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

10/03
16 July 2003

FINAL ASSESSMENT REPORT

APPLICATION A475

HEXOSE OXIDASE AS A PROCESSING AID (ENYZME)

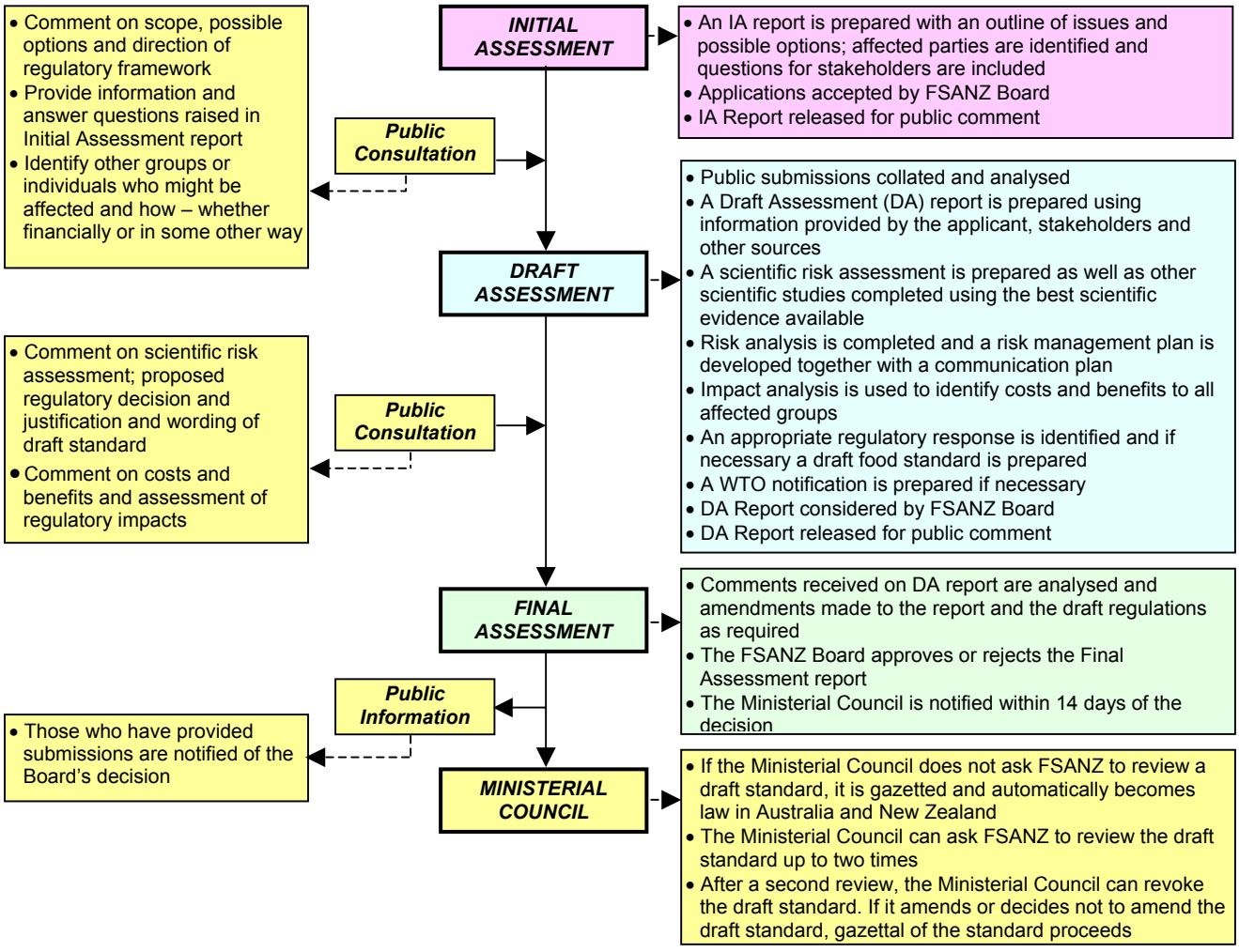
FOOD STANDARDS AUSTRALIA NEW ZEALAND (FSANZ)

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.



Final Assessment Stage

The Authority has now completed two stages of the assessment process and held two rounds of public consultation as part of its assessment of this Application. This Final Assessment Report and its recommendations have been approved by the FSANZ Board and notified to the Australia and New Zealand Food Regulation Ministerial Council (Ministerial Council).

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

In New Zealand, the New Zealand Minister for Food Safety gazettes the food standard under the *New Zealand Food Act (1981)*. Following gazettal, the standard takes effect 28 days later.

Further Information

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

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Executive Summary and Statement of Reasons

FSANZ received an Application from Danisco A/S to amend Standard 1.3.3 of the *Australia New Zealand Food Standards Code* (the Code) to approve the use of an enzyme, hexose oxidase as a processing aid. Work commenced on 13 September 2002. The hexose oxidase is produced, using recombinant DNA techniques, from the host yeast *Hansenula polymorpha* which, contains the donor gene coding for hexose oxidase from the algae *Chondrus crispus*. The abbreviation HOX is used in this report to refer to the above enzyme.

