	.84	.74	.08	.49
Digestibility and absorption coefficients												
DM	.14	.12	.21	.11	.12	.05	.16	.15	.15	.10	.05	.11
P	.01	.01	.06	.01	.01	.01	.01	.02	.01	.02	.01	.01
Ca	.01	.19	.12	.01	.01	.03	.31	.25	.13	.05	.25	.13
N	.18	.02	.12	.12	.24	.20	.06	.05	.10	.05	.01	.11
Fecal P excretion	.01	.07	.01	.01	.02	.01	.01	.01	.01	.01	.06	.10
Bone characteristics												
Metacarpal												
Shear force	.01	.01	.87	.01	.01	.01	.64	.93	.47	.02	.04	.72
Shear stress	.04	.15	.88	.01	.03	.03	.68	.31	.67	.35	.32	.56
Shear energy	.01	.09	.38	.01	.01	.01	.64	.51	.99	.55	.01	.45
Ash percent	.01	.01	.69	.01	.01	.01	.10	.10	.48	.01	.01	.46
Tenth rib												
Shear force	.01	.01	.01	.01	.01	.01	.01	.31	.01	.07	0.05	.57
Shear stress	.18	.95	.71	.22	.85	.12	.64	.44	.92	.47	0.44	.30
Shear energy	.01	.01	.05	.01	.01	.01	.23	.72	.19	.01	0.02	.84
Ash percent	.01	.01	.12	.01	.01	.01	.01	.01	.01	.86	0.01	.11

[a]PY = added phytase, U/kg diet.

[b]Lin = linear phytase effect; Lin1 = linear phytase effect at .07% aP; Lin 2 = linear phytase effect at .16% aP.

[c]Quad = quadratic phytase effect; Quad1 = quadratic phytase effect at .07% aP; Quad 2 = quadratic phytase effect at .16% aP.

[d]Con1 = contrast for diet 11 vs 12; Con2 = contrast for diet 1, 6 and 11, linear; Con3 = contrast for diet 1, 6 and 11, quadratic.

digestibility of DM and N, ash percent of 4th metacarpal, and shear force and ash percent of 10th rib had r^2 values ranging from .43 to .85 ($P < .04$); whereas, the r^2 of the remainder of the response equations were low (< .38) with P-values more than .10.

The nonlinear response equations of graded levels of phytase at each of the two aP levels were developed for performance, apparent digestibility coefficients, and bone characteristics (Table 5). High r^2 (> .8) were obtained for all response equations except shear force and shear stress and energy of both metacarpal and rib. The r^2 of the linear equations generated for the effects of only aP on ADG, Ca and P digestibility, shear force and ash percent of both metacarpal and rib, and shear energy of rib were .95 or greater (Table 6). Other measurements had lower r^2 values.

Replacement of available phosphorus by phytase. To evaluate microbial phytase efficacy, P-equivalency equations were developed using nonlinear and linear functions of phytase and aP (Table 7). Equivalency equations with high r^2 (>.9) were found for ADG, apparent digestibility and percentage of ash for metacarpal and rib. The equations for ADG and apparent digestibility of P were used for determining P-equivalency values of phytase (Table 8) because both parameters are nondestructive and practical to use. Averaged across the two aP levels, added microbial phytase of 250, 500, 750, and 1,000 U/kg diet could release 26.7, 52.0, 75.1 and 91.1% of phytate P in the diet, respectively. The amount of P released per 100 U of phytase decreased as the total level of phytase increased. Using the P-equivalency equation developed for the average of two aP levels and two response measurements (ADG and P absorption), $Y = .2622 - .2559e^{-0.00185X}$ [where X = added phytase (U/kg), and Y = aP (%)] (Table 8), 246 U of microbial would equal 1 g of inorganic P added as defluorinated phosphate.

Table 4. Second order translog functions for performance, apparent digestibility coefficients and bone characteristics of young pigs fed corn soybean meal diets containing two aP and five phytase levels

Item	Coefficients of equations[a]						
	a_0	a_1	a_2	a_3	a_4	P-value	r^2
Performance, 1-5 wk							
ADG, g	5.2900	.1280	.0190	.0070	.0036	.01	.43
ADFI, kg	-.7480	.0719	.0120	.0039	-.0050	.44	.11
G:F, g/kg	6.0380	.0560	.0070	.0030	.0087	.02	.28
Apparent digestibility coefficients, %							
DM	4.4640	-.0052	-.0003	.0001	-.0002	.03	.85
P	3.1320	.1940	.0511	.0077	-.0062	.01	.98
Ca	4.2320	-.0098	.0097	.0016	-.0023	.01	.94
N	4.4078	-.0148	-.0004	.0004	.0010	.04	.83
Fecal P excretion, g/d	1.2800	-.7500	-.0198	-.0049	-.0035	.01	.99
Bone characteristics[b]							
Fourth metacarpal							
Shear force	-.9650	.1460	.0370	.0096	.0010	.02	.26
Shear stress	-.5380	.0820	.0301	.0090	.0050	.05	.15
Shear energy	-.5910	.2660	.0770	.0170	-.0190	.01	.31
Ash percent	-1.3460	.0460	.0138	.0032	.0029	.01	.48
Tenth rib							
Shear force	-.7890	.1630	.0350	.0067	.0077	.01	.55
Shear stress	1.0680	.0465	.0135	.0007	.0012	.20	.09
Shear energy	-.9800	.1500	.0380	.0100	.0140	.01	.38
Ash percent	-.9500	.0810	.0156	.0010	-.0030	.01	.69

[a]Model: $LnY = a_0 + a_1D_1 + a_2LnX + a_3(LnX)^2 + a_4D_1LnX$.
D_1 = available P (aP), %. When aP = .07 %, D_1 = 0; When aP = .16 %, D_1 =1. X = added phytase, U/kg diet.

[b]Units are N, N/cm^2 and N-mm, respectively for shear force, stress and energy.

Table 5. Nonlinear functions of supplemental phytase for performance, apparent digestibility and bone characteristics of young pigs fed corn soybean meal diets containing two levels of P and five levels of phytase

Item	Equations[a]			
	.07 % aP	r^2	.16 % aP	r^2
Performance, 1-5 wk				
ADG, g	$Y = 376.71(1 - .354e^{-.00095X})$	.97	$Y = 383.6(1 - .304e^{-.00266X})$	.91
ADFI, kg	$Y = 0.859(1 - .387e^{-.0004X})$	.99	$Y = 0.648(1 - .105e^{-.00451X})$	.99
G:F, g/kg	$Y = 520(1 - .107e^{-.00210X})$	.88	$Y = 718(1 - .344e^{-.00061X})$	.94
Apparent digestibility coefficients, %				
P	$Y = 58.08(1 - .51e^{-.00107X})$	.97	$Y = 56.0715(1 - .373e^{-.00284X})$	.99
Ca	$Y = 81.508(1 - .1122e^{-.00160X})$	.95	$Y = 78.0877(1 - .068e^{-.00212X})$	.88
Fecal P excretion, g/d	$Y = 1.3461e^{-.00011X}$	.84	$Y = 1.6028e^{-.00019X}$	.90
Bone characteristics[b]				
Fourth metacarpal				
Shear force	$Y = 2.97(1 - .794e^{-.00008X})$	.99	$Y = 0.95(1 - 0.25e^{-.00188X})$	.99
Shear stress	$Y = 2.10(1 - .541e^{-.00013X})$	.68	$Y = 1.475(1 - 0.309e^{-.00061X})$	.95
Shear energy	$Y = 3.575(1 - .641e^{-.00067X})$	.95	$Y = 6.752(1 - 0.7118e^{-.00009X})$	.71
Ash percent	$Y = 33.06(1 - .132e^{-.00251X})$	.97	$Y = 39.06(1 - 0.219e^{-.00082X})$	.83
Tenth rib				
Shear force	$Y = 0.837(1 - .276e^{-.00197X})$	.98	$Y = 1.064(1 - 0.403e^{-.00197X})$	.65
Shear stress	$Y = 3.4345(1 - .1506e^{-.00569X})$	.39	$Y = 3.435(1 - 0.1506e^{-.00572X})$	.40
Shear energy	$Y = 1.076(1 - .443e^{-.00060X})$	.96	$Y = 1.206(1 - 0.464e^{-.00122X})$	.67
Ash percent	$Y = 45.06(1 - .1889e^{-.00452X})$	.97	$Y = 48.07(1 - 0.166e^{-.00360X})$	.93

[a]X = added phytase, U/kg diet; and Y = response obtained.

[b]Units are N, N/cm^2 and N-mm, respectively for shear force, stress and energy.

Table 6. Linear functions of aP for performance, apparent digestibility and bone characteristics of young pigs fed corn soybean meal diets containing two levels of phosphorus and five levels of phytase

Item	Equations[a]	r^2	P-value
Performance, 1-5 wk			
ADG, g	Y = 212.4 + 345.0X	.99	.04
ADFI, kg	Y = 0.5097 + .2709X	.99	.11
G:F, g/kg	Y = 366.4 + 427.8X	.91	.16
Apparent digestibility coefficients, %			
P	Y = 17.9323 + 120.55X	.99	.04
Ca	Y = 71.35 + 10.07X	.99	.02
Fecal P excretion, g/d	Y = 1.4500 + .1310X	.12	.77
Bone characteristics[b]			
Fourth metacarpal			
Shear force	Y = 0.6242 + .4678X	.98	.09
Shear stress	Y = 1.0828 + .6393X	.87	.21
Shear energy	Y = 1.2119 + 3.4626X	.87	.26
Ash percent	Y = 28.40 + 14.9000X	.99	.01
Tenth rib			
Shear force	Y = 0.6190 + .3690X	.98	.03
Shear stress	Y = 2.8717 + 2.6632X	.51	.32
Shear energy	Y = 0.5696 + .4751X	.99	.01
Ash percent	Y = 33.80 + 44.1900X	.99	.02

[a]X = aP levels, %, and Y = response obtained.
[b]Units are N, N/cm^2 and N-mm, respectively for shear force, stress and energy.

Table 7. Replacement functions of aP by microbial phytase for young pigs fed corn soybean meal diets containing two levels of P and five levels of phytase

Item	Equations[a]			
	.07 % aP	r^2	.16 % aP	r^2
Performance, 1-5 wk				
ADG	$Y = .4762 - .3865e^{-.00095X}$	.96	$Y = .4962 - .3380e^{-.00266X}$	.90
ADFI	$Y = 1.2894 - 1.2271e^{-.00400X}$	.99	$Y = .5105 - .2512e^{-.00451X}$	.99
G:F	$Y = .3599 - .1304e^{-.00210X}$	.80	$Y = .8238 - .5787e^{-.00063X}$	.86
Apparent digestibility coefficients				
P	$Y = .3331 - .2965e^{-.00108X}$	.96	$Y = .3164 - .1735e^{-.00284X}$	.98
Ca	$Y = 1.0087 - .9082e^{-.00161X}$	.95	$Y = .6691 - .5273e^{-.00212X}$	.87
Bone characteristics				
Fourth metacarpal				
Shear force	$Y = 5.0145 - 5.0410e^{-.00081X}$	.99	$Y = .6965 - .5077e^{-.00188X}$	.97
Shear stress	$Y = 1.5911 - 1.7771e^{-.00013X}$	.59	$Y = .6135 - .7129e^{-.00062X}$	.83
Shear energy	$Y = .6825 - .6618e^{-.00067X}$	.83	$Y = 1.6000 - 1.3880e^{-.00009X}$	.62
Ash percent	$Y = .3128 - .2929e^{-.00250X}$	.97	$Y = .7154 - .5741e^{-.00081X}$	.83
Tenth rib				
Shear force	$Y = .5908 - .6260e^{-.00197X}$	.96	$Y = 1.2060 - 1.1620e^{-.00197X}$	.64
Shear stress	$Y = .2113 - .1942e^{-.00569X}$	.20	$Y = .2115 - .1942e^{-.00570X}$	.20
Shear energy	$Y = 1.0659 - 1.0033e^{-.00061X}$	.96	$Y = 1.3395 - 1.1778e^{-.00123X}$	.67
Ash percent	$Y = .2548 - .1926e^{-.00452X}$	.97	$Y = .3229 - .1806e^{-.00362X}$	.93

[a]X = added phytase, U/kg diet; and Y = replaced aP, %; Equations were created using nonlinear and linear functions of supplemental phytase and inorganic P in tables 5 and 6.

Table 8. Phosphorus equivalency of iP by microbial phytase for young pigs fed corn soybean meal diets containing varying levels of P and phytase

Available P, %	.07				.16			
Added phytase, U/kg diet	250	500	750	1,000	250	500	750	1,000
Equivalent of aP, %								
ADG	.171	.236	.287	.326	.322	.407	.450	.473
Apparent P digestibility coefficient	.145	.189	.223	.249	.231	.274	.296	.306
Metacarpal ash percent	.156	.229	.268	.289	.245	.331	.400	.457
Rib ash percent	.192	.235	.248	.253	.249	.293	.311	.318
Mean equivalent[a]								
aP, %	.158	.212	.255	.285	.276	.340	.373	.89
Released P, %[b]	.088	.142	.185	.215	.116	.180	.213	.229
% of phytate P[c]	36.0	58.4	75.8	88.3	47.7	73.9	87.2	94.0
Mean equivalents of the two aP levels								
Released P, %[d]					.102	.161	.199	.222
% of phytate P					41.9	66.2	81.5	91.1
Released P, g/100 U of phytase					.040	.032	.027	.022

[a]Data of ADG and apparent P digestibility coefficients were used to calculate the mean equivalent.

[b]Released P = equivalent of aP - .07 (or .16).

[c]Plant P in diets is .356 %, phytate P is .244 % and nonphytate P is .110 %; released phytate P = (released P/phytate P) x 100.

[d]The function of released P (Y) by microbial phytase (X) was created using mean released P for pigs fed diets containing aP levels from .07 to .16 %: $Y = .2622 - .2559e^{-.00185X}$.

Discussion

This study reveals that supplemental microbial phytase is effective in improving performance, P digestibility and bone mineralization by enhancing hydrolysis of phytate P for young pigs fed diets of corn and soybean meal, which was also observed by other researchers (Simons et al., 1990; Cromwell et al., 1993b; Hoppe et al., 1993). The addition of 1,050 U of phytase/kg of diet at .07 or .16% aP level increased ADG by 38.5 and 39.5%; ADFI by 22 and 14.3%; and G:F by 14.3 and 24%, respectively (Table 2). More effects were found for ADG and G:F of pigs fed with .16 % aP than with .07%, which could be attributed to the high aP level being beneficial to the hydrolysis of phytate P. Average daily gain was found to be a good response indicator for graded supplemental levels of phytase and aP. These findings were consistent with other studies in pigs (Cromwell et al., 1993b; Lei et al., 1993a; Yi et al., 1995b).

Recently, much attention has been directed to P pollution from manure. Microbial phytase has been shown to be effective in reducing P excretion by increasing the utilization of phytate P (Simons et al., 1990; Jongbloed et al., 1992; Yi et al., 1995b). The data from this study further supported these finding. Adding 1,050 U of phytase per kg of diet decreased fecal P excretion by l2 and 22%, respectively for .07 and .16% aP diets, which resulted from increases in apparent P absorption by 72 and 59%, respectively.

The addition of 1,400 U of phytase to diets containing a recommended level of .32% aP further increased ADG, ADFI, apparent absorption of P, Ca and N, and bone strength and ash contents, which suggest that microbial phytase could be beneficial in the utilization of macro and trace minerals, protein and amino acids. Similar results also were found in young pigs fed a soybean meal based semi-purified diet (Yi et al., 1995b) or canola-grain sorghum basal diets (Veum et al., 1994). Supporting evidence also has been shown in the studies of Pallauf et al. (1992, 1994), Lei et al. (1993b), and Mroz et al. (1994). Ketaren et al. (1993b) found that the addition of phytase increased ADG, protein

deposition and retention, and energy retention, with no effects on the apparent digestibility of DM and CP. Mroz et al. (1994) reported that phytase supplementation enhanced the utilization of DM, OM, CP, amino acids and Ca. However, some researchers did not obtain these findings (Jongbloed et al., 1992). Clearly, further research needs to be conducted in this field. The effect of phytase on feed intake should be considered. The increased feed intake could relatively reduce the apparent absorption of some nutrients such as OM, N and minerals, and mask the effect of phytase on their responses.

Generally, the response curves for performance, apparent P absorption, and bone measurements indicated the inflection points were at phytase levels of 700 to 1,050 U/kg diet for pigs fed corn soybean meal diets with .07 and .16% aP, which suggested the maximum responses occurred at 700 or 1,050 U of phytase, respectively for .07 or .16% aP level. These results agreed with other phytase studies. The study of Yi et al. (1995b) reached the same conclusion with the semi-purified diets as this using corn soybean meal diets. Lei et al. (1993b) found that the inflection points of added Finase® phytase were at approximately 1,200 U/kg diet in weanling pigs fed corn soybean meal diet with .5% aP. For growing pigs, the maximum response of graded Natuphos® phytase ranged between 800 to 1,200 U/kg of diet (Veum et al., 1994). So, the maximum effect of phytase on pigs appeared at the supplemental level in the range of 700 and 1,200 U/kg diet. By comparing to the .32% aP diet (NRC recommended level), the maximum responses in performance, digestibilities and bone measurements of pigs fed with phytase supplementation in .07 or .16% aP diets were found close to or even better than the defluorinated phosphate addition.

Evaluation of nonlinear and linear functions for the various performance, digestibility and bone measurements suggested that ADG, apparent P digestibility, shear force and ash percent of bone were sensitive indicators, which was consistent with other studies (Cromwell et al., 1993ab; Yi et al., 1995b). Second order translog functions for

these indicators indicated similar results with relatively high r^2 and low P-value (Table 4). The apparent P digestibility was considered the best indicator to determine the effectiveness of feed phosphates and phytase (Dellaert et al., 1991; Beers and Jongbloed, 1993; Qian et al., 1994a). The data of apparent P digestibility in this study confirm this finding (Tables 4, 5 and 6). In addition, fecal P excretion also appeared highly sensitive to dietary supplemental phytase and P. In comparison to the .32% aP diet (NRC recommended level), fecal P excretion decreased by up to 25% by adding phytase for young pigs fed corn soybean meal diets, which was consistent with other research (Jongbloed et al., 1992; Cromwell et al., 1993a; Yi et al., 1995b).

Bone indicators are often used to evaluate P and Ca status in pigs (Koch and Mahan, 1985; NRC, 1988; Combs et al., 1991). Generally, metacarpal, metatarsal and femur of pigs were used. In this experiment, shear force and ash percent of metacarpal and tenth rib were sensitive measurements of the response to added dietary P. Shear energy is the energy required to deform a bone to the point of fracture, and represents the area under the force-deformation curve up to the point of fracture. Bone shear energy was less sensitive after shear force and ash percent. In this study, both metacarpal and tenth rib shear stress showed low sensitivity.

Using the P-equivalency function shown in Table 8 , the replacement of 1 g iP from defluorinated phosphate (**DFP**) would require about 245 U of phytase. The P-equivalency values are lower than those calculated from published data using the same type of diets, or in a soybean meal semi-purified diets for pigs (Yi et al., 1995b). Hoppe et al. (1993) reported 1 g P from monocalcium phosphate (**MCP**) was equivalent to 380 U of phytase when based on P-retention and 403 U of phytase when based on phalanx crude ash, when pigs were fed a corn, oat and soybean meal-based diet. In a review paper, Hoppe and Schwarz (1993) concluded that for diets based mainly on corn and soybean meal, 500 U of phytase was equivalent to 1 g P from MCP (= .8 g digestible P).

Using the P-equivalency equation generated in this study, and based on additions of 250, 500, 750, and 1,000 U of phytase/kg diet, 42, 66, 82 and 91% of the phytate P in corn-soybean meal was released. In a similar study using a soybean meal-based diet, Yi et al. (1995b) reported 40, 64, 83 and 93% of the phytate P was released, respectively, based on 250, 500, 750, and 1,000 U of phytase/kg diet. Cromwell et al. (1993b) observed that adding 250, 500, and 1,000 U of phytase/kg diet would release 14, 22, and 43% of the phytate P in a soybean meal-based semi-purified diet fed to growing pigs using bone shear force as the main criteria. Hoppe et al. (1993) in a study using pigs fed a grain and soybean meal diet reported that adding 250, 500, and 1,000 U of phytase/kg diet released 15, 42, and 57% of the phytate P, based on bone ash content, and P and Ca retention. Our results also support the observation of Jongbloed et al. (1992) who reported that the ileal digestibility of phytic acid increased from 9 to 59% by adding 1,500 U of phytase/kg diet to a corn and soybean meal diet fed to growing pigs.

From the data of this study, two important findings were achieved: 1) phytase was more effective for pigs fed with the higher aP level, which could be attributed to the beneficial effect of aP on the effectiveness of phytase; 2) phytase was more efficient at lower supplemental levels than at higher levels.

A wide Ca:tP ratio lowers P absorption, which results in decreased growth and bone calcification (Koch et al., 1984; Reinhart and Mahan, 1986; Pointillart et al., 1989). The wide Ca:tP ratio (2:1) used in this experiment probably reduced the overall response to phytase and inorganic P. Detrimental effects of the Ca:tP ratios on performance, bone characteristics and serum parameters generally were observed when the Ca:tP ratio exceeded 2.0:1; however, no significant effects were observed when the Ca:tP ratio was under 2.0:1, especially at the range of 1.0 to 1.6:1 (Koch et al., 1984; Reinhart and Mahan, 1986; Pointillart et al., 1989; Ketaren et al., 1993a). In some studies of the Ca:tP ratio, performance, bone measurements and serum parameters were not influenced by the

Ca:tP ratio even at a larger range than 2.0:1 such as 3:1 used by Koch et al. (1984). The adverse effects of a wide Ca:tP ratio may have reduced the efficacy of supplemental phytase in the present study when the dietary Ca:tP ratio increased from 1.2 to 2.0:1 (Qian et al., 1994a). The influence that Ca:tP ratio may have had was not taken into account in most recent phytase studies (Nasi, 1990; Jongbloed et al., 1992; Lei et al., 1994). In most studies, Ca:tP ratios ranged from 1.7 to 2.5:1. The effect of phytase on Ca release did not appear to have been given much attention.

Implications

In summary, microbial phytase is effective in improving performance, phosphorus utilization and bone mineralization, and decreasing fecal phosphorus excretion of pigs by enhancing the bioavailability of phytate phosphorus in corn and soybean meal. One gram of inorganic phosphorus as defluorinated phosphate could be released by 246 U of microbial phytase. In addition, phytase also appeared effective in improving the utilization of other nutrients such as crude protein, amino acids, and trace minerals.

CHAPTER IV

Adverse Effects of Wide Calcium:Phosphorus Ratios on Supplemental Phytase Efficacy for Weanling Pigs Fed Two Dietary Phosphorus Levels

ABSTRACT Ninety-six weanling pigs (initial BW = 9.3 kg, age = 30 d) were used in a 4-wk trial to evaluate the response to three Ca:total (t) P ratios (1.2:1, 1.6:1 and 2.0:1) fed in combination with two P levels [.07 or .16% available P (aP) or .36 and .45% tP] and two phytase levels (**PY**) (700 or 1,050 units/kg diet). A 3 x 2 x 2 factorial arrangement of treatments was employed using a corn-soybean meal diet. Performance, serum mineral concentrations and alkaline phosphatase (**ALP**) activity, P absorption and excretion, and bone mechanical measurements were examined. Average daily gain ($P <$ .001), ADFI ($P <$ .003) and gain:feed ($P <$ .02) were decreased linearly as the Ca:tP ratio became wider. The absorption of P ($P <$ .002) and Ca ($P <$ .02) was decreased linearly as the Ca:tP ratio became wider. The absorption of P and fecal P excretion was increased ($P <$ .001) and DM digestibility was decreased ($P <$ .005) for the higher level of aP. Increasing PY from 700 to 1,050 U/kg diet increased ($P <$ .02) P absorption, and decreased ($P <$ .03) P excretion, but did not improve bone measurements. Shear force, stress, energy and percentage of ash of both metacarpal and tenth rib decreased linearly ($P <$ .001 to .02) as the Ca:tP ratio became wider, and bone measurements generally were greater for pigs fed the higher P level. Serum Ca concentration increased ($P <$ .005) while the P level decreased ($P <$ .001) as the Ca:tP ratio increased, but Mg, Zn and ALP activity were not influenced. All serum measurements were affected by PY supplementation. Adverse effects of the Ca:tP ratio were greater at the lower P diet for all responses. In addition, the activity of supplemental PY in diets decreased as the Ca:tP ratio became wider ($P <$ .001) and this negative effect of Ca:tP ratio seemed greater at the low P diet, and seemed to parallel the effects of Ca:tP ratio on performance, P absorption, bone

measurements and serum criteria. Narrowing dietary Ca:tP ratio from 2.0:1 to 1.2:1 led to an increase in phytase efficacy by 16%.

Key Words: Phytase, Calcium, Phosphorus, Pigs

Introduction

Phytate can bind Ca and trace minerals, resulting in a decreased absorption of these minerals (Pallauf et al., 1992; Jongbloed et al., 1993). Supplemental phytase in swine diets has resulted in improved pig performance and bone mineralization by increasing the digestibility and retention of P and Ca, and resulted in decreased excretion of both P and Ca (Simons et al., 1990; Jongbloed, 1993; Lei et al., 1993ab). Nasi (1990) demonstrated that supplemental phytase improved not only P digestibility but also the digestibilities of Ca, Mg, Zn and Cu for pigs. Most studies (Nasi, 1990; Jongbloed et al., 1992; Yi et al., 1995b) have evaluated phytase-supplemented diets using Ca:total P (**tP**) ratios that ranged from 1.7 to 2.5:1. A narrow Ca:tP ratio may be important when microbial phytase is used in diets high in phytate because of three possible relationships: 1) a wide Ca:P ratio may lower P absorption as a result of the formation of an insoluble complex of Ca and P, especially when the diet is below the P requirement (NRC, 1988); 2) a high dietary level of Ca may decrease phytate-P bioavailability by forming a complex with phytic acid that is less available for degradation by phytase (Wise, 1983; Fisher, 1992); and 3) a high dietary level of Ca may directly reduce the activity of the phytase enzyme and limit phytase efficacy (Bhandari, 1980; Jongbloed et al., 1993; Lei et al., 1994). The objective of this study was to investigate the effect of Ca:tP ratios on the efficacy of supplemental phytase added to diets containing two levels of available P, both below NRC (1988) standards for weanling pigs.

Materials and Methods

Animals and feeding management. A total of 96 crossbred pigs (equal number of males and females) were utilized in this study to assess the effect of varying Ca:P ratio on phytase efficacy. Pigs were weaned at an average age of 30 d and allowed a 7-d adjustment period before diet treatments were started. During the adjustment period, pigs were fed a pre-starter formula (Maximum Wean, Southern States Cooperative, Richmond, VA) containing 22% CP for 4-d, then fed a 20% CP corn-soybean meal diet containing 10% dried whey for the remaining 3-d adjustment period. After the adjustment period, pigs were weighed (average weight, 9.3 kg) and assigned randomly to treatments from outcome groups established on the basis of gender and weight. Littermates were balanced across treatments as far as possible. The pigs were housed in groups of two in double deck nursery pens (.6 m x .9m) with expanded metal floors and a baffle between decks. Each pen was equipped with a nipple waterer and a stainless steel feeder. Room temperature was initially set at 29°C and was lowered approximately 2°C per wk after the 2nd wk. A continuous lighting regime was used. The care and treatment of pigs, and air ventilation rates followed the published guidelines (Consortium, 1988).

Treatments and diets. A 3 x 2 x 2 factorial arrangement of treatments was used to evaluate the response of weanling pigs to three Ca:tP ratios (1.2, 1.6 and 2.0:1), two levels of available P (.07 and .16%) and two supplemental phytase levels (700 and 1,050 unit/kg of diet; Table 1). A unit (**U**) is defined as the quantity of enzyme that liberates 1 μmole of inorganic phosphorus per min from 1.5 mM sodium phytate at pH 5.5 and 37°C. The total P (**tP**) levels were .36 and .45%, respectively for .07 and .16% available P (**aP**). These P levels were formulated below the current NRC (1988) standards for weanling pigs to ensure maximum response to phytase additions.

