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

25 February 2009
[2-09]

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

Executive Summary

Purpose

Food Standards Australia New Zealand (FSANZ) received an Application (A1004) from DSM Food Specialties Pty Ltd on 21 January 2008. The Applicant subsequently chose to expedite consideration of their Application by the payment of the relevant charge. 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 was 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 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 label their food as genetically modified as there will be no novel DNA and/or no novel proteins present in the final food product. The source organism is killed off and removed during the manufacturing process used for producing the enzyme preparation so there will be no novel protein or novel DNA in the final enzyme preparation. This is typical for enzymes sourced from genetically modified microorganisms approved in the Code.

The Application claims that the enzyme could be used in the production of foods certified as halal and kosher or called vegetarian. The Code does not define these terms and as such labelling issues relating to these aspects are outside the scope of this Application.

Phospholipase A2, is a normal constituent of wheat flour and phospholipase A2 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 requirements contained within clause 4 of 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.

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 provided for in section 29 of the *Food Standards Australia New Zealand Act 1991* (the FSANZ Act):

- whether costs that would arise from a food regulatory measure developed or varied as a result of the application outweigh the direct and indirect benefits to the community, Government or industry that would arise from the development or variation of the food regulatory measure;

- whether other measures (available to the Authority or not) would be more cost-effective than a food regulatory measure developed or varied as a result of the application;

- any relevant New Zealand standards; and

- any other relevant matters.

Decision

FSANZ approves the 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 Decision

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 approved. 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 food 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 were invited on the A1004 Assessment Report. Comments were specifically requested on the scientific aspects of the 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.

A total of 4 submissions were received. A summary of these is provided in **Attachment 2** to this Report.

As this Application is being assessed as a general procedure, there was only one round of public comment. Submissions to this Assessment Report were used to develop the Approval Report for this Application. The main issues raised in public comments are discussed in this Report. Neither the preferred approach nor the draft variation to the Code has altered from that proposed in the Assessment report.

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INTRODUCTION

Food Standards Australia New Zealand (FSANZ) received an Application (A1004) from DSM Food Specialties Pty Ltd on 21 January 2008. The Applicant subsequently chose to expedite consideration of their Application by the payment of the relevant charge. 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 (2006)[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.

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[1] Combined Compendium of Food Additive Specifications, FAO JECFA Monographs No. 3 (2006), Online Edition, at http://www.fao.org/ag/agn/jecfa-additives/search.html?lang=en Accessed on 7 January 2009

[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).

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.

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 (e.g., 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.

The Applicant claims the advantages of phospholipase A_2 to food 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 4**) 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, the Applicant 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 clause 4 of Standard 1.2.3.

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 Application claims that the enzyme could be used in the production of foods certified as halal and kosher or called vegetarian. The Code does not define these terms and as such labelling issues relating to these aspects are outside 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; and
- 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;

[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.

- 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 which FSANZ considered as part of the assessment were:

- 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:

[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.

- 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 3**.

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 4**.

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^{o}C. The temperature of the broth is then decreased to approximately 15^{o}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 the Application, the Applicant 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 product due to the requirements contained in the 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. Due to these conclusions there is no need to develop any particular unique risk management strategy other than to treat them as similar enzyme processing aids.

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.

Two options have been identified for this Application:

Option 1: Reject the Application, thus maintaining the *status quo*.

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) and 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.

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*, does not provide any additional benefit or cost to the food industry, consumers and government.

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 for option 2. Therefore Option 2 is the preferred option.

COMMUNICATION AND CONSULTATION STRATEGY

9. Communication

FSANZ has applied a basic communication strategy to Application A1004. This involved advertising in the national press the availability of the Assessment Report for public comment, which gave 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 have been notified at each stage of the Application. FSANZ will notify the Board's approval of the draft variation to the Ministerial Council. The Applicant and stakeholders, including the public generally, will be notified of the gazetted changes to the Code in the national press and on the FSANZ website.

10. Consultation

10.1 Issues Raised in Public Consultation

The Assessment Report was advertised for public comment between 28 October 2008 and 9 December 2008. Comments were specifically requested on the scientific aspects of this Application. As this Application is being assessed under a General Procedure, there was one round of public comment.

