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Phytase and Phytate Interactions

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Despite several thousand scientific papers and a rapidly growing market the use of phytase and the importance of phytate in practical poultry nutrition remains an area of some confusion. Initially phytases were offered as a means to improve the digestible phosphorus concentration of monogastric rations by the stepwise removal of orthophosphate from the *myo*-inositol ring of phytate. However, it was gradually understood that the digestibility of other minerals (notably calcium), carbohydrates and amino acids were also variably influenced by the ingestion of phytase (and phytate). The mechanisms at work are not entirely clear but recent evidence suggests that phytate is an antinutrient, beyond its effect on digestible phosphorus and influences secretory and absorptive processes in the gut. In addition to a new appreciation for the effect of phytate and phytase on digestive physiology, a rapidly growing market has attracted a variety of new phytase technologies with various proteolytic and thermal stability and improved kinetics. With increasingly effective phytases available it is more important than ever that the full range of effects are accommodated in diet formulation to ensure that value is optimised and the risk of nutrient imbalances reduced.

Keywords: phytate, phytase, nutrition, poultry

Occurrence and of phytate in feed ingredients

The concentration of total and phytate-phosphorus in plant material has been reported by several authors (Eeckhout & De Paepe, 1994; Selle et al, 2003) and varies considerably from source to source. For example, the phytate-P concentration of wheat (~0.22%), soybean meal (~0.45%) and rice bran (~1.58%) are quite different and thus will have considerable consequences on the total phytate-P concentration in poultry diets depending on their relative inclusion concentrations. Importantly the total phytate-P concentration may be misleading and it is the 'reactivity' and 'susceptibility' of the phytate under digestive processes that dictates both the antinutritive effect of the phytate and the phytase response. Leske & Coon (1999) demonstrated that though (for example) canola meal contains relatively high concentrations of phytate it is relatively poorly dephosphorylated by phytase compared with phytate from soybean meal or maize. This principle is important as different feed ingredients may contribute substantially to the total phytate-P 'pool' but in real terms this may mean little for the substrate available for phytase. It is suggested that users of phytase carefully consider not only the total phytate content of their diets but the relative solubility of this phytate and modify their phytase matrix values appropriately.

Chemical characteristics of phytate

A variable but large proportion of the phosphorus (P) in plant material is in the form of phytate-P (*myo*-inositol hexakis phosphate) (Haug and Lantzsich, 1983; Eeckhout and De Paepe, 1994; Selle et al., 2003). Phytate-P is largely unavailable for utilisation by poultry due to a lack of effective endogenous phytase, the enzyme responsible for the hydrolysis of phytate. Inositol hexaphosphate is extremely electrostatically reactive having 12 dissociable protons with pKa values that range from about 1.5-10 (Fig. 1; Costello, *et al*, 1976). At pH below 1.1, phytate will be neutrally charged and so relatively unreactive. However, between pH 1 and 2 phytate will lose 6 protons, becoming negatively charged and so able to react with basic amino acid residues of dietary protein. It is suggested that throughout the intestinal tract phytate will continue to carry a net negative charge (-6) as loss of any subsequent protons would take ambient pH conditions above 6.85. The reactivity of inositol phosphate isomers is thus highly dependent on the conformation and configuration of the molecule and the pH of its environment (Costello *et al*, 1976; Cheryan, 1980). *Myo*-inositol hexakis dihydrogen phosphate (IP₆) is a potent chelator of many mineral ions (Maenz *et al.*, 1999), forming insoluble salts at intestinal pH, reducing the availability of these minerals for absorption (Lonnerdahl, *et al*, 1999). Furthermore, the availability of dietary amino acids and energy is reduced with the presence of IP₆ in the ration (Selle & Ravindran, 2007). The interaction between IP₆ and protein is dependant to a large extent on pH, with binary protein-IP₆ complexes being formed at low pH and ternary protein-IP₆-mineral complexes formed as pH increases toward neutrality (Selle & Ravindran, 2007). The complexing of minerals by phytate is likely to reduce their participation as co-factors in enzymes, especially under suboptimal supplies. Knuckles *et al.* (1989) demonstrated *in vitro* that esters of inositol phosphate inhibited the digestion of casein by pepsin, the extent of inhibition being a function of the degree of phosphorylation of the inositol ring. This inhibition may be a function of reduced enzyme activity and/or an interaction between inositol phosphate and protein.