Table 1. Dietary treatments used to compare Ca:tP ratios, P levels and phytase levels for weanling pigs

Diets	tP[a] %	aP[a] %	Ca %	Ca:tP	Ca:aP[b]	Phytase U/kg[c]
1	.36	.07	.43	1.2:1	6.1:1	700
2	.36	.07	.58	1.6:1	8.3:1	700
3	.36	.07	.72	2.0:1	10.3:1	700
4	.45	.16	.54	1.2:1	3.4:1	700
5	.45	.16	.72	1.6:1	4.5:1	700
6	.45	.16	.90	2.0:1	5.6:1	700
7	.36	.07	.43	1.2:1	6.1:1	1,050
8	.36	.07	.58	1.6:1	8.3:1	1,050
9	.36	.07	.72	2.0:1	10.3:1	1,050
10	.45	.16	.54	1.2:1	3.4:1	1,050
11	.45	.16	.72	1.6:1	4.5:1	1,050
12	.45	.16	.90	2.0:1	5.6:1	1,050

[a]Total phosphorus = tP, and available phosphorus = aP.
[b]The values for aP do not include P released from phytate by phytase.
[c]Phytase (Natuphos®) was supplied by BASF Co. (3000 Continental Drive North, Mount Olive, NJ 07822-1234).

The basal diet contained corn and soybean meal as the protein sources that supplied all the P (.07% aP or .36% tP) contained in the basal diet (Table 2). The desired level of aP was achieved by the addition of defluorinated phosphate. The desired Ca:tP ratios were obtained by the addition of ground limestone. The dietary content of phytate P (.244%) was calculated using data presented in NRC (1988), and was similar in all diets. Chromic oxide was included at a level of .10% in diets as an indigestible indicator for digestibility measurements. To obtain a homogenous distribution of the indicator in the diet, chromic oxide first was mixed in a small mixer with corn starch at a ratio of 1:3 (wt/wt) and then ground in a laboratory mill to pass through a 1-mm sieve.

Each dietary treatment was fed to four replicate pens of two pigs each (one barrow and one female). Feed and water were available *ad libitum*. Pigs were weighed

Table 2. Percentage of composition of basal diets for weanling pigs

Item	Total P (aP), % / Ca:total P	.36 (.07)[a] 1.2:1	.36 (.07)[a] 1.6:1	.36 (.07)[a] 2.0:1	.45 (.16)[a] 1.2:1	.45 (.16)[a] 1.6:1	.45 (.16)[a] 2.0:1
Ingredients, %							
Corn (8.8% CP)		73.68	73.29	72.92	73.31	72.84	72.36
SBM (48.5% CP)		24.50	24.50	24.50	24.50	24.50	24.50
Limestone (38.0% Ca)[b]		.92	1.31	1.68	.79	1.26	1.74
Defluorinated phosphate[c]		--	--	--	.5	.5	.5
Salt		.30	.30	.30	.30	.30	.30
Vitamin premix[d]		.25	.25	.25	.25	.25	.25
Trace mineral premix[e]		.10	.10	.10	.10	.10	.10
Selenium premix[f]		.05	.05	.05	.05	.05	.05
Cr_2O_3		.10	.10	.10	.10	.10	.10
L-Lysine•HCl		.10	.10	.10	.10	.10	.10

[a]700 or 1,050 U phytase per kilogram was added to each diet. The basal diets contained 18.3% crude protein, 1.05% lysine, .61% methionine plus cystine, and a calculated level of .244% phytate P.

[b]Limestone Dust Corp., Bluefield, VA.

[c]Fine CDP, Southern Bag Corp., Valdosta, GA.

[d]Supplied per kilogram of diet: 4,400 IU of vitamin A, 440 IU of vitamin D_3, 11 IU of vitamin E, 2.2 mg of vitamin K (menadione), 4.4 mg of riboflavin, 22 mg of Ca-d-pantothenate, 22 mg of niacin, .022 mg of vitamin B_{12}, 440 mg of choline chloride, .44 mg of d-biotin, 3.9 mg of folic acid, 10 mg of thiamin•HCl, and 3.9 mg of pyridoxine•HCl.

[e]Supplied per kilogram of diet: 44 mg of Mn, 47.5 mg of Zn, 50 mg of Fe, 6.25 mg of Cu, and 2 mg of I.

[f]Supplied .3 mg of Se per kilogram of diet.

individually at weekly intervals during the 4-wk experimental period. Pen feed intakes were also recorded weekly.

Sampling and analysis. Approximately equal amounts of feces were collected from each pen twice daily (morning and evening) on three alternate days during the 3rd and 4th wk. Collections from each of the 3 d within a wk were pooled and frozen at -20°C in airtight plastic bags for subsequent analysis. Blood samples were collected initially and every 2 wk. Serum was collected and stored at 4°C for 3 to 4 h before determining alkaline phosphatase activity and then mineral concentration.

At the end of wk 4, all barrows (four per treatment) were slaughtered for collection of bone. The front foot and the 10th rib on the left side were removed and frozen. The foot samples later were thawed, extraneous tissue was removed and the 4th metacarpal was retained. The fleshy parts of the rib samples were removed. The width of bones at the narrow and wide dimensions of the bone shaft were measured. The bones then were refrozen in airtight plastic bags for shear force determination as described by Combs et al. (1991).

The shear force and energy of the 4th metacarpal and the 10th rib were determined using an Instron Universal Testing Machine (Model 1123, Instron Corp., Canton, MA). The shear energy is the energy required to shear a bone, and represents the area under the force-deformation curve up to the point of shear. Bones were thawed immediately prior to testing to prevent desiccation. After the shear force and energy test, wall thickness was measured using dial calipers at two sites (widest and narrowest). Shear stress values were calculated according to the formula of Wilson (1991). After the shear test, the bones were oven-dried at 100°C for 24 h and ashed in a muffle furnace at 600°C for 24 h. Metacarpal ash and 10th rib ash were expressed as a percentage of dried bone weight.

After thawing, fecal samples were dried in an oven at 65°C. The dried samples, along with representative samples of diets, were ground to pass through a 1-mm sieve and

analyzed for DM according to AOAC procedure (1990). Fecal and feed samples were wet digested using a mixture of nitric acid and perchloric acid (5:3, v/v), then total P concentrations in fecal, feed and serum were assayed photometrically (AOAC, 1990). Calcium and Cr concentrations of feces and feed, and serum concentrations of Ca, Mg and Zn were determined with a Perkin Elmer atomic absorption spectrophotometer (Model 5100 PC, The Perkin-Elmer Corp., 761 Main Ave., Norwalk, CT). The apparent digestibility of Ca and P was calculated by the indirect method (Dellaert, et al., 1990). The assay of serum alkaline phosphatase activity was based on the method outlined by Sigma Chemical Company (1987). Phytase activity of each diet (from a single batch) was determined (quadruplicates) according to the method of Simons, et al. (1990).

Statistical analysis. The data were analyzed as a 3 x 2 x 2 factorial arrangement of treatments by the GLM procedure of the SAS (1990). Pen means were the experimental unit for performance, serum criteria, and apparent digestibility data. Individual pig values were used as the experimental unit for bone measurements. The model included the main effects of Ca:tP ratios, phytase levels and P levels, and two- and three-way interactions. Linear and quadratic effects of Ca:tP ratios across and within each aP level and across phytase levels were tested using orthogonal polynomials. Nonlinear and linear equations were derived across phytase levels for Ca:tP ratio within each P level. Percentage of the decreased supplemental phytase efficacy or activity was calculated as the Ca:tP ratios became wider in the diet: the difference in the responses between the Ca:tP ratios of 2.0:1 and 1.2:1 was divided by the response value of the 2.0:1 ratio.

Results

The activity of supplemental phytase in diets. Assayed phytase activity of the diets was decreased as the Ca:tP ratio became wider (Table 3) and activity was higher for the

Table 3. Influence of dietary Ca:P ratio, P level and added phytase on phytase activity of the diet, performance, digestion coefficents, and fecal P excretion for weanling pigs[a]

Phytase, U/kg	700						1,050						
tP (aP), %	36 (.07)			.45 (.16)			.36 (.07)			.45 (.16)			
Ca:tP ratio	1.2:1	1.6:1	2.0:1	1.2:1	1.6:1	2.0:1	1.2:1	1.6:1	2.0:1	1.2:1	1.6:1	2.0:1	SEM
Phytase activity of diets, U/kg diet[b]													
	702	649	589	692	640	592	986	942	841	999	934	905	14
Performance													
ADG, g[c]	488	428	396	486	458	419	504	455	376	500	435	433	27
ADF, g[d]	913	880	829	871	865	809	893	870	732	922	865	868	40
G:F, g/kg[e]	532	488	478	559	528	517	565	522	528	547	444	498	26
Digestion coefficients, %													
Ca[f]	76.0	74.5	70.4	77.4	76.0	73.6	75.8	74.0	73.0	76.5	76.9	72.9	1.7
P[g]	53.4	49.2	44.4	57.2	57.9	54.5	52.9	52.8	49.4	61.2	60.1	55.6	1.4
DM[h]	86.1	86.4	86.5	84.5	85.9	86.4	85.2	86.1	86.5	85.4	85.6	85.6	.4
Excretion of fecal P, g/d[i]													
	1.65	1.80	1.82	1.80	1.84	1.87	1.66	1.63	1.69	1.80	1.74	1.97	.05

[a]Four pens (two pigs/pen) per treatment for performance, digestion coefficients. Average initial wt was 9.3 kg, and final wt was 22.85 kg.

[b]Each mean represents four independent assays of each diet from a single batch.

[c]Ca:tP ratio linear effect ($P < .001$; at .07% aP, $P < .001$; at .16% aP, $P < .02$).

[d]Ca:tP ratio linear effect ($P < .003$; at .07% aP, $P < .005$; at .16% aP, $P < .14$).

[e]Ca:tP ratio linear effect ($P < .02$; at .07% and .16% aP, $P < .08$); and phytase x P interaction ($P < .05$).

[f]Ca:tP ratio linear effect ($P < .001$; at .07% aP, $P < .02$; at .16% aP, $P < .03$); and Ca:tP ratio quadratic effect ($P < .02$).

[g]Ca:tP ratio linear effect ($P < .001$; at .07% aP, $P < .001$; at .16% aP, $P < .007$); and Ca:tP ratio quadratic effect ($P < .005$); P effect ($P < .001$); and phytase effect ($P < .02$).

[h]Ca:tP ratio linear effect ($P < .01$; at .07% aP, $P < .02$); and Ca:tP ratio quadratic ($P < .02$); and P effect ($P < .005$).

[i] Phosphorus effect ($P < .001$), and phytase effect ($P < .003$); phytase x P interaction ($P < .02$); Ca:tP ratio x P interaction ($P < .06$); and Ca:tP ratio x P x phytase interaction ($P < .002$).

1,050 vs the 700 U/kg of diets. The effect of the Ca:tP ratio appeared greater at the lower aP level.

Growth performance. Average daily gain was linearly decreased ($P < .001$) as the Ca:tP ratio became wider (Table 3). This decrease in ADG was associated with a linear decrease in ADFI ($P < .003$) and in gain:feed (G:F) ($P < .02$). The maximum responses in ADG, ADFI and G:F occurred at a Ca:tP ratio of 1.2:1. Average daily gain, ADFI and G:F were not affected ($P < .10$) by aP or phytase levels. A phytase x aP interaction ($P < .05$) indicated that G:F was higher for pigs fed the diets with the higher level of phytase and .07% aP. Although the effect of Ca:tP ratio seemed to be independent of aP or phytase levels since most of the two-way interactions were not significant, the adverse effect of Ca:tP ratio on ADG and ADFI appeared to be greater in .07% aP diets ($P < .001$ and .005, respectively) than of .16% diet ($P < .02$ and .14, respectively).

Apparent digestibility coefficients. Apparent digestibility of Ca and P decreased ($P < .001$) as the Ca:tP ratio became wider, but the digestibility of DM linearly increased ($P < .05$) (Table 3). A quadratic response for Ca, P, and DM digestibility showed a larger effect between a Ca:tP ratio of 1.6:1 and 2.0:1 than between 1.2:1 and 1.6:1. The apparent digestibility of P was higher ($P < .001$) and that of DM was lower ($P < .005$) for the higher level of aP. The digestibility of Ca only tended to be higher ($P = .12$) for the higher level of P. The higher level of phytase resulted in a greater absorption of P ($P < .02$), but the apparent digestibility of Ca and DM were not different ($P > .10$) between phytase levels. The two-way interactions between Ca:tP ratios, P levels and phytase levels and the three-way interaction were not significant ($P > .10$).

Fecal P excretion was increased ($P < .001$) as the level of aP fed was increased (Table 3), whereas, increasing the level of phytase from 700 to 1,050 U/kg of diet decreased ($P < .003$) fecal P excretion. Varying the Ca:tP ratio did not affect fecal P excretions. A phytase x aP interaction ($P < .02$) showed decreased fecal P excretion for

the lower aP level when the phytase level was increased, but a small increase for the higher phytase level when the higher aP level was fed. The Ca:tP ratio x aP interaction indicated ($P < .06$) a linear increase in fecal P excretion at the lower aP level as the Ca:tP ratio became wider; whereas, P excretion was only increased at the widest Ca:tP ratio for the higher aP level.

Bone measurements. Shear force, stress, energy and percentage of ash of both metacarpal and 10th rib linearly decreased ($P < .001$ to .02) as the Ca:tP ratio became wider (Table 4). Shear force ($P < .01$) and percentage ash ($P < .001$) of both metacarpal and 10th rib ($P < .001$) and shear energy of 10th rib were larger for pigs fed the higher P levels. Metacarpal shear energy and shear stress for both metacarpal and 10th rib were not influenced by aP levels in this study. The interactions of Ca:tP ratio x aP and Ca:tP ratio x phytase for all bone measurements were not significant indicating that the effects of Ca:tP ratio, aP and phytase levels were independent. However, aP x phytase interactions ($P < .02$ to .08) for the 10th rib measurements indicated a response to the higher phytase level when added to the diet containing the higher aP level.

Serum criteria. Serum Ca concentration increased ($P < .004$) while serum P level decreased ($P < .001$) as the Ca:tP ratio became wider (Table 5) at wk 2 and 4. Serum Ca concentration was lower ($P < .03$) at the higher aP level at both wk 2 and 4 and was lower for the higher phytase level at wk 4. In contrast, serum P was higher ($P < .005$) when the dietary aP or phytase level was higher. Serum Mg and Zn concentrations and alkaline phosphatase activity were not affected by dietary treatments. Mean values (± SEM) for initial, wk 2 and wk 4 were 19.79 ± .09, 23.49 ± .42, and 25.15 ± .53 mg/L for Mg; .50 ± .02, .52 ± .02 and .85 ± .03 mg/L for Zn; and 177 ± 10.52, 166 ± 9.54 and 144 ± 7.40 U/L for alkaline phosphatase activity, respectively. Serum Ca, P ,Mg and Zn concentrations increased and serum ALP activity decreased over the 4-wk trial (data not shown).

Table 4. Influence of dietary Ca:P ratio, P level and added phytase on bone measurements of weanling pigs[a]

Phytase, U/kg diet	700						1,050						
tP (aP), %	36 (.07)			.45 (.16)			.36 (.07)			.45 (.16)			
Ca:tP ratio	1.2:1	1.6:1	2.0:1	1.2:1	1.6:1	2.0:1	1.2:1	1.6:1	2.0:1	1.2:1	1.6:1	2.0:1	SEM
Metacarpal													
Shear force, N[b]	.79	.75	.75	.98	.96	.77	.81	.75	.70	.95	.83	.81	.08
Shear stress, kg/mm^2[c]	1.25	1.15	1.13	1.33	1.17	1.10	1.24	1.09	1.07	1.36	1.10	1.03	.11
Shear energy, kcal[d]	2.48	2.11	1.82	2.11	2.09	1.80	2.37	2.21	1.62	2.38	2.19	1.95	.27
Ash percent[e]	31.6	28.3	28.1	32.3	31.3	30.9	30.5	29.1	27.7	33.9	33.6	31.9	1.1
Tenth rib													
Shear force, N[f]	.95	.92	.73	.98	.95	.83	.84	.75	.71	1.08	.93	.89	.8
Shear stress, kg/mm^2[g]	3.34	3.19	2.88	3.37	3.01	2.63	3.03	2.89	2.46	3.65	3.59	2.97	.33
Shear energy, kcal[h]	1.14	.95	.92	1.71	1.53	1.28	1.13	1.08	1.02	1.42	1.11	1.07	.13
Ash percent[i]	48.6	47.9	46.9	51.2	49.3	48.6	48.9	46.8	45.2	51.0	50.8	50.5	.9

[a]Four pigs per treatment mean.
[b]Ca:tP ratio linear effect ($P < .02$; at .07% aP, $P < .23$; at .16% aP, $P < .02$); P effect ($P < .01$).
[c]Ca:tP ratio linear effect ($P < .006$; at .07% aP, $P < .17$; at .16% aP, $P < .01$).
[d]Ca:tP ratio linear effect ($P < .005$; at .07% aP, $P < .009$; at .16% aP, $P < .16$).
[e]Ca:tP ratio linear effect ($P < .004$; at .07% aP, $P < .007$; at .16% aP, $P < .16$); and P effect ($P < .001$).
[f]Ca:tP ratio linear effect ($P < .001$; at .07 or .16% aP, $P < .01$); P effect ($P < .001$); and phytase x P interaction ($P < .05$).
[g]Ca:tP ratio linear effect ($P < .01$; at .07% aP, $P < .03$); and phytase x P interaction ($P < .06$).
[h]Ca:tP ratio linear effect ($P < .007$; at .16% aP, $P < .007$); P effect ($P < .001$) and phytase x P interaction ($P < .02$).
[i]Ca:tP ratio linear effect ($P < .002$; at .07% aP, $P < .005$; at .16% aP, $P < .09$); P effect ($P < .001$); and phytase x P interaction ($P < .08$).

Table 5. Influence of dietary Ca:P ratio, P level and added phytase on serum Ca and P concentrations of weanling pigs[a]

Phytase, U/kg diet	700						1,050						
tP (aP), %	.36 (.07)			.45 (.16)			.36 (.07)			.45 (.16)			
Ca:tP ratio	1.2:1	1.6:1	2.0:1	1.2:1	1.6:1	2.0:1	1.2:1	1.6:1	2.0:1	1.2:1	1.6:1	2.0:1	SEM
Ca, mg/L													
Initial	90.3	93.7	95.7	91.0	84.0	94.5	84.8	88.0	88.0	89.3	89.7	96.0	4.8
Wk 2[b]	101.1	111.5	115.8	95.8	109.4	110.5	107.4	106.4	119.8	109.0	95.5	107.9	3.9
Wk 4[c]	112.3	103.3	120.3	104.6	95.6	106.1	102.1	98.8	108.1	100.0	93.6	105.5	4.9
P, mg/L													
Initial	68.7	71.0	62.8	68.7	72.9	68.3	75.0	73.0	73.9	63.6	69.2	70.7	5.5
Wk 2[d]	70.3	70.9	69.3	76.9	75.4	69.8	77.8	67.9	67.3	86.4	81.8	67.6	5.7
Wk 4[e]	103.6	85.4	65.1	106.4	100.7	87.0	104.5	92.8	89.5	116.9	102.4	98.4	5.4

[a]Eight pigs per treatment mean.

[b]Ca:tP ratio linear effect ($P < .004$; at .07% aP, $P < .006$); Ca:tP ratio quadratic effect ($P < .05$); and P effect ($P < .02$).

[c]Ca:tP ratio quadratic effect ($P < .001$; at .07 and .16% aP, $P < .02$); P effect ($P < .03$); and phytase effect ($P < .05$).

[d]Ca:tP ratio quadratic effect ($P < .04$; at .16% aP, $P < .08$); and P effect ($P < .08$).

[e]Ca:tP ratio linear effect ($P < .02$; at .07% aP, $P < .06$), and Ca:tP ratio quadratic effect ($P < .001$; at .07% aP, $P < .001$; at .16% aP, $P < .008$); P effect ($P < .001$); and phytase effect ($P < .005$); and Ca:tP ratio x phytase interaction ($P < .05$).

The effect of Ca:tP ratios. It was observed that a wider Ca:tP ratio decreased phytase efficacy on all measurements. Reduced phytase efficacy was calculated using the main effects of Ca:tP ratios across phytase and aP levels (Table 6). It was assumed that all significant responses to the adverse effect of Ca:tP ratio resulted from the decreased phytase efficacy. Increasing Ca:tP ratio .1 unit between 1.2:1 and 2.0:1 in diets resulted in 1.99 ± .27% reduction in the supplemental phytase efficacy, which was close to the value calculated for the reduced phytase activity (1.95%) of the diet. This adverse effect of Ca:tP ratio was greater at the lower dietary aP level, but was not different between the two phytase levels.

Discussion

Calcium is thought to be a key factor that influences the activity of mucosal phytase in poultry and rats (Bhandari, 1980; Wise, 1983). However, the influence of Ca on phytase activity of a microbial phytase product, added in a corn-soybean meal diet is not known. Results of the phytase assay of the mixed diets showed that the wider Ca:tP ratios decreased the phytase activity of diets with added phytase. This decrease in activity as the Ca:tP ratio became wide might be due to: 1) the extra Ca binding to phytate to form an insoluble complex that is unaccessible to phytase; or 2) the extra Ca that could directly repress phytase activity by competing for the active sites of the enzyme (Wise, 1983; Pointillart et al., 1985). This depressing effect was greater at the lower aP level, which suggested that phytase can also be influenced by the P level.

A wide Ca:tP ratio lowers P absorption, which results in decreased growth and bone calcification (Koch et al., 1984; Reinhart and Mahan, 1986; Pointillart et al., 1989). Adverse effects of the Ca:tP ratios on performance, bone characteristics and serum criteria were generally observed when the Ca:tP ratio exceeded 2.0:1; however, no significant effects were observed when the Ca:tP ratio was under 2.0:1, especially at the range of 1.0

Table 6. Phytase efficacy and activity as influenced by the adverse Ca:total P ratio effect

Total P (aP), % or phytase, U/kg diet	Percentage of the decrease in efficacy or activity of supplemental phytase as the Ca:tP ratios became wider [a]				
	Mean[b]	.36 (.07)[c]	.45 (.16)[c]	700[c]	1,050[c]
Performance					
ADG	2.71	3.56	1.97	2.76	2.99
ADF	1.37	1.92	.88	1.41	1.62
G/F	1.12	1.13	1.11	1.20	1.05
Digestion coefficient					
Ca	.70	.73	.63	.81	.55
P	1.30	1.68	.91	1.65	1.08
Metacarpal					
Shear force	2.00	2.88	1.20	2.17	2.17
Shear stress	2.43	3.32	1.68	2.04	2.96
Shear energy	3.70	5.19	2.46	3.31	4.14
Ash percent	1.01	1.34	.81	.83	.83
Tenth rib					
Shear force	2.70	2.95	2.47	3.04	2.50
Shear stress	2.84	3.17	2.38	2.77	2.85
Shear energy	3.30	4.17	2.21	3.64	2.76
Ash percent	.60	.82	.51	.58	.55
Serum mineral concentrations at wk 4					
Ca	.72	.84	.42	.74	.58
P	3.35	4.38	2.56	4.77	2.28
Mean (SEM)	1.99 (.27)	2.47 (.36)	1.48 (.20)	2.09 (.32)	1.93 (.28)
Phytase activity of diets	1.95	2.26	1.62	2.27	1.72

[a]Decreased supplemental phytase efficacy or activity (%) as the Ca:tP ratio increased .1 unit in the diet, which equals the difference in the responses between the Ca:tP ratios of 2.0:1 and 1.2:1 divided by the response value of the ratio of 2.0:1 and by 8 (the number of .1 units between 2.0 and 1.2). For example [ADG for .36 (.07)]: [(488 + 504)/2 - (396 + 376)/2] / [(396 + 376)/2] / 8 = 110/386/8 = 3.56%.

[b]Mean values across phytase and P levels.

[c]Averaged across phytase or P levels.

to 1.6:1 (Koch et al., 1984; Reinhart and Mahan, 1986; Pointillart et al., 1989; Ketaren et al., 1993a). In some studies evaluating the Ca:tP ratio, performance, bone measurements and serum criteria were not influenced by the Ca:tP ratio even at a higher ratios than 2.0:1 such as 3:1 used by Koch et al. (1984). In the current study, a portion of reduced responses observed for the wide Ca:tP ratio may have resulted from a reduction in the efficiency of supplemental phytase when the dietary Ca:tP ratio increased from 1.2 to 2.0:1. The effects of wide Ca:tP ratios may not have been taken into account in most recent phytase studies (Nasi, 1990; Jongbloed et al., 1992; Lei et al., 1993ab), since Ca:tP ratios ranged from 1.7 to 2.5:1. Lei et al. (1994) conducted a study to evaluate the adverse effect of Ca on phytase efficacy, in which two Ca:tP ratios calculated were approximately 1.6:1 and 3.1:1. The responses in their study may not be attributed to the decreased phytase efficacy by high dietary Ca because the Ca:tP ratio was above 2.0:1, at which level adverse Ca:tP effects did exist even without supplemental phytase in diets as discussed as above. In addition, they failed to consider that the adverse effects of Ca depended on the P level in the diet. In the present study, using Ca:tP ratios of 1.2:1, 1.6:1 and 2.0:1, significant effects of Ca:tP ratios on performance, digestibilities, bone characteristics and serum criteria could be attributed to the decreased supplemental phytase efficacy that resulted from the detrimental effect of a wide Ca:tP ratio.

Several studies have demonstrated that supplemental phytase significantly improved the performance and bone mineralization of pigs, and the optimal level in weaning pig diets was about 700 U/kg of diet (Simons et al., 1990; Lei et al., 1993b; Kornegay and Qian, 1994). However, these improvements were greatly reduced by the wider Ca:tP ratios in the present study, which may have been a result of the decreased phytase efficacy caused by the wide Ca:tP ratio. The adverse effect of a wide Ca:tP ratio on phytase efficacy may be independent of phytase levels because significant difference were not observed between the two phytase levels (700 and 1,050 U/kg of diet) for

performance and bone characteristics. Although independent of P levels, the adverse effect of Ca:tP on performance and bone measurements indicated apparently stronger responses at the lower dietary P level, which suggested that the Ca:tP ratio effect on supplemental phytase efficacy was more crucial at the lower dietary P level.

Two-way significant interactions between aP x phytase for G:F and 10th rib measurements indicated a better response to the higher phytase level when added to the diet containing the higher aP level (Yi et al., 1995b). Significant improvements in most bone measurements indicated a better response in bone mineralization by the higher aP level. No significant effects of an increase in phytase supplementation from 700 to 1,050 U/kg of diet further supported that the optimal level of supplemental phytase in weanling pigs was around 700 U/kg of diet (Simons et al., 1990; Lei et al., 1993b; Kornegay and Qian, 1994).

Recently, much effort has been made to increase the utilization of phytate P by using supplemental microbial phytase so as to decrease the excretion of P because of potential contamination of environment by fecal P from animals (Beers and Jongbloed, 1993). Apparent P digestibility that is inversely correlated with the P excretion was thought to be the most sensitive indicator for the evaluation of P utilization and phytase function in pig studies (Nasi, 1990; Pallauf et al., 1992; Beers and Jongbloed, 1993; Mroz et al., 1994). In agreement, P digestibility was significantly increased by the increased phytase supplementation from 700 to 1,050 U/kg of diet or aP level from .07 to .16%, and decreased by the wider Ca:tP ratio, which actually suggested that P digestibility was a highly sensitive indicator. Apparent Ca digestibility, which was found to be improved by supplemental phytase (Nasi, 1990; Pallauf et al., 1992; Kornegay and Qian, 1994), was diminished by the adverse effect of a wide Ca:tP ratio in this study. This reduction in Ca and P digestibilities could have resulted from a decrease in the efficacy of phytase which resulted from widening the Ca:tP ratio; this is consistent with the study by Jongbloed et al.

(1993). As P digestibility increased, a decrease in DM digestibility could be due to an increase in ADFI that resulted from the improvement in the P absorption, suggesting that an inverse relationship existed between DM and P digestibility. So, the wider Ca:tP ratio decreased P absorption, which reasonably resulted in an increase in DM digestibility. Quadratic responses in digestibilities of Ca, P and DM suggested that a greater effect of the Ca:tP ratio occurred at the lower dietary aP level.

Fecal P excretion was not significantly affected by the dietary Ca:tP ratio, but was decreased by added phytase or increased by increasing the aP level, which suggested that it was influenced by the dietary supplemental phytase and aP level more than by the Ca:tP ratio. The two- and three-way interactions between phytase, aP and Ca:tP ratio indicated a reduction in fecal P excretion for pigs fed diets with a combination of the higher phytase supplementation, lower P level and narrower Ca:tP ratio.

