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# Early Food Safety Assessment for the Phy02 Phytase Protein

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## Abbreviations

| | |
|---|---|
| ~ | Approximately |
| bwt | Body weight |
| FTU | Phytase activity unit |
| kDa | kilo Daltons |
| PMI | Phosphomannose isomerase |
| ™ | Trademark |

# 1. Name, Description and Function of the Phy02 Phytase Protein

The Phy02 phytase is derived from the native AppA phytase of *Escherichia coli* strain K-12. The *appA* gene and the AppA phytase it encodes have been previously described (Dassa, *et al.*, 1990). The AppA phytase is known to be structurally similar to the phytase from *Aspergillus niger* that is the phytase in the commercial phytase product Natuphos™ (Lim et al., 2000) that is an animal feed additive.

Phytases are a class of acid phosphatase enzymes that hydrolyze phosphates from phytic acid (also referred to as phytate) to produce free phosphate and inositol. Phytic acid (myo-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate) accounts for up to 80% of the phosphorus in the seeds of cereals and legumes and is the primary storage form of phosphate in these materials (Reddy et al., 1982). Phytate phosphorus is nutritionally unavailable to monogastric animals such as poultry and swine and therefore, inorganic forms of phosphorus are commonly added to animal feed to supply the nutritional needs for this important nutrient. The addition of inorganic phosphorus to animal feeds results in the generation of high-phosphorus manure that can contaminate rivers and streams resulting in algal blooms, oxygen depletion and the death of fish and aquatic animals due to eutrophication (Jongbloed and Lenis, 1998; Correll, 1999; Mallin, 2000; Poulsen, 2000). In addition, phytate also forms a complex salt with several mineral ions such as $K^{+}$, $Mg^{+2}$, $Ca^{+2}$ and $Zn^{+2}$ that is called phytin. In this complexed state, the minerals in phytin are nutritionally unavailable to monogastric animals (Lott, 1984; Harland and Morris, 1995; Minihane, 2002). For these reasons, phytate is considered an anti-nutrient.

One strategy for making phosphorus from phytate nutritionally available to monogastric animals is the addition of phytase to animal feeds (Jongbloed and Lenis, 1998; Onyango et al., 2005). The use of phytase in the diets of poultry and swine has been shown to improve feed and phosphorus utilization (Baker 2002; Nyannor et al., 2007 and 2009). A number of phytase products are currently marketed for this use and include Natuphos™ (BASF) a phytase derived from *Aspergillus niger*, Ronozyme™ (DSM) a phytase derived from *Peniophora lycii*, and Quantum (AB Vista) a phytase that is also derived from the AppA phytase of *Escherichia coli*. The use of phytase in animal feeds allows a reduction in the amount of inorganic phosphorus added to animal feeds and has been reported to result in reductions in fecal phosphorus as high as 56% (Nahm, 2002; Sharpley *et al.*, 1994; Wodzinski and Ullah 1996). In December, 2002, a regulation issued from the US EPA was implemented that regulates the application of manure from concentrated animal farming operations onto land based on the amount of phosphorus being applied (EPA, 2002). The use of phytase in poultry and swine feed results in a more efficient utilization of phosphorus and reduces phosphorus in animal wastes. Therefore, its use may assist concentrated animal farming operations in meeting the EPA guidelines without reducing the size of their operations or having to utilize other more expensive waste handling technologies.

All of the current phytase animal feed products are produced by genetically modified microorganisms through fermentation and purification of the phytase from the

fermentation medium. The Phy02 phytase that is being developed by Agrivida, Inc. is produced in the grain of maize (*Zea mays*). Genes encoding the Phy02 phytase under the expression of [CBI Deleted] specific promoters were introduced into maize to achieve the production of Phy02 phytase specifically in the grain of maize. The Phy02 phytase is derived from the native *E. coli* phytase AppA and differs from the AppA phytase by only [CBI Deleted] of the 412 total amino acid residues in the mature protein. The amino acid sequence of the Phy02 phytase is presented in Figure 1 and the [CBI Deleted] amino acid substitutions relative to the *E. coli* AppA phytase are indicated.

