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

4-04
26 May 2004

DRAFT ASSESSMENT REPORT

APPLICATION A501

PHOSPHOLIPASE A_2 AS A PROCESSING AID (ENZYME)

DEADLINE FOR PUBLIC SUBMISSIONS to FSANZ in relation to this matter:
7 July 2004
(See 'Invitation for Public Submissions' for details)

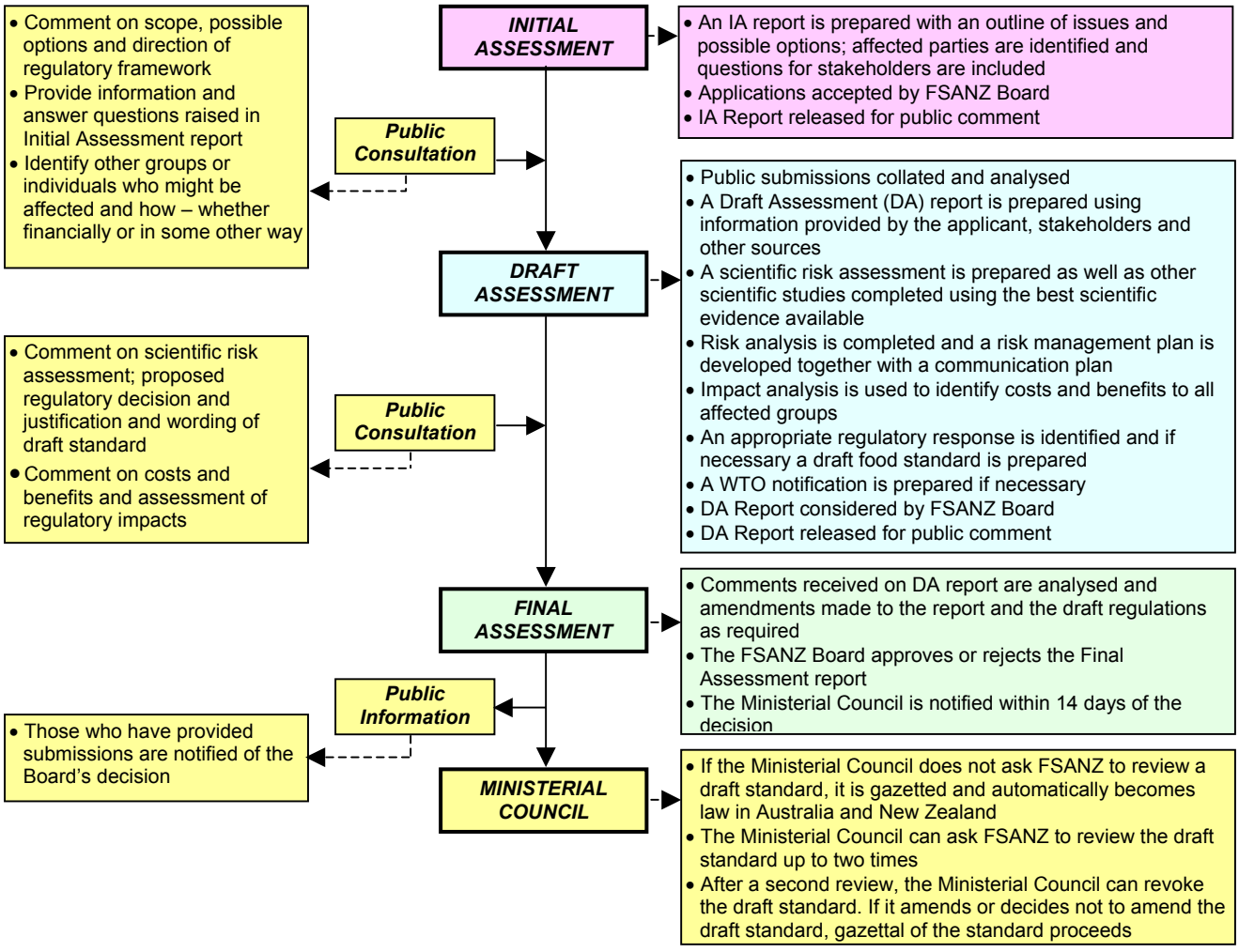
FOOD STANDARDS AUSTRALIA NEW ZEALAND (FSANZ)

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

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

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

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



INVITATION FOR PUBLIC SUBMISSIONS

FSANZ has prepared a Draft Assessment Report of Application A501 and prepared a draft variation to the *Australia New Zealand Food Standards Code* (the Code).

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

Written submissions are invited from interested individuals and organisations to assist FSANZ in preparing the Final Assessment for this Application. Submissions should, where possible, address the objectives of FSANZ as set out in section 10 of the FSANZ Act. Information providing details of potential costs and benefits of the proposed change to the Code from stakeholders is highly desirable. Claims made in submissions should be supported wherever possible by referencing or including relevant studies, research findings, trials, surveys etc. Technical information should be in sufficient detail to allow independent scientific assessment.

The processes of FSANZ are open to public scrutiny, and any submissions received will ordinarily be placed on the public register of FSANZ and made available for inspection. If you wish any information contained in a submission to remain confidential to FSANZ, you should clearly identify the sensitive information and provide justification for treating it as commercial-in-confidence. Section 39 of the FSANZ Act requires FSANZ to treat in-confidence, trade secrets relating to food and any other information relating to food, the commercial value of which would be, or could reasonably be expected to be, destroyed or diminished by disclosure.

Submissions must be made in writing and should clearly be marked with the word 'Submission' and quote the correct project number and name. Submissions may be sent to one of the following addresses:

Food Standards Australia New Zealand
PO Box 7186
Canberra BC ACT 2610
AUSTRALIA
Tel (02) 6271 2222
www.foodstandards.gov.au

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

Submissions should be received by FSANZ **by 7 July 2004**.

