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

2-05
23 March 2005

DRAFT ASSESSMENT REPORT

APPLICATION A544

ICE STRUCTURING PROTEIN AS A PROCESSING AID FOR ICE CREAM & EDIBLE ICES

DEADLINE FOR PUBLIC SUBMISSIONS: 6pm (Canberra time) 4 May 2005
SUBMISSIONS RECEIVED AFTER THIS DEADLINE
WILL NOT BE CONSIDERED
(See 'Invitation for Public Submissions' for details)

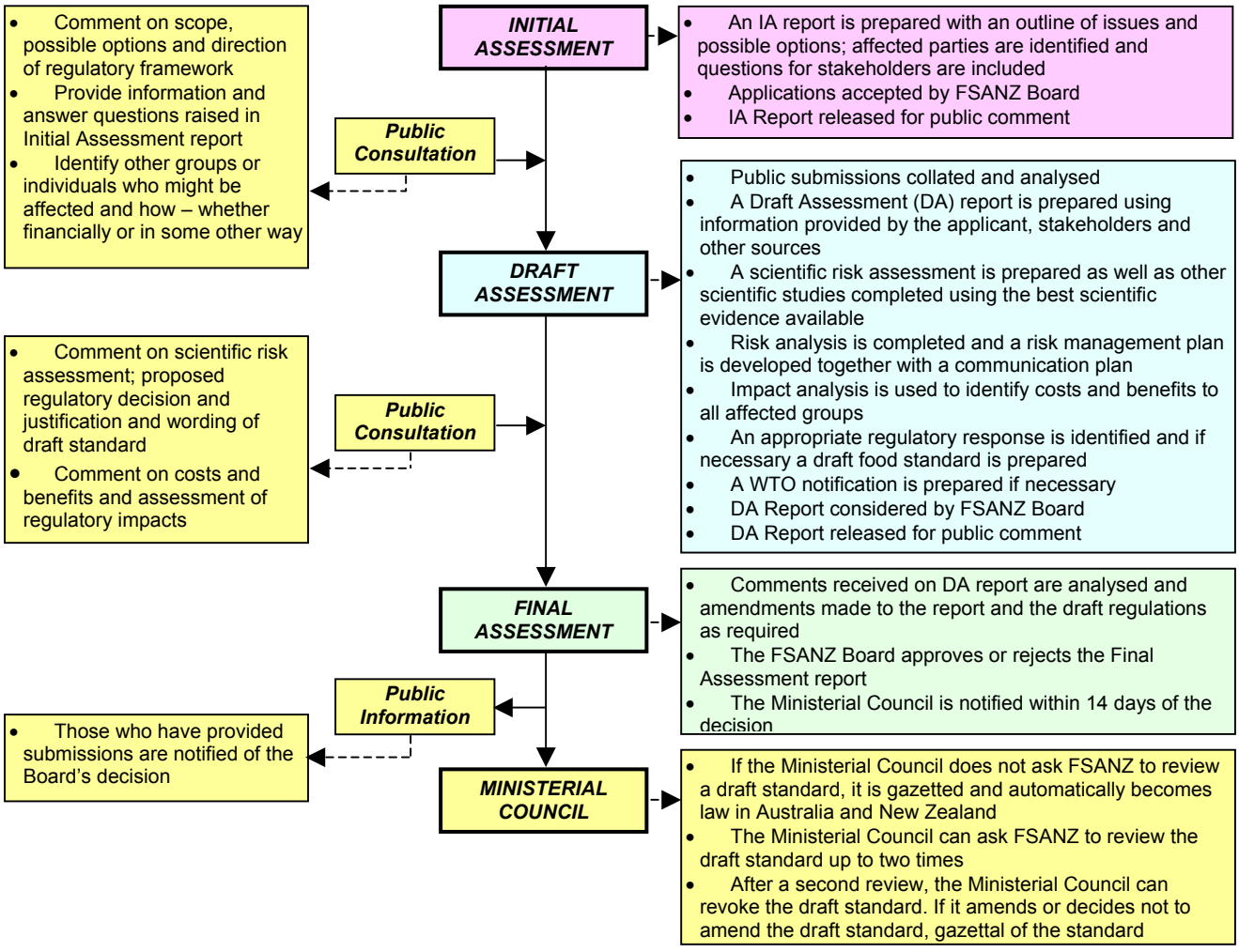
FOOD STANDARDS AUSTRALIA NEW ZEALAND (FSANZ)

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 Australian Government; Australian States and Territories; and New Zealand. It is a statutory authority under Commonwealth law and is an independent, expert body.

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

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

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



INVITATION FOR PUBLIC SUBMISSIONS

FSANZ has prepared a Draft Assessment Report of Application A544; 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 need to be received by FSANZ by 6pm (Canberra time) 4 May 2005.

Submissions received after this date will not be considered, unless agreement for an extension has been given prior to this closing date. Agreement to an extension of time will only be given if extraordinary circumstances warrant an extension to the submission period. Any agreed extension will be notified on the FSANZ Website and will apply to all submitters.

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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Introduction

FSANZ received an Application on 9 August 2004 from Unilever Australia Limited, to amend Standard 1.3.3 – Processing Aids of the *Australia New Zealand Food Standards Code* (the Code) to approve the use of Ice Structuring Protein Type III HPLC 12 (ISP) as a processing aid for the preparation of ice cream and edible ices. The Applicant requested that the term edible ices include frozen yoghurts and frozen fruit and/or vegetable juices and drinks.

Ice structuring proteins are naturally occurring proteins and peptides that are found in a variety of living organisms such as fish, plants, insects, fungi and bacteria which protect them from damage in very cold conditions that would normally cause organisms to freeze. A number of these products are consumed in foods so ice structuring proteins are a normal component of the human diet.

For use in manufacturing ice cream and edible ices, ice structuring proteins do not actually prevent freezing but influence the growth and structure of ice crystal formation and hence the physical properties of frozen foods. For ice cream and edible ices these include hardness, thermal stability (including improved heat shock resistance during storage and transportation), creaminess, mouth-feel, and flavour delivery.

Regulatory problem and objective

A regulatory problem to be considered as part of this Application is whether ISP should be regulated in the Code as a processing aid or a food additive. The Applicant requested that ISP be considered as a processing aid since it performs its technological function during manufacture of ice cream and edible ice products (where it alters the growth and shape of developing ice crystals and hence the ice structure and properties of the frozen products) and does not perform a technological function in the final food.

ISP does not fit neatly into any of the food additive functions, listed in Schedule 5 of Standard 1.3.1 – Food Additives, as required for ISP to be considered as a food additive.

The objective of this Draft Assessment Report therefore is to consider if it is appropriate to amend the Code to permit the use of ISP as a processing aid for the manufacture of ice cream and edible ices as requested by the Applicant.

Background

The ISP of this Application was originally isolated from ocean pout, a cold water fish found off the North American coast, which is consumed as part of the human diet. To produce commercial quantities of ISP, a synthetic gene encoding for ISP has been incorporated into a food grade yeast (baker's yeast, *Saccharomyces cerevisiae*) using standard genetic modification techniques. The protein expressed by the yeast is identical to the fish protein in amino acid sequence. No directly fish-derived protein is included in ISP, so it is not considered to be a fish product.

Risk assessment

A number of criteria have been addressed in the safety assessment including: a characterisation of the ISP gene transferred to the production organism, its origin, function and stability; a characterisation of the functional protein present in the ISP preparation secreted by the genetically modified yeast (GM yeast); and the potential for the ISP preparation to be either toxic or allergenic to humans.

No potential public health and safety concerns have been identified in the safety assessment of ISP. On the basis of the data provided in the present Application, and other available information, the ISP preparation derived from fermentation of GM baker's yeast can be considered safe for human consumption.

International permissions

The US FDA (Food and Drug Administration) has accepted this ISP as Generally Recognized As Safe (GRAS). Commercial ice creams and edible ices incorporating ISP have been sold in USA since June 2003, as well as in the Philippines. ISP is also approved for use in Hong Kong, Mexico and Indonesia.

Issues from submissions

The Initial Assessment Report was circulated for a round of public comment from the period starting form 20 October until 1 December 2004. Eight submissions were received including one providing further information from the Applicant. Two submitters supported the Application and two opposed it. Four submissions either did not state a position or tentatively supported further consideration but raised issues and concerns they believed needed to be addressed during further assessment. The major issues raised in submissions relate to whether ISP used for the proposed purpose should be considered as a processing aid or a food additive, and various labelling aspects, including labelling to provide consumer choice.

Risk management

Clause 3 (d) of Standard 1.2.4 – Labelling of Ingredients, exempts processing aids from ingredient labelling requirements.

The presence of ISP in the final food would not require labelling under the requirements of Standard 1.2.3 – Mandatory Warning and Advisory Statements and Declarations, since ISP is not directly obtained from fish and is not a fish product.

The presence of ISP in the final food would also not require labelling under the requirements of Standard 1.5.2 – Food Produced Using Gene Technology, because the ISP protein is not a novel protein since it is identical in amino acid sequence to the counterpart protein obtained from fish.

If ISP is approved FSANZ proposes to regulate ISP within Table to clause 14 – Permitted processing aids with miscellaneous functions of Standard 1.3.3 – Processing Aids, giving specific functions of how it can be used, for which products it can be used for, a detailed name of the protein and a maximum permitted level.

Submissions are now invited on this report to assist FSANZ to complete the Final Assessment.

Statement of Reasons

The draft variation to Standard 1.3.3 – Processing Aids of the Code to permit the use of ISP as a processing aid for the manufacture of ice cream and edible ice products is recommended for the following reasons.

- The safety assessment concluded that no public health and safety concerns associated with using ISP as a processing aid for the manufacture of ice cream and edible ice products have been identified.
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- The use of ISP is technologically justified to alter the properties of ice cream and edible ice products. ISP binds to and influences the growth and structure of the developing ice crystals during manufacture, which alters the physical and sensory properties of the final products.
-
- As concluded by the regulatory impact analysis, the costs that would arise from a variation to Standard 1.3.3 to permit the use of ISP as a processing aid for the manufacture of ice cream and edible ice products do not outweigh the direct and indirect benefits to the community, Government or industry that would arise from the variation.
-
- The proposed draft variation to the Code is consistent with the section 10 objectives of the FSANZ Act.
-
- To achieve what the Application seeks, namely permission to use ISP as a processing aid for the manufacture of ice cream and edible ices, there are no alternatives that are more cost effective than a variation to Standard 1.3.3.

1. Introduction

FSANZ received an Application on 9 August 2004 from Unilever Australia Limited, to amend Standard 1.3.3 – Processing Aids of the *Australia New Zealand Food Standards Code* (the Code) to approve the use of Ice Structuring Protein Type III HPLC 12 (ISP) as a processing aid for the preparation of ice cream and edible ices. The Applicant requested that edible ices include frozen yoghurts and frozen fruit and/or vegetable juices and drinks. For this report, ISP refers to the specific ice structuring protein of the Application and not a generic class of proteins. The Application is for the approval of the specific ISP product, rather than approval for the broad class of ice structuring proteins that may exist.

Work on this Group 3 (cost-recovered) Application commenced on 20 August 2004.

Ice structuring proteins are naturally occurring proteins and peptides that are found in a variety of living organisms such as fish, plants, insects, fungi and bacteria. These proteins help to protect the organisms from damage in very cold conditions that would normally cause them to freeze. A number of these products are present in commonly consumed foods, so ice structuring proteins are already a natural component of the human diet.

The Applicant wishes to use ISP during the manufacture of frozen ice products. For use in manufacturing ice cream and edible ices, ice structuring proteins do not actually prevent freezing but influence the growth and structure of ice crystal formation and hence physical and sensory properties of frozen foods. Properties relevant for frozen ice products include thermal stability, hardness, creaminess, mouth-feel and flavour delivery.

2. Regulatory Problem

Processing aids must not be added to food unless expressly permitted under Standard 1.3.3. In deciding whether to approve a new processing aid, FSANZ conducts a pre-market safety assessment.

The Applicant has requested that ISP be considered as a processing aid having a technological function during manufacture of the edible ice products, but not performing a technological function in the final food for the stated purpose of the Application.

ISP is proposed to be used in very low levels (maximum proposed concentration 0.01%, usual concentration 0.005%) and is therefore not considered to be a food or a food ingredient in this Application.

For ISP in this Application to be considered a processing aid it needs to be used during the manufacture of the edible ice products and not performing a technological function in the final food.

Under Standard 1.3.3, a processing aid is defined as:

> a substance listed in clauses 3 to 18, where –
>
> (a) the substance is used in the processing of raw materials, foods or ingredients, to fulfil a technological purpose relating to treatment

or processing, but does not perform a technological function in the final food; and

(b) the substance is used in the course of manufacture of a food at the lowest level necessary to achieve a function in the processing of that food, irrespective of any maximum permitted level specified.

ISP is a processing aid and not a food additive for the purposes of this Application since it fulfils its technological purpose during the manufacture of the frozen ice products and does not perform a technological function of a food additive in the final food. ISP does not fit neatly into any of the possible food additive functions listed in Schedule 5 of Standard 1.3.1 – Food Additives.

ISP 'binds' to and influences the growth and structure of the developing ice crystals during production of such products. This different ice structure alters the properties of the food products. The altered properties of the ice structure are not due to the presence of ISP by itself, but the effect ISP has on the ice structure formation during processing. Stability of iced products containing ISP is due to the ice structure that has been formed, rather than the residual presence of ISP.

3. Objective

The objective of this assessment is to determine whether it is appropriate to amend the Code to permit the use of ISP as a processing aid for the manufacture of ice cream and edible ices. The assessment is to ensure that there are no public health and safety concerns, and that ISP is technologically justified as a processing aid.

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

Various naturally occurring proteins and peptides have been extracted and identified from the blood of fish living in very cold water. These proteins and peptides protect the fish from the damage that would be caused by freezing and allow them to survive and were identified over thirty years ago. Similar proteins were subsequently also found in many other organisms that survive in very cold environments, such as plants, insects, fungi and bacteria. A number of these proteins are already consumed in foods that have been significant parts of the human diet, such as fish and carrots.

These proteins have been known as thermal hysteresis proteins or antifreeze proteins. However, since they do not prevent ice forming but modify the structure and growth of ice crystals they have been given the name 'ice structuring proteins'.

Ice structuring proteins affect the growth and structure of ice crystals by directly 'binding' (or more correctly 'adsorbing' or 'accumulating') to the growing ice crystals and inhibiting the growth (particularly in one direction) resulting in modification of the resulting ice structure and hence its physical properties. The mechanisms of the binding to ice crystals for different types of ice structuring proteins has been postulated by various groups to include hydrogen bonding, and hydrophobic and hydrophilic interactions. Regardless of how the proteins work, their addition during manufacture causes changes to ice crystal size and structure which also alters the ice's physical properties. For food products based on ice, addition of ISP also has important impacts on the sensory properties of the resultant ice products. Such altered sensory properties include resultant hardness, thermal stability, creaminess, alterations to mouth-feel and flavour delivery.

5. Relevant Issues

5.1 Risk Assessment

5.1.1 Safety assessment

5.1.1.1 Background

As natural fish sources are limited, the Applicant has developed a method of producing commercial quantities of ISP by fermentation of genetically modified (GM) baker's yeast that has been to manufacture and secrete the fish ISP. The ISP preparation is a mixture of functionally active ISP, an inactive form of ISP, proteins and peptides from common baker's yeast, as well as sugars, acids and salts commonly found in foods.

A number of criteria have been addressed in the safety assessment including: a characterisation of the gene transferred to the production organism, its origin, function and stability; a characterisation of the functional protein present in the ISP preparation secreted by the GM yeast; and the potential for the ISP preparation to be either toxic or allergenic to humans. The Safety Assessment Report is at **Attachment 4**.

5.1.1.2 History of Use

Humans have previously been exposed to ice structuring proteins in the diet through the consumption of certain fish and vegetable species. ISP is present in the blood of ocean pout, a species of cold-water fish found off the northeast coast of North America, that is harvested commercially for human food.

Food-grade yeasts are used widely in the manufacture of beer, wine, and for production of enzymes including those used in cheese manufacture. The production organism for ISP is baker's yeast (*Saccharomyces cerevisiae*) which has a long history of safe use in the leavening of bread.

5.1.1.3 Description of the Genetic Modification

The gene encoding ISP is a synthetic version of the gene derived from ocean pout. The gene from ocean pout was resynthesised to improve production and secretion of the protein in the production organism (baker's yeast). The synthetic gene in yeast encodes the identical amino acid sequence to that of the native ISP derived from ocean pout. The gene cassette did not contain any antibiotic resistance marker genes or any bacterial DNA.

5.1.1.4 Characterisation of ISP

ISP type III HPLC 12 consists of a known sequence of 66 amino acids, and studies on its properties and the physical structure of the protein have been published. Biochemical analysis of the yeast-derived ISP confirms that the protein is the same as the native ISP from ocean pout.