**Figure 1. Amino acid sequence of the full length Phy02 phytase**

The amino acid sequence of the *E. coli* AppA phytase is presented. The [CBI Deleted] amino acid substitutions in the Phy02 phytase relative to the AppA phytase are indicated above the AppA sequence in bold font and gray shading. The consensus phytase active site (RHGxRxP) is shown in bold font and yellow shading (Lim et al., 2000; Tomschy et al., 2000). Other residues that are involved in substrate binding that are conserved in other phytases are shown in bold font with blue shading.
[Amino acid substitutions in Phy02 redacted; CBI Deleted]

```
1    MAQSEPELKLESVVIVS**RHGVRAP**TKATQLMQDVTPDAWPTWPVKLGWLTPRGGELIAYL   60

61   GHYQRQRLVADGLLAKKGCPQSGQVAIIADVDERTRKTGEAFAAGLAPDCAITVHTQADT   120

121  SSPDPLFNPLKTGVCQLDNANVTDAILSRAGGSIADFTGHRQTAFRELERVLNFPQSNLC   180

181  LKREKQDESCSLTQALPSELKVSADNVSLTGAVSLASMLTEIFLLQQAQGMPEPGWGRIT   240

241  DSHQWNTLLSLHNAQFYLLQRTPEVARSRATPLLDLIKTALTPHPPQKQAYGVTLPTSVL   300

301  FIAGHDTNLANLGGALELNWTLPGQPDNTPPGGELVFERWRRLSDNSQWIQVSLVFQTLQ   360

361  QMRDKTPLSLNTPPGEVKLTLAGCEERNAQGMCSLAGFTQIVNEARIPACSL   412
```

## 2. Description of the Intended Effect of the Phy02 Phytase Protein

The Phy02 phytase is produced in the grain of maize but due to the relatively low content of water in grain it is not enzymatically active in the grain nor has it any obvious effect on the grain or the maize plant. The grain producing Phy02 phytase will be harvested and ground into a course meal that will be added as a feed additive at relatively low inclusion levels ([CBI Deleted] /ton of feed) to the feed of poultry and swine. The intended effect of the Phy02 phytase in animal feed is to enzymatically remove phosphate from phytic acid and phytin that is in the diet in order to provide enhanced phosphorus availability thereby reducing the need to add exogenous mineral phosphate and to

degrade phytin in order to increase the nutritional availability of other key minerals such as iron, calcium and magnesium.

## 3. Identity and Source of Introduced Genetic Material

The native *E. coli appA* phytase gene was optimized using Gene Site-Saturation Mutagenesis (Short, 2001) to generate a gene encoding the NOV9X phytase with increased thermotolerance. Thermotolerance is a desirable trait for commercial feed enzymes since many animal feeds are produced by a pelleting process that involves a heat treatment that inactivates thermolabile enzymes. The Phy02 phytase gene was derived from the NOV9X gene by further optimization to create additional specific amino acid substitutions. The NOV9X phytase is the active phytase in the commercial phytase product named Quantum that is produced by the yeast *Pichia pastoris* and that was approved by FDA-CVM for inclusion in animal diets since 2008. The NOV9X phytase has 8, and the Phy02 phytase has [CBI Deleted], amino acid substitutions relative to the AppA phytase from *E. coli* that consists of 412 amino acids. The Phy02 phytase demonstrates considerable tolerance to high temperatures, maintaining significant activity after incubation in aqueous conditions at temperatures up to 90° C for 5 minutes.

A transformation gene cassette containing three copies of the Phy02 phytase gene, each with a different monocot derived promoter and NosT terminator was constructed in plasmid pAG4758. The genetic elements of the T-DNA fragment that was used to transform maize are shown in Figure 2. The individual genetic elements within the T-DNA fragment are described in Table 1. This plasmid was transformed by *Agrobacterium*-mediated transformation into immature corn embryo tissue as described by Negrotto *et al.* (2000) and transformants were selected based on the presence of the plant selectable marker *manA* gene on the transformed DNA fragment that encodes the enzyme phosphomannose isomerase (PMI). The PMI enzyme enables corn tissue to grow on mannose as a sole source of carbon (Negrotto *et al.*, 2000). The *pmi* gene has been used as a selectable gene in several genetically modified corn varieties that have completed review by the USDA, FDA, and EPA for food and feed safety, including corn events 5307 and Mir604 corn with resistance to corn rootworm, lepidoptera resistant Mir162, and α-amylase expressing 3272, all products of Syngenta Seeds. Corn plants containing the Phy02 phytase gene were cultivated and were found to produce more than [CBI Deleted] units of phytase activity (FTU) per gram of grain.

