ORIGINAL SUBMISSION

AB Enzymes GmbH – Feldbergstrasse 78 , D-6412 Darmstadt

AB Enzymes
an ABF ingredients company

April 26, 2014

Office of Food Additive Safety (HFS-255),
Center for Food Safety and Applied Nutrition,
Food and Drug Administration,
5100 Paint Branch Parkway, College Park, MD 20740.

RE: GRAS NOTICE FOR PHOSPHOLIPASE A2 ENZYME PREPARATION FROM A GENETICALLY MODIFIED STRAIN OF *TRICHODERMA REESEI*

Pursuant to proposed 21 C.F.R § 170.36, AB Enzymes GmbH is providing in electronic media format (determined to be free of computer viruses), based on scientific procedures – a generally recognized as safe (GRAS) notification for phospholipase enzyme preparation from *Trichoderma reesei* (*T.reesei)* strain RF8793 expressing the gene encoding phospholipase A2 from *Aspergillus nishimurae* (*A. nishimurae)* (formerly *Aspergillus fumigates* (*A. fumigates*) for use in edible oil degumming at a recommended dose of 120 – 3,000 phospholipase units (PLU) per kilos of oil treated and preferably around 500 PLU per kilos of oil. The phospholipase A2 enzyme preparation described herein when used as described above and in the attached GRAS notice is exempt from the premarket approval requirements applicable to food additives set forth in Section 409 of the Food, Drug, and Cosmetic Act and corresponding regulations.

Please contact the undersigned by telephone or email if you have any questions or additional information is required.

(b) (6)

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AB Enzymes
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GRN 000524

RECEIVED
MAY 05 2014
OFFICE OF
FOOD ADDITIVE SAFETY

April 26, 2014

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5100 Paint Branch Parkway, College Park, MD 20740.

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Pursuant to proposed 21 C.F.R § 170.36, AB Enzymes GmbH is providing in electronic media format (determined to be free of computer viruses), based on scientific procedures – a generally recognized as safe (GRAS) notification for phospholipase enzyme preparation from *Trichoderma reesei* (*T.reesei)* strain RF8793 expressing the gene encoding phospholipase A2 from *Aspergillus nishimurae* (*A. nishimurae)* (formerly *Aspergillus fumigates* (*A. fumigates*) for use in edible oil degumming at a recommended dose of 120 – 3,000 phospholipase units (PLU) per kilos of oil treated and preferably around 500 PLU per kilos of oil. The phospholipase A2 enzyme preparation described herein when used as described above and in the attached GRAS notice is exempt from the premarket approval requirements applicable to food additives set forth in Section 409 of the Food, Drug, and Cosmetic Act and corresponding regulations.

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GRN 000524

RECEIVED
MAY 29 2014
OFFICE OF
FOOD ADDITIVE SAFETY

April 26, 2014

RE: GRAS Notification – Exemption Claim

Dear Sir or Madam:

Pursuant to the proposed 21C.F.R. § 170.36 (c)(1) AB Enzymes GmbH hereby claims that phospholipase enzyme preparation from *Trichoderma reesei* (*T.reesei)* strain RF8793 expressing the gene encoding phospholipase A2 from *Aspergillus nishimurae* (*A. nishimurae)* (formerly *Aspergillus fumigates* (*A. fumigates*) produced by submerged fermentation is Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

The following information is provided in accordance with the proposed regulation:
Proposed 21C.F.R. § 170.36 (c)(i) *The name and address of notifier.*
AB Enzymes GmbH
Feldbergstr. 78
D-64293 Darmstadt, Germany

Proposed 21C.F.R. § 170.36 (c)(ii) *The common or usual name of notified substance.*
Phospholipase enzyme preparation from *Trichoderma reesei* expressing the gene encoding a phospholipase A2 from *Aspergillus nishimurae* (formerly *Aspergillus fumigates).*

Proposed 21C.F.R. § 170.36 (c)(iii) *Applicable conditions of use.*
The phospholipase A2 is intended for use in edible oil refining as a processing aid in oil degumming. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices.

Proposed 21C.F.R. § 170.36 (c)(iv) *Basis for GRAS determination.*
This GRAS determination is based upon scientific procedures.

Proposed 21C.F.R. § 170.36 (c)(v) *Availability of information.*
A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The package includes **a** safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying at reasonable times at a specific address set out in the notice or will be sent to FDA upon request.

(b) (6)

April 26, 2014

Karl-Heinz Maurer
Director, Business Development

Date

GRAS NOTIFICATION FOR PHOSPHOLIPASE A2 FROM A GENETICALLY MODIFIED STRAIN OF *TRICHODERMA REESEI*

AB ENZYMES GmbH

April 26, 2014

AB Enzymes
an ABF Ingredients company

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1. GENERAL INTRODUCTION AND CLAIM OF EXEMPTION FROM PREMARKET APPROVAL REQUIREMENTS

Pursuant to the regulatory and scientific procedures established by proposed regulation 21 C.F.R. § 170.36 (see 62 Fed. Reg. 18,938 (April 17, 1997)), AB Enzymes GmbH ("AB Enzymes") has determined that its Phospholipase A2 (PLA2) enzyme preparation from *Trichoderma reesei* (*T.reesei)* strain RF8793 expressing the gene encoding PLA2 from *Aspergillus nishimurae* (*A. nishimurae)* (formerly *Aspergillus fumigatus* (*A. fumigatus*)), is a GRAS substance for the intended food applications based on scientific procedures and is therefore exempt from the requirement for premarket approval. Information on the enzyme and the production organism providing the basis for this GRAS determination is described in the following sections. General and specific information identifying and characterizing the enzyme, its applicable conditions for use, AB Enzymes' basis for its GRAS determination and the availability of supporting information and reference materials for FDA's review can be found here in Section 1.

The production organism, *T. reesei*, has a long history of safe use; this is discussed in Section 2. The FDA has previously affirmed as GRAS several enzyme preparations from *T. reesei* and subsequently received GRAS notifications for additional enzyme preparations, including several produced from genetically modified *T. reesei* strains.

Section 2 also describes the genetic modifications implemented in the development of the production microorganism to create a safe standard host strain resulting in a genetically well-characterized production strain, free from known harmful sequences.

In Section 3, data are presented that show the substantial equivalence of PLA2 to naturally occurring PLA2. The safety of the materials used in manufacturing, and the manufacturing process itself is described in Section 4. Section 5 reviews the hygienic measurements, composition and specifications as well as the self-limiting levels of use for PLA2. Section 6 provides information on the mode of action, applications, use levels of PLA2 and enzyme residues in final food products. The safety studies outlined in Section 7 indicate that PLA2 enzyme preparation from *T. reesei* show no evidence of pathogenic or

toxic effects. Estimates of human consumption and an evaluation of dietary exposure are also included in Section 7.

1.1. Name and Address of Notifier

Notifier:
AB Enzymes GmbH
Feldbergstr. 78
D-64293 Darmstadt
Germany

Manufacturer:
Roal Oy[1]
Tykkimäentie 15
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And

Dr. Hans-Juergen Schepers
AB Enzymes GmbH
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[1] ROAL is a Joint Venture between Associated British Foods (UK) and Altia OY (Finland). Manufacturing and some research and development activities are performed for AB Enzymes by ROAL OY in Finland. ROAL coordinates its R&D activities independently while taking into account the market requirements reported by their sole distributor AB Enzymes GmbH.

1.2. Common or Usual Name of Substance

Phospholipase A2 (PLA2) enzyme (formerly called lecithinase A) preparation from *Trichoderma reesei* strain RF8793 expressing the gene encoding PLA2 from *A. nishimurae* (formerly *A. fumigatus*).

Synonyms: lecithinase A; phosphatidase; phosphatidolipase; phospholipase A, phosphatidylcholine 2-acylhydrolase

1.3. Applicable Conditions of Use

pH value: Optimum: 3.5 – 5.5 Range: 3.0 – 6.0

Temperature: Optimum: 50 - 55°C Range: 50 – 58°C

1.3.1. Food Products Used in

PLA2 will be used in fats and oil processing (vegetable oil degumming including lecithin production) and egg processing (egg yolk modification and lecithin production).

1.3.2. Levels of Use

The maximal recommended use levels (mg TOS/kg raw material (RM)) for oil and egg processing respectively *are 0.5 mg TOS/kg RM and 6 mg TOS/kg RM*.

1.3.3. Purposes

Crude vegetable oil extracted from soya, rape seeds, and sunflower or rice bran has to be refined to remove impurities such as phospholipids (referred to as gums), colours, free fatty acid, odouring and flavouring substances, S-compounds and metal ions. These impurities have a negative influence on downstream processing, taste and storage stability of vegetable oils and therefore have to be reduced in the edible oil. A large part of the gums can be removed with water; however the remaining non-water soluble gums can either be removed by a caustic process or through an enzymatic process using phospholipases.

PLA2 degrades phospholipids into lysophospholipds (a derivative of a phospholipid in which one or both acyl derivatives have been removed by hydrolysis) during oil degumming. The formed lysophosholipids can easily be separated with the water phase from the oil due to their emulsifying properties. Lysolecithin, a type of lysopholipid is a by-product of oil degumming and can be used in food products whereby they help to boost the wetting capabilities, solubility and stabilization of emulsions.

Egg yolk is used world-wide as an effective food emulsifier to make sauces due to its high content of lecithin (type of phospholipid). Partial hydrolysis of the lecithin with PLA2 results in modification of lecithin to lysolecithin (more commonly called Lysophosphatidylcholine). Lysolecithin is used as an important ingredient in foods for their long-term storage and is an effective emulsifier as it enhances emulsion stability and also solubility of hydrophilic compounds in oil phase (Kim et al., 2009).

1.3.4. Consumer Population

PLA2 was the first phospholipase to be recognized. This enzyme is ubiquitous in nature and occurs naturally in animal and plant cells. It has been isolated from a number of food sources (including wheat flour) and is a natural constituent of the digestive pancreatic juice of mammals, including humans (*de Haas et al. (1968); Rossiter (1968); Johnson and McDermott (1974)*). PLA2 is a component of many animal and plant derived foods.

Similar PLA2 preparation, from *Aspergillus niger* and from *S. violaceumber* have already been the subject of a GRAS notification (respectively GRN 000183 - 2005, and GRN 000145-2004), and phospholipase A1s from different organisms are approved world-wide (*Appendix # 1*). Since the PLA2 produced in a genetically engineered *T. reesei* strain is a protein composed of natural amino acids, it will be digested in the human gastrointestinal tract just as any other food protein/enzyme.

PLA2 catalyses the hydrolysis of an ester bond (sn-2 ester bond) between a fatty acid and glycerol in phospholipids. Both, the substrate (phospholipids) and the products of this enzymatic reaction (lysophospholipids and free fatty acids) play important roles in a number of metabolic processes in all

organisms, from bacteria to mammals. As a result, lysophospholipids and free fatty acids are quite abundant in the human diet. The natural occurrence of phospholipids in crude soybean oil is 2%, of which 90% is removed during the degumming process to improve color and enhance oxidative stability (*Nzai and Proctor (1998)*). Hence, there is no basis to believe that conversion of phospholipids to lysophospholipids and free fatty acids will have a significant effect, if any, on processed foods or on the human body.

As is shown in Section 6.4 of this dossier, the enzyme is expected to be denatured during the oil degumming and egg processing. It is important to note that the use refined oils and modified egg yolk lecithin are mainly used in the food industry as an ingredient, lowering even less the final amount of (denatured) enzyme in final foods. Since PLA2 may be present in food products at only very low levels as an inactive protein, and because it is a naturally occurring substance in cells and tissues commonly ingested by humans without any harm, it is assumed that the consumer population will be unaffected by the presence of denatured PLA2 in foodstuffs when used as processing aids.

1.4. Basis for GRAS Determination

Pursuant to 21 C.F.R. § 170.30, AB Enzymes GmbH has determined, through scientific procedures, that its PLA2 enzyme preparation from *T. reesei* expressing the gene encoding PLA2 from *A. nishimurae/A. fumigatus* is GRAS for use as an enzyme for the degradation of phospholipids in edible oil degumming and in enzymatic egg processing.

1.5. Availability of Information for FDA Review

A notification dossier providing a summary of the information that supports this GRAS determination is enclosed herein. The dossier includes a safety evaluation of the production strain, the enzyme and the manufacturing process, as well as an evaluation of dietary exposure. The complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration (FDA) for review and copying at reasonable times at a specific address set out in the notice or will be sent to FDA upon request.

Please direct all inquiries regarding this GRAS determination to:

Candice Cryne

AB Enzymes GmbH

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Toronto, Ontario M6J1E5

647-919-3964

Candice.cryne@abenzymes.com

2. PRODUCTION MICROORGANISM

2.1. Donor, Recipient Organism and Production Strain

Donor:

The PLA2 gene was isolated from the *Aspergillus fumigatus* strain RH3949. The donor strain RH3949 was first identified as *Aspergillus fumigatus* (*Appendix #2*) and more recently as *Aspergillus nishimurae* (*Appendix #3*). As the name *Aspergillus fumigatus* has been used in our publications, both names *Aspergillus fumigatus* and *Aspergillus nishimurae* are used interchangeably in this dossier for the donor organism.

Aspergillus nishimurae belongs to the section Fumigati of *Aspergillus*. The taxonomic lineage of *Aspergillus nishimurae* is shown below (according to http://www.uniprot.org/taxonomy/1220166):

- Genus: *Aspergillus*
- Species: *Aspergillus nishimurae*
- Subspecies (if appropriate): not applicable
- Generic name of the strain: RH3949
- Previous or other name(s) (if applicable): *Aspergillus fumigatus*
- Commercial name: Not applicable. The organism is not sold as such

Aspergillus fumigatus is a ubiquitous filamentous fungus which plays an important role under natural conditions in the aerobic decomposition of organic materials. At the same time, *A. fumigatus* is an opportunistic fungal pathogen. However, *A. fumigatus* infections occur in susceptible patients who have breaches in their defence systems (*Tomee and Kauffman,* 2000). According to *Latgé (*1999), "all humans will inhale at least several hundred *A. fumigatus* conidia per day" and "inhalation of conidia by immunocompetent individuals rarely has any adverse effect, since the conidia are eliminated relatively efficiently by innate immune mechanisms".

Aspergillus fumigatus strains secrete multiple extracellular phospholipases (PL), including PLA, PLB, PLC and PLD. Several strains of *Aspergillus fumigatus* are known to be pathogenic. However, in the recent scientific literature it is also discussed that phospholipid acyl hydrolysase activity from environmental strains of *A. fumigatus* may be more important for growth in the environment than it is for clinical isolates growing in the body (*Birch et al., 2004*). According to *Rementeria et al.* (2005) "there is no unique essential virulence factor for development of this fungus in the patient and its virulence appears to be under polygenetic control". In another review article of *Abad et al. (*2010) the involvement of phospholipases of *A. fumigatus* as virulence factor is almost excluded: "Although these enzymes [phospholipases] have been considered virulence factors for other species such as *Candida albicans* or *C. neoformans*, in clinical isolates of *A. fumigatus* the production of B-phospholipases is lower than in environmental isolates, making unlikely, if not excluding, their involvement in the virulence of the fungus. PLA2 from *Aspergillus nishimurae* (ex-*fumigatus*) is produced by the transformed strain *Trichoderma reesei* RF8798 under controlled conditions. After use of the enzyme as processing aid in the manufacture of fats and oils, the enzyme is inactivated by heat treatment or even removed. There will be no residual enzyme activity in foodstuffs. Accordingly, there will be no putative virulence activity from this enzyme in food.

Recipient Organism:

The recipient organism used in the construction of the PLA2 production strain is a *T. reesei* host strain, designated RF7720. *T. reesei* RF7720 is a classical low protease mutant derived from the well characterized QM6a strain (Rut series) by conventional mutagenesis (*Nevalainen et al. 1994*). *T.reesei* strains have been cultivated in the production plant of Alko Oy/Roal Oy starting from year 1987 and original parental strain has been used from year 1994.

T. reesei is an aerobic filamentous fungus (an ascomycete). It grows in mycelium form but starts to sporulate when cultivation conditions do not favor growth (e.g. due to lack of nutrients). *T. reesei* is a mesophilic organism which means that it prefers to grow at moderate temperatures. The cultures are typically fast growing at about 30° C (above 20°C and below 37°C). *T. reesei* prefers acidic to neutral pH (about 3.5 to 6) for growth. The colonies are at first transparent or white on agar media such as potato

dextrose agar (PDA). The conidia are typically forming within one week of growth on agar in compact or loose tufts in shades of green. Sporulation is induced by daylight. Yellow pigment may be secreted into the agar by the growing fungal colonies, especially on PDA.

The classification of RF7720 (also referred to as RH32439) as *T. reesei* has been confirmed by the Centraalbureau voor Schimmelcultures, Baarn, Holland (*Appendix #4*).

The taxonomic classification of the *T. reesei* is: Hypocreaceae, Hypocreales, Hypocreomycetidae, Sordariomycetes, Pezizomycotina, Ascomycota, Fungi, according to Index Fungorum database.

AB Enzymes GmbH has been using *T. reesei* as an enzyme producer for many years without any safety problems. A GRAS notice was filed for pectin lyase enzyme preparation produced with *T. reesei* containing a gene from *Aspergillus niger* and FDA had no question and designated it as GRAS Notice No. GRN 000032 (*Appendix #5*). *T. reesei* has a long history (more than 30 years) of safe use in industrial-scale enzyme production (e.g. cellulases and xylanases produced by this fungus are used in food, animal feed, pharmaceutical, textile, detergent, bioethanol and pulp and paper industries). Currently, various *Trichoderma* enzymes are also used in the brewing process (β-glucanases), as macerating enzymes in fruit juice production (pectinases, cellulases, hemicellulases), as a feed additive to livestock (xylanases, endoglucanases) and for pet food processing. *T. reesei* - wild type or genetically modified - is widely accepted as safe production organism for a broad range of food enzymes.

Based on the available data, it is concluded that the organism *T. reesei* is non-pathogenic and non-toxigenic and is safe to use as the production organism for PLA2 from *Aspergillus fumigates/nishimurae* RH3949.

Production Strain:

The production organism, designated strain RF8793 from *T.reesei*, has been genetically modified by insertion of the PLA2 gene from the *Aspergillus fumigatus/nishimurae strain* RH3949 (*Appendix #2*). The

T. reesei strain RF8793 was constructed by transformation of the strain *T. reesei* RF7720 with a purified DNA fragment isolated from the plasmid pAB500-PL3949.

The techniques used in transforming and handling *T. reesei* were as described in *Penttilä et al*. (1987) and *Karhunen et al*. (1993). The production organism also meets the criteria for safe production microorganism as described by *Pariza and Johnson* (2001) (Decision Tree Analysis - *Appendix #6*). *T.reesei* strains are non-pathogenic and non-toxigenic and have been shown not to produce fungal toxins or antibiotics under conditions used for industrial enzyme production. Further they are considered a safe host for other harmless gene products (*Nevalainen et al.,* 1994).

The seed culture for the fermentation is inoculated with spores that have been stored at -80 ^{0}C. No additional growth cycles have been performed after the *T. Reesei* RF8793 strain deposition to the culture collection.

2.2. Genetic Modification

The construction of the phospholipase production stain was achieved by the use of an expression cassette.

PLA2 Expression Cassette

The PLA2 gene was isolated from the *Aspergillus fumigatus/nishimurae* strain RH3949. The phospholipase gene was then inserted into the plasmid pAB500 resulting in the plasmid pAB500-PL3949, in which the phospholipase was placed under the control of the *T. reesei* cbhI promoter and cbhI-terminator. The plasmid pAB500-PL3949 was characterized by restriction endonucleases and the expression cassette was confirmed by DNA sequencing. For the construction of the phospholipase production strain, the plasmid pAB500-PL3949 was digested with NotI and the phospholipase expression cassette was isolated.

The purified expression cassette fragment is free from any harmful sequences and contains the following genetic materials:

- *T. reesei cbh*I/cel7 promoter
- *Aspergillus fumigatus/nishimurae pla gene*
- *T. reesei cbhI/cel7* terminator
- PacI-SacII-BamHI linker
- *Aspergillus nidulans* amdS gene. The gene has been isolated from *Aspergilus nidulans* VH1-TRSX6 . The gene codes for the enzyme acetamidase that enables the strain to grow on acetamide as a sole nitrogen source. This characteristic has been used for selecting the transformants (*Hynes et al* (1983); *Kelly and Hynes* (1985)). The product of the *amd*S gene, acetamidase, can degrade acetamide and is neither harmful or nor dangerous. The *amd*S marker gene has been widely used as selection marker in fungal transformation.

2.3. Stability of the Transformed Genetic Sequence

T. reesei strains are widely used in biotechnological processes because of their known stability. The transformed DNA does not contain any antibiotic resistance genes. Southern blot analysis performed after 10 generations revealed that the *T.*reesei host strain RH7720 and production strain RF8793 stay genetically stable over the time necessary for the industrial fermentation process of the RF8793 production strain.

The fermentation process starts always from the identical replica of the RF8793 seed ampoule. Production preserves at -80°C ("Working Cell Bank") are prepared from the "Master Cell Bank" (culture collection maintained at -150°C) in the following manner: A Petri dish is inoculated from the culture collection preserve in such a way that single colonies can be selected upon germination. Altogether 30 individual colonies are inoculated into shake flasks. The shake flasks constitute the culture stage.

From each shake flask, one oblique tube and another shake flask are inoculated. The latter is subjected to a so-called productivity test, i.e. shake flask cultivation being completed; the enzymatic activity is measured, which must correspond to a given value. If this value is not reached, the culture is discarded. This test serves to determine the characteristic metabolic efficiency of each strain, i.e. to establish its

identity. The tubes whose parallel shake flasks show the highest results are then flushed with glycerol solution. The suspensions thus obtained are frozen and stored divided into 0.5 ml aliquots at -80°C (20 productivity preserves each).

The annual production starts from these production preserves. Six of them are thawed for inoculation of six shake flasks and subsequent inoculation of the first process bioreactor is from these flasks.
Mutation frequencies are low and they only occur in the vegetative state during cell division. Owing to the above-described procedure, this vegetative state of the cultures is reduced to an inevitable minimum during production.

Potential changes in the genome of the production strain could theoretically occur during the propagation in the fermentation process. The presence of the expression cassette integrated into the chromosome of the *T. reesei* strain RF8793 was confirmed by Southern blot hybridization. DNA labelling and detection were carried out according to the manufacturer´s protocols (Amersham Gene Images AlkPhos Direct Labelling and Detection system. GE Healthcare). Southern blot analysis showed that the phospholipase gene had successfully integrated into the *T. reesei* chromosome. The absence of the pUC plasmid containing the Ap selectable marker was also confirmed by Southern blot analysis.

Thus, the transferred DNA contains no mobilizable elements, is stably integrated into the *T. reesei* chromosome and is mitotically stable.

2.4. Good Industrial Large Scale Practice (GILSP)

The PLA2 production strain RF8793 complies with all criteria for a genetically modified GILSP organism.

T. reesei is listed as a Class 1 Containment Agent under the National Institute of Health (NIH) Guidelines for Recombinant DNA Molecules since. Class 1 Containment Agents are microorganisms with the lowest safety concern, such as baker's yeast.

As a result, *T. reesei* can be used under the lowest containment level at large scale, GILSP, as defined by Organization of Economic Cooperation and Development (*OECD, 1992*).

The host organism is non-pathogenic, does not produce adventitious agents under the fermentation conditions employed and has an extended history of safe industrial use (see Section 7.1). The host *T. reesei* RF7720 strain was developed by conventional mutagenesis from the wild type strain QM6a strain (Rut series) that has been isolated from soil only at low altitudes and within a narrow belt around the equator (*Kubicek et al. (2008)*). The mycoparasitism-specific genes have been shown to be lost in *T. reesei* (*Kubicek et al. (2011)*).

Overall, industrial microorganisms modified to produce high levels of enzymes, in fermentation conditions (e.g. no competitive microorganisms, optimal nutrients and aeration that are not present in the natural environment) are not expected to have any competitive advantage against other microorganisms in nature, which themselves are well-adapted in their natural environment. The fitness of the industrial strains to survive is very likely reduced by their high performance characteristic: most of the energy is needed for the production of proteins in high amounts. We consider that the colonization capacity of *T. reesei* RF8793 in the environment is rather low because of its adaptation to fermentation conditions.

From the genetic modification performed, there is no reason to believe that the survival of the genetically modified production organism would be different when compared to its ancestor. The DNA insert is fully characterized and is free from known harmful sequences. No antibiotic resistance markers or other heterologous markers are present in the strain.

Therefore, the *T. reesei* RF8793 enzyme production organism is considered to be of low risk and can be produced with minimal controls and containment procedures in large-scale production. This is the concept of Good Industrial Large Scale Practice (GILSP), as endorsed by the OECD. The production organism has been approved by the Finnish competent authorities for large-scale productions, under containment conditions not exceeding the GILSP level of physical containment.

2.5. Absence of the Production Organism in the Product

The down-stream process following the fermentation includes unit operations to separate the production strain. The procedures are executed by trained staff according to documented standard operating procedures complying with the requirements of the quality system.

The PLA2 enzyme produced from *T. reesei* strain RF8793 is recovered from the fermentation broth by a widely used process that results in a cell-free enzyme concentrate. The absence of the production strain is confirmed for every production batch.

2.6. Absence of Transferable rDNA Sequences in the Enzyme Preparation

As described above the expression cassette is well characterized and does not contain any undefined or harmful sequences (see section 2.2). The absence of the pUC plasmid containing the Ap selectable marker in the fungal genome of RF8793 was characterized by Southern blot analysis. No hybridizing band was detected in all samples of host strain and production strain indicating the absence of the pUC-plasmid in the fungal genome. Furthermore, the colonization capacity of the strain in the environment is rather low due to its adaptation to fermentation conditions. Samples of the PLA2 enzyme preparation were tested for the presence of recombinant DNA using highly sensitive and specific PCR techniques. Two primer sets were first tested using pAB500-PL3943 plasmid DNA and *T. reesei* RF8793 genomic DNA as controls. The results indicated that there is no recombinant DNA (rDNA) of the production strain present above the detection limits in the enzyme samples investigated.

2.7. Absence of Antibiotic Genes and Toxic Compounds

As noted above, the transformed DNA does not contain any antibiotic resistance genes. Further, the production of known mycotoxins according to the specifications elaborated by the General Specifications for Enzyme Preparations Used in Food Processing Joint FAO/WHO Expert Committee on Food Additives, Compendium of Food Additive Specifications, FAO Food and Nutrition Paper (Rome, 2006) has been also tested from the fermentation product of the *T. reesei* strain RF8793. Adherence to

specifications of microbial counts is routinely analyzed based on 3 batches[2] (1 concentrate and 2 final products). PLA2 samples (1 concentrate and 2 final products) as produced by the production strain *T. reesei* RF8793 were analyzed and no antibiotic or toxic compounds were detected.

3. ENZYME IDENTITY AND SUBSTANTIAL EQUIVALENCE

3.1. Enzyme Identity

Systematic name	phosphatidylcholine 2-acylhydrolase
Common name	PLA2
Other names	lecithinase A; phosphatidase; phosphatidolipase; PLA2 A
Enzyme Commission No.	3.1.1.4
CAS number	9001-84-7

3.2. Amino Acid Sequence

The PLA2 is a mature protein of 270 amino acids. The amino acid sequence was confirmed by SDS-page, amino-terminal protein sequencing, and MALDI-MS analysis.

3.3. Sequence Comparison to Other Phospholipase Enzymes

The *Aspergillus* PLA2 sequence shows identity of 59% with the *Aspergillus niger* phospholipase (accession no. CAF05457, patent WO03097825), 59% with *Aspergillus niger* phospholipase (accession no. CAB69391, patent WO 9831790), 53% with *Thermomyces lanuginosus* phospholipase (AB Enzymes strain), 34% with *Fusarium oxysporum* lipase/phospholipase (accession no. CAB69359, patent EP0869167), 60% with *Aspergillus foetidus* lysophospholipase (accession no CAB69552, patent EP 0808903), 62% with *Aspergillus japonicus* glyceroglycolipid lipase (accession no AGD01642, patent

[2] Generally speaking, the typical batch sizes range from 1 000 L to 150 000 L and depending on the market demand, the frequency of production of the food enzyme varies from once a week, to once in two years. In this particular case, this enzyme is not frequently produced and does not represent high volume of production. Analysis has been run on only 1 batch at this time.

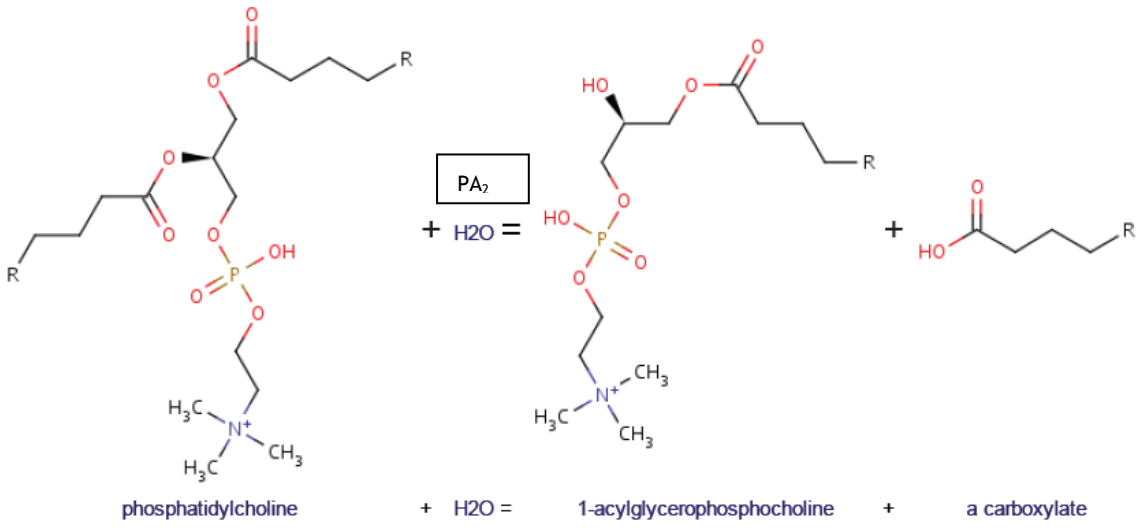
US8338133), 58% with *Aspergillus tubingensis* lipase (accession no.CAB69336, patent WO9845453) and 53% with *T. lanuginosus lipase*, accession no. AAC08588)

3.4. Enzymatic Activity

Phospholipases are a ubiquitous class of enzymes which degrade phospholipids. Phospholipase A1 and A2 hydrolyse the fatty acyl chains of phospholipids at positions sn1 and sn2, respectively, to form lysophospholipids. Phospholipase B can remove both fatty acyl chains simultaneously or it hydrolyses the single remaining fatty acid from lysophopsholipids to produce a glycerophosphoryl compound. Phospholipase C hydrolyses the phosphodiester bond between the glycerol and phosphate groups to form diacylglycerides. Similarly, Phospholipase D hydrolyses the phosphodiester bond between the polar head group and phosphate moiety to form phosphoglycerides.

The phospholipase subject for this dossier is a PLA2, and as such, hydrolyzes phospholipids to form free fatty acids and lysophospholipids. Phospholipids (PLs) are critical constituents of most biological membranes; they are amphipathic in nature. Phospholipases are characterized by a
glycerol backbone to which a polar phosphodiester group is linked at the sn-3 carbon, while the polar head group defines the class of the phospholipase. Further, two fatty acid-derived acyl residues are linked at the sn-1 and sn-2 carbons.

Reaction catalyzed by phospoholipase A2[3]:



PLA2 has an optimum temperature for activity of 50 ^{0}C, a broad pH optimum between 3 - 5 and a very low activity of lipase. The PLA2 enzyme preparation produced by *T. reesei* RF8793 also contains other *T. reesei* enzymes such as endogenous cellulase, xylanase and protease. These enzymes are not used to hydrolyze the phospholipids and therefore do not contribute to the technical effect in the application.

PLA2 activity is represented as PLU, or units of phospholipase, and PLU g^{-1} is the definition for phospholipase activity. 1 Unit of phospholipase corresponds to that amount of enzyme, which releases 1 µmol of fatty acids per minute under prescribed conditions. PLA2 activity is determined using in-house validated methods.

4. MANUFACTURING PROCESS

4.1. Overview

Like all food enzymes, the PLA2 enzyme described in this dossier is manufactured in accordance with current Good Manufacturing Practices for Food (cGMPs) and the principals of Hazard Analysis of Critical

[3] Brenda. The Comprehensive Enzyme Information System. Diagram: Reaction catalyzed by PLA2. Retrieved March 18, 2014, from http://www.brenda-enzymes.org/php/result_flat.php4?ecno=3.1.1.4

Control Points (HACCP). Compliance to Food Hygiene Regulation is regularly controlled by relevant food inspection services in Finland.

The PLA2 described herein is produced by controlled fed-batch submerged fermentation. The production process involves the fermentation process, recovery (downstream processing) and formulation and packaging. Finally, measures are taken to comply with cGMPs and HACCP. A manufacturing flow-chart is given in *Appendix 7*.

It should be noted that the fermentation process of microbial food enzymes is substantially equivalent across the world. This is also true for the recovery process: in a vast majority of cases, the enzyme protein in question is only partially separated from the other organic material present in the food enzyme.

4.2. Fermentation

The production of food enzymes from microbial sources follows the process involving fermentation as described below. Fermentation is a well-known process that occurs in food and has been used for the production of food enzymes for decades. The main fermentation steps are:

- Inoculum
- Seed fermentation
- Main fermentation

4.2.1. Raw materials

The raw materials used in the fermentation and recovery processes are standard ingredients that meet predefined quality standards controlled by Quality Assurance for ROAL OY. The safety is further confirmed by toxicology studies (Section 7.4). The raw materials conform to either specifications set out in the Food Chemical Codex, 6th edition, 2008 or The Council Regulation 93/315/EEC, setting the basic principles of EU legislation on contaminants and food, and Commission Regulation (EC) No 1881/2006 setting maximum limits for certain contaminants in food.

The antifoam agents and flocculants used in the fermentation and recovery processes are used as described in the Enzyme Technical Association submission to FDA on antifoam and flocculants (April 24, 1998, *Appendix 8*). The maximum use levels of antifoam and flocculants are ≤0.15% and ≤1.5% respectively.

4.2.2. Materials used in the fermentation process (inoculum, seed and main fermentation)

- Potable water
- A carbon source
- A nitrogen source
- Salts and minerals
- pH adjustment agents
- Foam control agents

4.2.3. Inoculum

A suspension of a pure culture of *T. reesei* RF8793 is aseptically transferred to a shake flask (1 liter) containing fermentation medium.

In order to have sufficient amount of biomass, the process is repeated several times. When a sufficient amount of biomass is obtained the shake flasks are combined to be used to inoculate the seed fermentor.

4.2.4. Seed fermentation

The inoculum is aseptically transferred to a pilot fermentor and then to the seed fermentor. The seed fermentation is run at a constant temperature and a fixed pH. At the end of fermentation, the inoculum is aseptically transferred to the main fermentation.

4.2.5. Main fermentation

Biosynthesis of the PLA2 enzyme by the production strain occurs during the main fermentation.

The fermentation in the main fermentor is run as normal submerged fed-batch fermentation. The content of the seed fermentor is aseptically transferred to the main fermentor containing fermentation medium.

As in all fed-batch processes, additional fermentation medium is added during the fermentation. In order to control the growth of the production organism and the enzyme production, the feed-rate of this medium is based upon a predetermined profile or on deviation from defined set points for CO2 or dissolved oxygen concentration. In case of PLA2, the carbon source is fed into the fermentor in controlled feed-rate during the fermentation.

The fermentation process is continued for a predetermined time or until laboratory test data show that the desired enzyme production has been obtained or that the rate of enzyme production has decreased below a predetermined production rate. When these conditions are met, the fermentation is completed.

4.3. Recovery

The purpose of the recovery process is:

- to separate the fermentation broth into biomass and fermentation medium containing the desired enzyme protein,
- to concentrate the desired enzyme protein and to improve the ratio enzyme activity/Total Organic Substance (TOS).

During fermentation, the enzyme protein is excreted by the producing microorganism into the fermentation medium. During recovery, the enzyme-containing fermentation medium is separated from the biomass.

This Section first describes the materials used during recovery (downstream processing), followed by a description of the different recovery process steps:

- Pre-treatment
- Primary solid/ liquid separation

- Concentration
- Polish and germ filtration

The nature, number and sequence of the different types of unit operations described below may vary, depending on the specific enzyme production plant.

4.3.1. Materials

Materials used, if necessary, during recovery of the food enzyme include:

- Flocculants
- Filter aids
- pH adjustment agents

Potable water can also be used in addition to the above mentioned materials during recovery.

4.3.2. Pre-Treatment

Flocculants and/or filter aids are added to the fermentation broth, in order to get clear filtrates, and to facilitate the primary solid/liquid separation. Typical amount of filter aids is 2.5 %.

4.3.3. Primary solid/liquid separation

The purpose of the primary separation is to remove the solids from the enzyme containing fermentation medium. The primary separation is performed at a defined pH and a specific temperature range in order to minimize loss of enzyme activity.

The separation process may vary, depending on the specific enzyme production plant. This can be achieved by different operations like centrifugation or filtration.

4.3.4. Concentration

The liquid containing the enzyme protein needs to be concentrated in order to achieve the desired enzyme activity and/or to increase the ratio enzyme activity/TOS before formulation. Temperature and pH are controlled during the concentration step, which is performed until the desired concentration has

been obtained. The filtrate containing the enzyme protein is collected for further recovery and formulation.

4.3.5. Polish and germ filtration

After concentration, for removal of residual cells of the production strain and as a general precaution against microbial contamination, filtration on dedicated germ filters is applied at various stages during the recovery process. Pre-filtration (polish filtration) is included if needed to remove insoluble substances and facilitate the germ filtration. The final polish and germ filtration at the end of the recovery process results in a concentrated enzyme solution free of the production strain and insoluble substances.