5.1.1.5 Safety assessment of ISP

The Applicant conducted a number of studies to determine whether ISP is potentially toxic in mammals and is likely to act as an allergen.

Bioinformatic analyses of the amino acid sequence of the protein was conducted to determine whether ISP shares any sequence similarity with known toxins or allergens. The results indicate that ISP is highly characteristic of other fish ice-structuring proteins and shows little similarity with that of any other proteins, in particular known allergens, including fish allergens.

The results of a 13-week sub-chronic rat feeding study using a concentrated form of the ISP preparation from yeast showed no toxicity at doses up to 580 mg/kg/day.

The genotoxic activity of ISP was assessed using four different assays: The results of these experiments indicate that ISP is not genotoxic.

The potential allergenicity of ISP was investigated systematically using a number of established methods using sera from fish-allergic subjects and skin prick tests. The conclusion from these investigations was that ISP is not likely to be allergenic in humans.

In studies using human volunteers, ingestion of ISP preparation for eight weeks at a high daily dose did not result in specific antibody formation, indicating that ISP is not likely to be any more immunogenic than the majority of dietary proteins.

Additional biochemical analyses simulating gastric fluid digestion with pepsin in an *in vitro* test system showed that both ISP and its inactive form would be readily degraded in the human digestive system. In addition, amino acid sequence analysis showed a susceptibility to proteolytic breakdown by intestinal enzymes such as trypsin. These results indicate that ISP is therefore unlikely to be absorbed intact or accumulate in the body.

Based on a thorough assessment of allergic potential, and the results of the analytical, animal, human, and *in vitro* data presented in this application, ISP preparation is not toxic and is unlikely to evoke an allergic reaction in fish-sensitised individuals, or to sensitise potentially susceptible individuals in the wider population.

5.1.1.6 Conclusion

No potential public health and safety concerns have been identified in the assessment of ISP. On the basis of the data provided in the present Application, and other available information, the ISP preparation derived from fermentation of GM baker's yeast can be considered safe for human consumption.

5.1.2 Dietary exposure assessment

A dietary exposure assessment was undertaken to estimate dietary exposure to ISP for the Australian and New Zealand populations. The maximum ISP usage concentration of 0.01% (100 mg/kg) as stated in the Application was used for the dietary modelling though typical concentrations are stated to be 0.005%. The population sub-groups examined were the whole population (2 years and above for Australia; 15 years and above for New Zealand), toddlers (2-4 years for Australia), primary school aged children (5-12 years for Australia), and teenagers (13-19 years for Australia; 15-19 years for New Zealand). Food consumption data based on the 1995 National Nutrition Survey (NNS) and 1997 New Zealand NNS were used to estimate ISP dietary exposure.

The estimated mean dietary exposures for consumers of ISP for Australia were:

- 12 mg/day for the whole population aged 2 years and above;
- 8 mg/day for toddlers aged 2-4 years;
- 13 mg/day for primary school aged children aged 5-12 years; and
- 17 mg/day for teenagers aged 13-19 years.

The estimated mean dietary exposures for consumers of ISP for New Zealand were:

- 10 mg/day for the whole population aged 15 years and above; and
- 15 mg/day for teenagers aged 15-19 years.

The 95th percentile dietary exposures for consumers of ISP for Australia were estimated as:

- 33 mg/day for the whole population aged 2 years and above;
- 23 mg/day for toddlers;

- 34 mg/day for primary school children aged 5-12 years; and
- 49 mg/day for teenagers aged 13-19 years.

The 95th percentile dietary exposures for consumers of ISP for New Zealand were estimated as:

- 26 mg/day for the whole population aged 15 years and above; and
- 38 mg/day for teenagers aged 15-19 years.

Of the population groups assessed, teenagers from both countries (aged 13-19 years for Australia and 15-19 years for New Zealand) had the highest estimated dietary exposures to ISP (in mg/day). When estimated mean dietary exposures are considered in mg/kg bw/day, Australian toddlers aged 2-4 years have the highest dietary exposures to ISP.

5.1.3 Risk characterisation

The Applicant advises that the typical level of ISP in consumer products will be 0.005% (50 ppm), with the maximum concentration for some uses of 0.01% (100 ppm). The dietary exposure assessment performed by FSANZ of the 95th percentile exposures for teenagers for Australia and New Zealand produces comparable figures of 0.9 mg/kg bw/day (teenagers aged 13-19 years) and 0.6 mg/kg bw/day (teenagers aged 15-19 years) respectively. This conservatively assumes a use level of 100 ppm, that the entire ice cream and edible ices category contains ISP, and that the body weight is 60 kg.

The dietary modelling performed by FSANZ and summarised above, using similar assumptions of maximum usage concentrations calculated the highest exposure to be for toddlers aged 2-4 years in Australia, with a 95th percentile of consumption of 1.3 mg ISP/kg bw/day.

Commercial ISP preparation is a solution of proteins – ISP (active component), glyco-ISP (inactive component), proteins and peptides from baker's yeast and sugars, acids, and salts commonly found in food. The safety assessment has focused primarily on the potential toxicity and allergenicity of the ISP protein itself. In evaluating these safety parameters, consideration was given to the history of its presence in the human diet primarily from consumption of fish, and the body of scientific evidence to show that ISP is not toxic and is unlikely to be allergenic. The highest dose that could be tested in the 13-week rat toxicity study, 580 mg ISP/kg body weight/day by gavage, showed no adverse effects. The Applicant refers to this level of exposure as the no-observed-adverse-effect-level (NOAEL).

Using the NOAEL derived in the rat study and a theoretical safety factor, the Applicant has determined an acceptable daily intake (ADI) for ISP, however expression of an ADI is not considered of primary relevance to this safety assessment for several reasons.

Firstly, according to JECFA (Joint FAO/WHO Expert Committee on Food Additives) guidelines, an ADI is based on toxicological information from animal studies in which a dose-response relationship has been established, allowing determination of a NOAEL. To achieve this, the highest doses of the test substance administered to the animals should elicit some detectable effect. There were no adverse effects detectable at the highest dose administered in the rat study using ISP preparation, and therefore the NOAEL has been inferred from these results.

Secondly, the ISP preparation is a protein-rich mixture that has been shown to be readily degraded in the gastrointestinal system, as expected of normal dietary protein.

Finally, given the available data on ISP (chemical, biochemical, toxicological and allergenicity), the intended low level of use, and its acceptable background in food, its use as a processing aid in frozen products such as ice cream does not raise any safety concerns.

5.2 Nature of the ice structuring protein

The ice structuring protein of this Application was originally found in a cold water fish, ocean pout, *Macrozoarces americanus,* found along the North American coast. The ocean pout is consumed by humans as fish, although the Application states that current stocks are over-fished.

The Application states that the serum of the ocean pout contains at least 12 different types of ice structuring proteins which can be separated by high performance liquid chromatography (HPLC). The protein of this Application is one of these 12 proteins which has been separated and purified and which the Applicant calls ISP type III HPLC 12. This protein is the most abundant and has the most active functionality from *in vitro* ice-structuring tests. It is made up of 66 amino acids in a known sequence with a molecular weight of approximately 7 kDa. The protein is heat tolerant, with an isoelectric point between 6 – 10, is stable between pH 2 – 12 and is not glycoconjugated (that is the protein is not bound with carbohydrates).

The identified ISP was selected for commercial production due to its good functionality and thermal and pH stability. The Application states that it was considered not economic or commercially feasible to produce the amount of ISP required from fish stocks, especially with the seriously depleted stocks that currently exist. The strategy that was commercialised to overcome these difficulties was to produce the protein via fermentation of a genetically modified food grade yeast *Saccharomyces cerevisiae* (baker's yeast) containing an inserted synthetic gene encoding the ISP protein. Such technology is well proven and developed and used for many commercial food enzymes. The production process used is typical industrial scale batch fed fermentations.

The nature of the ISP, its commercial production and discussion of the technological justification for its use as a processing aid for the manufacture of ice cream and edible ice products is provided in the Food Technology Report (**Attachment 3**).

A large amount of the information about the ISP of this Application is contained in publicly available peer-reviewed references written in collaboration with researchers from the Applicant.[1,2,3]

[1] Baderschneider, B.; Crevel, R.W.R.; Earl, L.K.; Lalljie, A.; Sanders, D.J. and Sanders, I.J. (2002) Sequence analysis and resistance to pepsin hydrolysis as part of an assessment of the potential allergenicity of ice structuring protein type III HPLC 12. *Food and Chem. Toxicol.,* **40:**965-978.
[2] Bindslev-Jensen, C.; Sten, E.; Earl, L.K.; Crevel, R.W.R.; Bindslev-Jensen, U.; Hansen, T.K.; Stahl Skov, P. and Poulsen, L.K. (2003) Assessment of the potential allergenicity of ice structuring protein type III HPLC 12 using the FAO/WHO 2001 decision tree for novel foods. *Food and Chem. Toxicol.*, **41**:81-87.
[3] Hall-Manning, T.; Spurgeon, M.; Wolfreys, A.M. and Baldrick, A.P. (2004) Safety evaluation of ice-structuring protein (ISP) type III HPLC 12 preparation. Lack of genotoxicity and subchronic toxicity. *Food and Chem. Toxicol.*, **42**:321-333.

5.3 Proposed food use

The Applicant proposes to use ISP to alter the properties of a number of ice creams and edible ice products, some of which may be new or unique compared to those that are currently available or possible with present technology and ingredients. The Applicant has stated the products they wish to use ISP for are those contained (standardised) under item 3 – Ice cream and edible ices in Schedule 1 of Standard 1.3.1 – Food Additives. The Applicant requested that such items include ice creams, frozen yoghurts and frozen fruit and/or vegetable juices and drinks.

As discussed above, ISP 'binds' to and influences the growth and structure of the developing ice crystals during production of such products. This different ice structure alters the properties of the food products. According to the Applicant, one important advantage is that the frozen ice products have improved resistance to melting which is a major advantage against temperature abuse and also allows the development of innovative new products. As well the ice crystal structure is altered which offers improved sensory delivery of flavours and colours. That is, flavours and colours are not so easily drawn out of the ice crystal structure by a consumer of a frozen ice product, as the new altered ice structure impedes this and allows for more even distribution.

The Applicant states another possible advantage that the use of ISP offers is the commercial production of new innovative products with consumer benefits. Such new products include consumer acceptable low/zero fat products, products with higher fruit content and products with low added sugar content. The altered ice structure provides opportunities to develop products due to the altered physical properties, texture and mouth-feel.

The ISP technology is different to that currently used in ice cream manufacture where stabilisers (food gums) and emulsifiers are used to slow ice cream melt, and alter mouth-feel and texture of products.

5.4 Relevant international or national regulatory standards

There is no Codex Alimentarius Commission standard that covers ice structuring proteins. The Joint FAO/WHO Expert Advisory Committee on Food Additives (JECFA) has not evaluated ISP.

The US FDA (Food and Drug Administration) has accepted this specific ISP as Generally Recognized As Safe (GRAS). Commercial ice creams and edible ices treated with ISP have been sold in USA since June 2003. The US FDA GRAS notification from the Applicant's expert panel is supplied along with the letter of no objection (GRAS notice no. GRN 000117) in the Application. For the US GRAS notification system ISP is not required to be designated as acting as a processing aid or food additive, just that its use for the proposed purpose is safe. The GRAS expert panel summary suggested ISP may be identified on the ingredients label of final products as the common or usual name (that is 'ice structuring protein'). The Applicant confirmed that this labelling is used for product treated with ISP in the USA and the Philippines.

ISP has also been approved for use in Hong Kong, Mexico, the Philippines and Indonesia. Commercial product is sold in the USA and the Philippines. The Applicant is also applying for approval in a number of other countries. The Applicant states that where approval has been sought, no rejections have been made.

5.5 Labelling issues

There are a number of relevant labelling issues for this Application, which could arise from regulating ISP as a processing aid. These include consideration of labelling for processing aids which are produced using gene technology and labelling for foods derived from substances that may cause adverse reactions.

The following sections outline the relevant labelling issues for the different aspects of this Application.

5.5.1 Processing aid

Clause 3 (d) of Standard 1.2.4 – Labelling of Ingredients, exempts processing aids from ingredient labelling requirements. However, there are other possible labelling requirements relevant for this Application that must be considered. Clause 4 of Standard 1.2.3 – Mandatory Warning and Advisory Statements and Declarations, requires the labelling of substances that may cause adverse reactions to food (see section 5.5.2). Standard 1.5.2 – Food Produced using Gene Technology requires labelling for processing aids or food additives produced using gene technology if the food contains novel DNA and/or novel proteins (see section 5.5.3).

5.5.2 Allergen labelling

Clause 4 – Mandatory declaration of certain substances in food of Standard 1.2.3 requires the presence of 'fish and fish products' in a food to be labelled.

The relevant section of Clause 4 of Standard 1.2.3 is provided below.

4 Mandatory declaration of certain substances in food

(1) The presence in a food of any of the substances listed in the Table to this clause, must be declared in accordance with subclause (2), when present as -

(a) an ingredient; or
(b) an ingredient of a compound ingredient; or
(c) a food additive or component of a food additive; or
(d) a processing aid or component of a processing aid.

Table to clause 4

Cereals containing gluten and their products, namely, wheat, rye, barley, oats and spelt and their hybridised strains other than where these substances are present in beer and spirits standardised in Standards 2.7.2 and 2.7.5 respectively
Crustacea and their products
Egg and egg products

Fish and fish products
Milk and milk products
Peanuts and soybeans, and their products
Added Sulphites in concentrations of 10 mg/kg or more
Tree nuts and sesame seeds and their products

As ISP is not a fish or fish product, but is produced from yeast there is no requirement to label under the requirements of Clause 4 of Standard 1.2.3. The Applicant also asserts that there are no allergenicity concerns with ISP, although it is identical to a protein from a fish source. FSANZ has assessed this aspect as part of the Safety Assessment Report (Attachment 4). The Safety Assessment Report confirms that ISP itself is not allergenic but the yeast extract was allergic for several fish-allergic people tested. Yeast allergenicity is not considered a food safety issue, nor is there a requirement of the Code for yeast allergen labelling within Standard 1.2.3 – Mandatory warning and advisory statements and declarations. Severe reactions to yeast ingestion is extremely rare, despite extensive exposure to common foods containing yeast. Most individuals allergic to yeast appear able to tolerate foods containing yeast[4,5].

5.5.3 Gene technology labelling provisions

Division 2 – Labelling etc of food produced using gene technology in Standard 1.5.2 – Food Produced using Gene Technology, requires that processing aids and food additives be labelled where novel DNA and/or novel protein from the processing aid or food additive remains present in the food to which it has been added.

Division 2 of Standard 1.5.2 states that:

> **novel DNA and/or novel protein** means DNA or a protein which, as a result of the use of gene technology, is different in chemical sequence or structure from DNA or protein present in counterpart food which has not been produced using gene technology.

ISP is stated by the Applicant to be the same as the protein found in ocean pout, which is a fish consumed by humans, although the ISP of this Application is derived from yeast. Because the ISP protein of this Application is identical to the counterpart protein found in nature it is not a novel protein under this definition and would not need to be labelled under this provision of the Code. This situation is an analogous case to that of chymosin, which is an enzyme used in cheese manufacture. Chymosin can be derived from natural sources and from genetically modified sources but the chymosin enzyme is identical in both cases and the enzyme from the genetically modified source does not need to be labelled under the requirements of Standard 1.5.2. The situation with chymosin is the same for other enzymes derived from genetically modified sources, which also do not need to be labelled even if present, provided they are used as processing aids, not performing an additive function in the final food.

[4] Kortekangas-Savolainen, O., Savolainen, J., Lantto, R. and Kalimo, K. (1994) Immediate hypersensitity to bakery, brewery and wine products in yeast-sensitive atopic dermatitis patients. *Clin. Exp. Allergy,* **24**:836-842.
[5] Savolainen, J., Kortekangas-Salolainen, O., Nermes, M., Viander, M., Kiovikko A., Kalimo K. and Terho E.O. IgE, IgA, and IgG responses to common yeasts in atopic patients. *Allergy* **53**:506-512.

5.6 Issues addressed from submissions

5.6.1 Issues raised to the Initial Assessment Report

Public comment on the Initial Assessment Report was sought from 20 October until 1 December 2004.

A number of issues were raised by submitters who did not support the Application, which are discussed below. The Australian Food and Grocery Council (AFGC) supported the Application and made a number of comments addressing issues.

The issues that submitters raised for the Application to approve ISP as a processing aid can be summarised as:

- Whether ISP has characteristics and functions for the proposed purpose as a food additive rather than a processing aid.
- Whether ISP protein contains novel DNA/protein.
- Various labelling aspects, including labelling requirements if ISP is considered a processing aid or a food additive, allergen labelling, GM labelling and labelling to allow consumer choice.
- Possible allergenicity issues.

