

Key considerations for phytase discussed

The true value of a phytase is a manifestation of the interaction between in vitro-determined traits and the intestinal environment.

By N.E. WARD*

THE lofty valuation of phosphate is a strong motivator for nutritionists to wring out excess phosphorus from feed formulations. Slimmed-down safety margins are now being considered with greater scrutiny.

This coincides with new developments and advancements in phytase research. A recent literature review on phytase recouped a remarkable number of studies, most from new and different sources.

In the market, descriptors such as “novel” and “new generation” buoy the perception that the latest product is now the better mousetrap. In truth, new generation may more accurately depict how recently that product entered the marketplace, potentially void of the improvements typically associated with a progression of generations, compared to those proven by years of commercial use.

Claims of meteoric phosphorus replacement levels spark questions on their ability to perform at such a grand pace.

Yet, the task facing the nutritionist is no different from that for other products: Due diligence is required to separate marketing from science.

The evaluation of enzymes is not always straightforward, and the interpretation of efficacy is influenced by a number of factors. The biochemical characterization of enzymes is essential but, by itself, can be sorely inaccurate to predict enzyme performance in poultry or swine under a commercial setting. Feeding trials are essential as the gatekeeper for enzyme bioefficacy.

Microbial phytases

Phytases are derived from more than 200 fungi, many bacteria, yeasts, numerous

plants and various animal tissues (Liu et al., 1998). Each can be characterized for molecular weight, optimal pH and temperature and kinetic properties, as well as other traits.

Oh et al. (2004) describe two subclasses of phosphatases (acid and alkaline phytases) based on optimal pH activity. Acid phytases (also known as histidine acid phytases) are common to the feed industry. These exhibit good catalytic activities, have an acidic pH optima (pH of about 4.0-6.0) and require phytate as substrate in the form of the free acid.

Most acid phytases can hydrolyze five of the six phosphate groups to yield mono-phosphate inositol and inorganic phosphates (Wyss et al., 1999a). Widely distributed in nature, this group includes fungal, bacterial and plant phytases.

The alkaline phytases are also abundant in nature (Liu et al., 1998) but are less known. These require calcium ions (Ca^{+2}), are active at alkaline pH and much prefer the calcium-phytate complex as substrate. Generally, they can hydrolyze three or more of

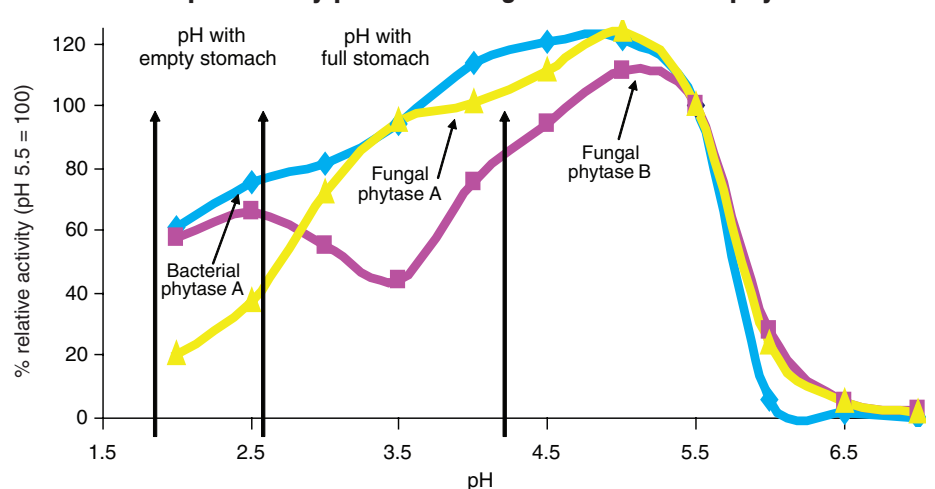
the six phosphate groups, and their narrow substrate specificity excludes the phosphoesters, which can act as substrates for the acid phytases (Oh et al., 2004).