**Figure 2. Diagram of the genetic elements within the 11,265 bp T-DNA with three copies of the Phy02 gene that was used to transform maize.**

[CBI Deleted]

**Table 1. Description of the genetic elements in the 11,265 bp T-DNA fragment containing three copies of the Phy02 phytase gene.**

[CBI Deleted]

| Genetic Element | Description | Size (bp) | Reference |
|---|---|---|---|
| [CBI Deleted] | Promoter [CBI Deleted] | | |
| [CBI Deleted] | Signal peptide [CBI Deleted] | | |
| Phy02 | Coding sequence of the Phy02 phytase gene that was derived from the *E. coli appA* phytase gene with the ER retention peptide SEKDEL. | 1257 | |
| SEKDEL | Sequence encoding an endoplasmic reticulum retention peptide | 18 | Semenza *et al.*, 1990 |
| NosT | Terminal sequence of the *nos* gene of *Agrobacterium tumefaciens* that terminates gene transcription by providing polyadenylation signals | 276 | Depicker *et al.*, 1982 |
| [CBI Deleted] | Promoter sequence [CBI Deleted] | | |
| [CBI Deleted] | Promoter [CBI Deleted] | | |
| ZmUbi1 promoter | Promoter derived from the *Zea mays* ubiquitin 1 gene including the first intron (ZmUbi1 intron); directs expression in all tissues of *Z. mays.* | 1992 | Christensen & Quail, 1996 |
| PMI | Gene sequence of the *manA* gene encoding phosphomannose isomerase derived from *E. coli;* Selectable marker gene | 1176 | Negrotto *et al.*, 2000 |

# 4. Assessment of Allergenicity Potential of Phy02 Phytase

## 4.1. Amino Acid Sequence Homology of Phy02 Phytase to Known Protein Allergens

Bioinformatic analyses were conducted to evaluate the potential allergenicity of the Phy02 phytase protein. The amino acid sequence of the Phy02 phytase protein was compared on March 9, 2015 to a database of allergens from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (version 15.0, January 12, 2015), which contains the amino acid sequences of known and putative allergenic proteins. Potential identities between the Phy02 amino acid sequence and those of proteins in the allergen database were evaluated using the FASTA sequence alignment algorithm (Pearson and Lipman, 1988) with a scoring matrix = BLOSUM62, gap extension penalty = 2, and gap creation penalty = 12. The resulting alignments were returned and reviewed for identities greater than or

equal to 35% over 80 or greater residues. None of the alignments met or exceeded the 35% threshold.

The Phy02 amino acid sequence was also evaluated for any eight or greater contiguous identical amino acid matches to the same database of allergens noted above. The use of a match of eight contiguous, identical amino acids appears to have relevance based upon the minimum peptide length for an IgE-binding epitope (Metcalfe *et al.*, 1996, Bannon and Ogawa, 2006). Results of the evaluation showed there were no contiguous identical amino acid matches of eight or more amino acids observed with the Phy02 amino acid sequence. These data indicate the lack of both amino acid identity and immunologically relevant similarities between the Phy02 protein and known or putative protein allergens.

### 4.2. Lability of Phy02 Phytase to Pepsin in Simulated Gastric Fluid (SGF)

One factor that is considered in relation to an assessment of potential allergenicity of novel proteins is their digestibility in a simulated gastric environment. Therefore, the susceptibility of purified, maize expressed, Phy02 protein to proteolytic digestion by pepsin in simulated gastric fluid (SGF) was evaluated. The International Life Sciences Institute (ILSI) has standardized the pepsin digestibility assay protocol in a multi-laboratory evaluation (Thomas *et al.*, 2004). The SGF formulation, time course, and experimental parameters followed in the evaluation of Phy02 were similar to conditions used in the ILSI multi-laboratory evaluation.