As indicators of the utilization of the dietary P or supplemental phytase, serum parameters such as serum Ca, P, and alkaline phosphatase were found less sensitive (Koch et al., 1984; Koch and Mahan, 1985; Reinhart and Mahan, 1986), except the report of Lei et al. (1993ab, 1994). In the present study, serum Ca and P concentrations were found more sensitive than ALP activity, Zn, and Mg concentrations for the weanling pigs fed diets with phytase supplementation. Increases in serum Ca, P, Zn and Mg for all treatments over the feeding time could be attributed to the positive effect for the optimal level of phytase supplementation on the Ca, P, Zn, and Mg utilization (Pallauf et al., 1992; Lei et al., 1993ab; 1994; Kornegay and Qian, 1994). However, these increases in serum Ca and P levels were also diminished as the Ca:tP ratio became wider, which was consistent with results for adverse effects of Ca:tP on performance, bone characteristics and P absorption. This detrimental effect finally resulted in an increase in serum Ca and a decrease in serum P levels, suggesting an inverse relationship between serum Ca and P which is regulated by dietary Ca and P levels in addition to hormones (Mahan, 1982; Koch

et al., 1984; Reinhart and Mahan, 1986). Furthermore, the magnitude of the Ca:tP ratio effects was greater at the lower dietary P level. Studies by Koch et al. (1984) and Koch and Mahan (1985) suggested that serum P concentration and ALP activity were stable over time for young pigs. A reduction in serum ALP activity and an increase in P concentration in the present study might result from the effect of supplemental phytase. In a similar study, Lei et al. (1994) found an increase in serum P over 30 d for weanling pigs fed diets with 750 and 1,200 U/kg of supplemental phytase. Serum P concentration is inversely correlated with ALP activity. Serum Zn, Mg and ALP were not affected by dietary Ca:tP ratio but influenced by supplemental phytase, suggesting that they were affected more by phytase supplementation than by the Ca:tP ratio.

Within the range of the ratios of 1.2, 1.6, and 2.0:1, the decreases in performance, bone characteristics, P digestibility and excretion, and serum measurements could be attributed to a reduction in phytase efficacy that was affected by an adverse effect of the wide Ca:tP ratio as observed for the assayed activity of phytase. The degree of reduction in phytase efficacy for increasing .1 unit of Ca:tP ratio was about 1.99%, which is very close to 1.95% reduction in assayed activity of phytase. This finding supports our hypothesis that a decrease in the efficacy of phytase by a wide Ca:tP ratio results from a decrease in phytase activity. On the other hand, a narrow Ca:tP ratio from 2.0 to 1.2:1 will lead to a 15.92% increase in phytase efficiency. Based on our previous study (Kornegay and Qian, 1994), .176 and .221% aP at .07% aP diet could be released from feedstuffs by supplementation of phytase of 700 and 1,050 U/kg diet, and .208 and .231% aP at .16% aP diet. The amount of released P could be increased to .211 and .265% aP for .07% aP diet, and to .233 and .296% aP for .16% aP diet by a decrease in the Ca:tP ratio from 2.0 to 1.2:1. So, in consideration of the released aP by phytase, the Ca:aP ratio in diets should be decreased to within 1.54 to 2.56: 1. This range of Ca:aP ratio was beneficial to growth and bone mineralization of pigs (NRC, 1988; Ketaren et al., 1993a).

Implications

Beneficial effects of microbial phytase supplementation in weanling pig diets were adversely effected by wide calcium:total phosphorus ratios, especially in the lower phosphorus diets. On the other hand, a decrease in calcium:total phosphorus ratios from 2.0 to 1.2:1 enhanced phytase efficiency approximately 16% by increasing the availability of phosphorus in plant ingredients. Supplemental phytase efficacy *in vivo* and *in vitro* favored the narrower calcium:total phosphorus ratio environment.

CHAPTER V

Utilization of Phytate Phosphorus and Calcium as Influenced by Microbial Phytase, Vitamin D_3 and the Calcium:Total Phosphorus Ratio in Broiler Diets

ABSTRACT The present study was performed to evaluate the potential of microbial phytase and vitamin D_3 ($\mathbf{D_3}$) for improving the utilization of phytate P and Ca and the influence of the Ca:total (t) P ratio in a corn soybean meal diet fed to broilers from hatch to 21 d of age. A 4 x 4 x 2 factorial arrangement of treatments was used: 1.1, 1.4, 1.7, and 2.0:1 Ca:tP ratio; 0, 300, 600, and 900 unit (U) of phytase/kg of diet; and 66 and 660 µg of vitamin D_3/kg of diet. Another four treatments were included: the four Ca:tP ratios with 6,600 µg of D_3 addition, but without phytase. Added phytase linearly increased ($P <$ .001) BW gain, feed intake, toe ash content, and P and Ca retention; these measurements were negatively influenced by widening the dietary Ca:tP ratio, and synergistically improved by addition of D_3. Widening the Ca:tP ratio decreased ($P <$ .001) all measurements in the presence or absence of supplemental phytase and D_3. Dietary Ca:tP ratios between 1.1:1 to 1.4:1 appears critical to the efficient use of supplemental phytase and D_3 for improving the utilization of phytate P and Ca. The addition of D_3 in corn-soybean meal diets indicated a potential for improving the utilization of phytate P and Ca by increasing Ca and P retention by about 5 to 12% in birds, which led to an increase in toe ash content ($P <$ .03). The enhanced phytate P utilization ($P <$ .001) was also observed during assay of the phytase activity in the mixed diets with an addition of D_3 and without added phytase. In summary, the findings of this study suggested that phytase, vitamin D_3, and Ca:tP are important factors in degrading phytate and improving phytate P and Ca utilization in broilers.

Key Words: Phytase, Vitamin D_3, Calcium, Phosphorus, Broilers

Introduction

Supplemental dietary microbial phytase has been shown to increase the availability of phytate P for poultry and pigs fed a commercial corn soybean meal diet. The P-equivalency of microbial phytase for 1 g of nonphytate (**n**) P is reported to be 650 to 750 unit (**U**) of phytase in broilers (Schoner et al., 1991; Kornegay et al., 1994, Yi et al., 1995c), 520 to 950 U of phytase in turkey poults (Qian et al., 1995; Ravindran et al., 1995), and 250 to 400 U of phytase in young pigs (Simons et al., 1990; Kornegay and Qian, 1994). These P-equivalent values of phytase were achieved when dietary Ca:total P (**tP**) ratios were formulated using a 2.0:1 Ca:tP ratio. Furthermore, the equivalence of phytase for nP was influenced by dietary Ca:tP ratios and nP levels because these two factors could affect not only phytate P release and P absorption in the small intestine, but also phytase activity (Wise, 1983; Schoner et al., 1993; Qian et al., 1995). A wider Ca:tP ratio was reported to decrease the performance and utilization of P in pigs (Qian et al., 1994a) and turkey poults (Qian et al., 1995) which was accompanied by a decrease in phytase activity of the diet. However, limited data are available examining the effect of Ca:tP ratios on the phytase efficacy in broilers.

It has been suggested that vitamin D_3 (**D_3**) can improve the phytate P and Ca absorption by stimulating the hydrolysis of phytate (Shafey et al., 1990; Mohammed et al., 1991). Edwards (1993) conducted two experiments in which the phytate P utilization greatly was enhanced by the addition of 5 to 10 μg of 1, 25-$(OH)_2D_3$/kg diet in the presence or absence of supplemental phytase; an interaction between phytase and 1, 25-$(OH)_2D_3$ only was found for the metabolizable energy value in one experiment. Mohammed et al. (1991) found that supplemental D_3 dramatically increased phytate P digestibility and the retention of P and Ca of the chick. However, this improvement by D_3 addition was also influenced by dietary Ca because the addition of D_3 could not totally overcome the P depletion except when dietary Ca was simultaneously lowered.

The purpose of the present study was to investigate the influence of supplemental phytase, vitamin D_3 and dietary Ca:tP ratios on the utilization of phytate P and Ca in broilers. Furthermore, the interaction of these three factors was examined.

Materials and Methods

Birds and dietary treatments. Day-old Peterson x Arbor Acres male broiler chicks (n = 864) were used in a 21-d experiment to investigate the utilization of phytate P and Ca in corn-soybean meal diets as influenced by microbial phytase (Natuphos; BASF Corp., 3000 Continental Drive North, Mount Olive, NJ), vitamin D_3 and dietary Ca:tP ratios. Three replicate pens (eight birds/pen) of a completely randomized design were used in a 4 x 4 x 2 factorial arrangement of treatments with 1.1, 1.4, 1.7, and 2.0:1 of Ca:tP ratios, with 0, 300, 600, and 900 U of phytase/kg of diet, and with 66 and 660 μg of D_3/kg of diet. In addition, another four treatments included the four Ca:tP ratios with 6,600 μg of D_3 added without supplemental phytase. The dietary P level was formulated at .27% of nonphytate P (.51% tP) for all diets, which was below the current NRC (1994) recommendations to ensure maximum responses with phytase additions. Composition of the basal diet is given in Table 1. The dietary percentage of phytate P (.24%) was calculated by using the data of NRC (1994) and was similar in all diets. The Ca:tP ratios were formulated by varying limestone at the expense of corn starch.

A U of phytase activity is defined as the quantity of enzyme that liberates 1 μmole of inorganic P per minute from 1.5 mM sodium phytate at pH 5.5 and 37°C. Supplemented microbial phytase activity in diets was determined using a modification of the method of Engelen et al. (1994). A suspension of supplemental phytase enzyme was extracted from about 5 g of dietary samples when mixed with 50 mL of .25 M, pH 5.5 buffer solution. Two milliliters of the suspension was cultured with 4 mL of 8.4 g/L sodium phytate solution (Sigma # P-3168) at 37°C for 1 h, and then colored and stopped

Table 1. Composition of the basal diet[a]

Ingredients, %	
Soybean meal (48.5% CP)	37.10
Corn (8.8% CP)	57.39
Canola oil	2.0
Limestone[b]	.601
Deflourinated phosphate[c]	.69
Vitamin premix[d]	.20
Trace mineral premix[e]	.20
Salt	.40
DL methionine	.20
Corn starch	1.219
Calculated analysis, %	
Ca	.561
Total P	.510
Nonphytate P	.27
CP	23.04
Lys	1.32
Met & Cys	.93

[a]Dietary Ca:tP ratios were formulated at 1.1, 1.4, 1.7 and 2.0 with defluorinated phosphate and limestone supplied at the expense of corn starch, and each Ca:tP ratio was supplemented with 0, 300, 600 and 900 U phytase/kg diet at D_3 levels of 66 and 660 µg/kg diet. Phytase (Natuphos®-5,000 U/g) was supplied by BASF Corp., 3000 Continental Drive North, Mount Olive, NJ 07828-1234.

[b]Limestone Dust Corp., Bluefield, VA 24605.

[c]Fine CDP, Southern Bag Corp., Valdosta, GA 31083.

[d]Supplied per kilogram of diet: retinyl acetate, 908µg ; cholecalciferol, 66µg ; dl-α -tocopheryl acetate, 26.5 mg•menadione sodium bisulfate complex, .75 mg; riboflavin, 7.5 mg; d-Ca-pantothenate acid, 9.7 mg; niacin, 26.4 mg; cyanocobalamin, 11µg; choline chloride, 1,013 mg; biotin, .31 mg; folic acid, 3.1 mg; thiamin•HCl, 8 mg; pyridoxine•HCl, 3.1 mg; ethoxyquin, 50 mg; virginiamycin, 2.9 mg.

[e]Supplied per kilogram of diet: Mn, 88 mg; Zn, 95 mg; Fe, 100 mg; Cu, 12.5 mg; I, 4 mg; Se, .6 mg.

by a mixed color-stop solution of ammoniummolybdate, ammoniumvanadate and nitric acid. The concentration of P released from sodium phytate by supplemental phytase was colorimetrically determined at 415 nm.

Feeding management. At 1 d of age, chicks were wingbanded and randomly assigned to pens in electrically heated, raised wire-floored battery brooders in an environmentally controlled room. Birds were exposed to continuous fluorescent light. All diets were provided for *ad libitum* consumption in mash form and birds had free access to water. Body weights and feed consumption were recorded on a pen basis at weekly intervals. The care and treatment of birds followed published guidelines (Consortium, 1988).

Sampling and analysis. During the 3rd wk (d 18 to 20), all excreta was collected from each pen and stored in plastic bags at -20 °C. Feed intake and production of excreta were measured quantitatively per pen over three consecutive days. After thawing, excreta were dried at 65 °C and weighed. Excreta and diet samples, were ground to pass a 1-mm sieve. Dry matter was determined according to AOAC (1990) procedures. Following a nitric-perchloric acid wet digestion, P concentrations were determined colorimetrically (AOAC, 1990) using the computer program "Microkinetics" and vertical photometer (Titertek Multiskan MCC/340: serial #1EEE-448). Concentrations of Ca were determined using an atomic absorption spectrophotometer. Apparent retention of P and Ca were calculated.

On d 21, all surviving birds were sacrificed. Toe samples were obtained by severing the middle toe through the joint between the 2nd and 3rd tarsal bones from the distal end. The left and right middle toes of all birds within a pen were pooled respectively, yielding two samples of toes per pen. The pooled samples were dried to a constant weight at 100 °C and ashed in a muffle furnace at 600 °C for 6 h. Toe ash was expressed as a percentage of dry weight.

Statistical analysis. Data were analyzed by the GLM procedure of the SAS® (1990) using pen as the experimental unit. Linear and quadratic effects of the Ca:tP ratio and supplemental phytase were tested with orthogonal polynomials. Second order

translog functions were developed for the 2 x 4 factorial arrangement of treatments of D_3 and phytase levels or Ca:tP ratios with the model: $LnY = a_0 + a_1D_1 + a_2LnX + a_3(lnX)^2 + a_4D_1LnX$. Where, Y = the response measurements; $D_1 = 0$ at $D_3 = 66$ μg/kg diet, and $D_1 = 1$ at $D_3 = 660$ μg/kg diet; X = added phytase, U/kg diet, or X = Ca:tP ratios. For the phytase and Ca:tP ratio effect, the model was used as $LnY = a_0 + a_1LnX_1 + a_2LnX_2 + a_3(LnX_1)^2 + a_4(LnX_2)^2 + a_5(LnX_1LnX_2)$, where Y = the response measurements; X_1 = added phytase, U/kg diet; X_2 = Ca:tP ratios. Nonlinear functions were created for the phytase effect at each D_3 level or Ca:tP ratio using the model: $Y = a(1 - b\ e^{-kX})$, where Y = the response measurement; X = added phytase, U/kg diet. For the Ca:tP ratio effect, the model for nonlinear functions was : $Y = a\ (1 - be^{-c+kX})$, or $Y = ae^{-kX}$, where, Y = the response measurement; X = Ca:tP ratios. Also, linear functions were derived for the phytase or Ca:tP ratio effect corresponding to different D_3 levels, Ca:tP ratios, or supplemental phytase levels. The model for linear functions was: $Y = a + bX$, where Y = the response measurement; X = added phytase levels, or Ca:tP ratios.

Results

Growth performance. Main effects of phytase and Ca:tP ratio on BW gain were observed. Body weight gain increased ($P < .001$) as phytase level increased, and decreased ($P < .001$) as the Ca:tP ratio became wider, and tended to increase ($P < .09$) as D_3 addition increased from 66 to 660 μg of D_3/kg of diet (Table 2). The magnitude of the responses to phytase was greatest for the narrowest Ca:tP ratio at both D_3 levels. The increase in BW gain by phytase supplements was linear up to 900 and 600 U of phytase/kg diet at 66 and 660 μg of D_3/kg diet, respectively. The effect of the Ca:tP ratio was independent of phytase and D_3 levels because two- and three-way interactions were not significant. This deterimental effect of a wide Ca:tP ratio was, however, greatest for the widest ratio and at the lower D_3 level with no added phytase. The maximum BW gains

Table 2. Body weight gain (g/bird) of broilers fed corn-soybean meal diets with four phytase levels, four Ca:tP ratios and three vitamin D_3 levels from hatch to 21 d of age[a]

Added phytase, U/kg diet[c]	Ca:tP ratio[b] 1.1:1	1.4:1	1.7:1	2.0:1	Mean
66 µg of D_3/kg diet[d]					
0	553	550	499	420	506
300	576	547	533	496	538
600	576	570	566	528	560
900	615	568	560	541	571
Mean	580	559	539	496	544
660 µg of D_3/kg diet[e]					
0	563	553	496	462	518
300	561	568	532	493	538
600	611	590	581	498	570
900	611	567	549	542	569
Mean	588	569	539	499	549
6,600 µg of D_3/kg diet	566	543	508	451	517

[a]Each mean represents three pens (eight birds per pen). The root MSE were 23.3 for BW gain and the pooled SEM for a single treatment mean would equal MSE/$\sqrt{3}$. Main effect: Ca:tP ratio and phytase ($P < .001$); D_3 ($P < .09$). Ca:tP ratio x phytase interaction ($P = .11$).

[b]Phytase effect: linear ($P < .001$) and quadratic ($P = .11$); at Ca:tP ratio = 1.1, linear ($P < .001$); at Ca:tP ratio = 1.4, linear ($P < .008$); at Ca:tP ratio = 1.7, linear ($P = 12$); at Ca:tP ratio = 2.0, linear ($P < .003$) and quadratic ($P < .08$).

[c]Ca:tP ratio effect: linear ($P < .003$), and quadratic ($P < .001$); at phytase = 0, linear ($P < .02$) and quadratic ($P < .001$); at phytase = 300, linear ($P = .14$) and quadratic ($P < .001$); at phytase = 600, linear ($P < .02$) and quadratic ($P < .006$); at phytase = 900, quadratic ($P < .01$).

[d]Ca:tP ratio effect: linear ($P < .07$) and quadratic, ($P < .001$). Phytase effect: linear ($P < .001$).

[e]Ca:tP ratio effect: linear ($P < .09$) and quadratic ($P < .001$). Phytase effect: linear, ($P < .01$).

Table 3. Feed intake (g/bird) of broilers fed corn-soybean meal diets with four phytase levels, four Ca:tP ratios and three vitamin D_3 levels from hatch to 21 d of age[a]

Added phytase, U/kg diet[c]	Ca:tP ratio[b] 1.1:1	1.4:1	1.7:1	2.0:1	Mean
66 µg of D_3/kg diet[d]					
0	797	763	785	644	756
300	823	763	779	726	773
600	859	844	835	787	831
900	835	832	817	777	816
Mean	828	826	804	734	803
660 µg of D_3/kg diet[e]					
0	896	807	731	717	788
300	854	851	801	765	817
600	936	885	855	757	858
900	927	917	813	805	865
Mean	903	865	780	760	832
6,600 µg of D_3/kg diet	909	773	755	673	777

[a]Each mean represents three pens (eight birds per pen). The root MSE were 38.9 for feed intake and the pooled SEM for a single treatment mean would equal MSE/$\sqrt{3}$. Main effect: Ca:tP ratio ($P < .001$); phytase ($P < .01$); D_3 ($P < .04$).

[b]Phytase effect: linear ($P < .002$); at Ca:tP ratio = 1.1, linear ($P < .005$); at Ca:tP ratio = 1.7, linear ($P = .14$); at Ca:tP ratio = 2.0, linear ($P < .07$).

[c]Ca:tP ratio effect: linear ($P < .03$) and quadratic ($P < .001$); at phytase = 0, linear ($P = .12$) and quadratic ($P < .001$); at phytase = 300, quadratic ($P < .05$); at phytase = 600, linear ($P = .13$) and quadratic ($P < .01$); at phytase = 900, quadratic ($P < .008$).

[d]Ca:tP ratio effect: linear ($P < .04$) and quadratic, ($P < .001$). Phytase effect: linear ($P = .13$).

[e]Ca:tP ratio effect: quadratic ($P < .001$). Phytase effect: linear, ($P < .02$).

resulted when dietary Ca:tP ratio was narrowed to 1.1:1 at each supplemental level of phytase or D_3.

Main effects of phytase, Ca:tP ratio and D_3 on feed intake were similar to those for BW gain (Table 3). Feed intake increased ($P < .001$) as the phytase level increased, decreased ($P < .01$) as the Ca:tP ratio became wider, and increased ($P < .04$) from 66 to 660 μg of D_3/kg. The magnitude of the response to added phytase was greater at the lower levels of phytase and the wider Ca:tP ratios in both levels of D_3. Widening the Ca:tP ratios decreased feed intake, the effect being independent of both phytase and D_3 levels since two-way interactions were not significant ($P > .10$).

Increasing D_3 above 660 μg/kg appeared to have no additional benefit for improving BW gain and feed intake (Tables 2 and 3). No effects were observed on feed efficiency (data not shown).

Toe ash content. The influence of phytase addition, Ca:tP ratio, and D_3 on toe ash percentage tended to parallel that of BW gains and feed intake ($P < .001$, .001, and .03, respectively) (Table 4). The effects of these three factors appeared independent because two- and three-way interactions were not significant. Supplemental phytase linearly increased toe ash percent at each D_3 level ($P < .001$) and Ca:tP ratio ($P < .004$). The magnitude of the response to supplemental phytase tended to be greatest at widest Ca:tP level at each level of D_3. Optimum dietary Ca:tP ratio was 1.4:1 for toe mineralization at 66 μg of D_3/kg diet, and 1.1:1 at 660 μg of D_3/kg diet. The highest values for toe ash were obtained at the two lower Ca:tP ratios and at the higher levels of phytase. By comparing only means without added phytase, toe ash percent linearly increased ($P < .05$) as the level of D_3 increased for all Ca:tP ratios.

Retention of P and Ca. The retention of P was increased linearly as added phytase increased ($P < .001$), was increased ($P < .001$) quadratically as the Ca:tP ratio became narrower, and was higher ($P < .001$) for 660 vs 66 μg D_3/kg diet (Table 5). Two- or

Table 4. Toe ash content (%) of broilers fed corn-soybean meal diets with four phytase levels, four Ca:tP ratios and three vitamin D_3 levels from hatch to 21 d of age[a,b]

Added phytase, U/kg diet[d]	Ca:tP ratio[c] 1.1:1	1.4:1	1.7:1	2.0:1	Mean
66 µg of D_3/kg diet[e]					
0	11.7	11.3	10.9	9.9	10.9
300	11.9	12.0	11.8	11.1	11.7
600	11.9	12.1	11.9	11.4	11.8
900	12.5	12.9	11.9	11.6	12.2
Mean	12.0	12.1	11.6	11.0	11.7
660 µg of D_3/kg diet[f]					
0	11.9	11.7	11.2	10.8	11.4
300	12.2	12.0	11.3	11.1	11.7
600	12.8	12.4	11.8	11.4	11.8
900	12.9	12. 9	12.1	12.0	12.5
Mean	12.5	12.2	11.6	11.1	11.8
6,600 µg of D_3/kg diet	12.4	12.2	11.8	11.7	12.0

[a]Each mean represents three pens (eight birds per pen). The root MSE were .43 for toe ash content and the pooled SEM for a single treatment mean would equal MSE/$\sqrt{3}$. Main effect: Ca:tP ratio ($P < .001$); phytase ($P < .001$); D_3 ($P < .03$).

[b]D_3 effect by comparing treatments of three levels of D_3 without phytase supplements: linear ($P < .05$); at Ca:tP ratio = 1.1, linear ($P < .001$); at Ca:tP ratio = 1.4, linear ($P < .005$); at Ca:tP ratio = 1.7, linear ($P < .09$); at Ca:tP ratio = 2.0, linear ($P < .07$).

[c]Phytase effect: linear ($P < .001$); at Ca:tP ratio = 1.1, linear ($P < .001$); at Ca:tP = 1.4, linear ($P < .004$); at Ca:tP ratio = 1.7, linear ($P < .001$); at Ca:tP ratio = 2.0, linear ($P < .002$).

[d]Ca:tP ratio effect: linear ($P < .001$) and quadratic ($P < .001$); at phytase = 0, linear ($P < .08$) and quadratic ($P < .001$); at phytase = 300, quadratic ($P < .03$); at phytase = 600, linear ($P < .02$) and quadratic ($P < .001$); at phytase = 900, quadratic ($P < .001$).

[e]Ca:tP ratio effect: linear ($P < .02$) and quadratic, ($P < .001$). Phytase effect: linear ($P < .001$).

[f]Ca:tP ratio effect: linear ($P = .10$) and quadratic ($P < .001$). Phytase effect: linear, ($P < .001$).

Table 5. Phosphorus retention (%) of broilers fed corn-soybean meal diets with four phytase levels, four Ca:tP ratios and three vitamin D_3 levels from hatch to 21 d of age[a, b]

Added phytase, U/kg diet[d]	Ca:tP ratio[c] 1.1:1	1.4:1	1.7:1	2.0:1	Mean
66 µg of D_3/kg diet[e]					
0	54.60	54.15	52.02	50.87	52.91
300	58.30	57.90	54.19	52.81	55.80
600	59.25	59.26	58.23	51.75	57.02
900	59.58	59.10	58.71	56.09	58.36
Mean	57.93	57.60	55.57	52.88	56.02
660 µg of D_3/kg diet[f]					
0	58.53	55.50	53.35	54.29	55.42
300	61.47	57.88	54.33	54.40	57.02
600	61.77	61.11	60.14	55.16	59.55
900	67.98	63.00	59.66	55.05	61.42
Mean	61.03	60.78	56.87	54.72	58.35
6,600 µg of D_3/kg diet	61.30	59.31	57.23	54.75	58.15

[a]Each mean represents three pens (eight birds per pen). The root MSE were 2.34 for P retention and the pooled SEM for a single treatment mean would equal MSE/$\sqrt{3}$. Main effect: Ca:tP ratio ($P < .001$); phytase ($P < .001$); D_3 ($P < .001$).

[b]D_3 effect by comparing treatments of three levels of D_3 without phytase supplements: linear ($P < .003$); at Ca:tP ratio = 1.1, linear ($P = .16$); at Ca:tP ratio = 1.4, linear ($P < .05$); at Ca:tP ratio = 1.7 ($P = .12$); at Ca:tP ratio = 2.0, linear ($P = .1$).

[c]Phytase effect: linear ($P < .001$); at Ca:tP ratio = 1.1, linear ($P = .2$); at Ca:tP = 1.4, linear ($P < .004$); at Ca:tP ratio = 1.7, linear ($P < .001$); at Ca:tP ratio = 2.0, linear ($P < .005$).

[d]Ca:tP ratio effect: linear ($P < .05$) and quadratic ($P < .001$); at phytase = 0, quadratic ($P < .07$); at phytase = 300, quadratic ($P < .004$); at phytase = 600, linear ($P < .02$), and quadratic ($P < .05$); at phytase = 900, linear ($P < .08$); quadratic ($P < .001$).

[e]Ca:tP ratio effect: quadratic, ($P < .007$). Phytase effect: linear ($P < .005$).

[f]Ca:tP ratio effect: quadratic ($P < .001$). Phytase effect: linear, ($P < .001$).

Table 6. Calcium retention (%) of broilers fed corn-soybean meal diets with four phytase levels, four Ca:tP ratios and three vitamin D_3 levels from hatch to 21 d of age[a, b]

Added phytase, U/kg diet[d]	Ca:tP ratio[c] 1.1:1	1.4:1	1.7:1	2.0:1	Mean
66 µg of D_3/kg diet[e]					
0	58.44	54.06	44.76	42.58	49.96
300	61.37	52.12	45.29	43.67	50.61
600	61.98	56.03	55.84	44.34	54.43
900	61.85	59.95	49.30	45.54	54.16
Mean	60.91	55.54	48.16	44.03	52.25
660 µg of D_3/kg diet[f]					
0	60.47	52.74	47.09	44.43	51.18
300	66.11	54.88	43.87	43.13	52.00
600	62.85	57.95	52.46	48.10	55.34
900	67.63	65.36	53.66	46.27	58.23
Mean	64.26	57.73	49.27	45.47	54.19
6,600 µg of D_3/kg diet	63.35	57.57	49.67	48.67	54.82

[a]Each mean represents three pens (eight birds per pen). The root MSE were 3.13 for Ca retention and the pooled SEM for a single treatment mean would equal MSE/$\sqrt{3}$. Main effect: Ca:tP ratio ($P < .001$); phytase ($P < .001$); D_3 ($P > .07$).

[b]D_3 effect by comparing treatments of three levels of D_3 without phytase supplements: linear ($P < .02$); at Ca:tP ratio = 1.1, linear ($P < .06$); at Ca:tP ratio = 1.4, linear ($P = .19$).

[c]Phytase effect: linear ($P < .001$); at Ca:tP ratio = 1.1, linear ($P = .2$); at Ca:tP = 1.4, linear ($P < .04$); at Ca:tP ratio = 1.7, linear ($P < .004$); at Ca:tP ratio = 2.0, linear ($P < .003$).

[d]Ca:tP ratio effect: quadratic ($P < .001$); at phytase = 0, quadratic ($P < .001$); at phytase = 300, quadratic ($P < .001$); at phytase = 600, quadratic ($P < .001$); at phytase = 900, quadratic ($P < .001$).