A total of four submissions were received, three submissions supported the Application, of these, two requested issues that they would like to be addressed at the Approval stage. One submitter opposed the Application due to its GM aspects. A summary of the submissions is provided in **Attachment 2** to this Report. FSANZ has taken the submitters' comments into account in preparing the Approval Report for this Application.

Specific issues related to the GM aspects of the Application, the removal of the source organism from the enzyme preparation, reference to vegetarian, kosher, halal terms as used in the Assessment report and the use of allergenic fillers in food enzymes are further discussed below.

10.1.1 Issues relating to the GM aspects of the application

One private submitter objected to the Application on the basis of the GM aspects of the Application. Issues raised included an opposition in general to GM food products and their derivatives, stating that there are 'definite findings against the long term health provided by these products'. The Submitter also stated that natural, non-GM alternatives should be used where available and that GM products should be clearly labelled.

10.1.1.1 FSANZ response

The safety aspects of both the enzyme and its source organism have been thoroughly addressed in the Safety Assessment Report (**Attachment 3**). In the case of enzymes produced from GM micro-organisms, the enzyme itself is not a novel protein since it is identical to other enzymes sourced from non-GM sources. The refinement process for the enzyme preparation removes all the source organisms from the preparation so there is no novel DNA remaining in the enzyme preparation.

This is the case for a number of enzymes sourced from GM micro-organisms in the Code. Further information relating to GM foods is also available from the FSANZ website[5].

10.1.2 Removal of source organism from enzyme manufacture

One submitter questioned the case of the enzyme manufacturing process failing to completely remove the source organism and noting that there has been no mention of tests or criteria to ensure the complete removal of the source organism from the enzyme preparation.

10.1.2.1 FSANZ response

The Applicant has stated that the manufacturing process ensures that there are no production micro-organisms (genetically modified *A. niger*) present in the final enzyme preparation.

The separation of biomass from fermentation fluid, under a tightly controlled GMP environment ensures that the commercial enzyme preparation is free from the production microorganism. The refinement process for the enzyme preparation as described in the Application effectively kills and physically removes all the source organisms from the enzyme preparation. Specifically this includes the separation of cellular material from the broth containing the phospholipase A_2 enzyme by means of filtration and/or centrifugation.

The fermentation broth is effectively filtrated with a filter aid to eliminate cellular materials. Any remaining particles are removed with a polish filtration and a germ reduction filtration step and then further concentrated by ultra-filtration (UF) methods. The commercial liquid formulation is further purified by chromatography methods.

These refinement processes, as describe in the Application, are considered adequate to completely remove the production organism from the enzyme preparation and are standard practices in food grade enzyme production. As such it is not necessary for a criterion and/or test to be described to ensure the complete removal of the source organism in this Approval report.

The Applicant has additionally stated in their product specifications that the source organism is 'not detected' in the final enzyme preparation and that the enzyme preparation complies with the international specifications relevant for food enzymes, compiled by the Joint FAO/WHO Expert Committee on Food Additives (JECFA), in the Compendium of Food Additives Specifications (2006) and the Food Chemical Codex (2004).

10.1.3 Reference to 'vegetarian', 'kosher' and 'halal' terms

There was some ambiguity noted in the Assessment Report, with clarification requested for stating that the Applicant claimed the enzyme 'would be suitable for use in vegetarian, kosher or halal food products' in one instance, and 'may be suitable for use in vegetarian, kosher or halal food products' in another.

[5] FSANZ (2008) Frequently Asked Questions on Genetically Modified Foods http://www.foodstandards.gov.au/foodmatters/gmfoods/frequentlyaskedquest3862.cfm, Accessed on 18 December 2008

The Food Technology Association of Australia and Queensland Health have noted in their submissions that vegetarian, kosher or halal should not be canvassed within the Application as these terms are not defined in the Code and have no bearing on public health and safety issues.