There is currently no approval for the use of hexose oxidase as a food enzyme in Australia and New Zealand. The objective of this assessment is to determine whether it is appropriate to amend the Code to permit the use of hexose oxidase produced from this source.

The only regulatory options considered were to approve or not approve this application. Approval of the use of this enzyme has advantages for food manufacturers by providing them with a new enzyme, which can perform a range of functions for their food manufacturing. There are no significant disadvantages to food manufacturers, consumers or government agencies.

Hexose oxidase catalyses the oxidation of various mono- and oligosaccharides to lactones and hydrogen peroxide. The enzyme's main application is in bread making to increase dough strength and bread volume. The enzyme acts in a similar way to glucose oxidase for this purpose, however it has added advantages, since it acts on a wider range of substrates. Other applications in the food industries are in cheese and tofu manufacture where it aids curd formation, limiting undesirable browning by limiting Maillard reactions in food and as an oxygen scavenger during production of dressings and sauces. Use of HOX is technologically justified.

The enzyme can be isolated from the red algae *Chondrus crispus*. However this source is not a suitable production organism since recoveries are low. The gene for the enzyme was therefore inserted into the host yeast *Hansenula polymorpha* from which the enzyme can be recovered in economic quantities using a submerged fermentation process.

The gene, the vector and the host organism are all well characterised. The donor organism, *Chondrus crispus* (a seaweed commonly called Irish moss), has a long history of safe use in food. The host organism, *Hansenula polymorpha* is non-toxigenic and non-pathogenic. *Hansenula polymorpha* is used for the production of pharmaceutical products with two hepatitis B vaccines produced by recombinant techniques commercially available.

The safety assessment of HOX concluded that:

- the host organism and donor organisms are safe and demonstrate no evidence of toxicity and/or pathogenicity;
- the hexose oxidase gene is stably integrated into the host genome;
- the enzyme preparation complies with international specifications; and
- the enzyme preparation causes no mutagenic effects in *in vitro* studies and there were no acute or subchronic toxicity effects in animals studies.

The safety assessment concludes that HOX, used as a processing aid poses no significant public health and safety risk.

In the USA a GRAS (Generally Recognized As Safe) expert panel has concluded that the enzyme is safe for food use as a processing aid. The enzyme is approved for use in baked goods (at a level up to 150 enzyme units/kg) in Denmark.

Public comment on the Initial Assessment Report for this application was sought from 9 October till 20 November 2002. Four submissions were received. Three (Goodman Fielder, Australian Food and Grocery Council (AFGC) and Food Technology Association of Victoria) supported approval of the use of the enzyme – subject to an appropriate safety assessment as part of the Draft Assessment. The fourth submission, from the Western Australian Department of Health raised a number of issues, which have been addressed.

Public comment on the Draft Assessment Report was sought from 19 March till 30 April 2003. Two submissions were received that both supported the application to approve the use of the enzyme as a processing aid.

The Final Assessment Report concludes that approval of the use of HOX as a food processing aid is technologically justified and does not pose a risk to public health and safety.

Statement of Reasons

The draft variation to Standard 1.3.3 – Processing Aids of the Food Standards Code, thereby giving approval for the use of HOX as a processing aid is recommended for the following reasons.

- There are no significant public health and safety concerns associated with the use of the enzyme.

- The use of the hexose oxidase enzyme is technologically justified since it has a role in food manufacturing, primarily with bread making to improve dough strength. It acts in a similar way to glucose oxidase but has wider substrate specificity. The enzyme may have potential applications in pasta and noodle manufacture where it acts in a similar way to bread making. Other potential food applications are in producing sauce and dressings, cottage cheese and tofu and limiting unwanted browning reactions.

- The safety assessment of HOX found that the donor and the source organisms are safe and demonstrate no evidence of toxicity and/or pathogenicity and the hexose oxidase gene is stably integrated into the host organism.

- The enzyme complies with the Joint Expert Committee on Food Additives (JECFA) specifications.

- The proposed draft variation to the Food Standards Code is consistent with the section 10 objectives of the FSANZ Act. FSANZ has addressed the protection of public health and safety by undertaking a safety assessment on the enzyme. The assessment is based on the best available scientific data. An approval will give food manufacturers access to a broader range of enzymes, so encouraging an efficient and internationally competitive industry. Approval also promotes consistency with international food standards.

- The benefits permitting use of the enzyme outweigh any costs associated with its use,

giving manufacturers the benefits of an alternative to glucose oxidase, and an enzyme that has wider substrate specificity.

1. Introduction

FSANZ received an application on 8 August 2002, from Danisco A/S to amend Standard 1.3.3 of the Code to approve the use of an enzyme, hexose oxidase as a processing aid. Work on the application commenced on 13 September 2002. The hexose oxidase is produced, using recombinant DNA techniques, from the host yeast *Hansenula polymorpha* that contains the donor gene coding for hexose oxidase from the algae *Chondrus crispus*.

