PHYTASE FROM *ASPERGILLUS NIGER* EXPRESSED IN *A. NIGER*

Chemical and Technical Assessment (CTA)

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1. Summary

This Chemical and Technical Assessment (CTA) summarizes data and information on the 3-phytase enzyme preparation submitted to JECFA by the World Food Programme and the Global Alliance (WFP and GAIN, 27 May 2011). The Committee evaluated the enzyme preparation 3-phytase (myo-inositol-hexakisphosphate-3-phosphohydrolase; Enzyme Commission number 3.1.3.8) derived from a genetically modified strain of *Aspergillus niger*, manufactured by DSM Nutritional Products, the Netherlands. In this report, the expression '3-phytase' is used when referring to the 3-phytase enzyme and its amino acid sequence, and the expression '3-phytase enzyme preparation' is used when referring to the 3-phytase enzyme preparation used for toxicological studies. This document also discusses published information relevant to 3-phytase and the production organism, *Aspergillus niger*.

Phytase (myo-inositol-hexakisphosphate-3-phosphohydrolase) is an enzyme which catalyses the hydrolysis of myo-inositol hexakiphosphate (phytate) to inositol penta-phosphate (IP5), and further to give a mixture of *myo*-inositol di-phosphate (IP2), *myo*-inositol mono-phosphate (IP1) and free orthophosphate. Phytases are natural constituents of plants (cereals and legumes) which are used as foods and can be readily produced from a number of microbial and fungal sources during the fermentation of certain foods.

The phytase enzyme preparation is intended to be used to degrade phytate found in plant foods, particularly cereal grains and legumes. Phytate/phytic acid is a recognized anti-nutrient (Rimbach et al 2008) which strongly inhibits the absorption from food of both native and added minerals such as iron, zinc, calcium, magnesium and manganese, by forming insoluble complexes with them in the stomach, thus reducing their availability for absorption.

The enzyme 3-phytase is produced by submerged fermentation of an *Aspergillus niger* strain containing multiple copies of the *Aspergillus niger* gene encoding the enzyme in question (van Dijck et al 2003). The *Aspergillus niger* strain is genetically modified to contain several copies of the wild type *Aspergillus niger* phytase gene and is referred to as a "self-cloned" microorganism. *A. niger* is a filamentous fungus that commonly occurs in the environment and is considered to be nonpathogenic.

Prior to the introduction of the 3-phytase gene, the *A. niger* host strain ISO-500 was genetically modified by deletion of the genes encoding glucoamylase activity. The modified host strain was then transformed with an amplifiable DNA cassette containing the phytase gene from *A. niger*, and the *Aspergillus nidulans* acetamidase (*amdS*) gene, which was the selectable marker. The recombinant production strain is genetically stable and does not contain any antibiotic resistance markers, or any other heterologous DNA

3-Phytase is secreted into the fermentation broth and is subsequently isolated by filtration to remove the biomass and concentrated by ultrafiltration. The enzyme concentrate is subjected to

germ filtration and is subsequently formulated and standardized to the desired activity using food-grade compounds. The phytase enzyme preparation complies with the General Specifications and Considerations for Enzymes Used in Food Processing (JECFA, 2006).

The 3-phytase is intended to be used as both processing aid and as an active enzyme to improve mineral bioavailability, particularly from foods and home fortification products.. The 3-phytase enzyme preparation is used as a food additive, in the processing of phytate-rich food such as cereal grains and legumes, and as a dietary supplement, for co-consumption with phytate-rich foods.

Batch analysis demonstrated that the 3-phytase preparations from *A. niger* were free of aflatoxin B1, T2 toxin, ochratoxin A, zearalenone and sterigmatocystin. 3-Phytase protein has no relevant match with known food allergens and is not likely to produce an allergenic or sensitization response upon oral consumption.

2. Description

Two product forms are currently produced; a liquid product form (EUPHOVIDA 5,000L – brownish colour) and a micro-granulated product form (EUPHOVIDA 20,000G – yellow to light brown). These products comply with the General Specifications for Enzyme Preparations used in Food Processing as established by the 67th meeting of the Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO, 2006 (JECFA 2006a).

3. Method of Manufacture

3.1 Aspergillus niger

The source of 3-phytase is an *Aspergillus niger* microorganism genetically modified to contain several copies of the wild type *Aspergillus niger* phytase gene ("self-cloned" microorganism).
A. niger is a filamentous fungus that commonly occurs in the environment and is generally regarded as nonpathogenic. It grows aerobically on organic matter and tolerates a wide range of temperature and pH. *A. niger* has a long history of use as a source of citric acid and enzymes used in food processing.