[e]Ca:tP ratio effect: quadratic, ($P < .001$). Phytase effect: linear ($P = .16$).

[f]Ca:tP ratio effect: quadratic ($P < .001$). Phytase effect: linear, ($P < .02$).

three-way interactions of these three factors were not significant. At Ca:tP ratios of 1.1:1 and 1.4:1, the magnitude of the response to added phytase was much larger for diets with 660 μg of D_3 than for those with 66 μg of D_3. By comparing only means without added phytase, the retention of P increased linearly ($P < .001$) as the level of D_3 increased. The maximum retention of P at both levels of D_3 was achieved when dietary Ca:tP ratio was formulated at 1.1:1 with 600 to 900 U of phytase/kg of diet.

Similar to P retention, the retention of Ca increased ($P < .001$) linearly as the level of phytase increased, was increased ($P < .001$) quadratically as the Ca:tP ratio became narrower, and was higher ($P < .07$) for 660 vs 66 μg of D_3/kg (Table 6). The increase in Ca retention by supplemental phytase was more dramatic in combination with a narrow Ca:tP ratio and high D_3 level. The two-way interactions were not significant suggesting that the response to phytase, Ca:tP ratio and D_3 were independent. The optimum response to the three dietary factors was observed when dietary Ca:tP ratio was formulated at 1.1:1 with the supplementation of 900 U of phytase and 660 μg of D_3 per kg of diet.

The activity of supplemental phytase in diets. The supplemented phytase activity of the diet was decreased ($P < .001$) linearly as the Ca:tP ratio became wider at each phytase or D_3 level (Table 7). The negative effect of the Ca:tP ratio was greater at lower levels of phytase supplementation, but was independent of D_3 levels. Vitamin D_3 also linearly increased ($P < .001$) the release of P from phytate in the absence of supplemental phytase in diets. At each phytase level, the assayed phytase activity was higher than the supplemental value. The increase in the determined values of phytase activity was greater at high levels of phytase and D_3 supplementation. All two-way interactions between phytase, Ca:tP ratios and D_3 were significant ($P < .01$).

Response curves. Coefficients, P-values and r^2 values for second order translog equations of performance, toe ash content, P and Ca retention are shown in Table 8 for

Table 7. Dietary phytase activity (U/kg diet) of the corn-soybean meal diets formulated with four phytase levels, four Ca:tP ratios and three vitamin D_3 levels[a, b]

Added phytase, U/kg diet[d]	Ca:tP ratio[c] 1.1:1	1.4:1	1.7:1	2.0:1	Mean
66 µg of D_3/kg diet[e]					
0[f]	0	0	0	0	0
300	385	358	350	306	350
600	956	915	855	804	882
900	1,264	1,235	1,154	1,103	1,189
Mean	651	627	589	553	605
660 µg of D_3/kg diet[g]					
0	174	146	114	108	135
300	451	400	378	331	390
600	1,018	974	902	843	934
900	1,319	1,263	1,200	1,151	1,233
Mean	741	696	649	606	673
6,600 µg of D_3/kg diet					
	249	222	185	184	210

[a]Each mean represents four replicates. The root MSE were 15.5 for phytase activity and the pooled SEM for a single treatment mean would equal MSE/$\sqrt{4}$. Main effect: Ca:tP ratio ($P < .001$); phytase ($P < .001$); D_3 ($P < .001$). D_3 x phytase interaction ($P < .001$); D_3 x Ca:tP ratio ($P < .008$); phytase x Ca:tP ratio ($P < .001$).

[b]D_3 effect by comparing treatments of three levels of D_3 without phytase supplements: linear ($P < .001$) and quadratic ($P < .001$); at Ca:tP ratio = 1.1, linear ($P < .001$); at Ca:tP ratio = 1.4, linear ($P < .001$), quadratic ($P < .001$); at Ca:tP ratio = 1.7 ($P < .001$), quadratic ($P < .004$); at Ca:tP ratio = 2.0, linear ($P < .001$), quadratic ($P < .07$).

[c]Phytase effect: linear ($P < .001$); at Ca:tP ratio = 1.1, linear ($P < .001$); at Ca:tP = 1.4, linear ($P < .001$); at Ca:tP ratio = 1.7, linear ($P < .001$); at Ca:tP ratio = 2.0, linear ($P < .001$).

[d]Ca:tP ratio effect: linear ($P < .03$) and quadratic ($P < .001$); at phytase = 300, quadratic ($P < .02$); at phytase = 600, linear ($P < .001$); at phytase = 900, linear ($P < .05$), quadratic ($P < .001$).

[e]Phytase effect: linear ($P < .001$).

[f]The diets without phytase supplementation were used as blank or '0' value for the assay of phytase activity in diets with microbial phytase supplementation.

[g]Phytase effect: linear, ($P < .001$).

Table 8. Second order translog functions for performance, toe ash content and P and Ca retention of broilers fed corn-soybean meal diets with four Ca:tP ratios, four phytase and two vitamin D_3 levels from hatch to 21 d of age

Item	Coefficients of equations						P-value	r^2
	a_0	a_1	a_2	a_3	a_4	a_5		
Phytase and D_3 effect[a]								
BW gain, g/bird	6.1286	.0156	.0094	.0032	-.0018		.001	.283
Feed intake, g/bird	6.5167	.0233	.0040	.0037	.0039		.023	.216
Toe ash content, %	2.2891	.0204	.0087	.0032	-.0032		.001	.272
P retention, %	3.8515	.0416	.0077	.0035	-.0004		.001	.239
Ca retention, %	3.6675	.0316	.0082	.0060	.0008		.101	.071
Ca:tP ratio and D_3 effect[b]								
BW gain, g/bird	6.3504	.0217	.0861	-.4227	-.0278		.001	.461
Feed intake, g/bird	6.7420	.0651	.0425	-.3441	-.0732		.001	.458
Toe ash content, %	2.4701	.0346	.1555	-.3995	-.0549		.001	.350
P retention, %	4.0493	.0568	.1213	-.3511	-.0382		.001	.283
Ca retention, %	4.1550	.0525	-.4163	-.1940	-.0403		.001	.543
Phytase and Ca:tP ratio effect[c]								
BW gain, g/bird	6.2174	.0028	.0308	.0032	-.4227	.0138	.001	.666
Feed intake, g/bird	6.6244	-.0016	-.0484	.0037	-.3441	.0181	.001	.501
Toe ash content, %	2.3539	.0053	.0990	.0029	-.3786	.0043	.001	.598
P retention, %	3.9016	.0091	.1210	.0035	-.3591	-.0038	.001	.501
Ca retention, %	3.9028	.0097	-.4215	.0059	-.2019	-.0026	.001	.591

[a]Model: $LnY = a_0 + a_1D_1 + a_2LnX + a_3(LnX)^2 + a_4D_1LnX$. At 66 µg of D_3/kg diet, $D_1 = 0$; At 660 µg of D_3/kg diet, $D_1 = 1$. X = added phytase, U/kg diet; or X = Ca:tP ratios.

[b]Model: $LnY = a_0 + a_1 LnX_1 + a_2LnX_2 + a_3(LnX_1)^2 + a_4(LnX_2)^2 + a_5(LnX_1LnX_2)$. X_1 = added phytase, U/kg diet. X_2 = Ca:tP ratios.

phytase and D_3, Ca:tP ratio and D_3, and phytase and Ca:tP ratio. Equations for phytase and D_3 or Ca:tP ratios and D_3 generally had low r^2 values (< .5), whereas, equations for phytase and Ca:tP ratio had relatively higher r^2 (> .50) and low P-values ($P < .001$). Nonlinear response equations and r^2 values are shown in Table 9, and linear response r^2 values and P-values are shown in Table 10 for D_3 levels or each of the four phytase levels. For BW gain, toe ash content and P retention, almost all equations had high r^2 (mostly r^2 > .90). It was observed that the phytase effect seemed more nonlinear, whereas, the Ca:tP ratio effect was more linear to responses because nonlinear equations to phytase and linear equations to the ratio relatively had higher r^2 and lower P-values. Based on the r^2 and P-value of developed equations, BW gain, toe ash contents and P retention were found to be the most sensitive indicators to assess the effects of the three factors in broilers.

In the evaluation of the negative effect of widening the Ca:tP ratio, the main effects of Ca:tP ratios on BW gain and toe ash contents were used. Narrowing the Ca:tP ratio from 2.0:1 to 1.4:1 in average resulted in an increase in the phytase efficacy of 11.1 and 12.2%, respectively for the 66 and 660 μg of D_3/kg diet, which was close to the change in phytase activity observed in diets (13.4 and 14.9%, respectively).

Discussion

The high content of phytate P in corn and soybean meal leads to limited availabilities of P, Ca, and trace minerals for poultry fed corn-soybean meal diets (Nelson et al., 1968a; Reddy et al., 1982). Nelson et al. (1968b) first reported that microbial preparation containing phytase greatly improved utilization of phytate P when supplemented in broiler diets. Phytase has also been reported to improve the utilization of phytate Ca (Simons et al., 1990; Qian et al., 1994b; Schoner et al., 1994). Schoner et al. (1994) suggested that 500 U of phytase diet is equivalent to .35 to .45 g of Ca in diets fed to broilers. Phytic acid, a cation chelator, makes Ca unavailable for intestinal absorption.

Table 9. Nonlinear functions for performance, toe ash content and P and Ca retention of broilers fed corn-soybean meal diets with four Ca:tP ratios, four phytase and two vitamin D_3 levels from hatch to 21 d of age

Item	Phytase effect[a]		Ca:tP ratio effect[b]	
	Equation	r^2	Equation	r^2
	66 µg of D_3/kg diet		66 µg of D_3/kg diet	
Weight gain, g/bird	$Y = 590.89 (1 - .1444 e^{-.00160 X})$	1.000	$Y = 607.13 (1 - .9443 e^{-4.64700 + 1.49990 X})$	.995
Feed intake, g/bird	$Y = 1228.7 (1 - .3623 e^{-.00008 X})$	.534	$Y = 857.2 (1 - 2.5262 e^{-8.08421 + 2.60780 X})$	.993
Toe ash content, %	$Y = 12.46 (1 - .1280 e^{-.00170 X})$	.957	$Y = 12.08 (1 - .2.5231 e^{-9.08422 + 2.93040 X})$	.974
P retention, %	$Y = 59.82 (1 - .1148 e^{-.00165 X})$	.986	$Y = 65.50 e^{-.10153 X}$	.993
Ca retention, %	$Y = 60.86 (1 - .1840 e^{-.00065 X})$	.834	$Y = 92.11 e^{-.37187 X}$	.999
	660 µg of D_3/kg diet		660 µg of D_3/kg diet	
BW gain, g/bird	$Y = 578.09 (1 - .1054 e^{-.00210 X})$	.925	$Y = 639.22 (1 - .7529 e^{-3.47142 + 1.11981 X})$	1.000
Feed intake, g/bird	$Y = 913.04 (1 - .1389 e^{-.00120 X})$	.974	$Y = 9440.9 (1 - .9392 e^{-.05890 + .01901 X})$	.992
Toe ash content, %	$Y = 55.720 (1 - .8007 e^{-.000025 X})$	.746	$Y = 13.55 (1 - .5305 e^{-2.85850 + .92211 X})$	.992
P retention, %	$Y = 326.94 (1 - .8383 e^{-.00003 X})$	.818	$Y = 71.26 e^{-.12949 X}$	.984
Ca retention, %	$Y = 277.06 (1 - .8226 e^{-.00003 X})$	.800	$Y = 100.09 e^{-.40178 X}$	.984
	Ca:tP ratio = 1.1:1		phytase = 0, U/kg diet	
BW gain, g/bird	$Y = 992.75 (1 - .442 e^{-.00012 X})$	.954	$Y = 591.93 (1 - .9618 e^{-5.72070 + 1.8454 1X})$	.989
Feed intake, g/bird	----		$Y = 1186.0 (1 - .8175 e^{-1.83021 + .59040X})$	.999
Toe ash content, %	$Y = 37.14 (1 - .6865 e^{-.00004 X})$	.936	$Y = 12.40 (1 - .7234 e^{-3.97920 + 1.28360 X})$	.999
P retention, %	$Y = 61.15 (1 - .0744 e^{-.00373 X})$	.875	$Y = 61.99 e^{-.08732 X}$	.846
Ca retention, %	$Y = 385.20 (1 - .8486 e^{-.00002 X})$	.828	$Y = 88.94 e^{-.36924 X}$	.981
	Ca:tP ratio = 1.4:1		phytase = 300, U/kg diet	
BW gain, g/bird	$Y = 570.69 (1 - .0353 e^{-.00330 X})$	.654	$Y = 597.94 (1 - .9442 e^{-4.78361 + 1.54313 X})$	1.000
Feed intake, g/bird	$Y = 1271.5 (1 - .3561 e^{-.00012 X})$	.562	$Y = 910.08 (1 - .4682 e^{-2.70631 + .87323 X})$	.982
Toe ash content, g/g	$Y = 71.74 (1 - .8415 e^{-.00025 X})$	.962	$Y = 12.48 (1 - .5236 e^{-4.25572 + 1.37281 X})$	.983
P retention, %	$Y = 428.90 (1 - .8720 e^{-.00003 X})$	.943	$Y = 69.30 e^{-13339 X}$	.991
Ca retention, %	$Y = 158.14 (1 - .6949 e^{-.00007 X})$	.645	$Y = 104.92 e^{-.46976 X}$	.948

	Ca:tP ratio = 1.7:1		phytase = 600, U/kg diet	
BW gain, g/bird	$Y = 567.19 (1 - .1253\ e^{-.00330\ X})$	.875	$Y = 705.24\ e^{-.14391\ X}$	.816
Feed intake, g/bird	$Y = 833.33 (1 - .0930\ e^{-.00311\ X})$	.780	$Y = 913.41 (1 - 1.5759\ e^{-6.55991 + 2.11610\ X})$	.988
Toe ash content, %	$Y = 12.09 (1 - .0959\ e^{-.002\ 21\ X})$	.999	$Y = 12.4107 (1 - 2.9492\ e^{-8.82230 + 2.84591\ X})$	1.000
P retention, %	$Y = 65.93 (1 - .206\ e^{-.00088\ X})$	.970	$Y = 70.17\ e^{-.11916\ X}$	.956
Ca retention, %	$Y = 74.20 (1-.3945\ e^{-.00034\ X})$	.653	$Y = 88.4658\ e^{-.31178\ X}$	.971
	Ca:tP ratio = 2.0:1		phytase = 900, U/kg diet	
BW gain, g/bird	$Y = 571.15 (1 - .2259\ e^{-.00150\ X})$	.985	$Y = 696.43\ e^{-.13220\ X}$	.863
Feed intake, g/bird	$Y = 804.16 (1 - .1531\ e^{-.00240\ X})$	.999	$Y = 998.97 (1 - .4595\ e^{-2.18241 + .70401\ X})$	.932
Toe ash content, %	$Y= 73.07 (1 - .8599\ e^{-.00002\ X})$	.666	$Y = 13.66 (1 - .3906\ e^{-2.79310 + .90102\ X})$	.819
P retention, %	$Y = 140.29 (1 - .6366\ e^{-.00004\ X})$	.993	$Y = 71.79\ e^{-.11739\ X}$	.992
Ca retention, %	$Y = 60.29 (1 - .2830\ e^{-.00022\ X})$	.735	$Y = 102.83\ e^{-.39540\ X}$	.972

[a]Phytase effect at two D_3 levels or four Ca:tP ratios, X = added phytase, U/kg diet. The equations were generated across four Ca:tP ratios or two D_3 levels.

[b]Ca:tP ratio effect at two D_3 levels or four phytase levels, X = Ca:tP ratios. The equations were generated across four phytase levels or two D_3 levels.

Table 10. Linear functions for performance, toe ash content and P and Ca retention of broilers fed corn-soybean meal diets with four Ca:tP ratios, four phytase and two vitamin D_3 levels from hatch to 21 d of age

Item	Phytase effect[a]			Ca:tP ratio effect[b]		
	Equation	r^2	P-value	Equation	r^2	P-value
	66 µg of D_3/kg diet			66 µg of D_3/kg diet		
BW gain, g/bird	Y = 511.03 + .0726 X	.954	.023	Y = 683.50 - 90.1808 X	.960	.020
Feed intake, g/bird	Y = 783.81 +.0427 X	.417	.354	Y = 992.08 - 121.9557 X	.905	.049
Toe ash content, %	Y = 10.97 + .0014 X	.922	.040	Y = 13.45 - 1.2112 X	.844	.082
P retention, %	Y = 53.38 + .0059 X	.953	.024	Y = 62.59 - 5.4466 X	.936	.033
Ca retention, %	Y = 49.83 + .0055 X	.826	.090	Y = 82.14 - 19.3390 X	.989	.005
	660 µg of D_3/kg diet			660 µg of D_3/kg diet		
BW gain, g/bird	Y = 522.93 + .0563 X	.841	.083	Y = 701.30 - 98.7259 X	.979	.010
Feed intake, g/bird	Y = 791.19 + .0910 X	.944	.029	Y = 1086.42 - 164.0398 X	.989	.006
Toe ash content, %	Y = 11.24 + .0011 X	.901	.050	Y = 14.26 - 1.6275 X	.973	.013
P retention, %	Y = 55.27 + .0068 X	.993	.003	Y = 71.35 - 9.0820 X	.987	.007
Ca retention, %	Y = 50.51 + .0082 X	.953	.024	Y = 87.66 - 21.5961 X	.981	.010
	Ca:tP ratio = 1.1:1			phytase = 0, U/kg diet		
BW gain, g/bird	Y = 556.38 + .0617 X	.992	.004	Y = 721.92 - 135.4115 X	.920	.041
Feed intake, g/bird	----			Y = 1149.18 - 231.6640 X	.992	.004
Toe ash content, %	Y = 11.71 + .001 X	.994	.003	Y = 13.62 - 1.6560 X	.972	.014
P retention, %	Y = 57.31 + .0048 X	.842	.082	Y = 59.79 - 4.8066 X	.790	.101
Ca retention, %	Y = 60.41 + .0048 X	.668	.183	Y = 79.15 - 18.4383 X	.966	.017
	Ca:tP ratio = 1.4:1			phytase = 300, U/kg diet		
BW gain, g/d	Y = 554.40 + .0189 X	.352	.406	Y = 671.87 - 85.6415 X	.960	.020
Feed intake, g/d	Y = 818.79 + .0589 X	.555	.255	Y = 947.84 - 98.4975 X	.971	.015
Toe ash content, g/d	Y = 11.37 + .0015 X	.966	.017	Y = 13.37 - 1.1287 X	.947	.027
P retention, %	Y = 54.87 + .0096 X	.999	.001	Y = 65.49 - 6.8831 X	.985	.008
Ca retention, %	Y = 51.95 + .0104 X	.862	.072	Y = 87.44 - 23.3104 X	.916	.043

	Ca:tP ratio = 1.7:1			phytase = 600, U/kg diet		
BW gain, g/bird	Y = 507.68 + .0705 X	.703	.162	Y = 693.00 - 82.6383 X	.807	.101
Feed intake, g/bird	Y = 768.06 + .0756 X	.625	.209	Y = 1048.91 - 131.7910 X	.924	.039
Toe ash content, %	Y = 11.03 + .0011 X	.940	.031	Y = 14.23 - 1.6051 X	.887	.058
P retention, %	Y = 51.27 + .0081 X	.881	.062	Y = 66.85 - 6.4311 X	.809	.090
Ca retention, %	Y = 52.68 + .0082 X	.622	.211	Y = 79.15 - 16.6946 X	.956	.022
	Ca:tP ratio = 2.0:1			phytase = 900, U/kg diet		
BW gain, g/bird	Y = 449.47 + .1068 X	.952	.024	Y = 682.81 - 74.1220 X	.844	.081
Feed intake, g/bird	Y = 693.67 + .119 X	.919	.042	Y = 1011.07 - 110.0384 X	.921	.040
Toe ash content, %	Y = 10.30 + .0015 X	.790	.111	Y = 14.16 - 1.2148 X	.795	.109
P retention, %	Y = 50.99 + .0031 X	.993	.003	Y = 75.59 - 10.7370 X	.974	.013
Ca retention, %	Y = 41.40 + .0040 X	.476	.310	Y = 94.78 - 25.7634 X	.978	.011

[a]Phytase effect at two D_3 levels or four Ca:tP ratios, X = added phytase, U/kg diet. The equations were generated across four Ca:tP ratios or two D_3 levels.

[b]Ca:tP ratio effect at two D_3 levels or four phytase levels, X = Ca:tP ratios. The equations were generated across four phytases or two D_3 levels.

Phytase releases Ca from the insoluble salts of phytic acid, and potentially makes Ca available for absorption in birds.

Recently, strong evidence indicates that microbial phytase is highly effective in degrading phytate (Simons., et al., 1990; Schoner et al., 1991; Denbow et al., 1993). In our previous study, Kornegay et al. (1994) reported that 735 U of phytase/kg diet was equivalent to 1 g of nonphytate P for broilers fed corn-soybean meal diets, and that about 20 to 60% of phytate P was hydrolyzed by graded levels of supplemental phytase. Findings of the present study are in agreement with the results reported by Kornegay et al. (1994). Graded levels of phytase increased Ca and P retention by up to 18% and 14% respectively for 66 and 660 μg of D_3/kg diet. That increased utilization of phytate Ca and P results in increased Ca and P retention is also supported by our findings of an increase in Ca, P, Zn, and Mg in bone ash and improved bone calcification and histological development (Qian et al., 1994b,c) when phytase was added to broiler and turkey poult diets.

Calcium is thought to be a key factor influencing the activity of mucosal phytase in small intestines of poultry and rat (Bhandari, 1980; Wise, 1983). This effect was also observed in the present study in which the activity of the microbial phytase added in the mixed diets was greatly decreased by a wide Ca:tP ratio. The decrease in the phytase activity as the Ca:tP ratio became wider could be explained as follows: 1) phytate P utilization of corn-soybean diets by broilers is influenced by Ca and P levels in the diet (Edwards and Veltmann 1983; Ballam et al., 1984); 2) extra Ca binds with phytate to form an insoluble complex that is less accessible to phytase; 3) the extra Ca could directly repress phytase activity by competing for the active sites of the enzymes (Wise, 1983; Pointillart et al., 1985). The negative effect is stronger at lower levels of supplemental phytase as well as at lower levels of available P. At lower levels of phytase, a limited amount of P would be released from phytate, which led to a P deficient environment

leading to influencing phytase activity (Qian et al., 1994a; 1995). The present study indicates that a widening Ca:tP ratios from 1.4:1 to 2.0:1 reduced phytase activity by 13.4 and 14.9%, respectively, for the mixed diets of 66 and 660 μg of D_3/kg diet, which was consistent with our two previous studies (Qian et al., 1994a; 1995).

In agreement with results of the phytase activity of diets, a wider Ca:tP ratio negatively influenced the phytase efficacy in broilers and decreased all measurements at each phytase and D_3 level. Schoner et al. (1991; 1993) also observed that the addition of Ca, thus widening the Ca:tP ratio, decreased all measurements whereas narrowing the Ca:tP ratio improved all measurements. In turkeys, widening the dietary Ca:tP ratios from 1.4:1 to 2.0:1 resulted in a decrease in the phytase efficacy by 7.4 and 4.9%, respectively for .27 and .36% nP diets (Qian et al., 1995). Widening the dietary Ca:tP ratio from 2.0:1 to 1.2:1 in young pigs (Qian et al., 1994a) reduced the phytase efficacy by 19.8 and 11.8%, respectively for .07 and .16% aP diets. Results of the present broiler study suggest that phytase efficacy was decreased by 11.1 and 12.2%, respectively for the diets with 66 and 660 μg of D_3/kg diet when BW gain and toe ash content were considered as the criteria to response dietary Ca:tP ratios.

In consideration of the equivalency of supplemental phytase for nP in broilers, results of our previous study (Kornegay et al., 1994) suggested that supplementation of 250, 500, 750 and 1,000 U of phytase/kg diet could be equivalent to .43, .75, 1.02 and 1.24 g of nP/kg diet, respectively, for 0 to 21-d old chicks fed a corn soybean meal diet containing .27% nP, 66 μg of D_3/kg diet and 2.0:1 of Ca:tP ratio (Figure 1). In most studies reported, the effects of extra Ca on phytate and phytase activity has not received much attention, and ratios of Ca:tP ranged from 1.7:1 to 2.5:1. In agreement with previous findings (Qian et al., 1995), the optimum dietary Ca:tP ratios appears to be 1:1 to 1.4:1 even for diets that are deficient in nP. Adjusted by 11.1% for the effect of narrowing dietary Ca:tP ratio from 2.0 to 1.4, the equivalent values of phytase to nP

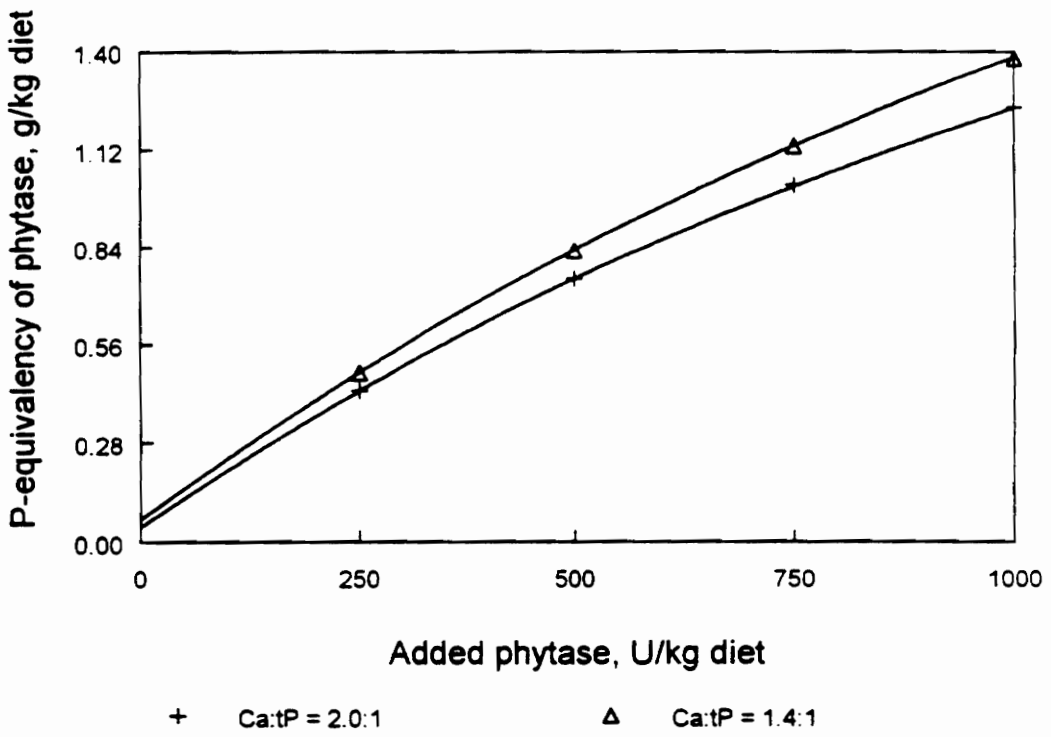

Figure 1. Equivalence of microbial phytase to nP as influenced by narrowing dietary Ca:tP ratio for broilers fed a corn soybean meal diet containing 66 μg of D_3/kg diet and .27% nP. At Ca:tP ratio = 2.0:1, $Y = 2.3300(1 - .9818e^{-.00074X})$; at Ca:tP ratio = 1.4:1, $Y = 2.7670(1 - .9768e^{-.00067X})$, where X = added phytase, U/kg diet, Y = equivalency value of nP, g/kg diet.

would be increased to .48, .83, 1.13, and 1.38 g of nP/kg diet, respectively, for the supplementation of 250, 500, 750, and 1,000 U of phytase/kg diet. This is very important for the practical utilization of phytase in poultry industry.

The adverse effect of a wide Ca:tP ratio seemed to be independent of supplemental phytase and D_3 because all two- and three-way interactions were not significant, which was consistent with our pig study (Qian et al., 1994a). However, almost all two-way interactions between Ca:tP and phytase were significant, as observed using turkey poults (Qian et al., 1995). The two-way interaction between Ca:tP and phytase was also observed in assay of phytase activity in the mixed diets. No clear explanation of this influence is available.

Vitamin D enhances the enterocytes of the small intestine to transport P into the plasma compartment, which appears independent of intestinal Ca transport (Deluca et al., 1989; Edward, 1993). As a result, dietary P absorption and retention is increased. Recent reports have indicated that the addition of D_3 or its derivatives greatly increased phytate P utilization when the supplementation was at very high levels (Shafey et al., 1990; Mohammed et al., 1991). This improvement in the utilization of phytate P by D_3 supplementation might result from an increase in the phytase activity in the small intestine of chicks (Pointillart et al., 1985).