Hexose oxidase catalyses the oxidation of various mono- and oligosaccharides to lactones and hydrogen peroxide. Its main application is in bread making to increase dough strength and bread volume. It acts in a similar way to glucose oxidase for this purpose however the applicant claims it has added advantages because it has wider substrate specificity. Other applications in the food industries are in cheese and tofu manufacture where it aids curd formation, limiting undesirable browning by limiting Maillard reactions in food and as an oxygen scavenger in dressings and sauces. It can also be used in the pasta and noodle-manufacturing industries where its use strengthens the structure of the dough, resulting in reduced loss of starch and protein on cooking and a firmer bite and better texture.

2. Regulatory Problem

2.1 Current Regulations

Processing aids are required to undergo a pre-market safety assessment before approval for use. A processing aid (defined within Standard 1.3.3 – Processing Aids) 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 may also be used in the course of manufacture of a food at the lowest level necessary to achieve a function in the processing of that food.

There is currently no approval for the use of hexose oxidase as a food enzyme in the Code. Hexose oxidase is not listed in the Table to clause 17 of Standard 1.3.3 – Processing Aids.

3. Objective

The objective of this assessment is to determine whether it is appropriate to amend the Code to permit the use of HOX. 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 *Food Standards Australia New Zealand Act 1991.* 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

It has been known for some time that hexose oxidase can be extracted from a number of red algae. However, due to the difficulty in recovering the small amounts of the enzyme from such algae, little use has been made of its properties. This has been overcome in recent times by using recombinant DNA technologies to produce greater quantities of enzyme to enable more complete characterisation. Such techniques have allowed industrial processes to be used to produce commercially viable enzyme preparations. The gene encoding for the enzyme has been isolated from the algae *Chondrus crispus* and inserted into the yeast host *Hansenula polymorpha*. The subsequent organism produces the enzyme in commercial quantities during a submerged fermentation process.

5. Relevant Issues

5.1 Nature of the enzyme

The common name of the enzyme is hexose oxidase while its chemical name is D-hexose:oxygen 1-oxidoreductase. The Enzyme Commission number is EC 1.1.3.5 and the CAS registry number is 9028-75-5.

HOX is produced by fermentation of a selected strain of the yeast *Hansenula polymorpha* modified with the hexose oxidase encoding gene isolated from the algae *Chondrus crispus*.

The enzyme catalyses the oxidation of various mono- and oligosaccharides (principally glucose, but also maltose, lactose, D-galactose, D-mannose and cellobiose) with oxygen to produce lactones and hydrogen peroxide. In aqueous solutions the lactones hydrolyse over time to the corresponding acids. The hydrogen peroxide acts as an oxidant with other food components.

5.2 Efficacy and technological justification

Hexose oxidase acts in a comparable way to glucose oxidase, but the applicant claims it is more effective and can be used in a wider range of products compared to glucose oxidase, which only acts on glucose.

The enzyme, hexose oxidase, can be used as an alternative to glucose oxidase in the baking industry to strengthen dough. The enzyme could replace chemical bread improvers. The applicant claims it could be used in a similar way in the pasta and noodle industries to improve pasta or noodles producing a firmer structure.

The applicant claims hexose oxidase could be used in foods where the browning Maillard reactions that normally occur with heating are not desirable. Also the enzyme could be used in cheese and tofu manufacture to improve curd formation. It is claimed the enzyme could be

used as an oxygen scavenger in sauces and dressings to improve appearance and shelf life of the products.

Goodman Fielder Baking sent a submission in response to the Initial Assessment Report supporting the application. As a major manufacturer of baked products in Australia/New Zealand they believe the new enzyme, hexose oxidase may offer significant advantages and benefits in processing and end-product quality compared to current enzymes used. They are particularly referring to the bread improver system for baking, specifically the oxidant system.

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

As with most enzymes there are not expected to be any dietary considerations since hexose oxidase is used as a processing aid in very low levels during the manufacture of food. Enzymes are proteinaceous and any heating steps which occur in most food manufacture would inactivate the enzyme and leave any residues as protein, which would be digested as normal protein.

5.3 Safety assessment

Application A475 to approve the use of hexose oxidase from a genetically modified microorganism involves the use of two organisms - *Hansenula polymorpha* (the host organism) and *Chondrus crispus* (the donor organism). A well-characterized DNA fragment from the donor strain is used in the construction of the genetically modified strain. In addition, the production strain is not detectable in the final enzyme product and the toxicological evaluation also confirmed the safety of HOX. The DNA used for transforming the HOX host strain does not contain antibiotic resistant genes.

Historically, enzymes used in food processing are considered to be non-toxic. 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 is non-toxic and non-pathogenic. The enzyme preparation contains no contaminants as it 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 acute, subchronic and genotoxicity studies.