4.4. Formulation and Packaging

Following formulation, the final product is defined as a 'food enzyme preparation.' Food enzymes can be sold as dry or liquid preparations, depending on the final application where the enzyme is intended to be used. Typically, food enzyme preparations sold to the oil degumming industry are sold in liquid form. For all kinds of food enzyme preparations, the food enzyme is standardized and preserved with food ingredients or food additives which are approved in the USA according to ruling legal provisions.

PLA2 enzyme preparation from *T. reesei* RF8793 is typically sold as a liquid food enzyme preparation and is thus standardized with food grade ingredients and adjusted to the desired activity. The preparation is in certain cases stabilized with food-grade preservatives.

PLA2 enzyme preparation from *T. reesei* RF8793 is tested by Quality Control for all quality related aspects, like expected enzyme activity and the general testing requirements for Food Enzyme Preparations, and released by Quality Assurance (see section 4.5). The final product is packed in suitable food packaging material before storage. Warehousing and transportation are performed according to specified conditions mentioned on the accordant product label for food enzyme preparations. Labels conform to relevant legislation.

4.5. Quality Control of Finished Product

The final enzyme product complies with the recommended General Specifications for Enzyme Preparations Used in Food Processing Joint FAO/WHO Expert Committee on Food Additives, Compendium of Food Additive Specifications, FAO Food and Nutrition Paper (Rome, 2006) and the Monograph "Enzyme Preparations" Food Chemicals Codex (FCC) 6th edition (2008) for food-grade enzymes. Specifications for the food enzyme preparation have been defined as follows:

Property	Requirement	Testing rate
Total viable counts	< 50000 g^{-1}	every lot
Yeasts and fungi	each < 1000 g^{-1}	every lot
E. coli	not present in 25 g	Semifinals, spot sampling in finals every 10th lot
Salmonella	not present in 25 g	Semifinals, spot sampling in finals every 10th lot
Coliform counts	< 30 g^{-1}	every lot
Arsenic	< 3 ppm	Spot samples from raw materials and semifinals
Lead	< 2 ppm	Spot samples from raw materials and semifinals
Heavy metals	< 30 ppm	Spot samples from raw materials and semifinals
Antibacterial Activity	not detectable	at least 1 lot/y

In addition, the following specifications were set:

Property	Requirement	
Phospholipase Activity (kPLU g^{-1})	min.	10
pH	3.6 – 4.0	
Appearance	Light brown liquid	
Density	1.1-1.2 g/mL	

5. COMPOSITION AND SPECIFICATIONS

5.1. Formulation

The composition of the final enzyme product is as follows:

Composition of Final Product	
Constituent	**%**
PLA2	2-10%
Glycerol	
Sorbitol	
Water	

5.2. General Production Controls and Specifications

In order to comply with cGMPs and HACCP principles for food production, the following potential hazards in food enzyme production are taken into account and controlled during production as described below:

Identity and purity of the producing microorganism:

The assurance that the production microorganism efficiently produces the desired enzyme protein is of utmost importance to the food enzyme producer. Therefore it is essential that the identity and purity of the microorganism is controlled.

Production of the required enzyme protein is based on a well-defined Master (MCB) and Working Cell Bank (WCB). A Cell Bank is a collection of ampoules containing a pure culture. The cell line history and the production of a Cell Bank, propagation, preservation and storage is monitored and controlled. The MCB is prepared from a selected strain. The WCB is derived by sub-culturing of one or more ampoules of the MCB. A WCB is only accepted for production runs if its quality meets the required standards. This

is determined by checking identity, viability, microbial purity and productivity of the WCB. The accepted WCB is used as seed material for the inoculum.

Microbiological hygiene:

For optimal enzyme production, it is important that hygienic conditions are maintained throughout the entire fermentation process. Microbial contamination would immediately result in decreased growth of the production organism, and consequently, in a low yield of the desired enzyme protein, resulting in a rejected product.

Measures utilized by ROAL OY to guarantee microbiological hygiene and prevent contamination with microorganisms ubiquitously present in the environment (water, air, raw materials) are as follows:

- Hygienic design of equipment:
  - all equipment is designed, constructed and used to prevent contamination by foreign micro-organisms
- Cleaning and sterilization:
  - Validated standard cleaning and sterilization procedures of the production area and equipment: all fermentors, vessels and pipelines are washed after use with a CIP-system (Cleaning in Place), where hot caustic soda and nitric acid are used as cleaning agents. After cleaning, the vessels are inspected manually; all valves and connections not in use for the fermentation are sealed by steam at more than 120°C; critical parts of down-stream equipment are sanitized with disinfectants approved for food industry
- Sterilization of all fermentation media:
  - all the media are sterilized with steam injection in fermentors or media tanks (at 121°C for at least 20 min at pH 4.3 – 4.8.).
- Use of sterile air for aeration of the fermentors:
  - Air and ammonia water are sterilized with filtration (by passing a sterile filter).
- Hygienic processing:
  - Aseptical transfer of the content of the WCB ampoule, inoculum flask or seed fermentor

  - Maintaining a positive pressure in the fermentor
- Germ filtration

In parallel, hygienic conditions in production are furthermore ensured by:

- Training of staff:
  - all the procedures are executed by trained staff according to documented procedures complying with the requirements of the quality system.
- Procedures for the control of personal hygiene
- pest control
- Inspection and release by independent quality organization according to version-controlled specifications
- Procedures for cleaning of equipment including procedures for check of cleaning efficiency (inspections, flush water samples etc.) and master cleaning schedules for the areas where production take place
- Procedures for identification and implementation of applicable legal requirements
- Control of labelling
- Requirements to storage and transportation

Chemical contaminants:

It is also important that the raw materials used during fermentation are of suitable quality and do not contain contaminants which might affect the product safety of the food enzyme and/or the optimal growth of the production organism and thus enzyme yield.

It is ensured that all raw materials used in production of food enzymes are of food grade quality or have been assessed to be fit for their intended use and comply with agreed specifications.

In addition to these control measures in-process testing and monitoring is performed to guarantee an optimal and efficient enzyme production process and a high quality product (cGMPs). The whole

process is controlled with a computer control system which reduces the probability of human errors in critical process steps.

These in-process controls comprise:

Microbial controls:

Absence of significant microbial contamination is analyzed by microscopy or plate counts before inoculation of both the seed and main fermentation and at regular intervals and at critical process steps during fermentation and recovery.

Monitoring of fermentation parameters may include:

- pH
- Temperature
- Dissolved oxygen content
- CO2

The measured values of these parameters are constantly monitored during the fermentation process. The values indicate whether sufficient biomass or enzyme protein has been developed and the fermentation process evolves according to plan.

Deviations from the pre-defined values lead to adjustment, ensuring an optimal and consistent process.

Enzyme activity and other relevant analyses (like dry matter, refraction index or viscosity):

This is monitored at regular intervals and at critical steps during the whole food enzyme production process.

6. APPLICATION

6.1. Mode of Action

Like any other enzyme, PLA2 acts as a biocatalyst: with the help of the enzyme, a certain substrate is converted into a certain reaction product. It is not the food enzyme itself, but the result of this conversion that determines the effect in the food or food ingredient. After the conversion has taken place, the enzyme no longer performs a technological function.

6.2. Application

PLA2 is used in the enzymatic conversion of phospholipids to stable lysophospholipids during processing of animal or vegetable based foods and food ingredients that naturally contain phospholipids. This technique is useful for both crude and crude degummed oils (oils that were previously water degummed).

It is important to note that Röhm GmbH founded in 1907 (now called AB Enzymes), jointly held the first patent on enzymatic oil degumming called "EnzyMax®" (*Aarlus et al*., 1993) using phospholipase A1, A2 or B from porcine pancreas to reduce the amount of phosphorus-containing components in edible oil and then separating the aqueous phase from the treated oil. The final level of phosphorus in the oil is used to assess the efficiency of removal of metals, but also on the specificity of the enzyme and the conversion of the phospholipids to lysopholipids, fatty acids and diglycerides.

Fats and Oils Processing

Enzymatic Oil Degumming:

Crude vegetable oil extracted from soy, rape seed, sunflower or rice bran contains impurities. They consist of phospholipids (gums), colours, free fatty acid, odouring and flavouring substances, S-compounds and metal ions. If these impurities are not removed before the oil is exposed to the high temperatures used in deodorization quality problems can well emerge. Colour, taste and stability of the oil would be affected as well due to the hydrolytic and oxidative modification of lipids during storage. The degumming process to remove phospholipids is the most important step in the oil refining process.

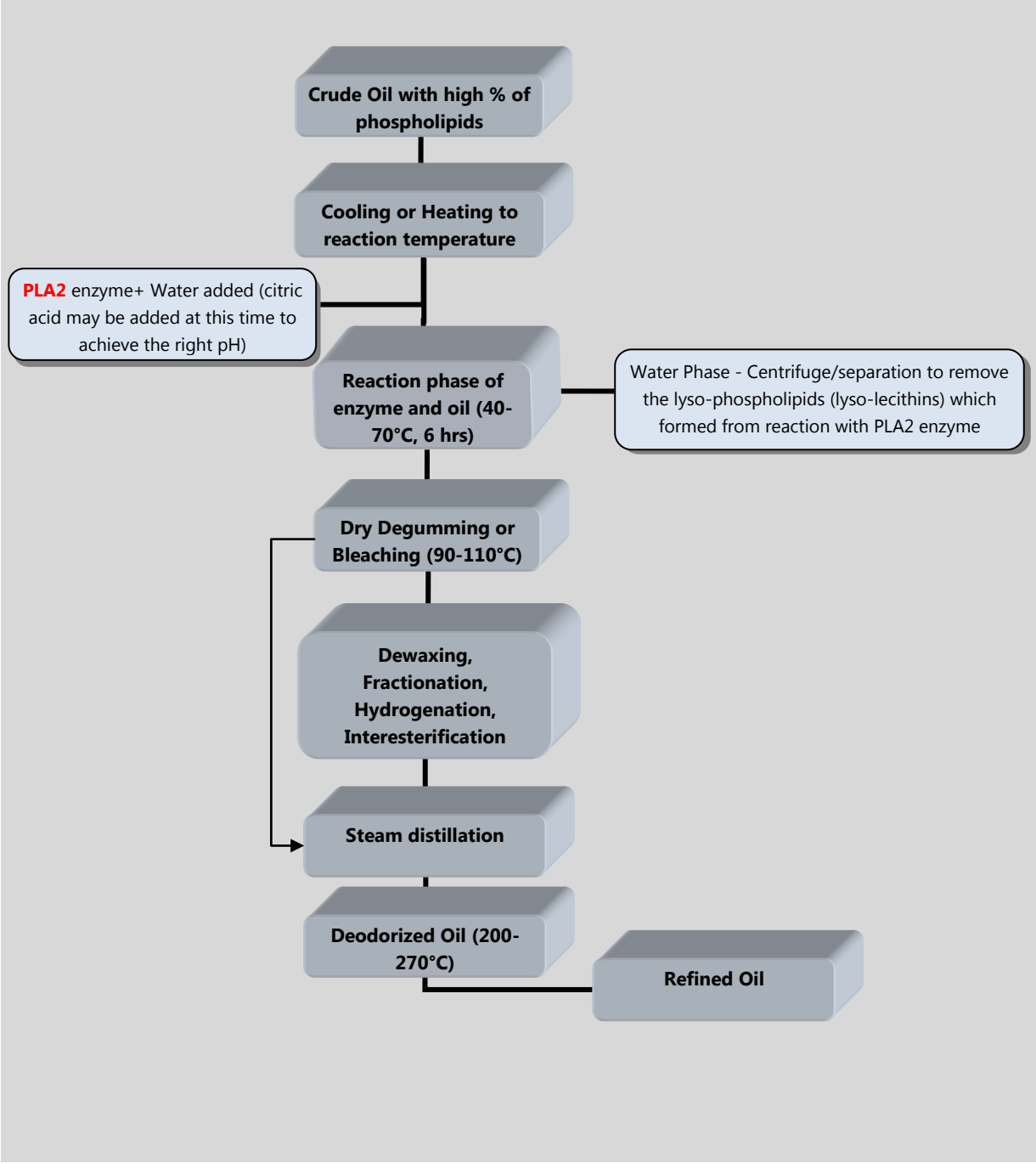
The two major oil refining processes are *chemical* refining and *physical* refining[4]. The refining method of choice is determined based upon the characteristics of the oil. The main purpose of *chemical* refining is to saponify the phospholipids and impurities by an alkaline solution and dilute the resulting soaps in a water phase. These soaps are removed by separators. Chemical refining of oil uses about 10-15% of water to total crude oil and sulphuric acid for neutralization of fatty acids, the resultant waste water (soap stock) is highly acidic. It is during the separation of the soap stock that results in high losses of oil and subsequently oil yield. Furthermore, the soap stock needs to be treated before disposal; this involves the use of harsh chemicals with a potentially negative impact on the environment (*Yang et al., 2005*). *Physical* refining is able to remove any free fatty acids and other impurities by steam stripping, which can totally eliminate the use of acids and keeps neutral oil loss to a minimum. However, to achieve this, pre-treatment (degumming) is required to remove impurities (such as phospholipids) that cause quality issues. In traditional physical crude oil refining, phospholipid removal is normally accomplished by water degumming as one of the first steps in the process, in addition to acid treatment in certain types of oils being refined. Water added to the oil causes hydration of the phosphate ester (phospholipids are comprised of diacylglycerol coupled to a phosphate ester). The most hydratable phospholipid, phosphatidylcholine, acts as a strong emulsifier and causes great loses in oil yield due to residual phospholipids left in the oil after water degumming.

As an efficient alternative, PLA2 can be used for the enzymatic hydrolysis of phospholipids (see diagram below) as compared to traditional degumming methods as the enzyme acts directly on phospholipids, leaving similar molecules untouched. PLA2 is able to break the phospholipid molecule into oil and water soluble fragments. The formed lysophosholipids from enzymatic hydrolysis can easily be separated with the water phase from the oil due to its poor emulsifying properties. Constant phosphorus contents < 10 ppm can be achieved after centrifugation with PLA2. The enzymatic degumming process has 3 important steps; adjustment of the pH by a buffering agent, enzymatic reaction in the production tanks, and separation of the sludge from the oil (*see diagram below*).

[4] Degumming is optional in chemical refining, but critical in physical refining of oils.

Advantages of using PLA2 in oil degumming are an increase in oil yield and efficiency, effective removal of most of the phospholipids from crude oil thereby reducing the consumption of excess caustic acid over the calculated amount during the subsequent refining operation and minimizing refining loss (*Mukherjee et al., 2013*). Furthermore, low and constant gum contents, typically below 10 ppm, can be achieved after centrifugation. Finally the PLA2 enzyme is highly specific, small quantities of the enzyme can be utilized, and enzyme reactions are carried out under mild conditions.

Enzyme Degumming Process Flow in Physical Vegetable Oil Refining:



Plant Lecithin Production/Modification

Plant (lyso-) lecithin is a by-product in the refining/degumming of vegetable oils (see diagram above) and form a portion of the lysophosopholipids. During the degumming process of vegetable oils, the oil

is heated to approximately 70 °C and mixed with water. The liquid is then stirred to ensure that the water hydrates the polar lipids in the oil, making them insoluble. The lyso-lecithin and other lysophospholipids are separated from the mixture by centrifugation. Modified lecithin can be used in a variety of food products, such as a viscosity agent in bakery products, an emsulfying agent in diary, fats and beverage products and a flavouring agent in various types of foods.

Egg Processing

Egg Yolk Processing:

Hen's egg yolk is a widely used and effective food emulsifier, and is used predominantly in the preparation of mayonnaise, sauces, and salad dressings. The lipid fraction of egg yolk contains mainly lecithin (approximately 70%), which is a major component of the phospholipid portion of cell membranes. The amphiphilic nature of lecithin enables its use as an effective and natural emulsifier. The main components of egg-yolk lecithin are phosphatidylcholine (PC, 80.5%) and 4-phosphatidylethanolamine (PE, 11.7%), along with other minor lipids. To modify the performance of egg yolk for suitability in different food technical applications, enzymatic hydrolysis can be utilized. PLA2 is able to remove one fatty acid at the C2 position from the lecithin molecule, resulting in a stable lysolecithin (along with free fatty acids and proteins), which has been shown to have improved emulsion properties and heat stability than untreated egg yolk (*Kim et al., 1997 and 2009*).

Furthermore, egg yolk lecithin can be chemically or mechanically extracted from the egg yolk and then further processed with PLA2 for other food uses. Modified lecithins have many uses in foods including, but not limited to, bakery, confectionary, dairy foods, fats, and beverage products. In these products, the modified lecithin can act as an emulsifying agent, a mixing/blending aid, a releasing agent, an egg replacer, and as a flavor in food systems.

6.3. Use Levels

Dosage shall be determined by means of tests in real processing conditions by manufacturers since many factors can influence the performance of the enzyme including the raw materials variations, temperature, pH, reaction time, and equipment etc and thus follow the *Quantum Satis (*QS) principle (at

a level not higher than the necessary dosage to achieve the desired enzymatic reaction). Dosage of a food enzyme is dependent on the enzyme activity present in the final enzyme preparation. Normally use levels of enzymes are expressed in enzyme activity units/mg TOS (total organic solids).

The presence of all organic materials in the enzyme preparation is expressed as Total Organic Solids[5]. This distinguishes the proportion of the enzyme preparation derived from the source material from that contributed by diluents, and other additives and ingredients.

The use levels for PLA2 from *T.reesei* RF8793 are expressed in mg TOS/kg raw material using the following values:

- TOS = 3.5%
- PLA2 enzyme concentrate has an activity of 1,177 PLU/g
- Final enzyme preparation has a minimum activity of 10,000 PLU/g

Based on the above values, the amount of enzyme concentrate required to achieve a final enzyme preparation activity of 10,000 PLU/g is calculated to be 8.5 mg TOS/g enzyme preparation.

Recommended use Levels:

- Oil refining/Lecithin Modification: Maximal use is 0.5 mg TOS/kg raw material
- Egg/Lecithin Processing: Maximal use is 6 mg TOS/kg raw material

6.4. Enzyme Residues in the Final Food

6.4.1. Residues of Inactive Enzyme in Various Applications

In general, enzymes perform their technological function during food processing. Like the endogenous enzymes present in food, they do not perform any technological function in the final food. The reasons why the enzymes do not typically exert enzymatic activity in the final food could be due to a combination of various factors, depending on the application and the process conditions used by the

[5] TOS: *% TOS = 100-(ash+moisture+diluents)*

individual food producer. These factors include depletion of the substrate, denaturation of the enzyme during processing, lack of water activity, inadequate pH, etc. In some cases (e.g. after alcohol distillation, products that result from processing of starch, and oil degumming), the enzymes may no longer be present in the final food.

In the production of high-quality edible oils, where the PLA2 is mainly intended to be used, the refining process comprises the steps of degumming, bleaching and deodorization. After hydrolysis of phospholipids by PLA2, the lysophospholipid fractions dissolve in aqueous phase and the fatty acids produced remain in the oil phase, i.e – degumming step. This aqueous phase will contain PLA2 protein, either inactive or not functional due to depletion of the substrate, and subsequence reaction products. All PLA2 protein is expected to remain only in the water phase. After centrifugation the extracted lyso-lecithins are dried with temperatures above 80°C, which will further denature any potential active enzymes that are left over.

The next step following the degumming step is a bleaching step typically with bleaching earth, a strong absorbent which would remove proteins in case they are not removed by the previous centrifugation step.

Finally, a distilling step is carried out in order to remove free fatty acids: this step is performed at a temperature above 190°C, inactivating all potentially remaining protein.

In egg processing PLA2 is added prior to pasteuratization and drying. Once the enzyme has been mixed with the egg yolk/lecithin, the mixture is pasteurized for a minimum of 3-5 minutes at 60°C and then rapidly cooled to below 7°C prior to spray-drying. Spray drying conditions are chosen to retain as much of the functional properties of the egg yolk as possible, typical conditions are:

— Air inlet temperature: 200 °C

— Air outlet temperature: 80 °C

— Feed temperature: > 10 °C

These processes will lead to the inactivation of the enzyme. Therefore, it can be assured based on the current evidence, that the above aforementioned processes (see section 6.2) results in the removal or inactivation of the food enzyme product during processing, with no resultant PLA2 activity present in the final food product.

6.4.2. Possible Effects on Nutrients

As the catalytic activity of the enzyme preparation is very specific, i.e. hydrolysis of the sn-2 ester bond between a fatty acid and glycerol in phospholipids, it is not to be expected that the enzyme preparation will have any effect on other constituents or nutrients in food.

7. SAFETY EVALUATION

7.1. Safety of the Production Strain

The insertion of the expression cassette into the genome of the recipient strain *T. reesei* RF7720, results in the recombinant *T. reesei* strain RF8793. The production strain only differs from its recipient strain by its production of phospholipase due to expression of the PLA2 gene from *A. fumigatus/nishimurae*.

T. reesei is an industrially important filamentous fungus and has been used as producer of different hydrolases such as xylanase and cellulase for food, animal feed, and pulp and paper industries. It is also used as host for production of heterologous proteins in the same areas. Like many other organisms with a long safe history of industrial use, *T. reesei* strains have been and are being used by many commercial companies in the construction of production strains by genetic engineering.

Trichoderma are metabolically versatile aerobic mesophilic imperfect fungi and are common in soil in all climate zones (*Nevalainen et al. (*1994*)*). According to *Kuhls et al*. (1996), *T. reesei* is a clonal, asexual derivative of the ascomycete *Hypocrea jecorina* and can be identified by PCR-fingerprinting assay and sequence analyses of the nuclear ribosomal DNA region containing the internal transcribed spacers (ITS-1 and ITS-2) and the 5.8S rRNA gene (*Kuhls et al. (*1996*)*).

T. reesei is classified as a Biosafety Level 1 (BSL-1) microorganism by the American Type Culture Collection (ATCC) based on assessment of the potential risk using U.S. Public Health Service guidelines. BSL-1 microorganisms are not known to cause disease in healthy adult humans. *T. reesei* is regarded as non-pathogenic and non-toxigenic. The safety of this organism as an enzyme producer has been reviewed by *Nevalainen et al.* (1994), *Blumenthal* (2004), and *Olempska-Beer* (2006).

The transformed expression cassette, fully characterized and free from potential hazards, is stably integrated into the fungal genome (see section 2.3) and is no more susceptible to any further natural mutations than any other genes in the fungal genome. Also, the transformation does not increase the natural mutation frequency. If there were any mutations in the genes affecting the relevant characteristics of the fungus, this would likely be noticed in the growth characteristics in the fermentation and/or in the product obtained, and no such changes have been observed. The possibility of mutations is further decreased by inoculating the seed culture for the fermentation with controlled spore stocks that have been stored at -80°C. There is no indication that this genetic modification will have a negative effect on the safety properties. Therefore, it can be concluded that the *T. reesei* strain RF8793 can be regarded as safe as the recipient or the parental organism to be used for production of enzymes for food and feed processing.

7.2. Safety of the PLA2 Enzyme

This enzyme hydrolyzes the phospholipids to form fatty acid and lysophospholipid. It has an optimum temperature for activity of 50°C, a broad pH optimum between 3- 5 and a very low activity of lipase. These properties are very useful for enzymatic degumming of vegetable oils.

The PLA2 enzyme preparation produced by *T. reesei* RF8793 also contains other *T. reesei* enzymes such as endogenous cellulase, xylanase and protease at very low levels. These enzymes are not used to hydrolyze the phospholipids and therefore do not contribute to the required effect in the application.

PLA2 PL3949 described in this application was isolated from an environmental isolate strain *A. nishimurae* RH3949.

A. nishimurae is a ubiquitous filamentous fungus which plays an important role under natural conditions in the aerobic decomposition of organic materials. *A. nishimurae* secretes multiple extracellular phospholipases, including PLA, PLB, PLC and PLD.

A. nishimurae RH3949 is an environmental isolate. *Birch et al*. (2004) suggested, "Phospholipid acyl hydrolase activity for the environmental isolates may be more important for growth in the environmental than it is for clinical isolates growing in the body". According to *Rementeria et al. (*2005*)*, "there is no unique essential virulence factor for development of this fungus in the patient and its virulence appears to be under polygenetic control". While phospholipases have been considered as virulence factors in *C. albicans* or *C. neoformans*, "in clinical isolates of *A. fumigatus* the production of phospholipases is lower than environmental isolates, making unlikely, if not excluding, their involvement in the virulence of the fungus" (*Abad et al. (*2010)).

Furthermore, a wide range of food enzymes, including phospholipases, have been on the market for decades and have been approved on the market for use in food on the basis of safety documentation. Phospholipases are used to make emulsifier-like products such as lysolecithin and monoglyceride. Phospholipase is used to improve the yield in cheese production. In bread making phospholipases are used to improve bread volume and dough stability. For degumming of vegetable oils PLA2s are used in the refining process that influences the stability of refined oils (*De Maria et al*. (2007)).

The use of PLA2 as a food processing aid has been approved in Australia and New Zealand isolated from *S. violaceoruber* (*Appendix #9*), and isolated from porcine pancreas expressed in *Aspergillus niger* (*Appendix #10*). Canada has approved the use of phospholipases as a processing aid expressed in *Steptomyces violaceoruber* (used in modified lecithin, unstandardized egg products)*, Aspergillus oryzae* (used in cheddar cheese)*, and Aspergillus niger* (used in bread, unstandardized bakery products, unstandardized egg whole and yolk, and modified lecithin). Phospholipase has also been approved as a

processing aid expressed in *Aspergillus oryzae* (GRN 142), *Streptomyces violaceoruber* (GRN 145), *Aspergillus niger* (GRN 183), and *Pichia pastoris* (GRN 204).

Finally phospholipases are natural constituents of foods: phospholipase is a normal constituent of wheat flour (*Verlotta et al*. (2013)), and is one of the digestive enzymes present in the pancreatic juice of mammals, including humans (*de Haas et al. (*1968); *Rossiter*, (1968); *Johnson and McDermott*, (1974)).

Based on these points and the long history of safe use of *T. reesei* strains in the production of food enzymes it is concluded that PLA2 from production strain *T. reesei* RF8793 is safe for use as a food processing aid.

7.2.1. Allergenicity

Virtually all food allergens are proteins, although only a small percentage of proteins are allergens. Any food containing protein has the potential to cause allergic reactions, however a few food groups are known to cause allergies more frequently than others. These major allergenic food groups are: milk, eggs, fish, crustacea (shrimp, lobster, and crab), soybeans, peanuts, tree nuts and wheat. Allergens from these food groups account for more than 90% of food allergic reactions. The prevalence of allergic sensitivities to specific foods varies between countries, depending on the frequency with which the foods are consumed and the age at which it is introduction into the diet. Although no general characteristics can be defined that make a protein an allergen, size and structure, glycosylation, solubility, resistance to heat and sensitivity to enzymatic and acidic degradation are believed to play a role. Most food allergens, perhaps especially those that do cause systemic effects, are resistant to digestion, proteolysis, and other forms of hydrolysis.

To evaluate the potential allergenicity of the PLA2 enzyme from *T. reesei* RF8793, the sequence comparison with known allergenic proteins was determined using a database of allergens from the Food Allergy Research and Resource Program (FARRP), University of Nebraska, Allergen Database (Version 12, February 7, 2012), which contains the amino acid sequences of known and putative allergenic proteins. The analyses were performed using the full-length alignment program, by searching

for 80 amino acid alignment program to find identities greater than 35% and by searching for a stretch of eight amino acids program.

The resulting alignment of the full-length PLA2 protein sequence to any allergenic proteins in Allergen Database showed no matches greater than 34% identity. According to *Aalberse R.C.* (2000) "cross-reactivity is rare below 50%. In most situations, cross-reactivity requires more than 70% identity".

In addition, the PLA2 protein sequence showed no similarity to the known allergens by searching for 80 amino acid alignments to find identities greater than 35% or by searching for a stretch of eight amino acids.

Therefore, it is concluded the PLA2 enzyme from *T. reesei* expressing the gene encoding PLA2 from *A. nishimurae/A. fumigatus* is considered non-allergenic.

7.2.2. Leading Publications on the Safety of Phospholipase Enzymes or Enzymes that are Closely Related

The production organism *T. reesei* has been demonstrated to be non-toxigenic and non-pathogenic and any food ingredient (enzyme) from that organism will exhibit the same safety properties if manufactured under current Good Manufacturing Practices ("cGMPs"). *Pariza and Foster* (1983) noted that a non-pathogenic organism was very unlikely to produce a disease under ordinary circumstances. In their publication, *T. reesei* is included in the authors' listing of the organisms being used in the industry. The evaluation of the safety of the genetic modification should be examined based on the concepts outlined in the *Pariza and Foster* (1983) paper. Their basic concepts were further developed by the JFBC in 1990, the EU Scientific Committee for Food in 1991, the OECD in 1993, ILSI Europe Novel Food Task Force in 1996 and FAO/WHO in 1996. Basically, the components of these evaluations start with an identified host strain, descriptions of the plasmid used and the source and fraction of the material introduced, and an outline of the genetic construction of the production strain. This information is found in Section 2.

The FDA has also accepted the GRAS Notifications stating that pectinlyase (GRN 32), chymosine (GRN 230), transglucosidase (GRN 315), protease (GRN 333), glucoamylase (GRN 372) enzyme preparations from *T. reesei* are generally recognized as safe. *T. reesei* is listed as a production organism for enzymes (*Pariza and Johnson* (2001)) and has a long history of safe use (also see Section 7.1).

As is clear from the information provided in this notification, there have been genetic modifications to the *T. reesei* used by AB enzymes, but these genetic modifications are thoroughly well characterized and specific in that the DNA encoded does not express any harmful or toxic substance. The safety studies described in Section 7.4 of this dossier support the fact that the genetic modification did not result in any toxic effects.

7.2.3. Substantial Equivalence

The substantial equivalence is determined by comparing the structure of enzymes**,** the enzyme activities and intended uses, the production organism and production process. Lipases and phospholipases type A are triacylglycerol hydrolyzing enzymes and belong to the members of carboxylic ester hydrolases (EC 3.1.1), which catalyze the hydrolysis of the carboxylic ester bonds of triglycerides or phosphoglyceride lipids to release carboxylic acids. Lipases and phospholipases are enzymes which have been used widely in food industry. Several lipases and phospholipases were notified as GRAS for use in baking applications, in the production of cheese, in egg processing, and for enzymatic degumming of vegetable oils.

Some lipases also have phospholipase activity. *Thermomyces lanuginosus* lipase (accession no. AAC08588) has no phospholipase activity; however, it can be modified for example by changing several amino acids near the active site to have activity towards phospholipids. The new lipolytic enzyme contains the amino acid residues 1-284 originated from the N-terminal region of *T. lanuginosus* lipase with three amino acids substitutions ($G^{113}A$, $D^{118}W$ and $E^{121}K$) and the catalytic triad consisting of Ser^{167}, His^{280} and Asp^{223}. The C- terminal 7 residues of the *T. lanuginosus* lipase were substituted by 55 residues of the C-terminal of the *Fusarium oxysporum* lipase. This lipolytic enzyme with both lipase and phospholipase activities is a commercial enzyme preparation from Novozymes (Lecitase Ultra) used for

enzymatic degumming of vegetable oils and is the subject of GRAS notification GRN No. 103 to which FDA has no question.

The PLA2 enzyme from *T. reesei* RF 8793 is 53% homologous to the *T. lanuginosus* lipase as well as to the enzyme core (modified *T. lanuginosus* lipase sequence) of the new lipolytic enzyme (Lecitase Ultra). Generally a sequence homology greater than 50% would indicate a similar structure. The additional sequence alignment shows the pentapeptide consensus Gly-X-Ser-X-Gly sequence and the catalytic residues Asp and His are well conserved in all enzymes.

PLA2 is substantially equivalent to the lipolytic enzyme from *T. lanuginosus* (Lecitase Ultra) based on the sequence homology and structure similarity, the functionally enzymatic equivalence and intended uses and the safety of the production strain. It can be conclude that the PLA2 enzyme preparation from *T. reesei* RF8793 is safe.

7.3. Safety of the Manufacturing Process

PLA2 enzyme preparation from *T. reesei* RF8793 meets the general and additional requirements for enzyme preparations as outlined in the monograph on Enzyme Preparations in the Food Chemicals Codex.

As described in Section 4, the PLA2 preparation is produced in accordance with cGMPs using ingredients that are acceptable for general use in foods, under conditions that ensure a controlled fermentation. These methods are based on generally available and accepted methods used for the production of microbial enzymes.

7.4. Safety Studies

This section describes the studies performed to evaluate the safety of the PLA2 preparation. All safety studies were performed according to internationally accepted guidelines (OECD or FDA) and are in compliance with the principles of Good Laboratory Practice (GLP) according to the FDA/OECD.

7.4.1. Summary of Safety Studies

The following studies were performed with the PLA2 enzyme preparation from *T. reesei* RF8793:

- Ames test - Bacterial reverse mutation test
- In-vitro mammalian chromosome aberration test
- 90-day oral toxicity study in rats

These safety studies were conducted using the dry enzyme concentrate, not with the diluted final product. All studies were conducted using the same production batch, Batch No. LF 10082 B3, which is representative of the commercial product. Dose calculations for the experiments were adjusted to account for TOS.

7.4.2. Results of the Safety Studies

7.4.2.1. Ames Test

The test, based on OECD Guidelines No. 471 (OECD, 2000a), was run at Harlan, Cytotest Cell Research GmbH (Harlan CCR) Rossdorf – Germany, November 25 – December 15, 2010.

This study was performed to investigate the potential of PLA2 enzyme preparation from *T. reesei* RF8793 to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, TA 100, and TA 102.

The assay was performed in two independent experiments both with and without S9 liver microsomal activation. Each concentration, including the controls, was tested in triplicate.

The test item was tested at the following concentrations calculated to the total organic substance (TOS):

Pre-Experiment/Experiment I: 3; 10; 33; 100; 333; 1,000; 2,500; and 5,000 µg/plate

Experiment II: 33; 100; 333; 1,000; 2,500; and 5,000 µg/plate

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation. No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with the phospholipase enzyme preparation at any concentration level, neither in the presence nor absence of metabolic activation (S9 mix). A minor but dose dependent increase in revertant colony numbers was observed following treatment with the phospholipase enzyme preparation in the absence of metabolic activation in strain TA 98 in experiment I and in strain TA 1537 in experiment II. In both strains the threshold of two (TA 98) and three times (TA 1537) the number of the corresponding solvent control was not reached. Therefore this minor increase is judged as biologically irrelevant. Appropriate reference mutagens were used as positive controls. They showed a distinct increase in induced revertant colonies.

These studies show that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frame shifts in the genome of the strains used. Therefore, the PLA2 enzyme preparation from *T. reesei* RF8793 is considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay.

7.4.2.2. Chromosomal Aberration Test

The test, based on OECD Guidelines No. 473 (OECD, 2000b), was run at Harlan, Cytotest Cell Research GmbH (Harlan CCR), Rossdorf – Germany, during 10 November 2010 – 20 January 2011.

This test assesses the potential of the PLA2 enzyme preparation from *T. reesei* RF8793, to induce structural chromosome aberrations in V79 cells of the Chinese hamster *in vitro* in two independent experiments. Phenobarbital/β-naphthoflavone induced rat liver S9 was used as the metabolic activation system.

Two independent experiments were performed. In Experiment I, the exposure period was 4 hours with and without metabolic activation. In Experiment II the exposure period was 18 hours without S9 mix.

The chromosomes were prepared 18 hours after start of treatment with the PLA2. In each experimental group two parallel cultures were set up. At least 100 metaphases per culture were evaluated for structural chromosome aberrations. The highest applied concentration 5495.0 µg/mL (5,000 µg/mL adjusted to TOS) was chosen with respect to the current OECD Guideline 473. Appropriate mutagens were used as positive controls. They induced statistically significant increases ($p < 0.05$) in cells with structural chromosome aberrations

No relevant increase in polyploidy metaphases was found after treatment with the test item as compared to the frequencies of the control cultures. In conclusion, no biologically relevant increases of chromosomal aberrations were observed and the PLA2 enzyme preparation from *T. reesei* RF8793 is classified as non-clastogenic.

Thus, can be stated that during the described mutagenicity test and under the experimental conditions reported, PLA2 enzyme preparation from *T. reesei* RF8793 did not induce gene mutations by base pair changes or frame shifts in the genome of the strains used.

7.4.2.3. 90-Day Sub-Chronic Toxicity Study (Gavage study)

The test was performed according to the following guidelines: OECD No. 408 (OECD, 2000c) at Harlan Laboratories Ltd (Itingen, Switzerland). Report was completed on 28 March 2012.

In this sub-chronic toxicity study, the PLA2 enzyme preparation from *T. reesei* RF8793 was administered daily by oral gavage to SPF-bred Wistar rats of both sexes at dose levels of 100, 300 and 1,000 mg/kg body weight/day for a period of 91/92 days. A control group was treated similarly with the vehicle, bidistilled water, only.

Clinical signs, outside cage observation, food consumption and body weights were recorded periodically during the acclimatization and treatment periods. Functional observational battery, locomotor activity and grip strength were performed during week 13.

At the end of the dosing period, blood samples were withdrawn for hematology and plasma chemistry analyses. Urine samples were collected for urinalyses. All animals were killed, necropsied and examined post mortem. Histological examinations were performed on organs and tissues from all control and high dose animals, and all gross lesions from all animals.

Oral administration of the PLA2 enzyme preparation to Wistar rats at doses of 100, 300 and 1,000 mg/kg/day, for 91/92 days resulted in no test item-related deaths of definitive toxicological relevance, no test item-related clinical signs at daily or weekly observations (weeks 1-12), no effects on the functional observational battery (week 13, including no effects upon grip strength or locomotor activity), no test item-related effects upon mean absolute or relative food consumption, no changes in mean absolute body weight or body weight gain, no ophthalmoscopic findings, no effects upon the hematology, clinical biochemistry or urinalysis parameters, no differences in organs weights and no macroscopic or microscopic findings of toxicological relevance.

Based on the results of this study, *1,000 mg/kg body weight/day* of the PLA2 enzyme preparation from *T. reesei* RF8793 was established as the no-observed effect-level (NOEL) and as the no-observed-adverse-effect-level (NOAEL).