10.1.3.1 FSANZ response

Within the Application, the Applicant has stated that the enzyme 'can be used in' foods certified as halal and kosher and in vegetarian foods and 'is particularly suitable for use in' the production of vegetarian, halal and kosher foodstuffs. As the Code does not define vegetarian, kosher and halal it is not possible and not in the scope of this Approval Report to canvass these definitions, other than to note the Applicant's claims.

10.1.4 Reference to the FSANZ Vegetarian Labelling Application (A545)

One submitter noted that in previous FSANZ reports (e.g. A490) the Application, A545 – Vegetarian Labelling had been noted, whereas in this Application, the Assessment Report did not make reference to Application A545. It was noted that the Code does not define the terms vegetarian, halal or kosher and as such issues relating to these aspects are outside of the scope of this Application.

10.1.4.1 FSANZ response

This is the current situation; the Code does not define the terms vegetarian, halal or kosher. It is noted that FSANZ is currently considering Application A545.

10.1.5 Issues relating to the management of food allergens used as 'fillers' in processing aids

One submitter reiterated that any enzyme preparations containing fillers utilising allergenic sources such as wheat flour will require an allergen declaration on the label of the final food product produced using such forms of enzymes. It was noted that this issue of using allergens as fillers in enzyme preparations needs to be addressed under the proposed work for the Regulatory Management of Food Allergens.

10.1.5.1 FSANZ response

In accordance with the current clause 4 of Standard 1.2.3, the presence of allergenic substances must be declared on the label of the final food product, which the Applicant has duly noted in the Application. These requirements apply to ingredients, additives and processing aids and, therefore, would capture fillers in enzyme preparations including phospholipase A_2 derived from genetically modified *A. niger* with a gene isolated from the porcine pancreas. For unpackaged foods, allergens must be declared on or in connection with the display of a food or declared to the purchaser upon request.

Manufacturers are required to label when an allergen is contained in processing aids (includes enzyme preparations) utilised in food manufacture. FSANZ requires applicants to address this issue as part of an application as provided for in the FSANZ *Application Handbook*.

The submission suggesting that the topic of 'allergens used as fillers in enzyme preparations' should be addressed under the proposed FSANZ work on the review of the Regulatory Management of Food Allergens has been passed on to the relevant FSANZ team members.

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 decided not to notify the WTO under either the Technical Barriers to Trade or Sanitary and Phytosanitary Measures Agreements.

CONCLUSION

11. Conclusion and Decision

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 Approval 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 (which is the same as that proposed at Assessment) is provided in **Attachment 1**. FSANZ has not made any amendments to the draft variation as a result of consideration of the submissions received.

Decision

FSANZ approves the 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 Decision

FSANZ approves the draft variation to Standard 1.3.3 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

The FSANZ Board's decision will 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. Summary of issues raised in public submissions
3. Safety Assessment Report
4. 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 in the* Table to clause 17 *for the enzyme* Phospholipase A_2 EC 3.1.1.4 the Source –

Aspergillus niger, containing the gene isolated from porcine pancreas

Attachment 2

Summary of Issues Raised in Public Submissions

Submitter	Option	Comments
Food Technology Association of Australia	**2**	• Supports progression. • Noted that the possible acceptability of this enzyme being acceptable either as vegetarian, kosher or halal, should not be canvassed as these terms are not defined in the Code and has no bearing on public health and safety issues.
New Zealand Food Safety Authority	**2**	• Supports progression. • Supports the conclusion that the use of the enzyme is technologically justified and that no public health or safety concerns were identified.
Queensland Health	**2**	• Clarification requested for an ambiguity identified in the Assessment Report for the Applicant claiming that the enzyme 'would be suitable for use in vegetarian, kosher or Halal food products' and 'may be suitable for use in vegetarian, kosher or Halal food products'. • Noted that information relating to Application A545 – Vegetarian Labelling had been addressed in previous FSANZ assessment reports (e.g. A490), yet in this Application it is stated that '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'. • Questions the case of the enzyme manufacturing process failing to completely remove the source organism and notes that there has been no mention of tests or criteria to ensure complete removal of the source organism. • Notes that the FSANZ work proposed for the 'Regulatory Management of Food Allergens' should address the use of allergens used as bases in food enzyme formulations.
Val Mesh (Private)	**1**	• Opposes approving the enzyme due to the GM aspects of the Application. • Opposes GM products and derivatives for use in the food supply in general based on proposed 'definite findings against the long term health provided by these products'. • States that where natural alternatives are available, profit should not be used as a reason for allowing a GM alternative. • Notes that GM products should be clearly labelled.