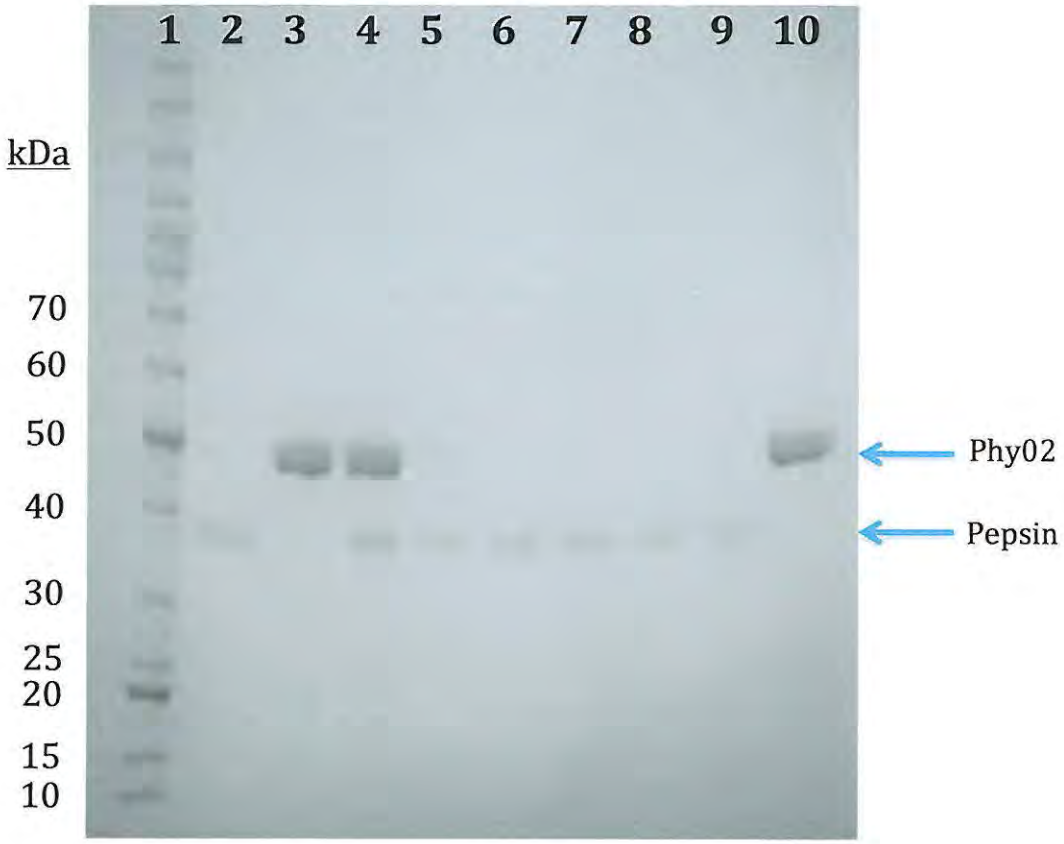
The Phy02 protein was incubated in SGF containing pepsin at pH 1.2 for 0, 1, 5, 10, and 30 min. and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining of the proteins with Coomassie Blue. The reaction conditions and molar ratio of pepsin to Phy02 protein in the study were similar to those described by Thomas *et al.* (2004).

Results of the SGF study shown in Figure 3 demonstrate that the Phy02 phytase protein is rapidly degraded in SGF containing pepsin at pH 1.2, as shown by SDS-PAGE analysis. Trace amounts of the Phy02 protein remained after 1 min. of incubation in the SGF environment (Figure 3, lane 5) and after 5 min. it was completely digested (Figure 3, lane 6). Aliquots of the reactions from the same SGF incubations were also subject to Western blot analysis (NuPage 4-12% Bis-Tris Gel). After electrophoresis the proteins were electro-blotted onto a membrane and a Western blot was performed using a rabbit anti-Phy02 phytase antibody followed by a goat anti-rabbit antibody. The results of the Western blot (Figure 4) confirm the previous results shown in the SDS-PAGE gel. After 1 min. of incubation in the SGF environment a small amount of Phy02 protein was present (Figure 4, lane 4) but after 5 min. the protein was completely digested and could not be detected (Figure 4, lane 5). These results demonstrate that the Phy02 protein is readily digested within 5 minutes in the SGF environment and therefore that it is unlikely to be a potential allergen.

## Figure 3. Lability of the Phy02 Phytase to Pepsin in SGF: Image of SDS-PAGE Gel.

The SGF reactions were loaded onto an SDS-polyacrylamide gel and subjected to electrophoresis followed by staining with Coomassie Blue. The sizes of individual proteins in the molecular weight standards (lane 1) are indicated in kDa on the left side of the gel.