5.6.1.1 Appropriate nomenclature

The Food technology association of Victoria (FTAV) made the point that ISP is an unacceptable abbreviation and should not be used in documentation, and certainly not on labels or in advertising for any product that contains the protein.

The submitter states that it is not appropriate that if the protein was assessed as a food additive or was required to be labelled on packages that 'ISP' could be used as a name, but probably a more specific term 'ice structuring protein' would need to be used (as is the case in the USA, as recommended by the USA GRAS expert group for this protein).

Discussion

ISP was used in the FSANZ Initial Assessment Report (and will be used in this and subsequent assessment reports) for brevity, as is usually the case for such assessments of applications. The term ISP was also used to indicate the specific ice structuring protein of the Application, rather than the generic class of proteins that have been given the designation 'ice structuring protein' and have been abbreviated in a number of technical articles as ISP.

The Applicant indicated that the term ice structuring protein would be used on food products as is the voluntary practice in the USA and the Philippines.

5.6.1.2 Processing aid or food additive?

There have been a variety of responses to the issue of whether ISP should be regulated as a processing aid (as the Applicant contends) or as a food additive.

Queensland Health noted an apparent inconsistency of the arguments supporting ISP acting as a processing aid in the Initial Assessment Report, and the definition of a processing aid in Standard 1.3.3. The submitter underlined the relevant wording to emphasis the point, which is reproduced below.

In the Regulatory Problem section of the Initial Assessment Report it was stated that:

> 'For ISP in this Application to be considered a processing aid it needs to be performing its major technological function during the processing or manufacture of the edible ice products and no, or a minor, technological function in the final food.'

While the definition of a processing aid is defined in Standard 1.3.3 as:

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

A number of submitters argued that ISP is functioning more as a food additive than a processing aid for the purposes requested in the Application. That is, the protein may have a continuing technological function in the final formed ice product.

For example, FTAV contends that ISP is expected to still be able to perform its function of modifying ice crystal formation during a thaw/freeze cycle, as it will have not been inactivated, which is often the case with processing aids.

Other aspects raised in the submission that are more comparable to food additives than processing aids are:

- It is not removed, inactivated or destroyed after it has performed its technological function.
- It is still present in the final food in exactly the same quantity originally added.
- It is added at similar levels to food additives that are currently added for a similar purpose such as stabilisers (food gums) and emulsifiers.

Queensland Health mentioned that ISP can be considered a food additive, even if it is having only a minor technological function in the final food and therefore needs to be labelled in the ingredients list.

PB Foods Ltd states that currently stabilisers (food gums) and emulsifiers are traditionally used in ice cream manufacture to alter the ice cream texture and mouth-feel, and that these products are considered food additives. The submission suggests that ISP is performing a similar function and should also be considered as a food additive not as a processing aid.

The AFGC proposed a different view, believing ISP fulfils the requirements of a processing aid since it is performing its technological function during the freezing process (manufacture of the ice products) and has no technological function in the final food. That is, ISP induces a physical reaction during the freezing process that modifies the ice crystal structure. The altered ice crystal structure provides the changes in texture, flavour and colour retention. The enhanced stability occurs because of the action of ISP to alter the ice crystal structure during processing and is not due to the presence of ISP in the final product.

Discussion

The divergent views on this issue received in submissions indicate that ISP does not fit neatly into the usual processing aid categories. It should also be pointed out that many food additives are also considered to be generally permitted processing aids. This permission is provided by subclause 3(b) of Standard 1.3.3. Food additives listed in Schedule 2 of Standard 1.3.1 – Food Additives can be considered to be generally permitted processing aids if they meet the definitional requirements of Standard 1.3.3.

There is a wide overlap between food additives and processing aids, and an important criterion used to decide into which group a substance best fits is how the compound is performing its technological function; during processing and manufacture or in the final food.

ISP is used to perform its technological purpose during the processing of the ice products and is not clearly performing any of the listed technological functions of food additives in Schedule 5 of Standard 1.3.1. The altered physical properties of the ice products would not be apparent if ISP was added after manufacture of the products. The altered properties are not due to the presence of ISP by itself, but the effect ISP had on the ice structure formation during processing.

The argument is raised that ISP may continue to perform a technological function in freeze/thaw cycles or during temperature abuse.

ISP performs its technological function in a different way to that of the additives acting as stabilisers and emulsifiers, which are traditionally used to alter sensory and melt properties of frozen ice products. Stabilisers (often food gums) alter the viscosity of the ice cream matrix (including water, fats, sugars and flavours; not the frozen ice) and slow down diffusion during melting. In this case the stabilisers are performing their function in the final food and not during processing.

Emulsifiers in ice cream manufacture act to improve the mixing of different phases (water and fats) making them miscible and preventing the different phases separating out. Emulsifiers are important to ensure air bubbles are stabilised in ice cream mixtures where added air is used.

These two food additive classes have quite different mechanisms on the molecular level to that of ISP. Stabilisers and emulsifiers do not have an effect on the ice crystal structure. The final effect may well be similar, that is amending final product texture, mouth-feel and melt properties, but the process is different.

For a substance to be considered a food additive it needs to achieve a technological function as listed within Schedule 5 of Standard 1.3.1. ISP does not seem to fit neatly in any of the food additive functions listed in Schedule 5. Possible food additive functions listed in Schedule 5 for ISP are stabiliser or firming agent. Stabiliser includes binder, firming agent, water binding agent, foam stabiliser, and is defined as 'maintains the homogeneous dispersion of two or more immiscible substances in a food'. Firming agent is defined as 'contributes to firmness of food or interact with gelling agents to produce or strengthen a gel'. ISP does not conform to these Schedule 5 definitions or behave in a manner of either a stabiliser or firming agent.

In this Application, ISP is more correctly regarded as a processing aid rather than a food additive.

5.6.1.3 Labelling issues

Labelling requirements for the use of ISP to produce ice cream and edible ices was raised as an issue in a number of submissions including that of the New Zealand Food Safety Authority. As stated in section 5.6.1.2 above there was discussion about whether the protein should be considered a processing aid or food additive for the proposed purpose of the Application. This decision has labelling implications as food additives are required to be labelled, while in general processing aids are not (section 5.5.1).

FTAV believed that if the protein is considered a food additive then the proposed labelling name 'ice structuring protein' is rather unique and if used by itself may not comply with the requirements of the Code.

A further statement was made that if ISP is considered a processing aid and is not therefore labelled on products, consumers would be denied a choice, whether to purchase the product containing ISP or not.

Another point raised was that the USA GRAS expert panel for this product recommended that the protein should be identified on the ingredients label of final products as its common or usual name (that is 'ice structuring protein').

The AFGC stated it expected FSANZ will find ISP will be safe for the proposed purpose since it has already been approved and used in a number of other countries, including the USA (where it is considered GRAS), Hong Kong, Mexico, the Philippines and Indonesia.

The AFGC stated that ISP is a processing aid and so is exempt from labelling provisions because of the exemption for ingredient labelling in subclause 3 (d) of Standard 1.2.4. The submitter believes there is no need for the mandatory declaration of allergens within clause 4 of Standard 1.2.3 for 'fish and fish products' since ISP is not derived from fish. ISP is produced from a genetically modified yeast and though is identical to a protein from fish, is not a fish product but derived from yeast. Fish allergenicity studies on fish allergic people provided in the Application support that there is no need to label ISP as a fish product.

Submissions from the Department of Human Services Victoria and Paula Young expressed the view that not labelling the presence of a substance produced from a genetically modified source was misleading to consumers and did not allow consumers to make choices on whether to purchase product containing ISP.

Discussion

Standard 1.2.4 - Labelling of Ingredients, covers the declaration of food additives on labels in Clause 8 and would apply if ISP were to be regulated as a food additive in Standard 1.3.1. If a food additive cannot be classified in one of the prescribed classes of food additives, then it needs to be listed by its prescribed name. If it can be classified as one of the classes (such as firming agent or stabiliser) then it needs to be labelled with the name of the class followed by the additive's specific name in brackets (or an INS number, if applicable).

The USA GRAS expert panel report for ISP stated that the protein 'may be' identified on labels by the common or usual name of 'ice structuring protein'. The relevant extract relating to labelling is:

> *The ISP type III preparation covered by this GRAS evaluation may be identified on the label of frozen novelties simply by the common or usual name declared in the designation of ingredients pursuant to 21 CFR 101.4 (e.g., "ice structuring protein"). There is no need for commercial products to be labeled with the word "fish" or any other designation as a condition of safe use.*

FSANZ has confirmed with the Applicant that commercial product produced using ISP in the USA and the Philippines is labelled with 'ice structuring protein', although this is not a mandatory requirement. The Applicant confirmed that a similar labelling approach would be followed if ISP were approved for use in Australia and New Zealand.

The USA food regulatory system is different to the situation in Australia and New Zealand. The USFDA did not make a decision as to whether the ISP was a food additive or a processing aid, just that ISP is safe to use for its proposed purpose.

The term 'ice structuring protein' would not necessarily be understood by consumers, if ISP were to be regulated as a food additive, with a labelling requirement.

5.6.1.4 Novel DNA/protein aspects

The FTAV thought the comparison made in the Initial Assessment Report between the enzyme chymosin and the ISP protein may not be valid. The submission argued that chymosin is degraded after it has performed its technological function while the ISP protein is unchanged.

An alternative view was expressed by the AFGC supporting the position stated in the Initial Assessment Report. That is, that ISP is not a novel protein since it is identical to a fish protein which is consumed as part of a human diet, and has been for many years. Therefore it does not come under the labelling requirements of Standard 1.5.2.

Along with the situation of chymosin mentioned in the Initial Assessment Report, this submitter believes the ISP situation is analogous to that of a number of enzyme processing aids which are also derived from genetically modified micro-organisms, which are identical to those from non-genetically modified sources and they also do not require labelling under Standard 1.5.2.

Two submissions stated they believed there are consumer choice issues if labelling under Standard 1.5.2 is not required, since this removes consumer's choice to make decisions to not purchase products that contain ingredients derived from genetically modified organisms.

Discussion

Gene technology labelling requirements in Standard 1.5.2 do not apply to ISP because ISP does not contain novel DNA nor is it a novel protein as defined in the Standard. The ISP of the Application is identical in amino acid sequence to the counterpart ISP found in nature (that is isolated from ocean pout). This is an analogous situation to that of chymosin (or other enzymes sourced from genetically modified micro-organisms). The chymosin sourced from genetically modified sources is identical to that obtained from natural sources, and does not need to be labelled under the requirements of Standard 1.5.2. The important point is whether novel DNA or novel protein is in the final food. The issue of whether ISP is unchanged during processing is irrelevant for labelling purposes. Cheese is usually not heat processed after the addition of chymosin, and the continued presence of undegraded chymosin has been argued to be performing an additive function as a flavouring in ripened cheese.

The situation for consumer choice concerning the identification of food that contains substances sourced from genetically modified organisms is similar to that for many currently approved enzymes produced from genetically modified sources. These issues have been addressed in a recent FSANZ report, Report on the Review of Labelling of Genetically Modified Food, December 2003. This report is publicly available on the FSANZ website[6] or from FSANZ.

FSANZ has also produced a user guide that provides advice on labelling of genetically modified products called 'Labelling Genetically Modified Food' which is also available on the FSANZ website [7].

5.6.1.5 Impacts analysis

The AFGC believed there would be only small impacts in terms of costs to food manufacturers which should be able to be absorbed into their costs. The benefit to manufacturers is that they should be able to produce new innovative products, which should be a benefit to consumers. Consumers should also receive benefits from improved quality products. There may be some cost associated with government agencies if they need to perform analyses if a maximum permitted level is imposed, rather than good manufacturing practice (GMP).

[6] http://www.foodstandards.gov.au/_srcfiles/GM_label_REVIEW%20REPORT%20(Final%203).doc
[7] http://www.foodstandards.gov.au/assistanceforindustry/userguides/index.cfm

Discussion

This matter is addressed under section 7 - Impact Analysis.

5.6.1.6 Drafting issues

The AFGC suggested that care would be needed to ensure correct legal drafting is provided to ensure the Applicant achieves the permissions they are requesting, and GMP permission or higher than requested levels should be considered if the Application is accepted and there are no safety issues.

The Applicant states that they wish to use ISP during the manufacture of ice cream and edible ices, which includes products under item 3 of Schedule 1 of Standard 1.3.1. The Applicant requested permission to include such products as frozen yoghurts and frozen fruit and/or vegetable juices and drinks. The submission states they believe frozen yoghurt do not actually come under this item but would be considered a yoghurt, which has been frozen. The same situation exists for frozen fruit and/or vegetable juices and drinks.

Discussion

If ISP is regulated as a processing aid, a drafting amendment is not required for Schedule 1 of Standard 1.3.1. If permission is provided for ISP in Standard 1.3.3 – Processing Aids, approvals can be provided to explicitly cover the product categories the Applicant has requested. The low levels of use proposed in this Application limit consideration of ISP as a food or food ingredient and the safety assessment also considered the low levels of use proposed.

5.6.1.7 Allergenicity issues

A number of submitters expressed concern about allergenicity issues, and asked that the risk assessment at Draft Assessment fully address allergenicity. Discussion of this topic is in the Safety Assessment Report at Attachment 4.

NZFSA suggested that the ISP is stated to be a fish protein and needs to be labelled because of the mandatory declarations of allergens within Standard 1.2.3.

Discussion

ISP, although identical to a fish protein for the purposes of Standard 1.5.2 is not directly derived from fish but from fermentation of a yeast. ISP is not captured by the mandatory declarations required in Clause 4 because it is not a fish product within the meaning of Standard 1.2.3. The Applicant contends, and has provided data to show, that ISP is not an allergen.

5.7 Risk management

Section 5.1.3 – Risk characterisation has summarised the risk assessments including the safety assessment and dietary modelling calculations from using ISP to treat ice cream and edible ices. The use of ISP as proposed does not raise any safety concerns. ISP is technologically justified as a processing aid for the proposed purpose (see section 5.3).

ISP functions as a processing aid as it is performing its technological purpose during the manufacturing step of making ice cream and edible ices, and does not perform a technological function of a food additive in the final products. ISP 'binds' to the developing ice crystal structure and modifies it during formation. The modified structure of the ice is responsible for the stability of the products containing ISP. This is different in the case of stabilisers and emulsifiers which are used in the traditional method of modifying mouth-feel and slowing product melt, where these chemicals act as food additives since they have a technological function in the final food consistent with Schedule 5 of Standard 1.3.1.

As a processing aid ISP does not need to be labelled on final foods. ISP also does not meet the labelling requirements for substances that cause adverse reactions to foods (Standard 1.2.3) or foods derived from genetically modified sources (Standard 1.5.2).

ISP is more consistently considered as a processing aid and therefore is most appropriately regulated within Standard 1.3.3. ISP is not considered a food additive since it does not perform its technological function in the final food or meet one of the technological functions of a food additive in Schedule 5 of Standard 1.3.1. It is not a stabiliser or a firming agent.

There are two risk management options available:

1. Regulate ISP within Table to clause 14 – Permitted processing aids with miscellaneous functions of Standard 1.3.3 – Processing Aids, giving specific functions of how it can be used, for which products it could be used for and a detailed name of the protein, that is Ice structuring protein Type III HPLC 12.

2. Do not permit the use of ISP.

A final drafting requirement is that a specification for ISP should be included in the Code, since it is not covered by any of the monographs (primary or secondary sources listed in clauses 2 and 3) of Standard 1.3.4 – Identity and Purity.

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.

There are no options other than a variation to the Code for this Application. Therefore the two regulatory options available for this Application are:

Option 1 Not approve the use of ISP in the manufacture of ice cream and edible ice products.

Option 2 Approve the use of ISP in the manufacture of ice cream and edible ice products as a processing aid under Standard 1.3.3 and list the specification in Standard 1.3.4.

7. Impact Analysis

7.1 Affected Parties

The affected parties to this Application include the following:

1. those sectors of the food industry wishing to market the food products subject to the Application, specifically companies who wish to produce ice cream and edible ice products;

2. consumers; and

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

7.2 Impact analysis

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

The following is an assessment by FSANZ of the costs and benefits of the two regulatory options identified so far. This is based on information supplied by the Applicant and experience FSANZ has gained from consideration of previous applications. Interested parties to this Application are also welcome to make comments on the costs and benefits identified for the options below.

Option 1.

Industry: Cost in terms of restricting innovation in manufacture of new and improved ice cream and edible ice products, especially in comparison to manufacturers in other countries where the technology is approved and has been commercialised.

Cost to industry groups in the supply chain of ice cream and edible ice products where new technology is not available to limit shelf life losses due to melting of product.