A. niger has no endogenous antibiotic resistance (Pel et al 2007) and in the construction of the strain no antibiotic resistance markers have been used, nor does the strain carry any other heterologous DNA. 3-Phytase is produced by a controlled submerged fermentation of a selected, pure culture of *A. niger*.

The host, *A. niger* strain ISO-500, was obtained from strain GAM-53 (DS 03045) by genetic modification. By further genetic modification of strain ISO-500, the present phytase producing *A. niger* strain NPH54 (DS35387) was obtained. The production microorganism does not contain any foreign recombinant-DNA. Instead, the production microorganism contains multiple copies of the endogenous gene coding for phytase. The *A. niger* strain GAM-53 (DS 3045), is a descendant of the original *A. niger* strain NRRL 3122 purchased from the Agricultural Research Service Culture Collection, National Center for Agricultural Utilization and Research, Peoria, IL, USA. The GAM-53 strain was selected by DSM in 1982 for its enhanced production of glucoamylase, an enzyme widely used in the starch processing industry. The strain was

taxonomically identified as *A. niger* by the Dutch culture collection, the Centraalbureau voor Schimmelcultures.

For the construction of the phytase production strain, two plasmids were used: one to derive the expression cassette, containing the phytase gene, and the other to derive the cassette containing a selectable marker.

The phytase and selectable marker expression units, both completely free of any *E. coli* DNA, were integrated into the genome of the host ISO-500 by co-transformation and the transformants were selected on agar plates containing acetamide as the sole carbon source. Both GAM53 (DS3045), which is the direct parent of the host strain ISO-500 (DS 30620), and the current recombinant production strain NPH54 (DS35387; CBS101672) have been taxonomically identified by the Dutch culture collection, the Centraalbureau voor Schimmelcultures (CBS). The parental strain GAM53 (DS 3045) was determined by the usual classical identification as *Aspergillus niger* according to the description of v. Tieghem. Also the recombinant phytase production strain NPH54 (DS35387) was determined as *Aspergillus niger*.

3.2 Fermentation, Recovery and Formulation

3-Phytase is produced by fed-batch submerged, aerobic, pure culture fermentation of the genetically modified *A. niger* production strain. The enzyme is secreted into the fermentation broth and is subsequently purified and concentrated by ultra-filtration. The enzyme concentrate is formulated with glycerol (liquid form) or with maltodextrin (powder form) to achieve the desired phytase activity and stability. Food grade raw materials are used for fermentation and formulation. The whole process is performed in accordance with the US Food and Drug Administration (FDA) current good manufacturing practice (GMP), as described in the Code of Federal Regulations (CFR) title 21, part 110.The phytase enzyme preparation conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing

4. Chemical Characterization

4.1 Phytase enzyme

Phytase is described by the International Union of Biochemistry and Molecular Biology (IUBMB) as follows:

Common Name:	3-phytase
Other name(s):	myo-Inositol-hexakisphosphate-3-phosphohydrolase
Reaction:	Catalyses the hydrolysis of *myo*-inositol hexakisphosphate (phytate) to inositol penta-phosphate (IP5), and further to give a mixture of *myo*-inositol di-phosphate (IP2), *myo*-inositol mono-phosphate (IP1) and free orthophosphate.
Secondary enzyme activities	No significant levels of secondary enzyme activities
IUB Nomenclature:	Phytase
EC No.:	3.1.3.8

Chemical Abstract Service Number (CAS No.): 37288-11-2

The enzyme activity is expressed in phytase units (FTU) per gram or ml. One FTU is defined as the amount of enzyme that liberates 1 micromole of inorganic phosphate per minute from 0.0051 mol/l sodium phytate at 37° C and pH 5.5. This method is used for quality assurance and product release.

The physical chemical characteristics of the 3-phytase have been extensively studied. The enzyme is active between pH 2-6.5, with a maximum activity at pH 5.5. The isoelectric point (IEP) of the enzyme is about 5.2. The optimum temperature, when measured in the standard assay protocol with a 30 minutes incubation period, is approximately 55° C and the enzyme is inactivated at temperatures above 60° C. The 3-phytase is a glycoprotein with a molecular weight of approximately 85 kDa. Based on the known complete amino-acid sequence of the peptide chain it can be deduced that the protein part is about 50 kDa, whereas the carbohydrate part is about 35 kDa. The enzymatic activity of 3-phytase is about 105 FTU/mg total organic solids (TOS). TOS consists of the enzyme of interest and residues of organic materials, such as proteins, peptides, and carbohydrates, derived from the production organism and the manufacturing process.