The safety assessment of HOX concluded that:

- the host organism and donor organisms are safe and demonstrate no evidence of toxicity and/or pathogenicity;

[1] Prepared at the 25th JECFA (1981), published in FNP 19 (1981), FNP 52 (1992) - FAO (1992) and FNP 52 Addendum 9 2001 (with amendments to the Appendix B to Annex 1), and General Specifications for Enzyme Preparations. Compendium of Food Additives Specifications, Vol. 1, Annex 1.

- the HOX gene is stably integrated into the host genome;
- the enzyme preparation complies with international specifications;
- the enzyme preparation causes no mutagenic effects in *in vitro* studies and there were no acute or subchronic toxicity effects in animal studies.

From the available information, it is concluded that the use of HOX as a processing aid poses no significant public health and safety risk. The full safety assessment is at Attachment 3.

5.4 Other international regulatory standards

In the USA a GRAS (generally recognised as safe) expert panel has concluded that HOX is safe for food use as a processing aid. The US Food Drug Administration (FDA) has not raised any issues in relation to this GRAS notification.

The enzyme is approved for use in baked goods (at a level up to 150 enzyme units/kg) in Denmark.

The applicant states that the hexose oxidase enzyme preparations comply with the specifications for food enzyme preparations in Food Chemicals Codex, 4th Edition 1996, and also JECFA (footnote 1 on previous page).

5.5 Commercial-in-Confidence Data

The Applicant has made commercial-in-confidence claims in relation to this Application. They relate to the genetic modification and the method of production of the enzyme. FSANZ has accepted the Applicant's reason for requesting commercial-in-confidence as they meet the requirements of section 39 of the FSANZ Act.

5.6 Issues addressed from submissions

A submission to the Initial Assessment Report received from the West Australian Department of Health (Western Australian Food Advisory Committee) raised a number of issues which are discussed below.

The submission noted that hexose oxidase is a new enzyme that has been derived using genetic manipulations. They wished to know if there were any novel DNA and/or protein present in the enzyme preparation, and if there was, whether there needed to be any labelling required. There is no novel DNA or novel protein in the enzyme preparation. The genetic manipulation has been performed on the production organism but not on the enzyme itself. The hexose oxidase enzyme is substantially equivalent to the native hexose oxidase from *Chondrus crispus*. The enzyme preparation is purified through a number of purification steps, including a final sterile filtration step, which removes the production organism. This ensures there is no novel DNA or novel protein in the enzyme preparation. Therefore there is no requirement for GM labelling under Standard 1.5.2 - Food Produced Using Gene Technology of the Food Standards Code.

Also while the gene coding for hexose oxidase from the donor strain is novel for the production organism, neither the gene nor the enzyme is expected to be in the final food, and the enzyme itself is not considered novel since it is found in seaweed which has been consumed in the diet for many years.

The second issue raised by WA Health relates to whether the enzyme is being used as a food additive or processing aid for all its proposed possible uses, specifically when used to act as an oxygen scavenger during sauce and dressing manufacture. Hexose oxidase acts as a processing aid during manufacture of sauces and dressings since it has its effect (oxygen scavenging) during manufacture and has no technological function in the final packaged foods. The purpose of the enzyme during manufacture of these products is to reduce the amount of active oxygen during manufacture. The enzyme is inactivated by heating and pasteurisation of products once packaged so has no activity in the final product. The applicant has said they believe such proposed uses may be only minor uses of the enzyme as other preservative or anti-oxidant systems may be more effective. It is envisaged that the major use of the enzyme will be for bread improving.

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 Food Standards 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 HOX as a food processing aid.

Option 2. Approve the use of HOX as a food processing aid.

7. Impact Analysis

The affected parties to this application include:

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

2. consumers; and

3. State, Territory, Commonwealth and New Zealand government 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 hexose oxidase enzyme.

7.2 Option 2

There are advantages to food manufacturers to be able to use hexose oxidase. It can be used as an alternative to glucose oxidase as well as having advantages due to wider substrate specificity.

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

Option 2, which supports the approval of HOX 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 this Initial Assessment Report for this application was sought from 9 October till 20 November 2002. Four submissions were received. The first three submissions (Goodman Fielder Baking, Australian Food and Grocery Council and Food Technology Association of Victoria) all supported option 2 – to approve the use of HOX – subject to an appropriate safety assessment as part of the Draft Assessment Report.

A late submission from the West Australian Department of Health (Western Australian Food Advisory Committee) raised a number of issues (which have been discussed and addressed within section 5.6 above).

Public comment on the Draft Assessment Report for this application was sought from 19 March till 30 April 2003. Two submissions were received who both supported the approval of the application and so use of the enzyme as a processing aid.

Attachment 2 summarises the submissions received during the first and second rounds of public comment.

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 HOX 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. FSANZ considers there is no need to notify the WTO.

9. Conclusion and Approval

The Final Assessment Report concludes that approval of the use of HOX as a food processing aid is technologically justified and does not pose a risk to public health and safety.