Consumers: Costs in terms of not having access to new and improved ice cream and edible ice products, with different sensory properties and take longer to melt.

Government: No immediate impact.

Option 2

Industry: Benefit to industry allowing the manufacture of new innovative and improved ice cream and edible ice products, especially in comparison to manufacturers in other countries where the technology is approved and has been commercialised. Such possible new products could include low fat, low sugar and higher fruit products.

Benefit to importers and distributors of overseas food products as the product range is extended.

Benefit to industry groups in the supply chain of ice cream and edible ice products where new technology is available to limit shelf life losses due to melting of product.

Benefit to food retailers in an increased product range.

Consumers: Possible benefit being able to purchase new innovative ice cream and edible ice products with improved sensory properties and improved shelf life of existing products (i.e. the products stay firmer longer and take longer to melt). Some possible new products with consumer benefits are low fat, low sugar and higher fruit products.

Possible cost may be paying a higher price for new premium innovative ice creams and edible ice products.

Possible perceived concern that foreign proteins have been added into ice cream and edible ice products.

Government: There may be a slight cost in terms of any analyses regulatory agencies may need to perform if a maximum permitted level for treated products are required rather than to permit the use of ISP to GMP.

8. Consultation

8.1 Public consultation

Public comment on the Initial Assessment Report was sought from 20 October until 1 December 2004. Eight (including one supporting submission from the Applicant providing more information and justifications for the Application) submissions were received. The Applicant plus one other submitter supported the Application. Two submissions rejected the Application while 4 did not state a position or tentatively supported further evaluation but raised concerns and issues they believed needed to be addressed during further assessment.

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 the Application at Final Assessment.

Comments on, but not limited to, the following would be useful.

-
- Is there technological justification for the use of ISP for the manufacture of ice cream and edible ice products?
- What additional safety considerations would be associated with its proposed use?
- What are the likely costs and benefits to food manufacturers, consumers and government if ISP is approved?
- Who are the affected parties relating to this Application?

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.

There are not any relevant international standards and amending the Code to allow ISP to be approved to manufacture ice cream and edible ice products is unlikely to have a significant effect on international trade. For this reason it is not FSANZ's intention to recommend relevant agencies notify the WTO.

9. Conclusion and Recommendation

The draft variation to Standard 1.3.3 – Processing Aids of the Code to permit the use of ISP as a processing aid for the manufacture of ice cream and edible ice products is recommended for the following reasons.

The draft variation to Standard 1.3.3 – Processing Aids of the Code to permit the use of ISP as a processing aid for the manufacture of ice cream and edible ice products is recommended for the following reasons.

- The safety assessment concluded that no public health and safety concerns associated with using ISP as a processing aid for the manufacture of ice cream and edible ice products have been identified.
-
- The use of ISP is technologically justified to alter the properties of ice cream and edible ice products. ISP binds to and influences the growth and structure of the developing ice crystals during manufacture, which alters the physical and sensory properties of the final products.
-
- As concluded by the regulatory impact analysis, the costs that would arise from a variation to Standard 1.3.3 to permit the use of ISP as a processing aid for the manufacture of ice cream and edible ice products do not outweigh the direct and indirect benefits to the community, Government or industry that would arise from the variation.
-

- The proposed draft variation to the Code is consistent with the section 10 objectives of the FSANZ Act.
-
- To achieve what the Application seeks, namely permission to use ISP as a processing aid for the manufacture of ice cream and edible ices, there are no alternatives that are more cost effective than a variation to Standard 1.3.3.

ATTACHMENTS

1. Draft variations to the *Australia New Zealand Food Standards Code*
2. Summary of public submissions
3. Food technology report
4. Safety assessment report
5. Dietary modelling 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 in the* Table to clause 14 –

Ice Structuring Protein type III HPLC 12	Manufacture of ice cream and edible ices	0.01

[2] ***Standard 1.3.4*** *of the Australia New Zealand Food Standards Code is varied by inserting in the* Schedule –

Specification for ice structuring protein type III HPLC 12 preparation.

Ice structuring protein type III HPLC 12 preparation is a protein excreted from the fermentation of a genetically modified yeast (*Saccharomyces cerevisiae*) to which a synthetic gene encoding for the protein has been inserted into the yeast's genome.

Assay	Not less than 5 g/L active ice structuring protein type III HPLC 12
pH	3.0+/-0.5
Ash	Not more than 2%
Appearance	Light brown aqueous preparation
Heavy metals	Not more than 2 mg/L
Microbial limits	
Total microbial count	<3000 per g
Coliforms	<10 per g
Yeast and mould count	<100 per g
Listeria sp.	Absent in 25 g
Salmonella sp.	Absent in 25 g
Bacillus Cereus	<100 per g

Attachment 2

Summary of public submissions

Round One

#	Submitter Organisation	Name
1	Food Technology Association of Victoria	David Gill
2	New Zealand Food Safety Authority	Carole Inkster
3	Australian Food and Grocery Council	Tony Downer
4	PB Foods Ltd	Monica Witsch
5	Department Human Service Victoria	Victor Di Paola
6	Unilever Australasia	Julie Newlands
7	Queensland Health	Gary Bielby
8	Individual	Paula Young

Submitter	Position	Comments
Food Technology Association of Victoria	Tentative support, with issues needing to be addressed.	They supported the Application, but they did have a number of issues which they believed needed addressing. • ISP is not an acceptable abbreviation for the protein and should not be used in any documentation, specifically not on labels or advertising for any food products containing it. Consumers would have no understanding of what ISP is. • They argue that ISP has properties that make it more like a food additive than a processing aid. o It has a recognised technological function forming the desired ice crystals during manufacture. o It is not changed once its function has occurred, in fact it may be able to repeat its function in thaw/freeze cycles. o It is not removed/destroyed/inactivated once its function has been achieved. o It is still present in the final food in the same quantity as initially added. o The levels added are similar to those of food additives used for a similar technological purpose. • If it is considered as a food additive then there are labelling issues, that is its presence in food needs to be labelled. However the proposed name of 'ice structuring protein; does not comply with the standard labelling philosophy in the Code, and is rather a unique situation. • The USA GRAS expert panel supported labelling as 'ice structuring protein' on food containing it. • If the Applicant does not label because the product is considered a processing aid then consumer's choice is limited, they will have no way of knowing if ISP has been used and is contained in the product. The argument about novel DNA/protein comparison between ISP and chymosin are believed may not be valid since chymosin is degraded after its technological function is completed while ISP is unchanged, and the DNA/protein is unchanged.

New Zealand Food Safety Authority	Not specifically stated, but do have a number of issues for consideration	They believe there are a number of labelling issues which need resolution. • They have expressed the opinion that ISP can be considered a food additive, if it is having a minor technological function in the final food, which then requires labelling in the ingredients list. • They also suggest that consideration for the presence of fish protein may require labelling provisions under Standard 1.2.3 (as ISP is a fish protein sourced from a genetically modified yeast).
Australian Food and Grocery Council	Supports	The AFGC supports the Application, subject to a satisfactory safety assessment. They expect that ISP will be considered safe due to: • International approvals; • established long-term human consumption; • ISP is a simple protein, and as such will be broken down and digested as any other protein; and • other information supplied by the Applicant. They also made a number of other comments. • They believe ISP is a processing aid since it is performing its technological function during the freezing process (manufacture) and has no function in the final food. The AFGC states that ISP induces a physical reaction during the freezing process that, together with the rate of the freezing, modifies the ice crystal structure that is formed at sub-zero temperatures. Once this process has occurs, ISP has no further physical action. The changes in texture and flavour and colour retention are caused by the altered ice crystal structure brought about by the action of ISP during processing, not by its presence in the final food. • Being a processing aid, ISP is exempt from labelling, due to subclause 3 (d) of Standard 1.2.4. • Although identical to a fish protein, ISP is derived from a genetically modified yeast and so allergen labelling for the presence of fish or fish products is not required, under subclause 4 (1) of Standard 1.2.3. A further analogy to that of chymosin listed in the Initial Assessment Report is that for many other enzyme processing aids derived from genetically modified sources, which also do not require labelling. • Likewise, though produced from a genetically modified yeast, ISP is not considered a novel protein since it is identical to a fish protein, consumed by humans as part of a diet, so does not come under the labelling requirements of Standard 1.5.2. • The AFGC recommends broad drafting to ensure permission to use ISP in the manufacture of ice cream, edible ices, frozen yoghurt and other potentially new innovative ice products. They state that frozen yoghurt is not an edible ice product (but would come under the yoghurt category) so care with drafting will be required, so as to not exclude some products which the Applicant may wish to use ISP for. The same situation may be the case with frozen fruit and/or vegetable juices and drinks which the Applicant requested approval for. The AFGC suggest ISP should be permitted to GMP, or if not, after consultation with the Applicant a maximum slightly above the level of 0.01% stated in the Application to allow for manufacturing variations and potential use for new products.

		• They believe there will be only minor impacts as costs to manufacturers should not need to be passed onto consumers. Manufacturers should be able to produce new innovative products while consumers should also benefit from improved quality products. There may be minor costs to government agencies if they need to perform analyses to check for maximum limits if such are imposed rather than GMP.
PB Foods Ltd	Support further consideration but they have a number of issues and concerns	As a manufacturer of ice cream and dairy products they have an interest in this Application. In summary they support further consideration of the Application, but they do have a number of issues and concerns which they believe need to be addressed. • Currently various stabilisers and emulsifiers are added to ice cream to modify ice crystal growth and size which alters ice cream texture and mouth-feel. They state these agents acts as processing aids but they also have a technological function in the final products so act also as food additives. They believe the same is the case with ISP, that is it also has a food additive function in the final food. • They also have concerns about the allergenicity of ISP, and believe further risk assessment on the allergen aspects be performed. • They believe ISP should be labelled on the final products.
Department of Human Service Victoria	Do not support the Application	• They believe ISP is a food additive and can be considered a stabiliser. They believe the protein has an effect upon the texture of the final product, so has a technological function in the final food. Being a food additive it would be required to be labelled. • It is declared as an ingredient in the USA. • They believe this Application would set a dangerous precedent if a protein that is identical to a protein requiring allergen declaration (fish protein) and produced from a GMO did not require allergen labelling. • All possible allergen and intolerance risks should be fully considered. • They also believe that not requiring a GMO declaration would also remove consumer choice to those who do not wish to purchase products containing ingredients derived from GMO. They believe this Application appears to be a deliberate attempt to bypass the requirements of the Code.
Unilever Australasia (the Applicant)	Supports	The Applicant has provided more supporting information for their Application. Some of this information is new, or an elaboration of earlier justifications. New or expanded issues are as summarised below. • The technological justification has been expanded to claim further consumer, customer and manufacturer benefits: o improved product quality with improved cold chain tolerance (less temperature abuse, better shape retention). o able to produce innovative products with different texture, flavour and structure. o Improved manufacturing efficiencies, during processing (extrusion). o Production of healthier (lower fat, sugar and higher fruit content) products.

		• They have provided quite a deal more information explaining how ice cream (and edible ice products) are manufactured and how ISP performs its technological function during manufacture. ISP has no effect on the temperature at which ice forms or the ice content, but it does alter the size and shape of the crystals and so the final ice structure. This information is to continue to justify that they believe ISP behaves as a processing aid for the proposed purpose not as a food additive. • They expanded on the justification for believing that ISP does not need to be labelled under Standard 1.5.2. They reiterated that they believe ISP is not a novel protein, since it is identical to a fish protein which has a history of safe use. Also they state the situation is analogous to that for enzymes (including chymosin, as stated in the Initial Assessment) which have been derived from genetically modified organisms which do not require labelling under Standard 1.5.2.
Queensland Health	No position at this stage but made some comments	They stated they neither accept nor reject the Application at this stage but will review once the safety assessment (including allergenicity aspects) has been performed. • However they did point out an inconsistency in the Initial Assessment Report (IAR) justification of ISP acting as a processing aid and the definition of a processing aid in the Code. The IAR stated (underlined in the submission to highlight the differences): 'For ISP in this Application to be considered a processing aid it needs to be performing its major technological function during the processing or manufacture of the edible ice products and no, or a minor, technological function in the final food'. While the definition of a processing aid in Standard 1.3.3 includes: (a) the substance is used in the processing of raw materials, foods or ingredients, to fulfil a technological purpose relating to treatment or processing, but does not perform a technological function in the final food' • They understand it will be difficult to perform dietary exposure assessments using out of date 1995 data, so they reiterate the call for a new comprehensive national nutrition monitoring and surveillance program to update the data.
Paula Young	Rejects	Believes consumers have a right to know if a substance that has been produced using genetically modified techniques has been added to food (whether as a processing aid or an ingredient). That requirement for consumer information means the substance so produced should be listed on the label so that consumers can make an informed choice. If the current labelling regulations in the Code do not require this then they should be amended.

Attachment 3

Food Technology Report

General Introduction for Ice Structuring Proteins

Cells of living organisms are usually irreversibly damaged during freezing causing cell death. Freezing deprives cells of their aqueous medium which they require for functioning, causes ion and solute concentration in the plasma, causes denaturation of biomolecules and can rupture cell membranes (Harding *et al.*, 1999). However a number of various organisms including fish, plants, insects, fungi and bacteria have been identified that are able to survive at temperatures below freezing (Barnett, 2001). Such diverse organisms have been found to contain molecules (essentially proteins and peptides) which assist survival by depressing the freezing point of cell liquids. Over thirty years of research has been performed on these proteins. Such proteins were first identified in 1969, in the blood of fish living in areas where the sea froze (De Vries and Wohlschlag, 1969).

These proteins have been given various names such as antifreeze proteins, ice growth modifiers, thermal hysteresis proteins and now more recently ice structuring proteins (Clarke *et al.*, 2002). The term ice structuring proteins has been proposed because regardless of their source and structure all the proteins bind to and influence the growth of ice crystals.

The term thermal hysteresis is defined as (Harding *et al*, 1999):

the difference between

(a) the equilibrium melting point and
(b) the ice growth temperature, the temperature at which seed ice crystals will grow in the solution.

For pure water the difference is zero, 0ºC is the temperature at which ice melts and also when ice crystals grow, i.e. ice forms from solution. Thermal hysteresis proteins have a positive measure of the thermal hysteresis and are greater (300-500 times) than the freezing point depression due to concentration effects of solutes (freezing point depression which is proportional to molar concentration of the solute) (Harding *et al.*, 1999). An example of freezing point depression is the well known use of salt (in reasonably high molar concentrations) to depress the freezing point of water. Thermal hysteresis proteins are therefore able to depress the freezing point to a much greater extent than could be estimated purely from their molar concentration.

How ice structuring proteins function

There has been a large amount of research effort and papers written trying to fully understand the mechanism of how ice structuring proteins work to prevent blood and cell fluids freezing. The general understanding has been revealed but not the exact chemistry at the molecular level. A number of general review articles have recently postulated about the mechanism of action of ice structuring proteins (Harding *et al.*, 1999; Barrett, 2001; and Griffith and Ewart, 1995).

The summary of the agreed understanding is that the ice structuring protein 'binds' to the developing ice crystal in one particular axis, so limiting growth in this direction. Also it is believed that ice structuring protein adsorbs preferentially onto a specific face of the developing ice crystal. There is a variety of quite detailed analyses of possible mechanisms for binding. These analyses detail crystal structure geometries and the various proteins' X-ray crystal structures of classes of ice structuring proteins but for the purposes of the Application it is sufficient to know that ice structuring proteins accumulate (if not strictly chemically 'bind') to specific faces of the ice crystal and so alters the growth patterns and growth rates of the ice structures. This alteration also changes the physical properties of the ice products formed by their use in commercial ice products (discussed below). It has been postulated that the adsorption of the proteins on the ice crystal structure is due to favourable intermolecular steric interactions and van der Waals forces. It is also believed that both hydrophobic and hydrophilic interactions are involved.

Specific background on the ice structuring protein of the Application

The various ice structuring proteins which have been identified from a variety of different organisms have been classified into different groups which have similar protein structures and properties. For ice structuring proteins isolated from fish varieties the groups have been termed type I, II, III and IV (Crevel *et al.*, 2002). The ice structuring protein of this Application is categorised as a type III protein. Fish type III ice structuring proteins have been found in the following fish: ocean pout, eelpout and wolffish.

The ice structuring protein of this Application was originally isolated and is found naturally in ocean pout (*Macrozoarces americanus*), which is a cold water fish found off the northeast coast of North America, in or near Arctic waters. High performance liquid chromatography (HPLC) extraction of the protein extract identified 12 isoforms. The most abundant and functionally active fraction from *in vitro* ice structuring tests is the isoform which has been labelled by the researchers as ISP type III HPLC 12 and is the protein of this Application. A number of recent references have provided information about this specific protein, relating to sequence analysis and allergenicity (Baderschneider *et al.*, 2002), allergenicity (Bindslev-Jensen *et al*., 2003) and safety (Hall-Manning *et al*., 2004).