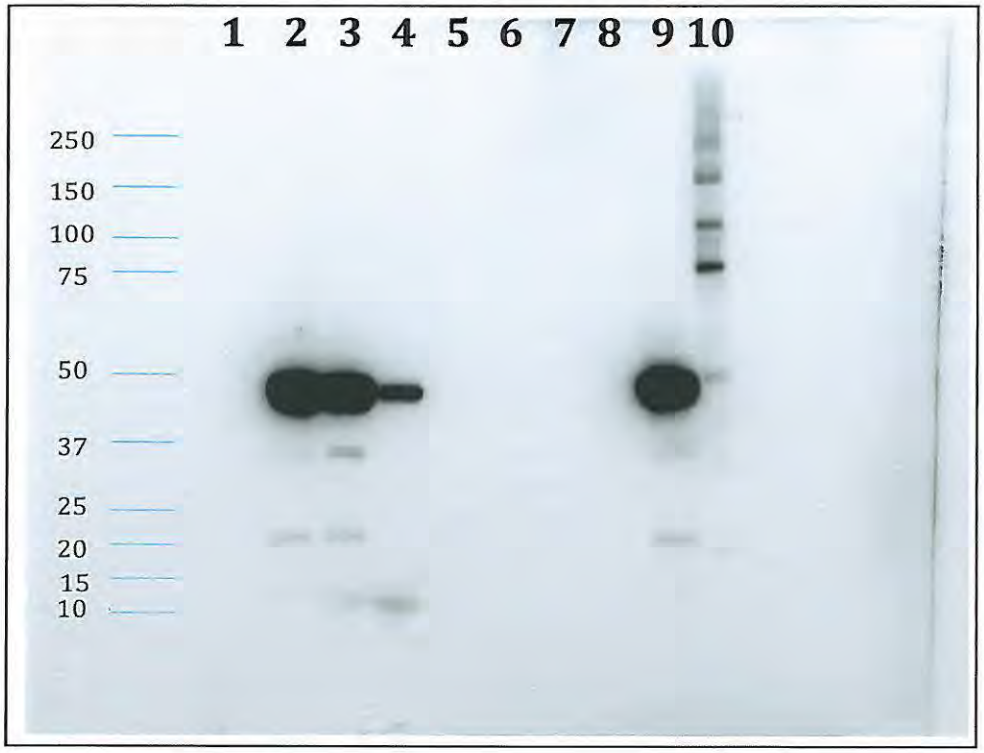
Lanes: 1. Protein Mol. Wt. Standards
2. Pepsin only
3. Phy02 only (44,982 kDa)
4. Pepsin + Phy02 after 0 min.
5. Pepsin + Phy02 after 1 min.
6. Pepsin + Phy02 after 5 min.
7. Pepsin + Phy02 after 10 min.
8. Pepsin + Phy02 after 30 min.
9. Pepsin only
10. Phy02 only (44,982 kDa)



## Figure 4. Lability of the Phy02 Phytase to Pepsin in SGF: Image of a Western Blot

A protein gel with aliquots from the Phy02 SGF reactions was electro-blotted onto PVDF membrane (BIO-RAD Laboratories, Inc.) and incubated with rabbit anti-Phy02 phytase and goat anti-rabbit antibodies to detect the Phy02 protein.

Lanes:
1. Pepsin only control
2. Phy02 only control (44,982 kDa)
3. Pepsin + Phy02 after 0 min.
4. Pepsin + Phy02 after 1 min.
5. Pepsin + Phy02 after 5 min.
6. Pepsin + Phy02 after 10 min.
7. Pepsin + Phy02 after 30 min.
8. Pepsin only control
9. Phy02 only control (44,982 kDa)
10. Biotinylated protein marker



### 4.3. Source of the *phy02* Gene and History of Exposure

The *phy02* gene was derived from the *appA* phytase gene of *Escherichia coli* strain K-12 and optimized using Gene Site-Saturation Mutagenesis (Short, 2001) and directed sequence modifications to achieve specific amino acid substitutions. The AppA phytase and the *appA* gene have been well characterized (Dassa *et al.*, 1990).