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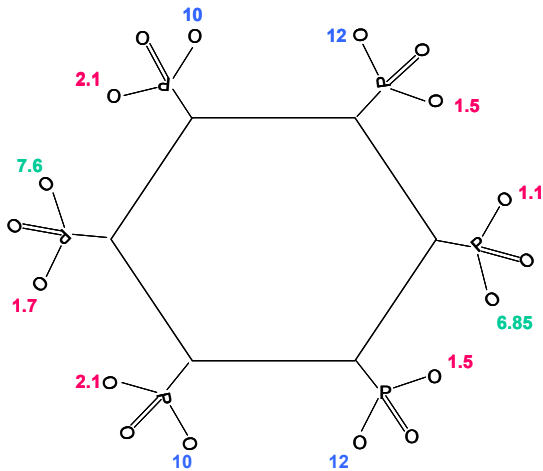


Fig. 1 pKa values of dissociable protons of *myo*-inositol hexakisdi-hydrogen phosphate (adapted from Costello et al., 1976).

Antinutritive effects of phytate and consequences on digestion

In dietary ingredients phytate exists as a salt of K and Mg and is relatively unreactive (Lott et al., 2000). However, when feed is exposed to the low pH conditions in the proximal gut, phytate becomes soluble as H^+ ions replace K and Mg (Cosgrove, 1966). Though protonated, phytate still carries a net negative charge and can react electrostatically with basic amino acid residues in dietary protein. The extent of this reaction depends on the concentration and solubility of phytate, ambient pH, the isoelectric point of the protein, and also its tertiary and quaternary structure (i.e. the degree of steric hindrance between reactive amino acids and phytate). These phytate-protein complexes are variably refractory to digestion by pepsin and solubilisation with HCl (Vaintrub & Bulmaga, 1991), leading to an increase in the secretion of both, and consequently also gastric mucin (Cowieson et al., 2004; Kies, 2005), by the animal. On gastric emptying, the distal gut is faced with a luminal challenge to maintain favourable conditions for optimal functioning of the pancreatic and brush border enzyme array, and for a satisfactory ion balance for nutrient transport. Hyper-secretion of mucin and sodium bicarbonate, commensurate with variation in proteolytic and H^+ antagonism follows, increasing the presence of endogenous amino acids and sodium in the lumen (Fig. 1; Cowieson et al., 2004; Cowieson & Ravindran, 2007) and presumably altering maintenance requirements.

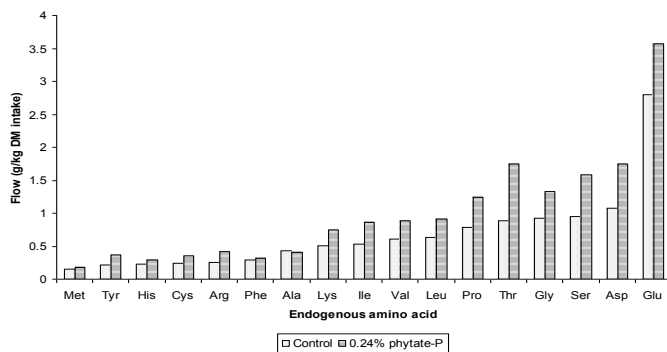


Fig. 1: Effect of 2.4g/kg phytate-P on the flow of endogenous amino acids (g/kg DM intake) in the ileum of broiler chickens (adapted from Cowieson & Ravindran, 2007).