The draft variation to Standard 1.3.3 – Processing Aids, thereby giving approval for the use of HOX as a processing aid is approved for the following reasons.

- There are no significant public health and safety concerns associated with the use of the enzyme.

- The use of the hexose oxidase enzyme is technologically justified since it has a role in food manufacturing, primarily with bread making to improve dough strength. It acts in a similar way to glucose oxidase but has wider substrate specificity. The enzyme may have potential applications in pasta and noodle manufacture where it acts in a similar way to bread making. Other potential food applications are in producing sauce and dressings, cottage cheese and tofu and limiting unwanted browning reactions.

- The safety evaluation of HOX found that the donor and the source organisms are safe and demonstrate no evidence of toxicity and/or pathogenicity and the hexose oxidase gene is stably integrated into the host organism.

- The enzyme complies with the Joint Expert Committee on Food Additives (JECFA) specifications.

- The proposed draft variation to the Code is consistent with the section 10 objectives of the FSANZ Act. FSANZ has addressed the protection of public health and safety by undertaking a safety assessment on the enzyme. The assessment is based on the best available scientific data. An approval will give food manufacturers access to a broader range of enzymes, so encouraging an efficient and internationally competitive industry. Approval also promotes consistency with international food standards.

- The benefits of permitting use of the enzyme outweigh any costs associated with its use, giving manufacturers the benefits of an alternative to glucose oxidase, and an enzyme that has wider substrate specificity.

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 variations 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 into the* Table to clause 17 –

Hexose oxidase EC [1.1.3.5]	*Hansenula polymorpha,* containing the gene for Hexose oxidase isolated from *Chondrus crispus*

ATTACHMENT 2

Summary of Public Submissions

Round One

Submitters

#	Submitter Organisation	Name
1	Goodman Fielder Baking	Greg Pointing
2	Australian Food and Grocery Council	Tony Downer
3	Food Technology Association of Victoria	David Gill
4	Department of Health, Western Australia (Western Australian Food Advisory Committee)	Dr Margaret Stevens

Submitter	Comments
Goodman Fielder Baking	They support the application, believing the enzyme would appear to offer significant advantages and benefits in processing and in end-product quality (for bread making) compared to the current enzymes used.
Australian Food and Grocery Council	It supports the application, subject to a satisfactory safety assessment as part of the Draft Assessment. It believes the enzyme is safe for food use and that FSANZ will find it thus. The US GRAS Expert Panel concluded it was safe for use as a processing aid. It does not believe the host organism *Hansenula polymorpha* is normally associated with food production but believe it is non-toxigenic. While the donor organism *Chondrus crispus* has a long history of safe use in food. The enzyme also complies with appropriate international enzyme specifications. There are currently no hexose oxidases approved. The application is technologically justified since it has use in bread making, in products such as cheese, egg white and whey powder to limit browning reactions and as an aid in precipitation in cheese and tofu manufacture.
Food Technology Association of Victoria	It supports option 2, to approve the use of the enzyme as a processing aid.
Department of Health, Western Australia (Western Australian Food Advisory Committee)	It believes there are two main issues of significance. 1. It is a new enzyme which needs approval, and has been derived through GM techniques 2. Is it always acting as a processing aid rather than a food additive, especially when used for sauce and dressing manufacture? Because it is derived from GM techniques they wished the applicant to provide details about the enzyme purity and whether there are any GM labelling requirements (i.e. any novel DNA present in food derived by using the enzyme). It would like further safety assessment information on the enzyme preparations. (This is discussed as part of the Safety Assessment Report and within section 5.6 of the report. There is no novel DNA or protein and therefore no need for GM labelling; the enzyme is identical to native enzyme). It also questions whether the enzyme can be correctly termed a processing aid for all its proposed uses, and not as a food additive. Such cases as when used in sauce and dressing manufacture. (The enzyme is used as a processing aid during manufacture and packaging and has no technological function in the final food). It would like more information before they can support either option.

Round Two

Submitters

#	Submitter Organisation	Name
1	Australian Food and Grocery Council	Tony Downer
2	Food Technology Association of Victoria	David Gill

Submitter	Comments
Australian Food and Grocery Council	It agrees with FSANZ's assessment and supports approval for use of the enzyme.
Food Technology Association of Victoria	The Technical Sub Committee accepts option 2 to support the acceptance of the use of the enzyme as a processing aid.

ATTACHMENT 3

Safety Assessment Report

Safety of hexose oxidase (HOX) from a genetically modified yeast strain, *Hansenula polymorpha* containing a donor gene coding for HOX from *Chondrus crispus*.

INTRODUCTION

Application A475 to approve the use of hexose oxidase (HOX) from a genetically modified microorganism involves the use of two organisms – *Hansenula polymorpha* (the host organism) and *Chondrus crispus* (the donor organism). The HOX is produced intracellularly in *Hansenula polymorpha* cells during a fermentation process. The enzyme is recovered, concentrated and purified in a sequence of filtration steps.