Attachment 3

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 PLA2, 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'.

Introduction

Application A1004 concerns the use of *A. niger* containing the gene coding for porcine phospholipase A_2 (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.

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.

Toxicity

Single-Dose Toxicity of Phospholipase A_2 in Rats

Study15.750. 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.

Study15.751. 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

Study15.234. 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

Study15.234. 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, justification 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.

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.

Study details	Method	Results	Validity
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.
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, phytohaemagglutinin 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 (per 1000 cells, duplicate cultures) and chromosome aberrations were determined (per 100 metaphase cells, duplicate incubations). Concn.-ranging test: Concs. tested 100, 333, 1000, 3330, 5000 µg/mL for 3 (±S9), 24 (-S9) and 48 h (-S9) incubations. Cytogenetics test #1: Concs. tested (µg PLA_2 (active enzyme)/mL): -S9: 1000, 1800, 3330, 4200, 5000 for 24 and 48 h incubations. For 24 h, 4200 and 5000 were not scored for chromosome aberrations. For 48 h, 1000 was not scored for chromosome aberrations. +S9: 333, 1000, 3330, 5000 for 3 h incubations (333 was not scored for chromosome aberrations). Cytogenetics test #2: Concs. tested 560, 1000, 1300, 1800, 2400, 3330 µg/mL using EDTA-inactivated PLA2 (see legend) for 48 h incubations (-S9).	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 in chromosomal aberrations was observed in the presence of S9. Positive controls gave expected results (±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 concn.-dependent increase in chromosome aberrations was observed at 560 to 1300 µg/mL ($p<0.01$). The aberrations included chromatid gaps,	GLP compliant. Negative controls and positive controls (mitomycin C and cyclophosphamide) elicited significant increases in cells with chromosome aberrations and S9 metabolism. Results continued: Number of metaphase cells (mitotic index, % of control) for test #1 (24/48h) were: 81/122%, 56/86%, 46/74%, 33/55% and 36/45% for 1000, 1800, 3330, 4200, and 5000 µg/mL. Mitotic index (% of control) in test #2 (48h): 86%, 62%, 29%, 12%, 3%, 2%. Mitotic index for mitomycin C treated cells were 98% (24 h) and 146% (48 h) of control for test #1 and 93% (48 h) for test #2. 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

Study details	Method	Results	Validity
	Concs ≥1800 were not scored for chromosome aberrations. PLA_2 batch PLA9901, purity 14.7%, vehicle, MilliQ water.	chromosome gaps, minutes, double minutes and increased miscellaneous findings such as polyploidy, endo-reduplication multiple aberrations and chromosome intrachange. Positive control gave expected results. Continued…	caused by the low concn. of EDTA. Under these *in vitro* conditions, PLA_2 should be considered as potentially 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).

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.

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'.

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Attachment 4

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 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 in the same manner as 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.

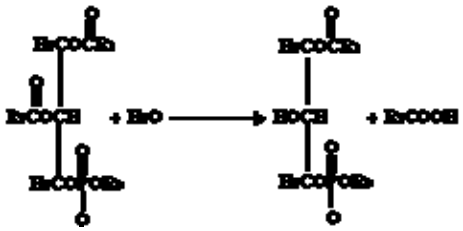
Microbial enzyme preparations have been widely used for a variety of purposes in the production of numerous food products for many years. The Code currently lists a number of enzymes produced from *A. niger* as permitted processing aids of microbial origin. Their practical application in fermented products 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 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[6]. 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) by the US FDA in 1996 (Code of Federal Regulations Title 21, section 184.1063).

[6] 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

(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

Enzymatic 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.

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