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Further, the loss of endogenous protein from the ileum has a direct effect on the digestible energy value of the diet, depending on the amino acid composition of the endogenous protein. This direct energetic cost has been estimated to be as much as 0.1MJ/kg DM intake for every 1g/kg dietary phytate-P (Cowieson et al., 2008), without including effects on net energy associated with protein synthesis and turnover. Thus, the ingestion of dietary phytate influences endogenous loss indirectly via a reduction in the solubility of dietary protein with a subsequent cascade altering intestinal dynamics via secretive and absorptive mechanisms.

Phytases

Whilst there are a multitude of phytases described in the literature, there are only 3 source organisms from which the most commonly encountered phytases in the animal feed industry emanate. They are *Peniophora lycii* (6-phytase), *Aspergillus niger* (3-phytase) or *Escherichia coli* (6-phytase). Despite the fact that all of these enzymes are capable of degrading phytic acid rapidly under ideal conditions, they differ significantly in several properties relevant to their suitability and therefore efficacy for use in animal feed. These properties are discussed below;

Classification as a 6 or 3 phytase

Phytases are phosphomonoesterases, as are acid and alkaline phosphatases, but what sets them apart is their specific ability to hydrolyse myo-inositol hexakisphosphate (phytic acid, (Greiner & Farouk, 2007)). The classification as a 6 or 3 phytase refers to the phosphate which is the initial target for hydrolysis by the enzyme. In the case of the commercial phytases, the sequential hydrolysis of phosphate groups following the initial attack appears to follow a numerical sequence, with the 6 phytases preferentially attacking the 1 position after the initial attack, and the 3 phytases, after initiation on the 3 phosphate, then attack the 4 position, followed by 5,6 and 1 (Wyss et al., 1999; Greiner et al., 2000; Greiner & Alminger, 2001; Greiner et al., 2001). As described in the previous section on phytate, the phytate molecule consists of 5 phosphate groups attached equatorially to the inositol ring (phosphate in positions 1,3,4,5,6) and one (the 2 position) attached axially (Irvine & Schell, 2001). The axially attached phosphate group seems to be resistant to hydrolysis by most phytases and as a result, once the 1-position phosphate has been removed from the phytate molecule, the phytases tend to seek other substrates rather than continuing on the current inositol ring (Wyss et al., 1999; Greiner et al., 2000). In the case of the 3 phytases, this results in rapid, sequential reduction of IP6 to IP1, whereas in the case of the 6 phytases, only the 6 and 1 position phosphates are removed before the 2 position phosphate is encountered. There is then a build up of IP4 as the enzyme tends to prefer IP6 compared with IP4 as a substrate. Eventually the concentration of IP4 reaches a level for the effective K_M of the enzyme to be surpassed and the 6 phytases attack and remove the 3 position phosphate and there follows a rapid reduction in IP4 concentrations as IP3, IP2 and ultimately IP1 are subsequently produced (Greiner et al., 2000). Given that phytic acid is a significant antinutrient, and given that phytases are assayed not by the rate of IP6 hydrolysis but rather the rate of phosphate release, it is likely that when dosed on similar "units" of activity, 6 phytases will degrade more IP6 than 3 phytases in order to achieve the P release necessary to satisfy the assay. Wyss et al., (1999) demonstrated that in the first 10-20 minutes of attack, less than 20% of the phytate had been depolymerised as far as IP3 by an *E.coli* phytase (6-phytase) compared with almost 60% by an *Aspergillus* (3-phytase). Moreover, in the same work, the *E.coli* phytase had completely destroyed all available IP6 in 5 minutes as opposed to 15-18 minutes for the *Aspergillus*. Thus for equivalent phosphorus release the *E.coli* phytase will deplete a greater proportion of the phytate (read antinutrient) pool than an *Aspergillus* phytase. Consequentially, it is likely that the energy and amino acid matrices of a 6 phytase are likely larger per unit of enzyme activity compared with the 3 phytases.