7.5. Estimates of Human Consumption and Safety Margin

7.5.1. Estimate Dietary Exposure

As described herein the PLA2 enzyme preparation from *T. reesei* RF8793 can be used in the manufacturing of a wide variety of crude oils, lecithin production and egg processing. The most appropriate way to estimate the human consumption in the case of food enzymes is using the so-called Budget Method (*Hansen* (1966); *Douglass et al.* (1997)). This method enables one to calculate a Theoretical Maximum Daily Intake (TMDI) based on conservative assumptions regarding physiological requirements for energy from food and the energy density of food rather than on food consumption survey data.

The Budget Method was originally developed for determining food additive use limits and is known to result in conservative estimations of the daily intake.

The Budget Method is based on the following assumed consumption of important foodstuffs and beverages (for less important foodstuffs, e.g. snacks, lower consumption levels are assumed).

Consumption patterns of food and beverages:

Average consumption over the course of a lifetime/kg body weight/day	***Total solid food (kg)***	***Total non-milk beverages (l)***	***Processed food (=50% of total solid food) (kg)***	***Soft drinks (=25% of total beverages) (l)***
	0.025	0.1	0.0125	0.025

In Section 6.3., the recommended use levels of PLA2 from *T. reesei* RF8793 are given, based on the raw materials used in the food processes. For the calculation of the TMDI, the maximum use levels are chosen. Furthermore, the calculation takes into account how much food (or beverage) is obtained per kg raw material and it is assumed that all the TOS will end up in the final product and the wide variety of food products based on edible oils that are available to consumers[6]. It is therefore assumed that refined oils are used in the manufacturing of all solid and liquid foods.

[6] A wide variety of products based on edible oils is available to the consuming public. Salad and cooking oils, salad dressings, mayonnaise, deep frying oils, margarines and spreads, chocolate fats, ice cream fats, bakery fats, confectionery filling and coating fats, vegetable fats for dairy products and fats for infant nutrition are some of the widely available products that are based entirely on fats and oils or contain fat or oil as a principal ingredient. Many of these products also are sold in commercial quantities to food processors, snack food manufacturers, bakeries and restaurants.

Application		Raw material (RM)	Maximal recommended use level (mg TOS/kg RM)	Final food or food ingredient	Ratio RM/final food[7]	Maximal level in final food (mg TOS/kg food)
Solid	Oil degumming	Crude oil	0.5	Refined oil used in solid foods	0.28	0.14
	Egg processing	Egg Yolk	6	Modified lecithin used in solid foods	0.1	0.6
Liquid	Oil degumming	Crude oil	0.5	Refined oil used in liquid foods	0.28	0.14
	Egg processing	Egg yolk	6	Modified lecithin used in liquid foods	0.1	0.6

[7] ***Assumptions behind ratios of raw material to final food:***

Fats and oils processing

Lipids are found in variable proportions in different foods resulting in variability of fat intake within and between populations. There is no recommended dietary intake of Total Fat in the United States (https://fnic.nal.usda.gov/dietary-guidance/dietary-reference-intakes/dri-tables), however the Adverse effects of excessive consumption value of 20-35 (to encompass all ages) was used for the calculation.

The assumption used for calculation of dietary exposure is an average intake of 28 % of the total diet. The corresponding RM/FF ratio will therefore be 0.28 kg fat / kg final food.

Assuming very conservatively that all fats are refined oils, we suggest to use the same RM/FF ratio as being 0.28 kg oil/ kg final food.

Egg processing:

Food ingredients obtained from egg processing are modified egg yolk and modified lecithin. These food ingredients can be used in the making of both solid and liquid final foods. Typical final foods where those ingredients maybe used are sauces, dressings, fine bakery wares, etc.

Egg yolk is typically added at level 5-10 % to foods whereas lecithin is typically added to foods at level 0.5 - 1 %. In respect to dietary exposure calculation for the 'worst case' scenario the corresponding RM/FF ratio is 0.1 kg enzyme modified egg derived ingredient per kg final food is used.

The Total TMDI can be calculated on basis of the **maximal** values found in food multiplied by the average consumption of food /kg body weight/day. Consequently, the Total TMDI will be:

TMDI in food (mg TOS/kg body weight/day)	TMDI in beverages (mg TOS/kg bodyweight/day)	Total TMDI (mg TOS/kg body weight/day)
0.6x0.0125=0.1375	0.6 x 0.025=0.015	**0.0225**[8]

It should be stressed that this Total TMDI is based on very conservative assumptions and represents a highly exaggerated value because of the following reasons:

- It is assumed that ALL solid and liquid foods contain edible oils that have been processed with this enzyme
- It is assumed that ALL producers apply the highest use level per application;
- It is assumed that the amount of TOS does not decrease as a result of the food production process;
- It is assumed that the final food and beverages containing the calculated theoretical amount of TOS is consumed daily over the course of a lifetime;
- Assumptions regarding food (and beverage) intake of the general population are overestimates of the actual average levels (*Douglass et al.* (1997)).

7.5.2 Safety Margin

Summarizing the results obtained from the toxicity studies (see section 7.4) the following conclusions can be drawn:

- No mutagenic or clastogenic activity under the given test conditions were observed;
- The sub-chronic oral toxicity study showed a No Observed Adverse Effect Level (NOAEL) of at least 1,000 mg TOS/kg body weight/day.

[8] Please note that the maximal values of the enzyme found in food and beverages are used to calculate TDMI – in this case, egg processing has the maximal value of 0.6 mg TOS/kg food, as compared to oil degumming with a value of 0.14 mg TOS/kg food. Thus, 0.6 mg TOS/kg food was used to calculate TDMI.

The Margin of Safety (MoS) for human consumption can be calculated by dividing the NOAEL by the Total Theoretical Maximal Daily Intake (TMDI). The Total TMDI of the food enzyme is **0.0225** mg TOS/kg body weight/day. Consequently, the MoS is:

MoS = 1,000/0.0225 = 44,444

As is explained above, the Total TMDI is highly exaggerated. Moreover, the NOAEL was based on the highest dose administered, and is therefore to be considered as a minimum value. Therefore, the actual MoS in practice will be some magnitudes higher. Consequently, there are no safety reasons for laying down maximum levels of use.

8. Conclusion

Results of the toxicity and mutagenicity tests described in Section 7.4 demonstrate the safety of PLA2 preparation from *T. reesei* RF8793, which showed no toxicity or mutagenicity across a variety of test conditions. The data resulting from these studies is consistent with the long history of safe use for *T. reesei* and PLA2 in food processing, and in keeping with the conclusions found in a review of relevant literature. Based upon these factors, as well as upon the limited and well characterized genetic modifications allowing for safe production of the enzyme preparations, it is AB Enzymes' conclusion that PLA2 enzyme preparation from *T. reesei* RF8793 expressing the gene encoding PLA2 from *A. nishimurae/A. fumigauts* is GRAS for the intended conditions of use described herein.

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FOOD STANDARDS Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

3-06
31 May 2006

FINAL ASSESSMENT REPORT

APPLICATION A561

PHOSPHOLIPASE A_1 AS A PROCESSING AID (ENZYME)

For information on matters relating to this Assessment Report or the assessment process generally, please refer to http://www.foodstandards.gov.au/standardsdevelopment/

EXECUTIVE SUMMARY

FSANZ received an Application on 26 April 2005, from Novozymes A/S, to amend Standard 1.3.3 – Processing Aids of the *Australia New Zealand Food Standards Code* (the Code) to approve the use of a new enzyme, phospholipase A_1, as a processing aid. Phospholipase A_1 is produced, using recombinant DNA techniques, from the host *Aspergillus oryzae* containing the gene coding for phospholipase A_1 from *Fusarium venenatum*.

Processing aids are required to undergo a pre-market safety assessment before approval for use in Australia and New Zealand. There is currently no approval for the use of phospholipase A_1, but there is approval for phospholipase A_2. The specific objective is to protect public health and safety by ensuring that phospholipase A_1 from the host micro-organism *A. oryzae* containing the gene coding for phospholipase A_1 from the fungus *F. venenatum* will only be permitted to enter the food supply if it is safe for human consumption.

The main use of phospholipase A_1 would be in the dairy industry for cheese manufacture to improve process efficiencies and cheese yields. The enzyme acts on phospholipids to form a lysophospholipid and a free fatty acid. These reaction products have improved emulsifying properties which produce an approximate 2% increase in cheese yield.

The enzyme preparation meets the international specifications for enzymes, namely the Food Chemicals Codex (5th Edition, 2004) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA), Compendium of Food Additive Specifications, FAO Food and Nutrition Paper 52, Volume 1, Annex 1, Addendum 9, 2001 (General Specifications and Considerations for Enzyme Preparations Used in Food Processing).

Phospholipase A_1 is already approved in Argentina, Germany, Great Britain, Italy, Sweden, Ireland, Egypt, Iran and Turkey. It has been self-affirmed as a Generally Recognized As Safe (GRAS) notification to the US Food and Drug Administration (FDA), GRAS notification GRN 000142 (FDA response letter June 2004). It has been submitted for approval in Denmark and will be submitted in France in the near future.

The safety assessment of phospholipase A_1 from the source *A. oryzae*, containing the gene for phospholipase A_1 isolated from *F. venenatum* concluded that:

- the production organism has a history of safe use as a production strain for food-grade enzyme preparations and has been shown not to produce toxic metabolites;
- the recombinant DNA in the production organism is considered to be stable and poses no safety concern;
- the enzyme preparation complies with international specifications;
- there was no evidence of toxicity in the sub-acute toxicity study or in the sub-chronic toxicity study in rats;
- in a sub-chronic study in rats, the No Observed Effect Level (NOEL) was 575.1 mg Total Organic Solids (TOS)/kg bodyweight (bw) per day. This is equivalent to 10 ml liquid enzyme concentrate (or approximately 110,000 LEU (enzyme activity))/kg bw per day; and
- the enzyme preparation produced no evidence of genotoxic potential in *in vitro* assays.

From the available information, it is concluded that the use of phospholipase A_1 from the source *A. oryzae*, containing the gene for phospholipase A_1 isolated from *F. venenatum* as a processing aid in food would not raise any public health and safety concerns.

The only regulatory options considered were to approve or not approve the use of phospholipase A_1 as a processing aid. Approval of the Application provides advantages to manufacturers of cheese, by improving cheese yields. There should be no added costs to government regulators or consumers.

Public comment on the Initial Assessment Report was sought from 3 August 2005 to 14 September 2005. Seven submissions were received; four supporting the Application, one reserved their opinion until the Draft Assessment, one raising issues which have been addressed in this report and one opposing it. Public comment on the Draft Assessment Report was sought from 7 December 2005 to 1 February 2006. Nine submissions were received, with seven supporting the Application and two opposing it. One issue was raised that has been addressed in this report.

The Final Assessment Report concludes that approval of phospholipase A_1 from the source *A. oryzae*, containing the gene for phospholipase A_1 isolated from *F. venenatum* as a processing aid is technologically justified and does not raise any public health and safety concerns.

Purpose

The purpose of the Application is to seek an amendment to the Code permitting the enzyme, phospholipase A_1 from the source *Aspergillus oryzae*, containing the gene for phospholipase A_1 isolated from *Fusarium venenatum*.

Decision

Approval is given for the enzyme, phospholipase A_1 from the source *Aspergillus oryzae*, containing the gene for phospholipase A_1 isolated from *Fusarium venenatum*. Permission is provided by adding this enzyme into the Table to clause 17 of Standard 1.3.3 – Processing Aids of the Code.

Reasons for Decision

The draft variation to Standard 1.3.3 – Processing Aids, thereby giving approval for the use of phospholipase A_1 from the source *A. oryzae*, containing the gene for phospholipase A_1 isolated from *F. venenatum* as a processing aid is agreed for the following reasons:

- The proposed draft variation to the Code is consistent with the section 10 objectives of the FSANZ Act. In particular, it does not raise any public health and safety concerns, the safety assessment of the enzyme is based on the best available scientific evidence and it helps promote an efficient and internationally competitive food industry.

- Use of the enzyme is technologically justified since it has a role in improving the yield efficiency of cheese manufacture.

- The regulation impact assessment concluded that the benefits of permitting use of the enzyme outweigh any costs associated with its use.

- The most cost-effective means to achieve what the Application seeks, namely permission to use phospholipase A_1 from the source *A. oryzae*, containing the gene for phospholipase A_1 isolated from *F. venenatum* as a processing aid, is a variation to Standard 1.3.3.

Consultation

FSANZ performed two rounds of public comment for this Application. These periods of public comment were on the Initial Assessment Report (which occurred between 3 August and 14 September 2005) and the Draft Assessment Report (between 7 December 2005 and 1 February 2006).

These periods of public comment were to assist FSANZ to complete its assessment of the Application.

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INTRODUCTION

FSANZ received an Application on 26 April 2005, from Novozymes A/S, to amend Standard 1.3.3 – Processing Aids of the Code to approve the use of a new enzyme, phospholipase A_1, as a processing aid. Phospholipase A_1 is produced, using recombinant DNA techniques, from the host micro-organism *A. oryzae* containing the gene coding for phospholipase A_1 from the fungus *F. venenatum*.

The Applicant claims that this new enzyme would be used in the dairy industry for cheese manufacture to improve process efficiencies and cheese yields. The phospholipase A_1 enzyme preparation catalyses the hydrolysis of diacylphospholipids to form a 2-acyl-1-lysophospholipid and a free fatty acid. The modified phospholipids from the milk are claimed to have improved emulsifying properties to keep more of the milk components in cheese and reduce losses into the waste whey stream, improving process efficiencies.

1. Background

1.1 Current Standard

Processing aids are required to undergo a pre-market safety assessment before approval for use. A processing aid is a substance used in the processing of raw materials, foods or ingredients, to fulfil a technological purpose relating to treatment or processing, but does not perform a technological function in the final food.

The Table to clause 17 of Standard 1.3.3 – Processing Aids contains a list of permitted enzymes of microbial origin. There is currently no approval for the use of phospholipase A_1 as a food enzyme in the Code. Phospholipase A_2 has recently been approved as a permitted enzyme of microbial origin and is listed in the Table to clause 17 (Application A501, gazetted in the Code on 16 December 2004).

1.2 Historical Background

The Applicant claims that phospholipase A_1 is found naturally in animal and plant tissues. The major sources in animals are found in the pancreas and the brain[1]. The enzyme selectively acts on the fatty acid in position 1 (sn-1) in phospholipids to cleave a free fatty acid and form a lysophospholipid. The enzyme, and reaction by-products, fatty acids and lysophospholipids, are claimed to be natural components of food and as such have a history of safe use, and are no different to other constituents in food.

Phospholipase A_2 (EC number [3.1.1.4]) is currently approved as an enzyme. It is listed in the Table to clause 15 – Permitted enzymes of animal origin, being sourced from porcine pancreas. It has recently been listed in the Table to clause 17 – Permitted enzymes of microbial origin, being sourced from *Streptomyces violaceoruber*. Phospholipase A_2 is used to hydrolyse lecithin to produce a modified lecithin which has improved emulsifying properties, especially for aqueous systems.

[1] Encyclopedia of Food Sciences and Nutrition, Phospholipids, Second Edition, Academic Press, (2003), 4528-4529.

1.3 Other International Regulatory Standards

The Applicant states that the enzyme can be legally sold in Argentina, Germany, Great Britain, Italy, Sweden, Ireland, Egypt, Iran and Turkey.

The same enzyme from the same Applicant has recently been deemed self-affirmed GRAS in the USA. GRAS notice No. GRN 000142 is a letter of no objection dated June 23 2004 for this enzyme.

It has been submitted for approval in Denmark and will be submitted in France in the near future.

The enzyme preparation is also claimed to comply with the proposed guidelines of the Scientific Committee on Food (SCF) of the European Union for food enzyme preparations.

2. The Issue

Enzymes, which are proposed to be used as processing aids in food manufacture, are required to undergo an appropriate assessment process to ensure they are safe for human consumption and appropriate for their proposed purpose.

A risk assessment is required to assess whether the enzyme is safe for use as a processing aid in food.

An assessment is required to ensure that there is a technological justification for approval of the enzyme.

A microbiological assessment of the nomenclature of the enzyme and source organism is required to ensure that it is consistent with current and appropriate terminology.

Dietary modelling is not required for the use of the enzyme since it will be used as a processing aid and the majority of the enzyme will be removed from the final product, and any remaining enzyme would be metabolised as any other protein.

3. Objectives

In developing or varying a food standard, FSANZ is required by its legislation to meet three primary objectives which are set out in section 10 of the FSANZ Act. These are:

- the protection of public health and safety;
- the provision of adequate information relating to food to enable consumers to make informed choices; and
- the prevention of misleading or deceptive conduct.

In developing and varying standards, FSANZ must also have regard to:

- the need for standards to be based on risk analysis using the best available scientific evidence;
- the promotion of consistency between domestic and international food standards;
- the desirability of an efficient and internationally competitive food industry;
- the promotion of fair trading in food; and
- any written policy guidelines formulated by the Ministerial Council.

The specific objective is to protect public health and safety by ensuring that phospholipase A_1 from the host micro-organism *A. oryzae* containing the gene coding for phospholipase A_1 from the fungus *F. venenatum* will only be permitted to enter the food supply if it is safe for human consumption.

4. Key Assessment Questions

Is phospholipase A_1, sourced from the host micro-organism *A. oryzae* containing the gene coding for phospholipase A_1 from the fungus *F. venenatum* a safe enzyme that can be approved for food use as a processing aid? Is there a specification for the enzyme in one of the references listed in Standard 1.3.4 – Identity and Purity, and does the enzyme meet it?

The Application needs to provide appropriate safety data to allow an independent assessment to be made. This includes information on the source organism and the nature of the genetic modification. In the case of an enzyme it is also warranted to assess whether there are any limitation on the foods for which the enzyme can be used. That is, does the enzyme catalyse any reactions that may produce unsafe by-products in certain foods.

Is there a technological justification for seeking the approval for the enzyme?

Is the source organism nomenclature provided by the Applicant consistent with current practice and is it correct?

RISK ASSESSMENT

5. Risk Assessment Summary

5.1 Safety Assessment

Phospholipase A_1 will be used as a processing aid only, and is not expected to be present in significant quantities in the final food. Any residue would be in the form of inactivated enzyme, which would be metabolised like any other protein. There are no nutritional or dietary implications in approval of the enzyme since there will be no or very little residual inactivated enzyme in the final foods.

The Safety Assessment Report of phospholipase A_1 (**Attachment 2**) concluded that:

- the production organism has a history of safe use as a production strain for food-grade enzyme preparations and has been shown not to produce toxic metabolites;
- the recombinant DNA in the production organism is considered to be stable and poses no safety concern;
- the enzyme preparation complies with international specifications;
- there was no evidence of toxicity in the sub-acute toxicity study or in the sub-chronic toxicity study in rats;
- in a sub-chronic study in rats, the No Observed Effect Level (NOEL) was 575.1 mg Total Organic Solids (TOS)/kg bodyweight (bw) per day. This is equivalent to 10 ml liquid enzyme concentrate (or approximately 110,000 LEU)/kg bw per day; and
- the enzyme preparation produced no evidence of genotoxic potential in *in vitro* assays.

From the available information, it is concluded that the use of this enzyme as a processing aid in food would not raise any public health and safety concerns.

5.2 Source Organism Nomenclature

A search was performed to assess the nomenclature of the source micro-organism (*Aspergillus oryzae* containing a gene encoding for phospholipase A_1 from *Fusarium venenatum*). The conclusion of this search is that the nomenclature of both organisms is correct. It was noted that the FDA GRAS Notice No. GRN 000142 for the same enzyme from Novozymes uses the same nomenclature, that is GRAS status of phospholipase A_1 (PLA1) enzyme preparation from *Aspergillus oryzae* expressing a gene encoding a PLA1 from *Fusarium venenatum*.

5.3 Technological Justification

The Food Technology Report (**Attachment 3**) provides detail about the nature and technological justification for the enzyme.

The Applicant claims that the enzyme preparation is used to improve process efficiencies in cheese manufacture with lower losses of fat and other solids into the whey stream. The phospholipase A_1 enzyme preparation is added to the milk used for cheese manufacture before the coagulant is added. The phospholipids produced after the enzyme treatment have better emulsifying properties compared to untreated milk and as such keep more of the milk components in the cheese with reduced losses to the whey stream. The Applicant claims the cheese yields are increased by approximately 2.0%, without any significant change to the quality or composition of the cheese.

The phospholipase A_1 enzyme preparation is produced by submerged fermentation of the microbial source *A. oryzae* that has the gene coding for phospholipase A_1 from *F. venenatum* inserted by recombinant DNA techniques. The enzyme preparation is manufactured in accordance with Good Manufacturing Practices, using standard enzyme manufacturing practices. The enzyme preparation is stabilised with common approved stabilisers and standardised to company specifications.

It is unlikely that there will be any dietary or nutrition implications resulting from approval of this Application. The enzyme is to be used as a processing aid and the majority of the enzyme will be removed from the final product as part of the process.

Some small proportion of the enzyme may remain in the final product (cheese) but it has no technological function once there is no substrate to act on. Any remaining substrate will be unavailable to react with the enzyme since it will be bound in the resultant solid cheese matrix. Enzymes and their reaction by-products, lysophospholipids and fatty acids are natural components of food.

The Application states that the enzyme preparations meet the international specifications for enzymes contained in the Food Chemical Codex (5th Edition, 2004)[2], and the Joint FAO/WHO Expert Committee on Food Additives (JECFA), in the Compendium of Food Additives Specifications, Vol 1 Annex 1, FAO 1992 (Addendum 9, 2001)[3].

The use of the enzyme phospholipase A_1 from the source *Aspergillus oryzae*, containing the gene for phospholipase A_1 isolated from *Fusarium venenatum*, is technologically justified to improve process efficiencies in cheese manufacture. It achieves this by improving the emulsifying properties of the treated phospholipids of the cheese, keeping more of the milk fat components in the cheese with less lost into the whey stream.

RISK MANAGEMENT

6. Options

FSANZ is required to consider the impact of various regulatory (and non-regulatory) options on all sectors of the community, which includes consumers, food industries and governments in Australia and New Zealand.

The two regulatory options available for this Application are:

Option 1. **Not approve** the use of phospholipase A_1 from *A. oryzae* containing the gene coding for phospholipase A_1 from *F. venenatum* as a processing aid.

Option 2. **Approve** phospholipase A_1 from *A. oryzae* containing the gene coding for phospholipase A_1 from *F. venenatum* as a processing aid.

7. Impact Analysis

7.1 Affected Parties

The affected parties to this Application include the following:

1. those sectors of the food industry wishing to produce and market food products produced using this enzyme, specifically cheese manufacturers;

2. consumers; and

[2] Food Chemicals Codex (2004). National Academy of Sciences, Food and Nutrition Board, Committee on Food Chemical Codex, 5th Edition, National Academy Press, Washington DC.
[3] Joint FAO/WHO Expert Committee on Food Additives (JECFA) (2001). General specifications and considerations for enzyme preparations used in food processing. FAO Food and Nutrition Paper 52, Addendum 9, pp 37-39.

3. Australian, State, Territory and New Zealand Government agencies that enforce food regulations.

7.2 Benefit Cost Analysis

In the course of developing food regulatory measures suitable for adoption in Australia and New Zealand, FSANZ is required to consider the impact of all options on all sectors of the community, including consumers, the food industry and governments.

7.2.1 Option 1 – Not approve the use of the enzyme

There are no perceived benefits to industry, government regulators or consumers if this option is taken.

There are disadvantages to those food industries, specifically cheese manufacturers, if this option is taken, since they will not have an enzyme available to them that may improve their process efficiencies.

7.2.2 Option 2 – Approve the use of the enzyme

There are advantages to manufacturers of cheese as the availability of this enzyme should provide efficiency gains for their manufacturing process which is an economic advantage.

There should be no added costs to government food regulators or consumers.

7.3 Comparison of Options

Option 2 has advantages for manufacturers of cheese, while there are no advantages for option 1.

Option 2, which supports the approval of phospholipase A_1 from *Aspergillus oryzae* containing the gene coding for phospholipase A_1 from *Fusarium venenatum* as a processing aid is the preferred option, since it has advantages for the food industry but has no significant cost for government regulators, consumers or food manufacturers.

COMMUNICATION

8. Communication and Consultation Strategy

This is a routine approval matter. As a result, FSANZ has applied a basic communication strategy to Application A561. This involved advertising the availability of assessment reports for public comment in the national press and making the reports available on the FSANZ website. FSANZ issued a media release drawing journalists' attention to the matter, which received coverage in trade and industry newsletters.

Once the FSANZ Board has approved the Final Assessment Report, FSANZ will notify the Ministerial Council, the Applicant and individuals and organisations who made submissions on this Application. Stakeholders, including the public, will be notified of the gazettal of changes to the Code in the national press and on the website. FSANZ provides an advisory service to the jurisdictions on changes to the Code.

9. Consultation

9.1 Public Consultation

Public comment on the Initial Assessment Report for this Application was sought from 3 August 2005 till 14 September 2005. Seven submissions were received, of which four supported the Application, one reserved comment until the Draft Assessment, one raised issues and one opposed the Application with these issues addressed in section 12.1.1. Public comment on the Draft Assessment Report for this Application was sought from 7 December 2005 till 1 February 2006. Nine submissions were received, of which seven supported the Application and two opposed it. **Attachment 4** summarises the submissions received during both rounds of public comment.

9.2 Issues Arising from Submissions

9.2.1 GM labelling requirements

One submitter questioned whether products containing phospholipase A_1 will be required to be labelled as genetically modified under subclause 4(1) of Standard 1.5.2 – Food Produced Using Gene Technology.

9.2.1.1 FSANZ response

Although the source organism (*A. oryzae*, containing the gene for phospholipase A_1 isolated from *F. venenatum*) is produced using recombinant DNA techniques, the phospholipase A_1 enzyme is not genetically modified. This enzyme has the same amino acid sequence and structure as phospholipase A_1 isolated from *F. venenatum*. Genetically modified micro-organisms are used as sources for enzymes as they are more economic, being able to produce greater quantities of commercial enzymes than can be obtained by other means.

The relevant section of the Code relating to labelling of genetically modified food is contained in Division 2 – Labelling etc of food produced using gene technology of Standard 1.5.2. This requires that processing aids (and food additives) be labelled where novel DNA and/or novel protein from the processing aid or food additive remains present in food to which it has been treated.

In the case of enzymes produced from genetically modified micro-organisms the enzyme is not a novel protein since it is identical to other enzymes sourced from non-genetically derived sources. The refinement process for the enzyme preparation removes all the source organism from the preparation so there is no novel DNA in the enzyme preparation. Therefore small amounts of enzymes (inactivated or not) from a genetically modified source remaining in food do not require labelling under the gene technology labelling requirements. This is the case for all enzymes sourced from a genetically modified micro-organism (of which there are a number approved in the Code).

9.2.2 Safety of phospholipase A_1 from a GM source

A submitter expressed concern that there may be public health and safety issues over the use of recombinant DNA techniques, where there could be known or unknown genetic attachments to the enzyme, or in the final product.

The submitter also considers the labelling required is insufficient for consumers to be able to make informed choices about what food products they buy.

9.2.2.1 FSANZ response

The safety of phospholipase A_1 produced in *A. oryzae* containing the phospholipase A_1 gene from *F. venenatum* has been fully evaluated by FSANZ and is detailed in the Safety Assessment Report (summarised in section 5.2 and in full in Attachment 2). The labelling requirements for food (including processing aids) produced using gene technology are contained in Division 2 of Standard 1.5.2 and have been discussed above. These requirements are among the most stringent labelling requirements in the world and represent a balance between the consumer's right to know and the jurisdictions' ability to enforce.

9.2.3 Approval of phospholipase A_2 before phospholipase A_1

A submitter questioned the approval of phospholipase A_2 (approval gazetted in December 2004) and suggested that if phospholipase A_1 had already been approved at this time, there would be no need for an application to approve phospholipase A_2. The submitter stated that this was misleading and deceptive conduct by the enzyme manufacturers.

9.2.3.1 FSANZ response

There is no intention to mislead or deceive on the part of FSANZ or the enzyme manufacturers by approving phospholipase A_2 before phospholipase A_1 was submitted. They are two different enzymes, submitted by two different companies. They, and any other enzyme Application, are treated on their own merits. Approving one enzyme first does not invalidate the case for another similar enzyme. It is up to the food industry which, of a number of alternatives, suits them for a particular food application. There are a number of considerations, including cost and availability as well as performance which determines which enzyme is suitable for use in different products.

9.2.4 GRAS status of phospholipase A_1 in the USA

A submitter raised the concern that FSANZ may be relying on the GRAS status of phospholipase A_1 in the USA to show that this enzyme is safe for use in food. The submitter also noted that an error was made in the Initial Assessment Report regarding the US FDA GRAS notice of GRN 000054, stating that *F. venenatum* is the host organism in this case, not the donor.

9.2.4.1 FSANZ response

The GRAS status of phospholipase A_1 was provided in the Initial Assessment Report for information on international approvals of this enzyme. In conducting the safety assessment on this enzyme, FSANZ has done an independent analysis of the data supplied by the Applicant, as well as any relevant information from other sources. No weight has been given to the fact that this enzyme is considered GRAS in the USA.

It has been noted that *F. venenatum* is the host and not the donor in the US FDA GRAS notice of GRN 000054.

9.2.5 Production of aflatoxins from the source organism

A submission noted that particular strains of the source organism (*A. oryzae*) have been found to produce aflatoxins. However, as discussed in the safety assessment report (**Attachment 3**), the particular strain used as a source organism (*A. oryzae* PFJo42) does not. Further, the genetic modification to the source organism has removed the genes involves in aflatoxin production and therefore any potential for the strain to revert to producing toxins is removed.

The submitter has requested that this issue be further explained in the Final Assessment Report.

9.2.5.1 FSANZ response

The issue of the production of mycotoxins by the production organisms, *A. oryzae*, was investigated in the Safety Assessment Report (**Attachment 3**). Although some strains of this organism are known to produce toxins, many strains do not. This particular strain, *A. oryzae* PFJo42, has been shown to not produce aflatoxins and lacks the gene cluster involved in aflatoxin biosynthesis.

Phospholipase A_1 from the production organism, *A. oryzae* must comply with the recommended purity specifications for food-grade enzymes[2,3].

It was concluded in the safety assessment that the production strain, *A. oryzae* PFJo42, does not produce secondary metabolites of toxicological concern to humans. Further, *A. oryzae* strains have a history of safe use in the production of food enzymes. The use of *A. oryzae* PFJo42 to produce phospholipase A_1 does not pose any concern to human health and safety.

9.2.6 Lack of consumer choice for food produced using GM technology

One submitter was of the opinion that the current labelling requirements of the Code for food produced using GM technology are not rigorous enough to ensure products such as this enzyme are labelled as genetically modified.

9.2.6.1 FSANZ response

The labelling requirements for genetically modified foods in Standard 1.5.2 of the Code are among the most comprehensive labelling requirements in the world. They were written following extensive public consultation and represent a fair balance between what industry and consumers want and what governments can enforce. They are not there for any safety concern, but rather to allow consumers to purchase or avoid products depending on their own beliefs.

Foods produced using this enzyme will not be required to be labelled as genetically modified.

9.3 World Trade Organization (WTO)

As members of the World Trade Organization (WTO), Australia and New Zealand are obligated to notify WTO member nations where proposed mandatory regulatory measures are inconsistent with any existing or imminent international standards and the proposed measure may have a significant effect on trade.

Amending the Code to approve the enzyme phospholipase A_1 from *A. oryzae* containing the gene coding for phospholipase A_1 from *F. venenatum* as a processing aid is unlikely to have a significant effect on trade. Most countries do not regulate enzymes as processing aids as in Australia and New Zealand. Also since the enzyme is considered a processing aid there is no requirement to label final food for the presence of the enzyme. The enzyme preparation is consistent with the international specifications for food enzymes of Food Chemicals Codex (5th Edition, 2004) and JECFA so it was determined that there was no need to notify the WTO under either the Technical Barrier to Trade (TBT) or Sanitary and Phytosanitary Measure (SPS) Agreements.

CONCLUSION

10. Conclusion and Preferred Option

The Final Assessment Report concludes that the approval of the use of phospholipase A_1 from the source *A. oryzae*, containing the gene for phospholipase A_1 isolated from *F. venenatum* as a processing aid is technologically justified and does not pose a risk to public health and safety.

Approval is given for the enzyme, phospholipase A_1 from the source *A. oryzae*, containing the gene for phospholipase A_1 isolated from *F. venenatum*. Since the source for this enzyme is of microbial origin, approval will be listed in clause 17 – Permitted enzymes of microbial origin. The enzyme name, EC number and source will be listed. The drafting is included in **Attachment 1**.

11. Implementation and Review

If approved it is proposed that the draft variation come into effect on the date of gazettal.

ATTACHMENTS

1. Draft variation to the *Australia New Zealand Food Standards Code*
2. Safety Assessment Report
3. Food Technology Report
4. Summary of Submissions

Attachment 1

Draft variation to the *Australia New Zealand Food Standards Code*

To commence: on gazettal

[1] ***Standard 1.3.3*** *of the Australia New Zealand Food Standards Code is varied by –*

[1.1] *inserting in the* Table to clause 17 –

Phospholipase A_1 EC [3.1.1.32]	*Aspergillus oryzae*, containing the gene for phospholipase A_1 isolated from *Fusarium venenatum*

Attachment 2

Safety Assessment Report

A561 – PHOSPHOLIPASE A_1 AS A PROCESSING AID

Summary and Conclusion

Application A561 seeks approval for the use of phospholipase A_1 from *Aspergillus oryzae* as a processing aid. This strain of *A. oryzae* contains a gene encoding phospholipase A_1 from *Fusarium venenatum*.

The enzyme is used as a processing aid only, and is not expected to be present in the final food. Any residue would be in the form of inactivated enzyme, which would be metabolised like any other protein.

The safety assessment concluded that:

- the production organism has a history of safe use as a production strain for food-grade enzyme preparations and has been shown not to produce toxic metabolites;
- the recombinant DNA in the production organism is considered to be stable and poses no safety concern;
- the enzyme preparation complies with international specifications;
- there was no evidence of toxicity in the sub-acute toxicity study or in the sub-chronic toxicity study in rats;
- In a sub-chronic study in rats, the NOEL was 575.1 mg TOS /kg bw per day. This is equivalent to 10 ml liquid enzyme concentrate (or approximately 110,000 LEU)/kg bw per day; and
- the enzyme preparation produced no evidence of genotoxic potential in *in vitro* assays.

From the available information, it is concluded that the use this enzyme as a processing aid in food would not raise any public health and safety concerns.

1. Introduction

Application A561 seeks approval for the use of the enzyme phospholipase A_1 from Aspergillus oryzae as a processing aid. The production organism, A. oryzae, contains a phospholipase A_1 gene derived from Fusarium venenatum.

Phospholipase A_1 is found in animal and plant tissues. It is also known as phosphatidylcholine 1-acylhydrolase (EC 3.1.1.32, CAS No. 9043-29-2). The phospholipase enzyme preparation produced by the Applicant is called YieldMAX PL and has a typical activity of 2000 Lecitase Units (LEU)/g. Phospholipase A_1 specifically acts on the fatty acid in position 1 in phospholipid substrates resulting in formation of lysophospholipids and free fatty acid. These are natural constituents in food and therefore both the enzyme itself and its reaction products have a history of safe use and are not different from other constituents in the food.

The applicant's intent is for the enzyme preparation to be used as a processing aid in the dairy industry, mainly in the manufacture of cheese. The enzyme not expected to be present in the final food. Any residue would be in the form of inactivated enzyme, which would be metabolised like any other protein.

Information on the production organism, *A. oryzae*, and its potential to produce undesirable metabolites was provided. Information on the introduced DNA and the process by which the recombinant *A. oryzae* strain were produced were also provided.

Two toxicity studies (14-day and 90-day studies) in rats and three *in vitro* mutagenicity studies were submitted by the applicant.

2. Purity of enzyme preparation and proposed specifications

Historically, enzymes used in food processing have been found to be non-toxic, and the main toxicological consideration is in relation to possible contaminants. The production organism in this case is considered to be non-toxic and non-pathogenic. The specifications to which the preparation conforms are shown in Table 1.

Table 1: Complete specification of phospholipase A_1 preparation

Criteria	Specification
Phospholipase A_1 activity (LEU/g)	according to declaration
Total viable count/g	not more than 1 x 10^4
Total coliforms/g	not more than 30
Enteropathogenic E. coli/25 g	negative
Salmonella/25 g	negative
Production strain/g	negative
Heavy Metals	< 30 ppm
Arsenic	< 3 ppm
Lead	< 5 ppm
Mycotoxins	negative

Phospholipase A_1 from the production organism, *A. oryzae* complies with the recommended purity specifications for food-grade enzymes (JECFA, 2001; NAS FNB, 2004).

Phospholipase A_1 produced by *A. oryzae* is approximately 14 kDa. The Applicant states that the n-terminal sequence of this protein was analysed and found to be 100% homologous to the *F. venenatum* phospholipase.

3. The production organism – *Aspergillus oryzae*

The safety of the production organism is an important consideration in the safety assessment for enzymes used as processing aids. The primary issue is the toxigenic potential of the production organism, that is, the possible synthesis by the production strain of toxins, and the potential for the carryover of these into the enzyme preparation (Pariza and Johnson 2001).

A. oryzae is not considered to be a pathogen and has a history of safe use as a production organism for food enzymes and is a permitted source of a number of enzymes in the *Australia New Zealand Food Standards Code*[4].

The strain of *A. oryzae* in which phospholipase A_1 is produced is designated PFJo142. This strain was produced by transformation of the BECh2 strain with the phospholipase A_1 expression plasmid pPFJo142.

BECh2 was produced from strain IFO 4177 (synonym A1560) obtained from the Institute for Fermentation, Osaka (IFO), by a series of gene deletions and mutations to remove the ability to produce unwanted side activities (including amylase and protease activities) and secondary metabolites.