The alkaline phytases are inherently more thermostable, and some can withstand 80-95°C temperatures. DSM investigations found that fungal phytase *Aspergillus fumigatus* could endure temperatures of 100°C for 20 minutes (Pasamontes et al., 1997). In addition, the DSM consensus phytase derived from several fungal phytases survived 90°C for 10 minutes (Lehman et al., 2000).

Work by DSM discovered a mixture of an acid and alkaline phytase to remove all six phosphates from phytic acid (Wyss et al., 1999a), whereas some fungal phytases and the bacterial *Escherichia coli* phytase removed five of the six phosphates. A protein-engineered commercial *E. coli* phytase capably removed four of the six phosphates (Garret et al., 2004).

Based on which of the six phosphate groups on the phytic acid molecule that is first hydrolyzed, the NC-IUBMB classifies microbial phytases into at least two major groups: three-phytase (EC 3.1.3.8) and six-phytase (EC 3.1.3.26). Fungal *Peniophora lycii* and bacterial *E. coli* phytases are categorized as six-phytases, while *Aspergillus niger* is a three-phytase. The preference of the third or sixth phosphate group is not known to

In vitro pH activity profile of fungal and bacterial phytases



*Dr. N.E. Ward is with DSM Nutritional Products, Parsippany, N.J.

affect phytase efficacy.

Fungal, bacterial phytases

Of the phosphatases, the fungal and bacterial acid phytases (on the basis of genetic origin) are the most relevant forms in the feed industry. Differences exist within each group, but the overlap is so significant that general characterizations are reckless.

As with the three- and six-phytase terminology, the universal classification of “fungal” and “bacterial” provides no meaningful generalizations on efficacy and utility. Instead, various biochemical and biophysical traits are phytase specific (Liu et al., 1998).

Vats and Banerjee (2004) noted that across all phytases, the optimum pH for bacterial phytases is neutral to alkaline, while that for fungal phytases is acidic (pH between 2.5 and 6.0). This distinction is more applicable to native or wild-type phytases because genetically engineered phytases expand such confines.

Indisputably, phytase discussions have grown far more sophisticated since early commercial interest (Ward, 1993). Protein engineering and recombinant activities have broadened the field of phytases not envisioned when phytase was discovered 100 years ago (Suzuki et al., 1907).

Variants of a phytase developed through selective molecular modification can launch new characteristics. If deemed to be a significant improvement over the “parent,” the variant can find itself competing in the marketplace with “relatives” borne into the same category.

Further burdening any general statements about these two groups is that post-fermentation activities — and, more precisely, product formulation and optimization — can influence efficacy and stability. Certainly, fermentation, enzyme stabilization and product formulation are sciences within themselves.

Expression systems

The choice of the expression system for phytases is not incidental. Large-scale commercial production can change the enzymatic character of fungal or bacterial phytases through “glycosylation” (Brugger et al., 2004; Han and Lei, 1999).

Glycosylation is the enzymatic attachment of sugars to amino acid side chains of the enzyme molecule. Glycosylation can be positive; hence, the inability to glycosylate hinders widespread use of bacterial expression systems (Lei and Stahl, 2001).

During the 1990s, development work at DSM with several phytases revealed that significant glycosylation patterns were not necessarily identical across phytases (Wyss et al., 1999a; Wyss et al., 1999b). Modifications in pH profile, function and

thermal stability can occur (Guo et al., 2008; Han and Lei, 1999) and change the character of phytases.

The marriage of an expression system with a unique phytase gene is critical. Three expression systems for fungal *P. lycii* resulted in pH optima of 5.0, 4.0-4.5 and 4.5, and all three had unique thermal stabilities (Xiong et al., 2006).

After exhaustive testing, today’s product is heavily glycosylated (Bagger et al., 2003) and expressed by *Aspergillus oryzae* to optimize traits such as specific and pH activities (Lassen et al., 2001).

At least three bacterial *E. coli* phytases exist in the marketplace. All three are engineered uniquely, and two share the same expression system (*Schizosaccharomyces pombe*, *Pichia pastoris*). With the intended molecular manipulations, along with different production systems, these phytases theoretically would perform differently.