The present study shows that the addition of D_3 at the higher levels increased the toe ash content perhaps by improving P and Ca retention of birds. The improved P and Ca retention were attributed to the enhanced P and Ca utilization of the Ca salt of phytic acid by D_3 addition. Vitamin D_3 increased the utilization of phytate P and Ca in the presence or absence of supplemental phytase in diets in our study. This is supported by the report of Edward (1993) who observed that the addition of 1,25-$(OH)_2D_3$ at 5 or 10 μg/kg diet increased the utilization of phytate P from 30 to 80% when the basal diet contained 27.5 μg of D_3 with or without graded levels of phytase supplementation. The

NRC (1994) recommends that the requirement for D_3 is 5 μg/kg diet for broilers. Edwards (1993) indicated that 3 to 5 μg/kg of 1,25-$(OH)_2D_3$ will give maximum biological responses in broilers fed diets either adequate or deficient in D_3. Recently, however, addition of D_3 or its derivatives at very high levels have shown a large increase in phytate P utilization (Shafey et al., 1990; Mohammed et al., 1991). Mohammed et al. (1991) also reported phytate P digestibility was about 50% when the diet contained normal amounts of inorganic P (.45%), Ca (1.0%), and D_3 (12.5 μg/kg diet). Increasing the addition of D_3 to 1,250 μg/kg diet increased phytate P digestibility from 59 to 77%. Increasing the addition of D_3 from 66 to 660 to 6,600 μg/kg diet in our study enhanced P retention by approximately 10% in the presence or absence of phytase supplementation.

A large increase in the utilization of phytate P that resulted from very high levels of D_3 addition is normally explained as increased intestinal phytase activity from the addition of D_3 (Pointillart et al., 1985; Edward, 1993). Pointillart et al. (1985; 1989) observed that D supplementation increased the level of phytase activity in chickens, rats and pigs which led to an improvement in phytate P utilization when diets were low or devoid of D_3. However, supplementation of D_3 at very high levels has not been shown to increase phytase activity in small intestines when diets contained adequate amount of D_3. The results of studies by Shafey et al. (1990), Mohammed et al. (1991) and Edward (1993) suggest that D_3 or its derivatives have a potential in hydrolysis of phytate. Evidence in the present study tended to support this hypothesis. An increase in the release of P from phytate was observed by graded levels of D_3 addition in diets when assaying the phytase activity in the mixed diet that contained no supplemental phytase and very limited vegetable-source phytase. In addition, a further increase in P release from phytate was also achieved when diets were simultaneously formulated with microbial phytase, which indicated some synergetic effect between D_3 and phytase on hydrolysis of phytate. This synergetic effect was also observed in toe ash content, and P and Ca retention, although

the two-way interaction between D_3 and phytase was not significant, which suggested that both effects were independent of each other. More studies are needed to investigate the potential role of D_3 supplementation in hydrolysis of phytate, and its relationship with microbial and intestinal phytase.

Findings in the present study demonstrated that addition of D_3 to corn-soybean diets for broilers increased Ca retention by 5 to 12% in the presence or absence of supplemental phytase. Calcium combined with phytic acid has low availability when it is present as the Ca salt of phytic acid. The addition of D_3 has been shown to improve the utilization of phytate Ca, which may be due to degradation of phytic acid by D_3. When broilers receive diets of 1,25-$(OH)_2D_3$, the maximum bone ash was achieved for diets containing approximately two-thirds as much Ca as needed to obtain maximum bone ash when no 1,25-$(OH)_2D_3$ presents in the diet (Edwards et al., 1992). Vitamin D_3 deficient in laying hens, on the other hand, decrease Ca absorption, which leads to bone loss even with adequate dietary Ca (Ruschowski and Hart, 1991). Vitamin D repletion of D_3 deficient birds and pigs restores bone mineralization by improving the utilization of phytate Ca (Pointillart and Fontaine, 1986; Shafey et al., 1990).

The efficacy of D_3 in improving the utilization of phytate P is influenced by dietary Ca:tP ratios in the presence or absence of supplemental phytase. Widening dietary Ca:tP ratios reduced all measurements and the phytase activity of the diets. Therefore, dietary Ca:tP ratios are critical to the efficacy of microbial phytase as well as D_3 in the hydrolysis of phytic acid. A possible mechanism is that the solubility of phytate, a salt of phytic acid, determines the utilization of phytate P. Phytate with high solubility is more accessible to phytase and D_3 whereas the solubility of phytate is, on the other hand, greatly influenced by Ca and P concentrations. This has been indicated *in vitro* (Wise, 1983) and in broilers (Ballam et al., 1984; Edwards and Veltmann, 1983). This hypothesis is also supported by the recent study of Mohammed et al. (1991) who observed that the elevation of D_3 alone

dramatically increased phytate digestibility and the retention of P and Ca, but, this improvement could not totally overcome the P depletion due to a high phytate P, and that simultaneously lowering of dietary Ca and elevation of D_3 in a phytate P diet restored all variables to the levels for the control. Similar results were reported in pigs (Pointillart et al., 1986; 1989) and in poultry (Shafey et al., 1990).

The NRC (1994) sets the requirements of Ca and nP for 0-3 wk broilers to be 1.0 and .45 %, respectively, to meet the optimal growth of birds. In the present study, nP levels in diets were below the requirement, and most Ca levels were also below the requirement to provide a more favorable situation for measuring the effect of phytase supplementation on increasing the bioavailability of the phytate P and Ca. As discussed above, supplementation of phytase and D_3 dramatically increased the utilization of phytate P and Ca. However, on the other hand, the efficacy of phytase and D_3 were influenced by dietary Ca and P levels, and the Ca:tP ratio. Further studies are needed to determine the optimal Ca levels in the broiler diet containing supplemental phytase and D_3. In addition, dietary Ca:tP ratio seems more critical than the amount of Ca or P alone in diets in the practical utilization of microbial phytase and D_3 in broilers. Dietary Ca:tP ratios that are formulated in the range of 1.1:1 to 1.4:1 appear to provide the best efficacy of supplemental phytase and D_3 in broilers.

Implications

The effectiveness of microbial phytase for improving the utilization of phytate phosphorus and calcium by broilers is influenced by the calcium:total phosphorus ratio and the vitamin D_3 level. Supplemental phytase improved body weight gain, feed intake, toe ash content, and calcium and phosphorus retention of broilers fed a corn-soybean based diet. These improvements were negatively influenced by wide calcium:total phosphorus ratios, and positively influenced by higher levels of vitamin D_3. High levels of vitamin D_3

added to the diets resulted in an increase in the retention of phosphorus and calcium which seemed independent of supplemental phytase but additive with it. Maximum responses to supplemental phytase were achieved when broiler chicks were fed diets with 600 to 900 U of phytase/kg diet, a dietary calcium;total phosphorus ratios of 1.1:1 to 1.4:1, and a vitamin D_3 level of 660 µg/kg diet.

CHAPTER VI

Phosphorus Equivalence of Microbial Phytase in Turkey Diets as Influenced by Calcium:Phosphorus Ratios and Phosphorus Levels

ABSTRACT Male day-old turkey poults (n = 768) were fed 0, 300, 600 or 900 units (**U**) of phytase/kg of a corn-soybean meal diet in combination with four Ca:total P (**tP**) ratios of 1.1, 1.4, 1.7, and 2.0:1, and two levels of nonphytate P (**nP**) of .27 and .36% in a 21-d trial. Dietary Ca:tP ratios were obtained by varying defluorinated phosphate and limestone at the expense of corn starch. The calculated dietary percentage of phytate P was .266 for all diets. Phytase additions linearly increased ($P < .05$) BW gain, feed intake, gain:feed, toe ash content, and apparent retentions of Ca and P at each Ca:tP ratio and nP level, but the response was influenced by dietary Ca:tP ratios and P levels. Two- and three-way interactions for most measurements were significant, which indicated that the Ca:tP ratios were not independent of dietary P and phytase levels. The detrimental effect ($P < .02$) of widening the Ca:tP ratio was observed for all measurements at each phytase and P level, and was greatest at lower phytase and P levels. Widening the Ca:tP ratio from 1.4 to 2.0 decreased the phytase efficacy by 7.4 and 4.9%, respectively for .27 and .36% nP diets, which was close to the decrease in the phytase activity *in vitro* by 7.5 and 6.7%, respectively. The largest responses to supplemental phytase were achieved when poults were fed diets with 600 and 900 U of phytase/kg diet, respectively for .36 and .27% nP, and for Ca:tP ratios ranging from 1.1 to 1.4:1. Second-order translog equations were generated for the phytase, Ca:tP ratio and P effect, and nonlinear and linear equations for the phytase and Ca:tP ratio effect. Based on an assessment for the r^2 and P-values of equations, BW gain, feed intake, toe ash content and P retention were sensitive measurements of the response to phytase addition. Equivalent equations were developed

to determine the P-equivalency of supplemental phytase. About 340 and 511 U of phytase were equivalent to 1 g nP, respectively for .27 and .36% nP diets in turkey poults from hatch to 21 d of age.

Key Words: Phytase, Calcium, Phosphorus, Turkey, Equivalent

Introduction

It has been well documented that microbial phytase is effective in releasing a significant portion of the P bound in phytate present in corn soybean meal diets and making it available to broilers (Schoner et al., 1991; Vogt, 1992; Denbow et al., 1995), pigs (Simons et al., 1990; Kornegay and Qian, 1994) and turkeys (Ravindran et al., 1995a). The effectiveness of phytase in broiler and pig diets has been shown to be reduced as the level of total and nonphytate P (**nP**) are increased and as the Ca:total P (**tP**) ratio becomes wider (Schoner et al., 1993; Qian et al., 1994a). The detrimental effect of Ca:tP ratio may reduce phytase efficacy by influencing phytase activity in the digestive tract of broilers and pigs (Qian and Kornegay, 1995). However, published data on the use of phytase in turkey poult diets is scanty. Ravindran et al. (1995a) reported 699 U of phytase is equivalent to 1 g of P from defluorinated phosphate; poults were fed a soybean meal-based semi-purified diet with various levels of nP and supplemental phytase. Turkey poults were fed tiered levels of phytase in combination with tiered levels of nP for 14 d following a 7 d adjustment after hatch; varying Ca:nP ratios were observed to influence bone mineralization as well as the optimal phytase dosage required for maximum performance (Zyla and Ledoux, 1994).

The objectives of the present study were 1) to determine the effects of dietary Ca:tP ratios and nP levels on the efficacy of phytase in turkey poult diets, and 2) to calculate P-equivalency values of phytase by derived equivalent equations.

Materials Methods

Birds and treatments. Male day-old British United turkey poults (n = 768) were used in a 21-d experiment to investigate the effects of various Ca:tP ratios on the efficacy of microbial phytase for improving the phytate P availibility of corn soybean meal diets. A 4 x 4 x 2 factorial arrangement of treatments with three replicates (8 birds/pen) was used. Dietary Ca : tP ratios were formulated at 1.1:1, 1.4:1, 1.7:1 and 2.0:1, and each Ca : tP ratio was supplemented with 0, 300, 600 and 900 unit of phytase/kg of diet at nP levels of .27 and .36% (or .54 and .63% tP). The dietary P levels were formulated below the current NRC (1994) recommendations to ensure maximum responses with phytase additions. The Ca : tP ratios were formulated by varying defluorinated phosphate and limestone at the expense of corn starch. The dietary percentage of phytate P (.266%) was similar in all diets. The composition of the basal diets is given in Table 1. Microbial phytase activity of diets was determined using a modification of the method of Simons et al. (1990). A unit (U) of phytase activity was defined as the quantity of enzyme that liberated 1 μmol of inorganic P per min from 1.5 mM sodium phytate at pH 5.5 and 37 °C.

Feeding management. At 1 d of age, poults were wingbanded and randomly assigned to pens in electrically heated, raised wire-floored battery brooders in an environmentally controlled room. Poults were exposed to continuous fluorescent light. All diets were provided for *ad libitum* consumption in mash form, and birds had free access to water. Body weights and feed consumption were recorded on a pen basis at weekly intervals. The care and treatment of birds followed published guidelines (Consortium, 1988).

Sampling and analysis. During the 3rd wk (d 18 to 20), all excreta from each pen was collected. Feed intake and production of excreta were measured quantitatively per pen over the 3 consecutive d. Excreta from each pen were stored in plastic bags at -20 °C.

Table 1. Percentage of composition of the basal diet (as-is basis)[a]

Ingredients	Percentage
Soybean meal (48.5% CP)	49.6
Corn (8.8% CP)	43.0
Canola oil	3.0
Limestone	.522
Deflourinated phosphate	.589
Vitamin premix[b]	.20
Trace mineral premix[c]	.20
Salt	.40
DL methionine	.20
Corn starch	2.289
Calculated analysis[d]	
Ca	.530
Total P	.536
Nonphytate P	.266

[a]Dietary Ca:tP ratios of 1.1:1, 1.4:1, 1.7:1 and 2.0:1 and nP levels of .27 and .36% (.54 and .63% tP) were formulated using defluorinated phosphate and limestone substituted for corn starch, and each Ca:tP ratio and nP level was supplemented with 0, 300, 600 and 900 U phytase/kg diet. Phytase (Natuphos®-5,000 U/g) was supplied by BASF Corp., 100 Cherry Hill Road, Parsippany NJ 07054. Analyzed levels of Ca were .648, .763, .896 and 1.073%, respectively, for the analyzed P level of .57%, and .718, .987, 1.169 and 1.310%, respectively, for the analyzed P level of .64%.

[b]Supplied per kilogram of diet: retinyl acetate, 908 µg; cholecalciferol, 66 µg; dl-α -tocopheryl acetate, 26.5 mg, menadione sodium bisulfate complex, .75 mg; riboflavin, 7.5 mg; d-Ca-antothenate acid, 9.7 mg; niacin, 26.4 mg; cyanocobalamin, 11 ug; choline chloride, 1,013 mg; biotin, .31 mg; folic acid, 3.1 mg; thiamin•HCl, 8 mg; pyridoxine•HCl, 3.1 mg; ethoxyquin, 50 mg; virgiamycin, 2.9 mg.

[c]Supplied per kilogram of diet: Mn, 88 mg; Zn, 95 mg; Fe, 100 mg; Cu, 12.5 mg; I, 4 mg; Se, .6 mg.

[d]Diet was formulated to contain 28.0% crude protein, 1.69% lysine, and 1.69% methionine plus cystene.

After thawing, excreta were dried in an oven at 65 °C and weighed. Excreta, along with diet samples, were ground to pass a 1-mm sieve. Dry matter was determined according to AOAC (1990) procedures. Following a nitric-perchloric wet digestion, P concentrations were determined colorimetrically (AOAC, 1990) with the computer program

"Microkinetics (Catalog no. 78-588-00, Flow Laboratories, Inc., 7655 Old Springhouse Road, Mclean, Virginia)" and the vertical photometer (Titertek Multiskan MCC/340: serial #1EEE-488). Concentrations of Ca were determined with an atomic absorption spectrophotometer. Apparent retention of P and Ca were calculated.

On d 21, all surviving birds were euthanatred. Toe samples were obtained by severing the middle toe through the joint between the 2nd and 3rd tarsal bones from the distal end. The left and right middle toes of all birds within a pen were pooled respectively, yielding two samples of toes per pen. The pooled samples were dried to a constant weight at 100 °C and then ashed in a muffle furnace at 600 °C for 6 h. Toe ash was expressed as a percentage of dry weight.

Statistical analysis. Data were analyzed by the GLM procedure of SAS (1990) using pen as the experimental unit. The model included main effects of Ca:tP ratios, phytase levels, nP levels, all two-way interactions, and the three-way interaction. Linear and quadratic effects of the Ca:tP ratio and supplemental phytase were tested with orthogonal polynomials. Second order translog functions were developed for the 2 x 4 factorial arrangement of nP levels and phytase levels, or nP levels and Ca:tP ratios with the model: $LnY = \alpha_0 + \alpha_1 D_1 + \alpha_2 LnX + \alpha_3 (LnX)^2 + \alpha_4 D_1 LnX$, where, Y = the response measurements; $D_1 = 0$ at nP = .27%, and $D_1 = 1$ at nP = .36%; X = added phytase (U/kg diet), or X = Ca:tP ratios. The second order translog model was used to determine the response surfaces to a combination of tiered levels of phytase, nP, and various ratios of Ca:tP, and to evaluate the sensititivity of measurements to dietary Ca:tP ratios, nP levels and supplemental phytase levels. The second order translog function for the 4 x 4 factorial of phytase level and Ca:tP ratio effect was: $LnY = \alpha_0 + \alpha_1 LnX_1 + \alpha_2 LnX_2 + \alpha_3 (LnX_1)^2 + \alpha_4 (LnX_2)^2 + \alpha_5 LnX_1 LnX_2$, where Y = the response measurements; X_1 = added phytase (U/kg diet); X_2 = Ca:tP ratios.

Nonlinear functions were created for the phytase effect at each P level or Ca:tP ratio using the model: $Y = a(1 - be^{-kX})$, where Y = the response measurement; X = added phytase (U/kg diet). For the Ca:tP ratio effect, the nonlinear model was : $Y = a (1 - be^{-c + kX})$, or $Y = ae^{-kX}$, where, Y = the response measurement; X = Ca:tP ratios. Also, linear functions were derived for the phytase or Ca:tP ratio effect corresponding to different P levels, Ca:tP ratios, or supplemental phytase levels. The model of linear functions was: $Y = a + bX$, where Y = the response measurement; X = added phytase levels, or Ca:tP ratios. The nonlinear and linear functions that were developed were also used to compare both response functions for dietary phytase levels and Ca:tP ratios and to determine which models were best fitted for these factors.

Based on the assumption that the data obtained at .27 and .36% nP levels were representative of a wider range of P levels, linear response functions of BW gain and toe ash percentage were developed across the four Ca:tP ratios but without supplemented phytase. The slopes of the equations across Ca:tP ratios were slightly lower than obtained when the unpublished data of Ravindran for BW gain and toe ash percentage were plotted at .27, .36 and .45; the slopes were 988 vs 20.78 for toe ash percentage, respectively. If only the data for a Ca:tP ratio of 2.0:1 are used, the slopes are slightly greater than obtained with Ravindran's data (1211 vs 1122 for BW gain and 22.22 vs 20.78 for toe ash percentage, respectively). The slopes for BW gain in this study and for Ravindran's data are higher than slopes derived for data reported by Potchanakorn and Potter (1987) and Potter (1988) (500 and 467, respectively, but slopes for toe ash percentage are lower for our data and the data of Ravindran than for slopes of data reported by Potchanakorn and Potter (1987) and Potter (1988) (26.60 and 25.78, respectively).

The use of a regression line (equation) generated from graded levels of P or phytase provides a more accurate means of estimating a response than a single number.

Also, the generation of a P equivalency equation from the equations for P and for phytase allow for the calculation of the equivalency of phytase for P for any point on the line. Further, the use of mathematical equations allow for the easy incorporation of this information in computer models.

Results

Growth performance. Main effects of phytase, Ca:tP ratio and nP were observed for BW gain ($P < .001$); the magnitude of the responses to phytase was greatest for the lowest level of phytase, for the widest Ca:tP ratio, and for the lower P level (Table 2). Interactions of Ca:tP ratio x phytase, Ca:tP ratio x nP, phytase x nP, and Ca:tP ratio x phytase x nP were observed for BW gain ($P < .001$, .003, .001 and .004, respectively). The detrimental effect of Ca:tP ratio was greatest for the widest ratio and at the lower P level with no added phytase. Widening the Ca:tP ratio had less of an effect as the amount of phytase added was increased (at phytase = 0, linear, $P < .02$, quadratic, $P < .001$; at phytase = 300 U/kg diet, linear, $P < .01$, quadratic, $P < .05$; at phytase = 600 U, linear, $P < .01$; at phytase = 900 U, quadratic, $P < .03$). At .27% nP, BW gains increased (linear, $P < .001$; quadratic, $P < .001$) up to 900 U phytase/kg diet; whereas, at .36% nP, BW gains improved (linear, $P < .001$; quadratic, $P < .06$) up to 600 U and then reached a plateau. At .27% nP, BW gains were similar for dietary Ca:tP ratios of 1.1:1 and 1.4:1 and decrease afterwards as the ratio became wider; however, the largest BW gain occurred at 1.1:1 ratio for .36 nP.

The main effects ($P < .001$) of Ca:tP ratio, phytase level and nP level on feed intake were similar to those observed for BW gains (Table 3). Interactions of Ca:tP ratio x phytase, phytase x nP, and Ca:tP ratio x phytase x nP were also observed ($P < .05$, .001 and .1, respectively). Adding phytase improved feed intake (linear, $P < .001$; quadratic, P

< .001) across Ca:tP ratios; the magnitude of the response to phytase addition was greatest for the widest Ca:tP level at .27% nP (linear, $P < .001$, quadratic, $P < .002$). The detrimental effect of widening the Ca:tP level was greater at .27% nP (.54% tP) and at the lower levels of supplemental phytase.

Table 2. Body weight gain (g/bird) of turkey poults fed corn soybean meal diets with four phytase levels, four Ca:tP ratios and two nP levels from hatch to 21 d of age[a,b]

Ca:tP ratio[d]	Supplemental phytase (U/kg of diet)[e]				
	0	300	600	900	Mean
nP = .27% (tP = .54%)[c]					
1.1:1	435	501	506	524	492
1.4:1	442	511	492	534	495
1.7:1	357	476	506	519	464
2.0:1	261	459	477	483	420
Mean	374	487	496	515	
nP = .36% (tP = .63%)[f]					
1.1:1	508	510	552	547	529
1.4:1	448	495	513	517	493
1.7:1	444	507	504	509	491
2.0:1	415	466	497	487	466
Mean	454	495	517	515	

[a] Three pens of eight birds each per mean. The root MSE was 12.75. The pooled SEM for a single mean would equal MSE/$\sqrt{3}$.

[b] Main effect of Ca:tP ratio, phytase, and tP ($P < .001$); Ca:tP ratio x phytase interaction ($P < .001$); Ca:tP ratio x tP interaction ($P < .003$); phytase x tP interaction ($P < .001$); Ca:tP ratio x phytase x tP interaction ($P < .004$).

[c] Phytase effect: linear and quadratic ($P < .001$); at Ca:tP ratio = 1.1, linear and quadratic ($P < .001$); at Ca:tP ratio = 1.4, linear ($P < .001$); at Ca:tP ratio = 1.7, linear ($P < .001$); at Ca:tP ratio = 2.0, linear ($P < .001$) and quadratic ($P < .005$).

[d] Ca:tP ratio effect: linear and quadratic ($P < .001$); at phytase = 0, linear ($P < .02$) and quadratic ($P < .001$); at phytase = 300, linear ($P < .1$) and quadratic ($P < .05$) at phytase = 900, quadratic ($P < .03$).

[e] Ca:tP ratio effect: linear ($P < .05$) and quadratic ($P < .001$). Phytase effect: linear and quadratic ($P < .001$).

[f] Ca:tP ratio effect: quadratic ($P < .04$). Phytase effect: linear ($P < .001$) and quadratic ($P < .06$).

Table 3. Feed intake (g/bird) of turkey poults fed corn soybean meal diets with four phytase levels, four Ca:tP ratios and two nP levels from hatch to 21 d of age[a,b]

Ca:tP ratio[d]	Supplemental phytase (U/kg diet)[c]				Mean
	0	300	600	900	
nP = .27% (tP = .54%)[e]					
1.1:1	673	797	807	774	763
1.4:1	659	723	736	812	733
1.7:1	555	716	767	807	711
2.0:1	496	655	695	742	672
Mean	596	748	751	784	
nP = .36% (tP = .63%)[f]					
1.1:1	827	820	786	834	817
1.4:1	721	752	753	772	750
1.7:1	651	766	748	727	723
2.0:1	673	703	736	723	708
Mean	718	760	756	764	

[a]Three pens of eight birds each per mean. The root MSE was 27.39. The pooled SEM for a single mean would equal MSE/$\sqrt{3}$.

[b]Main effect of Ca:tP ratio, phytase, and tP ($P < .001$); Ca:tP ratio x phytase interaction ($P < .05$); phytase x tP interaction ($P < .001$); Ca:tP ratio x phytase x tP interaction ($P < .1$).

[c]Phytase effect: linear and quadratic ($P < .001$); at Ca:tP ratio = 1.1, linear ($P < .001$) and quadratic ($P < .01$); at Ca:tP ratio = 1.4, linear ($P = .13$); at Ca:tP ratio = 1.7, linear ($P < .005$); at Ca:tP ratio = 2.0, linear ($P < .001$) and quadratic ($P < .01$).

[d]Ca:tP ratio effect: quadratic ($P < .001$); at phytase = 0, quadratic ($P < .001$); at phytase = 300, quadratic ($P < .1$); at phytase = 600, quadratic ($P = .12$); at phytase = 900, quadratic ($P < .05$).

[e]Ca:tP ratio effect: quadratic ($P < .01$). Phytase effect: linear ($P < .001$) and quadratic ($P < .002$).

[f]Ca:tP ratio effect: quadratic ($P < .003$). Phytase effect: linear ($P < .1$) and quadratic ($P < .002$).

The main effects of Ca:tP ratio, phytase and nP on gain:feed ($P < .02$, .001, and .08, respectively) and the interactions of Ca:tP ratio x phytase, Ca:tP ratio x nP, and Ca:tP ratio x phyatse x nP ($P < .02$, .05, and .03, respectively) are usually not as strong as observed for BW gain (Table 4). There appeared to be no improvement of gain:feed from adding phytase beyond 600 U/kg of diet for most Ca:tP ratios. Maintaining optimum Ca:tP ratios seemed to be more critical for turkey poults fed with .27% nP (.54% tP) and lower phytase addition.Supplemental phytase linearly increased toe ash contents at each Ca:tP ratio and at each nP level ($P < .001$), and quadratic responses to the phytase addition were also observed.

Toe ash content. The influence ($P < .001$) of dietary Ca:tP ratio, nP levels and phytase addition on toe ash contents tended to parallel that of BW gains and feed intake (Table 5). Interactions among the three factors were significant ($P < .001$, .001, and .05, respectively for Ca:tP ratio x phytase, phytase x nP, and Ca:tP ratio x phytase x nP interaction). The magnitude of the response to supplemental phytase was dependent on the Ca:tP ratio and the level of nP; the detrimental effect of widening the Ca:tP ratio was greatest at the lower nP level.

Retention of P and Ca. The retention of P decreased as the nP levels increased ($P < .001$) and as the Ca:tP ratios were widened ($P < .001$; at .27% nP, quadratic, $P < .01$; at .36% nP, quadratic, $P < .001$). Adding phytase increased the retention of P at all ratios of Ca:tP and levels of nP ($P < .001$; at .27% nP, linear, $P < .001$; at .36% nP, linear, $P < .005$; Table 6). Only a phytase x nP intereaction was observed ($P < .05$); phytase linearly increased P retention to 900 U/kg for .27% nP diets and to 600 U/kg for .36% nP diets. The highest response to the supplemental phytase and dietary Ca:tP ratios was achieved when dietary Ca:tP ratio was formulated at 1.1:1 with 900 U of phytase added per kg of diet for both levels of nP, although retention of P was higher for the .27% nP diets.

Table 4. Gain:feed (g/kg) of turkey poults fed corn soybean meal diets with four phytase levels, four Ca:tP ratios and two nP levels from hatch to 21 d of age[a,b]

Ca:tP ratio[d]	Supplemental phytase (U/kg diet)[c]				
	0	300	600	900	Mean
nP = .27% (tP = .54%)[e]					
1.1:1	648	629	628	678	645
1.4:1	621	708	669	658	677
1.7:1	644	666	660	645	654
2.0:1	522	617	688	651	620
Mean	621	655	661	658	
nP = .36% (tP = .63%)[f]					
1.1:1	615	623	703	657	650
1.4:1	623	658	683	670	658
1.7:1	655	662	674	701	680
2.0:1	617	664	676	673	658
Mean	635	652	684	675	

[a]Three pens of eight birds each per mean. The root MSE was .02. The pooled SEM for a single mean would equal MSE/$\sqrt{3}$.

[b]Main effect: Ca:tP ratio ($P < .02$); phytase ($P < .001$); tP ($P < .08$). Ca:tP ratio x phytase interaction ($P < .02$); Ca:tP ratio x tP interaction ($P < .05$); Ca:tP ratio x phytase x tP interaction ($P < .03$).

[c]Phytase effect: linear ($P < .001$) and quadratic ($P < .05$); at Ca:tP ratio = 1.1, linear and quadratic ($P < .001$); at Ca:tP ratio = 1.4, linear ($P < .03$) and quadratic ($P < .06$); at Ca:tP ratio = 1.7, quadratic ($P < .1$).