This was done because certain strains of *A. oryzae* may produce one or more of the moderately toxic secondary metabolites cyclopiazonic acid, kojic acid and β-nitropropionic acid (Burdock *et al*., 2001a; Burdock *et al*., 2001b; Blumenthal, 2004). *A. oryzae* strains used in the production of food enzymes need to be routinely screened for the production of cyclopiazonic acid and other undesirable metabolites. Also, *A. oryzae* is closely related to the aflatoxin-producing fungus *Aspergillus flavus*, and contains some genes from the aflatoxin biosynthesis pathway. However, these genes are inactive in *A. oryzae* and it is generally agreed that *A. oryzae* does not produce aflatoxin (Blumenthal 2004).

In BECh2, it has been shown that one arm of a chromosome, containing the genes suspected to be involved in cyclopiazonic acid synthesis and the whole aflatoxin gene cluster has been deleted, making BECh2 unable to produce, or revert to a strain that is capable of producing, these mycotoxins. The synthesis of kojic acid is also impaired in this strain. Although BECh2 has the metabolic pathway necessary to produce β-nitropropionic acid, it appears that it is expressed only very weakly under specific circumstances.

This was verified by testing the ability of *A. oryzae* strains IFO 4177 and BECh2 to produce cyclopiazonic acid, β-nitropropionic acid and kojic acid when grown on optimal media. While strain IFO 4177 produced cyclopiazonic acid, β-nitropropionic acid and traces of kojic acid, only kojic acid was detected from the fermentations with the BECh2 strain, estimated to be present in quantities of only 15% of the level detected in IFO 4177.

Absence of these secondary metabolites under enzyme production conditions was confirmed for the phospholipase A_1 production strain, *A. oryzae* PFJo42. Two batches of phospholipase A_1 were analyzed and the results shown below.

[4] The following enzymes sourced from *A. oryzae* are permitted in the Code: aminopeptidase; α-amylase; carboxyl proteinase; β-glucanase; glucoamylase; a-glucosidase; xylanase; lactase β –galactosidase; triacylglycerol lipase; metalloproteinase; mucorpepsin; pectin methlyesterase; 6-phytase; polygalacturonase; and serine proteinase.

Analysis of Phospholipase A_1 produced in *A. oryzae* PFJo42

	Batch ID: PPW 22837	Batch ID: PPW 23436
Aflatoxin B1 (<0.0004 mg/kg) [a]	ND [b]	ND
Ochratoxin A (<0.001 mg/kg)	ND	ND
Sterigmatocystin (<0.02 mg/kg)	ND	ND
T-2 toxin (<0.02 mg/kg)	ND	ND
Zearalenon (<0.02 mg/kg)	ND	ND
Cyclopiazonic acid (<0.6 mg/kg)	ND	ND
Kojic Acid (<6 mg/kg)	ND	ND
3-Nitropropionic acid (<13 mg/kg)	ND	ND

a) Limit of detection is given in brackets
b) ND = Not Detected

It is concluded that the production strain, *A. oryzae* PFJo42, does not produce secondary metabolites of toxicological concern to humans. Further, *A. oryzae* strains have a history of safe use in the production of food enzymes. The use of *A. oryzae* PFJo42 to produce phospholipase A_1 does not pose any concern to human health and safety.

4. Characterisation of the genetic modification

A. oryzae strain PFJo142 was produced by transformation of BECh2 with the *A. oryzae* expression plasmid pPFJo142. This plasmid contains the phospholipase gene from *F. venenatum* strain CC1-3, a morphological mutant of a wild type isolate of *F. venenatum* Nirenberg sp. nov. *F. venenatum* is a saprophytic fungi found in soil and also found to occur on some plants. The only DNA from *F. venenatum* that has been introduced into *A. oryzae* PHJo142 is the protein coding sequence of the phospholipase gene.

In addition to the phospholipase gene, other genetic information contained on plasmid pPFJo142 to enable the efficient expression of this gene in *A. oryzae* includes:

- the neutral amylase II promoter (NA2) from *Aspergillus niger* strain BO-1;
- the 5' untranslated leader of the triose phosphate isomerase (TPI) gene, which is synthetic and corresponds to the sequence of the *Aspergillus nidulans* TPI gene; and
- the glucoamylase transcriptional terminator from *A. niger* strain BO-1.

In addition to the phospholipase gene and regulatory elements, pPFJo142 contains two marker genes, *amdS* and *URA3*. The acetamidase gene (*amdS*) from *A. nidulans* allows selection in *A. oryzae* as it allows growth on media with acetamide as the sole nitrogen source. The *URA3* gene from *Saccharomyces cerevisiae* allows selection in *E. coli* as it confers uridine prototrophy in auxotrophic *pyrF E. coli*. The *A. oryzae* expression plasmid also contains a bacterial origin of replication (from pUC19).

The gene cassette does not contain any antibiotic resistance marker genes.

A. oryzae PFJo142 was produced by transformation of BECh2 protoplasts with plasmid pPFJo142. Transformants were selected by growing on a medium with acetamide as the sole nitrogen source and screening for co-expression of phospholipase activity. The selected transformant was designated PFJo142 and this is the strain that is used for enzyme production.

Genetic stability

The applicant states that *A. oryzae* PHJo142 is stable during production fermentation, as the inserted DNA is integrated into the chromosome. This was tested after large-scale fermentation. The strain stability during fermentation was analyzed by Southern blotting and no instability of the strain was observed.

5. Evaluation of the safety studies

Bioinformatics analysis and five toxicological studies were submitted in support of this application. These were:

1. an analysis for homology of the phospholipase A_1 protein sequence with known protein toxins and allergens;
2. a 14-day repeat dose oral toxicity study in rats;
3. a 90-day sub-chronic oral toxicity study in rats;
4. a Salmonella/*E. coli* Reverse Mutation Assay (Ames test);
5. a human lymphocyte assay; and
6. an *in vitro* cytotoxicity test.

5.1 Potential toxicity and allergenicity of phospholipase A_1

The *F. venenatum* phospholipase A_1 protein sequence was compared to the sequences of known toxins and allergens to assess if there was any significant sequence homology.

No significant homology to any toxin sequence was found. No matches greater than 5 contiguous residues were found between known allergens and phospholipase A_1. It has been reported that an immunologically significant sequence similarity requires a match of at least 8 contiguous identical residues.

These data demonstrate that phospholipase A_1 is unlikely to share structurally or immunologically relevant sequence similarities with known protein toxins or allergens.

5.2 Short term toxicity

A 14-day oral (gavage) drf study in rats. Study director A Christensen and Z El-Salanti Scantox, Denmark. Study No 546625 May 2004

Test material	Phospholipase A_1 liquid enzyme concentrate (11000 LEU/g, Total Organic Solids 5.6% w/w)
Control and vehicle material	Tap water
Test Species	SPF Sprague Dawley rats (4 groups of 5 males and 5 females)
Dose	0, 57.5, 189.8 and 575.1 mg TOS/kg bw/day by gavage
GLP	OECD (1997)

Groups of 5 male and 5 female rats received a daily dose of phospholipase A_1 administered orally by gavage for 14 days. Clinical signs were recorded daily. Body weight and food consumption were recorded weekly. At termination, animals were killed and subjected to macroscopic examination. No treatment related signs were recorded at the clinical examinations, on food consumption or body weight gain. No macroscopic findings were noted at necropsy.

Under the conditions of this study, fourteen days of oral (gavage) treatment of rats with phospholipase A_1 at dose levels up to 575.1 mg TOS/kg bw/day resulted in no treatment related effects.

5.3 Sub-chronic toxicity

A 13-week oral (gavage) toxicity study in rats. Study Director: Z. Salanti, Scantox Study No: 54663, Sponsor Reference No: Novozymes Ref No 20046002. 5 October 2004

Test material	Phospholipase A_1 liquid enzyme concentrate (11000 LEU/g, Total Organic Solids 5.6% w/w)
Control and vehicle material	Tap water
Test Species	SPF Sprague Dawley rats (4 groups of 10 males and 10 females)
Dose	0, 1, 3.3 or 10 mL liquid enzyme concentrate/kg bw/day (equivalent to 0, 57.5, 189.8, 575.1 mg TOS/kg bw/day) by gavage
GLP	OECD (1997)
Guidelines	OECD guideline 408 (1998)

Study conduct

Four groups of rats (4-5 weeks old, 10/sex/group) were treated with phospholipase A_1 by gavage at doses of 0, 57.5, 189.8, 575.1 mg/kg bw per day for 90 days. Groups were designated group 1 (control), 2 (low dose), 3 (medium dose) and 4 (high dose).

Animals were checked daily for visible signs of ill health or any behavioural changes. Morbidity/mortality checks were performed at least twice daily. Detailed clinical examination was performed weekly. Once during the last two weeks of the study all animals were examined for sensory reactivity, grip strength and motor activity.

Bodyweight and food consumption were recorded weekly; haematology and clinical chemistry near the end of the study; and ophthalmology performed on all animals before the start of the study and on animals from groups 1 and 4 near termination. At the end of the study, all animals were sacrificed and necroscopy performed (gross examination, organ weights and histopathology on selected organs). See Appendix A for details of haematology, clinical chemistry, urinalysis and organs and tissues sampled.

Results

One female from group 2 was sacrificed in extremis on day 20 and another female from group 4 died immediately following dosing on day 73. Necropsy and microscopic examination confirmed that both deaths were due to intubation errors and as such not due to the test article. No other deaths occurred during the study.

No treatment-related clinical signs were noted. In motor activity tests, group 4 females spent significantly less time moving and had fewer moves per count compared to the control group. As these findings were observed only in one sex and as mean values for both parameters were within the 95% confidence interval for historical controls, this result was not considered test article related.

No treatment related effects were observed on body weights or body weight gain. Males in group 4 (week 13) and females in group 4 (week 9) had significantly lower food consumption compared to the control group, however as this was sporadic and occurred without any related decrease in body weight, these results were considered to be incidental.

Ophthalmoscopy revealed no treatment related effects.

Females in groups 3 and 4 had statistically significantly increased fibrinogen concentration ($p<0.05$ and $p<0.01$ respectively). This appeared to be dose dependant, however was determined by the study director not to be test article related as values were within the 95 % confidence interval for the historical control data and similar changes had not occurred in the male rats. This finding was therefore considered incidental.

Group 2 males had significantly decreased alanine amino transferase and group 3 males had significantly increased serum sodium. These changes were considered to be incidental as they were not dose dependent and were seen only in males. Females in groups 3 and 4 had significantly increased blood glucose concentration, this was also considered incidental as values were within the 95 % confidence interval for the historical control data and similar changes were not seen in the male rats.

Urinary sodium was statistically significantly increased in group 2 males and decreased in group 2 females. This was not considered to be test related. A statistically significant decrease in urinary potassium was noted in group 4 females. A decrease was also observed in group 4 males but was not significant. The mean urinary potassium in group 4 females was within the 95 % confidence interval for the historical control data and combined with the absence of pathological findings in relevant organs, this finding was considered incidental.

Group 4 females had statistically significantly increased relative heart weight ($p<0.05$) compared to the control group. The mean value was within the 95 % confidence interval for the historical control data and coupled with an absence of pathological findings in the heart and the finding was considered incidental.

Macroscopic and microscopic examination of organs and tissues revealed no treatment related findings. All reported findings were considered to be with the background incidence of findings reported in rats of this age and strain.

Conclusion

No treatment related changes were observed in rats treated with up to 575.1 mg/kg bw/ day phospholipase A_1 for 13 weeks.

Under the conditions of this study, the NOEL of phospholipase A_1 was shown to be 575.1 mg/kg bw per day, based on the maximum dose tested in this study. This is equivalent to 10 ml liquid enzyme concentrate (or approximately 110,000 LEU)/kg bw per day.

5.4 Genotoxicity studies

Test for Mutagenic Activity with Strains of *Salmonella typhimurium* and *Escherichia coli*. Study Director Peder Bjarne Pedersen. Safety and Toxicology, Novozymes A/S. Study no. 20048085. 10 January 2005.

Test article

Phospholipase A_1 liquid enzyme concentrate (batch # PPW 23436), sterilized and standardized at 5% w/v dry matter.

Study design

Phospholipase A_1 was examined for mutagenic activity in four strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537) and a strain of *Escherichia coli* (WP2uvrApKM101). Experiments were performed with and without metabolic activation using liver S9 fraction from chemically pre-treated rats.

The phospholipase A_1 preparation contains a variety of unspent medium residues, including low concentrations of free amino acids like histidine and tryptophan, which poses a problem during mutagenicity tests in vitro. For this reason, all strains were exposed to the test article in liquid culture for three hours, before the bacteria were collected, washed and plated on minimal agar plates for 48 – 72 hours. The study comprised of negative and positive controls with and without S9 metabolising system. Viability determination and estimation of mutant numbers were carried out in triplicates at each test point. Five doses of test substance were applied with 5 mg/incubation as the highest dose level. The sensitivity of the individual bacterial strains was confirmed by significant increases in the number of revertant colonies induced by diagnostic mutagens (2-nitrogluorene, 9-aminoacridine, n-methyl-n'-nitrosoguanidine, n-ethyl-n'-nitro-n-nitrosoguanidine, 2-aminoacnthracene, benzo(α)pyrene).The study was conducted in accordance with OECD guideline 471, however the exposure of the culture to the test substance in liquid culture is not specifically described in any guideline.

A third experiment with lower doses of phospholipase A_1 was conducted with *S. typhimurium* strains TA1537 and TA100 in the presence of S9 activation as in the previous experiments there was a distinct reduction in viability of the cultures at the high dose levels. Lower revertant numbers were also observed with these strains compared to the negative controls.

Test	*Test material*	*Concentration*	*Test object*	*Result*
Reverse mutation (*In vitro*)	phospholipase A_1	**test 1 and 2**: 156, 625, 1250, 2500 and 5000 µg **test 3**: TA100 78, 156, 313, 625, 1250 and 2500 µg and TA1537 15.6, 31.3, 62.5, 125, 250 and 500 µg.	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* WP2uvrApKM101	-ve

Results and conclusion

No dose-related increases in mutation frequency were observed in the strains tested. It was concluded that phospholipase A_1 produced by *A. oryzae* did not exhibit mutagenic activity under the conditions of the test.

Induction of chromosome aberrations in cultured human peripheral blood lymphocytes. Study director James Whitwell. Safety and Toxicology, Novozymes. Covance Study no. 1974/22-D6172. 17 November 2004.

Test article

Phospholipase A_1 liquid enzyme concentrate (batch no. 23436) with a purity of 14500 LEU/g.

Study design

Phospholipase A_1 was tested in an *in vitro* cytogenetics assay using duplicate human lymphocyte cultures prepared from the pooled blood of three female donors in two independent experiments. Treatment was performed in the absence and presence of metabolic activation (S9). The study was conducted in accordance with OECD guideline 473 (1997).

In the first experiment, treatment was for 3 hours followed by a 17-hour recovery period prior to harvest. The dose levels (2812, 3750 and 5000 ug/mL) were selected by evaluating the effect of phospholipase A_1 on mitotic index. The highest concentration chosen for analysis induced approximately 3% and 15% mitotic inhibition in the absence and presence of S9 respectively.

In the second experiment, treatment in the absence of S9 was continuous for 20 hours. Treatment in the presence of S9 was for 3 hours followed by a 17-hour recovery period. Three dose levels were chosen (3200, 4000 and 5000 ug/mL) based on mitotic inhibition at the highest dose of 33% and 45% with and without activation.

In both experiments all treatments were performed in duplicate. 4-nitroquinoline 1-oxide (-S9) and cyclophosphamide (+S9) were used as positive controls.

Following harvesting, lymphocytes were fixed and slides prepared and stained. Slides were examined microscopically and cells with structural aberrations (including and excluding gaps) and polyploid, endoreduplicated or hyperdiploid cells were scored.

Test	*Test material*	*Concentration*	*Test object*	*Result*
chromosome aberration (*In vitro*)	Phospholipase A1 solution (5.6 w/w TOS)	Experiment 1: 2812, 3750 and 5000 µg/mL Experiment 2: 3200, 4000 and 5000 µg/mL	human lymphocyte cultures	-ve

Results and Conclusion
Treatment did not produce statistically significant increases in the frequency of aberrant chromosomes at any concentration tested when compared to control values, either in the presence or absence of S9 metabolic activation. Positive controls induced statistically significant increased in the number of cells with structural aberrations, indicating the efficacy of the metabolic activation mix and the sensitivity of the test procedure.

5.5 Cytotoxicity

***In vitro* cytotoxicity test: Neutral Red Uptake in L929 Monolayer Culture. Study director SG Elvig-Jørgensen. Safety and Toxicology, Novozymes. Study no. 20048047. 18 November 2004**

Phospholipase A_1 (batch PPW 23436, activity of 11000 LEU/g) was tested for cytotoxicity using the L929 established mouse fibroblast cell line. The study was conducted under GLP. Cells were grown in 96 well plates for 24 hours to establish a confluent monolayer. Phospholipase A_1 was added to the wells at a concentration of 0, 0.3, 3, 10 and 30 g/ml growth medium (4 replicates of each dose) and incubated for 24 hours. The test material was then replaced by Neutral Red dye and incubated for 3 hours. Following washing and the application of Neutral Red desorb solution, the absorbance at 540 nm of each well was measured to determine the number of cells surviving exposure to the test material. Sodium dodecyl sulphate (SDS) was used as a positive control at concentrations of 80, 100 and 120 µg/mL.

Test substance	**Concentration mg/mL**	**Viability %**
Phospholipase A_1	0	100
	0.3	103
	1	106
	3	99
	10	101
	30	96
Positive control	**Concentration ug/mL**	**Viability %**
SDS	0	100
	80	89
	100	56
	120	1

Under the conditions of this study, phospholipase A_1 is non-cytotoxic in mouse fibroblast cells at levels up to 30 mg/mL.

6. Overall Conclusion

Following the safety assessment of phospholipase A_1 from *A. oryzae*, it was concluded that:

- The production organism has a history of safe use as a production strain for food-grade enzyme preparations and has been shown not to produce toxic metabolites;
- The recombinant DNA in the production organism is considered to be stable and poses no safety concern;
- The enzyme preparation complies with international specifications;
- there was no evidence of toxicity in the sub-acute toxicity study or in the sub-chronic toxicity study in rats;
- The NOEL from the sub-chronic feeding study was greater than 575.1 mg/kg bw per day, the highest dose level. This is equivalent to 10 ml liquid enzyme concentrate (or approximately 110,000 LEU)/kg bw per day; and
- The enzyme preparation produced no evidence of genotoxic potential in *in vitro* assays.

From the available information, it is concluded that the use of phospholipase A_1 as a processing aid in food would pose no public health and safety risk.

References:

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APPENDIX A

Analyses performed in 13-week oral toxicity study in rats

Haematology parameters	
Haemoglobin	Red blood cell count
Heamocrit	Mean cell volume
Mean cell haemoglobin	Mean cell haemoglobin concentration
White blood cell count	Differential leucocyte count (Neutro, Lympho, Eos, Baso and Mono)
Platelet count	Prothrombin time
Fibrinogen	
Clinical chemistry	
Alanine aminotransferase	Sodium
Aspartate aminotransferase	Potassium
Alkaline phosphatase	Calcium
Bilirubin	Magnesium
Gamma-glutamyl transferase	Inorganic phosphorus
Cholesterol	Chloride
Triglycerides	Protein (total)
Carbamide	Albumin
Creatinine	Globulin
Glucose	Albumin/Globulin
Urinalysis	
Volume	Colour
Creatinine	Protein
N-acetyl-β-D-glucosamine	Leucocytes
Gamma-glutamyl transferase	Nitrite
Sodium	Blood
Potassium	Glucose
Chloride	Ketones
Specific gravity	Bilirubin
pH	Urobilinogen
Organs and tissues sampled for microscopic examination	
Abnormalities (gross lesions)	Adrenals
Aorta	Brain
Bone marrow smear	Epididymides
Eyes with lens/optic nerve	Heart
Small intestine	Large intestine
Kidneys	Liver
Lungs	Lymph nodes
Mammary gland	Oesophagus
Ovaries	Pancreas
Pituitary	Prostate
Salivary gland	Sciatic nerve
Seminal vesicles	Skeletal muscle
Skin	Spinal cord
Spleen	Sternum
Stomach	Testes
Thymus	Thyroids
Trachea	Urinary bladder
Uterus	Vagina

Attachment 3

Food Technology Report

Phospholipase A_1 as a processing aid (enzyme)

Introduction

The phospholipase A enzymes are acyl hydrolases, which means they remove one acyl group from a phospholipid, yielding one fatty acid and lysophospholipid. Phospholipase A_1 and phospholipase A_2 remove fatty acids at positions sn-1 and sn-2, respectively. Phospholipase A_1 is widely distributed in nature, being found in animal and plant tissues. The major sources are found in the pancreas and the brain of animals (Encyclopedia of Food Sciences and Nutrition, 2003, p 4528). Phospholipase A_1 selectively acts on the fatty acid in position 1 (sn-1) in phospholipids to cleave a free fatty acid and form a lysophospholipid.

Enzyme details

The common name for the enzyme is phospholipase A_1. The systematic name for the enzyme is phosphatidylcholine 1-acylhydrolase (IUBMB Enzyme Nomenclature, 2005).

The phospholipase A_1 enzyme has the Enzyme Commission (EC) number of [3.1.1.32].

It has the Chemical Abstracts Service (CAS) Registry Number of 9043-29-2.

The molecular weight of the enzyme is listed by the Applicant as 110 -115 kDa. The enzyme preparation is a clear pale yellow liquid which is water soluble. The recommended pH range for reaction is 3.5-11.

Phospholipase A_1 catalyses the reaction of:

phosphatidylcholine +H_2O = 2-acylglycerophosphocholine + carboxylate (fatty acid).

This reaction is comparable to that catalysed by the enzyme phospholipase A_2:

phosphatidylcholine +H_2O = 1-acylglycerophosphocholine + carboxylate (fatty acid).

Figure 1 indicates the different reaction sites of a phospholipid of the two enzymes, phospholipase A_1 and phospholipase A_2. The carbon atoms of the glycerol backbone have been labelled 1, 2, and 3 for clarity. This figure has been adopted from a reference (Thomson et al, 2004).

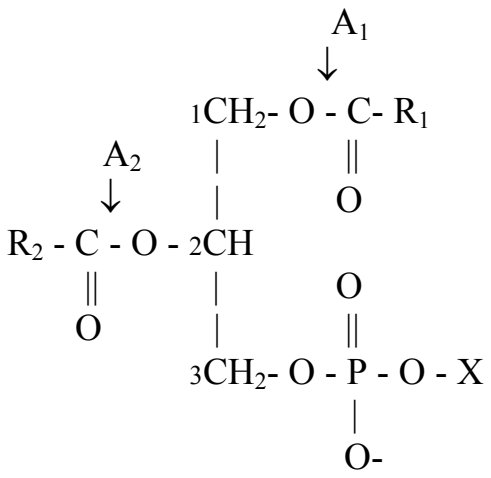


Figure 1. Diagram indicating the two different reaction sites of a phospholipid molecule by the two different phospholipase enzymes, phospholipase A_1 and A_2.

Phospholipase A_1 attacks and cleaves the fatty acid from the number 1 position (sn-1) of the glycerol backbone of lecithin (so leaving the acyl group remaining on the number 2 position), while phospholipase A_2 attacks the number 2 position (sn-2). Phospholipase A_1 is stated to have much broader specificity than phospholipase A_2 (Encyclopedia of Food Sciences and Nutrition, 2003, pp 4528-4529).

Enzyme manufacture

The phospholipase A_1 enzyme preparation is produced by submerged fermentation of the microbial source *Aspergillus oryzae* that has the gene coding for phospholipase from *Fusarium venenatum* inserted by recombinant DNA techniques. The enzyme preparation is manufactured in accordance with Good Manufacturing Practices, using standard enzyme manufacturing practices. The enzyme preparation is stabilised with common approved stabilisers and standardised to company specifications.

Enzyme specification

The Application states that the enzyme preparations meet the international specifications for enzymes contained in the Food Chemical Codex (5th Edition, 2004), and the Joint FAO/WHO Expert Committee on Food Additives (JECFA), in the Compendium of Food Additives Specifications, Vol 1 Annex 1, FAO 1992 (Addendum 9, 2001).

Criteria	**Specification (meets or exceeds JECFA)**
Heavy Metals as Pb	not more than 30 ppm
Arsenic	not more than 3 ppm
Lead	not more than 5 ppm
Total viable count (cfu/g)	not more than 10,000
Total coliforms (cfu/g)	not more than 30
Mycotoxins	negative by test
Antimicrobial activity	negative by test
Salmonella (/25 g)	negative by test
Escherichia coli (/25 g)	negative by test
Production organism	negative by test

Food uses and technological justification

The enzyme preparation is used to improve process efficiencies in cheese manufacture with lower losses of fat and other solids into the whey stream. The phospholipase A_1 enzyme preparation is added to the milk used for cheese manufacture before the coagulant is added. The phospholipids produced after the enzyme treatment have better emulsifying properties compared to untreated milk and as such keep more of the milk components in the cheese with reduced losses to the whey stream. The cheese yields are increased by approximately 2.0%, without any significant change to the quality or composition of the cheese.

Natural cheese is basically an oil-in-water emulsion, stabilised by cheese protein (Encyclopedia of Food Sciences and Nutrition, 2003, p1111). Improving the emulsifying properties of the naturally occurring fats and lipids in milk, by reacting phospholipase A_1 with the milk phospholipids to produce a fatty acid and a lysophospholipid assists to keep more milk components in the cheese.

The Applicant, Novozymes A/S, has a number of US patents related to the use of phospholipases (phospholipase A_1, as well as other phospholipases). A patent search on the US Patent & Trademark Office website (http://www.uspto.gov/) picked up 16 documents. Four patents that are relevant to this current Application are:

United States Patent Application	**Title**
20050106665	Phospholipase
20050019471	Whey protein emulsion
20050069607	Process for producing cheese
20040253680	Phospholipase

Conclusion

The use of the enzyme phospholipase A_1 from the source *Aspergillus oryzae*, containing the gene for phospholipase A_1 isolated from *Fusarium venenatum*, is technologically justified to improve process efficiencies in cheese manufacture. It achieves this by improving the emulsifying properties of the treated phospholipids of the cheese, keeping more of the milk fat components in the cheese with less lost into the whey stream.

References

Encyclopedia of Food Sciences and Nutrition, Phospholipids, Second Edition, Academic Press, (2003), p 1111 and pp 4528-4529.

Food Chemical Codex, (2004). National Academy of Sciences, Food and Nutrition Board, Committee on Food Chemical Codex, 5th edition, National Academy Press, Washington DC

International Union of Biochemistry and Molecular Biology (IUBMB) Enzyme Nomenclature http://www.chem.qmul.ac.uk/iubmb/enzyme/EC3/1/1/32.html, accessed on 5 May 2005.

Joint FAO/WHO Expert Committee on Food Additives (JECFA) (2001). Compendium of Food Additives Specifications, General specifications and considerations for enzyme preparations used in food processing. FAO Food and Nutrition Paper 52, Addendum. 9, pp37-39.

Thompson, J.E., Lo, M., Taylor, C., Wang, L., Nowack, L., and Wang, T.W., (2004), Amelioration of sensitivity to UV-B in *Arabidopsis* by suppression of a putative phospholipase. In: 16th International Plant Lipid Symposium, Budapest, Hungary 1-4 June 2004, pp 63-69. http://www.mete.mtesz.hu/pls/proceedings/eloadasok-pdf/16pls-proceeding-20041116.pdf. Accessed on 17 August 2005.

US Patent & Trademark Office, at http://www.uspto.gov/, accessed on 25 August 2005.

Attachment 4

Summary of public submissions

Round One

Submitter Organisation	Name
Food Technology Association Vic	David Gill
Australian Food and Grocery Council	Kim Leighton
New Zealand Food Safety Authority	Carole Inkster
Department of Human Services, Victorian Government	Victor Di Paola
Department of Health, South Australia	Joanne Cammans
New South Wales Food Authority	Chris Chan
Private	Paul Elwell-Sutton

Submitter	Position	Comments
Food Technology Association Vic	Agrees, supports option 2	It supports the application without further comment.
Australian Food and Grocery Council	Agrees, supports the Application subject to an appropriate safety assessment.	Other comments are: • The AFGC considers it likely that FSANZ will determine that the enzyme phospholipase A_1 is safe. • Makes note that the host and donor organisms are used for other enzymes (in the Code and in the US FDA GRAS system). • Notes that it is not the organisms which are used but the enzyme, most of which does not remain in the final product and is digested like other protein by gastrointestinal enzymes. • Notes the enzyme has other overseas approvals, GRAS acceptance and compliance with specifications. • The use of the enzyme is technologically justified, including in relation to production efficiencies. • States that the Application appears to fulfil the objectives of food standards and meets the section 10 objectives of the FSANZ Act.
New Zealand Food Safety Authority	No position at this stage, may do so at Draft Assessment	It will review the safety data at the Draft Assessment stage.
Department of Human Services, Victorian Government	Raised an issue it asked to be addressed at Draft Assessment	It understands the enzyme is manufactured using recombinant DNA techniques. It also understands some enzyme may remain in the final cheese products, but with no technological function. It appears to them from the Initial Assessment Report that the enzyme contains altered genetic material and so is a genetically modified organism, so would require labelling under subclause 4(1) of Standard 1.5.2. They ask that the Draft Assessment address this point to clarify: (a) that altered genetic material remains in the final product, and (b) that GMO labelling will be required if any of the altered genetic material remains.

Submitter	Position	Comments
Department of Health, South Australia	No objection	It has no objection to the progression of the Application.
New South Wales Food Authority	Supports further consideration	It agrees there seems sufficient technological justification for approval of the enzyme based on the potential for increasing cheese yield. It raised a specific issue to be considered as part of the safety assessment. Approval, if given, does not restrict the use of the enzyme to any specific application, so it asks that all probable uses of the enzyme be considered and not just those specific applications nominated by the Applicant.
Private, Paul Elwell-Sutton	Does not support the Application.	The submitter opposes the Application for a number of reasons. He points out an error in the Initial Assessment report that *Fusarium venenatum* is the host not the donor in the US FDA GRAS notice of GRN 000054. He disputes that an FDA GRAS notice for the enzyme of this Application is an assessment based on consumer health and safety. He believes the approval process is based on political considerations rather than scientific ones. He questions whether the encoding gene and the enzyme have any attachments, so therefore questions the safety of the enzyme. He questions the trade implications of approving or not this enzyme. If approving it will have no impact on trade, then not approving it will also have no impact on trade. He questions the strategy of approving the less effective phospholipase A_2 before phospholipase A_1. If the timing was reversed there would be little reason to approve phospholipase A_2 after the more effective A_1 was approved. This he claims is 'misleading and deceptive conduct' (one of FSANZ's objectives) by the enzyme manufacturers. He also believes FSANZ fails its objective of 'the provision of adequate information relating to food to enable consumers to make informed choices' over the failure of the labelling regime for GM foods (including enzymes derived from GM sources).

Round Two

Submitter Organisation	Name
Food Technology Association Vic	David Gill
New South Wales Food Authority	Kelly Boulton
Australian Food and Grocery Council	Kim Leighton
Individual	Ivan Jeray
South Australia Department of Health	Joanne Cammans
New Zealand Food Safety Authority	Carole Inkster
Department of Human Services Victoria	Victor Di Paola
Environmental Health Unit, Queensland Health	Gary Bielby
Private	Paul Elwell-Sutton

Submitter	Position	Comments
Food Technology Association Vic	Agrees, supports option 2	It supports the application without further comment.
New South Wales Food Authority	Agrees, supports option 2	Supports the Application. It is pleased that the Safety Assessment Report, in the Draft Assessment Report concluded that use of the enzyme in food in general (not requiring any restriction to any specific application, which was an issue it raised at Initial Assessment), would not raise any public health and safety concerns.
Australian Food and Grocery Council	Agrees, supports option 2	Supports the Application without reservation. It agrees with the conclusion of the safety assessment that use of the enzyme does not pose any health or safety concerns. It states on this basis alone, the Application should be approved. It noted that the enzyme is approved in other countries and approval would ensure consistency between domestic and international standards. Approval will provide Australian and New Zealand manufacturers the same opportunity to use the enzyme as their overseas competitors.
Ivan Jeray	Opposes	He opposes all genetically modified food on safety and environmental grounds.
South Australia Department of Health	Agrees, supports option 2	It has no objection to the progression of the Application
New Zealand Food Safety Authority	Agrees, supports option 2	Supports the Application.
Department of Human Services Victoria	Agrees, supports option 2	Supports the Application
Environmental Health Unit of Queensland Health	Agrees, supports option 2	It gives qualified support for the Application. It acknowledges that the safety assessment concludes that it does not raise any public health and safety concerns. The enzyme is also technologically justified. It does have a concern they asked be addressed in the Final Assessment report. That is, they note the genetically modified source micro-organism is related to a mould that produces aflatoxins, but that the Application source does not produce any toxins as by-products. Can this be further explained as to why there are no safety concerns?

Submitter	Position	Comments
Paul Elwell-Sutton	Rejects	Opposes the Application (for the same reasons indicated in his submission to A548 (GM rootworm protected corn). That is, the lack of consumer choice since processing aids (in this case an enzyme) that has been produced using GM technology does not need to be labelled on food produced using this enzyme. Requests a more robust and inclusive food labelling regime.

DSMZ
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Identification of Fungus cultures

Sent by: Röhm Enzyme GmbH Darmstadt, Dr. B. Winter

Strain designation: RH 3949

Substrate: waste water

Colony habit:

Colony on Czapek-Dox-agar growing up to 6 mm/day at 25°C, bluish-green. Mycelium colourless, unconspicuous. Very good growth at 45°C.

Morphology:

Conidiophores smooth-walled, 200-300 µm long, up to 7 µm Ø, enlarging gradually into vesicles of 18-20 µm Ø. No metulae; phialides ampulliform, 7 µm long, with a short neck; conidia mostly subglobose globose to ellipsoidal, 2.5-3 µm in length, smooth-walled, adhering in long compact columns.

Identity: *Aspergillus fumigatus* Fresenius

DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
(b) (6)

Braunschweig, 31.03.99

Centraalbureau voor Schimmelcultures

Fungal Biodiversity Centre

Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW)

AB Enzymes GmbH
i.V. Dr. Patrick Lorenz
Feldbergstraße 78
D-64293 Darmstadt
Germany

MGT	FC	SC	HR	GBO
R&D	AB Enzymes GmbH			KAM
QM	II			RM
IPM	29. OKT. 2012			BUFS
RA	MGT			BUTE
				BUFE

Utrecht, October 25 2012

CBS IDENTIFICATION SERVICE

Your ref.: **RH3949**

Our ref.: **Det 12.090** Please state always our reference number when you contact us.

Dear Dr. Patrick Lorenz,

Herewith we inform you about the results of our identification of your strain(s).

On arrival the strain was cultivated on several media; Malt Extact Agar (two plates), Oatmeal Agar, Synthetic Nutrient Agar and Dichloran 18% Glycerol Agar.
DNA was extracted from one MEA plate after an incubation period of 3 days in the dark at 25°C using the MoBio - UltraClean™ Microbial DNA Isolation Kit. Fragments containing of part of the β-tubulin gene was performed using the primers Bt2a (GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (ACCCTCAGTGTAGTGACCCTTGGC, (Glass & Donaldson 1995)). The PCR fragments were sequenced with the ABI Prism® Big DyeTM Terminator v. 3.0 Ready Reaction Cycle sequencing Kit. Samples were analyzed on an ABI PRISM 3700 Genetic Analyzer and contigs were assembled using the forward and reverse sequences with the programme SeqMan from the LaserGene package.
The sequences were compared on GenBank using BLAST and in a large fungal database of CBS-KNAW Fungal Biodiversity Centre with sequences of most of the type strains. The results had a 100% match in all databases with the type strain of ***Aspergillus nishimurae*** Takada, Y. Horie & Abliz.

RH3949 = ***Aspergillus nishimurae*** Takada, Y. Horie & Abliz (previously known as ***Neosartorya nishimurae*** Takada, Y. Horie & Abliz

The invoice for this identification will be sent separately.

Yours sincerely,

(b) (6)

M. Meijer, Bsc.

Centraalbureau voor Schimmelcultures

Fungal Biodiversity Centre

Institute of the Royal Netherlands Academy of Arts and Sciences (KNAW)

AB Enzymes GmbH
i.V. Dr. Patrick Lorenz
Feldbergstraße 78
D-64293 Darmstadt
Germany

Utrecht, May 15, 2012

CBS IDENTIFICATION SERVICE

Your ref.: **RH32439**

Our ref.: **Det 12-032** Please state always our reference number when you contact us.

Dear Dr. Patrick Lorenz,

Herewith we inform you about the results of our identification of your strain(s).

RH32439 = ***Trichoderma reesei*** E.G. Simmons

The invoice for this identification will be sent separately.

Yours sincerely,

(b) (6)

M. Meijer, Bsc.

U.S. Department of Health & Human Services

U.S. Food & Drug Administration

Food

Home Food Food Ingredients & Packaging Generally Recognized as Safe (GRAS)

Agency Response Letter GRAS Notice No. GRN 000032

CFSAN/Office of Premarket Approval

April 20, 2000

Gary L. Yingling
McKenna and Cuneo, L.L.P.
1900 K Street, N. W.
Washington, D.C. 20006-1108

Re: GRAS Notice No. GRN 000032

Dear Mr. Yingling:

The Food and Drug Administration (FDA) is responding to the notice, dated October 13, 1999, that you submitted on behalf of Rohm Enzyme GmbH in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)). FDA received the notice on October 14, 1999 and designated it as GRAS Notice No. GRN 000032.

The subject of the notice is the pectin lyase enzyme preparation obtained from a *Trichoderma reesei* (formerly classified as *Trichoderma longibrachiatum*), which contains a recombinant gene encoding pectin lyase (also called pectin transeliminase) from *Aspergillus niger* var. *awamori*. The notice informs FDA of the view of Rohm Enzyme GmbH that the pectin lyase enzyme preparation is GRAS, through scientific procedures, for use as a processing aid for the preparation of fruit and vegetable juices, purees and concentrates by various production processes, including the pulp wash processes and in-line pulp wash processes in the processing of citrus fruits. According to Rohm Enzyme GmbH, the pectin lyase preparation will be used for the degradation of the pectin to lower the viscosity and to clarify juices at a dose of 5 to 100 parts per million (ppm) to accomplish the intended technical effect in accordance with current good manufacturing practices.