Lei and Stahl (2001) gave an overview on expression systems and how they can affect phytases. Indeed, merely with expression systems, diverse biochemical properties can occur in phytases, further undermining attempts to separate fungal and bacterial phytases.

Important characteristics

Cornell University investigators (Lei and Stahl, 2001) noted that an ideal phytase is composed of many factors, but three are

especially important:

(1) Effectively release phosphorus.

The ability to hydrolyze phosphorus from phytic acid in the intestinal tract is influenced by a number of traits.

(2) Feed processing heat stability.

All native fungal and bacterial phytases inherently have some thermal stability, but thus far, this is minor compared to the demands of commercial conditions.

(3) **Economical production.** Many factors influence production costs, and these are beyond the objectives to be discussed here.

Effective release

Individual phytases are distinguished by a long list of biochemical traits. These traits cannot predict phytase performance in the animal, but they can provide guidelines on potential functionality in animal feeds and digestive systems. At best, these traits can give direction. They might eliminate a phytase from additional research or serve as a springboard into commercial consideration that requires confirmation by animal studies.

Certainly, numerous variables can short-circuit expectations based on *in vitro* enzyme traits. If these and all potential interactions are known, animal trials would no longer be needed, which would permit complete reliance on *in vitro*-derived traits.

1. Intestinal pH of chickens

	---Gao et al., 2008---		Murai et al., 2001,	Rynsburger & Classen, 2007,*
	21 days	49 days	17 days	days 2-15
	-----pH-----			
Crop	4.9	—	5.9	5.0-6.0
Proventriculus	5.4	5.3	3.2	5.2-3.4
Gizzard	4.9	4.6	3.4	3.5-3.3
Duodenum	6.1	5.7	—	6.6-6.4
Jejunum	6.0	6.2	—	6.8-6.5
Intestine	—	—	6.4	—

*Rounded to nearest 10th.

2. Effect of lighting program and phytase source on 21-day broiler performance

Dietary program	Number	Apparent phosphorus, %	Weight gain, g/bird	Feed: gain	Tibia ash, %
Constant lighting					
Pos. control	8	0.45	596 ^a	1.44 ^{cd}	51.99 ^{ab}
Neg. control	8	0.28	533 ^{cd}	1.55 ^{ab}	46.52 ^f
Fungal <i>P. lycii</i>	8	0.28	562 ^{abc}	1.52 ^{ab}	50.78 ^{bc}
Bacterial <i>E. coli</i>	8	0.28	549 ^{cd}	1.52 ^{ab}	50.24 ^{cd}
Intermittent lighting					
Pos. control	8	0.45	585 ^{ab}	1.40 ^d	51.29 ^{ab}
Neg. control	8	0.28	527 ^d	1.49 ^{bc}	45.59 ^g
Fungal <i>P. lycii</i>	8	0.28	568 ^{bcd}	1.45 ^{cd}	49.48 ^d
Bacterial <i>E. coli</i>	8	0.28	558 ^{bcd}	1.45 ^{cd}	48.50 ^e
Std. error of means	—	—	12	0.02	0.26

a,b,c,d,e,f,g Different superscripts within a column (P < 0.05)

Source: Saylor et al., 2009.

Optimal pH activity. The pH profile of phytases is important since the phytate “window of solubility” in the intestinal tract is short-lived. Fungal *P. lycii* has a pH optimum activity of 4.0-5.0 (Lassen et al., 2001; Xiong et al., 2006) based on sodium-phytate substrate. Other commercial phytases have similar pH optima, and all current commercial phytases decline precipitously at pH 6.0-7.0.

The pH curves for three commercial phytases show some differences, particularly at pH 2.0-3.0 (Figure). Much has been promoted about bacterial *E. coli* phytases being active in this region.

However, for the proventriculus and gizzard, pH is usually 3.0-4.0 when feed is present (Table 1). Feed can buffer and increase pH, being that it contains calcium carbonate, phosphate and other components. Limestone increases intestinal pH (Lawlor et al., 2005) and crop pH of poultry.