The ice structuring protein type III HPLC 12 isoform which is the ice structuring protein of this Application will be now abbreviated for convenience for the rest of this report as ISP. The DNA sequencing of ISP has been performed and shown to comprise 66 amino acids in a known sequence with a molecular weight of approximately 7kDa. The protein is heat tolerant, with an isoelectric point between 6 and 10, is stable between pH 2-12 and is not glycoconjugated (that is the protein is not bound with carbohydrates).

The Applicant does not consider it is acceptable or economic to produce ISP in commercially viable quantities by extracting from the fish, ocean pout, especially since the fish is in danger of being over-fished. Therefore the Applicant produces ISP by fermentation techniques using recombinant baker's yeast (*Saccharomyces cerevisiae*). This utilises a synthetic gene coding for ISP, which is inserted into the yeast. The gene is not identical to that obtained from fish because codon usage is different between fish and yeast. If the gene were taken from the fish the resultant protein obtained from the yeast fermentation would be a slightly different protein. Tests have revealed the obtained protein to be 'nature identical' (in terms of amino acid sequence) to that extracted from ocean pout.

More detailed discussion about the molecular biological and safety aspects, including the genetic stability of the modified yeast, are contained in the Safety Assessment Report (**Attachment 4**).

The commercial production of ISP from the modified yeast occurs using standard industrial scale batch fermentations, with subsequent isolation using microfiltration, concentration and packaging steps. This is very similar to production processes for commercial enzymes used for food manufacture. The commercial ISP preparation is a mixture of ISP, glycosylated ISP (ISP bound to the sugar mannose), proteins and peptides from the yeast and sugars, acids and salts commonly found in food. The ISP preparation is standardised and stabilised in citric acid buffer.

Technological justification for ISP

As mentioned in an above section ice structuring proteins affect the growth and structure of ice crystals by directly accumulating or adsorbing (if not strictly chemically 'binding') to the growing ice crystals and inhibiting the crystal growth (particularly in one direction or axis) resulting in modification of the resulting ice crystal size and structure and hence its physical properties. For food products based on ice, addition of ISP also has important impacts on the sensory properties of the resultant ice products. Such altered sensory properties include resultant hardness (and how long before the ice product melts), creaminess and alterations to flavour delivery. These aspects were postulated in some of the recent references concerning ice structuring proteins (specifically Griffith and Ewart, 1995).

Some of the suggested advantages of using ISP during the manufacture of ice cream and edible ice products are:

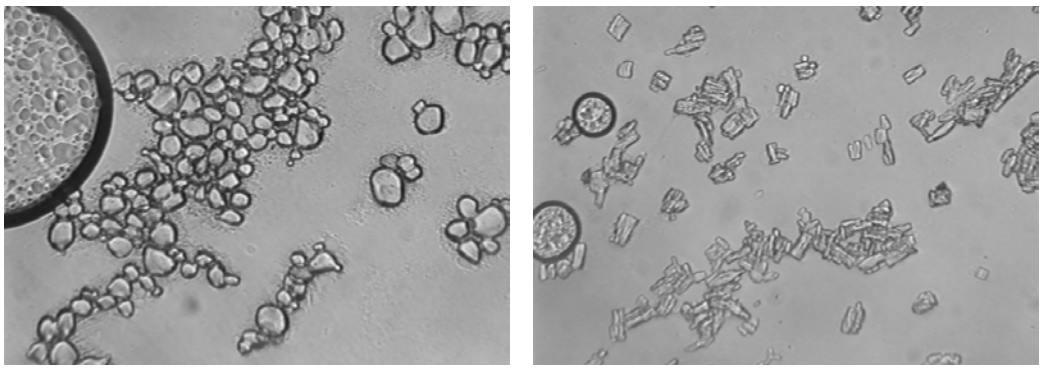
- Assist in limiting melt drip of ice products, so providing a longer lasting product for consumers.
- Ensure a firmer product with improved product integrity, which is less affected by temperature fluctuations during the transport chain (i.e. more resistant to temperature abuse).
- The formed ice crystal structure is different, being not as regular and not allowing the easy removal of added flavours or colours from the ice structure. That is it limits flavours and colours being 'sucked' out of ice products as they are being consumed.
- The changed sensory aspect of the products allows commercially acceptable low fat products to be produced. Sensory aspects of low fat products will be comparable to standard products. Such possible new products are higher quality low/zero fat products, products with higher fruit content and ones with lower added sugar content.
- Wider range of novel textures, and more complicated and intricate shapes are possible.

These postulated aspects are no longer just theories since commercial ice cream and edible ice products containing ISP have been available in the USA since June 2003, and also sold in the Philippines.

ISP is added to the ice cream or water ice mixture where it has no effect until freezing starts. ISP does not affect the quantity of ice present at any given temperature but it does have an impact on the size and shape of the ice crystals formed.

Commercial manufacture of ice cream or edible ices occurs in a standard freezer where cold ice cream or water ice mix enters and is cooled on the cold walls of the freezer. The ice, which forms on the walls, is scraped off back into the mixture. Nearly all the ice crystals present in the final products are formed in the freezer stage. The ice crystals/water mix continues through the freezer stage where the ice crystals formed increase in size. It is stated that typical manufacture of ice cream and edible ices has the product mix entering the freezer at 5ºC and extruded at approximately –6ºC where approximately 60% of the final ice structure has been formed. Colder extruder temperature increases the percentage of ice formed.

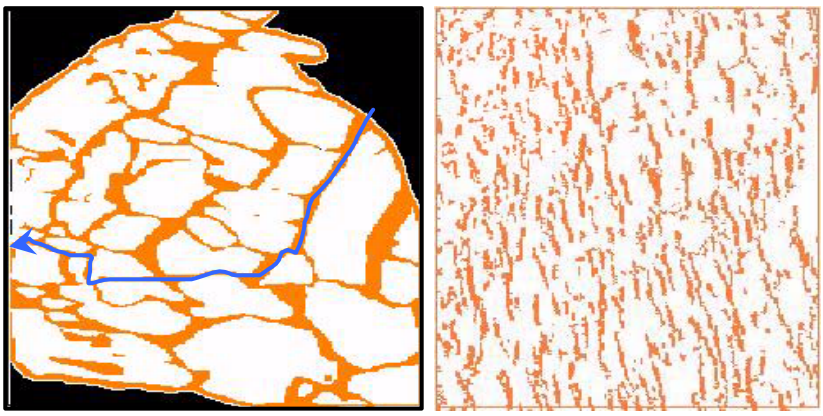
During the freezer stage the addition of ISP alters the shape and size of the ice crystals; with crystals produced with the addition of ISP being rod shaped rather than the usual round shape. The resultant smaller rod shaped ice crystals produce a product from the extruder that is firmer and has higher viscosity (see pictures below in Figure 1 provided by the Applicant in their submission to the Initial Assessment Report).



No ISP ISP

Figure 1. Differences in ice crystal shape in extruded ice cream at –5ºC with and without ISP.

After the ice cream has been extruded it is hardened at storage temperatures (-20ºC) where the ice crystals grow so increasing the ice content, but no new crystals are formed. The final ice crystal structure of product produced without ISP is quite different to that produced with the addition of ISP during processing (see pictures below in Figure 2, again taken from the Applicant's submission).



No ISP ISP

Figure 2. Ice crystal structures in hardened water ice with and without ISP (the dark grey colour is the mixture matrix and white is the ice).

The situation with using ISP is different to the technology, which is traditionally used to alter the physical properties of ice cream and edible ice products including texture, mouth-feel and melt resistance. The traditional method uses food additives called stabilisers (food gums) and emulsifiers to alter the properties. Stabilisers alter the viscosity of the ice cream matrix, which modify the gel network at the interface between the ice structure and the water matrix. This increased viscosity slows down the diffusion during melting so slowing down melting effects. Emulsifiers improve the miscibility of two different phases; water and fat in the ice cream mixture. Emulsifiers also improve stability of air bubbles in mixtures where air is added to ice cream products to improve their properties.

Specification of ISP

The Applicant states that there is no international standard for ISP. That is there is no Codex standard, and JECFA (Joint FAO/WHO Expert Committee on Food Additives) has not assessed ISP.

There is no specification specific for ISP in any of the monographs (primary and secondary sources) within Standard 1.3.4 – Identity and Purity of the Code.

The Application states that specification requirements for the commercial ISP protein preparation are based on those for enzymes within the Food Chemicals Codex 4th Edition (2001) (which has now been updated to the 5th Edition (2004)) since there is similarity of the production processes (submerged batch fermentations of a micro-organism) and use levels in food.

The Application supplied the following specification for the commercial ISP preparation. This will be included in Standard 1.3.4, as a stand-alone specification since there are no specifications covering it in the monographs referenced in Standard 1.3.4.

Specification for ice structuring protein type III HPLC 12 preparation.

Ice structuring protein type III HPLC 12 preparation is a protein excreted from the fermentation of a genetically modified yeast (*Saccharomyces cerevisiae*) to which a synthetic gene encoding for the protein has been inserted into the yeast's genome.

Assay	Not less than 5 g/L active ice structuring protein type III HPLC 12
pH	3.0+/-0.5
Ash	Not more than 2%
Appearance	Light brown aqueous preparation
Heavy metals	Not more than 2 mg/L
Microbial limits	
Total microbial count	<3000 per g
Coliforms	<10 per g
Yeast and mould count	<100 per g
Listeria sp.	Absent in 25 g
Salmonella sp.	Absent in 25 g
Bacillus Cereus	<100 per g

Conclusion

The Application to use ISP as a processing aid during the manufacture of ice cream and edible ices is technologically justified.

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Attachment 4

Safety assessment report

APPLICATION A544 – ICE STRUCTURING PROTEIN AS A PROCESSING AID IN ICE CREAM AND EDIBLE ICES

SUMMARY AND CONCLUSIONS

Background

Ice Structuring Protein type III HPLC 12 (ISP), derived from a northern hemisphere fish species, has been assessed in terms of safety for human consumption. Naturally occurring ice structuring proteins can bind to and influence the growth and structure of ice crystals, resulting in a modified ice structure. When used in the manufacture of certain frozen food products, these properties affect the physical and sensory properties of the foods, as well as improve temperature stability. In this application, permission is sought to use ISP type III HPLC 12 as a processing aid in the manufacture of dessert products such as ice cream and water ices.

As natural fish sources are limited, the Applicant has developed a method of producing commercial quantities of ISP by fermentation of baker's yeast that has been genetically modified (GM) to manufacture and secrete the fish ISP. The ISP preparation is a mixture of functionally active ISP, inactive mannose-conjugated ISP, proteins and peptides from common baker's yeast, and sugars, acids and salts commonly found in food.

A number of criteria have been addressed in the safety assessment including: a characterisation of the gene transferred to the production organism, its origin, function and stability; a characterisation of the functional protein present in the ISP preparation secreted by the GM yeast; and the potential for the ISP preparation to be either toxic or allergenic to humans.

History of Use

Humans have previously been exposed to ice structuring proteins in the diet through the consumption of certain fish and vegetable species. ISP is present in the blood of ocean pout, a species of cold-water fish found off the northeast coast of North America, that is harvested commercially for human food.

Food-grade yeasts are used widely in the manufacture of beer, wine, and for production of enzymes including those used in cheese manufacture. The production organism for ISP is baker's yeast (*Saccharomyces cerevisiae*), which has a long history of safe use in the leavening of bread.

Description of the Genetic Modification

The gene encoding ISP (derived from ocean pout) was re-synthesised in the laboratory using a yeast-optimised gene sequence to improve production and secretion of the protein. The gene expression cassette consisting of the synthetic ISP gene, together with appropriate regulatory elements derived from *S. cerevisiae*, was introduced as a stable, multi-copy insert into baker's yeast using osmotic shock.

The synthetic gene in yeast encodes the identical amino acid sequence to that of the native ISP derived from ocean pout. The gene cassette did not contain any antibiotic resistance marker genes or any bacterial DNA.

Molecular analysis of the yeast showed that the genetic modification was stable over more than 70 generations of culture, and further analysis demonstrated that the protein produced by the GM yeast was of the expected profile and activity.

Characterisation of ISP

ISP, consisting of 12 isoforms, was originally isolated from ocean pout. Using high performance liquid chromatography (HPLC) to separate the isoforms, HPLC 12 was identified as the largest peak and the most functionally active in ice-structuring studies. ISP type III HPLC 12 consists of a known sequence of 66 amino acids, and studies on its properties and the physical structure of the protein have been published. Biochemical analysis of the yeast-derived ISP demonstrated that the protein is the same as the native ISP from ocean pout.

Safety assessment of ISP

The Applicant conducted a number of studies to determine whether ISP is potentially toxic in mammals and is likely to act as an allergen.

Bioinformatic analyses of the amino acid sequence of the protein was conducted to determine whether ISP shares any sequence similarity with known toxins or allergens. Careful examination of the results of these analyses showed that the structure of ISP is highly characteristic of other fish ice-structuring proteins and shows little similarity with that of any other proteins. In particular, the results showed no primary sequence similarity between ISP and the sequence of any known allergens, including fish allergens.

The results of a 13-week sub-chronic rat feeding study using a concentrated form of the ISP preparation from yeast showed no toxicity at doses up to 580 mg/kg/day. The food consumption of the animals receiving the ISP preparation was similar to that of the controls and there were no behavioural differences observed throughout the study. On conclusion of the study, there were no detected differences between test and control groups in haematological parameters, ophthalmology, organ weights, or on macroscopic or microscopic examination of organs. ISP shows no indication of toxicological or histopathological changes in rats.

The genotoxic activity of ISP was assessed using four different assays: the bacterial reverse mutation assay, the *in vitro* chromosome aberration assay in human peripheral blood lymphocytes, the gene mutation assay in mouse lymphoma L5178Y cells, and the *in vivo* rat bone marrow micronucleus assay. The results of these experiments showed that ISP is not genotoxic in this series of mutagenicity and cytogenetic studies.

The potential allergenicity of ISP was investigated systematically using a number of established methods. ISP did not bind IgE from fish-allergic subjects in the RAST assay, nor did it show any activity in a functional biological assay using basophils from the same fish-allergic individuals. Absence of IgE binding was confirmed visually by immunoblotting.

Skin prick testing with ISP did not produce any positive reactions to the protein, although four reactions to yeast proteins were observed and confirmed by *in vitro* tests. A confirmatory skin prick test with a highly purified ISP (yeast protein content <1%) was negative. The conclusion from these investigations was that ISP is not likely to be allergenic in humans.

In studies using human volunteers, ingestion of ISP preparation for eight weeks at a high daily dose did not result in specific antibody formation, indicating that ISP is not likely to be any more immunogenic than the majority of dietary proteins.

Additional biochemical analyses simulating gastric fluid digestion with pepsin in an *in vitro* test system showed that both ISP and its glycoconjugated form would be readily degraded in the human digestive system. In addition, amino acid sequence analysis showed a susceptibility to proteolytic breakdown by intestinal enzymes such as trypsin. These results indicate that ISP is therefore unlikely to be absorbed intact or accumulate in the body.

Based on a thorough assessment of allergic potential, and the results of the analytical, animal, human, and *in vitro* data presented in this application, ISP preparation is not toxic and is unlikely to evoke an allergic reaction in fish-sensitised individuals, or to sensitise potentially susceptible individuals in the wider population.

Conclusion

No potential public health and safety concerns have been identified in the assessment of ISP. On the basis of the data provided in the present application, and other available information, the ISP preparation derived from fermentation of GM baker's yeast can be considered safe for human consumption.

1. INTRODUCTION

Unilever Australia Limited is seeking to vary Standard 1.3.3 – Processing Aids – in the Code, to permit the use of Ice Structuring Protein Type III HPLC 12 (ISP) as a processing aid for the preparation of ice cream and edible ices.

Ice structuring proteins occur in nature in a wide range of species including animals, plants, insects, fungi and bacteria. This Application relates to a specific ice structuring protein that occurs naturally in ocean pout, an arctic fish. Ice structuring proteins are also known as thermal hysteresis proteins (THPs), or antifreeze proteins. The sole function of ice structuring proteins in nature is to protect organisms from the cellular damage that occurs by freezing.

Ice is a major component of ice cream and water ice and, as such, has a major effect on the physical and sensory properties of these products. In addition, the size and structure of the ice crystals affects temperature stability. Ice structuring proteins lower the temperature at which ice crystals grow, and modify the shape and size of the ice crystals that are formed. These properties have potential uses in the manufacture of ice cream and edible ice products.

When used in food products, ISP does not actually prevent ice formation but instead binds to and directly influences the growth and structure of ice crystals. This modifies the resulting ice structure and its physical properties, imparting new physical and sensory characteristics to the products.