The notice describes (1) published information pertaining to the safety of the various components of the production organism, including the host (*T. reesei*) and the donor (*Aspergillus niger*) organisms; (2) published information about the safety evaluation of microbial-derived food-grade enzyme preparations, including commercial pectinase enzyme preparations; (3) published information about the technical effect of the enzyme preparation; (4) published information related to the production process of the pectin lyase enzyme preparation; (5) scientific publications and recommendations issued by international organizations on the safety of enzymes used in food processing including enzymes derived from genetically modified microorganisms; and (6) unpublished studies conducted with the production strain and the pectin lyase enzyme preparation from *A. niger*.

The notice states that the pectin lyase preparation meets the specifications for enzyme preparations provided in the Food Chemicals Codex (4th ed., 1996) and the specifications for enzyme preparations provided by the Joint Expert Committee on Food Additives (JECFA; a joint committee of the Food and Agriculture Organization/World Health Organization).

Based on the information provided by Rohm Enzyme GmbH, as well as other information available to FDA, the agency has no questions at this time regarding the conclusion of Rohm Enzyme GmbH that the pectin lyase enzyme preparation is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of the pectin lyase enzyme preparation. As always, it is the continuing responsibility of Rohm Enzyme GmbH to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in your notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the Office of Premarket Approval's homepage on the World Wide Web.

Sincerely,

Alan M. Rulis, Ph.D.
Director
Office of Premarket Approval
Center for Food Safety and Applied Nutrition

Links on this page:

- Accessibility
- Contact FDA
- Careers
- FDA Basics
- FOIA
- No Fear Act
- Site Map
- Transparency
- Website Policies

U.S. Food and Drug Administration
10903 New Hampshire Avenue
Silver Spring, MD 20993
Ph. 1-888-INFO-FDA (1-888-463-6332)

Appendix 8 - ANALYSIS OF SAFETY BASED ON PARIZA/JOHNSON DECISION TREE

Pariza and Johnson have published guidelines for the safety assessment of microbial enzyme Preparations (2001). The safety assessment of a given enzyme preparation is based upon an evaluation of the toxigenic potential of the production organism. The responses below follow the pathway indicated in the decision tree. The outcome of this inquiry is that the Phospholipase A2 (produced in *T. reesei* from Aspergillus fumigates/nishimurae strain RH3949) enzyme preparation is "ACCEPTED" as safe for its intended use.

1. **Is the production strain genetically modified?** Yes go to #2;

2. **Is the production strain modified using rDNA techniques?** Yes go to #3a;

3a. Does the expressed enzyme product which is encoded by the introduced DNA have a history of safe use in food? Yes, phospholipase A2 from *Aspergillus nishimurae* (formerly *Aspergillus fumigatus*) and *Trichoderma reesei* have a safe history of use. Phospholipase A2 has been approved as a processing aid in Australia and New Zealand. Go to 3c;

3c. Is the test article free of transferable antibiotic resistance gene DNA? Yes, transferable DNA was not detected in the phospholipase A2 enzyme preparation manufactured using *T. reesei* and production process described herein. Additionally, no antibiotic resistance gene has been integrated. Go to 3e;

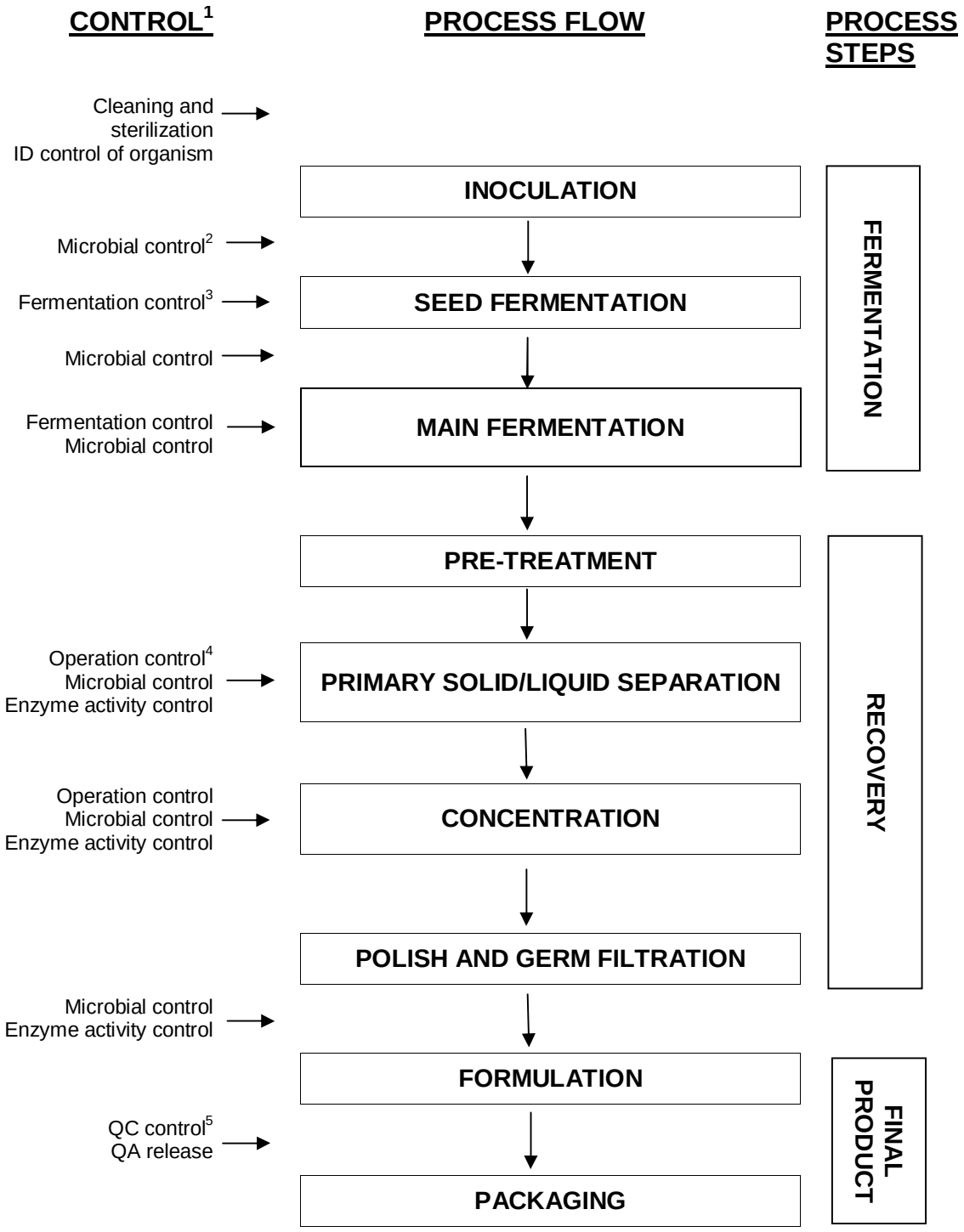
3e. Is all other introduced DNA well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products? Yes, inserted DNA is well characterized. Go to 4;

4. **Is the introduced DNA randomly integrated into the chromosome?** Yes, go to #5;

5. **Is the production strain sufficiently well characterized so that one may reasonably conclude that unintended pleiotropic effects which may result in the synthesis of toxins or other unsafe metabolites will not arise due to the genetic modification method that was employed?** Yes, there is no concern for pleiotropic effects. Go to #6;

6. **Is the production strain derived from a safe lineage, as previously demonstrated by repeated assessment via this evaluation procedure?** Yes, *T. reesei* has been demonstrated as a safe production host and methods of modification have been well documented. Safety of this organism has been evaluated and confirmed through toxicological testing as described herein. **ACCEPTED**

Production Process of Food Enzymes from Fermentation



[1] The controls shown on the flow chart may vary depending on the production set-up. Controls are conducted at various steps throughout the production process as relevant.

[2] Microbial control: Absence of significant microbial contamination is analyzed by microscope or plate counts

[3] During fermentation parameters like e.g. pH, temperature, oxygen, CO_2, sterile air overflow are monitored / controlled.

[4] Operation control in downstream processes cover monitoring and control of parameters like e.g. pH, temperature

[5] Final QC control will check that product does live up to specifications like e.g. enzyme activity as well as chemical and microbial specification.

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington DC 20204

September 11, 2003

Mr. Gary Yingling
Kirkpatrick & Lockhart LLP
1800 Massachusetts Avenue, NW
Second Floor
Washington, DC 20036-1221

Dear Mr. Yingling:

You requested, on behalf of the Enzyme Technical Association, that OFAS review the use of certain defoaming and flocculating agents in the manufacture of enzyme preparations used in food. You provided information related to these compounds in your letters of December 20, 1996 (to Dr. Alan Rulis), 4-24-1998 (to Dr. Zofia Olempska-Beer), and 11-30-99 (to Dr. Zofia Olempska-Beer). You also arranged for a teleconference between ETA members and OFAS representatives, facilitated telephone contacts with technical experts from ETA member companies, and responded to numerous requests for clarification. We appreciate your and ETA's cooperation.

We reviewed the information on defoaming and flocculating agents that you submitted as well as the information provided in GRAS affirmation petitions and GRAS notices for enzyme preparations. The enclosed attachment provides a brief overview of our evaluation and itemizes the evaluated defoamers (Table 1) and flocculants (Table 2). We conclude that these compounds are used by enzyme manufacturers in accordance with the principles of good manufacturing practice (GMP).

Sincerely yours,

(b) (6)

Laura M. Tarantino, Ph.D.
Acting Director
Office of Food Additive Safety, HFS-200
Center for Food Safety and Applied Nutrition

Defoaming and Flocculating Agents Used in the Manufacture of Enzyme Preparations Used in Food

Enzyme Preparations

Most enzymes currently used in food are derived from microorganisms. The manufacturing process of such enzymes includes three major steps: fermentation, enzyme recovery, and enzyme formulation. The formulated products are generally referred to as enzyme preparations. In addition to the enzymes of interest, enzyme preparations contain added substances such as diluents, preservatives, and stabilizers. They may also contain metabolites derived from the production microorganism and the residues of substances used in the manufacturing process, such as components of the fermentation medium or defoaming and flocculating agents used during fermentation and recovery. When FDA reviews safety data on enzyme preparations, it considers all components of the preparation.

Defoaming Agents

Defoaming agents (defoamers) are used by enzyme manufacturers to reduce or prevent foaming during fermentation and recovery. They are formulated with ancillary ingredients such as surface-active agents or carriers. Defoamers currently used in the manufacture of food enzymes are listed in Table 1. The Table includes five major defoamers that are identified by a double asterisk and several compounds that are used either as secondary defoamers or ancillary ingredients in defoamer formulations.

The major defoamers are added to the fermentation broth at levels within the range of 0.05-1% on a weight basis. Some of these defoamers, for example, polyoxyethylene-polyoxypropylene block copolymer, may contain trace levels of ethylene oxide, propylene oxide, and 1,4-dioxane which are known to cause cancer in laboratory animals. The Office of Food Additive Safety (OFAS) has evaluated the use of defoamers listed in Table 1 and determined that human exposure to the residues of these defoamers in enzyme preparations does not present human safety concern.

Flocculating Agents

Flocculating agents (flocculants) are used in the enzyme recovery step to separate microbial cells and cell debris from the fermentation broth containing the dissolved enzyme. The flocculation typically consists of two steps - primary flocculation and secondary flocculation. In the primary flocculation, inorganic salts (such as calcium chloride or aluminum sulfate) or "low molecular weight" polymers (such as polyamines) are used to agglomerate the cellular debris. The primary flocculation is usually followed by the secondary flocculation in which "high molecular weight" polymers are used to aid the formation of larger agglomerates that are subsequently removed by centrifugation or filtration. The polymers used as flocculants can be either cationic or anionic. The cationic polymers are added to the fermentation broth at levels not higher than 1% on a

weight basis. The anionic polymers are used at levels at or below 0.025%.

The flocculants used in the manufacture of food enzymes are listed in Table 2. They include inorganic salts, polyamines, and polyacrylamides. Several of these compounds are regulated in 21 CFR either as food additives or GRAS substances. Certain polyamines may contain traces of epichlorohydrin and 1,3-dichloro-2-propanol. Polyacrylamides usually contain very low levels of acrylamide. These contaminants of polyamines and polyacrylamides are known to cause cancer in laboratory animals. OFAS has evaluated all polymers included in Table 2 and determined that human exposure to the residues of these flocculants in enzyme preparations does not present human safety concern.

Sources of Information on Defoamers and Flocculants

OFAS compiled data on defoamers and flocculants listed in Tables 1 and 2 using information voluntarily submitted by the Enzyme Technical Association. OFAS also relied on the information provided in GRAS affirmation petitions and GRAS notices for enzyme preparations. Other sources of information included published articles, computer searches, and Material Safety Data Sheets issued by manufacturers of defoamers and flocculants.

Table 1. Defoamers Used in the Manufacture of Food Enzymes

Compound	CAS Reg. No.	Supplemental Information
Polypropylene glycol**	25322-69-4	Average MW: 2000
Polyglycerol polyethylene-polypropylene glycol ether oleate**	78041-14-2	
Polyoxyethylene-polyoxypropylene block copolymer**	9003-11-6	Average MW: 2000
Polypropylene glycol monobutyl ether**	9003-13-8	
Polydimethylsiloxane**	63148-62-9 68083-18-1	
Silica	7631-86-9 63231-67-4	
Stearic acid	57-11-4	
Sorbitan sesquioleate	8007-43-0	
Glycerol monostearate	123-94-4	
Polysorbates (polyoxyethylene sorbitan fatty acid esters)		Polysorbate 60 (CAS No. 9005-67-8), Polysorbate 65 (CAS No. 9005-71-4), and polysorbate 80 (CAS No. 9005-65-6) are regulated as food additives and components of defoamer formulations
Rape oil mono- and diglycerides	93763-31-6	
White mineral oil	64742-47-8	

Table 2. Flocculants Used in the Manufacture of Food Enzymes

Compound	CAS Reg. No.	Supplemental Information
Dimethylamine-epichlorohydrin copolymer	25988-97-0	Cationic polyamine
Methylamine-epichlorohydrin copolymer	31568-35-1	Cationic polyamine
Dimethylamine-epichlorohydrin-ethylenediamine terpolymer	42751-79-1	Cationic polyamine
Polyacrylamide modified by condensation with formaldehyde and dimethylamine	67953-80-4	Cationic polyacrylamide
Acrylamide-acryloxyethyl-trimethyl-ammonium chloride copolymer	69418-26-4	Cationic polyacrylamide
Acrylamide-acrylic acid copolymer	25987-30-8 9003-06-9	Anionic polyacrylamide
Aluminum sulfate	10043-01-3	
Calcium chloride	10035-04-8 10043-52-4	

FOOD STANDARDS Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

8-04
20 October 2004

FINAL ASSESSMENT REPORT

APPLICATION A501

PHOSPHOLIPASE A_2 AS A PROCESSING AID (ENZYME)

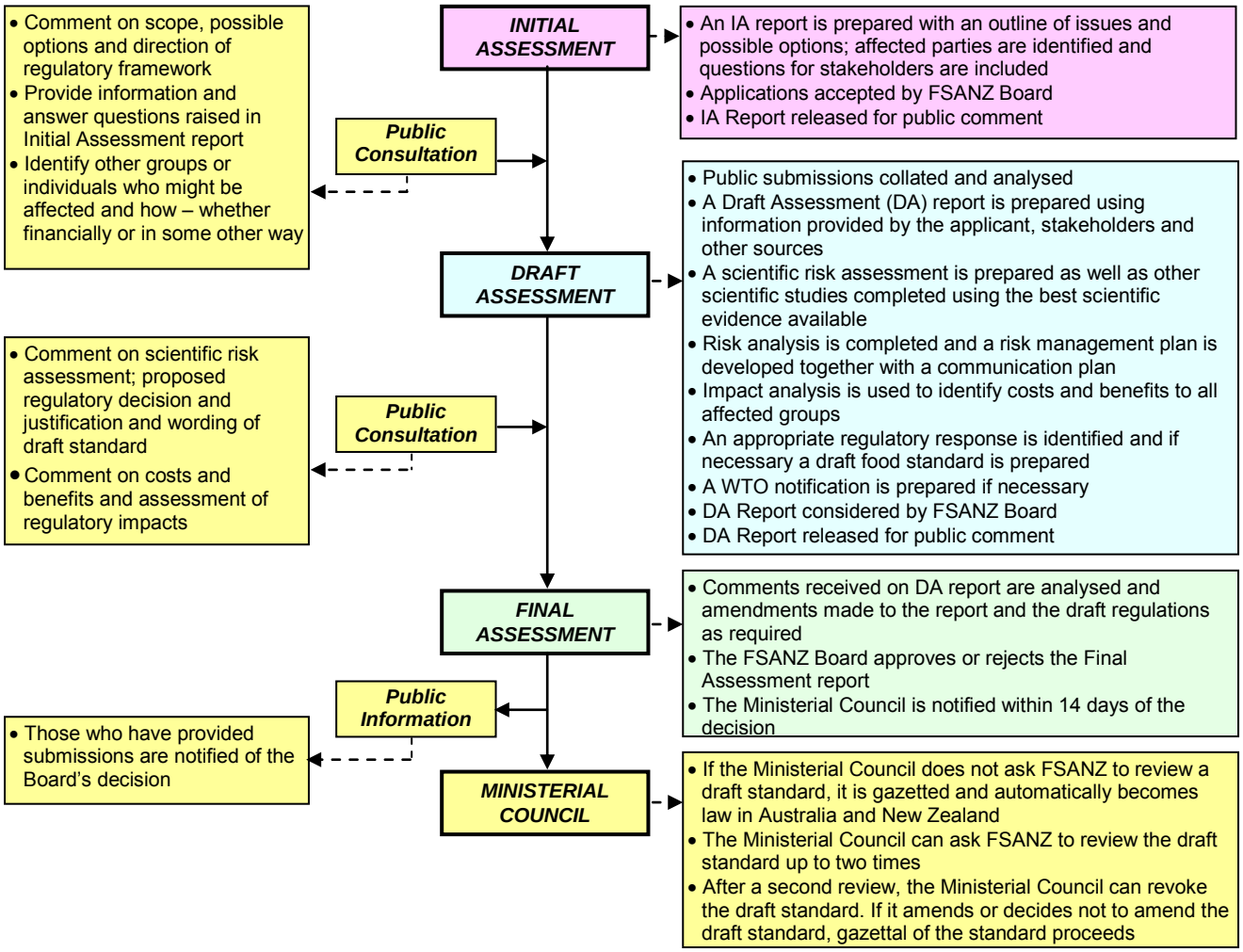
FOOD STANDARDS AUSTRALIA NEW ZEALAND (FSANZ)

FSANZ's role is to protect the health and safety of people in Australia and New Zealand through the maintenance of a safe food supply. FSANZ is a partnership between ten Governments: the Australian Government; Australian States and Territories; and New Zealand. It is a statutory authority under Commonwealth law and is an independent, expert body.

FSANZ is responsible for developing, varying and reviewing standards and for developing codes of conduct with industry for food available in Australia and New Zealand covering labelling, composition and contaminants. In Australia, FSANZ also develops food standards for food safety, maximum residue limits, primary production and processing and a range of other functions including the coordination of national food surveillance and recall systems, conducting research and assessing policies about imported food.

The FSANZ Board approves new standards or variations to food standards in accordance with policy guidelines set by the Australia and New Zealand Food Regulation Ministerial Council (Ministerial Council) made up of Australian Government, State and Territory and New Zealand Health Ministers as lead Ministers, with representation from other portfolios. Approved standards are then notified to the Ministerial Council. The Ministerial Council may then request that FSANZ review a proposed or existing standard. If the Ministerial Council does not request that FSANZ review the draft standard, or amends a draft standard, the standard is adopted by reference under the food laws of the Australian Government, States, Territories and New Zealand. The Ministerial Council can, independently of a notification from FSANZ, request that FSANZ review a standard.

The process for amending the *Australia New Zealand Food Standards Code* is prescribed in the *Food Standards Australia New Zealand Act 1991* (FSANZ Act). The diagram below represents the different stages in the process including when periods of public consultation occur. This process varies for matters that are urgent or minor in significance or complexity.



Final Assessment Stage

FSANZ has now completed two stages of the assessment process and held two rounds of public consultation as part of its assessment of this Application. This Final Assessment Report and its recommendations have been approved by the FSANZ Board and notified to the Ministerial Council.

If the Ministerial Council does not request FSANZ to review the draft amendments to the Code, an amendment to the Code is published in the *Commonwealth Gazette* and the *New Zealand Gazette* and adopted by reference and without amendment under Australian State and Territory food law.

In New Zealand, the New Zealand Minister of Health gazettes the food standard under the New Zealand Food Act. Following gazettal, the standard takes effect 28 days later.

Further Information

Further information on this Application and the assessment process should be addressed to the FSANZ Standards Management Officer at one of the following addresses:

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Assessment reports are available for viewing and downloading from the FSANZ website www.foodstandards.gov.au or alternatively paper copies of reports can be requested from FSANZ's Information Officer at info@foodstandards.gov.au including other general enquiries and requests for information.

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Executive Summary and Statement of Reasons

FSANZ received an Application on 12 May 2003 from Genencor International to amend Standard 1.3.3 – Processing Aids - of the *Australia New Zealand Food Standards Code* (the Code) to approve the use of an enzyme, phospholipase A_2 (EC number 3.1.1.4) sourced from *Streptomyces violaceoruber*, as a processing aid. The enzyme is not sourced from a genetically modified organism. Work commenced on this cost-recovered application on 9 July 2003.

Processing aids are required to undergo a pre-market safety assessment before approval for use in Australia and New Zealand. There is currently approval for the use of phospholipase A_2 derived from porcine pancreas in the Code. The objective of this assessment is to determine whether the Code should be amended to permit the use of phospholipase A_2 sourced from *S. violaceoruber*.

Phospholipase A_2 is used to hydrolyse lecithin to produce lysolecithin, which has improved emulsifying properties. Lysolecithin can perform as an emulsifier in non-fat based systems, unlike normal lecithin. It is anticipated by the applicant that the use of this enzyme derived from a microbial source may lead to foods with a broader acceptability than those produced with a porcine derived enzyme. The source organism, *S. violaceoruber,* does not have a history of safe use in the production of food enzymes.

Phospholipase A_2 preparations meet both the current Food Chemical Codex (FCC) specifications and the Joint WHO/FAO Expert Consultation on Food Additives (JECFA) Compendium of Specifications for Food Grade Enzyme Preparations.

Safety assessment

FSANZ has completed a comprehensive safety assessment of phospholipase A_2 derived from *S. violaceoruber*. The safety assessment concluded that:

- although the source organism does not have a history of safe use as a production strain for food-grade enzyme preparations, the pathogenicity study demonstrates that *S. violaceoruber* is non-pathogenic and non-toxigenic;
- the enzyme preparation complies with international specifications;
- the enzyme is not mutagenic in *in vitro* studies; and
- an acute toxicity study and a sub-chronic study in rats produced no evidence of toxicity.

The approval of phospholipase A_2 sourced from *S. violaceoruber* as a processing aid is technologically justified and does not raise any public health and safety concerns.

Impact of regulatory options

Two regulatory options were considered in the assessment: either (1) no approval; or (2) approval of the use of phospholipase A_2 sourced from *S. violaceoruber* as a processing aid based on the conclusions of the safety assessment. Following an assessment of the potential impact of each of the options on the affected parties (consumers, the food industry and government), option 2 is the preferred option as it potentially offers significant benefits to all sectors with very little associated cost.

The proposed amendment to the Code, giving approval to the use of phospholipase A_2 sourced from *S. violaceoruber* as a processing aid, is therefore considered to be of net benefit to both food producers and consumers.

Consultation

FSANZ undertook two rounds of public consultation in relation to this Application. In response, four submissions were received during the first round, and two submissions were received in the second round. Three of the first-round submitters expressed support for the Application, contingent on a satisfactory safety assessment, and the remaining submitter informed FSANZ that it would comment once the Draft Assessment Report was available. After the second round of consultation, both submissions were in support of the application. There were no specific comments on this application.

Statement of Reasons

The draft variation to Standard 1.3.3 – Processing Aids of the Code, thereby giving approval for the use of phospholipase A_2 sourced from *S. violaceoruber* as a processing aid is agreed for the following reasons.

- Use of the enzyme does not raise any public health and safety concerns.
- Use of the enzyme is expected to provide technological benefit to manufacturers.
- The source organism, *S. violaceoruber* is regarded as non-pathogenic and non-toxigenic.
- The proposed draft variation to the Code is consistent with the section 10 objectives of the FSANZ Act.
- The regulation impact assessment has concluded that the benefits of permitting use of the enzyme outweigh any costs associated with its use.

1. Introduction

FSANZ received an Application on 12 May 2003, from Genencor International to amend Standard 1.3.3 – Processing Aids – of the *Australia New Zealand Food Standards Code* (the Code) to approve the use of a enzyme, phospholipase A_2 (EC number 3.1.1.4), produced from a new source, as a processing aid. Work commenced on this cost-recovered application on 9 July 2003.

Phospholipase A_2 is sourced from *Streptomyces violaceoruber* (*S. violaceoruber*). The source organism does not have a history of safe use in the production of food enzymes. The organism has not been genetically modified.

The main function of phospholipase A_2 food manufacturing is to hydrolyse lecithin, producing a modified lecithin, called lysolecithin, with improved emulsifying power. Lysolecithin performs as an emulsifier in non-fat based systems, unlike normal lecithin. Lysolecithin can be used in the baking, confectionery, dairy fats and beverage industries but is not limited to these products. Currently, porcine pancreas is the only permitted source of phospholipase A_2. It is anticipated by the Applicant that the use of this enzyme derived from a microbial source may produce foods with a broader acceptability than those produced with a porcine derived enzyme.

2. Regulatory Problem

2.1 Current Standard

Under Standard 1.3.3 of the Code, processing aids are required to undergo a pre-market safety assessment before approval for use in Australia and New Zealand. A processing aid is a substance used in the processing of raw materials, foods or ingredients, to fulfil a technological purpose relating to treatment or processing, but does not perform a technological function in the final food. A processing aid used in the course of manufacture of a food must be used at the lowest level necessary to achieve a function in the processing of that food, irrespective of any maximum permitted level specified.

There is currently no approval for the use of phospholipase A_2 sourced from *S. violaceoruber* in the Code. Phospholipase A_2 is not listed in the Table to clause 17 of Standard 1.3.3 – Processing Aids, for permitted enzymes of microbial origin.

The source organism *S. violaceoruber* is not listed as an approved source for any other permitted enzymes listed in the Table to clause 17 of Standard 1.3.3.

3. Objective

The objective of this assessment is to determine whether the Code should be amended to permit the use of phospholipase A_2 derived from *S. violaceoruber*.

In developing or varying a food standard, FSANZ is required by its legislation to meet three primary objectives which are set out in section 10 of the FSANZ Act. These are:

- the protection of public health and safety;

- the provision of adequate information relating to food to enable consumers to make informed choices; and
- the prevention of misleading or deceptive conduct.

In developing and varying standards, FSANZ must also have regard to:

- the need for standards to be based on risk analysis using the best available scientific evidence;
- the promotion of consistency between domestic and international food standards;
- the desirability of an efficient and internationally competitive food industry;
- the promotion of fair trading in food; and
- any written policy guidelines formulated by the Ministerial Council.

4. Background

4.1 Historical Background

Phospholipase A_2 was the first phospholipase to be recognised. The enzyme is ubiquitous in nature and occurs in virtually all types of cells that have been examined. Phospholipase A_2 is a component of many animal and plant derived foods and thus has always been consumed by humans.

The *S. violaceoruber* sourced phospholipase A_2 is similar to the porcine pancreatic phospholipase A_2, which is a currently permitted enzyme of animal origin in the table to clause 15 of Standard 1.3.3 of the Code.

5. Relevant Issues

5.1 Nature of the Enzyme

The common name of the enzyme is phospholipase A_2. Other alternative names include lipase, lecithinase, lecithinase A, phosphatidase, phosphatidolipase, and phospholipase A, while the systematic name is phosphatidylcholine 2-acylhydrolase.

The Enzyme Commission number is EC 3.1.1.4 and the CAS registry number is 9001-84-7. The molecular weight of the enzyme is approximately 10-12 kDa.

The enzyme is characterised by its ability to catalyse the reaction:

phosphatidylcholine + $H_2O \rightarrow$ 1–acylglycerophosphocholine + carboxylic acid.

The products of lecithin hydrolysis are normal constituents of food and there are no known unintended reaction products formed by either enzymatic or chemical reaction of the components of the enzyme preparation with food.

5.2 Technological purpose of the enzyme

Phospholipase A_2 is used as a processing aid for the hydrolysis of lecithin, which results in the production of a modified lecithin with improved emulsifying power. Commercial lecithin is a naturally occurring mixture of phosphatides of choline, ethanolamine, and inositol, with smaller amounts of other lipids and is widely used in many categories of foods. The benefits of lecithin as an emulsifier in food processing are well known; however, the functionality of 'unmodified' lecithin is limited to fat-based systems. In aqueous systems, i.e., baked goods, lecithin must be structurally altered, either chemically or enzymatically, to exhibit good emulsifying properties. Chemical modification can be costly and non-specific, generating undesired hydrolysis products. Phospholipase A_2 hydrolyses the ester bond between the glycerol backbone and the fatty acid at the number 2 position of the glycerol backbone of lecithin, producing one molecule of lysolecithin and one molecule of fatty acid from one molecule of lecithin. The resulting lysolecithin product is a compound with emulsifying capabilities in many foods that are superior to that of the unmodified lecithin.

Pasteurisation and drying steps (if required) will inactivate the enzyme. The enzyme is to be used as a processing aid only and any residue would be in the form of inactivated enzyme, which would be metabolised like any other protein.

The Food Technology Report (Attachment 4) provides more information about the purpose and efficacy of the enzyme.

5.3 Safety assessment

Enzyme preparations used in food processing are generally considered to have low potential toxicity. The main toxicological consideration is in relation to possible contaminants arising from the host organism and the enzyme preparation production processes.

From the available data, the production organism *S. violaceoruber* is non-toxic and non-pathogenic. The enzyme preparation complies with international standards for enzyme preparations and with the recommended purity specifications for food-grade enzymes issued by the Joint FAO/WHO Expert Committee on Food Additives (JECFA)[1].

Six toxicological studies were submitted in support of this Application. These consisted of an acute toxicity study, a sub-chronic oral toxicity study, a bacterial mutation test, a mammalian cell mutagenicity test, a DNA repair assay and a study of the pathogenicity of *S. violaceoruber*.

The safety assessment of phospholipase A_2 from *S. violaceoruber* concluded that:

- although the source organism does not have a history of safe use as a production strain for food-grade enzyme preparations, the pathogenicity study demonstrates that *S. violaceoruber* is non-pathogenic;

1. Joint FAO/WHO Expert Committee on Food Additives (JECFA), 2001. General specifications and considerations for enzyme preparations used in food processing. FAO Food and Nutrition Paper 52, Add. 9, pp. 37-39.

- the enzyme preparation complies with international specifications;

- there was no evidence of toxicity in the acute toxicity study or in the sub-chronic toxicity study in rats;

- the NOEL from the sub-chronic feeding study was greater than 23 mg/kg bw per day, the highest dose level. Phospholipase A_2 is safe at the levels at which it is anticipated to be used; and

- the enzyme preparation produced no evidence of genotoxic potential in *in vitro* assays;

From the available information, it is concluded that the use of phospholipase A_2 as a processing aid in food would pose no public health and safety risk. The full toxicological evaluation is in Attachment 3.

5.4 Other International Regulatory Standards

The phospholipase A_2 preparations comply with specifications for enzyme preparations set forth in the *Food Chemical Codex* (FCC), 4th edition (National Academy of Sciences, 1996) and by the FAO/WHO Joint Expert Committee on Food Additives (JECFA, 2001, General specifications and considerations for enzyme preparations used in food processing; FAO Food and Nutrition Paper 52, Add. 9. pp. 37-39).

Phospholipase A_2 derived from *S. violaceoruber* was given GRAS status by the US Food and Drug Administration in July 2004. An application has also been made to Health Canada.

6. Regulatory Options

Option 1. Not approve the use of phospholipase A_2 sourced from *Streptomyces violaceoruber* as a food processing aid.

Option 2. Approve the use of phospholipase A_2 sourced from *Streptomyces violaceoruber* as a food processing aid.

7. Impact Analysis

7.1 Affected Parties

The affected parties to this Application include:

1. those sectors of the food industry wishing to produce and market food products produced using phospholipase A_2 as a processing aid;

2. consumers; and

3. Australian, State, Territory and New Zealand Government enforcement agencies that enforce food regulations.

7.2 Impact Analysis

7.2.1 Option 1: Maintain the status quo and not approve the use of phospholipase A_2 sourced from Streptomyces violaceoruber as a food processing aid.

There are no perceived benefits to industry, government regulators or consumers if this option is taken.

There are disadvantages to those food industries that wish to use the phospholipase A_2 enzyme.

7.2.2 Option 2: Amend the Code to approve the use of phospholipase A_2 sourced from Streptomyces violaceoruber as a food processing aid.

There are advantages to food manufacturers to be able to use phospholipase A_2. It is from a novel source and may allow kosher certification for foods produced using this enzyme, which would provide a variety of foods from which consumers could choose.

There should be no added costs to government regulators or consumers.

7.2.3 Discussion

Option 2, which supports the approval of phospholipase A_2 as a food processing aid is the preferred option, since it has advantages for the food industry and consumers but has no significant cost for government regulators, consumers or manufacturers.

8. Consultation

8.1 Public Consultation

The Initial Assessment of this Application was advertised for public comment between 13 August 2003 and 24 September 2003. Four submissions were received during this period with three expressing support for the application and one deferring comment until the Draft Assessment Report. A summary of these is included in **Attachment 3** to this Report.

Following the first round of consultation, FSANZ carried out an Draft Assessment of the Application, including a safety assessment of the processing aid, taking into account the comments received in the first round of consultation.

On completion of the Draft Assessment Report, further public comment was invited between 26 May 2004 and 7 July 2004. In response, FSANZ received two submissions that are summarised in **Attachment 3** to this Final Assessment Report. Both submissions supported the Application.

FSANZ has now completed the assessment of the Application, involving a safety evaluation and consideration of comments received in two rounds of public consultation. FSANZ will notify the outcomes of this Final Assessment Report to the Ministerial Council.

8.2 World Trade Organization (WTO)

As members of the World Trade Organization (WTO), Australia and New Zealand are obligated to notify WTO member nations where proposed mandatory regulatory measures are inconsistent with any existing or imminent international standards and the proposed measure may have a significant effect on trade.

Amending the Code to approve phospholipase A_2 as a processing aid is unlikely to have a significant effect on trade. The enzyme preparations are also consistent with the international specifications for food enzymes of Food Chemicals Codex (4th Edition, 1996) and JECFA, so the WTO was not notified.

9. Conclusion

The Final Assessment Report concludes that approval of the use of phospholipase A_2 sourced from *S. violaceoruber* as a processing aid is technologically justified and does not pose a public health and safety risk.

An amendment to the Code to give approval to the use of phospholipase A_2 sourced from *S. violaceoruber* as a processing aid in Australia and New Zealand is agreed on the basis of the available scientific information for the following reasons:

- Use of the enzyme does not raise any public health and safety concerns.
- Use of the enzyme is expected to provide technological benefit to manufacturers.
- The source organism, *S. violaceoruber* is regarded as non-pathogenic and non-toxigenic.
- The proposed draft variation to the Code is consistent with the section 10 objectives of the FSANZ Act.
- The regulation impact assessment has concluded that the benefits of permitting use of the enzyme outweigh any costs associated with its use.

The proposed draft variation is provided in **Attachment 1**.

10. Implementation and review

The draft variation will come into effect on the date of gazettal.

ATTACHMENTS

1. Draft variation to the *Australia New Zealand Food Standards Code*
2. Summary of Public Submissions
3. Safety Assessment Report
4. Food Technology Report

Attachment 1

Draft Variation to the *Australia New Zealand Food Standards Code*

To commence: on gazettal

[1] ***Standard 1.3.3*** *of the Australia New Zealand Food Standards Code is varied by inserting in the* Table to clause 17 –

Phospholipase A_2 EC [3.1.1.4]	*Streptomyces violaceoruber*

Attachment 2

Summary of Public Submissions

Summary of First Round of Public Submissions

Submitter	Position	Comments
1. Food Technology Association of Victoria	Supports Option 2	• Supports the approval of phospholipase A_2 as a processing aid.
2. New Zealand Food Safety Authority	-	• No comments at this stage. • Will review the safety data when it is prepared for the Draft Assessment Report.
3. Australian Food and Grocery Council	Supports Option 2	• Supports the approval of phospholipase A_2 as a processing aid.
4. Confectionery Manufacturers of Australasia Limited	Supports Option 2	• Supports the approval of phospholipase A_2 as a processing aid.

Summary of Second Round of Public Submissions

Submitter	Position	Comments
1. Food Technology Association of Victoria	Supports Option 2	• Supports the approval of phospholipase A_2 as a processing aid.
2. Australian Food and Grocery Council	Supports Option 2	• Supports the approval of phospholipase A_2 as a processing aid.

Attachment 3

Safety Assessment Report

A501 – PHOSPHOLIPASE A_2 DERIVED FROM *STREPTOMYCES VIOLACEORUBER*

1. Introduction

Application A501 seeks approval for the use of phospholipase A_2 from a non-genetically modified *Streptomyces violaceoruber* as a processing aid.

The enzyme is used as a processing aid only, and is not expected to be present in the final food. Any residue would be in the form of inactivated enzyme, which would be metabolised like any other protein.