Phytate is a naturally occurring phosphorus storage molecule in the seed of many plants, including major food/feed grain crops such as maize, soybeans, wheat, rice and barley. Phytase is produced in the seed during normal germination to release the phytate bound phosphorus and make it available to the growing plant. Phytase enzymes are widespread in nature, occurring in plants, microorganisms and in some animal tissues (Konietzny, 2002). Significant levels of endogenous phytase activity (>1000 FTU $kg^{-1}$) have been reported in rye, wheat, rye bran and wheat bran (Viveros, 2000). Multiple forms of phytase have been reported in barley, maize, rice, wheat, spelt, soybean, rape seed, pumpkin, lily, as well as in *Aspergillus niger, A. oryzae, Escherichia coli*, and *Saccharomyces cerevisiae* (Konietzny, 2002). Phytase has been shown to be produced by microorganisms used in food fermentations, including yeasts such as *S. cerevisiae* (Nakamura, 2000) and *Schwanniomyces castellii* (Lambrechts, 1992). Bacteria that inhabit the intestinal tracts of animals are known to produce phytase and phytase activity has been measured in the gastrointestinal tracts of animals, including humans (Iqbal, 1994). In ruminants, production of phytase by anaerobic ruminal bacteria is most likely responsible for the increased rate of phytate degradation that has been noted in these animals (Yanke, 1998).

Phytases are included in human dietary supplements currently marketed in the U.S. and are claimed to improve the digestion of foods and the absorption of minerals. The absorption of iron in humans has been shown to be dramatically improved when at least 2 of the 6 phosphate groups of phytic acid are removed (Sandberg *et al.*, 1996), thereby demonstrating the positive nutritional affects of phytase in alleviating the anti-nutritive properties of phytic acid. General Nutrition Centers (Pittsburgh, PA) markets a dietary supplement (GNC Natural Brand™ Supa Digestive Enzymes; GNC, 2015) consisting of a mixture of different enzymes including phytase. Nurtiteck-Ultra Bio-Logics Inc. (Montreal, Canada) markets a dietary supplement called Phytase NSP Blend that contains 200 FTU/g of a phytase derived from *Aspergillus niger*. Global Healing Center (Houston, TX) markets a phytase containing enzyme mixture named VeganZyme® (GHC, 2015). CereCalase (NEC, 2015) is another phytase-containing human dietary supplement. It is produced by the National Enzyme Company (Forsyth, MO) and contains a phytase from *A. niger*. Most of the phytase enzymes included in the abovementioned dietary supplement products are derived from *Aspergillus niger*. The AppA phytase that is nearly identical to the Phy02 phytase has been shown to be structurally similar to the phytase from *Aspergillus niger* (Lim et al., 2000).

The NOV9X phytase in the FDA-approved animal feed additive Quantum is nearly identical to the Phy02 phytase, differing in only [CBI Deleted] of 412 amino acids. None of these amino acid changes occur in either the active site nor any other motifs associated with catalytic activity. After many years of inclusion of Quantum in poultry and swine diets, there have been no reports of toxicity or adverse effects. Likewise, maize expressed NOV9X phytase has been tested in poultry (Nyannor and Adeola, 2008; Nyannor *et al.*, 2009) and swine (Nyannor *et al.*, 2007) and has been demonstrated to be effective and safe. Similarly, poultry feeding trials have been conducted with maize expressed Phy02 phytase and these demonstrated the Phy02 phytase to be effective and safe.

### 4.4. Conclusions on the Allergenicity Potential of the Phy02 Phytase Protein

Bioinformatic analyses of the Phy02 amino acid sequence revealed no similarities to known or putative protein allergens. None of the sequence matches identified met or exceeded the threshold of greater than or equal to 35% identity over 80 or more contiguous amino acid residues. Furthermore, no contiguous stretches of eight or greater identical amino acids were shared between the Phy02 protein and proteins in the allergen database. These data indicate the lack of both amino acid identity and immunologically relevant similarities between the Phy02 protein and known or putative protein allergens. The Phy02 protein was rapidly degraded in simulated gastric fluid demonstrating its sensitivity to digestion in the gastric environment. Further, the source organism for the Phy02 protein, *E. coli*, is a well known member of the human intestinal microflora and has been demonstrated to produce the AppA phytase in this environment (Iqbal, 1994). Humans have consumed dietary supplements containing phytase enzymes that are structurally similar to the Phy02 phytase for many years with no reports of toxicity or allergenicity. Taken together, these data support the conclusion that the Phy02 protein is not a potential allergen.

## 5. Assessment of Toxicity Potential of the Phy02 Phytase Protein

### 5.1. Assessment of Amino Acid Homology of Phy02 Phytase to Known Protein Toxins

A global sequence similarity search of the Phy02 amino acid sequence was conducted on February 17, 2015 against the NCBI Protein dataset using the BLASTP algorithm (Altschul *et al.*, 1997). A sequence file comprising the translation of the *phy02* gene was queried using the BLASTP 2.2.30+ algorithm against the "nr" dataset, which incorporates non-redundant entries from all GenBank nucleotide translations along with protein sequences from SWISS-PROT, PIR, PRF, and PDB.