The enzyme is to be 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.

The Host Organism - Hansenula polymorpha

HOX is the first enzyme product produced by *Hansenula polymorpha* by the company Danisco Cultor**.** Therefore, specific information about the strain lineage and safety of products from *Hansenula polymorpha* is not publicly available.

However, from previous literature searches conducted by the Applicant (Toxline and Medline) detailed in Application A475 and confirmed by FSANZ, *Hansenula polymorpha* was not considered to be pathogenic and is widely distributed in nature. Furthermore, the commercial enzyme product (HOX) has undergone several filtration steps and the presence of the production organism *Hansenula polymorpha* will be close to zero.

Hansenula polymorpha is used for the production of pharmaceutical products with 2 hepatitis B vaccines produced by recombinant *Hansenula polymorpha* commercially available.

The donor organism – *Chondrus crispus*

The organism from which the HOX gene is derived, *Chondrus crispus,* has a long history of safe use in foods, in particular, its use as food grade carrageenan.

There have been no previous descriptions in the scientific literature that *Chondrus crispus* is pathogenic nor contains toxins, or vectors for transmission of disease.

Nature of the genetic modification

The gene encoding HOX is isolated from the seaweed *Chondrus crispus* and is inserted into the yeast *Hansenula polymorpha*. A full description of the process, gene and vectors is described in the application. The applicant has provided information to indicate that the recombinant organism was found to be stable during production fermentations and that the inserted DNA was stably integrated into the host genome. The DNA used for transforming the host strain does not contain antibiotic resistance genes.

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 is non-toxic and non-pathogenic and preparation complies with current international standards for enzyme preparations and contains no contaminants.

TOXICOLOGY STUDIES

Acute studies

Acute oral toxicity in the rat. Study No. 36523 and 36524. Kari Kaaber Study Director, Scantox test report. 30 November 1999.

Test material	Fermented sample of hexose oxidase (HOX); activity 300μg/mL
Vehicle material	Sterile water
Test Species	5 female and male Wistar rats; administration by gavage
Dose	2000 mg HOX/kg bw
GLP/guidelines	OECD guideline No. 420 Acute Oral Toxicity-fixed dose method

Groups of five 6-7 week-old male and female Wistar rats received single doses of HOX administered orally by gavage (in a sterile water vehicle) and were observed for 14 days post-dose. There were no deaths and the only clinical sign observed was piloerection 3-6 hours post-treatment in 2 males and 3 females. Gross examination revealed no abnormalities.

A second acute toxicity study (36524) was conducted in five male and female 6-7 week old Wistar rats. Single doses (2,000 mg) of HOX (activity 400 μ/ML) were administered orally by gavage (in a sterile water vehicle) and rats were observed for 14 days post-dose. No clinical signs were observed post-dosing. Necroscopy revealed hydronephrosis of the right kidney in one animal only, which appeared an incidental finding unrelated to treatment.

Short-term and sub-chronic toxicity

A two-week dose-ranging study in rats. Study No. 39143 Peter Glerup Study Director, Scantox test report. 31 October 2000.

Test material	HOX (500μ/mL)
Control and vehicle material	Sterile water
Test Species	Sprague-Dawley rats 5 males and females per test dose; administration by gavage
Dose	0, 500, 1250 or 5000 HOX/kg bw/day
GLP/guidelines	OECD; EEC; USFDA; Japanese Ministry of Health and Welfare

Study conduct

Four groups of rats (5/sex/group) were treated with HOX via gavage at 0, 500, 1250 or 5000 mg/kg bw/day for 2 weeks as a dose-ranging study for a subsequent 90-day study. Clinical observations were recorded daily, bodyweight and food consumption were recorded weekly. Following the dosing period, 2 animals/group were sacrificed and necroscopy performed.

Results

There were no deaths following treatment. There were no clinical signs, changes in either weight/food consumption or macroscopic findings attributable to treatment with HOX. The No Observed Adverse Affect Level (NOAEL) was 5000 mg/kg bw/day (the highest dose tested).

A 13-week oral gavage toxicity study in rats. Study No. 40232 Peter Glerup Study Director, Scantox test report. 4 October 2001.

Test material	HOX (500μ/mL)
Control and vehicle material	Sterile water
Test Species	Sprague-Dawley rats 10 males and females per test dose; administration by gavage
Dose	0, 500, 1250 or 5000 HOX/kg bw/day
GLP/guidelines	OECD guideline No. 408; EEC; USFDA; Japanese Ministry of Health and Welfare

Study conduct

Four groups of rats (10/sex/group) were treated with HOX via gavage at 0, 500, 1250 or 5000 mg/kg bw/day for 90-days.

Clinical observations were recorded daily. Bodyweight and food and water consumption were recorded weekly; haematology, clinical chemistry and urinalysis before the end of the treatment period; and ophthalmology performed on controls and highest dose before the start of the study and near termination. At the end of the study, all animals were sacrificed and a complete necroscopy performed (gross examination, organ weights and histo-pathology on selected organs).