The pH for chicks in one study was determined for the crop (5.5-5.9), proventriculus (3.2-3.6), gizzard (3.4-3.6) and intestine (6.4-6.5; Muari et al., 2001). With broilers at 21 and 49 days of age, the pH for the proventriculus and gizzard was even higher (Gao et al., 2008). While recognizing that intestinal pH is not always the same for the same regions, a pH of about 3.5-4.0 is generally found for the most acidic areas when broilers are fed corn/soybean meal diets.

In swine (Pagano et al., 2007), a level of 2,000 units of phytase per kilogram of feed resulted in higher pH than a level of 500 units did. Phytase was suggested to liberate inorganic phosphate that, in turn, buffered stomach pH. With broilers, however, dietary phytase and phosphorus seemed unrelated to intestinal pH (Murai et al., 2001).

New findings on phytase pH. Recently, with corn/soybean meal as the source of phytate (Pontoppidan et al., 2007a; Pontoppidan et al., 2007b), a steady-state equilibrium was found to exist between insoluble and soluble phytate. Despite finding very different profiles of soluble phytate with two levels of calcium in a corn/soybean meal blend, degradation of phytate by fungal *P. lycii* was similar. At pH 5.0, more phytate was degraded than expected based on what was originally soluble. As phytate was degraded, the equilibrium shifted to maintain a minimal level of soluble phytate, according to these investigators. Apparently, the observed pH optimum of the phytase is shifted toward more acidic pH (about one pH unit) when soybean meal/corn is used as the substrate compared to using pure sodium-phytate as a substrate.

These results also question the reliability of pH activity curves based on sodium-phytate to predict degradation of feed phytate in animals. The pH curves

4. Trial 3: Laboratory assay results for pellets:fines, PDI and THI assay results by dietary supplement (sand or coarse HSCAS) for pellet mills 1 and 2 combined

Dietary supplement	Number	Pellets: fines, %	PDI, %	THI
None, 0%	4	81.1	96.7	78.5
HSCAS, 0.4%	2	86.8	96.8	84.1
Sand, 0.4%	4	79.6	95.6	76.5
P-value	—	0.799	0.932	0.838

5. Corn/soy/bakery byproduct formulas

Ingredients (partial list)	---Amount of ingredient in diet, %---	
	Negative control	HSCAS (0.5%)
Bakery byproduct meal	8.08	8.02
DDGS	5.02	5.03
Meat and bone meal (50% CP)	2.02	3.29
Poultry fat	1.51	1.52
Phytase	+	+
Defluorinated phosphate	0.20	0
AZOMITE Feed-Grit	0	0.50
Cost difference, \$ per ton	—	-0.80
Summary of results by feed treatment		
Temperature mid-conditioner, °F	185	185
Tons per hour throughput achieved	30	45
Electrical amps	382	390
Electrical efficiency, amps per ton per hour	12.7	8.7
Hot pellets (after screening), %	94.1	92.3
	(at 30 tons/hour; 21 tons)	(at 38 tons/hour)
Hot pellets (after screen, tumbler), % (PDI)	76.6	82.1

of phytases are commonly determined with sodium-phytate largely because of convenience and high solubility. However, the trade-off might be that it lacks accuracy in predicting phytase efficacy in animal digestive tracts. Thus, this aspect of *in vitro* characterizations is dubious when questions on the methodology are so evident.

Crop activity. The crop has been suggested as the primary organ in which phytate is broken down by phytase. In part, this is due to the short residence time in the proventriculus and gizzard, which would minimize their importance for phytase function to occur. Liebert et al. (1993) noted that about 50% or more of the activity of fungal *A. niger* occurred in the crop, and Takemasa et al. (1996) reported similar results for a “yeast phytase.”