A cutoff expectation (*E*) score of 1.0 was used to generate biologically meaningful similarity between the Phy02 protein and proteins in the "nr" dataset. Although a statistically significant sequence similarity generally requires a match with an *E* score

of less than 0.01 (Pearson, 2000), a cutoff of $E < 1.0$ ensures that proteins with even limited similarity will not be overlooked in the search. Low complexity filtering was turned off and the maximum number of alignments returned was set to 2000.

The Phy02 protein similarity search identified 2000 protein accessions with an $E$ score of less than 1.0. This represents the maximum number of alignments that could be returned from the search and is indicative of the many amino acid sequences displaying similarity to Phy02 protein. A large number of the accessions returned by the search displayed complete significance ($E = 0$) and represented nearly identical or closely related phytase proteins from various microbial or plant species. The remaining sequences represented a variety of proteins that were all functionally related phosphatase proteins from many different organisms.

None of the similar proteins returned by the search were identified as toxins, demonstrating that the Phy02 protein is unlikely to share relevant sequence similarities with known protein toxins and is therefore unlikely to be a toxin itself.

## 5.2. Results of Toxicity Studies Using NOV9X Phytase, a Related Phytase

To date no safety studies have been completed with the Phy02 phytase. However, safety studies have been conducted using NOV9X which is the phytase in the animal feed additive Quantum that has been used commercially in the feed of poultry and swine since 2008. Feed enzymes are feed additives whose animal safety and efficacy are reviewed by the U.S. FDA Center for Veterinary Medicine. As such, studies were conducted to support the safety of the NOV9X phytase and these were reviewed by the FDA CVM. The NOV9X and Phy02 phytases are both derived from the *E. coli appA* phytase gene and Phy02 differs from NOV9X by only [CBI Deleted] amino acids out of a total of 412 amino acids in the mature protein. Therefore, the Phy02 phytase can be considered to be nearly identical to the NOV9X phytase and safety studies on the latter should be appropriate to support conclusions relative to the safety of the former. This is supported by the decision tree developed by Pariza and Johnson (2001) for the evaluation of the safety of enzymes used in food processing. They recognized that protein engineering techniques that are applied to food and feed enzymes to improve the performance of the enzymes and which result in a small number of amino acid changes will not result in any increased toxicity of the new enzyme relative to its progenitor.

An acute oral toxicity study in rats was conducted using NOV9X phytase produced by the yeast *Pichia pastoris* at the Central Toxicology Laboratory, Alderley Park, UK. This study was conducted according to the requirements as defined by the European Community Guidelines for the Assessment of Additives in Feeding Stuffs and the US FDA Redbook 2000, Toxicological Principles for the Safety of Food Ingredients. Groups of 5 male and 5 female rats were dosed with 2000 mg/kg body weight (bwt) of a test substance consisting of 78.5% NOV9X phytase and containing 231,000 units of phytase activity/g. The test animals were assessed daily for 14 days and at the end of the study all animals were sacrificed and blood samples were taken for hematology and clinical chemistry. Brain, kidney, liver, and spleen were weighed

and selected tissues were submitted for histopathological examination. In this test, there was zero mortality during the duration of the study and no signs of systemic toxicity. There were no toxicologically significant effects on body weight, food consumption, clinical pathology, or organ weights. There were no treatment related abnormalities at examination *post mortem*. In conclusion, NOV9X caused no adverse effects when administered to rats as a single oral dose of 2000 mg/kg bwt (equal to ~500,000 units phytase/kg). The complete study report was reviewed by the FDA Center for Veterinary Medicine as part of the submission for Quantum phytase, the *Pichia pastoris* produced NOV9X phytase.