The content of total organic solids (TOS) is calculated as follows:
% TOS = 100 - (A + W + D)
where:
A = % ash, W = % water and D = % diluents and/or other formulation ingredients (JECFA 2006b).

The phytase enzyme preparation contains food grade materials and conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing. 3-Phytase is expected to be inactivated during processing or cooking.

3-Phytase was evaluated for potential allergenicity according to the bioinformatics criteria recommended by FAO/WHO (FAO/WHO, 2001). The amino acid sequence of 3-phytase was compared with the amino acid sequences of known allergens. A similarity search using the Allermatch database did not produce a match with any sequence showing >35% identity over any sliding window of 80 amino acids.

5. Functional uses

The purpose of the addition of phytase to food is to reduce its natural phytic acid / phytate content. Highest phytate levels are found in grains, legume seeds and linseed and can be as high as 1- 2%. Phytate is a strong chelator of di- and trivalent mineral cations including iron, zinc, calcium and manganese, and it therefore occurs mainly as a mixed salt of calcium / magnesium / potassium phytate. These mineral-phytate complexes have a very low solubility under the pH conditions of the upper gastrointestinal tract, where most minerals are absorbed. Thus, these complex-bound minerals have very poor bioavailability in humans. Phytase can cleave off five of the six phosphate residues from IP6. With each phosphate residue removed, the mineral binding capacity of phytate decreases.

3-Phytase is intended to be used to degrade phytate found in plant foods, particularly cereal grains and legumes. Cereal- and legume-based complementary foods, such as corn soy blend (CSB) and wheat soy blend (WSB), distributed by the World Food Programme (WFP) and other agencies,

are good candidates for the removal of phytate, particularly because in developing countries infants over six months of age may depend on these foods as their main sources of dietary iron. Flour, cereal based beverages and ready to use foods may also be considered for removal of phytate by phytase.

Phytase can exert its effect in two general ways:

- Technological use: where the intended use of the enzyme takes place prior to ingestion, for example during processing of phytate-rich food such as cereals and legumes, in which case the phytase will be inactivated by the time of consumption.
- Nutritional use: where the intended use of the enzyme takes place post ingestion, for example its inclusion in supplements intended for co-consumption with phytate-rich foods, in which case the phytase is expected to remain functional during the residence time of a meal in the stomach.

Phytase from *Aspergillus niger* has been approved for use as a food processing aid in Australia and New Zealand (Food Standards Australia NZ 2011) and is listed in the Codex Alimentarius Inventory of Substances used as Processing Aids (CAC 2010b), and DSM´s 3-phytase specifically is approved for use in baking in France, and has self-affirmed GRAS status in the US for use as an active enzyme consumed together with cereal foods.

6. Reactions and fate in food

Phytase is a normal constituent of wheat flour and other cereals. During germination of seeds, the enzyme phytase is produced to liberate phosphate, which is subsequently used as building block for the young fast-growing plant. Ou *et al.* studied phytase activity in brown rice during steeping and sprouting (Ou et al 2011), and found that during the sprouting phase, phytase activity increased with prolonged time and gradually reached stable levels. Comparable results were obtained with barley during malting, wheat flour and standard bread doughs. Microbial phytase is produced during the fermentation of many traditional foods, including soybean products such as lao-chao, Koji, which is used to make sake, soy sauce and miso in Japan and China and tempeh in Indonesia, ogi from maize in Africa, kenkey and other fermented products made from corn, sorghum, cowpeas and soybeans in Africa, fermented flatbread in Egypt, pulque in Mexico. Therefore it can be concluded that the addition of extra phytase does not result in new or unintended reaction products in food.

The secondary effect of phytase activity is the release of minerals bound to phytate. Liberation of iron in processed foods could theoretically affect their stability, due to the promotion of lipid oxidation by free iron, but in reality the processed foods in which 3-phytase is intended to be used are stored dry (e.g. CSBs, pasta, breakfast cereals). Similarly, products containing active enzyme would either have a low moisture content in order to ensure the stability of the enzyme (e.g. flour and lipid based ready to use foods), would not contain phytate themselves (e.g. soft drinks, candies), or would not contain lipids (food supplements including multiple micronutrient powders).

At temperatures above 60 °C, the enzyme is denatured. Therefore in applications where the food is cooked, either during industrial preparation e.g. breakfast cereals or in the home e.g. during home baking or preparation of CSB, the 3-phytase would be inactive at the time of consumption.

In applications where DSM´s 3-phytase is consumed in its active form it is expected to remain functional in the stomach. Based on data available from pigs, the phytase is subsequently expected to be digested by proteases in the small intestine (Jongbloed et al 1992).

7. References

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