For fungal *P. lycii*, 78-80% of the phytate hydrolysis occurred in the crop — considerably more than two commercial bacterial *E. coli* phytases (Glitsso et al., 2007). The phytases were added separately to a corn/soybean meal feed to replace 0.1% phosphorus. The crop contents indicated a reduction of 37-40% in phytate with two different bacterial *E. coli* phytases, or roughly half that of the fungal phytase.

At crop pH (5.0-6.0), the bacterial *E. coli* phytases may be restricted. Yin et al. (2007) reported fungal *A. niger* to be more effective than *E. coli* phytases to release phosphorus from phytic acid at pH 5.5.

Close scrutiny of the various *in vitro*

determined biochemical traits does not resolve the findings that the fungal *P. lycii* degrades almost twice as much phytate in the crop. It would be premature to assume that all fungal phytases act in this manner, but this does appear to distinguish the fungal *P. lycii* from bacterial *E. coli* phytases.

New findings on effect of lighting on phytase efficacy. Based on the effectiveness of fungal *P. lycii* in the crop, two lighting programs were tested for phytase efficacy (Saylor et al., 2009). One program (intermittent) encouraged crop fill (meal feeding) while the other was the traditional (constant) lighting used in most battery studies. Fungal *P. lycii* and a bacterial *E. coli* phytase were supplemented to replace 0.1% phosphorus in separate diets.

Overall, the chicks on the intermittent lighting performed with increased efficiency (Table 2). The intermittent program resulted in a significant ($P < 0.05$) increase in 21-day tibia ash of *P. lycii* birds over those fed *E. coli*. There was a trend for fungal phytase birds to be heavier than those fed bacterial phytase across both lighting programs.

More work is needed, but this insinuates that studies with enzymes should be completed under lighting programs similar to commercial conditions to avoid “bias creep.” Practical conditions utilize some degree of intermittent lighting, all of which favor crop fill, as used in this experiment.

Pepsin stability. Pepsin stability of

phytases at low pH is important, but one that is affected by the ratio of pepsin to phytase, duration of test, etc. Work at DSM found the mere presence of soybean meal protein to markedly change pepsin degradation of phytases.

Igbasan et al. (2002) reported a 2% survival rate for *P. lycii* incubated at pH 2.0 and 40°C for 45 minutes in a solution of water, pepsin and phytase. However, these results are markedly different with actual intestinal contents from various segments of the intestines (Igbasan et al., 2002). Survival of *P. lycii* was plainly higher: 97% (crop), 59% (stomach), 95% (duodenum) and so forth.

In an *in vitro* model with pH, incubation times and enzyme concentrations similar to *in vivo* conditions, along with corn/soybean meal and exposure to pepsin and pH 3.0, about 80% of *P. lycii* survived (Pontoppidan et al., 2007).

Certainly, questions exist on the application of *in vitro*-determined pepsin stability of enzymes. Considerable differences exist in methodology, and none appear to accurately depict enzyme performance in the animal.

Feed processing

Phytase stability for pelleted feeds is dependent primarily on three variables: temperature, moisture and time during which the enzyme is exposed to heat and moisture. Other factors influence enzyme survival (Eeckhout, 2000), but DSM data indicate that they are secondarily important.

The inherent heat stability of today's fungal and bacterial phytases is inadequate for commercial feed production. Modifications with polyols show promise (Lamosa et al., 2000), as do sorghum liquor wastes with starch (Chen et al., 2001) and glycosylation (Han et al., 1999), as well as the introduction of disulfide bonds (Scott and Steven, 2000) and proline (Watanabe and Suzuki, 1998) into the phytase molecule. Inorganic salts with a bivalent ion, such as magnesium sulfate, act as stabilizers. The obvious hurdle is to avoid restricting phytase efficacy while improving its tolerance to heat and moisture.

DSM studies include the cloning and modification of thermophilic phytases for heat stability (Wyss et al., 1998; Mitchell et al., 1997; Pasamontes et al., 1997) and the use of stabilizers (Brugger et al., 1996). DSM's "consensus" phytase, which was developed from 13 different fungal phytase sequences, provided a 15-22°C increase in temperature stability over any "parent" (Lehmann et al., 2000).