2. HISTORY OF USE

The Applicant states that hundreds of kilograms of ISP would be required each year to generate commercial quantities of frozen dessert products. Obtaining these quantities directly from fish would be expensive and would result in serious depletion of ocean pout stocks. To ensure a consistent, reproducible supply, ISP has been produced by fermentation using a genetically modified (GM) microorganism.

2.1 Production organism

The production process consists of fermentation with a GM food-grade baker's yeast, *Saccharomyces cerevisiae*. This technique has been used for the production of many other food ingredients, particularly enzymes such as amylase, pectinase, xylanase and chymosin used in the manufacture of cheese.

There is a long history of safe use of *Saccharomyces cerevisiae* associated with the production of food for human consumption. It is the most widely used yeast in the food industry employed for the manufacture of wine, beer and bread. All strains of *Saccharomyces cerevisiae* are GRAS (Generally Regarded As Safe) under the United States Food and Drug Administration (US FDA) system. In 1994, the US Environmental Protection Agency (EPA) evaluated the risk associated with industrial use of *Saccharomyces cerevisiae*, including GM strains, and concluded that human health and environmental release risks associated with this organism are low, and that it poses no significant health hazard.

2.2 Donor organism

Most food use of ocean pout (*Macrozoarces americanus*) has occurred in the US and Canada. This species was marketed as food during World War II, but consumer demand waned with the outbreak of a protozoan parasite that caused lesions on the fish. From 1964 onwards, there have been significant fluctuations in the scale of commercial interest in this species. Currently, the ocean pout is considered to be over-fished. Notwithstanding their current status, ocean pout have a long history of use as food for humans.

2.3 Ice structuring proteins in nature

Ice structuring proteins are naturally occurring proteins and peptides that are already consumed as part of the human diet. They were first identified over thirty years ago in the blood of fish, such as cod and herring, living in areas where the sea freezes. Since this time, ice structuring proteins have been found in a wide variety of organisms that protect themselves against freeze damage, including many plants, insects, fungi and bacteria. Edible plants in which ice structuring proteins occur include common food sources such as oats, barley, wheat, carrot and potato (Griffith and Ewart, 1995). In many plants, ice structuring proteins are found in the edible parts such as the carrot tap root, potato tuber, or leaves of Brussels sprouts (Urrutia *et al*. 1992; Smallwood *et al.* 1999).

ISP prevents freezing of the blood of ocean pout by binding directly to ice crystals and subsequently controlling the way in which the ice crystal grows, thus preventing cellular damage. The level of ISP type III naturally present in the fish is estimated to be about 30 mg/ml in blood. Assuming the blood volume of modern bony fishes is about 30-70 ml/kg, the ISP type III content of an ocean pout can be calculated at 900-2100 mg/kg.

Thus consumption of a 200g portion of ocean pout would result in an intake of between 180 mg and 420 mg of ISP type III from the diet. Fletcher *et al*. (1985) reported that ice structuring proteins are present in fish plasma all year round, and therefore consumption of ocean pout would always be associated with consumption of ISP type III.

3. DESCRIPTION OF THE GENETIC MODIFICATION

3.1 Method used in the genetic modification

The gene expression cassette encoding ISP type III HPLC 12 (derived from ocean pout) was introduced into baker's yeast using osmotic shock, which increases the permeability of the yeast cell membrane allowing the uptake of exogenous DNA. The gene cassette is then able to automatically integrate into the yeast chromosomal DNA, at the ribosomal DNA (rDNA) locus, as a stable, multi-copy insert.

Strain description

Producing strain: CENPK338 containing multi-copy integration fragment of plasmid pUR3993 integrated at the rDNA locus. (CENPK338 = *Saccharomyces cerevisiae MATa MAL2-8c SUC2 leu2-3, 112 gal1: URA3 pmt1 (201,2350): loxP*)

3.2 Function and regulation of the ISP gene

The gene expression cassette was constructed to contain a yeast-optimised synthetic ISP gene plus other genetic information to enable the efficient expression and secretion of the protein in yeast. The synthetic gene encodes ISP, the identical protein to that derived from ocean pout.

In order to facilitate adequate production of ISP protein in yeast, a synthetic gene was constructed in the laboratory, based on the known amino acid sequence of the protein originally identified in ocean pout. The amino acid sequence of ocean pout ISP was published in 1988 (Hew *et al*). Re-synthesising the gene sequence encoding ISP was necessary to ensure the preferred DNA codon usage by the yeast. The yeast-optimised synthetic gene sequence produces a protein of the same amino acid sequence as the native protein.

In addition to the synthetic gene, the expression cassette is composed of:

(1) a Pgal7 promoter (for galactose induction), allowing activation of gene expression by addition of this sugar to the medium;
(2) a TDH3 leader sequence to improve protein synthesis; and
(3) an invertase (SUC2) signal sequence to ensure secretion of the protein into the culture medium.

All of the above regulatory elements are derived from *S. cerevisiae.* The gene cassette does not contain any antibiotic resistance marker genes or any bacterial DNA.

3.3 Molecular characterisation of the yeast

Insert and copy number

Southern blot analysis was used to establish the site of integration of the inserted gene cassette and the number of copies. The presence of multiple copies shows that the integration has been targeted towards the ribosomal DNA locus as intended.

On the basis of the results from the Southern blot analysis, integration of between 30 and 50 copies of the 6.2 Kilobase (Kb) ISP expression cassette from pUR9339 has occurred at the rDNA locus in the yeast genome.

3.4 Stability of the genetic change

Genetic stability of the ISP-modified strain of *S. cerevisiae* was measured after more than 70 generations of growth under non-selective conditions. Plating cells on selective and non-selective media revealed the same amount of viable cells. Inductive growth (after 70 generations) showed identical expression levels of ISP when tested in liquid culture. Polymerase chain reaction (PCR) analysis on whole yeast cells (chromosomal DNA as template) demonstrated that the ISP gene was present. In addition, Southern blot analysis showed that the strain after 70 generations was identical to the initial modified strain with respect to the integration site.

These results demonstrate that the genetic modification in the engineered yeast strain is stable.

4. CHARACTERISATION OF THE ISP PROTEIN

4.1 Chemical properties

Native ISP is composed of 66 amino acids (sequence provided), and has a molecular weight of 7.027 kDa. The structure of the protein has been investigated and has been shown to have a fold in which eight beta strands form triple-stranded antiparallel sheets and one double-stranded antiparallel sheet, with the two triple-stranded sheets arranged as an orthogonal beta-sandwich (Sonnichsen *et al.* 1993; Chao *et al.* 1994). The protein is not glycoconjugated. The ISP is functional for ice structuring properties but the ISP commercial preparation also contains a glycoconjugated form of ISP, which is non-functional.

4.2 Protein expression analysis

The level of ISP expressed by the modified strain is determined by High Performance Liquid Chromatography (HPLC) of a yeast fermentation sample. The activity of the protein peak was demonstrated using the recrystallisation inhibition assay. These results showed that the protein identified on chromatograms as ISP is active, significantly reducing the amount of ice crystal growth compared to a control sucrose solution in the assay system.

4.3 Potential toxicity of ISP protein

Published Studies:
Hall-Manning, T., Spurgeon, M., Wolfreys, A.M. and Baldrick, A.P. (2004) Safety evaluation of ice-structuring protein (ISP) type III HPLC 12 preparation. Lack of genotoxicity and subchronic toxicity. Food and Chemical Toxicology 42, 321-333.

4.3.1 Sub-chronic toxicity study in rats

Stewart, J. (March 2002) Batch 201008: 13 Week Oral (Gavage Administration) Toxicity Study in the Rat. Covance Laboratories Ltd (Harrogate, England), Study Number 375/154, Report Number 375/154-D6154.

The Applicant submitted a sub-chronic (13 weeks) oral toxicity study in rats to support the safety of ISP. The study was performed at Covance Laboratories (UK) according to FDA guidelines[8] and OECD guidelines[9] for repeated dose oral toxicity studies in rodents, and in compliance with international regulations for Good Laboratory Practice[10].

The overall study design included two control groups of animals and three different testing doses of ISP. A comparison of treatments for each group of animals is presented in Table 1 below. Each group was comprised of 20 rats per sex per group, and animals were approximately six weeks old at the start of dosing. All animals were individually housed during the course of the study.

The test substance was ISP produced from yeast fermentation (*S. cerevisiae*). This material also contained inactive glyco-conjugated (mannose) ISP, as well as proteins and peptides from the fermentation and sugars, acids and salts commonly found in food. The preparation was concentrated by ultrafiltration without altering its properties compared to the commercial preparation. The concentrated material was characterised using HPLC, and stability and homogeneity measured.

Concentrated test material was administered as a single daily dose volume of 20 ml/kg delivering ISP levels of either 58, 290 or 580 mg/kg bodyweight/day respectively for three months. The lower doses were achieved by dilution with citric acid (to approximately pH 3), as this was present in high concentration in the ISP preparation. One control group received ultra-purified water and a second group received citric acid solution (0.12%), in order to control for acidity by administering a solution with a pH equivalent to that of the ISP type III preparation.

[8] Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food (1993), Redbook 2000: Toxicological Principles for the Safety of Food Ingredients (2001), United States Food and Drug Administration.
[9] Guideline for the Testing of Chemicals. Section 4: Health Effects Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents. Organisation for Economic Cooperation and Development (OECD), 1998a.
[10] Series on Principles of Good Laboratory Practice and Compliance Monitoring, OECD, 1998b.

Table 1: Dosing information for test and control groups in the 13-week rat study

Group	ISP type III HPLC 12	Total ISP	Total Solids
Water control	0 mg/kg/day	0 mg/kg/day	0 mg/kg/day
Citric acid control	0 mg/kg/day	0 mg/kg/day	100 mg/kg/day
Low dose	58 mg/kg/day	100 mg/kg/day	400 mg/kg/day
Intermediate dose	290 mg/kg/day	480 mg/kg/day	2000 mg/kg/day
High dose	580 mg/kg/day	960 mg/kg/day	4000 mg/kg/day

Parameters measured in the study included clinical observations, food consumption, neuro-behavioural testing, opthalmoscopic examination, clinical pathology (haematology, clinical chemistry, urinalysis, bone marrow smears), gross necropsy, selected organ weights and histopathology of specified organs/tissues.

Summary of experimental observations

Clinical signs: Animals were observed daily for signs of ill health or overt toxicity. Additional observations were conducted daily during Week 1 immediately post dosing, and 30 minutes, 1, 2, and 4 hours after dosing. Post dosing observations were made once weekly after Week 1.

Physical examination: Performed at weekly intervals

Mortality/morbidity: All animals were observed at the beginning and end of the working day.

Body weights: Individual body weights were recorded before treatment on the first day of dosing, at weekly intervals, and before necropsy.

Food consumption: The amount of food consumed by each animal was determined weekly.

Functional observation: Ten males and ten females were subjected to a battery of behavioral tests and observations before treatment and once weekly afterwards, including observations, open field and motor activity.

Opthalmoscopy: Investigations were performed on all rats before treatment and on control and high dose animals during week 12.

Clinical pathology: Blood samples were taken from ten male and ten female animals during weeks 4 and 8 and from all surviving animals at the end of the study. Urine samples were taken when possible from ten male and ten female rats from each group during week 12.

At termination: All animals were subjected to a necropsy. A full macroscopic examination was carried out and all lesions recorded. A full complement of tissues from all animals was retained in the appropriate preservatives.

Organ weights: The following organs were weighed before fixation; adrenals, brain, heart, liver, ovaries, spleen, testes and epididymides, thymus, and uterus.

Histopathology: Gross lesions from all animals and the following tissues from both control and the high-dose group were examined: adrenals, aorta, bone marrow smear, brain, cecum, colon, duodenum, eyes, femur, heart, ileum, jejunum, kidney, liver, lungs with bronchi, mammary gland, mandibular lymph nodes, mesenteric lymph nodes, muscle, esophagus, optic nerve, ovaries, pancreas, Peyers patches, pituitary, prostate, rectum, salivary glands, sciatic nerve, seminal vesicles, skin, spinal cord (cervical, lumber and thoracic), spleen, sternum and bone marrow, stomach, testes and epididymides, thymus, thyroids and parathyroids, trachea, urinary bladder, uterus, and vagina.

Results

One male receiving the highest dose was sacrificed during week 10 due to deterioration of his condition, which was not considered related to treatment. Salivation associated with dosing was seen from week 7 onwards in several animals given the highest dose. Animals given 290 or 580 mg/kg bodyweight/day gained slightly more body weight than the vehicle controls. Food consumption was similar among all groups. There were no persistent conditions, or trends in the functional observation battery of tests, or effects on ambulatory movements, attributable to treatment.

There were no differences between groups in haematological parameters, clotting potential, or in the biochemical composition of the blood. There were no inter-group differences in organ weights related to treatment. There were no macroscopic or microscopic findings due to the effects of the test material.

Due to the lack of treatment-related effects at all dose levels, it was concluded that the administration of the test material, ISP, to rats at dose levels up to 580 mg/kg/day for 13 weeks was well tolerated and without adverse signs of toxicity. The highest dose that could be tested, 580 mg ISP per kg body weight per day, was considered to be the NOAEL (no-observed-adverse-effect-level) in this study.

4.3.2 Assessment of Genotoxicity

The potential genotoxic activity of ISP was assessed using four different assays. These were (i) the bacterial mutation assay, (ii) the *in vitro* chromosome aberration assay in human peripheral blood lymphocytes, (iii) the gene mutation assay in mouse lymphoma L5178Y cells, and (iv) the *in vivo* rat bone marrow micronucleus assay. All assays were performed in compliance with the OECD and UK Regulations according to GLP. For the purposes of the mutagenicity studies, the sample was freeze-dried prior to testing and the concentrations are stated in terms of total weight of sample per unit volume, not as concentrations of ISP per unit volume.

Bacterial Reverse Mutation Assay

The bacterial reverse mutation assay was performed using *Salmonella typhimurium* histidine-requiring strains TA1535, TA1537, TA98, TA100, and TA102 and was compliant with OECD Guideline 471 (1997a) and ICH Tripartite Harmonised Guideline on Genotoxicity: Specific Aspects of Regulatory Tests (FDA, 1997). Three independent assays were performed in the presence and absence of rat liver derived S9 fraction (10%) and both plate-incorporation (using 1.6-5000 μg total solids/plate) and pre-incubation (using 156.25-5000 μg total solids/plate) methods were used. For all experiments, a freeze-dried preparation of microbially produced ISP was dissolved in water.

The test was negative with strains TA1537, TA98, TA100, and TA102, both in the presence and absence of rat liver S9 fraction. A small but statistically significant increase in the number of revertant colonies was observed with strain TA1535 only in experiments (both plate incorporation method and pre-incubation), which required further investigation.

In the repeat experiments, the maximum concentration of ISP preparation was increased to 8,000 μg/plate, above the conventional maximum concentration for this assay of 5,000 μg/plate. This increase in concentration revealed that the test material preparation was slightly contaminated, resulting in colonies that were not *Salmonella typhimurium* TA1535, the test organism. Following re-calculation of the number of revertant colonies, no statistically or biologically significant differences were observed between the numbers of colonies on plates exposed to the test material and those exposed to the control solvent.

Based on this assessment, it was concluded that ISP displays no mutagenic activity, as measured by the bacterial reverse mutation assay.

In Vitro *Chromosome Aberration Assay in Human Peripheral Blood Lymphocytes*

The *in vitro* chromosome aberration assay was performed using whole blood cultures of human peripheral blood lymphocytes and was compliant with OECD Guideline 473 (1997b) and the ICH Tripartite Harmonised Guideline on Genotoxicity: Specific Aspects of Regulatory Tests (FDA, 1997). As before, a freeze dried preparation of ISP was dissolved in water and assessed at concentrations up to, and including, 5000 μg total solids/ml or the limit of toxicity. The assay was performed on two independent occasions in the presence and absence of rat liver derived S9 fraction (2%). The whole blood cultures were exposed to ISP for either 3 h (with and without metabolic activation) or 20 h (without metabolic activation only). Cultures were harvested 20 hours after the initiation of treatment. A total of 200 cells were assessed for chromosome aberrations per concentration.

There was no evidence of either a biologically or statistically significant increase in the percentage of cells with aberrations in any of the treated cultures when compared to the solvent control cultures. In addition, the incidence of polyploid and endoreduplicated cells was assessed in 2000 mitotic cells per treatment. No numerical aberrations were observed in any of the treated cultures in comparison with the solvent control cultures.

Under the conditions of this study, ISP showed no evidence of genotoxic potential.