Results

Only 1 animal was found dead on day 2 post-treatment in the high-dose group. There were no observed changes in bodyweights, food consumption, haematological or blood chemistry parameters during the treatment period. No treatment related changes were observed in macroscopic or microscopic examination. The NOAEL was 5000 mg/kg bw/day (the highest dose tested).

Genotoxicity studies

HOX-TOX-3-99 Ames test. Study No. 42119; Study Director: C. Nicholas Edwards. Scantox test report. 22 August 2001.

Hexose oxidase. In vitro mammalian chromosome aberration test performed with human lymphocytes. Study No. 39720; Study Director: C. Nicholas Edwards. Scantox test report. 21 November 2001.

The following studies were performed and main features are included in the table (below). Protocols were carried out under GLP, with OECD guidelines No. 471 (Bacterial Reverse Mutation Test) and No. 473 (In Vitro Mammalian Chromosome Test).

Studies were designed with appropriate positive and negative control test substances and appropriate criteria were defined for positive and negative outcomes. Reference to historical control data was also included. Appropriate data are presented on dose ranging phases of experiments to assess cytotoxicity and solubility of test materials. Where S9 mix has been used as a metabolic activating system of test chemicals, the preparation of S9 is described, and the procedures were appropriate.

Test	*Test material*	*Concentration*	*Test object*	*Result*
Reverse mutation (*In vitro*)	HOX	50 to 5,000 μg/plate with and without S9 mix	*S. typhimurium* TA98, TA100, TA 102, TA1535, TA1537.	-ve
Chromosome aberrations (*In vitro*)	HOX	150 to 600 μg/mL with S9 mix and 4.7 to 300 μg/mL without S9 mix	Human lymphocytes	-ve

Recent safety evaluation of hexose oxidase

A recent safety evaluation was undertaken to establish the safety of HOX from *Chrondus crispus* expressed in *Hansenula polymorpha*[1]. The studies evaluated in this paper were the same as those assessed by FSANZ. It was concluded that no significant findings were observed in the toxicological studies and that HOX was a safe processing aid for use in the food industry.

CONCLUSION

The safety assessment of HOX from genetically modified *Hansenula polymorpha* containing a donor gene coding from *Chondrus crispus* for HOX concluded that:

- the host and donor organisms are safe and demonstrate no evidence of toxicity and/or pathogenicity;
- the HOX gene is stably integrated into the host genome;
- the enzyme preparation complies with international specifications; and
- the enzyme preparation causes no mutagenic effects in *in vitro* studies and there were

[1] Cook MW and Thygesen HV (2003) Safety evaluation of a hexose oxidase expressed in *Hansenula polymorpha*. Food and Chemical Toxicology, 41, 523-529

no acute or subchronic toxicity effects in animals studies.

From the available information, it is concluded that the use of HOX as a processing aid poses no public health and safety risk.

ATTACHMENT 4

Food Technology Report

Application A475 – Hexose Oxidase as a Processing Aid (Enzyme)

Introduction

A application has been received from Danisco A/S to approve a new food enzyme hexose oxidase as a processing aid in Standard 1.3.3 of the *Food Standards Code*. Hexose oxidase is produced by the yeast *Hansenula polymorpha*, containing the gene coding for hexose oxidase isolated from the seaweed *Chondrus crispus*.

Hexose Oxidase

The common name of the enzyme is hexose oxidase (HOX) while its chemical name is D-hexose:oxygen 1-oxidoreductase. The Enzyme Commission number is EC 1.1.3.5 and the CAS registry number is 9028-75-5.

The enzyme catalyses the oxidation of various mono and oligosaccharides (principally glucose, but also maltose, lactose, D-galactose, D-mannose and cellobiose) with oxygen to produce lactones and hydrogen peroxide. In aqueous solutions the lactones hydrolyse over time to the corresponding acids. The hydrogen peroxide acts as an oxidant with other food components.

Technological Justification

Commercial food production enzymes are proteins that are able to catalyse chemical reactions more economically than traditional chemical or thermal processes. They are very important for many food manufacturing processes. Enzymes are able to be quite specific in the reactions they catalyse. Enzymes are able to catalyse chemical reactions with one or more of the improved properties; faster time, reduce the required temperature of reaction so less energy used and greater specificity over reactions performed and products formed. Enzymes need to be stable for the conditions for their use.

For the case of the current enzyme, hexose oxidase, it meets the requirements of a food production enzyme, in that it is a protein, it catalyses desired reactions during food preparation and is able to perform these reactions quicker and cheaper than other alternatives, or is an alternative for other treatments.