Submissions received after this date may not be considered, unless the Project Manager has given prior agreement for an extension.

While FSANZ accepts submissions in hard copy to our offices, it is more convenient and quicker to receive submissions electronically through the FSANZ website using the Standards Development tab and then through Documents for Public Comment. Questions relating to making submissions or the application process can be directed to the Standards Management Officer at the above address or by emailing slo@foodstandards.gov.au.

Assessment reports are available for viewing and downloading from the FSANZ website. Alternatively, requests for paper copies of reports or other general inquiries can be directed to FSANZ's Information Officer at either of the above addresses or by emailing info@foodstandards.gov.au.

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Executive Summary and Statement of Reasons

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

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

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

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

The safety assessment concluded that:

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

The only regulatory options considered were to approve or not approve the use of phospholipase A_2 sourced from *S. violaceoruber* as a processing aid. Approval of the application provides advantages to manufacturers who wish to use an alternate source of this enzyme. There should be no added costs to government or consumers.

Public comment on the Initial Assessment Report had been sought from 13 August 2003 till 24 September 2003. Four submissions were received; three submissions supported the application, while one deferred comments until after the Draft Assessment Report.

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

Statement of Reasons

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

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

1. Introduction

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

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

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

2. Regulatory Problem

2.1 Current Standard

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

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

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

3. Objective

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

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

- the protection of public health and safety;

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

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

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

4. Background

4.1 Historical Background

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

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

5. Relevant Issues

5.1 Nature of the Enzyme

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

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

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

phosphatidylcholine + H_2O → 1–acylglycerophosphocholine + carboxylic acid.

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

5.2 Technological purpose of the enzyme

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

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

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

5.3 Safety assessment

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

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

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

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

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

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

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

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

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

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

5.4 Other International Regulatory Standards

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

A GRAS notification to the US Food and Drug Administration has been submitted. An application has also been made to Health Canada.

6. Regulatory 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 benefits and costs associated with the proposed amendment to the Code will be analysed using regulatory impact principles.

The following two regulatory options are available for this application:

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

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

7. Impact Analysis

7.1 Affected Parties

The affected parties to this application include:

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

2. consumers; and

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

7.1 Option 1

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

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

7.2 Option 2

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

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

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

8. Consultation

8.1 Public Consultation

Public comment on the Initial Assessment Report for this application was sought from 13 August till 24 September 2003. Four submissions were received, with three expressing support for the application and one deferring comment until the Draft Assessment Report. Attachment 2 summarises the submissions received during the first round of public comment.

FSANZ is seeking further public comment on this Draft Assessment Report to assist in assessing this application at Final Assessment.

Comments on the following topics would be useful:

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

8.2 World Trade Organization (WTO)

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

Amending the Code to approve phospholipase A_2 as a processing aid is unlikely to have a significant effect on trade. The enzyme preparations are also consistent with the international specifications for food enzymes of Food Chemicals Codex (4th Edition, 1996) and JECFA, so there is no need to notify the WTO.

9. Conclusion and Recommendation

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

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

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

10. Implementation and review

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. Summary of Public Submissions
3. Safety Assessment Report
4. Food Technology Report

ATTACHMENT 1

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

To commence on gazettal

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

Phospholipase A_2 EC [3.1.1.4]	*Streptomyces violaceoruber*

Attachment 2

Summary of First Round of Public Submissions

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

Attachment 3

Safety Assessment Report

A501 – PHOSPHOLIPASE A_2 DERIVED FROM *STREPTOMYCES VIOLACEORUBER*

1. Introduction

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

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

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

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

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

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

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

3. Purity of enzyme preparation and proposed specifications

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

Table 1. Complete specification of phospholipase A_2 preparation

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

Phospholipase A_2 from the source organism, *S. violaceoruber* complies with the recommended purity specifications for food-grade enzymes[2,3].

4. Evaluation of the submitted studies

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

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

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

[3] National Academy of Sciences, Food and Nutrition Board, Committee on Food Chemical Codex. 1996. *Food Chemical Codex*, 4th edition, National Academy Press, Washington DC.

4.1 Acute toxicity study

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

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

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

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

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

4.2 Sub-chronic toxicity

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

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

Study conduct

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

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

Results

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

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

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

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

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

4.3 Genotoxicity studies

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

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

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

Test article

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

Study design

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

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

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

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

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

Results

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

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

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

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

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

Conclusion

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

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

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

Test article

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

Study design

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

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

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

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

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

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

Results

Results are shown in tables 2 and 3.

Conclusions

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

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

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

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

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

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

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

4.4 Pathogenicity study.

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

Test article

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

Study design

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

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

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

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

Conclusion

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

5. Overall Conclusion

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

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

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

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Attachment 4

Food Technology Report

Phospholipase A_2

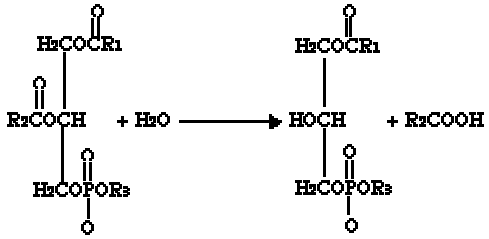
Introduction

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

Enzyme: 3.1.1.4 Phospholipase A_2

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

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



Other names: Lecithinase A; Phosphatidase' Phosphatidolipase

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

Enzyme source and production

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

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

Applications

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

Table 1. Important food applications for lecithins

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

(Van Nieuwenhuyzen, 1981)

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

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

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

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

Stability in processing

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

Conclusion

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

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

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

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