Gene Mutation Assay using Mouse Lymphoma L5178Y Cells

Gene mutation was assessed using the *thymidine kinase (tk)* locus in mouse lymphoma L5178Y cells and was compliant with OECD guideline 476 (1997d) and the ICH Tripartite Harmonised Guideline on Genotoxicity: Specific Aspects of Regulatory Tests (FDA, 1997). Freeze-dried ISP (same batch used in previous genotoxicity studies) was dissolved in water and assessed at concentrations up to, and including, 5000 μg total solids/ml or the limit of toxicity. The assay was performed on two independent occasions in the presence and absence of rat liver derived S9 fraction (2%). The mouse lymphoma L5178Y cells were exposed to this ISP for either 3 hours (with and without metabolic activation) or 24 hours (without metabolic activation only). There was no evidence of either a biologically significant or a statistically significant increase in mutation frequency in treated cultures in comparison with the solvent control cultures.

Under the conditions of this study, ISP showed no evidence of mutagenic potential.

In Vivo *Rat Bone Marrow Micronucleus Assay*

The rat bone marrow micronucleus assay was performed using groups of seven male rats of approximately 7 weeks of age, and was compliant with OECD Guideline 474 (1997c) and the ICH Tripartite Harmonised Guideline on Genotoxicity: Specific Aspects of Regulatory Tests (FDA, 1997). Induction of micronuclei is used as an indicator of chromosome damage in immature erythrocytes. A preliminary dose-range finding assay had shown no significant difference in the toxicity observed in male and female rats and thus only males were used for this study. Freeze-dried ISP was suspended in water and administered once daily on two consecutive days via gavage at 500, 1000, and 2000 mg total solids/kg. The animals were killed 24 hours after final dosing and slides were prepared from the bone marrow obtained from a single femur. The ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) was assessed in 1000 cells per animal.

Some increases in the PCE:NCE ratio were observed but these were not dose related and thus were not considered indicative of toxicity to the bone marrow.

4.3.3 Studies in humans

Information on human exposure to ISP is derived primarily from its history of consumption as a natural protein component in ocean pout, a species of fish that has a long history of safe consumption by humans. There are no epidemiological data on ISP.

Although there are no available studies in humans evaluating the long-term safety of the ISP preparation from yeast, the applicant has presented the details of a randomised, placebo-controlled clinical trial[11] to evaluate any possible adverse effects of a single ingestion of ISP. The test materials consisted of the ISP-based food component and a control product without ISP, delivered in a cherry flavoured water ice. No information was provided on the characterisation of the ISP preparation used in the experiment, nor on the amount of ISP present in the test material.

[11] Study Title: A Randomised, Placebo-controlled Trial to Evaluate a Single Ingestion of a New Protein-based Food Component. Principal Investigator: M. H. Davidson, MD. Affiliation: Chicago Centre for Clinical Research, Chicago, Illinois. Study ID: CCCR 2596 – Code KQ990234. Date of report: 30 March 2000.

The study involved the participation of sixty-nine healthy men and women who met particular age and health criteria determined at the commencement of the study. The participants received a single serving of either control food or test protein food at week1, and the opposite product at week 2 (cross-over). The control and test products were designed to be as similar as possible in composition.

Clinical monitoring of subjects

The safety and acceptability of the test material were assessed by monitoring treatment-emergent adverse experiences in the study participants, at each clinic visit (Weeks 1 and 2). At the screening visit (Week 0) and 4-hours following study product ingestion at each treatment clinic visit (Weeks 1 and 2), clinical laboratory testing, including serum chemistry and haematology profiles, were performed. Vital signs were measured at the screening visit (Week 0) and prior to and 4-hours following study product ingestion at each treatment clinic visit (Weeks 1 and 2). At the screening visit (Week 0), a urine sample was collected for routine testing (all subjects) and for a pregnancy test (all females of childbearing potential). At the screening visit (Week 0) and at the end of the study (Week 2), a brief physical examination was conducted.

Results and conclusion

There were no significant differences in the test product containing ISP and the control product in terms of effects on serum chemistry, haematology, vital signs, or occurrence of adverse events. These results indicate that a single ingestion of yeast-derived ISP in food does not elicit adverse reactions in otherwise healthy adults.

4.4 Potential allergenicity of ISP

Food allergies are caused by abnormal immunological responses to particular substances in food and affect between 1 and 2% of the population. The Codex Alimentarius Commission (CAC) has adopted a list of the most common allergenic foods – these include peanuts, soybean, milk, eggs, fish, crustacean, cereals and tree nuts. These foods account for over 90% of all moderate to severe allergic reactions to food.

Virtually all food allergens are proteins, but only a small fraction of the many hundreds of thousands of different proteins found in food are allergenic. Therefore the chances that a new protein will cause allergic reactions in some individuals are relatively small. However, prediction of the allergenic potential of new proteins is not straightforward. Unlike traditional toxicological parameters, there are no reliable animal models for assessing the allergenic potential of new proteins.

Nevertheless, the potential allergenicity of a protein can be evaluated using an integrated, step-wise approach relying on a body of evidence which, in totality, permits a judgment to be made regarding the potential to cause allergic reactions. Such an assessment focuses on criteria including (i) the source of the protein, (ii) any significant amino acid sequence similarity between the protein of interest and other proteins that are known allergens, and (iii) the biochemical and structural properties of the protein, including susceptibility to degradation in simulated digestion models. Applying such criteria systematically provides reasonable evidence concerning the potential of a new protein to act as an allergen.

The applicant's assessment of the potential allergenicity of ISP has considered two issues: (i) whether the protein is likely to sensitize potentially susceptible individuals and thereby increase the likelihood of a reaction on subsequent exposure to that protein, and (ii) whether the protein is likely to provoke a reaction in individuals allergic to the source from which the protein originated (or to structurally related proteins). This approach is consistent with recent international consensus documents, including the recommendations of a recent FAO/WHO Expert Consultation (FAO 2001) and those of the Codex Alimentarius Commission (CAC 2003). The information provided by each test is summarised in Table 2.

Table 2: Tests conducted to assess the allergenic potential of ISP preparation

TEST	INFORMATION PROVIDED WITH RESPECT TO	
	Potential to sensitize	**Potential to elicit reactions in sensitized individuals**
Sequence analysis	Identifies similarity to known allergens and classes of proteins containing known allergens	Identifies short sequences in common with known allergens (possible epitopes) Can provide information for additional serum screening
IgE binding in vitro – RAST and RAST inhibition		Indicates whether protein can bind specific IgE that might provoke reactions in individuals with a specific allergy
IgE binding in vitro – Immunoblotting		Indicates whether protein can bind specific IgE and might provoke reactions in individuals with a specific allergy and visualizes implicated proteins
IgE binding in vitro – Basophil histamine release		Indicates whether protein can bind specific IgE and might provoke reactions in individuals with a specific allergy and shows whether binding is biologically meaningful
Skin prick testing		Indicates whether protein could provoke reactions in individuals with a specific allergy
Antibody response to ingestion	Provides information on immunogenicity of protein	
Pepsin resistance	Ready hydrolysis by pepsin suggests lower probability of sensitization through GI tract	Ready hydrolysis by pepsin may indicate low probability of reactions in GI tract

4.4.1 Amino acid sequence analysis

Published studies:
Badershneider, B., Crevel, R.W.R., Earl, L.K., Lalljie, A., Sanders, D.J. and Sanders I.J. (2002) Sequence analysis and resistance to pepsin hydrolysis as part of an assessment of the potential allergenicity of ice structuring protein type III HPLC 12. Food and Chemical Toxicology, 40, 965-978.

Amino acid sequence analysis can identify regions in the linear sequence of a protein that resembles the sequence of known allergens. The absence of any similarity suggests that a protein does not possess any possible sequence epitopes resembling those present in known allergens. Sequence analysis can also indicate whether the protein shares any structural similarity with classes of proteins containing known allergens and thus provide guidance for subsequent serum screening.

Several algorithms have been proposed for this purpose, but the most frequently used are FASTA and BLAST (Basic Local Alignment Search Tool), from which computer programs of the same name have been generated. Both methods rely on assessing the probability that an alignment between a query sequence (the unknown protein) and a sequence in the database occurs by chance. The FASTA program automatically searches for and eliminates regions of low complexity, for example multiple repeats of one or two amino acids, which would otherwise result in apparently significant similarity, but without necessarily having any biological significance. Using BLAST, as for the FASTA program, low complexity regions, which would be expected to give very high alignment scores without biological significance, are screened out.

Sequence analysis of ISP type III was performed in line with the suggested procedures (FAO 2001), although with some differences described below. It consisted of three main steps:

1. Identification of similarity with other proteins using the programs BLAST (version 2.2.1, 13 April, 2001) and FASTA (version 3.2, 1998). Databases examined were the nr database of NCBI (all non-redundant GenBank CDS translations + PDB + Swiss-Prot + PIR + PRF) and PIR-NREF, a non-redundant protein database compiled from PIR, Swiss-Prot, TrEMBL, RefSeq, GenPept and PDB. A subset of the nr database was searched with the terms "allergen [ALL]" NOT "immunoglobulin [ALL]" to restrict the search space to entries relevant to allergens ("ALL" specifies the fields where the terms occur). The subset of the nr database served as the allergen database, although it is acknowledged that it has limitations compared to a dedicated allergen database prepared for the purpose. However, these limitations are balanced by the advantage that the databases used are the most up to date. In addition, ISP was also examined against the Food Allergy Research and Resource Program (University of Nebraska) allergen database[12].

2. Identification of local alignments also using the program BLAST 2.2.1. The database examined was the subset of the nr database described above.

3. All six-, seven-, and eight-amino acid peptides (61 hexamers, 60 heptamers, and 59 octamers) that could be produced from the 66-amino acid sequence of ISP were generated. The program "Peptide Match" (Barker et al., 2001) was then used to identify exact matches with sequences contained in the PIR-NREF database.

[12] University of Nebraska, Food Allergy Research and Resource Program allergen database: http: http://www.allergenonline.com/asp/members/fastasearch.asp

Results

A search for similarity to sequences contained in the whole NCBI nr (non-redundant) as well as the PIR-NREF database, using BLAST 2.2.1 with default parameters, produced 61 matches. All but four of the matches in the NCBI database and all but six of those in the PIR-NREF database were with ice-structuring protein sequences. None of the non-ISP matches was with known allergens or related proteins. The FASTA 3.2 search in PIR-NREF also did not reveal any matches with known allergens, nor did a search of the FARRP allergen database, using the same program. A BLAST search against the "allergen database" produced a single hit against allergen Asp f6 from the fungal micro-organism *Aspergillus fumigatus* (Crameri et al., 1996). The match only occurred over a very short part of the sequences and was therefore not considered to be significant.

A BLAST search of the "allergen database," using parameters optimised to detect short alignments, produced 355 alignments at the most sensitive settings. However, the longest contiguous sequence in any alignment was only five amino acids, and all but one alignment possessed four or fewer contiguous amino acids.

The number of exact matches obtained with octamers, heptamers, and hexamers was 1674, 1771, and 2442, respectively. An immunologically significant sequence identity requires a match of at least eight contiguous identical amino acids, or 35% identity over eighty amino acid residues. No such sequence identity was detected for the ISP sequence to known allergens. All the matches obtained with the octamers and most of the exact matches of seven contiguous amino acids identified by the program "Peptide Match" in the PIR database were with sequences within other ISPs. Matches with sequences in six unrelated proteins were not considered to be structurally meaningful in terms of similarity with known allergens.

Conclusions

The amino acid structure of ISP is highly characteristic of fish ice-structuring proteins and shows little structural similarity with any other proteins. In particular, the sequence analysis performed by the applicant clearly showed no primary sequence similarity between ISP and the sequence of any known allergens, including fish allergens. Using an eight-amino acid reading frame, the only matches were with other ice structuring proteins. Although narrowing the reading frame to seven or six amino acids increased the number of matches with unrelated proteins, there were still no matches with known allergens.

4.4.2 Investigations in individuals with established allergy to fish

Given that ISP is derived from the ocean pout, evidence is required concerning the potential of this protein to elicit an allergic response in individuals who are known to be allergic to the consumption of fish species. Fish allergy occurs from sensitization to a codfish muscle protein, known as Gad c1, which is extremely stable to heat and acid (Bindslev-Jensen and Poulsen, 1997) and partially resistant to proteases (Metcalfe, 1997). The protein Gad c1, a parvalbumin that controls calcium flow across cell membranes, has a high degree of sequence homology with parvalbumins from other fish species, and individuals allergic to Gad c1 will react upon ingestion of other fish (Hansen et al., 1996, 1997).

No specific data exist on allergy to ocean pout, however allergy to a closely related species, eel, has been described (Bruijnzeel-Koomen et al., 1995). The applicant therefore sought to demonstrate that fish-allergic individuals, who may be expected to react to ocean pout flesh (containing Gad c1), do not react to ISP preparation.

As allergy to fish is relatively common in Scandinavian countries (Hansen and Bindslev-Jensen, 1992), allergy experts in Denmark were used to carry out studies with fish-allergic volunteers. In order to ensure that the study participants were not placed at any risk from the investigation, a step-wise process was used. Investigations started with serological studies on the sera of fish-allergic patients (Phase I). Once data were available to attest to the toxicological safety of the ISP preparation, the testing was extended to skin prick testing and ingestion (Phase II). An outline of the experimental procedure is presented in Table 3.

Table 3: Approach to the allergological assessment of ISP using human subjects with documented allergy to fish

Phase I (20 subjects): Tests: ▪ Confirmatory skin prick test (eel, eel pout, and ocean pout) ▪ MaxiSorp radioallergosorbent test (RAST) using ocean pout and ISP ▪ MaxiSorp inhibition RAST, using ISP and ocean pout to inhibit ocean pout RAST ▪ Basophil histamine release
Phase II (22 subjects, 17 from Phase I): Tests: ▪ Skin prick tests with ISP preparation and yeast fermentation supernatant. In four individuals with positive results, skin prick test with ISP type III HPLC 12 standard (pure). ▪ MaxiSorp RAST using ISP type III preparation and, for selected samples, yeast fermentation supernatant. ▪ Immunoblotting ▪ Basophil histamine release (selected samples)

Phase I

Samples of blood from twenty subjects with confirmed allergy to codfish were used in the *in vitro* experiments. All patients demonstrated positive skin prick test reactions to eel, eel pout, and ocean pout.

Since the binding between an allergen and IgE is central to eliciting an allergic response, the RadioAllergoSorbent Test (RAST) plays an important role in allergen determination and standardisation, as well as measurement of specific IgE levels. Background binding was determined with pooled sera from non-allergic donors.

None of the fish-allergic patients' sera demonstrated binding of IgE to the freeze-dried ISP preparation, as determined using this method (Maxisorp™ RAST) when protein concentrations up to 200 μg/ml were used.

The Applicant also conducted experiments to test for histamine release in basophils to ascertain the potential biological significance of any IgE-binding of ocean pout extract or freeze-dried ISP preparation. Immunoglobulin E binding *in vitro* can sometimes occur without translating into any biologically meaningful event, such as mast cell degranulation (Taylor and Hefle, 2001). A release of >15 ng histamine/ml blood was considered positive. None of the basophils from the fish-allergic volunteers released histamine when exposed *in vitro* to the freeze-dried ISP preparation, whereas the test was positive with eel, eel pout, and ocean pout extracts in all patients.

Phase II

Thirty subjects were asked to participate in this phase of the study to supply information about the allergenic potential of ISP preparation. Of twenty-five who accepted, 22 agreed to participate in the skin prick testing using solutions of sterile ISP preparation (at 5.0, 1.0, 0.1, and 0.01 mg ISP /ml), as well as solutions of the parent yeast strain fermentation supernatant (at 3.0, 0.87, 0.087, and 0.0087 mg yeast protein/ml). The results showed that four individuals reacted to both the ISP preparation and the yeast fermentation supernatant and these were further investigated using the ISP standard, at the same concentrations of ISP as in the preparation. They did not react to the pure ISP, revealing that they were sensitized to other proteins in the preparation.

The serum used for the RAST was the same as that used in Phase I, with the additional five patients recruited as part of Phase II. The results of these experiments are presented in Table 4. Eight of the serum samples were judged to demonstrate specific binding of IgE to the freeze-dried ISP preparation (represented in bold in Table 4). Significant binding was largely confined to the samples from individuals who had positive skin prick tests to the whole ISP preparation and yeast fermentation supernatant. The applicant states that, in the light of the skin prick test results, these findings almost certainly reflect either sensitization to the yeast protein component of the preparation or non-specific binding. As skin prick tests are considered more sensitive than RAST in detecting marginal sensitisation (Bernstein et al., 1994), a positive result in the RAST in the presence of a negative skin prick test is almost certainly a false positive. Sensitisation to *Saccharomyces cerevisiae* was also confirmed in three of the subjects by the commercial CAP RAST method (Pharmacia, Sweden).