2. The source (production) organism – *Streptomyces violaceoruber*

The safety of the production organism is an important consideration in the safety assessment of food enzymes. *S. violaceoruber* has not been used as a source organism for food enzymes in the past and is not the source for any currently approved enzymes within the Code.

The strain used for production of the enzyme preparation is a derivative of *S. violaceoruber* strain ATCC 14980, the type strain of the species. The production strain was developed by Enzyme Bio-Systems Ltd. and then transferred to Genencor International Inc USA and is designated Genencor GICC03161. It has not been modified using recombinant DNA techniques. What is now classified as *S. violaceoruber* was first described in 1916 by Waksman and Curtis (1916) and named *Actinomyces violaceus ruber*.

Phospholipase A_2 is the first enzyme product produced by *S. violaceoruber*. Therefore, specific information about the strain lineage and safety of products from *S. violaceoruber* is not publicly available. However, the applicant conducted an evaluation of the *S. violaceoruber* virulence potential in Balb/c mice by oral intubation and intraperitoneal injection (described in Section 4.4) and found it to be neither pathogenic nor toxic by either route of administration.

In addition, literature searches conducted by the Applicant and confirmed by FSANZ indicate that *S. violaceoruber* is widely distributed in nature (Locci, 1986) and is considered to be non-pathogenic and a safe source of food grade phospholipase A_2 (Pariza and Johnson, 2001). Furthermore, the commercial enzyme product (phospholipase A_2) has undergone several filtration steps and the production organism, *S. violaceoruber*, is not present in the product at a detection limit of <1 CFU/ml.

3. Purity of enzyme preparation and proposed specifications

Historically, enzymes used in food processing have been found to be non-toxic, and the main toxicological consideration is in relation to possible contaminants. The production organism in this case is considered to be non-toxic and non-pathogenic. The detailed specifications to which the preparation was found to conform are shown in Table 1.

Table 1. Complete specification of phospholipase A_2 preparation

Criteria	Specification
Phospholipase A_2 activity (U/g)	Between 400 and 600
Alpha Amylase activity (U/kg)	Between 0 and 10
Total viable count (cfu/mL)	Not more than $5x10^4$
Total coliforms (cfu/mL)	Not more than 30
E. Coli	Negative by test
Salmonella	Negative by test
Moulds (cfu/mL)	Not more than 100
Yeasts (cfu/mL)	Not more than 100
Production strain	Negative by test
Antibacterial activity	Negative by test
Heavy Metals as Pb (mg/kg)	Not more than 30
Arsenic (mg/kg)	Not more than 3
Cadmium (mg/kg)	Not more than 0.50
Mercury (mg/kg)	Not more than 0.50
Lead (mg/kg)	Not more than 5
Mycotoxins	No data
Potassium Sorbate (% w/w)	Between 0.10 and 0.30
Sodium Benzoate (% w/w)	Between 1.3 and 1.7

Phospholipase A_2 from the source organism, *S. violaceoruber* complies with the recommended purity specifications for food-grade enzymes.

4. Evaluation of the submitted studies

Six toxicological studies were submitted in support of this application. These were:

1. an acute oral toxicity study in rats,
2. a 90-day sub-chronic oral toxicity study in rats,
3. a Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames test).
4. a mutagenicity test (Mouse lymphoma forward mutation assay)
5. a DNA repair assay on rat liver primary cell cultures (unscheduled DNA synthesis)
6. A study of the pathogenicity of *S violaceoruber* on mice.

4.1 Acute toxicity study

Acute oral toxicity in the rat. Study Director: Steven M. Glaza. Hazelton Wisconsin Inc. Report no. HWI 01200944. 28 March 1991.

Test material	Phospholipase A_2, batch number 757677 'Brown liquid'
Vehicle control	deionised water
Test Species	10 female and 10 male Crl: CD®BR rats; administration by gavage
Dose	20 g/kg body weight
GLP/guidelines	Guide for the Care and Use of Laboratory Animal. NIH Publication No. 86-23 (revised 1985)

Twenty male and twenty female fats were assigned to two treatment groups consisting of a test group and vehicle control group. The rats received single doses of phospholipase A_2 (or distilled water control) administered orally by gavage. Clinical signs and mortality checks were conducted at 1, 2.5, and 4 hours after the administration of the test material. The animals were observed daily thereafter for 14 days for clinical signs and twice a day (morning and afternoon) for mortality. Qualitative food consumption and the general appearance of the faeces were also noted on a daily basis. Body weights were measured prior to dosing, at day 7 and 14. All animals in the test group and vehicle control group appeared clinically normal and gained weight throughout the study. All animals survived until day 14 when they were sacrificed and necropsy was performed.

At necropsy, the large pelvis observed in the right kidney of two male rats (one control rat and one test rat) and the diffusely dark red mandibular lymph nodes of one female test rat were considered incidental findings. There were no visible lesions in any of the remaining animals.

The estimated LD_{50} for male and female rats was determined to be greater than 20 mg/kg body weight.

4.2 Sub-chronic toxicity

A 13-week oral (gavage) toxicity study in rats. Study Director: H. Voute, MDS Pharma Service, France. Study no. 75/005 19 November 2003.

Test material	Phospholipase A_2
Control and vehicle material	Sterile water
Test Species	Sprague-Dawley rats 10 males and females per test dose; administration by gavage
Dose	0, 5.75, 11.5, 23 mg phospholipase A_2 per kg body weight per day
GLP/guidelines	OECD guideline No. 408

Study conduct

Four groups of rats (10/sex/group) were treated with phospholipase A_2 by gavage at 0, 5.75, 11.5, 23 mg/kg bw per day for 90 days.

Morbidity/mortality checks were performed at least twice daily. Clinical observations were recorded daily and more detailed clinical examination performed once a week. Behavioural and functional tests were performed during week 13. In week 13 sensory reactivity and grip strength was assessed. Bodyweight and food consumption were recorded weekly; haematology and clinical chemistry at the beginning of week 14; and ophthalmology performed on all animals before the start of the study and on animals from groups 1 and 4 near termination. At the end of the study, all animals were sacrificed and a complete necroscopy performed (gross examination, organ weights and histopathology on selected organs).

Results

All animals survived until the end of the study period. There were no treatment-related clinical signs. There were no treatment-related effects in behavioural and functional tests and there were no treatment-related opthalmological findings. Corneal opacity was noted in three control males and two high dose group males and is commonly encountered in rats of this strain and age. Thus these findings were considered to be incidental. There was some variation in body weight gain among male and female rats in all groups, however these were neither dose-related nor of a sufficient magnitude to be considered to have any toxicological significance.

There were no treatment-related changes in serum clinical chemistry parameters evaluated. A statistically significant decrease (6%) in potassium levels in female rats in the high dose group was not considered to be toxicologically significant. A statistically significant increase in the level of ALAT in the male group treated at 11.5 mg/kg/day was considered to be incidental as it was not dose related and was the result of an individual elevated value (for male number 25). There were no treatment-related effects on all urinary parameters evaluated.

There was a statistically significant increase in the absolute weight of the prostate in male rats treated with 5.75 mg/kg bw/day and 23 mg/kg bw/day but not at 11.5 mg/kg bw/day. The increases were 25% (5.75 mg/kg bw/day), 20% (11.5 mg/kg bw/day, not statistically significant), and 27% (23 mg/kg bw/day) compared to the control group.

The prostate weights relative to body weight from all treated groups were statistically significantly higher than the control weights (increases of 27%, 27% and 35% respectively). However, the study authors considered the increase in relative prostate weight of no toxicological importance, because the mean absolute weight of the prostate in the control group was lower than the mean absolute weight of the prostate in males of the same age when compared to historical controls.

The NOAEL was 23 mg/kg bw per day, based on the maximum dose tested in this study.

4.3 Genotoxicity studies

Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test). Study Director: T. E. Lawlor, Hazelton Washington Inc., Report No. 14647-0-401R. 6 November 1991.

A Salmonella/Mammalian-Microsome Reverse Mutation Assay (Ames Test) was performed on phospholipase A_2, however, the test article could not be adequately evaluated using this system due to the test article's interference with the selective conditions of the assay.

Mouse Lymphoma Forward Mutation Assay. Study Director: M. A. Cifone, Hazleton Washington Inc, Maryland USA. Report No. 14647-0-431R. 9 October 1991.

Test article

The test article was phospholipase A_2, described as brown flakes and labelled as Lecithinase.

Study design

Phospholipase A_2 was examined for mutagenic activity using the mouse lymphoma forward mutation assay. The mouse lymphoma forward mutation assay evaluated the test article's mutagenic potential in a specific locus mutation assay using mammalian cells in culture. The objective of this study was to evaluate the ability of phospholipase A_2 to induce forward mutations at the thymidine kinase (TK) locus in L5178Y TK+/- mouse lymphoma cells as assayed by colony growth in the presence of 5-trifluorothymidine (TFT).

Phospholipase A_2 was dissolved in media and filter-sterilised. Filtered stock was analysed by the sponsor for enzyme activity. This was determined to be normal.

Positive controls were treated with the known mutagens ethylmethane sulfonate (EMS) and methylmethane sulfonate (MMS) at 0.25 µL/mL and 0.4 µL/mL, and 10.0 nL/mL and 15 nL/mL respectively.

A preliminary cytotoxicity experiment was performed to establish an appropriate concentration range for the mutation experiment. This study was performed with and without S9 metabolic activation since substantial shifts in toxicity can often occur for the two test conditions. Ten dose levels were used in each case that ranged from 9.75 µg/mL to 5000 ug/mL (4450 µg/mL +S9). The preliminary cytotoxicity test showed the test material to be toxic to mouse lymphoma cells in culture. The test material was more toxic with S9 metabolic activation than without. Without activation, moderate reductions in cell growth were observed at 313 µg/mL with total cell killing obtained at 625 µg/mL and higher. These results were used to select dose levels for the mutation assays.

Test	*Test material*	*Concentration*	*Test object*	*Result*
Reverse mutation (*In vitro*)	Phospholipase A_2	**Non-activation** Trial 1: 156 µg/mL to 3000 µg/mL Trial 2: terminated Trial 3: 62.5 µg/mL to 2500 µg/mL Trial 4: 150 µg/mL to 2500 µg/mL	*mouse lymphoma L5178Y cell line*	-ve
		Activation Trial 1: 25 µg/mL to 600 µg/mL Trial 2: terminated Trial 3: 12.5 µg/mL to 500 µg/mL Trial 4: 75 µg/mL to 500 µg/mL		-ve

Results

Four mutation assays were initiated with the test material using non-activation conditions, however only three trials were completed. One of the assays was terminated (trial two) before subculture due to excessive cyto-toxicity. Dose related toxicity was obtained in the three completed trials. In trial one, eight dose levels were used that ranged from 156 µg/mL to 3000 µg/mL. The highest dose level (3000 µg/mL) was not used in the evaluation due to less than 10% relative growth. The remaining treatments induced a good range of toxicities. In order for a culture to be evaluated as mutagenic, a mutant frequency of equal to or greater than two times the concurrent background mutant frequency is required. The background mutant frequency is defined as the average mutant frequency of the negative control cultures. No culture treated with the test material had a mutant frequency that was significantly mutagenic.

In trial three, nine dose levels were used that ranged from 62.5 µg/mL to 2500 µg/mL. The dose levels of 1500 µg/mL and higher induced excessive cyto-toxicity and were not acceptable for analysis. No culture treated with test material that had acceptable levels of toxicity had a mutant frequency that was significantly increased compared to the control frequency.

In trial four, nine treatments from 150 µg/mL to 2500 µg/mL were initiated and treatments at 2000 µg/mL and 2500 µg/mL were terminated because of excessive cyto-toxicity. The remaining seven treatments were cloned and a good range of cyto-toxicities were induced (95.9% to 10.6% relative growths). None of the treatments induced a mutant frequency that exceeded the minimum mutagenicity criterion. Since no significant, repeatable increases were observed in any of the three trials, phospholipase A_2 was evaluated as negative for inducing forward mutation at the TK locus in mouse lymphoma cells under non-activation test conditions.

In the presence of metabolic activation, four mutation assays were initiated, but only three completed. One of the assays (Trial 2) was terminated before subculture due to excessive cyto-toxicity. Dose related toxicity was obtained in the three completed trials. In the three completed trials, dose levels were used that ranged from 25 µg/mL to 600 µg/mL. However doses above 300 µg/mL were terminated because of excessive cyto-toxicity. No culture treated with test material that had acceptable levels of toxicity had a mutant frequency that was significantly increased compared to the control frequency.

The assays used in this study met all assay acceptance criteria. The average cloning efficiencies for the negative controls varied from 74.5% to 101.4% without activation and from 88.6% to 127.9% with S9 metabolic activation, which demonstrated good cloning conditions for the assays. In each trial the average negative control mutant frequency was within the acceptable range. The positive control cultures had mutant frequencies that were greatly in excess of the background and met assay acceptance criteria.

Conclusion

In the preliminary cytotoxicity assay, cells were exposed to the test material for four hours in the presence and absence of rat liver S9 metabolic activation. Under non-activation conditions, phospholipase A_2 was highly toxic at 2500 µg/mL and lethal at 5000 µg/mL. In the presence of S9 activation, toxicity was more pronounced: treatment at 313 µg/mL was moderately toxic and at the next highest dose level (625 µg/mL) and above the test material was lethal.

Three acceptable non-activation and S9 activation mutation assays were performed using single cultures per dose levels. The test material produced dose related increases in toxicity in all mutation trials. In the three non-activation trials, no significant increases were observed except for one small increase at excessively high toxicity. A similar effect was observed in the presence of metabolic activation. One small increase was observed at very high toxicity. These increases were not repeatable and were considered spurious. Therefore, the test material was evaluated as negative for inducing forward mutations at the TK locus in L5178Y mouse lymphoma cells under the non-activation and S9 metabolic activation conditions used in this study.

Unscheduled DNA Synthesis in Rat Liver Primary Cell Cultures with a Confirmatory Assay. Study Director: M.E. McKeon, Hazleton Washington Inc, Maryland USA. Report No. 14647-0-447. 11 October 1991.

Test article

The test article was phospholipase A_2, described as brown flakes and labelled as Lecithinase.

Study design

The objective of this assay was to detect DNA damage caused by the test material, or an active metabolite, by measuring unscheduled DNA synthesis (UDS) in rat primary hepatocytes *in vitro*. The existence and extent of DNA damage was inferred from an increase in net nuclear grain counts in treated hepatocytes when compared to untreated hepatocytes.

Fresh hepatocytes obtained from adult male Fischer 344 rats were treated with phospholipase A_2 at concentrations ranging from 5 µg/mL to 500 µg/mL and 1 mM ^{3}H-thymidine. A positive control experiment included the compound 2-acetylaminofluorene (2-AAF) in DMSO, which is known to induce UDS.

To determine the appropriate dose, a range of 15 concentrations of phospholipase A_2 was applied initially to the cells. A viable cell count was then obtained 20 hours after initiation of treatment. Toxicity data obtained was used to select 10 dose levels for a second trial of the UDS. In Trial 2 a viable cell count was performed at 20 hours after dosing and six concentrations were chosen for analysis of nuclear labelling, starting with the highest dose that resulted in a sufficient number of survivors with intact morphologies and proceeding to successively lower doses.

Two UDS assays were performed. Doses used were 5, 10, 25, 50, 100, and 250µg phospholipase/mL in assay one and 10, 25, 50, 100, 250, 500µg/mL in assay two. Cells were established in culture for 1.6 and 1.8 hours respectively, at approximately 37ºC in a humidified atmosphere containing 5% CO_2. Unattached cells were then removed and the cultures were refed with media. The UDS assays were initiated 2.1 hours (assay one) and 2.8 hours (assay two) later by replacing the media in the culture dishes with 2.5 mL media containing the test material at the desired concentration. Each treatment, including the controls, was performed on five cultures, two of which were used for cytotoxicity measurements. After treatment for 18.7 (assay one) and 18.2 hours (assay two) the UDS assay was terminated by washing the cell monolayers twice with fresh media. Three of the cultures from each treatment were washed with media containing 1 mM labelled thymidine.

The remaining two cultures used to monitor the toxicity of each treatment were refed with media and returned to the incubator. At 20.1 hours after the initiation of the treatments, trypan blue was added to the cultures and viable cell counts were determined to estimate cell survival relative to the negative control.

UDS in the labelled cells was measured by counting nuclear grains and subtracting the average number of grains in three nuclear-sized areas adjacent to each nucleus (cytoplasmic count). The net nuclear grain count was determined for at least fifty randomly selected cells for each culture. Only nuclei with normal morphologies were scored, and any occasional nuclei blackened by grains too numerous to count were excluded as cells in which replicative DNA synthesis occurred rather than repair synthesis.

Results

Results are shown in tables 2 and 3.

Conclusions

The test material, phospholipase A_2, did not induce significant changes in the nuclear labelling of rat primary hepatocytes in two independent trials for an analysed concentration range of 500 µg/mL to 5.0 µg/mL. Therefore, phospholipase A_2 was evaluated as inactive in the assay for UDS in Rat Primary Liver Cell Cultures with a Confirmatory Assay.

Table 2: Summary of data from trial one of the rat hepatocyte UDS assay

Test Condition	Concentration µg/mL	Mean Net Nuclear Grains (NNG)[1]	% Cells w/≥ 5 Mean NNG[2]	Mean Cyto Grains[3]	% Survival at 20.1 hours[4]
Negative Control	-	-2.30	6.00	10.64	100.0
Positive Control (2-AAF)	0.100	25.57[a]	96.00[a]	14.43[a]	81.2
Test material	250	-2.69	2.69	11.95	72.3
	100	-2.86	0.67	8.87	88.0
	50	-2.27	2.67	12.67	91.3
	25	-2.73	2.00	12.46	99.3
	10	-1.47	3.33	9.35	103.2
	5	-1.44	4.00	11.73	100.8

[1] Average of net nuclear grain counts on triplicate coverslips (150 total cells). Net nuclear grains = nuclear grain count – average cytoplasmic grain count.
[2] Average percentage of cells with greater than or equal to 5 net nuclear grains on triplicate coverslips (150 total cells).
[3] Average of cytoplasmic grain counts on triplicate coverslips (150 total cells).
[4] Survival = Number of viable cells per unit area relative to the negative control.
[a] 1 slide not analysed; UDS = average of mean NNG counts on 2 coverslips (100 total cells).
2-AAF = 2-acetylaminofluorene

Table 3: Summary of data from trial two of the rat hepatocyte UDS assay

Test Condition	Concentration µg/mL	Mean Net Nuclear Grains (NNG)[1]	% Cells w/≥ 5 Mean NNG[2]	Mean Cyto Grains[3]	% Survival at 20.1 hours[4]
Negative Control	-	-0.95	2.67	5.65	100.0
Positive Control (2-AAF)	0.100	15.43	95.33	8.86	95.9
Test material	500	-1.60	0.67	5.79	53.9
	250	-3.40	0.67	9.19	78.0
	100	-2.78	2.67	9.15	87.0
	50	-1.81	0.67	7.85	93.1
	25	-1.27	1.33	6.89	105.5
	10	-2.09	1.33	7.83	107.2

[1] Average of net nuclear grain counts on triplicate coverslips (150 total cells). Net nuclear grains = nuclear grain count – average cytoplasmic grain count.
[2] Average percentage of cells with greater than or equal to 5 net nuclear grains on triplicate coverslips (150 total cells).
[3] Average of cytoplasmic grain counts on triplicate coverslips (150 total cells).
[4] Survival = Number of viable cells per unit area relative to the negative control.
2-AAF = 2-acetylaminofluorene

4.4 Pathogenicity study.

Evaluation of *Streptomyces violaceoruber* for mouse toxicity. University of South Alabama. 1 August 1990.

Test article

The test article was the production organism, *S. violaceoruber*.

Study design

Viable cells of the production micro-organism, *S. violaceoruber*, were given to groups of eight male Balb/c mice each by either intraperitoneal injection (IP) or by gavage at nine doses ranging from 0.00025 mg/kg to 5000 mg/kg. Dry weights were used for dosage due to the filamentous nature of the organism.

Groups of eight control mice were dosed (by gavage or IP) with suspension buffer, sterile broth or filtered spent growth medium.

All mice were observed for signs of toxicity hourly for the first four hours following treatment, then daily for three weeks. After three weeks mice were sacrificed and a necropsy performed on two from each test group.

Following treatment, all mice injected with the test organism showed slight distress evidenced by ruffled fur and the huddling together of cage occupants. These symptoms disappeared within four hours and were more pronounced in those mice receiving doses greater than 250 mg/kg body weight. No other morbidity was observed for the remainder of the holding period. No abnormalities were seen in the mice necropsied at sacrifice.

Conclusion

Under the conditions of test, *S. violaceoruber* ATCC 14980 exhibited no evidence of pathogenicity of toxicity for Balb/c mice.

5. Overall Conclusion

The safety assessment of phospholipase A_2 from *S. violaceoruber* concluded that:

- although the source organism does not have a history of safe use as a production strain for food-grade enzyme preparations, the pathogenicity study demonstrates that *S. violaceoruber* is non-pathogenic;
- the enzyme preparation complies with international specifications;
- there was no evidence of toxicity in the acute toxicity study or in the sub-chronic toxicity study in rats;
- the NOEL from the sub-chronic feeding study was greater than 23 mg/kg bw per day, the highest dose level. Phospholipase A_2 is safe at the levels at which it is anticipated to be used; and
- the enzyme preparation produced no evidence of genotoxic potential in *in vitro* assays;

From the available information, it is concluded that the use of phospholipase A_2 as a processing aid in food would pose no public health and safety risk.

References

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Pariza, M. W. and Johnson E. A. (2001). Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Reg. Toxicol. Pharmacol. 33:173-186.

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Attachment 4

Food Technology Report

Phospholipase A_2

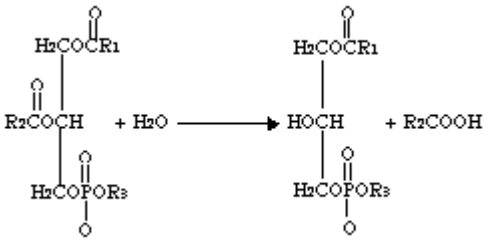
Introduction

Microbial enzyme preparations have been widely used of a variety of purposes in the production of numerous food products for many years. Their practical application in fermented products dates back many centuries long before the nature and function of enzymes or even the micro-organisms themselves, were known or understood (Bechhom, Labbee and Underkofler, 1965).

Enzyme: 3.1.1.4 Phospholipase A_2

Reaction: Phospholipase A_2 represents a class of heat-stable, calcium-dependent enzymes catalysing the hydrolysis of the 2-acyl bond of 3-n-phosphoglycerides.

Phosphatidylcholine + H_2O → 1-acylglycerophophocholine + carboxylic acid (fatty acid)



Other names: Lecithinase A; Phosphatidase; Phosphatidolipase

Systematic name: Phosphatidylcholine 2-acetylhydrolase (IUBMB, 1992)

Enzyme source and production

The application is for Phospholipase A_2 that is a food enzyme derived from *Streptomyces violaceoruber* that is used as a processing aid for the hydrolysis of lecithin, which results in the production of a modified lecithin, referred to as lysolecithin, with improved emulsifying power. Phospholipase A_2 has also been isolated from porcine pancreas, snake and bee. The applicant envisages that the cost of the microbial phospholipase A_2 will be similar, on an activity basis, to the animal derived version.

In the manufacture of any commercial microbial enzyme an important step is the selection of an organism, that when grown in pure culture, produces the desired enzyme in good yield. The reactions catalysed by any given active component are essentially the same, regardless of the source from which that component is derived (Food Chemicals Codex, 1996).

Applications

Commercial lecithin is a naturally occurring mixture of phosphatides of choline, ethanolamine and inositol, with smaller amounts of lipids. Lecithin is widely used in many categories of food as an emulsifier. Lecithin functions effectively as an emulsifier in fat-based food systems. For aqueous food systems such as baked goods, lecithin must be altered structurally either chemically or enzymatically, to function effectively as an emulsifier. If one of the fatty acids present on the middle or '2' position of the glycerol backbone is removed, leaving only one fatty acid at the '1' position, then lysolecithin is formed.

Table 1. Important food applications for lecithins

Application	Typical Function
Bakery goods	Improvement of volume
	Fat dispersion
	Anti-staling
Chocolate	Reduction of viscosity
	Prevention of crystallisation
Instant products	Wetting
	Dispersion
Margarine and edible oil spreads	Stabilization of product
	Prevention of spattering
	Browning and dispersion of sediment

(Van Nieuwenhuyzen, 1981)

Enzymic modification has advantages over chemical modification in that chemical modification, generate non-specific hydrolysis products and can be costly.

The use of lysolecithin for food applications has distinct advantages over lecithin. Lysolecithin is able to better stabilize the oil-in-water emulsions in many food products than lecithin.

Modified lecithins have many uses in foods (Meinhold, 1991; van Nieuwenhuyzen, 1981) including, but not limited to bakery, confectionery, dairy, edible oil and beverage products. In these products, the modified lecithin can act as an emulsifying agent, a mixing aid, a release agent, an egg replacer, and as a flavour in food systems.

For example, traditional mayonnaise can be considered as an acidic oil-in-water emulsion, which is stabilised by egg yolk. The stabilising power of egg yolk is due mainly to the presence of lipoproteins. One of the problems in mayonnaise production is the breaking of the emulsion, which leads to oil exudation. This occurs when the temperature is raised over 70 °C, or cooled below 0 °C or when too much shear is applied. Treatment of egg yolk with phospholipase A_2 results in hydrolysis of the phospholipids (lecithin). Egg yolk fermented with phospholipase A_2 has been shown to be a more potent emulsifier for mayonnaise than untreated egg yolk. Treated egg yolk in mayonnaise withstood heating at 100 °C for 30 min without the emulsion breaking (Dutilh and Groger 1981).

Stability in processing

Phospholipase A_2 can hydrolyse lecithin to lysolecithin under a wide range of conditions. The enzyme's activity rises with increasing temperature and is greatest about 40°C. Above 50 °C, thermal decay becomes increasingly significant as enzyme stability fails.
S. violaceoruber derived phospholipase A_2 is active over a wide pH range, depending on the specific application. The optimum pH for activity is near pH 8.5. The usage level will vary according to the application and desired degree of enzymic conversion.

Conclusion

Phospholipase is used as a processing aid to improve the emulsifying capabilities of lecithin which are then added to foods to improve the desired characteristics of the food.

At present, the only source of phospholipase A_2 permitted in the *Australia New Zealand Food Standards Code*, Standard 1.3.3 - Processing Aids, is an animal-derived enzyme from porcine pancreas. Approving phospholipase A_2 produced from *S. violaceoruber* will allow food manufactures an alternative source.

The advantage to the manufacturer and final consumer are in the benefits the lysolecithin imparts on food such as emulsification properties and improved heat stability in foods, including mayonnaise, ice-cream, margarine, and baked goods. Consumers may also benefit by having a greater choice of new, heat-stable foods that are developed by food manufacturers.

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Bechhom, E.J. M.D. Labbee, and L.A. Underkofler. 1965 Production and use of microbial enzymes for food processing. J. Agric. Food Chem. 13;30-34.

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Van Nieuwenhuyzen, W. The industrial uses of special lecithins: a review. JAOCS, Oct. 1981, p. 886-888.

FOOD STANDARDS Australia New Zealand
Te Mana Kounga Kai – Ahitereiria me Aotearoa

28 October 2008
[18-08]

APPLICATION A1004
PHOSPHOLIPASE A_2 AS A PROCESSING AID (ENZYME)
ASSESSMENT REPORT

Executive Summary

Purpose

Food Standards Australia New Zealand (FSANZ) received a paid Application (A1004) from DSM Food Specialties Pty Ltd on 21 January 2008. The Application seeks to amend Standard 1.3.3 – Processing Aids of the *Australia New Zealand Food Standards Code* (the Code) to include *Aspergillus niger* (*A. niger*), containing the gene for phospholipase A_2 isolated from porcine pancreas. This is a new microbial source of the enzyme, phospholipase A_2 (EC number 3.1.1.4), to be included in the Table to clause 17 – Permitted enzymes of microbial origin.

Processing aids are required to undergo a pre-market safety assessment before approval for use in Australia and New Zealand. Phospholipase A_2 derived from porcine pancreas is currently listed as a permitted processing aid in Standard 1.3.3 – Processing aids in the Table to clause 15 – Permitted enzymes of animal origin. Similarly phospholipase A_2 from the microbial source, *Streptomyces violaceoruber* (*S. violaceoruber*), is listed in the Table to Clause 17 – Permitted enzymes of microbial origin.

The phospholipase A_2 enzyme's primary use is to increase the efficacy of phospholipids, such as lecithin, used as an emulsifier in aqueous food products, such as bakery products, sauces and dressings. The Applicant claims that the phospholipase A_2 enzyme acts as a processing aid in exactly the same way as phospholipase A_2 enzyme derived from porcine pancreas and from other microbial sourced phospholipase A_2 enzymes.

The enzyme preparation meets the international specifications for enzymes. The enzyme has been approved for use in France and the Applicant has received a no-objection letter from the US Food and Drug Administration (FDA) after submitting a GRAS (Generally Recognised As Safe) notification. In addition to this Application, further applications have or will be made in Denmark, China, Mexico, Brazil and Canada, by DSM for the approval of this enzyme.

The Application is being assessed under the General Procedure.

Safety Assessment

FSANZ has completed a Safety Assessment Report for phospholipase A_2 derived from genetically modified *A. niger* with a gene isolated from the porcine pancreas. No toxicology or hazard-related concerns were identified as a result of this safety assessment.

The hazard assessment of the submitted studies concluded that:

- there was no evidence of toxicity in single or repeat-dose toxicity studies;
- bacterial reverse mutation and mouse micronucleus assays were negative; and
- the chromosomal aberration assay for the enzyme was positive (i.e., clastogenic) in the absence of S9 in human peripheral blood lymphocytes. The positive finding was not considered to be indicative of mutagenic potential *in vivo* based on the weight of evidence from the negative bacterial reverse mutation assay, negative *in vivo* micronucleus studies and submitted discussion and references.

Based on the available evidence, it was concluded that the submitted studies did not reveal any toxicology or hazard–related concerns with the phospholipase A_2 enzyme that would be a reason to not list the enzyme as a food processing aid. The absence of any specific hazards being identified is consistent with phospholipase A_2 undergoing normal proteolytic digestion in the gastrointestinal tract.

The Acceptable Daily Intake (ADI) for phospholipase A_2 is 'not specified'

Dietary Exposure Assessment

There are no nutritional or dietary implications in approval of the enzyme since there will be no or very little residual inactivated enzyme present in the final foods. Any remaining enzyme would be metabolised like any other protein. Extensive dietary modelling is not required for the use of the enzyme since it will be used as a processing aid and the majority of the enzyme will be removed from the final food product.

Labelling

If approved, food manufacturers using phospholipase A_2 sourced from genetically modified, *A. niger*, will not be required to be label their food as genetically modified as there are no novel DNA and/or no novel proteins present in the final food product. The source organism is killed off and removed during the formulation manufacturing process used for producing the enzyme preparation. This is the case for a number of enzymes sourced from genetically modified microorganisms approved in the Code.

Phospholipase A_2, is a normal constituent of wheat flour and phospholipase A_2 itself is not considered to be allergenic. However, the Applicant indicates that the granulated formulation (e.g. as used in bakery products) may be granulated on wheat flour. The use of this formulation would require wheat flour (gluten) to be declared in the product under the Standard 1.2.3 – Mandatory Warning and Advisory Statements and Declarations.

According to the Applicant, the liquid formulation is diluted with water; therefore there would be no labelling requirement under Standard 1.2.3. The liquid formulation does not contain any known allergens. The Code does not define the meaning of Vegetarian, Halal or Kosher and as such issues relating to these aspects are outside of the scope of this Application.

Assessing the Application

In assessing the Application and the subsequent development of a food regulatory measure, FSANZ has had regard to the following matters as prescribed in section 29 of the FSANZ Act:

- whether costs that would arise from an amendment to the Code to permit the use of the enzyme phospholipase A_2 sourced from *A. niger* expressing a gene isolated from porcine pancreas would outweigh the direct and indirect benefits to the community, Governments or industry;
- there are no other measures that would be more cost-effective than a variation to Standard 1.3.3 that could achieve the same end;
- there are no relevant New Zealand standards; and
- there are no other relevant matters.

Preferred Approach after Assessment

FSANZ recommends the proposed draft variation to the Table to clause 17 of Standard 1.3.3 – Processing Aids, to permit the use of the enzyme phospholipase A_2 sourced from *Aspergillus niger* containing the phospholipase A_2 gene isolated from porcine pancreas.

Reasons for Preferred Approach

An amendment to the Code to permit the use of phospholipase A_2 sourced from *A. niger* containing the gene isolated from porcine pancreas as a processing aid in Australia and New Zealand is recommended. This is on the basis of :

- A detailed safety assessment has concluded that there were no toxicology / safety related concerns with the enzyme phospholipase A_2 sourced from genetically modified *A. niger* with the gene isolated from porcine pancreas.
- Use of the enzyme from this source is expected to provide technological benefit to manufacturers.
- The source organism, *A. niger* is regarded as non-pathogenic and non-toxigenic.
- The regulation impact assessment has concluded that the benefits of permitting the use of this enzyme outweigh any costs associated with its use.
- There are no other measures that would be more cost-effective than a variation to Standard 1.3.3 that could achieve the same end.
- The proposed draft variation to the Code is consistent with the section 18 objectives of the FSANZ Act.
- There are no relevant New Zealand standards.

Consultation

Public submissions are now invited on this Assessment Report. Comments are specifically requested on the scientific aspects of this Application, in particular, information relevant to the safety assessment of the enzyme phospholipase A_2 sourced from *A. niger* containing the gene isolated from porcine pancreas as a processing aid.

As this Application is being assessed as a general procedure, there will be one round of public comment. Submissions to this Assessment Report will be used to develop the Approval Report for this Application.

Invitation for Submissions

FSANZ invites public comment on this Report and the draft variation to the Code based on regulation impact principles for the purpose of preparing an amendment to the Code for approval by the FSANZ Board.

Written submissions are invited from interested individuals and organisations to assist FSANZ in further considering this Application/Proposal. Submissions should, where possible, address the objectives of FSANZ as set out in section 18 of the FSANZ Act. Information providing details of potential costs and benefits of the proposed change to the Code from stakeholders is highly desirable. Claims made in submissions should be supported wherever possible by referencing or including relevant studies, research findings, trials, surveys etc. Technical information should be in sufficient detail to allow independent scientific assessment.

The processes of FSANZ are open to public scrutiny, and any submissions received will ordinarily be placed on the public register of FSANZ and made available for inspection. If you wish any information contained in a submission to remain confidential to FSANZ, you should clearly identify the sensitive information, separate it from your submission and provide justification for treating it as confidential commercial material. Section 114 of the FSANZ Act requires FSANZ to treat in-confidence, trade secrets relating to food and any other information relating to food, the commercial value of which would be, or could reasonably be expected to be, destroyed or diminished by disclosure.

Submissions must be made in writing and should clearly be marked with the word 'Submission' and quote the correct project number and name. While FSANZ accepts submissions in hard copy to our offices, it is more convenient and quicker to receive submissions electronically through the FSANZ website using the Standards Development tab and then through Documents for Public Comment. Alternatively, you may email your submission directly to the Standards Management Officer at submissions@foodstandards.gov.au. There is no need to send a hard copy of your submission if you have submitted it by email or the FSANZ website. FSANZ endeavours to formally acknowledge receipt of submissions within 3 business days.

DEADLINE FOR PUBLIC SUBMISSIONS: 6pm (Canberra time) 9 December 2008

SUBMISSIONS RECEIVED AFTER THIS DEADLINE WILL NOT BE CONSIDERED

Submissions received after this date will only be considered if agreement for an extension has been given prior to this closing date. Agreement to an extension of time will only be given if extraordinary circumstances warrant an extension to the submission period. Any agreed extension will be notified on the FSANZ website and will apply to all submitters.

Questions relating to making submissions or the application process can be directed to the Standards Management Officer at standards.management@foodstandards.gov.au.

If you are unable to submit your submission electronically, hard copy submissions may be sent to one of the following addresses:

Food Standards Australia New Zealand
PO Box 7186
Canberra BC ACT 2610
AUSTRALIA
Tel (02) 6271 2222

Food Standards Australia New Zealand
PO Box 10559
The Terrace WELLINGTON 6036
NEW ZEALAND
Tel (04) 473 9942

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INTRODUCTION

Food Standards Australia New Zealand (FSANZ) received a paid Application (A1004) from DSM Food Specialties Pty Ltd on 21 January 2008. The Application seeks to amend Standard 1.3.3 – Processing Aids of the *Australia New Zealand Food Standards Code* (the Code) to include *Aspergillus niger* (*A. niger*), containing the gene for phospholipase A_2 isolated from porcine pancreas. This is a new microbial source of the enzyme, phospholipase A_2 (EC number 3.1.1.4), to be included in the Table to clause 17 – Permitted enzymes of microbial origin.

The enzyme phospholipase A_2 sourced from porcine pancreas is currently listed as a permitted processing aid in the Table to clause 15 – Permitted enzymes of animal origin of Standard 1.3.3. Similarly, phospholipase A_2 from the microbial source, *Streptomyces violaceoruber*, is listed in the Table to clause 17 – Permitted enzymes of microbial origin.

The phospholipase A_2 enzyme's primary use is to increase the efficacy of phospholipids such as lecithin used as an emulsifier in aqueous food products such as bakery products, sauces and dressings. The Applicant has stated that the phospholipase A_2 enzyme acts as a processing aid in exactly the same way as phospholipase A_2 enzymes derived from porcine pancreas and from other microbial sources. The phospholipase A_2 enzyme may remain in the final product as an inactive protein or as an enzyme with no functionality once the substrate has been depleted. The Applicant claims that this processing aid may be suitable for use in vegetarian, Halal and Kosher food products and consequently widen the choice of food products available for these consumers.

1. The Issue / Problem

The Applicant proposes the use of the enzyme phospholipase A_2 as a processing aid. A processing aid is a substance used in the processing of raw materials, foods or ingredients, to fulfil a technological purpose relating to treatment or processing, but which does not perform a technological function in the final food.