The pelleting stability of enzymes can be improved significantly via a patented combination of core materials, carbohydrates and vegetable oil developed by coating technology (Jacobsen and Jensen, 1992). This coating

instrumentally improves the phytase pelleting stability in North America, with an average of 82.2% (standard deviation of 7.8) at a temperature of 85.9°C (standard deviation of 7.8; Ward and Wilson, 2002). Latin America averages are higher at 89.8% (standard deviation of 10.5) but at a lower temperature of 79.1°C (standard deviation of 5.7) in 20 feed mills (Sorbara et al., 2007). Retentions continue to get better as coating technology improves (Ward et al., 2006).

Analytical issues

The definition of FYT and FTU is the amount of phytase that catalyzes the release of one micromole of inorganic phosphorus per minute from 5.1 millimoles of sodium phytate in pH 5.5 buffer at 37°C. Hidden within this definition are important details about types and concentrations of buffers, incubation times, buffer additives and other aspects of phytase analytics that certainly change the expression of activity (Ward and Campbell, 2007).

A comparison of three phytase methods found a 2.8-fold difference attributable to buffer (Weaver and Lei, 2007), which is essentially identical with DSM's findings (Ward and Campbell, 2007).

The units of expressed phytase activity are confounded such that the phosphorus replacement value of phytase should be related to mass (grams per metric ton, pounds per ton, etc.) to account for product formulation and analytical differences. Ultimately, the cost to replace a given amount of phosphorus counts more than "bragging rights" associated with phytase units.

Matrix values

The high cost of phosphate has renewed efforts to replace more dietary phosphorus with higher levels of phytase, and typical corn/soybean meal broiler diets with some meat and bone meal contain 0.22-0.27% phytate phosphorus. Thus, room exists to remove phosphorus beyond the 0.1% that is the standard focal point of phytase recommendations.

Research in Brazil (Vieira et al., 2009) and the U.S. (Waldroup et al., 2008) has shown that higher phytase levels can replace increased amounts of dietary phosphate. At mega doses (15,000 FYT/kg), fungal *P. lycii* outperformed ($P < 0.05$) a commercial bacterial phytase for protein accretion (Puttress et al., 2006). Both products, however, exerted effects beyond the amount to replace 0.1% phosphorus.

Considerable interpretation exists in the development of matrix values, especially for metabolizable energy (ME) and amino acid values. Matrix values for phytase products usually include ME,

amino acids and protein (Ward, 2006). For the fungal phytases, ME commercial recommendations are 11-53 kcal/kg of final feed. For bacterial phytases, the disparity ranges from zero to 53 kcal ME/kg of final feed.

These substantial differences are a consequence of interpretation or philosophies as opposed to efficacy differences.

ME and amino acid matrix values are directly proportional to the degradation of the phytate by a phytase. Equivalent phosphorus liberation gives equivalent ME and amino acid values across phytases.

Especially high values for ME for phytases lack justification. This point will be rendered clear as carbohydrases and other enzymes enter a feed formulation with their own matrix values. The wide disparity in philosophies for ME values for phytases impedes this aspect of phytase from being useful and accurate when evaluating relative value.

Parting comments

Attempts to characterize phytase function and utility according to either fungal or bacterial classification are futile. Considerable overlap exists in characteristics of both groups. Marked changes through protein engineering and the effects induced by expression systems obscure proposed boundaries between the two genetic origins. Product formulation adds another dimension but was not discussed here.

Reliance on *in vitro* characterizations is dubious when questions on the methodology remain high on the list. Recent investigations question the use of phytate solubility for corn/soybean meal to estimate phytate degradation and the validity of sodium-phytate to develop phytase pH curves for estimated enzyme function in animal digestive tracts.

The true value of a phytase is a manifestation of the interaction between *in vitro* determined traits and the intestinal environment. Despite some unique biochemical differences, for example, no synergism or additive effect appears in animal performance when feeding combinations of current commercial fungal and bacterial phytases. Some differences do exist, however, and lighting programs potentially could be one of these.

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