[d]Ca:tP ratio effect: linear ($P < .005$); at phytase = 0, linear ($P < .001$); at phytase = 300, linear ($P < .06$).

[e]Ca:tP ratio effect: linear ($P < .01$) and quadratic ($P < .05$). Phytase effect: linear ($P < .001$) and quadratic ($P < .002$).

[f]Phytase effect: linear ($P < .008$).

Table 5. Toe ash percent (%) of turkey poults fed corn soybean meal diets with four phytase levels, four Ca:tP ratios and two nP levels from hatch to 21 d of age[a,b]

Ca:tP ratio[d]	Supplemental phytase (U/kg diet)[c]				Mean
	0	300	600	900	
nP = .27% (tP = .54%)[e]					
1.1:1	10.2	11.7	11.4	12.1	11.2
1.4:1	9.5	11.2	11.1	11.2	10.9
1.7:1	7.6	10.5	11.3	11.4	10.2
2.0:1	7.2	10.3	10.7	10.9	9.7
Mean	8.6	10.9	11.2	11.4	
nP = .36% (tP = .63%)[f]					
1.1:1	11.6	11.8	11.8	12.1	11.8
1.4:1	10.6	11.0	11.6	12.0	11.6
1.7:1	9.9	11.7	11.6	12.1	11.4
2.0:1	9.2	11.2	11.5	11.6	10.9
Mean	10.3	11.7	11.7	12.0	

[a]Three pens of eight birds each per mean. The root MSE was 30. The pooled SEM for a single mean would equal MSE/$\sqrt{3}$.

[b]Main effect of Ca:tP ratio, phytase, and tP ($P < .001$). Ca:tP ratio x phytase interaction ($P < .001$); Ca:tP ratio x tP interaction ($P < .07$); phytase x tP interaction ($P < .001$); Ca:tP ratio x phytase x tP interaction ($P < .05$).

[c]Phytase effect: linear and quadratic ($P < .001$); at Ca:tP ratio = 1.1; linear and quadratic ($P < .001$); at Ca:tP ratio = 1.4, linear ($P < .02$) and quadratic ($P < .06$); at Ca:tP ratio = 1.7, linear ($P < .001$) and quadratic ($P < .002$); at Ca:tP ratio = 2.0, linear and quadratic ($P < .001$).

[d]Ca:tP ratio effect: linear ($P < .04$) and quadratic ($P < .001$); at phytase = 0, quadratic ($P < .001$); at phytase = 300, quadratic ($P < .006$); at phytase = 600, quadratic ($P < .06$); at phytase = 900, linear ($P = .12$).

[e]Ca:tP ratio effect: quadratic ($P < .001$). Phytase effect: linear and quadratic ($P < .001$).

[f]Ca:tP ratio effect: quadratic ($P < .04$). Phytase effect: linear ($P < .001$) and quadratic ($P < .008$).

Table 6. Phosphorus retention (%) of turkey poults fed corn soybean meal diets with four phytase levels, four Ca:tP ratios and two nP levels from hatch to 21 d of age[a,b]

Ca:tP ratio[d]	Supplemental phytase (U/kg diet)[c]				Mean
	0	300	600	900	
nP = .27% (tP = .54%)[e]					
1.1:1	56.0	59.6	62.5	63.5	60.4
1.4:1	53.9	56.5	61.9	63.3	58.9
1.7:1	49.4	55.4	59.7	63.1	56.9
2.0:1	44.2	53.7	54.7	61.7	53.6
Mean	50.8	56.3	59.7	62.9	
nP = .36% (tP = .63%)[f]					
1.1:1	54.2	59.2	57.8	58.5	57.4
1.4:1	53.7	55.3	57.6	56.7	55.8
1.7:1	48.0	53.1	53.7	55.6	52.6
2.0:1	45.7	47.3	53.1	51.8	49.0
Mean	50.4	53.2	55.6	55.6	

[a]Three pens of eight birds each per mean. The root MSE was 2.39. The pooled SEM for a single mean would equal MSE/$\sqrt{3}$.

[b]Main effect of Ca:tP ratio, phytase, and tP ($P < .001$). Phytase x tP interaction ($P < .05$).

[c]Phytase effect: linear ($P < .001$); at Ca:tP ratio = 1.1; linear ($P < .001$); at Ca:tP ratio = 1.4, linear ($P < .03$); at Ca:tP ratio = 1.7, linear ($P < .008$); at Ca:tP ratio = 2.0, linear ($P < .001$).

[d]Ca:tP ratio effect: linear ($P < .05$), and quadratic ($P < .001$); at phytase = 0, quadratic ($P < .001$); at phytase = 300, quadratic ($P < .003$); at phytase = 600, quadratic ($P < .02$).

[e]Ca:tP ratio effect: quadratic ($P < .01$). Phytase effect: linear ($P < .001$).

[f]Ca:tP ratio effect: quadratic ($P < .001$). Phytase effect: linear ($P < .005$).

Similarly, supplemental phytase increased ($P < .001$) Ca retention, which was independent of dietary Ca:tP ratios and nP levels since interactions were not significant (Table 7). At both levels of nP the increase in Ca retention appeared to plateau at 600 U of phytase/kg of diet. Widening the Ca:tP ratios decreased Ca retention ($P < .001$) at both levels of nP, but the effect of widening the Ca:tP ratio appeared to be greater at the higher level of nP. The highest response to dietary Ca:tP ratio and supplemental phytase was observed when dietary Ca:tP ratio was 1.1:1 with phytase supplementation of 600 U/kg of diet.

Activity of supplemental phytase in diets. Phytase activity at each added phytase level was linearly decreased ($P <.001$) as the Ca:tP ratio became wider. The detrimental effect of phytase levels (Table 8). The phytase activity was higher at the lower P level than at the higher P level. All main effects and two-way interactions were significant ($P < .05$).

Effect of Ca:tP ratios. Narrowing the Ca:tP ratio from 2.0 to 1.4 resulted in an widening the Ca:tP ratio by increasing the level of Ca was more pronounced at lower P and increase in the phytase efficacy, averaged for BW gain and toe ash content, of 7.4 and 4.9%, respectively for .27 and .36% nP diets, which was close to the increase in the phytase activity of the diets (7.5 and 6.7%, respectively). However, further narrowing the ratio from 2.0 to 1.1 resulted in a great increase in the dietary phytase activity (17.4 and 16.2%, respectively), whereas the increase in the phytase efficacy *in vivo* was limited.

Response curves. Second order translog equations of performance, toe ash content, P and Ca retention which were generated for the effect of added phytase and nP level (across Ca:tP ratios), for Ca:tP ratios and nP (across phytase level), and for added phytase and Ca:tP ratios (across nP levels) are shown in Table 9. Equations for phytase

Table 7. Calcium retention (%) of turkey poults fed corn soybean meal diets with four phytase levels, four Ca:tP ratios and two nP levels from hatch to 21 d of age[a,b]

Ca:tP ratio[d]	Supplemental phytase (U/kg diet)[c]				Mean
	0	300	600	900	
nP = .27% (tP = .54%)[e]					
1.1:1	63.3	65.0	74.1	69.6	68.7
1.4:1	52.2	59.3	65.5	62.1	59.8
1.7:1	42.1	51.6	53.1	60.4	51.8
2.0:1	40.6	45.3	50.5	45.9	45.6
Mean	50.3	55.3	60.8	59.5	
nP = .36% (tP = .63%)[f]					
1.1:1	61.7	66.3	68.6	67.5	67.5
1.4:1	52.6	54.4	59.6	59.3	56.5
1.7:1	40.0	44.4	59.7	52.4	49.1
2.0:1	31.7	39.5	42.9	43.2	39.3
Mean	48.0	51.2	57.7	55.6	

[a]Three pens of eight birds each per mean. The root MSE was 3.10. The pooled SEM for a single mean would equal MSE/$\sqrt{3}$.

[b]Main effect of Ca:tP ratio, phytase, and tP ($P < .001$).

[c]Phytase effect: linear ($P < .001$) and quadratic ($P < .01$); at Ca:tP ratio = 1.1; linear ($P < .003$), and quadratic ($P < .05$); at Ca:tP ratio = 1.4, linear ($P = 1.1$); at Ca:tP ratio = 1.7, linear ($P < .004$), and quadratic (P = .16); at Ca:tP ratio = 2.0, linear ($P < .001$).

[d]Ca:tP ratio effect: linear ($P < .005$) and quadratic ($P < .001$); at phytase = 0, quadratic ($P < .001$); at phytase = 300, quadratic ($P < .001$); at phytase = 600, linear ($P < .05$), and quadratic ($P < .001$); at phytase = 900, linear ($P < .008$) and quadratic ($P < .001$).

[e]Ca:tP ratio effect: quadratic ($P < .001$). Phytase effect: linear ($P < .05$).

[f]Ca:tP ratio effect: linear ($P < .04$) and quadratic ($P < .001$). Phytase effect: linear ($P < .05$).

Table 8. Phytase activity (U/kg of diet) determined in the corn soybean meal diets formulated with four phytase levels, four Ca:tP ratios and two nP levels[a,b]

Ca:tP ratio[d]	Supplemental phytase (U/kg diet)[c] 0	300	600	900	Mean
nP = .27% (tP = .54%)[e]					
1.1:1	0	364	642	1053	515
1.4:1	0	340	566	973	470
1.7:1	0	312	520	950	446
2.0:1	0	287	560	940	447
Mean	0	326	573	979	
nP = .36% (tP = .63%)[f]					
1.1:1	0	307	523	971	450
1.4:1	0	275	490	886	413
1.7:1	0	289	458	891	410
2.0:1	0	260	440	834	384
Mean	0	283	478	896	

[a]Each mean represents four replicates. The root MSE was 11.3 for phytase activity. The pooled SEM for a single treatment mean would equal MSE/$\sqrt{4}$.

[b]Main effect of Ca:tP ratio, phytase, and nP ($P < .001$). Ca:tP ratio x phytase interaction ($P < .001$); Ca:tP ratio x nP interaction ($P < .05$); phytase x nP interaction ($P < .001$).

[c]Phytase effect: linear and quadratic ($P < .001$); at Ca:tP ratio = 1.1, linear ($P < .001$) and quatratic ($P < .002$); at Ca:tP ratio = 1.4, lineal ($P < .001$) and quadratic ($P < .007$); at Ca:tP ratio = 1.7, linear and quadratic ($P < .001$); at Ca:tP ratio = 2.0, linear and quadratic ($P < .001$).

[d]Ca:tP ratio effect: quadratic ($P < .001$); at phytase = 300, quadratic ($P < .05$); at phytase = 600, quadratic ($P < .007$); at phytase = 900, quadratic ($P < .001$).

[e]Phytase effect: linear and quadratic ($P < .001$).

[f]Phytase effect: linear and quadratic ($P < .001$).

Table 9. Second order translog functions for performance, toe ash content, and P and Ca retention of turkey poults fed corn soybean meal diets with four Ca:tP ratios, four phytase and two nP levels from hatch to 21 d of age

Item	Coefficients of equations							
	a_0	a_1	a_2	a_3	a_4	a_5	*P*-value	r^2
Phytase and nP effect[a]								
BW gain, g/bird	5.9679	.1136	.0251	.0021	-.0150		.001	.54
Feed intake, g/bird	6.4749	.0916	.0196	.00008	-.0146		.001	.48
Gain:feed, g/g	-.5070	.0219	.0055	.0013	-.0004		.004	.15
Toe ash content, %	2.2420	.1248	.0211	.0007	-.0103		.001	.54
P retention, %	3.7931	-.0519	.0146	.0050	-.0054		.001	.58
Ca retention, %	3.7559	-.0767	.0142	.0052	.0010		.018	.12
Ca:tP ratio and nP effect[a]								
BW gain, g/bird	6.2120	.0240	.0540	-.4610	.1090		.001	.22
Feed intake, g/bird	6.6660	.0520	-.2560	.0360	-.0100		.001	.23
Gain:feed, g/g	-.4540	-.0280	.3090	-.4970	.1190		.014	.13
Toe ash content, %	2.4280	.0380	-.1486	-.1523	.1327		.001	.22
P retention, %	4.0898	-.0325	.0747	-.3306	-.0884		.001	.32
Ca retention, %	4.2556	.0215	-.3586	-.4165	-.2247		.001	.62
Phytase and Ca:tP ratio effect[b]								
BW gain, g/bird	6.1240	.0036	.0070	.0021	-.4614	.0338	.001	.65
Feed intake, g/bird	6.6490	.0030	-.3284	.0008	.0356	.0226	.001	.56
Gain:feed, g/g	-.5250	.0006	.3350	.0010	-.4970	.0112	.001	.25
Toe ash content, %	2.4167	.0022	-.1269	.0005	-.2200	.0328	.001	.69
P retention, %	3.8407	.0073	.0226	.0050	-.3802	.0121	.001	.61
Ca retention, %	4.0379	.0031	-.5173	.0051	-.4967	.0307	.001	.72

[a]Model: $LnY = \alpha_0 + \alpha_1 D_1 + \alpha_2 LnX + \alpha_3 (LnX)^2 + \alpha_4 D_1 LnX$. At nP = .27%, D_1 = 0; At nP = .36%, $D_1 = 1$. X = added phytase, U/kg diet; or X = Ca:tP ratios.

[b]Model: $LnY = \alpha_0 + \alpha_1 LnX_1 + \alpha_2 LnX_2 + \alpha_3 (LnX_1)^2 + \alpha_4 (LnX_2)^2 + \alpha_5 LnX_1 LnX_2$. X_1 = added phytase, U/kg diet. X_2 = Ca:tP ratios.

and nP, and Ca:tP ratios and nP for most measurements had low r^2 although P values were significant. The response equations based on phytase and Ca:tP ratios had a relatively high r^2 (>.54) and were significant (P <.001) for all measurements, except gain:feed ratio.

Nonlinear response equations were developed for the phytase effect at each of two P levels or each of the four Ca:tP ratios, and for the Ca:tP ratio effect at each of the two P levels and at each of the four phytase levels (Tables 10 and 11). Nonlinear equations could not consistently be generated for feed intake and gain:feed and r^2 values of linear equations were very low (< .5). Based on r^2 values, nonlinear equations seemed to be a better fit of the effect of phytase at each nP level and Ca:tP ratio than linear equations. The difference in the fit of nonlinear and linear equations of the effect of Ca:tP ratios at each nP level or each phytase level was not as great as for the effect of phytase at each nP level or each Ca:tP ratio. The r^2 values were high for the effect of phytase by nP levels or by Ca:tP ratio, and for the effect of Ca:tP ratio by nP or by phytase levels for all measurements, except phytase at a Ca:tP ratios of 1.1:1 and Ca:tP ratio at 900 U/kg phytase for toe ash.

Based on the assumption that data obtained at .27 and .36% nP levels were linearly representative over a wider range, linear response equations to nP were generated for BW gain and toe ash percent (Table 12). Using nonlinear responses for the supplemental phytase and linear responses for nP level, P-equivalency equations of phytase were developed to determine the P-equivalent values (Y, %) of supplemental phytase (X, U/kg diet): $Y = .1244(1 - 1.8956e^{-.00668X})$ and $Y = .1210(1 - 1.8849e^{-.00467X})$, respectively for .27 and .36% nP. Approximately 340 and 511 U of phytase are required to replace 1 g of P for .27 and .36% nP diet, respectively.

Table 10. Nonlinear functions for BW gain, toe ash content, and P and Ca retention of turkey poults fed corn soybean meal diets with four Ca:tP ratios, four phytase and two nP levels from hatch to 21 d of age

Item	Phytase effect[a]		Ca:tP ratio effect[b]	
	Equation	r^2	Equation	r^2
	nP = .27%		nP = .27%	
BW gain, g/bird	$Y = 509.15\ (1-.2654\ e^{-.0056X})$	.99	$Y = 500.86\ (1-4.6024\ e^{-9.4237+3.0399X})$	.98
Toe ash content, %	$Y = 11.232\ (1-.2362\ e^{-.0071X})$	.99	$Y = 15.99\ (1-.5386\ e^{-.8869+.2861X})$	.99
P retention, %	$Y = 73.959\ (1-.3049\ e^{-.0008X})$	.99	$Y = 61.198\ (1-2.6384\ e^{-7.9481+2.5639X})$	.99
Ca retention, %	$Y = 61.734\ (1-.1712\ e^{-.0023X})$	.92	$Y = 118.112\ e^{-.4908X}$	.99
	nP = .36%		nP = .36%	
BW gain, g/bird	$Y = 524.63\ (1-.1407\ e^{-.0047X})$	.99	$Y = 1174\ (1-.6574\ e^{-.2542+.0820X})$	.89
Toe ash content, %	$Y = 12.6647\ (1-.2904\ e^{-.00482X})$	.99	$Y = 12.01\ (1-.7532\ e^{-5.744+1.8529X})$	.99
P retention, %	$Y = 56.692\ (1-.1115\ e^{-.0023X})$	.98	$Y = 62.469\ (1-.7458\ e^{-3.4813+1.1232X})$	.99
Ca retention, %	$Y = 58.296\ (1-.1821\ e^{-.0021X})$	.94	$Y = 127.774\ e^{-.5784X}$	.99
	Ca:tP ratio = 1.1:1		Phytase = 0, U/kg diet	
BW gain, g/bird	$Y = 548.14\ (1-.1407\ e^{-.0021X})$	.99	$Y = 528.72\ (1-1.5859\ e^{-4.1726+1.3461X})$	.99
Toe ash content, %	$Y = 13.071\ (1-.1589\ e^{-.0007X})$	.75	$Y = 15.796\ e^{-.3352X}$	.98
P retention, %	$Y = 60.923\ (1-.0954\ e^{-.0042X})$	.99	$Y = 57.592\ (1-3.3995\ e^{-6.9285+2.2350X})$	.99
Ca retention, %	$Y = 72.002\ (1-.0776\ e^{-.0009X})$	.69	$Y = 161.585\ e^{-.8055X}$	.99
	Ca:tP ratio = 1.4:1		Phytase = 300, U/kg diet	
BW gain, g/bird	$Y = 520.24\ (1-.1435\ e^{-.0054X})$	.94	$Y = 508.33\ (1-3.8126\ e^{-10.5515+3.4037X})$	.99
Toe ash content, g/g	$Y = 11.706\ (1-.1433\ e^{-.0086X})$	.99	$Y = 12.174\ (1-.5589\ e^{-4.3282+1.3962X})$	.99
P retention, %	$Y = 64.345\ (1-.1445\ e^{-.0011X})$	.95	$Y = 68.118\ (1-.6477\ e^{-2.4583+.7931X})$	.98
Ca retention, %	$Y = 62.617\ (1-.1668\ e^{-.0027X})$	.89	$Y = 113.382\ e^{-.4969X}$	.99
	Ca:tP ratio = 1.7:1		Phytase = 600, U/kg diet	
BW gain, g/bird	$Y = 512.596\ (1-.2188\ e^{-.0054X})$	.99	$Y = 956.36\ (1-.541\ e^{-.2725+.0879X})$	.83
Toe ash content, %	$Y = 11.809\ (1-.2617\ e^{-.0049X})$	.99	$Y = 11.547\ (1-4.7917\ e^{-13.29+4.2872X})$	.97

P retention, %	$Y = 71.476 (1-.3712\ e^{-.0007X})$	.99	$Y = 62.438 (1-.8163\ e^{-5.0015+1.6134X})$	.98
Ca retention, %	$Y = 60.309 (1-.3558\ e^{-.0022X})$	.98	$Y = 197.151 (1-.9429\ e^{-.6014+.1940X})$	.99
	Ca:tP ratio = 2.0:1		Phytase = 900, U/kg diet	
BW gain, g/bird	$Y = 487.857 (1-.3069\ e^{-.0061\ X})$	.99	$Y = 542.21 (1-1.2618\ e^{-6.9809+2.2519X})$	.99
Toe ash content, %	$Y = 11.177 (1-.2677\ e^{-.0062X})$	.99	$Y = 13.0406\ e^{-.0696X}$	.75
P retention, %	$Y = 59.921 (1-.3169\ e^{-.0020X})$	.99	$Y = 61.311 (1-1.1653\ e^{-7.7748+2.5079X})$	.99
Ca retention, %	$Y = 45.77 (1-.2601e^{-.0048X})$	.96	$Y = 83.086 (1-1.4124\ e^{-3.1651+1.0211X})$	.98

[a]Phytase effect at two nP levels or four Ca:tP ratios, X = added phytase, U/kg diet. The euqations were generated across four Ca:tP ratios or two nP levels.

[b]Ca:tP ratio effect at two nP levels or four phytase levels, X = Ca:tP ratios. The euqations were generated across four phytase levels or two nP levels.

Table 11. Linear functions for performance, toe ash content, and P and Ca retention of turkey poults fed corn soybean meal diets with four Ca:tP ratios, four phytase and two nP levels from hatch to 21 d of age

Item	Phytase effect[a]			Ca:tP ratio effect[b]		
	Equation	r^2	*P*-value	Equation	r^2	*P*-value
	nP = .27%			nP = .27%		
BW gain, g/bird	Y = 402.95 + .1440X	.77	.125	Y = 586.18 - 81.6800X	.84	.080
Toe ash content, %	Y = 9.2282 + .0028X	.71	.157	Y = 12.895 - 1.5851X	.97	.017
P retention, %	Y = 50.0544 + .0153X	.95	.024	Y = 71.6264 -9.4869X	.91	.047
Ca retention, %	Y = 50.0336 + .0131X	.80	.071	Y = 98.2259 - 27.2778X	.99	.003
	nP = .36%			nP = .36%		
BW gain, g/bird	Y = 464.07 + .0686X	.82	.090	Y = 593.82 - 63.79X	.91	.043
Toe ash content, %	Y = 10.679 + .0016X	.75	.137	Y = 13.026 - 1.053X	.95	.027
P retention, %	Y = 51.0099 + .0060X	.89	.055	Y = 68.4796 - 9.5314X	.97	.013
Ca retention, %	Y = 48.7161 + .0098X	.85	.098	Y = 100,6356 - 30.6611X	.99	.003
	Ca:tP ratio = 1.1:1			Phytase = 0, U/kg diet		
BW gain, g/bird	Y = 478.05 + .0721X	.92	.090	Y = 643.1 - 148X	.97	.016
Toe ash content, %	Y = 11.027 + .0011X	.64	.198	Y = 14.34 - 3.1513X	.98	.012
P retention, %	Y = 56.1388 + .0061X	.82	.092	Y = 74.1969 -15.8648X	.92	.043
Ca retention, %	Y = 66.5795 + .0035X	.79	.045	Y = 106.2219 - 37.4994X	.96	.018
	Ca:tP ratio = 1.4:1			Phytase = 300, U/kg diet		
BW gain, g/d	Y = 457.91 + .0904X	.82	.092	Y = 563.69 - 41.03X	.85	.071
Toe ash content, g/d	Y = 10.5 + .0017X	.61	.216	Y = 13.096 - 1.1556X	.95	.024
P retention, %	Y = 53.9368 + .0076X	.92	.042	Y = 70.9388 - 10.4432X	.97	.016
Ca retention, %	Y = 53.5314 + .0102X	.77	.122	Y = 93.8385 - 26.2026X	.99	.005
	Ca:tP ratio = 1.7:1			Phytase = 600, U/kg diet		
BW gain, g/bird	Y = 424.47 + .1182X	.76	.126	Y = 569.63 - 41.03X	.84	.085
Toe ash content, %	Y = 9.328 + .0033X	.79	.101	Y = 12.217 - .5421X	.89	.103

P retention, %	Y = 49.5811 +.0115X	.96	.021	Y = 68.8931 - 7.2723X	.93	.037
Ca retention, %	Y = 40.9197 + .02X	.89	.051	Y = 100.6813 - 26.7308X	.99	.003
	Ca:tP ratio = 2.0:1			Phytase = 900, U/kg diet		
BW gain, g/bird	Y = 373.61 + .1546X	.71	.155	Y = 599.88 - 54.81X	.93	.038
Toe ash content, %	Y = 8.88 + .0031X	.71	.161	Y = 12.977 - .8171X	.74	.198
P retention, %	Y = 42.472 + .0173X	.94	.030	Y = 66.1831 - 4.4562X	.91	.047
Ca retention, %	Y = 36.4687 + .012X	.80	.085	Y = 96.9812 - 25.445X	.97	.017

[a]Phytase effect at two nP levels or four Ca:tP ratios, X = added phytase, U/kg diet. The euqations were generated across four Ca:tP ratios or two nP levels.

[b]Ca:tP ratio effect at two nP levels or four phytase levels, X = Ca:tP ratios. The euqations were generated across four phytase levels or two nP levels.

Table 12. Equivalence of microbial phytase to the nP for turkey poults fed corn soybean meal diets

Added Phytase, U/kg diet	Equivalent values 250	500	750	1,000	Equivalent equations[a]
nP (tP) = .27 (.54) %					
Equivalent of nP, %					
BW gain	.346	.371	.377	.379	$Y = .3797(1 - 3603e^{-.0056X})$
Toe ash content	.354	.399	.407	.409	$Y = .4093(1 - .7791e^{-.0070X})$
Mean equivalent					
nP, %	.350	.386	.393	.394	$Y = .3952(1 - .5565e^{-.0063X})$
Released P, %[b]	.080	.116	.123	.124	$Y = .1244(1 - 1.8956e^{-.0067X})$
% of phytate P[c]	30.0	43.4	46.1	46.7	$Y = 47.07(1 - 1.6625e-.^{0061X})$
nP (tP) = .36 (.63) %					
Equivalent of nP, %					
BW gain	.392	.450	.468	.474	$Y = .4763(1 - .5662e^{-.0047X})$
Toe ash content	.426	.468	.480	.484	$Y = .4852(1 - .4013e^{-.0048X})$
Mean equivalent					
nP, %	.409	.459	.474	.479	$Y = .4809(1 - .4884e^{-0047X})$
Released P, %[b]	.050	.099	.114	.119	$Y = .1210(1 - 1.8849e^{-.0047X})$
% of phytate P[c]	18.6	37.2	42.9	44.6	$Y = 45.37(1 - 1.9354e^{-.0047X})$
Mean equivalent of two P levels					
nP, %	.380	.422	.433	.436	$Y = .4370(1 - .4948e^{-.0053X})$
Released P, %	.065	.107	.118	.121	$Y = .1220(1 - 1.7721e^{-.0053X})$
% of phytate P	24.3	40.3	44.5	.45.6	$Y = 45.99(1 - 1.7974e^{-.0054X})$
Released P, g/100	.26	.21	.16	.12	

[a]Equivalency equations were developed across four Ca:tP raios using nonlinear functions of phytase and linear functions of nP. At .27% nP, Y (BW gain, g/bird) = $509.15(1 - .2654e^{-.00561X})$, Y (toe ash percent) = $11.232(1 - .5362e^{-.00700X})$, and at .36% nP, Y (BW gain, g/bird) = $604.63(1 - .4407e^{-.00466X})$, Y (toe ash percent) = $12.6647(1 - .2904e^{-.00482X})$ where X = phytase (U/kg of diet). Linear functions of nP were Y = 134 + 988X for BW gain and Y = 3.5 + 18.89X for toe ash, where X = nP. In the equivalency equations, X = added phytase (U/kg of diet) and Y equivalent values of nP (%), or released P (%).

[b]Released P = equivalent of nP - .27 (or .36).

[c]Plant P in diets is .536%, phytate P is .266%, and nonphytate P is .27%; percentage of released phytate P = released P / .266.

Discussion

The results of the present study clearly support findings from numerous studies demonstrating that microbial phytase is very effective for improving P availability in corn-soybean meal-based diets and soybean meal-based diets when fed to pigs, broilers and turkeys. However, findings of the present study indicate that the response obtained with microbial phytase additions to diets for turkey poults is influenced by the Ca:tP ratio (or level of Ca in the diet) as well as the level of nP. Widening the Ca:tP ratio lowered all measurements at each nP and at each phytase level. At .27% nP level, the efficacy of microbial phytase, based on an average of BW gain and toe ash content, decreased 7.4% when the Ca:tP ratio (or level of Ca) was increased from 1.4:1 to 2:1; this decrease was 4.9% when a .36% nP level was fed. It was evident that the negative effect of widening the Ca:tP ratio was greater for lower levels of nP. In agreement, Schoner et al. (1993) reported for broilers that feeding high levels of Ca with a constant level of P (.35% nP) reduced the increase in BW gain, feed intake, and P and Ca retention that was observed when phytase was added. From their lowest (.6%) to highest (.9%) levels of Ca, the Ca:tP ratio varied from 1.7:1 to 2.57:1. In the present study, there appeared to be no difference in BW gain between Ca:tP ratios of 1.1:1 and 1.4:1 with .27% nP (.54% tP) level, but at .36% nP (.63% tP) the largest BW gain was obtained with the 1.1:1 ratio. Toe ash content, and P and Ca retention were always higher for the 1.1:1 Ca:tP ratio at both nP levels. Two- and three-way interactions for most measurements were significant, which indicated that the Ca:tP effects were not independent of dietary P and phytase levels. The detrimental effect of widening the Ca:tP ratio was strongest at lower dietary P levels and phytase levels.