Processing aids are prohibited from use in food in Australia and New Zealand unless there is a specific permission for them in Standard 1.3.3. Processing aids (which includes enzymes) are required to undergo a pre-market assessment before they are approved for use in food manufacture in Australia and New Zealand. Additionally, Standard 1.5.2 – Food produced using Gene Technology requires processing aids sourced from a genetically modified organisms to undergo a pre-market assessment.

Although the phospholipase A_2 enzyme is listed twice in Standard 1.3.3, and there is an already-permitted non-genetically modified microbial source of the enzyme, an assessment (which includes a safety assessment) of the use of phospholipase A_2 derived from this new genetically modified microbial strain of *A. niger* is required before an approval for its use can be given (i.e. listed in Standard 1.3.3).

2. Background

2.1 Historical background

Phospholipase A_2 is ubiquitous in nature and occurs in virtually all types of cells that have been examined. Phospholipase A_2 is a component of many animal and plant derived foods and thus has always been consumed by humans.

2.2 Current Standard

Standard 1.3.3 regulates the use of processing aids in food manufacturing. The Table to clause 17 – Permitted enzymes of microbial origin of Standard 1.3.3 contains a list of permitted enzymes of microbial origin for use as processing aids. Similarly, the Table to clause 15 – Permitted enzymes of animal origin contains a list of permitted enzymes of animal origin for use as processing aids

Clause 1 of Standard 1.3.3 defines a processing aid as:

> ***Processing aid*** *means a substance listed in clauses 3 to 18, where –*
>
> *(a) the substance is used in the processing of raw materials, foods or ingredients, to fulfil a technological purpose relating to treatment or processing, but does not perform a technological function in the final food; and*
>
> *(b) the substance is used in the course of manufacture of a food at the lowest level necessary to achieve a function in the processing of that food, irrespective of any maximum permitted level specified.*

Phospholipase A_2 from the microbial source *Streptomyces violaceoruber* was approved in 2004 (Application A501) and is listed in the Table to Clause 17 – Permitted enzymes of microbial origin of Standard 1.3.3. Phospholipase A_2 from animal origin (porcine pancreas) is listed in the Table to clause 15 – Permitted enzymes of animal origin in Standard 1.3.3. Phospholipase A_2 from the genetically modified microbial source organism, *A. niger,* is not currently listed in the Table to clause 17 or any other Table in Standard 1.3.3.

2.3 International Regulatory Standards

The phospholipase A_2 preparation complies with the international specifications relevant for enzymes, which include the Compendium of Food Additives Specifications (2001)[1] compiled by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemical Codex (2004)[2]. These specification references are both primary sources of specifications listed in clause 2 of Standard 1.3.4 – Identity and Purity.

Phospholipase A_2 produced from *A. niger* has been assessed as Generally Recognised As Safe (GRAS) based on a self-assessment process. A 'no objection' letter was received from the US Food and Drug Administration (FDA) in 2005. The enzyme has also been approved for use in France. An application has or will be made in Denmark, China, Mexico, Brazil and Canada for the approval of phospholipase A_2 produced from this genetically modified *A. niger*.

2.4 Nature of the Enzyme and Source of Organism

Phospholipase A_2 is a naturally occurring enzyme, has been isolated from a number of food sources (including wheat flour) and is a natural constituent of the digestive pancreatic juice of humans.

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[1] Combined Compendium of Food Additive Specifications, FAO JECFA Monographs No. 1, Online Edition, at http://www.fao.org/docrep/009/a0691e/A0691E00.htm

[2] Food Chemical Codex 5th edition, Enzyme Preparations – Monograph specifications: 129 -134 and 786 -788), published by the National Academy of Science and the National Research Council of the United States of America in Washington, D.C. (2004).

The phospholipase A_2 enzyme of this Application is produced via fermentation using a genetically modified *A. niger* strain containing multiple copies of the gene for the phospholipase A_2 enzyme originating from porcine pancreas. The DNA coding and the amino acid sequence of the enzyme expressed by *A. niger* is the same as that derived from the porcine pancreas.

The *A. niger* strain is killed off at the end of fermentation with the biomass being separated from the enzyme formulation, assuring the final enzyme preparation is free from the source micro-organism.

2.5 Technological purpose of the enzyme

Phospholipase A_2 is used as a processing aid for the hydrolysis of phospholipids (lecithin), which results in the production of lysolecithin with improved emulsifying power. Commercial lecithin is a naturally occurring mixture of phosphatides of choline, ethanolamine, and inositol, with smaller amounts of other lipids and is widely used in many categories of foods. The benefits of lecithin as an emulsifier in food processing are well known; however, the functionality of 'unmodified' lecithin is limited to fat-based systems.

In aqueous systems (i.e., baked goods) lecithin must be structurally altered, either chemically or enzymatically, to exhibit good emulsifying properties. Chemical modification can be costly and non-specific, generating undesired hydrolysis products. The enzyme phospholipase A_2 hydrolyses the ester bond between the glycerol backbone and the fatty acid at the number two position of the glycerol backbone of lecithin, producing one molecule of lysolecithin and one molecule of fatty acid from one molecule of lecithin. The resulting lysolecithin product is a compound with emulsifying capabilities in many foods that are superior to that of the unmodified lecithin.

Advantages of phospholipase A_2 to the manufacturers and final consumers are in the benefits that the lysolecithin imparts on food such as superior emulsification properties and improved heat stability in foods such as mayonnaise, ice-cream, margarine, and baked goods.

Consumers may also benefit by having a greater choice of new, heat-stable foods that are consequently developed by food manufacturers. After hydrolysis, the enzyme remains in the final product as an inactive protein or as an enzyme with no functionality once the substrate has been depleted.

Any inactive or non-functional enzyme that may result in the final food product would be metabolised like any phospholipase A_2 that is naturally present in other foods or from human pancreatic phospholipase A_2. The Food Technology Report (**Attachment 3**) provides more information about the technological purpose and efficacy of this food processing aid enzyme.

2.6 Labelling issues

Phospholipase A_2 is a normal constituent of wheat flour and is itself not considered to be allergenic. However, in its Application, DSM indicates that its granulated formulation (e.g. as used in bakery products) may be granulated on wheat flour. The use of wheat flour as a base in this formulation would require wheat flour (gluten) to be declared in the final product under the requirements within Standard 1.2.3 – Mandatory Warning and Advisory Statements and Declarations.

Other forms of phospholipase A_2 may not require labelling. According to the Applicant, the liquid formulation is diluted with water; therefore there would be no labelling requirement under Standard 1.2.3.

Standard 1.5.2 requires that all foods containing genetically modified DNA or novel protein must carry the statement 'genetically modified' in the ingredients list on the label. There are no genetically modified ingredient labelling requirements for this Application as it is the source organism that is genetically modified and not the phospholipase A_2 enzyme. The phospholipase A_2 enzyme is identical to that obtained from porcine pancreas and does not contain novel DNA or novel protein[3]. The Applicant has advised that the manufacturing process completely removes any source organisms, eliminating the trigger for GM labelling.

The Code does not define the meaning of Vegetarian, Halal or Kosher and as such labelling issues relating to these aspects are outside of the scope of this Application.

3. Objectives

The objective of this Assessment is to determine whether it is appropriate to amend the Code to permit the use of the enzyme phospholipase A_2 from the source *A. niger* expressing a gene isolated from porcine pancreas. The safety of any possible contaminants arising from the host organism and the enzyme production process will also be assessed.

In developing or varying a food standard, FSANZ is required by its legislation to meet three primary objectives which are set out in section 18 of the FSANZ Act. These are:

- the protection of public health and safety;
- the provision of adequate information relating to food to enable consumers to make informed choices; and
- the prevention of misleading or deceptive conduct.

In developing and varying standards, FSANZ must also have regard to:

- the need for standards to be based on risk analysis using the best available scientific evidence;
- the promotion of consistency between domestic and international food standards;
- the desirability of an efficient and internationally competitive food industry;
- the promotion of fair trading in food; and
- any written policy guidelines formulated by the Ministerial Council[4].

4. Questions to be answered

The key questions to be answered are:

[3] From Standard 1.5.2; novel DNA and/or novel protein means DNA or a protein which, as a result of the use of gene technology, is different in chemical sequence or structure from DNA or protein present in counterpart food which has not been produced using gene technology.

[4] In May 2008, the Australia and New Zealand Food Regulation Ministerial Council endorsed the Policy Guideline on Addition to Food of Substances other than Vitamins and Minerals. This includes policy principles in regard to substances added for technological purposes such as food additives and processing aids. FSANZ has given regard to each of these principles in assessing this Application.

- What is the risk to public health and safety from the use of phospholipase A_2 derived from this new, genetically modified, strain of *A. niger*?

- Are there any risk management measures required to protect public health and safety?

- Does the regulatory impact statement (RIS) conclude that the benefits of permitting use of the enzyme outweigh any costs associated with its use?

RISK ASSESSMENT

5. Risk Assessment Summary

5.1 Safety Assessment

Application A1004 seeks approval for the use of phospholipase A_2 from *A. niger* as a processing aid (only). This strain of *A. niger* was engineered to contain multiple copies of the gene sequence for porcine phospholipase A_2 (Applicant code PLA54). Phospholipase A_2 (from different sources) is currently approved for use as a food processing aid in the Code. *A. niger* has been approved as a host for a variety of different enzymes used as food processing aids. The purified phospholipase A_2 enzyme formulations are free of the production strain DNA and the production strain itself tests negative for the presence of impurities such as mycotoxins.

The Applicant submitted numerous studies including;

- two metabolism studies;
- two single dose toxicity studies in rats;
- one 14-day repeat-dose toxicity study in rats;
- one 3-month repeat-dose toxicity study in rats;
- one bacterial reverse mutation study in *Salmonella typhimurium* and *Escherichia coli;*
- one micronucleus assay in mice; and
- one chromosomal aberration assay in human lymphocytes *in vitro*.

All toxicity and genotoxicity studies were adequately documented to support the claims by the Applicant.

The hazard assessment of the submitted studies concluded that:

- there was no evidence of toxicity in single or repeat-dose toxicity studies;
- bacterial reverse mutation and mouse micronucleus assays were negative; and
- the chromosomal aberration assay for PLA54 was positive (i.e., clastogenic) in the absence of S9 in human peripheral blood lymphocytes. The positive finding was not considered to be indicative of mutagenic potential *in vivo* based on the weight of evidence from the negative bacterial reverse mutation assay, negative in vivo micronucleus studies and submitted discussion and references.

Based on the available evidence, it was concluded that the submitted studies did not reveal any toxicology or hazard–related concerns with the phospholipase A_2 enzyme that would be a reason to not list the enzyme as a food processing aid. The absence of any specific hazards being identified is consistent with phospholipase A_2 undergoing normal proteolytic digestion in the gastrointestinal tract.

The Acceptable Daily Intake (ADI) for phospholipase A_2 is 'not specified'. The full Safety Assessment Report is provided in **Attachment 2**.

5.2 Dietary Exposure Assessment of Phospholipase A_2

FSANZ reviewed the dietary exposure estimation for the enzyme phospholipase A_2 as provided by the Applicant. Taking into account that any phospholipase A_2 in the final food product is unlikely to be active and would be digested in the gastro-intestinal tract in a similar manner to any other ingested protein, FSANZ considers a dietary exposure assessment for phospholipase A_2 as unnecessary.

5.3 Technological Justification

The phospholipase A_2 enzyme's primary use is to increase the efficacy of phospholipids such as lecithin used as an emulsifier in aqueous food products. Phospholipase A_2 is used to hydrolyse natural phospholipids in food products, resulting in the formation of lyso-phospholipids (lysolecithin) that have surface active and emulsifying properties.

Phospholipase A_2 hydrolyses the ester bond between the glycerol backbone and the fatty acid at the number two position of the glycerol backbone of lecithin, producing one molecule of lysolecithin and one molecule of fatty acid from one molecule of lecithin. The resulting lysolecithin product is a compound with emulsifying capabilities in many foods that are superior to that of the unmodified lecithin. The Applicant has suggested that the main uses of their phospholipase A_2 enzyme formulations would be in bakery products, sauces and dressings and be particularly suitable for use in vegetarian, Halal and Kosher food products.

Microbial enzyme preparations have been widely used for a variety of purposes in the production of numerous food products for many years. The Code currently lists a number of enzymes produced from *A. niger* as permitted processing aids of microbial origin. The full Food Technology Report is provided in **Attachment 3**.

5.4 Production of the enzyme

The Applicant states that the Phospholipase A_2 enzyme preparation is produced by a fed-batch fermentation process using an *A. niger* strain, under contained conditions and conducted under Good Manufacturing Practices (GMP). The production process can be summarised as involving a fed-batch fermentation process, which produces the phospholipase A_2 enzyme, stopping the fermentation and effectively destroying the active production organisms. The next steps are separation and concentration of the phospholipase A_2 enzyme from the broth and formulation of the final enzyme preparation.

5.4.1 Standardisation

Food Chemical Codex lists a method to measure the activity of phospholipase A_2 called the egg-yolk test in which it uses egg yolk as a substrate. A disadvantage of egg-yolk is that its composition, due to the fact that it is a natural product, is not constant and activity measurements may vary depending on the nature of the egg yolk used. Therefore, the activity measurement has to be repeated on various egg-yolks to get a more accurate mean value. Activity is expressed in so-called Egg Yolk Units (EYU).

One EYU of phospholipase A_2 activity is defined as the amount of enzyme producing 1 micromole of free fatty acid per minute under the conditions described for the egg yolk test. The Applicant, DSM, utilises an alternative, relative method of analysis to prepare a calibrated and validated phospholipase A_2 standard.

This method utilises a synthetic substrate (namely; 1,2-dithiodioctanoyl phosphatidylcholine) instead of egg-yolk as it has a more constant composition and produces more accurate results. The results are expressed in Chromogenic Phospholipase Units (CPU) with one EYU being equal to one CPU.

5.4.2 Manufacturing Process

The fermentation process consists of inoculum fermentations and a main fermentation. Once the fermentation has been completed the active production organisms are destroyed by incubating with sodium benzoate (4.0 g/kg) at pH 4.0 for 6 hours at 30°C. The temperature of the broth is then decreased to approximately 15°C.

The Applicant has indicated that the separation of cell material from the broth containing the phospholipase A_2 is done by filtration and centrifugation processes. The desired enzyme is separated from the microbial biomass using simple filtrations (broth filtration with the help of a filter aid, followed by polishing and a germ reduction filtration) and then the enzyme is concentrated by an ultra-filtration (UF) process. After ultra-filtration the pH is adjusted to 8.0 and the UF concentrate is polish-filtered, followed by another germ reduction filtration.

In the case of the liquid formulation the UF concentrate is further purified by chromatography. The eluate is then diluted with water to a 1% solution and the pH adjusted; sodium benzoate is also added as a preservative. The final product is standardised with water to an enzyme concentration of 10,000 CPU/ml. This liquid product is used for certain applications like mayonnaise, dressings and sauces. Sodium benzoate (INS 211) is a permitted preservative in a number of foods specified in Schedule 1 of Standard 1.3.1. There are no specific requirements for food additives for enzyme preparations in the Code.

For other applications the UF concentrate may be dried and granulated as is or granulated on wheat flour, resulting in a product with an enzyme activity ranging between 5000 and 25000 CPU/g with a particle size (90%) between 63-225 µm. The final product is standardised with granulated flour.

The enzyme phospholipase A_2 preparations may also contain some harmless substances derived from the microorganism and the fermentation medium. These may include polypeptides, proteins, carbohydrates and salts.

5.5 Allergenicity

Phospholipase A_2 is a normal constituent of wheat flour and is itself not considered to be allergenic. However, in their Application, DSM indicate that their granulated formulation (e.g. as used in bakery products) may be granulated on wheat flour. The use of wheat flour as a base in this formulation would require wheat flour (gluten) to be declared in the product due to the requirements contained in Table to clause 4 of Standard 1.2.3.

Other carriers of the phospholipase A_2 may not require labelling. According to the Applicant, the liquid formulation is diluted with water and preserved with sodium benzoate; therefore there would be no labelling requirement under Standard 1.2.3.

RISK MANAGEMENT

6. Issues raised

6.1 Risk Management Strategy

The Risk Assessment concludes that the use of phospholipase A_2 sourced from genetically modified *A. niger* as a processing aid does not pose a public health and safety risk and its use is technologically justified by food manufacturers.

The phospholipase A_2 enzyme is identical to that obtained porcine pancreas and does not contain novel DNA and/or novel protein. The source organism, *A. niger*, which is genetically modified, is destroyed and physically removed during the manufacturing process and hence products utilising this processing aid will not be required to be labelled as genetically modified as there is no novel DNA and/or novel protein present in the final food. The separation process (including polish filtration, germ reduction filtration and ultra filtration) of the biomass from the fermentation fluid assures that the commercial enzyme formulation is completely free from the *A. niger* production strain. This is the case for a number of enzymes sourced from GM microorganisms approved in the Code.

7. Options

Processing aids used in Australia and New Zealand are required to be listed in Standard 1.3.3. The phospholipase A_2 enzyme acts as a processing aid when it is used to hydrolyse natural phospholipids (e.g. as an emulsifier) in food products, and requires a pre-market approval under Standard 1.3.3. It is not appropriate to consider non-regulatory options.

Two regulatory options have been identified for this Application:

Option 1: Reject the Application

Option 2: Permit the use of phospholipase A_2 sourced from genetically modified *A. niger*, containing the gene isolated from porcine pancreas, as a food processing aid.

8. Impact Analysis

In developing food regulatory measures for adoption in Australia and New Zealand, FSANZ is required to consider the impact of all options on all sectors of the community, including consumers, the relevant food industries and governments. The regulatory impact assessment identifies and evaluates, though is not limited to, the costs and benefits arising from the regulation and its health, economic and social impacts.

The regulatory impact analysis is designed to assist in the process of identifying the affected parties and the likely or potential impacts the regulatory provisions will have on each affected party. Where medium to significant competitive impacts or compliance costs are likely, FSANZ will seek further advice from the Office of Best Practice Regulation (OBPR) to estimate compliance costs of regulatory options.

FSANZ has conducted, with OBPR subsequently approving, a preliminary assessment of this Application which has concluded that there were no business compliance costs involved and/or minimal impact and consequently a Regulation Impact Statement (RIS) is not required.

8.1 Affected Parties

The affected parties to this Application include:

- those sectors of the food industry wishing to produce and market food products produced using phospholipase A_2 as a processing aid;
- consumers of food products utilising phospholipase A_2 as a processing aid; and
- Australian, State, Territory and New Zealand Government enforcement agencies that enforce food regulations.

8.2 Benefit Cost Analysis

8.2.1 Option 1: Reject the Application

This option is the *status quo*, with no changes to the Code.

Rejecting the Application would disadvantage consumers and relevant food industries where the enzyme could provide a technological function.

8.2.2 Option 2: Permit the use of the use of phospholipase A_2 sourced from genetically modified A. niger, containing the gene isolated from porcine pancreas, as a food processing aid

This option provides positive benefits to consumers and food manufacturers to be able to use phospholipase A_2 sourced from genetically modified *A. niger*. The Applicant has stated that this enzyme is from a non-animal source which may allow vegetarian, Halal, or Kosher certification for foods produced using this enzyme. This in turn would provide a wider variety of foods which consumers could consume. The use of the enzyme is technologically justified and there are no public health and safety concerns.

There should not be any significant compliance costs for government enforcement agencies since they would not need to analyse for the presence of the enzyme. The use of enzymes to treat food during their manufacture does not require labelling so it would not be expected that enforcement agencies would need to analyse for the presence or otherwise of the enzyme in any final food for compliance. There should also be no added costs to consumers.

Option 2, which supports the approval of phospholipase A_2 as a food processing aid is the preferred option, since it has advantages for the food industry and consumers but has no significant costs for government regulators, consumers or manufacturers.

8.3 Comparison of Options

In assessing applications, FSANZ considers the impact of various regulatory (and non-regulatory) options on all sectors of the community, including consumers, food industries and governments in Australia.

For this Application, Option 1, the *status quo*, is considered unacceptable because it rejects a technologically justified processing aid as an alternative source of the currently permitted and used processing aid.

Option 2 is favoured since there are potential benefits for the food manufacturing industry, as well as consumers. Such benefits are most likely to include providing manufacturers with an alternative source of the enzyme. No significant adverse costs have been identified with option 2 for government stakeholders. Overall, the benefits outweigh the costs.

No significant adverse costs have been identified with either option for consumer and government stakeholders. Overall, the benefits outweigh the costs.

COMMUNICATION AND CONSULTATION STRATEGY

9. Communication

FSANZ will apply a basic communication strategy to Application A1004. This will involve advertising in the national press the availability of the Assessment Report for public comment, which gives people without access to the internet a chance to participate in the process, as well as making the reports available on the FSANZ website.

The Applicant, individuals and organisations making submissions to this Application will be notified at each stage of the Application. If the FSANZ Board approves the draft variation to the Code, FSANZ will notify its decision to the Ministerial Council. The Applicant and stakeholders, including the public, will be notified of the gazetted changes to the Code in the national press and on the FSANZ website.

10. Consultation

10.1 Public consultation

FSANZ is seeking comments from the public and other interested stakeholders to help assess this Application. Once the public comment period has closed there will be no further round of public comment.

Comments on the following topics would be useful:

- technological justification
- safety considerations
- other scientific aspects
- costs and benefits

10.2 World Trade Organization (WTO)

As members of the World Trade Organization (WTO), Australia and New Zealand are obligated to notify WTO member nations where proposed mandatory regulatory measures are inconsistent with any existing or imminent international standards and the proposed measure may have a significant effect on trade.

Amending the Code to approve phospholipase A_2 as a processing aid is unlikely to have a significant effect on trade. The enzyme preparation is consistent with the international specifications for food enzymes of JECFA and Food Chemicals Codex, so there does not appear to be a need to notify the WTO. For these reasons FSANZ has decided not to notify the WTO under either the Technical Barriers to Trade (TBT) or Sanitary and Phytosanitary Measures (SPS) Agreements.

CONCLUSION

11. Conclusion and Preferred Option

This Application has been assessed against the requirements of section 29 of the FSANZ Act. FSANZ recommends the proposed draft variation to Standard 1.3.3. This Assessment Report concludes that the use of the enzyme phospholipase A_2 sourced from genetically modified *A. niger* as a processing aid is technologically justified and does not pose a public health and safety risk. An amendment to the Code to give approval to the use of the enzyme phospholipase A_2 sourced from *A. niger* containing the gene for phospholipase A_2 isolated from porcine pancreas as a processing aid in Australia and New Zealand is recommended on the basis of the available scientific information. The proposed draft variation is provided in **Attachment 1**.

Preferred Approach

FSANZ recommends the proposed draft variation to the Table to clause 17 of Standard 1.3.3 – Processing Aids, to permit the use of the enzyme phospholipase A_2 sourced from *Aspergillus niger* containing the phospholipase A_2 gene isolated from porcine pancreas.

11.1 Reasons for Preferred Approach

The preferred approach is recommended for the following reasons:

- A detailed safety assessment has concluded that the use of the enzyme does not raise any public health and safety concerns.
- The use of the enzyme sourced from genetically modified *A. niger* is expected to provide technological benefit to manufacturers.
- The source organism, *A. niger* is regarded as non-pathogenic and non-toxigenic.
- The regulation impact assessment has concluded that the benefits of permitting use of the enzyme outweigh any costs associated with its use.
- There are no other measures that would be more cost-effective than a variation to Standard 1.3.3 that could achieve the same end.
- The proposed draft variation to the Code is consistent with the section 18 objectives of the FSANZ Act.
- There are no relevant New Zealand standards.

12. Implementation and Review

Following the consultation period for this document, an Approval Report will be completed and the draft variation will be considered for approval by the FSANZ Board. The FSANZ Board's decision will then be notified to the Ministerial Council. Following notification, the proposed draft variation to the Code is expected to come into effect on gazettal, subject to any request from the Ministerial Council for a review of FSANZ's decision.

ATTACHMENTS

1. Draft variation to the *Australia New Zealand Food Standards Code*
2. Safety Assessment Report
3. Food Technology Report

Attachment 1

Draft variation to the *Australia New Zealand Food Standards Code*

Section 87 of the FSANZ Act provides that standards or variations to standards are legislative instruments, but are not subject to disallowance or sunsetting

To commence: on gazettal

[1] ***Standard 1.3.3*** *of the Australia New Zealand Food Standards Code is varied by inserting the following source in* Column 2 *of the* Table to clause 17 *for the enzyme* Phospholipase A_2 *in* Column 1 –

Aspergillus niger, containing the gene for phospholipase A_2 isolated from porcine pancreas

Attachment 2

Safety Assessment Report

A1004 – Porcine phospholipase A_2 derived from *Aspergillus niger* as a processing aid

SUMMARY AND CONCLUSION

Application A1004 seeks approval for the use of phospholipase A_2 from *Aspergillus niger* as a processing aid (only). This strain of *A. niger* was engineered to contain multiple copies of the gene sequence for porcine phospholipase A_2 (Applicant code PLA54). Phospholipase A_2 is currently approved for use as a food processing aid and the same strain of *A. niger* has been approved as a host for the production of asparaginase for use as a food processing aid.

The hazard assessment of the submitted studies concluded that:

- single-dose toxicity in rats (PO) was absent or minimal and not of concern;
- repeat-dose toxicity in rats was minimal and restricted to possible changes in several clinical chemistry parameters but overall was not of concern;
- bacterial reverse mutation and mouse micronucleus assays were negative; and
- the chromosomal aberration assay for PLA54 was positive (i.e. clastogenic) in the absence of S9 in human peripheral blood lymphocytes. The positive finding was not considered to be indicative of mutagenic potential *in vivo* based on the weight of evidence from the negative bacterial reverse mutation assay, negative *in vivo* micronucleus studies and submitted discussion and references.

Based on the available evidence, it was concluded that the submitted studies did not reveal any toxicology or hazard –related concerns with PLA54 that would impede listing PLA54 (porcine PLA_2, as sourced from *A. niger*) as a food processing aid. The absence of any specific hazards being identified is consistent with PLA54 undergoing normal proteolytic digestion in the gastrointestinal tract. The Acceptable Daily Intake (ADI) for phospholipase A_2 is 'not specified'.

1 Introduction

Application A1004 concerns the use of *A. niger* containing the gene coding for porcine phospholipase A2 (PLA2) which was isolated from the pig pancreas. The enzyme product from A. niger was identical to pig pancreatic PLA2 and the latter, when isolated from natural sources is already permitted to be used as a food processing aid (Standard 1.3.3, clause 15).

PLA2 is a natural constituent of pancreatic juice and certain foods. PLA2 hydrolyses phospholipids present in food stuffs with the formation of lyso-phospholipids which have surface active and emulsifying properties. The resultant purified PLA2 formulations are free of the production strain DNA and the production strain itself tests negative for the presence of mycotoxins.

The present preparation of PLA2 from *A. niger* was notified as GRAS in 2005, but has not been evaluated per se by the US Food and Drug Administrator (FDA).

Summary of Submitted Safety Studies

Submitted studies:

- two metabolism studies;
- two single dose toxicity studies in rats;
- one 14-day repeat-dose toxicity study in rats;
- one 3-month repeat-dose toxicity study in rats;
- one bacterial reverse mutation study in *S. typhimurium* and *E. coli;*
- one micronucleus assay in mice; and
- one chromosomal aberration assay in human lymphocytes *in vitro*.

All toxicity and genotoxicity studies were adequately documented to support the claims by the Applicant. The Applicant also demonstrated that *A. niger* was not capable of producing mycotoxins.

2 Metabolites

Two Metabolite Analysis Report summaries (no study numbers or data were included) were provided by the Applicant which were performed by the Institute of the Royal Netherlands Academy of Arts and Sciences for toxic metabolite formation by *A. niger* PLA54, the filtrate and PLA9901 UF concentrate (Reports were dated May and October 1999). Culture plates were incubated for 14 days in darkness at 24°C, extracted and analysed by HPLC with diode array detection and metabolites compared to spectral UV libraries of authentic standards analysed under the same conditions.

The *A. niger* PLA54 strain produced anticipated secondary metabolites including nigragilin, 'a few' naphtha-γ-pyrones and tetracyclic compounds. The Applicant reported that naphtha-γ-pyrones from extracts of *A. niger* isolated from stored cotton seeds, demonstrated toxic effects (not defined) when injected into female mice and chicken embryos. No known mycotoxins were detected in the extracts. The extract from the filtrate preparation led to the detection of only 3 tetracyclic compounds. No other metabolites were described. The Applicant stated that the analysis of the PLA9901 UF concentrate 'contained several metabolites but no compounds which could be identified as mycotoxins'. No additional analysis or description of the detected 'several metabolites' was provided.

3 Toxicity

Single-Dose Toxicity of Phospholipase A_2 in Rats

Study 15.750. Sponsor: Gist-brocades, Delft, The Netherlands. Contract sponsor: Notox B.V., 's-Hertogenbosch, The Netherlands (study number NTX 258209). GLP Yes (OECD). In-life: March 1999, Final Report: June 1999.

Rats (Wistar, Crl: (WI) BR outbred, SPF, 3/sex, 7 weeks old, group housing of 3/cage) received a single dose of phospholipase A_2 (batch PLA9901-enriched, 23.4 g/kg bw, 20 mL/kg bw, vehicle used was not defined) by Per Oral (PO) gavage after food was withheld for <20 h, and resumed 3-4 h post dosing. Rats were monitored twice daily for 2 weeks, clinical signs were graded daily and body weights weekly. The study was performed based on the guidelines described in: EC Commission Directive 96/54/EC, Part B.1 tris 'Acute Toxicity-Oral, Acute Toxic Class Method' and OECD No. 423. Macroscopic changes were recorded at necropsy.

Clinical signs of lethargy were noted in all males on day 1 and red staining on the neck on one female on days 1, 2 and 10. No mortalities were recorded. There were no changes in body weights or abnormal macroscopic findings at necropsy. The NOAEL was 23.4 g/kg bw, PO.

Study 15.751. Sponsor: Gist-brocades, Delft, The Netherlands. Contract sponsor: Notox B.V., 's-Hertogenbosch, The Netherlands (study number NTX 258064). GLP Yes (OECD). In-life: March 1999, Final Report: June 1999.

Rats (Wistar, Crl: (WI) BR outbred, SPF, 3/sex, 7 weeks old, group housing of 3/cage) received a single dose of phospholipase A_2 (batch PLA9901-inactivated, 21.2 g/kg bw, 20 mL/kg bw, vehicle was not defined) by PO gavage after food was withheld for <20 h, and resumed 3-4 h post dosing. The method of preparation of inactivated PLA9901 was not described. Rats were monitored twice daily for 2 weeks, clinical signs were graded daily and body weights weekly. The study was performed based on the guidelines described in: EC Commission Directive 96/54/EC, Part B.1 tris 'Acute Toxicity-Oral, Acute Toxic Class Method' and OECD No. 423. Macroscopic changes were recorded at necropsy.

Lethargy was observed in all rats on day 1. No changes in body weight or macroscopic findings were observed. The NOAEL was <21.2 g/kg bw.

Repeat-dose Toxicity of Phospholipase A_2 in Rats -2 Weeks

Study 15.234. Sponsor: Gist-brocades, Delft, The Netherlands. Contract sponsor: Notox B.V., 's-Hertogenbosch, The Netherlands (study number NTX 258029). GLP Yes (OECD). In-life: May-June 1999, Final Report: Jan 2000.

Rats (Wistar, Crl:(WI)BR outbred, SPF, 5/sex/group, 6 weeks old, group housing of 5/cage) received daily doses of phospholipase A_2 (0, 500, 2,000 or 10,000 mg/kg bw/day PLA54, batch PLA9901-enriched, 20 mL/kg bw, vehicle not defined) for 2 weeks by PO gavage. The study protocol was adapted from EEC Directive 96/54/EEC, B.7 Repeated dose (28 days) Toxicity (oral), 1996 and OECD 407, Repeated dose 28-day oral Toxicity Study in Rodents, 1995. Food was withheld for <20 h, and resumed 3-4 h post dosing. Dosing was not adjusted for changing volumes: group 1 control rats received Milli U water at 9.43 mL/kg bw; group 2 received 0.47 mL/kg bw PLA54, group 3 received 1.89 mL/kg bw; group 4 received 9.43 mL/kg bw for 0, 500, 2000 and 10000 mg/kg bw/day, respectively. Justification of doses tested was not provided in the reports. Nevertheless the top doses exceed the maximum recommended doses for these assays. Rats were monitored for mortality twice daily for 2 weeks, clinical signs were graded daily and body weights and food consumption were recorded weekly. Macroscopic changes and organ weights (adrenal glands, heart, kidneys, liver, spleen and testes) were recorded at necropsy. Clinical biochemistry and haematology samples were collected for analysis at autopsy. Microscopic examination of tissues was not performed.

No mortalities occurred during the 2 week study. No toxicological significant changes in clinical signs were observed. Minor observations of alopecia, scabs and red staining of fur were noted but were considered to be sporadic. The latter could be due to the group housing of the animals. No significant changes were noted in food consumption or body weights. No changes in macroscopic examination (except a hemorrhagic cyst in the ovaries of one control female rat), haematology, or selected organ weights were observed at autopsy. Clinical biochemistry endpoints were unchanged with the exception of cholesterol values which increased slightly (10-25%) but significantly above controls in males with a similar slight trend in females. The increase in males occurred in all male groups but not dose-dependently. Triglyceride levels were not determined but there were no changes in plasma albumin levels.

The NOAEL for PLA54 was 10,000 mg/kg bw/day for 2 weeks by PO gavage.

Repeat-dose Toxicity of Phospholipase A_2 in Rats -3 Months

Study 15.234. Sponsor: DSM Gist R&D, Delft, The Netherlands. Contract sponsor: Notox B.V., 's-Hertogenbosch, The Netherlands (study number NTX 258031). GLP Yes (OECD). In-life: July-Oct 1999, Final Report: April 2000.

Rats (Wistar, Crl:(WI)BR outbred, SPF, 10/sex/group, 6 weeks old, group housing of 5/cage) received daily doses of phospholipase A_2 (0, 500, 2,000 or
10,000 mg/kg bw/day PLA54, batch PLA9901-enriched, 20 mL/kg bw, vehicle was not defined) by PO gavage for 3 months.

The study protocol was adapted from EEC Directive 87/302/EEC, B Repeated dose (90 days) Toxicity (oral), 1988; OECD 408, Repeated dose 90-day oral Toxicity Study in Rodents, 1998 and EPA 712-C-96-199, 90-day Oral Toxicity, Draft 1996. Food was withheld for <20 h, and resumed 3-4 h post dosing.

Dosing was not adjusted for changing volumes: group 1 control rats received Milli U water at 9.43 mL/kg bw; group 2 received 0.47 mL/kg bw PLA54, group 3 received 1.89 mL/kg bw; group 4 received 9.43 mL/kg bw for 0, 500, 2000 and 10000 mg/kg bw/day, respectively. Dose selection was based on the previous 2 week study, however, jjustification of the doses tested was not provided in the report. Nevertheless the top doses exceed the maximum recommended doses for these assays.

Rats were monitored for mortality twice daily for 3 months, clinical signs were graded (1 to 4) daily and body weights and food consumption were recorded weekly. Ophthalmological assessments were performed before treatment and prior to autopsy. Functional tests were performed during weeks 12-13 (hearing, papillary reflex, static righting reflex, grip strength). Macroscopic changes and organ weights (extensive list) were recorded at necropsy. Extensive clinical biochemistry and haematology samples were collected for analysis at autopsy. Microscopic examination of tissues was performed on all lungs, livers and kidneys, all tissues from control and high dose animals and all gross lesions or animals which were terminated *in extremis*.

Two mortalities occurred during the study. One male rat that received the low dose died on day 22 after showing signs of abnormal posture, pilo-erection and emaciation. The second mortality (female) received the high dose and died after blood sampling (day not specified). Collectively, the mortalities were not considered to be treatment-related. No significant clinical signs of toxicity were observed. Occasional observations of blood staining on fur and in the cage were attributed to the group housing of animals. No changes in functional parameters or ophthalmological examinations and no toxicologically significant changes in body weights or food consumption were observed. Haematological parameters were unchanged with the exception of dose-dependent increases in WBC in males that received the mid and high doses (8.6, 10.1, 10.6*, 11.4** G/L for control, low, mid and high dose, *=p<0.05, ** p=<0.01, respectively). Partial thromboplastin time was increased in females that received the mid and high doses (16.3, 16.6, 17.7*, 17.9** sec for control, low, mid and high dose, *=p<0.05, ** p=<0.01, respectively). The absence of findings in both sexes and lack of histological findings consistent with inflammation suggest that these findings were not toxicologically significant. Changes in clinical biochemistry included slight but significant increases in bilirubin (1.9 vs. 2.5* µmol/L in males, 2.8 vs. 3.4* µmol/L in females, control vs. high dose, *=p<0.05, ** p=<0.01, respectively) and potassium (4.63 vs. 5.07* mmol/L in males, 4.26 vs. 4.58** mmol/L in females, control vs. high dose, *=p<0.05, ** p=<0.01, respectively and a slight increase in inorganic phosphate in males (but not females) that received the mid and high doses.

These changes were not considered to be toxicologically significant because they were not accompanied by other changes in clinical biochemistry and/or did not occur in both genders. No significant macroscopic, microscopic or changes in organ weights were noted in any treated groups.

The NOAEL was 10,000 mg/kg bw/day, PO for 3 months based on the absence of significant dose-dependent findings that were consistent between male and female rats. The observations of increased bilirubin and potassium levels in males were not accompanied by histological evidence of lesions in the liver, kidney or adrenal glands. However, because the group sizes were considered to be small, the NOEL was assigned to 2,000 mg/kg bw/day for 3 months based on the observed changes in serum bilirubin and potassium.