A 90-day subchronic oral toxicity study with rats was conducted using purified NOV9X phytase produced by *Pichia pastoris*. Groups of twelve male and twelve female Alpk:APfSD (Wistar-derived) rats were dosed orally by gavage with 0 (control), 40, 120 or 400 mg NOV9X protein/kg bwt/day for 90 consecutive days. Clinical observations, bodyweights and food consumption, were measured throughout the study and at the end of the study the animals were euthanized and subjected to a full examination post mortem. Cardiac blood samples were taken for clinical pathology, selected organs were weighed, and specified tissues were taken for subsequent histopathological examination. There were no test substance related effects on any of the measured parameters in the 40 and 120 mg/NOV9X protein/kg bwt/day dose groups. Male bodyweight was increased when compared to controls in the 400 mg/kg bwt/day group, from week 3 onwards. This was associated with increased food utilization due to the activity of the phytase and increased dietary protein due to the higher level of proteinaceous test material. The no observed adverse effect level (NOAEL) for this study was 400 mg/kg bwt/day. The results of this study and of the acute oral toxicity study cited above demonstrate that the NOV9X phytase is not toxic after oral exposure. The complete study report was reviewed by the European Food Safety Authority as part of the submission to the EU for Quantum phytase, the NOV9X phytase produced by *Pichia pastoris*.

In addition to the above cited safety studies with NOV9X phytase produced by *Pichia pastoris*, numerous animal trials have been conducted with broiler chickens (Nyannor and Adeola, 2008; Nyannor *et al.*, 2009) and feeder piglets (Nyannor *et al.*, 2007) that demonstrate the efficacy and enzyme functionality of the maize expressed NOV9X phytase and its safety for animals consuming feed treated with it. In all of these studies the maize expressed NOV9X phytase was demonstrated to be effective in delivering improved nutritional benefits to the animals and no safety concerns or issues were observed. Similar feeding trials using maize grain expressing the Phy02 phytase have been conducted in poultry by Agrivida. In these trials the Phy02 phytase provided a dose related improvement in animal performance as expected for a phytase feed additive and no adverse effects on the animals were noted.

### 5.3. Conclusions on Toxicity Potential of Phy02 Phytase

The Phy02 phytase is a well characterized phytase enzyme with a highly specific enzymatic activity. Humans have experienced oral exposure to phytase enzymes as they are naturally present in wheat, barley, rice and other food grains. Phytase

enzymes are also expressed in microbes such as yeasts that are used in the production of common foods. In addition, phytases from *Aspergillus nig*er that are structurally related to Phy02 are marketed as human dietary supplements to improve digestion and there have been no reports or incidents of toxicity as a result of this exposure. The only biological activity of the Phy02 phytase is that of a phytase enzyme and the only other dietary impact of its presence in food is the same as any other dietary protein that is digested to its constituent amino acids. The primary cause of oral toxicity of an enzyme would be due to its specific enzymatic activity. In the case of phytases it is clear that their biological activity is not orally toxic. A search of the non-redundant protein databases with the Phy02 amino acid sequence revealed that the Phy02 protein has no similarity to any known toxic proteins. The safety of the Phy02 phytase is further demonstrated by the results of safety studies with the nearly identical phytase NOV9X and by the poultry feeding trials with the Phy02 maize expressed phytase that support a conclusion that the Phy02 protein is safe and nontoxic.

# 6. Overall Conclusions

According to the guidance of the FDA for the early food safety evaluation of new proteins in new plant varieties that are under development, the Phy02 phytase protein was evaluated for its allergenicity and toxicity potential.

The allergenic potential of Phy02 was assessed by: 1) bioinformatic comparison of the amino acid sequence of the Phy02 protein with the amino acid sequences of well known protein allergens; 2) evaluation of the stability of the maize produced and purified Phy02 protein in a simulated gastric environment; and 3) assessment of the *phy02* gene source and history of use or exposure.

Bioinformatic analyses revealed no similarities between known protein allergens and the Phy02 protein sequence. These data indicate the lack of both amino acid identity and immunologically relevant similarities between the Phy02 protein and known allergens. The Phy02 protein was rapidly degraded in the simulated gastric fluid environment. Further, there has been a long history of human dietary exposure to phytases, including the phytase from *Aspergillus niger* that is structurally similar to the Phy02 phytase. Taken together, this information supports the conclusion that the Phy02 protein is not a potential allergen.

Bioinformatic analyses revealed Phy02 phytase to be similar to other phytase proteins. No biologically relevant sequence similarities were detected between known protein toxins and the Phy02 protein. There was no evidence of acute or subchronic toxicity in rats when treated with the NOV9X phytase that is nearly identical to Phy02. These data support the conclusion that the Phy02 protein is not toxic.

Based on the data and information provided in this submission, we have determined that the Phy02 protein is unlikely to cause an allergic reaction in humans or be a toxin in humans or animals.

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