The influence of added phytase and varying Ca:tP ratios on feed intake and gain:feed ratio were not as consistent as for BW gain, toe ash, or P retention. However,

feed intake and gain:feed were generally increased as phytase was added and as the Ca:tP ratio became narrower; most of the effect occurred at the lowest level of phytase addition and the widest Ca:tP ratios. Ravindran et al. (1995a) reported improved gain:feed ratios of poults fed soybean meal-based semi-purified diets containing .27 and .36% nP when the lowest levels of phytase were added. Some previous reports using broilers have shown improved gain:feed when phytase was added to the diet (Simons et al., 1990; Saylor et al., 1991). To the contrary, other studies in broilers (Swick and Ivey, 1990; Vogt, 1992; Kornegay et al., 1994; Denbow et al., 1995) have reported no effect or very inconsistent effects of supplemental phytase on gain:feed ratio.

The results presented here demonstrate that in addition to improving P availability, supplemental phytase improves Ca availability as suggested by increased Ca retention of turkey poults. This finding is in agreement with observations in broilers (Schoner et al., 1991, 1993; Kornegay et al., 1994). In a broiler study designed to measure the effect of phytase on Ca availability, Schoner et al. (1994) reported that 500 U of microbial phytase was equivalent to .35 g Ca as measured by BW gain and .56 g Ca as measured by phalanx ash. Phytic acid, a cation chelator, makes Ca unavailable for intestinal absorption. Phytase releases Ca from the insoluble salts by hydrolizing phytic acid, and potentially makes Ca available for absorption by poults. Increased Ca and P retention is also supported by our findings of an increase in bone ash Ca, P, Zn, and Mg contents and improved bone calcification and histological development (Qian et al., 1994b,c) when phytase was added to broiler and turkey poult diets.

In agreement with previous poultry studies (Kornegay et al., 1994; Denbow et al., 1995; Ravindran et al., 1995a), BW gain and toe ash percent of broiler and turkey poults were the most sensitive measurements to assess microbial phytase efficacy for the replacement of nP for turkey poults. These measurements were also sensitive for

assessing the effects of varying Ca:tP ratios and levels of nP. Body weight gain and toe ash were also reported by Ravindran et al. (1995b) to be equally or more sensitive for assessing P availability of several inorganic sources of phosphorus than tibia ash. They also reported that tibia specific gravity, tibia shear force, toe shear forces and metatarsal shear force were of limited value as response criteria for assessing P availability. Ravindran et al. (1995b) did not measure P or Ca retention. In the present study, both P and Ca retention appear to be very sensitive to the addition of phytase at varying nP levels or Ca:tP ratios, and varying Ca:tP ratios at different levels of phytase or nP.

Calcium is thought to be a key factor that influences the activity of mucosal phytase in poultry and rat (Bhandari, 1980; Wise, 1983). Similarly, the influence of Ca on phytase activity of a microbial product added to corn-soybean meal diets fed to poults was observed. Results of the phytase assay of the mixed diets, showed that the wider the Ca:tP ratios the greater the decrease in the phytase activity of diets with added phytase. The decrease in activity as the Ca:tP ratio became wider could be explained as follows: 1) the extra Ca binds with phytase to form an insoluble complex that is less accessible to phytase; 2) the extra Ca could directly repress phytase activity by competing for the active sites of the enzyme (Wise, 1983; Pointillart et al., 1985). This depressing effect was greater at the lower aP level, which suggested that the P level in the reacting mixture is also critical to the phytase activity. Maximum responses to phytase efficacy were achieved when diets of poults were formulated at 1.1 - 1.4 to 1 of Ca:tP.

In the calculation of P-equivalency values for phytase in this study, about 340 and 511 U of phytase are required to replace 1 g of P in corn soybean-meal diets for 1 to 21 d old turkey poults fed with .27 and .36% nP, respectively. This value is calculted across four Ca:tP ratios of 1.1 to 2.0:1, and is in accordance with the findings by Ravindran et al. (1995a) using turkey poults fed a soybean meal-based semi-purified diet; they also

reported a higher P-equivalency value for the .27% nP diets vs the .36% nP diets (602 vs 700 U). Denbow et al. (1995), using broilers fed a soybean meal-based semi-purified diet similar to that used by Ravindran et al. (1995a), reported that 609 U of phytase was equivalent to 1 g P when .20% nP diets were fed, but 1,133 U when the .27% nP diet was fed. Both studies of Ravindran et al. (1995a) and Denbow et al. (1995) used a 2.0:1 Ca:tP ratio in diets, which resulted in a higher P-equivalency values of phytase. Supplemental phytase seemed more effective in the lower dietary nP level than the higher nP level. Wise (1983) suggested extra inorganic P might inhibit mucosal phytase activity of chicken small intestines. Estimated P percentage released from phytate in this study is slightly lower than the values of their study, which could be attributed to the difference in the diet type.

Implications

In summary, the results show that supplemental phytase improved body weight gain, feed intake, gain:feed, toe ash content, and calcium and phosphorus retention of turkey poults fed a corn-soybean based diet; these improvements were negatively influenced by wider dietary calcium:total phosphorus ratios, and also by dietary nonphytate phosphorus levels. Maximum responses to supplemental phytase were achieved when poults were fed diets with 600 to 900 U of phytase/kg diet (900 U at .27% nonphytate phosphorus and 600 U at .36% nonphytate phosphorus), and to dietary calcium:total phosphorus ratios as diets were formulated at 1.1 to 1.4:1. Similar results were found *in vitro* in which phytase activity was inhibited by the wider calcium:total phosphorus ratios. In addition, various response curves to phytase, calcium:total phosphorus and total phosphorus were developed for the practical utilization of phytase. Also, phosphorus-equivalency equations of microbial phytase for nonphytate phosphorus were generated; 340 to 511 U of phytase per kilogram of diet could be equivalent to 1 g

nonphytate phosphorus for turkey poults fed corn soybean meal diets with nonphytate phosphorus levels from .27 to .36%.

CHAPTER VII

Characterization of *Aspergillus niger* Phytase and Investigation of the Inhibitory Effect of Cations on the Phytase Activity

ABSTRACT A discontinuous assay using malachite green as color reagent was adapted for the characterization of *Aspergillus niger* phytase and for evaluation of the influence of several cations on the phytase activity. The sensitivity of the malachite green assay for the inorganic phosphorous was approximately 50 fold higher than with molybdovanadate as color reagent. A K_m of 62 μM and V_{max} of 139 units of specific activity per mg of phytase protein were determined using the malachite green reagent. The cations, Mg^{2+}, Mn^{2+}, Ca^{2+}, Cr^{3+}, Fe^{3+}, Cu^{2+} and Zn^{2+}, inhibited microbial phytase activity, and were competitive or of mixed-type inhibitors. Binding of the cations with phytate may also inhibit the enzyme by decreasing the effective substrate concentration. A decreasing order of the inhibitory effect of cations on phytase activity was observed: $Zn^{2+} > Cu^{2+} > Fe^{3+} > Cr^{3+} > Ca^{2+} > Mn^{2+} > Mg^{2+}$. A competitive inhibition was observed for Ca^{2+}, Cr^{3+}, Fe^{3+}, Cu^{2+} and Zn^{2+}; whereas Mg^{2+} and Mn^{2+} gave a mixed-type inhibition. The inhibition by Ca^{2+} and Mg^{2+} caused only a partial inhibition because the enzymatic reaction rate was never reduced to zero and replots of slopes for Ca^{2+} and Mg^{2+} was hyperbolic. A pure inhibition was imposed by Zn^{2+}, Cu^{2+}, Fe^{3+}, Cr^{3+} and Mn^{2+}. Similar inhibitory effects of the cation also were observed *in vitro* using a commercial diet containing microbial phytase in the incubation system. The mineral concentrations tended to be more critical than the molar ratio of [mineral] to [phytate] for inhibition of phytase activity. In summary, the cations tested possessed a potential for decreasing *A. niger* phytase activity by competitive or mixed-type inhibition; and binding of cations to the phytate substrate might be also involved in the inhibition.

Key Words: Characterization, Phytase, Cations, Incubation

Introduction

Phytase, *myo*-inositol hexaphosphate phosphohydrolase, is a family of enzymes catalyzing the stepwise removal of inorganic orthosphate from phytate (Ullah, 1988a). Phytase is widely distributed in plant and animal tissues, and in various microorganisms (Bitar and Reinhold, 1972; Gibson and Ullah, 1988; Beers and Jongbloed, 1993). For example, the fungi *Aspergillus* such as *A. ficuum* and *A. niger* are of great interest at present, because they are able to produce relatively large amount of phytase (Ullah, 1988a; Zyla and Koreleska, 1993).

Characteristics of phytase from plant sources such as soybean, corn, wheat and rapeseed and from intestinal mucosa of chickens, rat, calf and man have been investigated extensively (Bitar and Reinhold, 1972; Gibson and Ullah, 1987; and Yang et al., 1991). Ullah (1988ab) reported that *A. ficuum* phytase had a K_m of 40 μM for phytate, and Simon et al. (1990) indicated that *A. ficuum* phytase had an optimum pH at 2.5 and 5.5. Han (1989) reported that hydrolysis of phytate in soybean meal by microbial phytase increased as the temperature increased from 20 to 55 °C. Similar results also were obtained from studies using phytase from plant and animal sources (Gibson and Ullah, 1987; Yang et al., 1991). Results of several *in vitro* studies of the influence of cations on phytase activity, however, are not consistent, and results from some *in vitro* studies are not consistent with those of *in vivo* studies. For example, calcium, which had a strong inhibitory effect on the phytase efficacy by decreasing phytase activity in pigs and poultry (Schoner et al., 1993; Lei et al., 1994; Qian and Kornegay, 1995), was reported to have no effect on *A. ficuum* phytase *in vitro* (Ullah, 1988a), but had a positive effect on soybean phytase *in vitro* (Gibson and Ullah, 1988). In commonly available methods for phytase assay, molybdovanadate reagent is used because it easily forms a yellow-color complex with inorganic phosphate (Simons et al., 1990; Engelen et al., 1994). However, the sensitivity of the molybdovanadate reagent is not as high as that of the malachite green

reagent (Hess and Derr, 1975). In assay of phosphates in trace concentrations, Hess and Derr (1975) observed that malachite green reagent had high sensitivity to inorganic phosphates because the developed color complex resulted from the interaction between the reagent and phosphate.

The objectives of the present study were to apply a discontinuous assay using malachite green reagent to the characterization of *A. niger* phytase, and to the investigation of the influence of several cations on the phytase activity.

Materials and Methods

Materials and reagents.

All of the following chemicals were reagent grade, and were purchased from Fisher Scientific Co. (Fair Lawn, NJ): ammonium molybdate [$(NH_4)_6Mo_7O_{24}\bullet 4H_2O$], ammonium vanadate ($NH_4VO_3$), calcium chloride ($CaCl_2\bullet 2H_2O$), chromium chloride ($CrCl_3\bullet 6H_2O$), cupric chloride ($CuCl_2$), ferric chloride ($FeCl_3$), malachite green, magnesium chloride ($MgCl_2\bullet 6H_2O$), manganese chloride ($MnCl_2\bullet 4H_2O$), sodium acetate ($NaC_2H_3O_2\bullet 3H_2O$), sodium phosphate (Na_2HPO_3), and zinc chloride ($ZnCl_2$). Bovine serum albumin, malachite green, sodium phytate ($C_6H_6O_{24}P_6Na_{12}$), and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). *Aspergillus niger* phytase, *myo*-inositol-hexaphosphate phosphohydrolase, was supplied by BASF Co. (Mount Olive, NJ),

The malachite reagent was prepared by mixing 3 parts of .045% malachite green solution with 1 part of 4.2% ammonium molybdate in 4 M hydrochloric acid solution; then, a 2% Triton X-100 stock solution was added to the mixture such that the solution contained .040% Triton X-100. This procedure was performed using a modification of the method of Hess and Derr (1975). Molybdovanadate reagent was prepared by mixing 250 mL of a 80 mM ammonium molybdate stock solution and 250 mL of a 20 mM ammonium vanadate stock solution. As the mixture was stirred, 165 mL of 65% nitric

acid was added before the volume was made up to 1 L with distilled water. A .25 M acetate buffer (pH 5.5) was prepared by adding 1.66 mL of 100% acetic acid, 30.02 g of sodium acetate, and .147 g of calcium chloride in 900 mL of water; the pH was adjusted to 5.5 with acetic acid (100%), and the volume was made up to 1 L with distilled water (Engelen et al., 1994). The sodium phytate solution (3.64 mM) was made by dissolving 3.36 g sodium phytate in 900 mL acetate buffer solution, adjusting pH to 5.5 using 4 M acetic acid, and making up to 1 L with the acetate buffer. The phytase solution also was prepared using the .25 M acetate buffer (pH 5.5).

The diet sample contained corn and soybean meal (~3:1) with added minerals and vitamins. It was calculated to contain 4.3 g of Ca/kg, 3.6 g of total P/kg, 2.44 g of phytate P/kg, 70 mg of Mn/kg, 80 mg of Zn/kg, 150 mg of Fe/kg, 25 mg of Cu/kg, and 700 units of supplemental phytase/kg of diet (Table 1).

Discontinuous assay of phytase activity.

The sodium phytate solution (3.64 mM) and the phytase solution (5.48 μg/mL) were added in appropriate amounts to obtain the desired final concentrations in the incubation mixture. The total volume of the incubation mixture was made up to 500 μL with acetate buffer (.25 M, pH 5.5). The enzymatic hydrolysis of phytate was performed at 37°C (the normal body temperature in animals) in a water-bath. After initiation of the reaction, typically with the addition of the enzyme, aliquots of 50 μL were withdrawn at 0, 5, 10, 15 and 20 minutes. The aliquots were immediately added to 1.0 mL of malachite reagent.

The enzymatic reaction were stopped by acidic denaturation of phytase, while the chromogen product of phosphomolybdate-malachite green complex were formed simultaneously. The green chromogen was measured spectrophotometically using a Split-Beam Spectrophotometer (Spectronic® 1001, The Bausch & Lomb Inc., 820 Linden Ave., Rochester, NY) at 645 nm after10 minutes of color development (Hess and Derr,

Table 1. Percentage composition of the practical diet[a]

Ingredients	%
Corn (8.8% crude protein)	73.68
Soybean meal (48.5% crude protein)	24.50
Limestone (38.0% Ca)	.92
Salt	.30
Vitamin premix[b]	.25
Trace mineral premix[c]	.10
Selenium premix[d]	.05
Cr2O3	.10
L-Lysine	.10
Calculated analysis of minerals	**mg/kg**
Ca	4,300
Total P	3,600
Phytate P	2,440
Mn	70
Zn	80
Fe	150
Cu	25

[a]The diet was a practical pig diet that was supplemented with 700 unit of microbial phytase/kg of diet. A unit is defined as the quantity of enzyme that liberates 1 μmol of inorganic phosphorus per minute from 1.5 mM sodium phytate at pH 5.5 and 37°C.

[b]Supplied per kilogram of diet: 4400 IU of vitamin A, 440 IU of vitamin D3, 11 IU of vitamin E, 2.2 mg of vitamin K, 4.4 mg of riboflavin, 22 mg of Ca-d-pantothenate, 22 mg of niacin, .022 mg of vitamin B12, 440 mg of choline chloride, .44 mg of d-biotin, 3.9 mg of folic acid, 10 mg of thiamin•HCl, and 3.9 mg of pyridoxine•HCl.

[c]Supplied per kilogram of diet: 44 mg of Mn, 47.5 mg of Zn, 50 mg of Fe, 6.25 mg of Cu, and 2 mg of I.

[d]Supplied .3 mg of Se per kilogram of diet.

1975). The amount of the chromogen in each aliquot was plotted as a function of the time. The reaction velocity was determined from the linear portion of the absorbance by time plots.

A standard curve using sodium phosphate was developed to estimate the amount of inorganic orthophosphate released from sodium phytate hydrolyzed by phytase. The specific activity of phytase was calculated based on the amount of inorganic

orthophosphate released from sodium phytate. One unit of specific activity of the enzyme phytase was defined as μmole of inorganic orthophosphate liberated per min by 1 mg of phytase protein. The protein content of phytase was determined by BCA assay using bovine serum albumin as standard (Smith et al., 1985).

For the evaluation of the sensitivity of malachite color reagent to the inorganic P, a photometric assay was performed using the molybdovanadate as color reagent to determine its response to inorganic P. The malachite green and molybdovanadate assays were photometrically conducted to determine the absorbence at 645 and 415 nm, respectively, and were compared for the sensitivity of the two color reagents to inorganic P.

Influence of cations on phytase activity.

The influence of several cations on phytase activity was studied by measuring the K_m, V_{max} and K_i values using the assay described above. Graded concentrations of Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Fe^{3+} and Cr^{3+} were obtained by the addition of stock solutions of the chloride salt of these cations to the incubation mixture. For each assay an incubation mixture without the enzyme, but containing sodium phytate, served as a blank. Sodium phytate was used as the substrate. The *A. niger* phytase was used as the enzyme for hydrolysis of the substrate.

Determination of the activity of supplemental phytase in the diet.

Suspension of the phytase enzyme from the diet. A 5 g sample of the diet (Table 1) was added to 50.0 mL of the .25 M acetate buffer. Graded concentrations of Ca^{2+}, Zn^{2+} and Cu^{2+} were obtained by adding appropriate amounts of the stock solution of the chloride salt of these cations to the mixture. The dietary sample was prepared by stirring on a magnetic stirrer at room temperature (approximately 20°C) for 75 minutes, filtered

through a folded filter (No. 595, Lot No. 0931, Schleicher & Schuell, Keene, NH). The suspension (filtrate) that contained supplemental phytase was kept in an ice bath until the incubation was started.

Determination of supplemental phytase activity using the added sodium phytate as substrate. A 200 μL aliquot of the suspension was added to 300 μL of an incubation mixture that contained 280 μL of the acetate buffer and 20 μL of 3.64 mM sodium phytate. The mixture was incubated for 15 minutes at 37 °C, then a 50 μL aliquot of the incubation mixture was withdrawn, and immediately added to 1.0 mL of the malachite reagent. After 10 min of color development, the chromogen was determined at 645 nm.

Determination of supplemental phytase activity using the dietary phytate as substrate. A 20 μl aliquot of the filtered suspension prepared above was withdrawn, and directly added in the 1.0 ml of the malachite reagent. The chromogen was measured at 645 nm after 10 minutes for color development. All other conditions were similar to the above discontinuous assay for determination of the amount of the inorganic orthophosphate produced by supplemental phytase in diets.

Results

Characterization of A. niger phytase by discontinuous assay. The effect of substrate on the rate of phytase-catalyzed reaction was studied in the concentration range of 0 to 2.88 mM for sodium phytate in .25 M sodium acetate buffer (pH 5.5) at 37°C (Figure 1). A Lineweaver-Burk plot was used to determine the Michaelis constant (K_m) and V_{max}. The K_m value of the *A. niger* phytase for phytate was estimated to be 62 μM with V_{max} of 139 units of specific activity.

The malachite reagent was used in the discontinuous assay to stop the enzyme reaction, and to measure the released free phosphate. The sensitivity of malachite reagent to inorganic P was compared to that of molybdovanadate reagent. Based on the

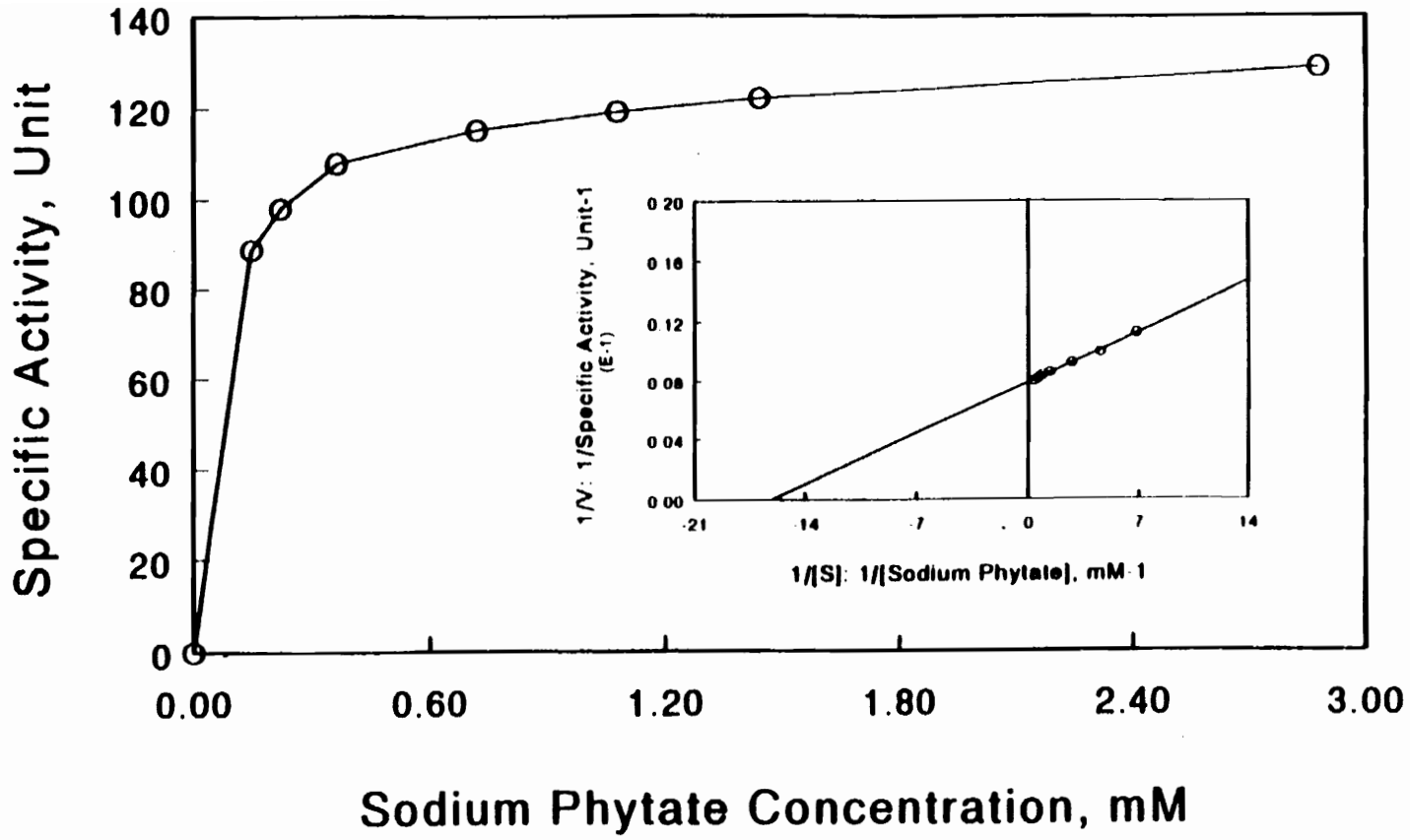

Figure 1. Effect of phytate concentration versus *A. niger* phytase activity. The enzyme concentration was fixed at .3 μg of the .5 ml incubation mixture. The discontinuous assays were performed as described in the materials and methods section. The K_m was 62 μM with a V_{max} of 139 units of specific activity. The inset depicts a Lineweaver-Burk plot of the data.

regression coefficient, the absorbency of the developed malachite chromogen was about 1.1 at 645 nm in response to 20 nmole of inorganic phosphate, whereas, the molybdovanadate chromogen product was only .022 at 415 nm (Figure 2). The absorbency maximum for malachite and molybdovanadate reagent is at 645 and 415 nm, respectively (Hess and Derr, 1975; Engelen et al., 1994). With inorganic phosphate, the sensitivity of the chromogen developed by malachite reagent was approximately 50 times greater than that of the molybdovanadate reagent.

Influence of cations on phytase activity. The sensitivity of *A. niger* phytase to the influence of different cations varied (Figures 3 and 4). The addition of Ca^{2+}, Cr^{3+}, Fe^{3+}, Cu^{2+} and Zn^{2+} caused an increase in the apparent K_m values but the apparent V_{max} values were not changed. The Lineweaver-Burk plots of the data of these cations showed a common intersect on the 1/v-axis, which indicated competitive inhibition. In contrast, the addition of Mg^{2+} and Mn^{2+} caused a mix-type inhibition because the common intersect occurred above the 1/[S] and to the left of the 1/v-axis, which resulted in an increase in the apparent K_m but a decrease in the apparent V_{max} values. The slope replots of data for Ca^{2+} and Mg^{2+} inhibition were hyperbolic (Figure 5), which indicated that a partial inhibition was involved in the competitive Ca^{2+} inhibition, and in the mixed-type Mg^{2+} inhibition of the *A. niger* phytase enzyme. All other cations imposed a pure competitive or pure mixed-type inhibition, resulting in a linear slope replot. In addition, the formation of a white precipitate was observed when cation solutions were added to the phytate incubation mixture before adding the enzyme. This phenomenon was more evident in the presence of Ca^{2+}, Zn^{2+}, and Fe^{3+}.

The following decreasing order of the inhibitory effect on phytase activity was observed in this study: $Zn^{2+} > Cu^{2+} > Fe^{3+} > Cr^{3+} > Ca^{2+} > Mn^{2+} > Mg^{2+}$, based on the K_i value that increased from a low value for Zn^{2+} to a high value for Mg^{2+} (Table 2). This

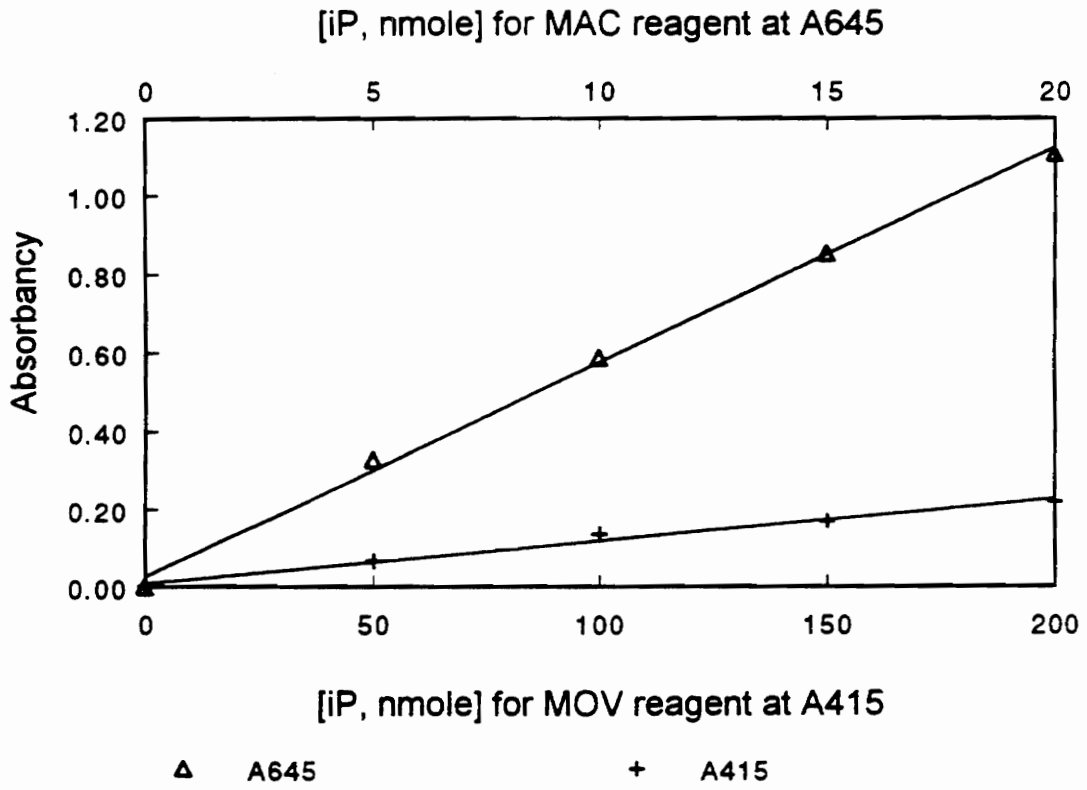

Figure 2. Comparison of the absorption of the phosphate-malachite (MAC) green complex at 645 nm with that of the chemically reductive phosphate-molybdovanadate (MOV) yellow complex at 415 nm.