Gastric suitability

Phytases are most active in the gastric region of the intestinal tract, principally as a result of the low pH being favourable for soluble, unchelated phytate (Bohn et al., 2008). This environment is intensely proteolytic, however, and for the phytase to be effective it must be capable of functioning at low pH and resisting hydrolysis by pepsin. The *E.coli* derived enzymes have been shown to exhibit a pH profile which is marginally superior to that of the *Aspergillus* and *Peniophora* phytases, and moreover to be the most stable to pepsin attack (Igbasan et al., 2000; Igbasan et al., 2002).

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Thermostability

Most commercially used phytases are not intrinsically thermostable enough to survive the harsh conditions encountered when feed is steam conditioned then pelleted. Three approaches have been employed in order to circumvent this problem ; 1) Genetic modification to produce a more thermotolerant enzyme ; 2) Coating the enzyme with a barrier to prevent contact of the enzyme with steam; 3) Spraying the enzyme onto feed after the pelleting process. To date, all of these solutions have limitations. The genetically modified products available are stable enough for most but not all pelleted feeds. The coated products may survive the pelleting process perfectly well, but then the coating incurs a delay in the release of the product and as a result performance per unit of enzyme in the animal is compromised. Post pellet application of a liquid is a good, but costly solution, and the accuracy of application is difficult to ensure on a consistent basis. The main consideration for users is that consistent delivery of enzyme to the animal is essential if the nutrient matrices suggested are to be delivered. Whilst the assay can be used as a direct QC in this regard to uncoated and liquid enzymes, care must be taken to ensure that the assay correlates with the bioefficacy of the product when using coated enzymes. The danger is that in order for the coating to protect the enzyme from the pelleting process, it will inevitably delay the rate of release in the intestine. Given that phytases are limited to the gastric region for their activity, and hence are under a time constraint (clearly implied by the log-linear dose response relationship (Rosen, 2001)), any delay in release will effectively reduce the efficacy of the enzyme. As a result it is quite possible that the analysis of the phytase activity present in the feed bears no relationship to the bioefficacy of the product if a coated product is used (Klein Holkenborg & Braun, 2001).

Assigning nutrient matrices to a given dose of phytase

The commercial usage of phytase is based on the assignment of a nutrient matrix to a given dose of the enzyme. In the past this has been on the basis of a fixed dose of phytase, most typically 500FTU/kg in broiler feed for example. Recently, the volatility of phosphorus and energy prices has stimulated interest in reducing phosphate and fat use through use of higher dosages of phytase. The relationship between phytase dose and the biological response has previously been established as log-linear, ie a logarithmic increase in dose is required to maintain a linear increment in response (Rosen, 2001; Kornegay, 2001; Rosen, 2002). This is in line with a similar relationship noted for NSP enzymes (Zhang *et al.*, 1996; Zhang *et al.*, 2000). Given this observation, it is possible to calculate the expected nutrient sparing effect of any dose of phytase from the equations published in the literature. If, for example, the phosphorus sparing effect of a 500 unit dose is stated at 0.1% Available phosphorus (AvP), then 1000 units would be expected to spare 0.13%, and 5000 units would be needed to spare 0.2%. The table below gives estimates of the multiple of the 500 unit matrix for dosages between 500 and 1000 units and shows that doubling the dose of phytase from 500 to 1000 units results in a matrix increment of only 30%. Since the assigned matrix values for individual amino acids and energy are related proportionally to the destruction of phytic acid, it is clear that the matrix values for these nutrients should be linked to the phosphorous value ascribed to any dose of the phytase in question. It is likely that any given dose of a 3 phytase may result in a lesser energy and amino acid matrix compared with a similar dose of a 6 phytase as a result of the latter hydrolysing proportionately more IP6 in order to deliver the same quantity of P. However, when 3 or 6 phytases are compared within a class, the energy and amino acid matrices for any given AvP matrix value should be identical. For example, if two *Aspergillus* phytases claim a 0.1 % AvP matrix at 500 units, then it does not make sense that one should claim a higher energy or amino acid matrix than the other. The AvP matrix relates to phytate hydrolysis, and phytate hydrolysis drives the energy and amino acid savings. Care must therefore be taken in interpreting some of the literature in the marketplace as there are many circumstances where the link between the phosphate released and nutrient benefits ascribed to the enzyme is counter-intuitive.