4 Genotoxicity

Study details	Method	Results	Validity
Bacterial reverse mutation Study number 15.757, Project 258042, Contract lab. Notox B.V., 's-Hertogenbosch, The Netherlands. Study dates: 9-26 April 1999; Final Report 5 July 1999. Strains tested: *S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP_2uvrA.	Ranging assay: TA100 and WP_2uvrA tested at 3, 10, 33, 100, 333, 1000, 3330, 5000 µg/plate ±S9 liver microsomes (Wistar, male). Mutation assay: Test #1 strains (TA1535, TA1537, TA98) and Test #2 (TA1535, TA1537, TA98, TA100, WP_2uvrA) were tested at 3-100 to 5000 µg/plate for each strain, ±S9, in triplicate. Bacteria strains were mixed with test PLA2, ±S9, plated and incubated at 37°, 48 h before revertant colonies were counted. PLA_2 batch PLA9901, purity 14.7%, vehicle, MilliQ water.	Overall: **NEGATIVE** Ranging assay: no decrease in revertants was observed. Mutation assay: Negative revertant responses were observed over all concns. tested. All responses were <2 fold increases and were not concn.- dependent in 2 independent tests.	GLP compliant. Precipitation of PLA_2 in the agar or evidence of toxicity/ decreased background lawn were not observed. Negative and positive controls within historical values. Metabolic activation system was active.
Mouse Micronucleus Assay Study number 15.233, Project 276942, Contract lab. Notox B.V., 's-Hertogenbosch, The Netherlands. Study dates: 19 Oct-14 Dec 1999; Final Report 20 Jan 2000.	Dose-ranging test: Mice (NMRI BR SPF 2/sex/gp) received 2000 mg/kg bw PLA_2 PO or IP. Main test: Mice (5/sex/gp) received 500, 1000 or 2000 mg/kg bw PLA_2 *via* PO intubation. The IP route was not tested in the main test. Groups were sacrificed and bone marrow smears collected at 24 and 48 h. Positive control mice received 50 mg/kg bw cyclophosphamide PO and were sampled after 48 h. The proportion of micronucleated polychromatic erythrocytes (PCE) in 2000 polychromatic erythrocytes (NPC) was determined. PLA_2 batch PLA9901, purity 14.7%, vehicle, MilliQ water.	Overall: **NEGATIVE** Dose-ranging test: no reaction to PO or IP treatment was observed. Main micronucleus test: No increase in polychromatic erythrocytes was observed in male (2.6-4.2/2000) or female (1.8-4.4/2000) mice at up to 2000 mg/kg bw (PO) PLA_2. PLA_2 did not affect the PCE/NCE ratio in male or female mice indicating no effect on erythropoiesis.	GLP compliant. Cyclophosphamide (positive control) induced a significant increase in polychromatic erythrocytes (55/2000 and 39/2000) but no change in PCE/NPC ratio (1.20 and 1.02, males, females, respectively). No mortalities were observed.

Study details	Method	Results	Validity
Chromosomal Aberration Assay – human lymphocytes *in vitro* Study number 15.928, Project 258053, Contract lab. Notox B.V., 's-Hertogenbosch, The Netherlands. Study dates: 20 May-27 Oct 1999; Final Report 10 Oct 2000.	Heparinised human blood (male) was diluted in F10 complete media with 20% foetal calf serum, phytohaemoagglutanin and ± rat liver S9 microsomes for 3, 24 or 48 h. Cell division was arrested using the spindle inhibitor colchicine during the last 3 h of incubation. Cells were processed and mounted on microscope slides and the mitotic index and chromosome aberrations were determined (per 1000 metaphase cells, duplicate incubations). Concn.-ranging test: Concs. tested 100, 333, 1000, 33300, 5000 µg/mL for 3 and 24 h incubations. Cytogenetics test #1: Concns. tested 1000, 1800, 3330, 4200, 5000 µg PLA_2 (active enzyme)/mL for 3, 48 h incubations. Cytogenetics test #2: Concns tested 560, 1000, 1300, 1800, 2400, 3330 µg/mL using EDTA-inactivated PLA2 (see legend) for 48 h incubations. PLA_2 batch PLA9901, purity 14.7%, vehicle, MilliQ water.	Overall: **POSITIVE** (in absence of S9) Concn.-ranging test: Concn-dependent decrease in metaphase index when cells cultured with ≥1000 µg/mL PLA_2 for 24 or 48 h. No change in the incidence of chromosome aberrations was observed. Cytogenetics test #1: In the absence of S9, a concn-dependent increase in incidence of cells with chromosomal aberrations was observed after 48 h culture with ≥4200 µg/mL ($p<0.05$). The metaphase index decreased $conc^n$-dependently in cells cultured with ≥1000 µg/mL PLA_2 for 24 or 48 h. No increase was observed in the presence of S9. Cytogenetics test #2: The positive result in test #1 was repeated using EDTA-inactivated PLA_2 with 48 h culture in the absence of S9. A significant (≥560 µg/mL) concn.-dependent increase in chromosome aberrations was observed. The aberrations included chromatid gaps, chromosome gaps, minutes, double minutes and increased miscellaneous findings such as polyploidy, endo-reduplication multiple aberrations and chromosome intrachange. Continued…	GLP compliant. Negative controls and positive controls (mitomycin C and cyclophosphamide) elicited significant increases in cells with chromosome aberrations and S9 metabolism. Results continued: Changes in percentage metaphase cells for test#1 (48h) were: 100%, 122%, 86%, 74%, 55% and 45% for control, 1000, 1800, 3330, 4200, and 5000 µg/mL. Changes in metaphase cells in test #2 (48h) were: 100%, 86%, 62%, 29%, 12, 3, 2%. Mitomycin C treated cells were 146% (test #1) and 93% (test 3#) of control. Cytotoxicity was not recorded. The absence of small amounts of EDTA in the formulation used in test #1 suggested that the positive result in test #2 when inactivated PLA54 was added was not caused by the low concn. of EDTA. Under these conditions, PLA_2 should be considered as clastogenic.

TA98 and TA1537 detect frame-shift mutagens. TA100, TA1535 and WP2uvrA detect base-pair substitution mutagens; low purity of test substance (14.7%) indicates possibility of effects caused by other substances present in the test formulation. Concentrations were based on 'dry matter' and adjusted. PLA2-inactivated enzyme was generated by incubation of bulk PLA2 enzyme (est 1 g/mL) with EDTA (5 mg/mL) for 6 h, 50°C. Dry weight substance proportions were PLA2 = 14.7%, EDTA = 0.5% (or 3.2% of PLA2. The molecular ratio of EDTA:PLA2 was about 35-40:1).

5 Discussion

Toxicity

Single-dose PO toxicity studies in rats with active and inactive PLA_2 did not reveal any significant adverse toxicological findings. In the repeat-dose PO studies in rats, there were few consistent toxicological findings. Several mortalities were observed but there was no evidence to indicate that they were treatment-related and therefore the deaths were considered to be incidental. No significant changes were noted in food consumption or body weights, macroscopic changes in organs or ophthalmology parameters. Some changes in haematological parameters (WBC) in males and (partial thromboplastin clotting time) females were observed but the absence of findings in both genders and lack of histological findings consistent with inflammation suggested that these findings were not toxicologically significant. Slight increases in bilirubin and potassium were observed in males and females that received the high dose and a slight increase in inorganic phosphate was noted in males (but not females) that received the mid and high doses in the 13 week study.

A NOAEL at the high dose of 10,000 mg/kg bw/day was assigned based on the absence of significant dose-dependent findings.

Genotoxicity

PLA54 was negative in the *in vitro* bacterial reverse mutation assay and the *in vivo* mouse micronucleus assay but positive for the chromosomal aberration/ clastogenicity in human peripheral lymphocytes in *in vitro*. The positive finding occurred in the absence of the S9 microsomal enzyme system and therefore indicated that the effect was not dependent upon hepatic metabolism.. The positive finding was accompanied by a marked decrease in the Mitotic Index.

The validity of the bacterial reverse mutation and the micronucleus assays were confirmed by appropriate positive control agents. While the micronucleus assay was negative and the internal positive control agent (cyclophosphamide) elicited an appropriate response when dosed by the PO route. No evidence was presented to confirm that systemic exposure (and therefore the bone marrow) had been achieved with PLA54 due to e.g., gastro-intestinal proteolysis. The negative micronucleus assay also indicates that if there were any impurities or other fermentation or soluble products present in the formulation that may have caused the positive chromosome aberration result, that any such substances were without effect upon the bone marrow when dosed PO in mice.

Exposure comparisons between *in vivo* and *in vitro* protocols can be uncertain, however, if only 5% of the high dose was absorbed (i.e., 100 mg/kg), the systemic exposure/blood concentration would have been about 20-fold higher than the *in vitro* concentrations at which the chromosome aberrations (≥4.2 mg/mL) were observed *in vitro*. Therefore, while not substantive evidence, it could be argued that the doses used in the micronucleus assay were at sufficiently high multiples of the comparable doses achieved in the chromosome aberration assay to over-ride the apparently positive chromosome aberration finding and indicate that the genotoxicity potential of PLA54 *in vivo* is absent.

The Applicant did not provide an adequate explanation for the positive clastogenicity findings (-S9) to be dismissed in the initial application and was therefore requested to justify the claim that the enzyme preparation showed no mutagenicity. However, the Applicant's follow-up response provided an adequate discussion to discount the findings based on 'weight of evidence' and plausibility of the findings.

The newly submitted references (including: Pariza & Johnson, 2001; Kirkland *et al*., 2007a, 2007b) provided an adequate review of the literature for the weight of evidence approach that may be applicable to discount unexpected positive mutagenicity results for enzyme preparations that are used in various stages of food preparation. Based on a survey of 49 Ames tests and 27 chromosome aberration tests performed on enzymes from genetically modified organisms (including *A niger*), false-positive results were found in 7 Ames tests and 6 chromosome aberration tests. The false-positive Ames test results were attributed to the growth enhancing effects of histidine in the enzyme preparations, but there was no evidence for this with PLA_2. From the literature survey results, positive chromosome aberration tests were attributed to:

(i) Inconsistent *in vitro* findings between Chinese hamster ovary cells vs. human lymphocytes but this situation is not relevant in this case because studies were only performed in one cell type.

(ii) Lack of confirmation of *in vitro* results by the *in vivo* cytogenetic assay which was difficult to ascribe because systemic exposure to PLA54 was not verified when dosed PO, (as described above).

(iii) Consideration of the production of e.g., hydrogen peroxide or another deleterious enzyme reaction product by the test preparation, which when used in cell culture systems, may cause clastogenic aberrations but would be metabolised or decomposed *in vivo*. The observation that the positive responses only occurred at the longer harvest time in the absence of S9 is consistent with damage to internal organelles or altering e.g., plasma membrane integrity when added directly to cells in culture.

The Applicant provided additional arguments in support of their case for the absence of a mutagenic capacity for PLA54. These were: (a) that *in vitro* genotoxicity tests on mammalian cells exhibit a high incidence of false-positive results compared to rodent carcinogenicity studies, possibly attributable to 'excessive or irrelevant' levels of the test agent or absence of metabolic or elimination pathways that are normally present *in vivo*; (b) that natural porcine PLA_2 is regarded as safe and is already permitted as a food processing aid (Standard 1.3.3); and (c) the enzyme is derived from a safe strain of *Aspergillus* that is not capable of producing mycotoxins and which is manufactured to specifications set by JECFA. FSANZ considers that the lack of confirmation of *in vitro* results by the *in vivo* cytogenetic assay, to be the major factor in favour of the dismissal of the positive chromosome aberration assay result.

The above arguments proposed by the Applicant, notably (b) and (c), markedly added to the weight-of-evidence case that PLA54 is not mutagenic.

In addition to the weight of evidence points presented by the Applicant, the Applicant was required to confirm whether i) the genotoxicity studies were performed using the final commercial grade material grade enzyme preparation; ii) a chemical analysis data sheet for PLA54 to ascertain the possible presence of contaminants that might also contribute to the mutagenicity findings and a statement on whether any additional GLP genotoxicity studies have been performed. The Applicant indicated that the material used in the toxicity studies was an 'Ultra-filtrate' preparation from a pilot plant fermentation process (e.g., 1-3 m^3) that was claimed to be representative of the fermentations performed on a larger scale. The 'Ultrafiltrate' was selected for use in animal and genotoxicity studies based on being the most concentrated (liquid) product from which the commercial products are derived by dilution with formulation agents suitable for use in food. This rationale was accepted as reasonable. The analysis results for batch PLA9901 did not detect any impurities of concern. The Applicant stated that no additional GLP genotoxicity studies had been performed.

Overall, FSANZ agrees with the weight of evidence approach as presented by the Applicant to indicate that there is no evidence from the available data for any mutagenic potential *in vivo* attributable to PLA54. The additional requests for information did not raise any additional concerns that might alter the weight of evidence approach as presented.

6 Conclusions

There were no toxicologically significant toxicity findings in rats after single-dose exposures. Repeat-dose toxicity in rats was minimal and was restricted to possible changes in several clinical chemistry parameters but overall these changes were not of concern. Mutagenicity tests were negative in the bacterial reverse mutation and mouse micronucleus assays. The chromosomal aberration test for PLA54 was positive (clastogenic) in human peripheral blood lymphocytes but this finding was dismissed based on the weight of evidence from the negative bacterial reverse mutation and micronucleus studies, submitted references and that the positive finding is likely to be an artefact of the test system. Therefore, on a weight of evidence basis, the PLA54 formulation was considered to be non genotoxic.

Collectively, no special hazards attributable to PLA54 were revealed in the submitted studies. Therefore, the use of PLA54 as a processing aid does not raise any concerns. The ADI for porcine PLA_2 from *A. niger* is 'not specified'.

7 References

Kirkland, D.J., Aardema, M., Banduhn, N., Carmichael, P., Fautz, R., Meuniere, J.-R., and Pfuhler, S. (2007a) *In vitro* approaches to develop weight of evidence (WoE) and mode of action (MoA) discussions with positive *in vitro* genotoxicity results. *Mutagenesis* 22:161-175.

Kirkland. D., Pfuhler, S., Tweats, D., Aardema, M., Corvi, R., Darroudi, F., Elhajouji, A., Glatt, H., Hastwell, P., Hayashi, M., Kasper, P., Kirchner, S., Lynch, A., Marzin, D., Maurici, D., Meunier, J.R., Müller, L., Nohynek, G., Parry, J., Parry, E., Thybaud, V., Tice, R., van Benthem, J., Vanparys, P., White, P. (2007b) How to reduce false positive results when undertaking *in vitro* genotoxicity testing and thus avoid unnecessary follow-up animal tests: Report of an ECVAM Workshop. *Mutat Res.* 628:31-55.

Pariza, M.W., Johnson, E.A. Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. *Regul. Toxicol. Pharmacol.* (2001) 33:173-86.

Attachment 3

Food Technology Report

A1004 – Phospholipase A_2 as a processing aid (enzyme)

Introduction

DSM Food Specialties (DSM) submitted an Application to amend Standard 1.3.3 – Processing Aids to include a genetically modified *Aspergillus niger* (*A. niger*) as a microbial source of the enzyme phospholipase A_2 (EC number 3.1.1.4) as a processing aid in the Table to clause 17 – Permitted enzymes of microbial origin.

The source microorganism *A. niger* is genetically modified to contain the same gene coding as the porcine pancreas. Consequently, the phospholipase A_2 contains the same 123 amino acid sequence as the phospholipase A_2 enzyme derived from porcine pancreas. Phospholipase A_2 derived from porcine pancreas is currently listed as a permitted processing aid in the Table to clause 15 – Permitted enzymes of animal origin of Standard 1.3.3. Phospholipase A_2 is also listed in the Table to clause 17 – Permitted enzymes of microbial origin of Standard 1.3.3 as a permitted processing aid from the microbial source *Streptomyces violaceoruber.* This microbial source is not genetically modified.

The phospholipase A_2 enzyme's primary use is to increase the efficacy of phospholipids such as lecithin used as an emulsifier in aqueous food products. DSM has suggested that the main uses of their phospholipase A_2 enzyme formulations would be in bakery products, sauces and dressings and would be particularly suitable for use in vegetarian, Halal and Kosher food products. The substrates for phospholipase A_2, phospholipids, are natural constituents of various foods as are also the reaction products, lyso-phospholipids, which form in the human body from the action of pancreatic phospholipase A_2 on dietary phospholipids (Rossiter, 1968; Johnson and McDermott, 1974).

The Application also states that the phospholipase A_2 acts as a processing aid in exactly the same way as phospholipase A_2 enzyme derived from porcine pancreas, which has been used for the hydrolysis of egg-yolk for more than 25 years (Dutilh and Groger, 1981). Phospholipase A_2 is used to hydrolyze natural phospholipids in food products, resulting in the formation of lyso-phospholipids that have surface active and emulsifying properties. After hydrolysis, the enzyme remains in the final product either as (1) an inactive protein in the case of products heated to over 65°C (e.g. in bakery products) or (2) as an enzyme with no functionality once the substrate has been depleted or there is a low pH (around 4) such as in sauces and dressings.

Although the enzyme may have no functionality at pH 4 or if there is no available substrate, theoretically, it may become functional again if the pH or substrate requirements are met. However, according to the Applicant, it is unlikely that the enzyme would become functional again as the manufacturing processes involved for products likely to use this enzyme would inactivate the protein. Any inactive or non-functional enzyme that may result in the final food would be metabolised like the phospholipase A_2 that is naturally present in other foods and human pancreatic phospholipase A_2.

Phospholipase A_2 hydrolyses the ester bond between the glycerol backbone and the fatty acid at the number two position of the glycerol backbone of lecithin, producing one molecule of lysolecithin and one molecule of fatty acid from one molecule of lecithin. The resulting lysolecithin product is a compound with emulsifying capabilities in many foods that are superior to that of the unmodified lecithin.

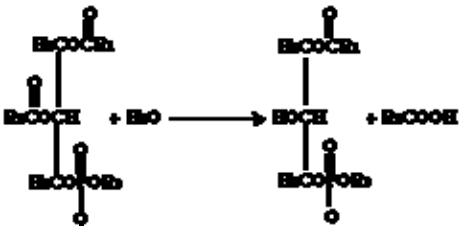
Microbial enzyme preparations have been widely used for a variety of purposes in the production of numerous food products for many years. The Code currently lists a number of enzymes produced from *A. niger* as permitted processing aids of microbial origin. Their practical application in fermented products dates back many centuries, long before the nature and function of enzymes or even the microorganisms themselves, were known or understood (Bechhom, Labbee and Underkofler, 1965).

Identity of the enzyme

Chemical name:	Phosphatidylcholine 2-acylhydrolase (IUBMB, 1992)
Common name:	Phospholipase A_2
Synonyms:	Lecithinase A; Phosphatidase; Phosphatidolipase
CAS Number:	9001-84-7
Enzyme Commission number:	3.1.1.4
Host organism:	*Aspergillus niger*

***Reaction*:** Phospholipase A_2 represents a class of heat-stable, calcium-dependent enzymes catalysing the hydrolysis of the sn-2-acyl bond of 3-*sn*-phospholipids.

Phosphatidylcholine + H_2O → 1-acyl-3-*sn-lyso*-phospholipid + carboxylic acid (fatty acid)



Enzyme production

The Application is for a new microbial source of the enzyme phospholipase A_2 for use as a food processing aid. This microbial source is a genetically modified *A. niger*, which produces the enzyme phospholipase A_2 with the gene coding the phospholipase A_2 enzyme obtained from porcine pancreas. Phospholipase A_2 has also been isolated from snakes and bees and is a natural constituent of digestive pancreatic juice of humans (Haas et al, 1968; Rossiter, 1968; Johnson and McDermott, 1974).

Phospholipase A_2 from porcine pancreas has been used for the hydrolysis of egg-yolk for more than 25 years (Dutilh and Groger, 1981). Phospholipase A_2 is also recognised as a normal constituent of wheat flour (Nolte et al., 1974).

The reaction product lysolecithin (i.e. a glycerol backbone with the fatty acid at position two removed) is naturally present in egg-yolk[5]. The phospholipids that are the substrate of phospholipase A_2 and the lysolecithin formed as the end product of hydrolysis of lecithin by phospholipase A_2 are both also normal constituents of wheat flour (Eliasson and Larsson, 1993; Hargin and Morrison, 1980; Morrison et al., 1975; Clayton and Morrison, 1972). Lysolecithin formed by the action of phospholipase A_2 on lecithin was affirmed as Generally Recognised As Safe (GRAS) in the USA in 1996 (21 C.F.R. 184.1063).

[5] Encyclopaedia of Food Science, Food Technology and Nutrition, 1993.

DSM provided information on the production of phospholipase A_2 from *A. niger*. The enzyme is produced by microbial fermentation under containment using food-grade raw materials. Once fermentation has been completed, the microbial biomass is killed off by addition of sodium benzoate (final concentration of 4.0 g/kg) at a broth temperature of 30 ^{0}C and pH 4.0. The microbial biomass is separated from the fermentation broth before the broth undergoes a purification and formulation process. The finished product, phospholipase A_2, is free from the production strain.

During production of the enzyme, *A. niger* also produces other enzymes which it uses for the breakdown of nutrients and other cell material. Although phospholipase A_2 is produced in excess, the initial enzyme preparation will contain other enzymes such as glucoamylase, amylase and protease. These enzymes do not assist in the technological function of the phospholipase A_2 enzyme and, according to the Applicant, these are separated and removed from the phospholipase A_2 formulations (e.g. the two commercial products produced by the Applicant) by column chromatography or by simple filtration, centrifugation, polish or ultra-filtration.

According to the Applicant, the fermentation process for the phospholipase A_2 enzyme is the same for the two commercial products being made and the Applicant envisages that the cost of the microbial phospholipase A_2 will be similar, on an activity basis, to the animal derived version. The difference between the Applicant's two products is the end formulation. One product is a liquid primarily for edible oil products and egg-based sauces and dressing, and the other a granulated product primarily used for bread, bakery and some egg-based products. Regardless of the formulation, the enzyme is used for the hydrolysis of lecithin, which results in the production of a modified lecithin, referred to as lysolecithin, with improved emulsifying power.

It is recognised that in the manufacture of a microbial enzyme the reactions catalysed by any given active component are essentially the same, regardless of the source from which that component is derived (Food Chemicals Codex, 1996). From the information provided by the Applicant, the reactions from the phospholipase A_2, from the genetically modified *A. niger* is the same as that produced by the phospholipase A_2 from other non-genetically modified microbial and animal sources.

Identity and purity

(a) Identity

The DNA coding for phospholipase A_2 is derived from the porcine pancreas. The amino acid sequence of the enzyme expressed by *A. niger* is exactly the same as that derived from the porcine pancreas. The amino acid sequence of the porcine pancreas enzyme has been published in the literature (Verheij et al. 1981). The porcine phospholipase A_2 has a primary sequence of 123 amino acids and a calculated molecular weight of 13980 Da. (Haas et al, 1968). The porcine pancreatic phospholipase A_2 is not glycosylated (Nieuwenhuizen et al, 1973).

The Applicant provided information to support that the phospholipase A_2 enzyme expressed by *A. niger* is identical to that of porcine derived phospholipase A_2.

The Applicant indicated that via electro-spray mass spectrometry, the *A. niger* phospholipase A_2 was shown to have a molecular weight of 13982 Da, which is in good agreement with the theoretical mass of 13980 Da. The last 6 amino acids at the N-terminus of the protein show the same sequence for both the *A. niger* and the porcine phospholipase A_2, namely Ala_1-Leu_2-Trp_3-Gln_4-Phe_5-Arg_6. This sequence is in full agreement with the mature form of phospholipase A_2 described in the literature (Verheij et al., 1981).

(b) Purity

The Application states that the enzyme preparation complies with the international specifications relevant for enzymes, which are compiled by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), in the Compendium of Food Additives Specifications (2001) and the Food Chemical Codex (2004). These specification references are both primary sources of specifications listed in clause 2 of Standard 1.3.4 – Identity and Purity.

The specification of a batch of un-standardised enzyme taken from the Application is provided in table 1 below compared to the JECFA specification.

Table 1: Specifications for phospholipase A_2

Criteria	**JECFA specification**	**Results for phospholipase A_2**
Heavy metals as Pb	Not more than 40 ppm	<30 ppm
Lead	Not more than 5 ppm	<1 ppm
Arsenic	Not more than 3 ppm	<3 ppm
Cadmium		<0.5 ppm
Mercury		<0. 5 ppm
Total viable counts (cfu/g)	Not more than 50,000	<400
Total coliforms (cfu/g)	Not more than 30	<1
Enteropathogenic *E. coli* (/25 g)	Negative by test	Not detected
Salmonella (/25 g)	Negative by test	Not detected
Antibiotic activity	Negative by test	Not detected
Production strain (/g)		Not detected

The Applicant states that the manufacturing process ensures that there are no production micro-organisms (the genetically modified *A. niger*) present in the final enzyme preparation.

The specification of the enzyme of this Application satisfies the relevant specification of the Code.

Applications

The substrates for phospholipase A_2, phospholipids, are natural constituents of various foods. The reaction products, lyso-phospholipids, form in the human body from the action of pancreatic phospholipase A_2 on dietary phospholipids (Rossiter, 1968; Johnson and McDermott, 1974).

Commercial lecithin is a naturally occurring mixture of phosphatides of choline, ethanolamine and inositol, with smaller amounts of lipids. Lecithin is widely used in many categories of food as an emulsifier. Lecithin functions effectively as an emulsifier in fat-based food systems. For aqueous food systems such as baked goods, lecithin must be altered structurally either chemically or enzymatically, to function effectively as an emulsifier.

Table 2: Important food applications for lecithins

Application	Typical Function
Bakery goods	Improvement of volume
	Fat dispersion
	Anti-staling
Chocolate	Reduction of viscosity
	Prevention of crystallisation
Instant products	Wetting
	Dispersion
Margarine and edible oil spreads	Stabilisation of product
	Prevention of spattering
	Browning and dispersion of sediment

(Van Nieuwenhuyzen, 1981)

The Applicant has envisaged that their phospholipase A_2 enzyme formulations will be used in products such as:

- breads and bakery products (tin breads, buns and rolls e.g. French sticks or batards, biscuits, crackers, doughnuts, muffins and a variety of breads like e.g. multi grain types of bread, raisin bread, etc);
- eggs and egg products (egg-yolk based fine bakery wares e.g. high-ratio cake, pound cake, Swiss rolls, snack cakes, etc);
- mixed foods (mayonnaise, salad dressings, sauces, etc); and
- edible oils and oil emulgations (margarine).

Enzymic modification has advantages over chemical modification in that chemical modification generates non-specific hydrolysis products and can be costly. The use of lysolecithin for food applications has distinct advantages over lecithin. Lysolecithin is able to better stabilise the oil-in-water emulsions in many food products than lecithin.

Modified lecithins have many uses in foods (Meinhold, 1991; van Nieuwenhuyzen, 1981) including, but not limited to bakery, confectionery, dairy, edible oil and beverage products. In these products, the modified lecithin can act as an emulsifying agent, a mixing aid, a release agent, an egg replacer, and as a flavour in food systems. For example, traditional mayonnaise can be considered as an acidic oil-in-water emulsion, which is stabilised by egg yolk. The stabilising power of egg yolk is due mainly to the presence of lipoproteins. One of the problems in mayonnaise production is the breaking of the emulsion, which leads to oil exudation. This occurs when the temperature is raised over 70°C, or cooled below 0°C or when too much shear is applied.

Treatment of egg yolk with phospholipase A_2 results in hydrolysis of the phospholipids (lecithin). Egg yolk fermented with phospholipase A_2 has been shown to be a more potent emulsifier for mayonnaise than untreated egg yolk.

Allergenicity

The enzyme, phospholipase A_2, is a normal constituent of wheat flour and phospholipase A_2 itself is not considered to be allergenic. However, in their Application, DSM indicates that their granulated formulation (e.g. used in bakery products) may be granulated on wheat flour. The use of this formulation would require wheat flour (gluten) to be declared in the product under the requirement contained in Standard 1.2.3. The liquid formulation is diluted with water; therefore there would be no labelling requirement under Standard 1.2.3.

Stability in processing

Phospholipase A_2 can hydrolyse lecithin to lysolecithin under a wide range of conditions. The enzyme's activity rises with increasing temperature and is greatest between 50°C and 60°C. The enzyme is inactivated at temperatures above 65°C.

Like *S. violaceoruber* derived phospholipase A_2 which is active over a wide pH range, phospholipase A_2 derived from *A. niger* is also active over a wide pH range depending on the specific application. This range is between 6 and 9.5 with the optimum pH for activity at or near pH 8.5. The usage level will vary according to the application and desired degree of enzymic conversion.

After hydrolysis, the enzyme remains in the final product as an inactive protein (i.e. if heated to at least 65°C) or as an enzyme with no functionality once the substrate has been depleted.

Conclusion

Phospholipase A_2 is a naturally occurring enzyme in a number of foods and is also produced by the human pancreas. Phospholipase A_2 from animal and microbial sources are currently used as a processing aid to improve the emulsifying capabilities of naturally present or added phospholipids (primarily lecithins) to improve the desired characteristics of the food.

At present, two sources of phospholipase A_2 are listed in the Code, Standard 1.3.3*;* one is an animal-derived enzyme from porcine pancreas, the other from a non-genetically modified microbial source, *Streptomyces violaceoruber*.

The advantage to the manufacturer and final consumer are in the benefits the lysolecithin imparts on food such as emulsification properties and improved heat stability in foods, including mayonnaise, ice-cream, margarine, and baked goods. Consumers may also benefit by having a greater choice of new, heat-stable foods that are developed by food manufacturers.

Phospholipase A_2 from this genetically modified, microbial source, *A. niger* is technologically justified and will provide food manufacturers with an alternative microbial source of this enzyme.

References

Bechhom, E.J. M.D. Labbee, and L.A. Underkofler. 1965 Production and use of microbial enzymes for food processing. J. Agric. Food Chem. 13;30-34.

Dutilh. C.E. and W. Groger. 1981 Improvement of product attributes of mayonnaise by enzymatic hydrolysis of egg yolk with phospholipase A02 EC 3.1.1.4. J. Sci. Food Agric. 32(5): 451-458.

Food and Nutrition Paper 52 Compendium of Food Additive Specifications Volume 1 and 2, including addenda 1 to 12, published by the Food and Agriculture Organisation f the United Nations in Rome (1992)

Food Chemical Codex 5th edition, Enzyme Preparations – Monograph specifications: 129 -134 and 786 -788), published by the National Academy of Science and the National Research Council of the United States of America in Washington, D.C. (2004).

Combined Compendium of Food Additive Specifications, FAO JECFA Monographs No. 1, Online Edition, at http://www.fao.org/docrep/009/a0691e/A0691E00.htm

International Union of Biochemistry and Molecular Biology (IUBMB) Nomenclature Committee. 1992. Enzyme Nomenclature Recommendations (1992) of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology. Academic Press, Inc., San Diego.

Meinhold, N. M. Lecithin: Development and applications. Food Processing, May 1991, p 130-134.

Van Nieuwenhuyzen, W. The industrial uses of special lecithins: a review. JAOCS, Oct. 1981, p. 886-888.

Pages 000173-000545 have been removed in accordance with copyright laws. Please see the bibliography list of the references that have been removed from this request on pages 000057-000061.

SUBMISSION END

Memorandum of to the File

Date: June 3, 2014

To: GRN 524 (RB) - Phospholipase A2 enyzme preparation from Trichoderma reesei carrying a phospholipase A2 gene from Aspergillus nishimurae

Subject: Revised GRAS Exemption Claim with live signature - Filing Date

FDA received a revised GRAS exemption claim from Karl-Heinz Maurer of AB Enzymes GbH regarding their GRAS determination for their submission.

The appropriate filing date is May 29, 2014, the date of the receipt of the amendment rendering the submission fillable as a GRAS notice.

Richard E. Bonnette

Cc: GRN 000522
FT:HFS-255:RBonnette:HFS-255:6/3/14

Richard E. Bonnette lii III -S	Digitally signed by Richard E. Bonnette lii III -S DN: c=US, o=U.S. Government, ou=HHS, ou=FDA, ou=People, 0.9.2342.19200300.100.1.1=1300212202, cn=Richard E. Bonnette lii III -S Date: 2014.06.04 09:48:10 -04'00'	

(b) (6)

6/6/14

Bonnette, Richard

From: Srinivasan, Jannavi
Sent: Thursday, May 15, 2014 8:25 AM
To: 'Cryne, Candice'
Cc: Bonnette, Richard
Subject: RE: Withdrawal of GRN 490

Dear Candice,

I just spoke with our GRAS Pre-filing Team, and there are a couple of items we needed to bring to your attention on the GRAS-Exemption claim letter in order to file the recent dossier you have sent to us for review (phospholipase A2 enzyme preparation from a genetically modified *T. reesei* for use in degumming oils):

1. It is not clear whether the signature (by Karl-Heinz Maurer) is original or a scanned copy of the same. We would need a letter with the original signature.
2. The letter is dated as October 21, 2013, while the cover letter for this dossier is dated April 26, 2014. These need to align.

We would appreciate if you can fix these two issues and send us back the same.

I am copying Richard Bonnette, who is on the GRAS Pre-filing Team, who you can send the corrected letter to, and contact if you have any further questions regarding these prefiling issues.

Thank you!

Jannavi

From: Cryne, Candice [mailto:Candice.Cryne@abenzymes.com]
Sent: Monday, May 12, 2014 6:54 PM
To: Srinivasan, Jannavi
Subject: RE: Withdrawal of GRN 490

Hi Jannavi,

Just wondering if my resubmission was processed yet?

Many thanks, Candice

From: Srinivasan, Jannavi [mailto:Jannavi.Srinivasan@fda.hhs.gov]
Sent: Wednesday, April 30, 2014 4:41 PM
To: Cryne, Candice
Subject: RE: Withdrawal of GRN 490

Thanks for the heads-up, Candace. I will keep a look out.
Best
Jannavi

From: Cryne, Candice [mailto:Candice.Cryne@abenzymes.com]
Sent: Wednesday, April 30, 2014 4:36 PM

To: Srinivasan, Jannavi
Subject: RE: Withdrawal of GRN 490

Hi Jannavi,

I just wanted to let you know, that I sent the resubmitted GRAS notice and it was received by your office today.

Please let me know if you need anything.

Kind regards, Candice

From: Srinivasan, Jannavi [mailto:Jannavi.Srinivasan@fda.hhs.gov]
Sent: Monday, April 14, 2014 11:50 AM
To: Cryne, Candice
Subject: RE: Withdrawal of GRN 490

Yes, all is well, Candice. I am sorry I could not meet you personally, but my colleagues felt likewise about the meeting with you ☺
Looking forward to hearing from you shortly!
Jannavi

Dr. Jannavi R. Srinivasan
FDA/CFSAN
Office of Food Additive Safety
Division of Biotechnology and GRAS Notice Review
College Park MD 20740
Ph: 240 402 1199

From: Cryne, Candice [mailto:Candice.Cryne@abenzymes.com]
Sent: Monday, April 14, 2014 11:48 AM
To: Srinivasan, Jannavi
Subject: RE: Withdrawal of GRN 490

Dear Jannavi,

I had a great meeting with your colleagues and I appreciate you setting this meeting up. We will be resubmitting the application shortly.

I hope all is well.

Kind regards, Candice

From: Srinivasan, Jannavi [mailto:Jannavi.Srinivasan@fda.hhs.gov]
Sent: Friday, March 28, 2014 1:15 PM
To: Cryne, Candice
Subject: RE: Withdrawal of GRN 490

Candice,

I am assuming you will drive as I send you these directions. Lease let me know if not.

We are in the University Station Building at 4300 River Road, College Park, MD 20740, and the meeting will be in Conf Room 2073. So we are in the building annex to Harvey Wiley Building, where majority of CFSAN offices are housed, and that is the address that comes up on Google (5100 Paintbranch Parkway, College Park MD). When facing the parking lot at FDA's College Park Campus, the University Station Building is the smaller building on the right hand side of the parking

AB Enzymes GmbH – Feldbergstrasse 78 , D-6412 Darmstadt

AB Enzymes
an ABF Ingredients company

October 21, 2013
RE: GRAS Notification – Exemption Claim
Dear Sir or Madam:

Pursuant to the proposed 21C.F.R. § 170.36 (c)(1) AB Enzymes GmbH hereby claims that phospholipase enzyme preparation from *Trichoderma reesei* (*T.reesei)* strain RF8793 expressing the gene encoding phospholipase A2 from *Aspergillus nishimurae* (*A. nishimurae)* (formerly *Aspergillus fumigates* (*A. fumigates*) produced by submerged fermentation is Generally Recognized as Safe; therefore, they are exempt from statutory premarket approval requirements.

The following information is provided in accordance with the proposed regulation:

Proposed 21C.F.R. § 170.36 (c)(i) *The name and address of notifier.*
AB Enzymes GmbH
Feldbergstr. 78
D-64293 Darmstadt
Germany

Proposed 21C.F.R. § 170.36 (c)(ii) *The common or usual name of notified substance.*
Phospholipase enzyme preparation from *Trichoderma reesei* expressing the gene encoding a phospholipase A2 from *Aspergillus nishimurae* (formerly *Aspergillus fumigates).*

Proposed 21C.F.R. § 170.36 (c)(iii) *Applicable conditions of use.*
The phospholipase A2 is intended for use in edible oil refining as a processing aid in oil degumming. The enzyme preparation is used at minimum levels necessary to achieve the desired effect and according to requirements under current Good Manufacturing Practices.

Proposed 21C.F.R. § 170.36 (c)(iv) *Basis for GRAS determination.*
This GRAS determination is based upon scientific procedures.

Proposed 21C.F.R. § 170.36 (c)(v) *Availability of information.*
A notification package providing a summary of the information which supports this GRAS determination is enclosed with this letter. The package includes **a** safety evaluation of the production strain, the enzyme, and the manufacturing process, as well as an evaluation of dietary exposure. Complete data and information that are the basis for this GRAS determination are available to the Food and Drug Administration for review and copying at reasonable times at a specific address set out in the notice or will be sent to FDA upon request.

(b) (6)

Oct 22, 2013

Karl-Heinz Maurer
Director, Business Development

Date

AB Enzymes GmbH – Feldbergstrasse 78 , D-6412 Darmstadt

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an ABF Ingredients company

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(b) (6)

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Date

Karl-Heinz Maurer
Director, Business Development