Hexose oxidase acts in a comparable way to glucose oxidase, but is claimed to be more effective by acting on a greater range of substrates (than just glucose). Therefore hexose oxidase is proposed as an alternative to glucose oxidase for a number of glucose oxidase's current uses as a food enzyme (processing aid). Glucose oxidase, from a number of sources, is listed as an approved enzyme in Table to clause 17 of Standard 1.3.3 – Processing Aids of the *Food Standards Code*. Glucose oxidase is used commercially in bread making. Glucose oxidase catalyses the conversion of glucose and oxygen into gluconolactone and hydrogen peroxide. The produced hydrogen peroxide then oxidises thiol groups of the gluten protein to form (or reform) disulphide bonds which strengthen the gluten structure of the dough which improves the dough strength during mixing and proofing of the dough.

Commercial availability of hexose oxidase has only just recently occurred so there is not a lot of history of use or references to commercial use. The various claims of the applicant are listed below.

- The enzyme can be used alone or in combination with other oxidants such as ascorbic acid in bread making where it is added to the dough. The enzyme catalyses the oxidation of thiol groups in the gluten structure forming disulphide bonds so strengthening the gluten network of the dough and improving the dough handling. The baked bread is larger and has better textual and mouth-feel properties. The enzyme can replace other chemical bread improvers such as potassium bromate which is no longer permitted for this purpose in Australia and New Zealand. It is claimed it can be used as an alternative to glucose oxidase for this function.

- The enzyme can also be used in the pasta and noodle manufacturing industries where its use strengthens the structure of the dough, resulting in reduced loss of starch and protein on cooking and a firmer bite and better texture of the resulting pasta or noodle.

- Hexose oxidase can be used in a number of foods to limit unwanted browning reactions (Maillard) that occur with the naturally occurring oligosaccharides, by oxidizing them so they are unable to react. Such a case is shredded cheese (such as Mozzarella used on the top of pizzas) where the lactose and galactose naturally occurring in cheese form strong Maillard reactions during baking so producing strong brown baked colours. The use of the enzyme limits colour generation which may be desirable for certain products.

- A similar case of limiting the formation of browning Maillard reactions in potato chip production. Sprouted potatoes have a higher concentration of reducing sugars, and chips produced from such potatoes form undesirable brown colours when fried. Treatment with the enzyme limits the browning.

- Hexose oxidase is claimed to also oxidise the reducing sugars so that whiter egg powders and whey powders can be produced due to limiting browning reactions.

- Lactose in skim milk and soya milk can be oxidised by hexose oxidase to form the acid, lactobionic acid, which aids in the precipitation of curd for cottage cheese and tofu manufacture respectively.

- The enzyme can be added as an oxygen scavenger during sauce and dressing manufacture reducing dissolved oxygen concentrations during manufacture and packaging of the products which improves their shelf life.

Hexose oxidase enzyme preparation

The enzyme preparation is produced by fermentation of a selected strain of the yeast *Hansenula polymorpha* modified with the hexose oxidase encoding gene isolated from the seaweed *Chondrus crispus* (also commonly called Irish moss).

The enzyme hexose oxidase is found in very low amounts in the seaweed *Chondrus crispus*.

There appears to have been a large amount of research in the last 5-10 years aimed at fully characterising the enzyme and then how to produce the enzyme in commercial quantities. This has been achieved by using recombinant technologies to add the gene encoding for hexose oxidase sourced from *Chondrus crispus* into a yeast host, *Hansenula polymorpha*.

Chondrus crispus is a common seaweed that is commercially harvested in Ireland, Spain, France, Portugal and North America for the extraction of carrageenan, for some places for centuries. Carrageenan is used in food for its gelling and thickening properties.

Hansenula polymorpha (also called *Pichia Angusta*) is a yeast that has recently been found that can act as a very good expression system for large scale production and secretion of protein and in this case enzymes.

The enzyme preparation is produced using a submerged fermentation process with the recombinant yeast and appropriate processes and nutrients that are common for producing food grade enzymes. Good Manufacturing Practice (GMP) is used throughout the production process meeting the requirements and specifications for food enzymes within Food Chemicals Codex (4th Edition, 1996) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in the Compendium of Food Additives Specifications, Vol 1, Annex 1 Addendum 9 (2001) (and earlier relevant Addenda). The specific requirements for genetically modified microorganisms are contained in appendix B to Annex 1 of addendum 6 titled 'General Considerations and Specifications for Enzyme Preparations from Genetically Modified Microorganisms'.

Conclusion

The use of the food enzyme hexose oxidase sourced from *Hansenula polymorpha*, containing the gene coding for hexose oxidase isolated from the seaweed *Chondrus crispus* as a processing aid is technologically justified. Hexose oxidase can act as a food enzyme, is an alternative to glucose oxidase and can act on a broader range of substrates than glucose oxidase (rather than just glucose).

References

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IUBMB (International Union of Biochemistry and Molecular Biology) Enzyme Nomenclature internet site: http://www.chem.qmul.ac.uk.iubmb/enzyme/EC1/1/3/5.html

Internet search site searching for *Hansenula polymorpha*: http://www.phaffcollection.org/about/about.asp