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

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FINAL ASSESSMENT REPORT

APPLICATION A561

PHOSPHOLIPASE A_1 AS A PROCESSING AID (ENZYME)

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

EXECUTIVE SUMMARY

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

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

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

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

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

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

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

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

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

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

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

Purpose

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

Decision

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

Reasons for Decision

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

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

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

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

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

Consultation

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

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

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INTRODUCTION

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

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

1. Background

1.1 Current Standard

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

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

1.2 Historical Background

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

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

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

1.3 Other International Regulatory Standards

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

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

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

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

2. The Issue

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

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

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

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

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

3. Objectives

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

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

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

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

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

4. Key Assessment Questions

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

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

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

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

RISK ASSESSMENT

5. Risk Assessment Summary

5.1 Safety Assessment

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

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

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

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

5.2 Source Organism Nomenclature

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

5.3 Technological Justification

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

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

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

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

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

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

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

RISK MANAGEMENT

6. Options

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

The two regulatory options available for this Application are:

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

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

7. Impact Analysis

7.1 Affected Parties

The affected parties to this Application include the following:

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

2. consumers; and

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

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

7.2 Benefit Cost Analysis

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

7.2.1 Option 1 – Not approve the use of the enzyme

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

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

7.2.2 Option 2 – Approve the use of the enzyme

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

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

7.3 Comparison of Options

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

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

COMMUNICATION

8. Communication and Consultation Strategy

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

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

9. Consultation

9.1 Public Consultation

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

9.2 Issues Arising from Submissions

9.2.1 GM labelling requirements

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

9.2.1.1 FSANZ response

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

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

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

9.2.2 Safety of phospholipase A_1 from a GM source

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

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

9.2.2.1 FSANZ response

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

9.2.3 Approval of phospholipase A_2 before phospholipase A_1

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

9.2.3.1 FSANZ response

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

9.2.4 GRAS status of phospholipase A_1 in the USA

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

9.2.4.1 FSANZ response

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

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

9.2.5 Production of aflatoxins from the source organism

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

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

9.2.5.1 FSANZ response

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

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

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

9.2.6 Lack of consumer choice for food produced using GM technology

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

9.2.6.1 FSANZ response

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

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

9.3 World Trade Organization (WTO)

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

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

CONCLUSION

10. Conclusion and Preferred Option

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

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

11. Implementation and Review

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

ATTACHMENTS

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

Attachment 1

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

To commence: on gazettal

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

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

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

Attachment 2

Safety Assessment Report

A561 – PHOSPHOLIPASE A_1 AS A PROCESSING AID

Summary and Conclusion

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

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

The safety assessment concluded that:

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

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

1. Introduction

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

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

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

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

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

2. Purity of enzyme preparation and proposed specifications

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

Table 1: Complete specification of phospholipase A_1 preparation

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

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

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

3. The production organism – *Aspergillus oryzae*

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

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

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

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

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

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

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

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

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

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

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

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

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

4. Characterisation of the genetic modification

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

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

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

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

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

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

Genetic stability

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

5. Evaluation of the safety studies

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

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

5.1 Potential toxicity and allergenicity of phospholipase A_1

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

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

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

5.2 Short term toxicity

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

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

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

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

5.3 Sub-chronic toxicity

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

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

Study conduct

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

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

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

Results

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

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

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

Ophthalmoscopy revealed no treatment related effects.

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

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

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

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

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

Conclusion

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

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

5.4 Genotoxicity studies

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

Test article

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

Study design

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

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

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

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

Results and conclusion

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

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

Test article

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

Study design

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

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

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

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

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

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

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

5.5 Cytotoxicity

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

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

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

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

6. Overall Conclusion

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

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

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

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APPENDIX A

Analyses performed in 13-week oral toxicity study in rats

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

Attachment 3

Food Technology Report

Phospholipase A_1 as a processing aid (enzyme)

Introduction

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

Enzyme details

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

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

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

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

Phospholipase A_1 catalyses the reaction of:

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

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

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

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

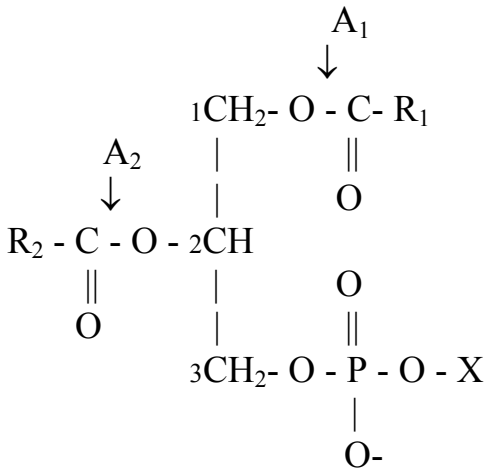

Figure 1. Diagram indicating the two different reaction sites of a phospholipid molecule by the two different phospholipase enzymes, phospholipase A_1 and A_2.

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

Enzyme manufacture

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

Enzyme specification

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

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

Food uses and technological justification

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

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

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

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

Conclusion

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

References

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

Attachment 4

Summary of public submissions

Round One

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

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

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

Round Two

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

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

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