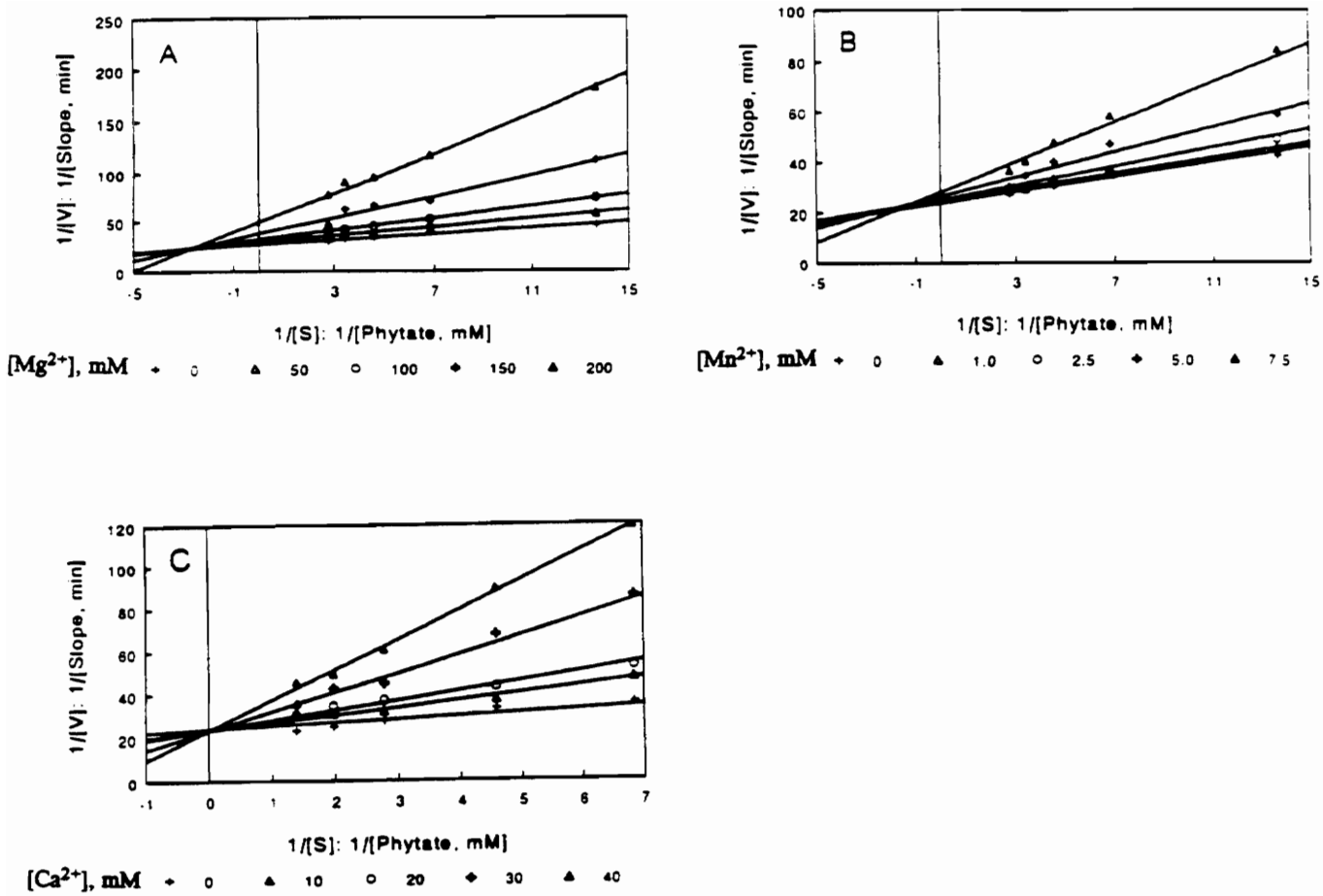

Figure 3. Lineweaver-Burk plots of data for effects of Mg^{2+} (A), Mn^{2+} (B), and Ca^{2+} (C) on the kinetic parameters for phytase hydrolyzing phytate: (A) an increase in Mg^{2+} concentrations in the incubation mixture resulted in an apparent K_m increase and an apparent V_{max} decrease: 62, 71, 94, 141 and 196 μM for apparent K_m, and 139, 112, 103, 88 and 67 U of specific activity for apparent V_{max}, respectively for 0, 50, 100, 150 and 200 mM of Mg^{2+}; (B) an increase in Mn^{2+} concentrations in the incubation mixture resulted in an apparent K_m increase and an apparent V_{max} decrease: 62, 68, 74, 92 and 139 μM for apparent K_m, and 139, 134, 132, 125 and 118 U of specific activity for apparent V_{max}, respectively for 0, 1.0, 2.5, 5.0 and 7.5 mM of Mn^{2+}; (C) an increase in Ca^{2+} concentrations in the incubation mixture resulted in an increase in apparent K_m and no change in the V_{max}: 62, 169, 232, 543 and 1121 μM for apparent K_m, respectively for 0, 10, 20, 30 and 40 mM of Ca^{2+}.

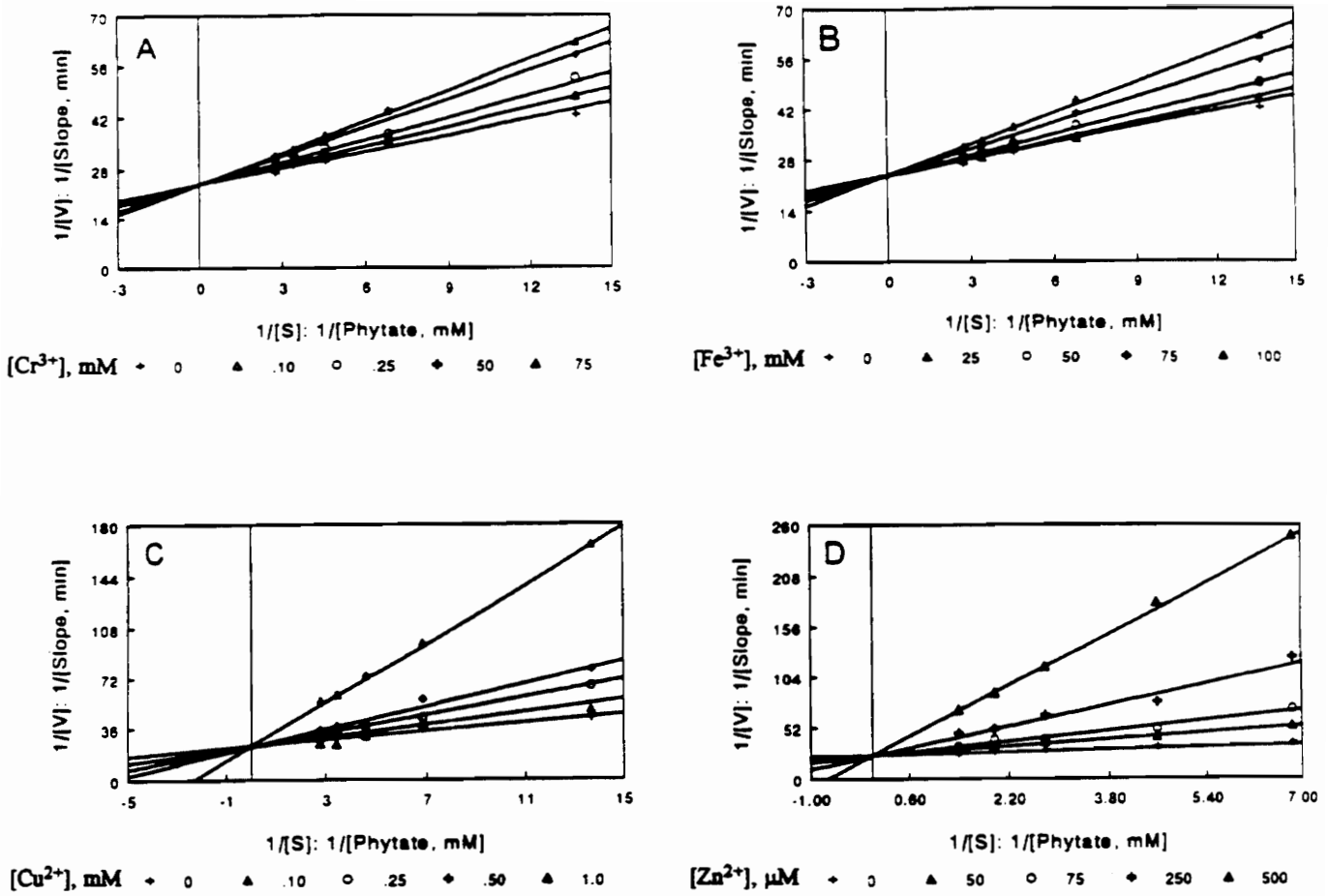

Figure 4. Lineweaver-Burk plots of data for effects of Cr^{3+} (A), Fe^{3+} (B), Cu^{2+} (C), and Zn^{2+} (D) on the kinetic parameters for phytase hydrolyzing phytate: (A) an increase in Cr^{3+} concentrations in the incubation mixture resulted in an apparent K_m increase and no change in the V_{max} value of 139 U of specific activity: 62, 73, 84, 107 and 119 µM for apparent K_m, respectively for 0, .10, .25, .50 and .75 mM of Cr^{3+}; (B) an increase in Fe^{3+} concentrations in the incubation mixture resulted in an increase in apparent K_m and no change in the V_{max} value: 62, 69, 83, 108 and 135 µM for apparent K_m, respectively for 0, 25, 50, 75 and 100 µM of Fe^{3+}; (C) an increase in Cu^{2+} concentrations in the incubation mixture resulted in an increase in apparent K_m and no change in the V_{max} value: 62, 97, 135, 175 and 431 µM for apparent K_m, respectively for 0, .10, .25, .50 and 1.0 mM of Cu^{2+}; (D) an increase in Zn^{2+} concentrations in the incubation mixture resulted in an increase in apparent K_m and no change in the V_{max} value: 62, 179, 283, 579 and 1358 µM for apparent K_m, respectively for 0, 50, 75, 250 and 500 µM of Zn^{2+}

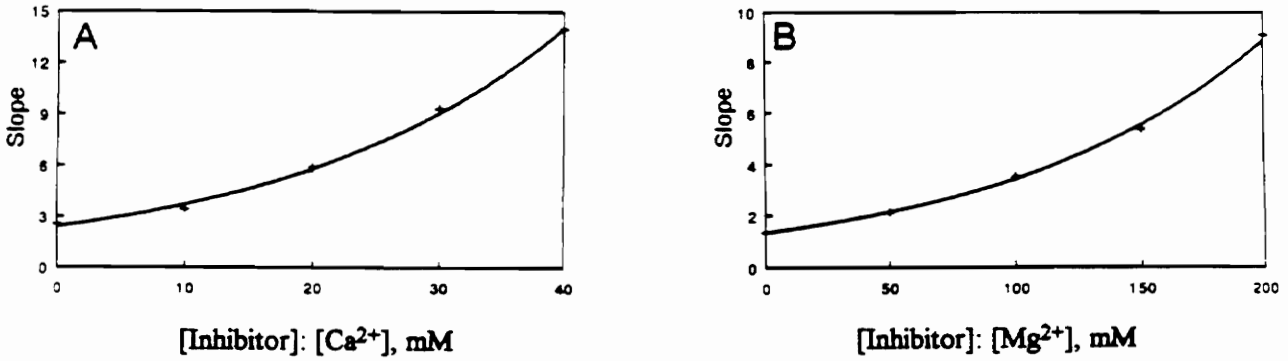

Figure 5. Replots of slopes for Ca^{2+} (A) and Mg^{2+} (B) inhibition: (A) slope replot for Ca^{2+} competitive inhibition was hyperbolic; (B) slope replot for Mg^{2+} mixed-type inhibition was hyperbolic.

Table 2. Inhibitory effect of cations on microbial phytase activity

	Cation Inhibitory Range[a]			
Cations	[Cation], mM	[Cation]/[Phytate]	K_i, M	Nature of Inhibition
Mg^{2+}	50 - 300	685 - 4,110	10.1×10^{-3}	Partial mixed-type
Mn^{2+}	1 - 50	14 - 685	3.9×10^{-3}	Pure mixed-type
Ca^{2+}	10 - 100	137 - 1,370	3.1×10^{-3}	Partial competitive
Cr^{3+}	.1 - 5	1.4 - 68.5	$.84 \times 10^{-3}$	Pure competitive
Fe^{3+}	.01 - 5	.014 - 68.5	$.11 \times 10^{-3}$	Pure competitive
Cu^{2+}	.1 - 2	1.4 - 27.4	$.07 \times 10^{-3}$	Pure competitive
Zn^{2+}	.05 - 2	.68 - 27.4	2.6×10^{-6}	Pure competitive

[a]Indication of cation concentrations and molar ratios of cation to phytate from initial inhibitory effect to complete loss of phytase activity (no measured activity) after 15 min of incubation in the test condition, except Ca^{2+} and Mg^{2+} in which activities did not reach zero.

also was consistent with the inhibitory range of cations, in which the minimum concentration of Mg^{2+} for inhibition was 50 mM and the molar ratio of [Mg^{2+}] to [phytate] was 685, whereas, the maximum concentration of Zn^{2+} was .05 mM and the molar ratio of [Zn^{2+}] to [phytate] was .68. Addition of Zn^{2+} to the incubation mixture at a level of 1 mM completely inhibited phytase activity, while a complete loss of phytase activity was not reached even by the addition of Mg^{2+} to 300 mM.

Evaluation of the inhibition of minerals on phytase activity in the diet. The addition of Ca, Cu, and Zn in the diet resulted in a decrease in phytase activity (Tables 3, 4 and 5). A dietary Ca content of 32.3 g/kg led to a 45% loss in phytase activity using dietary phytate and chemical sodium phytate as the substrate (Table 3). Furthermore, adding 44.3 g/kg of Ca resulted in a 85% loss of phytase activity when dietary phytate was used as the substrate but only a 54% loss of phytase activity when sodium phytate was used as the substrate. A very high Ca content in the diet did not completely inhibit phytase activity, whereas, high contents of Cu and Zn in the diet resulted in a complete loss of phytase activity. A dietary Cu content of 154 mg/kg resulted in about 18 to 23% loss of the phytase activity; and a very high level of Cu (1,315 mg/kg) led to complete loss of phytase activity using dietary phytate as the substrate, and approximately a 94% loss of phytase activity when sodium phytate was used as the substrate (Table 4). Similar effects were observed for Zn, in which 145 mg of Zn/kg led to 6 to 16% loss of the activity of phytase, and 1,388 mg of Zn/kg resulted in almost complete loss of phytase activity using both dietary phytate or sodium phytate as the substrate (Table 5).

The degree of the inhibitory effects of Ca, Cu and Zn seemed stronger when dietary phytate was used as the substrate than when sodium phytate was used. Although the molar ratio of [mineral] to [phytate] was narrower for the dietary phytate substrate, the concentrations of minerals were higher when dietary phytate was used as the substrate than when sodium phytate was used as the substrate in phytase assay.

Table 3. The *in vitro* activity of supplemental microbial phytase in a practical diet as influenced by calcium concentrations

Dietary Ca content, g/kg	Dietary phytate as the substrate			Chemical phytate as the substrate		
	[Ca], mM	[Ca]/[Phytate][a]	Activity, %[b]	[Ca], mM	[Ca]/[Phytate][a]	Activity, %[b]
4.3	10.8	41.5	100	4.3	58.9	100
6.3	15.8	60.8	87.8	6.3	86.3	87.5
8.3	20.8	80.0	82.9	8.3	113.7	82.3
10.3	25.8	99.2	74.5	10.3	141.0	79.6
12.3	30.8	118.5	74.1	12.3	174.0	72.5
16.3	40.8	156.9	59.4	16.3	223.3	71.6
24.3	60.8	233.8	54.1	24.3	332.9	70.7
32.3	80.8	310.8	52.7	32.3	442.5	57.2
44.3	110.8	426.2	14.7	44.3	606.8	46.2

[b]The molar ratio of Ca to phytate.

[a]Phytase activity of the diet without extra calcium supplementation is assumed to be 100%.

Table 4. The *in vitro* activity of supplemental microbial phytase in a practical diet as influenced by copper concentrations

Dietary Cu content, mg/kg	Dietary phytate as the substrate			Chemical phytate as the substrate		
	[Cu], μM	[Cu]/[Phytate][a]	Activity, %[b]	[Cu], μM	[Cu]/[Phytate][a]	Activity, %[b]
25	30	.11	100	10	.15	100
154	230	.88	77.4	90	1.34	82.2
283	430	1.65	49.5	170	2.33	80.6
412	630	2.42	38.7	250	3.42	72.5
541	830	3.19	27.5	330	4.52	45.1
670	1,030	3.96	9.9	410	5.62	41.5
1,315	2,030	7.81	--	810	11.10	6.1
2,605	4,030	15.5	--	1,610	22.05	--
6,475	10,030	38.58	--	4,010	54.93	--

[a]The molar ratio of Cu to phytate.

[b]Phytase activity of the diet without extra copper supplementation is assumed to be 100%.

Table 5. The *in vitro* activity of supplemental microbial phytase in a practical diet as influenced by zinc concentrations

Dietary Zn content, mg/kg	Dietary phytate as the substrate			Chemical phytate as the substrate		
	[Zn], μM	[Zn]/[Phytate][a]	Activity, %[b]	[Zn], μM	[Zn]/[Phytate][a]	Activity, %[b]
80	70	.28	100	30	.41	100
145	170	.65	94.4	70	.96	83.9
210	270	1.04	86.1	110	1.51	83.6
276	370	1.42	80.9	150	2.05	77.2
341	470	1.81	77.1	190	2.60	73.2
407	570	2.19	51.2	230	3.15	63.4
537	770	2.96	31.7	310	4.25	58.3
734	1,070	4.12	29.7	430	5.89	25.6
1,388	2,070	7.96	-	830	11.37	8.2
2,040	3,070	11.80	-	1,230	16.80	-

[a]The molar ratio of Zn to phytate.

[b]Phytase activity of the diet without extra zinc supplementation is assumed to be 100%.

Discussion

The characteristics of phytase from plant sources such as soybean, corn, wheat and rapeseed and from small intestinal mucosa of chicks, rat, calf and man have been investigated extensively (Bitar and Reinhold, 1972; Gibson and Ullah, 1988; Yang et al., 1991). The K_m value of phytase purified from the intestinal mucosa of rat was 210 μM for sodium phytate (Yang et al., 1991). The purified phytase from soybean seeds exhibited a high affinity for sodium phytate (K_m = 48 μM) (Gibson and Ullah, 1988). The Michaelis constant, K_m, is often associated with the strength of binding of substrate to enzyme (Mathews and van Holde, 1990). A large K_m value means that the enzyme possess a weak catalysis potential on the substrate. The present study showed that the *A. niger* phytase had a high affinity for the sodium phytate with a K_m of 62 μM using the discontinuous assay, which is in agreement with the study of Ullah (1988a). He pointed out that of all phosphoric substrates evaluated, phytase had the highest affinity for the sodium phytate, and that K_m was 40 μM when the assay was performed at pH 5.0 and 58° C using the laboratory-purified *A. ficcum* phytase. The kinetic constant, V_{max} that was determined to be 139 U of specific activity, also was consistent with the result of Ullah (1988a). The specific activity was 125 U by calculating V_{max} in the study conducted by Ullah (1988a).

The assays for phytase activity are usually conducted by using a 'one-time stop' assay, in which the phytase enzyme is denatured by a 'stop-color' reagent (molybdovanadate reagent that contains nitric acid) after the incubation with phytate substrate for one assigned time period (Ullah, 1988ab; Simons et al., 1990; Engelen et al., 1994). Molybdovanadate is utilized as the color reagent. Molybdovanadate reacts with the inorganic phosphate released from phytate by phytase, which leads to a formation of chemical reductive complex, the phosphomolybdate complex of yellow chromogen. However, the yellow chromogen is not sufficiently sensitive to detect low concentrations

of phosphorus (Hess and Derr, 1975). In the present study, malachite green was used as the color reagent that interacted with phosphomolybdate to form an intensive color complex. The formed chromogen complex was a highly stable green color because of the presence of Triton X-100 addition (Hess and Derr, 1975). The sensitivity of malachite green to phosphorus concentrations was about 50 times greater than that of molybdovanadate yellow. This was supported by the observation of Hess and Derr (1975), who indicated that the malachite green complex with phosphorus had a marked shift in absorption maximum and a high molar absorption coefficient.

For the 'one-time stop' assay, the absorbance achieved at one time period of incubation did not seem adequate for the calculation of kinetic constants because it is difficult to obtain an accurate slope or to establish a linear relationship of the increased amount of released phosphate with time. In the discontinuous assay, an analysis of multiple samples from each assay was performed to establish the linearity of product formation with time, which can supply a more accurate estimate of slope than the 'one-time stop' assay.

The results presented here demonstrate that increases in cation concentrations of Ca^{2+}, Mg^{2+}, Mn^{2+}, Cr^{3+}, Fe^{3+}, Cu^{2+} and Zn^{2+} depress microbial phytase activity. In agreement, Ullah (1988a) observed an inhibitory effect of Cu^{2+}, Zn^{2+} and Fe^{3+} on the *in vitro* phytase activity of *A. ficuum*. However, he found a positive effect of Mn^{2+} on the phytase, but did not report any effects of Ca^{2+}. In the study of phytase activity from rat intestinal mucosa, the addition of Zn^{2+} caused the activity to rise by about 40% as Zn^{2+} concentrations increased from 20 to 80 μM, whereas, a decrease in the activity was observed as Zn^{2+} exceeded 625 μM (Bitar and Reinhold, 1972). They did not obtain similar effects of Zn^{2+} on the intestinal phytase from chicken, calf and man. No effects of Cu^{2+} and Mn^{2+} were observed on the phytase activity from intestinal mucosa of rat, chicken, calf and man. In contrast, soybean phytase activity increased by 30 to 80% as

Ca^{2+}, Mg^{2+}, Mn^{2+} and Fe^{3+} concentrations increased from 200 to 1,000 μM but decreased by 25% as Zn^{2+} concentration increased from 200 to 1,000 μM (Gibson and Ullah, 1988). However, they also observed an inhibitory effect of all cations when they were increased to 5.0 mM in the *in vitro* assay system. It would appear that the influence of cations on phytase activity differed with different phytase sources.

Ullah (1988a) indicated that Cu^{2+} and Zn^{2+} were noncompetitive inhibitors of *A. ficuum* phytase. In disagreement, our study suggested that Cu^{2+} and Zn^{2+} were competitive inhibitors when *A. niger* phytase was used. The degree of inhibition by a competitive inhibitor depends on the concentrations of substrate and inhibitor, a high concentration of inhibitor can reduce the velocity of the reaction to zero (Segel, 1975). However, the inhibition caused by a noncompetitive inhibitor is not influenced by both substrate and inhibitor concentrations. At a constant concentration of Cu^{2+} and Zn^{2+}, we observed that the inhibition was decreased as sodium phytate concentrations increased. Furthermore, an important finding in our study was the formation of a white precipitate when cation solutions were added to the phytate incubation mixture before adding the enzyme. This phenomenon was more evident in the presence of Ca^{2+}, Zn^{2+} and Fe^{3+}. The precipitate started to be dissolved after phytase was added to the incubation mixture. Based on this observation, it was hypothesized that the inhibition caused by cations resulted not only from an interaction of cations with the enzyme, but also from a binding of cations with the substrate. The inhibition resulting from the binding of inhibitor with substrate is caused by a decrease in the effective substrate concentration. This inhibition resembles competitive inhibition when analysed by the Lineweaver-Burk plot (Segel, 1975). Of seven cations tested, five showed a competitive inhibition, which indirectly supported the hypothesis that phytase was inhibited by an interaction of cations with the enzyme as well as a binding of cations with the substrate.

Results of the present study showed a decreasing order of the inhibitory effect of various cations on phytase activity: $Zn^{2+} > Cu^{2+} > Fe^{3+} > Cr^{3+} > Ca^{2+} > Mn^{2+} > Mg^{2+}$. This is consistent with a decreasing order of stability of the metal phytate complex: $Zn^{2+} > Cu^{2+} > Co^{2+} > Mn^{2+} > Ca^{2+}$ (Maddaiah et al., 1964), or $Cu^{2+} > Zn^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Fe^{3+}$ (Vohra et al., 1965). This consistence further supported the hypothesis above.

The competitive inhibition by Ca^{2+} and the mixed-type inhibition by Mg^{2+} were partial in nature, because slope replots of data for the inhibition caused by Ca^{2+} and Mg^{2+} showed a hyperbolic curve (Figure 5). In contrast to the pure system, the velocity of the partial enzymatic reaction cannot be driven to zero by increasing inhibitor concentrations. This was observed by the study of the Ca^{2+} and Mg^{2+} inhibition of phytase in the animal diet. In a dietary incubation mixture with high concentration of Ca^{2+} (110.8 mM), there was an 85% loss of phytase activity compared with 100% activity at 10.8 mM of Ca^{2+} (Table 3). However, the enzymatic rate did not reach zero because activity could be detected even at a very high Ca^{2+} concentration. Ullah (1988a) did not observed a Ca^{2+} effect (10 mM of Ca^{2+}), which may have been partially due to a partial inhibition by Ca^{2+}, and also, due to the fact that the inhibition caused by Ca^{2+} was apparent at very high concentration of the inhibitor. Although no effects of Ca^{2+} and Mn^{2+} were observed in another *in vitro* study using rat intestinal mucosal phytase (Yang et al., 1991), our *in vivo* studies showed that extra Ca^{2+} apparently reduced phytase efficacy in pigs, broilers and turkey poults by decreasing phytase activity (Qian and Kornegay, 1995).

In the practical-type pig diet, the contents of Ca, Cu and Zn are usually formulated in the range of 4.3 to 12.3 g/kg, 25 to 283 mg/kg and 80 to 276 mg/kg, respectively. Within these ranges, increases in Ca, Cu and Zn contents led to a reduction in phytase activity of about 25, 50 and 20%, respectively, in these *in vitro* incubations using dietary phytate as the substrate. The degree of the inhibitory effect of Ca, Cu and Zn was more

apparent when dietary phytate was used as the substrate than when chemical sodium phytate was used as the substrate (Tables 3, 4 and 5). A higher level of Ca, Cu and Zn was present in the incubation mixture when dietary phytate was used as the substrate although the mixture contained a narrower molar ratio of [mineral] to [phytate]. This finding might suggest that the mineral concentrations were more critical than the molar ratio of [mineral] to [phytate] in the inhibition process, which could be explained by a dominant interaction between inhibitor and the enzyme.

Implications

Cations, such as Mg^{2+}, Mn^{2+}, Ca^{2+}, Cr^{3+}, Fe^{3+}, Cu^{2+} and Zn^{2+}, have an inhibitory potential in microbial phytase activity principally by the competitive or mixed-type inhibition system; a binding of the cations with phytate may be also involved in the inhibition by decreasing the effective substrate concentration.

General Implications

Supplemental microbial phytase was effective in improving performance, phosphorus and calcium digestibility and bone mineralization by enhancing hydrolysis of phytate phosphorus and calcium for young pigs, broilers and turkey poults fed a corn-soybean meal diet. The studies strongly demonstrated that the phytase efficacy in pigs and poultry was negatively influenced by a wide calcium:total phosphorus ratio but synergetically improved by adding a high level of vitamin D_3 in diets. Dietary calcium:total phosphorus ratio of 1.2:1 for young pigs and 1.1 to 1.4:1 for poultry is very critical for the maximum phytase efficacy. The studies suggest that 1 g of phosphorus (inorganic deflourinated phosphate) can be replaced by 250 to 400, 600 to 900 and 340 to 550 unit of phytase per kilogram of diet, respectively in young pigs, broilers and turkey poults when they fed a corn soybean meal diet. Besides effects on phosphorus and calcium, supplemental phytase seems to provide some benefits in improving the digestibility of dry matter, proteins and trace minerals for pigs and poultry.

The phytase of *Aspergillus niger* showed a high affinity for sodium phytate with K_m of 62 μM and V_{max} of 139 unit of specific activity per mg of phytase protein using a discontinuous assay method. Cations of Mg^{2+}, Mn^{2+}, Ca^{2+}, Cr^{3+}, Fe^{3+}, Cu^{2+} and Zn^{2+} had a potential for inhibiting the *in vitro* activity of phytase, which was dominantly controlled by the competitive or mixed-type inhibition system; a binding of cations with phytate was also involved in the inhibition by decreasing the effective substrate concentration. A decreasing order of the inhibitory effect from cations was clearly shown on the phytase activity: $Zn^{2+} > Cu^{2+} > Fe^{3+} > Cr^{3+} > Ca^{2+} > Mn^{2+} > Mg^{2+}$ because the K_i value was increased from a low value for Zn^{2+} to a high value for Mg^{2+}.

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