Table 1. Illustration of the log-linear dose response to microbial phytases.

Dose	Relative matrix
500	100%
600	108%
700	115%
750	118%
800	121%
900	126%
1000	130%

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Factors influencing the response to phytase

There are a multitude of factors which have been shown to influence the response to phytase including Ca levels, Ca: ratio, vit D levels, organic acid presence, interfering metal ions, feeding/lighting programs and many others (Kornegay, 2001; Selle & Ravindran, 2007). These have been reviewed elsewhere but the most relevant recent findings which have may play a significant role in mitigating the value of phytases when applied commercially include Pellet binders, calcium content of drinking water and the use of high levels of zinc along with phytase when diets are heat processed.

Pellet binders

Recent work in our laboratory indicated uncharacteristically low recoveries of enzyme activity not only from some pelleted feed samples but also from some vitamin/mineral premixes. It was ultimately found that mixing any one of several commercial pellet binders with any commercial phytase product resulted in considerable loss in recovered activity. E.coli phytases seemed to be slightly more susceptible than Aspergillus, and the coated E.coli phytase particularly vulnerable to this problem, losing 90% of activity within minutes of mixing with the binder. Whether such apparent inactivation is irreversible and thus influences performance in the animal or whether it is simply a problem in the conditions of the assay remains to be ascertained, but the results imply that the use of a pellet binder should be considered with due care if the diet contains a phytase.

Calcium content of drinking water

There is a great deal of evidence which suggests that the Ca:P ratio is a very significant factor in determining the efficacy and thus response to phytases (Selle *et al.*, 2009). As the ratio widens the efficacy of the enzyme falls and thus its ability to deliver the expected nutrient matrix diminishes. Much of the research in the literature and its subsequent application in the field fails to take into account that commercial feeds more often than not contain more calcium than was formulated, and that this is compounded by the fact that in areas with hard water, the equivalent of as much as an extra 0.1% dietary Ca can come through the drinking water. This means that, for example, where the targets may be 1% Ca and 0.45 AvP, the actual diet may deliver 1.1% Ca, plus 0.1% from the water to realise an effective dietary Ca content of 1.2%. This increases the Ca:P ratio from 2.22 to 1 up to 2.67 to one which will radically reduce the efficacy of the phytase and may leave the flock prone to wet litter as a result of calcium excess.

Metal ions

As antibiotic growth promoters have been systematically removed from animal feeds over the past 10-15 years, either legislatively or through consumer demand, feed manufacturers have looked for alternatives. Elemental copper and zinc (swine mostly) have been used at pharmacological levels in the past to control microbial populations and reduce the impact of removal of AGP's. High levels of zinc (200ppm) have been shown to reduce the efficacy of phytase when fed to chickens (Augspurger *et al.*, 2004). Copper (>200ppm), particularly in the form of copper sulphate, has been shown interfere not only with phosphate digestion and absorption but also to reduced tibia ash content of birds fed diets containing 600 U/kg phytase (Banks *et al.*, 2004a; Banks *et al.*, 2004b). Care must be taken when such ions are used in conjunction with a phytase since the expectations of efficacy will likely be moderated in presence of pharmacological levels of these metals.

Conclusions

The biological effect of phytase is very much dependent on the associated anti-nutritive effect of phytate. Removal of this nutritional obstacle has a substantial impact on the nutritional value of the diet both in terms of mineral bioavailability and energy and protein efficiency. However, there are a variety of factors which influence the scale and consistency of the response to phytase and these should be carefully considered in ration formulation. Substantial economic benefits arise when phytase has been appropriately accommodated in the diets of monogastric animals and the use of phytases, and ancillary enzymes is likely to continue well into the future.

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