Table 4: Skin prick test responses to ISP preparation and yeast fermentation supernatant, and RAST responses to ISP preparation (Phase II)

Subject	Skin prick tests responses (mm) [1]								RAST responses to ISP preparation (cpm)	
	ISP prep. (mg/ml)				Yeast fermentation supernatant (mg/ml)					
	5	*1*	*0.1*	*0.01*	*3*	*0.87*	*0.087*	*0.0087*	*Phase I* [2]	*Phase II*
1	Negative				Negative				32	34
2	Negative				Negative				26	32
4	Negative				Negative				46	63
7	Negative				Negative				81	33
9	Negative				Negative				375	46
11[3]	7.5	4.5	4	2.5	5	2.5	2	1	33	**1239**
12	Negative				Negative				83	**137**
13	Negative				Negative				279	**591**
15	Negative				Negative				42	35
16	Negative				Negative				162	**141**
17	Negative				Negative				47	**225**
18	Negative				Negative				52	41
19[3]	4	0	0	0	5	3	0	0	76	**243**
20	Negative				Negative				884	73
21	Negative				Negative				75	41
22	Negative				Negative				136	60
23	Negative				Negative				33	**140**
26	Negative				Negative				N.D.	29
27	4.5	3	0	0	4.5	2.5	0	0	N.D.	70
31[3]	7	6	6	4	6	4.5	0	0	N.D.	**1908**
32	Negative				Negative				N.D.	49
33	Negative				Negative				N.D.	95

1 Skin prick test values are the mean of largest perpendicular diameters, in mm.
2 RAST values obtained with the same sera in Phase I are reproduced for comparison.
3 Subjects determined to be sensitive to S. cerevisiae by CAP RAST method: Subject 11, Class 3; Subject 19, Class 4; Subject 31, Class 2.

Western blots were performed in order to investigate whether any of the sera from the fish allergic individuals would bind to proteins present in the ISP preparation. The positive control used in these experiments was purified ISP, detectable with anti-ISP monoclonal antibodies. The results of these immunoblotting experiments demonstrated that no binding of IgE from test sera to the ISP preparation could be detected.

In Phase II experiments, the basophil histamine release test was used only to investigate positive skin prick test results. Two of the four subjects who had a positive skin prick test showed a positive basophil histamine release when the ISP preparation was used as the antigen. Positive reactions in these two samples were also obtained when the yeast supernatant skin prick test reagent was used as the antigen. In contrast, no histamine release was observed when basophils from these subjects were exposed to pure ISP standard as the antigen, or when cord blood basophils were sensitised with their serum and subsequently exposed to pure ISP standard. The other two individuals with positive skin prick tests produced inconclusive results in the basophil histamine release test with ISP preparation and yeast fermentation supernatant.

Discussion

Studies on the allergenicity of ISP revealed the occurrence of several positive skin prick tests to yeast proteins, confirmed in three cases (out of four) by positive RAST. The Applicant claims that sensitisation to yeast as measured by specific IgE or skin prick testing is common, according to the fairly limited literature (Kortekangas-Savolainen et al., 1994; Savolainen et al., 1998, 2001). Clinical symptoms appear to be principally respiratory and cutaneous, while classical symptoms of food allergy are rare (Parker et al., 1990). Severe reactions to yeast following ingestion appear to be extremely rare, despite extensive exposure to common foods containing yeast. Most individuals allergic to yeast appear able to tolerate foods containing yeast (Kortekangas-Savolainen et al., 1994). The occurrence of reactions to the yeast protein component of the ISP preparation is therefore likely to be of little significance in terms of safety.

4.4.3 Additional assessment of potential allergenicity of ISP type III preparation

The applicant has undertaken additional investigations on the potential allergenicity of the ISP preparation based on research experiments that look at antibody production resulting from ingestion of proteins in man (reviewed by Husby, 2000). Studies such as these are additional to the standard assessment strategies for the assessment of possible allergenicity (FAO 2001, CAC 2003) and are included in this assessment as supplementary information only.

Normal, healthy adults were recruited for the study and allocated randomly to either the test group or the control group. Individuals (n=28) in the test group received ISP preparation providing 16.3 mg ISP in a flavoured drink daily for 5 days a week for 8 weeks. The selected amount corresponds to an estimate of ISP intake for 90th percentile consumers in USA. No correction was made for body weights. A control group (n=9) received the flavoured drink alone. Based on a pre-study questionnaire, seven members of the test group and four of the control group had an atopic predisposition. Blood samples (20 ml) were obtained immediately prior to the start of the test and at 4 and 6 weeks for the measurement of serum concentrations of IgG and IgE specific to ISP.

Results of IgG measurements

Specific IgG to ISP was measured by enzyme-linked immunosorbent assay (ELISA). Sera from 5 subjects displayed elevated IgG levels throughout the study, however as these values were elevated in the pre-test sera and did not increase as the study progressed, it was concluded that ingestion of the test material did not induce production of specific IgG antibody, nor did it stimulate any potential pre-existing response.

The binding of the sera showing the two strongest responses were further investigated in inhibition experiments with the test material (ISP preparation) or mannose (the sugar residue found on glycosylated ISP). Neither material produced any meaningful inhibition. These results therefore appear most likely to be due to a higher level of non-specific binding of IgG in some study participants.

Results of IgE measurements

Specific IgE to ISP preparation was measured using the MaxiSorp RAST system as used previously. The test revealed one weak specific IgE response, peaking at week 4, and possibly indicative of a physiological phenomenon. It was not accompanied by an IgG response, casting doubt on whether it was a true positive finding. Nonetheless, this response was further investigated using RAST inhibition, basophil histamine release, and immunoblots to identify the IgE binding components, as well as skin prick testing to confirm the result.

The test materials used were as described for the Phase I and Phase II allergenicity studies in the fish-allergic patients (see above). The subject showed a positive skin prick test to ISP preparation and yeast fermentation supernatant, but not to the more highly purified ISP standard. This subject also did not respond when skin prick tested with ocean pout extract. Immunoblots and basophil histamine release experiments were similarly negative.

As discussed previously, the skin prick test is generally considered more sensitive than *in vitro* methods in detecting low levels of sensitization (Bernstein et al., 1994), implying that a positive response in the RAST in the presence of a negative skin prick test is more likely to be a false positive. However, an additional MaxiSorp RAST using yeast fermentation supernatant as a solid phase was positive.

Additional screening for common allergens in this individual indicated they are sensitised to a multiplicity of common allergens. The applicant claims that given the negative results in the other investigations, including particularly the skin prick tests, together with the very marginal response to ISP preparation by this subject, this RAST inhibition result should be considered a false positive.

The results of this study do not indicate that ISP possesses any significant immunogenicity.

4.4.4 In vitro *digestibility studies*

In general, ingested proteins that are stable to gastric juices are more likely to come in contact with the intestinal mucosa where absorption and recognition by the immune system could occur, increasing the likelihood that they could be allergenic. Conversely, ingested proteins that are unstable in the acidic conditions of the digestive system are less likely to reach the intestine and therefore are considered less likely to elicit an allergic response. For example, the major fish allergen, Gad c1 (and analogs), is heat-stable, acid-stable, and resistant to proteolytic degradation.

The stability of ISP and its glycosylated form (mannose-conjugated ISP) was determined by incubating each with the enzyme pepsin and monitoring proteolytic degradation by taking samples for analysis at various time points. As controls, a protein susceptible to digestion (bovine serum albumin, BSA) and a protein resistant to digestion (bovine β-lactoglobulin, BLG), were also tested in this simulated gastric system.

Test forms of ISP were subjected to enzymatic degradation at different pH by pepsin (from porcine stomach) at 37˚C for defined intervals over a period of 120 minutes. The breakdown of ISP was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, as well as by reverse phase HPLC.

Gel filtration chromatography (GFC) was used to monitor hydrolysis of the glyco-ISP, while matrix assisted laser desorption ionization time of flight (MALDI-ToF) mass spectrometry (Chapman, 1996) was used in addition to densitometric analysis of SDS-PAGE gels to identify and quantify fragments generated by pepsin hydrolysis of ISP.

SDS-PAGE analysis

At pH 1.5, visible degradation of ISP had occurred by 15 minutes and by 60 minutes appeared to be complete. Densitometric analysis showed that the half-life of ISP, determined from several experiments, was approximately 4 minutes under these conditions. At pH 2.5 and 3.5, the test material was still detectable at 60 minutes and 120 minutes respectively. The corresponding half-lives were approximately 13 minutes at pH 2.5 and 28 minutes at pH 3.5. The control proteins, bovine serum albumin and β-lactoglobulin, behaved as expected – BSA was not detectable after 15 seconds, while BLG showed a half-life in excess of 2 hr.

Other analyses

The breakdown of ISP was also quantified by HPLC, a more reproducible method than scanned densitometric readings. The results were consistent with the SDS-PAGE analysis showing half-lives of approximately 6, 9 and 22 minutes at pH 1.5, 2.5 and 3.5 respectively.

As the glyco-conjugated proteins show poor resolution on SDS-PAGE gels, enzymatic breakdown by pepsin could not readily be detected by that method. The Applicant reports (data not provided) that GFC was used to investigate the digestive fate of glycosylated ISP by pepsin, and showed that it was readily broken down.

Use of bioinformatics

Bioinformatic tools are available to predict potential protease cleavage sites in a given protein sequence, for example PeptideCutter, 2002.

As well as predicting cleavage products from the preferred cleavage sites of pepsin, PeptideCutter was used to show that trypsin and chymotrypsin would also hydrolyse ISP, providing greater assurance that the protein will be extensively degraded to small peptides in the gastrointestinal tract.

4.4.5 Summary and conclusion of potential allergenicity assessment

The applicant has conducted a range of studies aimed at determining the likely allergenic potential of ISP type III derived from commercial yeast cultures. Each study on its own does not provide conclusive information concerning potential allergenicity, but when the results of all analyses are considered together as a whole, the weight of evidence indicates that ISP is unlikely to be allergenic to humans.

This conclusion is based on data and observations presented in the application, and summarised as follows:

- no history of allergenicity from human consumption of ocean pout;
- no structural indications for allergenicity;
- no similarity to known allergens;

- susceptibility to hydrolysis by pepsin;
- lack of binding of ISP to IgE;
- lack of histamine release from basophils of fish-allergic individuals in the presence of ISP;
- absence of skin prick test reactivity to ISP itself; and
- absence of immunogenicity, as measured by the lack of a definitive ISP-related antibody response in a two-month ingestion study.

5 RISK CHARACTERISATION

5.1 Applicant estimates of dietary exposures in consumers

The applicant advises that the typical level of ISP in consumer products will be 0.005% (50 ppm), with the maximum concentration for some uses of 0.01% (100 ppm). Using consumption data applicable to Australian consumers, the applicant has estimated the daily intake for the group that would have the highest exposure (16 to 18 year old males), at the 95th percentile of consumption on a single day, at 0.52 mg ISP /kg body weight. This conservatively assumes a use level of 100 ppm, that the entire frozen milk products category contains ISP, and that the body weight is 60 kg.

5.2 Safety assessment of ISP

Commercial ISP type III preparation is a solution of proteins – ISP (active component), glyco-ISP (inactive component), proteins and peptides from bakers yeast and sugars, acids, and salts commonly found in food. The safety assessment has focused primarily on the potential toxicity and allergenicity of the ISP protein itself. In evaluating these safety parameters, consideration was given to the history of its presence in the human diet primarily from consumption of fish, and the body of scientific evidence to show that ISP is not toxic and is unlikely to be allergenic. The highest dose that could be tested in the 13-week rat toxicity study, 580 mg ISP /kg body weight/day by gavage, showed no adverse effects. The Applicant refers to this level of exposure as the no-observed-adverse-effect-level (NOAEL).

Using the NOAEL derived in the rat study and a theoretical safety factor, the applicant has determined an acceptable daily intake (ADI) for ISP, however expression of an ADI is not considered of primary relevance to this safety assessment for several reasons.

Firstly, according to JECFA guidelines, an ADI is based on toxicological information from animal studies in which a dose-response relationship has been established, allowing determination of a NOAEL. To achieve this, the highest doses of the test substance administered to the animals should elicit some detectable effect. There were no adverse effects detectable at the highest dose administered in the rat study using ISP preparation, and therefore the NOAEL has been inferred from these results.

Secondly, the ISP preparation is a protein-rich mixture that has been shown to be readily degraded in the gastrointestinal system, as expected of normal dietary protein.

Finally, given the available data on ISP (chemical, biochemical, toxicological and allergenicity), the intended low level of use, and its acceptable background in food, its use as a processing aid in frozen products such as ice cream do not raise any safety concerns.

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Attachment 5

Dietary exposure assessment report

An application was received by FSANZ from Unilever Australia Limited requesting amendment of Standard 1.3.3 – Processing Aids of the *Australia New Zealand Food Standards Code* (the Code) to include the use of Ice Structuring Protein Type III HPLC 12 (ISP) as a processing aid for the preparation of ice cream and edible ices. Edible ices include frozen yoghurts and frozen fruit and/or vegetable juices and drinks.

A dietary exposure assessment was deemed necessary in order to determine the estimated dietary exposure to ISP for the Australian and New Zealand populations if ISP were added to ice creams and edible ice products.

Summary

A dietary exposure assessment was undertaken to estimate dietary exposure to ISP for the Australian and New Zealand populations. The population sub-groups examined were the whole population (2 years and above for Australia; 15 years and above for New Zealand), toddlers (2-4 years for Australia), primary school aged children (5-12 years for Australia), and teenagers (13-19 years for Australia; 15-19 years for New Zealand). Food consumption data based on the 1995 National Nutrition Survey (NNS) and 1997 New Zealand NNS were used to estimate ISP dietary exposure.

The estimated mean dietary exposures for consumers of ISP for Australia were:

- 12 mg/day for the whole population aged 2 years and above;
- 8 mg/day for toddlers aged 2-4 years;
- 13 mg/day for primary school aged children aged 5-12 years; and
- 17 mg/day for teenagers aged 13-19 years.

The estimated mean dietary exposures for consumers of ISP for New Zealand were:

- 10 mg/day for the whole population aged 15 years and above; and
- 15 mg/day for teenagers aged 15-19 years.

The 95th percentile dietary exposures for consumers of ISP for Australia were estimated as:

- 33 mg/day for the whole population aged 2 years and above;
- 23 mg/day for toddlers;
- 34 mg/day for primary school children aged 5-12 years; and
- 49 mg/day for teenagers aged 13-19 years.

The 95th percentile dietary exposures for consumers of ISP for New Zealand were estimated as:

- 26 mg/day for the whole population aged 15 years and above; and
- 38 mg/day for teenagers aged 15-19 years.

Of the population groups assessed, teenagers from both countries (aged 13-19 years for Australia and 15-19 years for New Zealand) had the highest estimated dietary exposures to ISP (in mg/day). When estimated mean dietary exposures are considered in mg/kg bw/day, Australian toddlers aged 2-4 years have the highest dietary exposures to ISP.

Background

ISP's are naturally occurring proteins and peptides that are found in a variety of living organisms such as fish, plants, insects, fungi and bacteria, which protect them from damage in very cold conditions that would normally cause organisms to freeze. Since a number of these organisms are consumed as food, ISP's are naturally a component of the human diet. ISPs do not actually prevent freezing but influence the growth and structure of ice crystals. They inhibit growth of ice crystals and modify the ice structure and hence its physical properties. Properties relevant for frozen ice products include thermal stability, hardness, creaminess and flavour delivery.

The ISP of this Application was originally isolated from ocean pout, a cold water fish found off the North American coast, which is consumed as part of the human diet. For commercial use, a synthetic copy of the gene responsible for producing ISP has been incorporated into yeast using standard genetic modification techniques. ISP is then produced by batch fermentations of this yeast. No actual fish derived protein is included in the ISP of this application.

The US FDA (Food and Drug Administration) has deemed this ISP as generally recognised as safe (GRAS). Commercial ice creams and edible ices incorporating ISP have been sold in USA since June 2003. ISP is also approved for use in Hong Kong, Mexico, the Philippines and Indonesia.

Dietary exposure assessment provided by the Applicant

The Application contains dietary exposure information, with the Applicant stating that there are no anticipated dietary implications from consumption of ISP as used in this Application. The Application also states that the use of ISP in the Applicant's products is not expected to significantly change the population consumption of ice creams and edible ices, but rather the choice of products.

The dietary consumption data in the application was taken from the publication *National Nutrition Survey Foods Eaten in Australia 1995* and shows that males aged 16-18 years have the highest mean consumption of ice cream (which includes other products such as thick shakes and frozen yoghurt). The mean ice cream consumption for this group is 224.4 g/day, with 95% of all consumers having ice cream consumption of between 133 and 316 g/day (as calculated by mean ± 2 standard errors).

The maximum amount of ISP in ice cream products is stated by the Applicant to be 0.01%. However the Applicant states this is conservative since, for many products, usage will be 0.005%.

The Applicant provided an Acceptable Daily Intake for ISP of 5.8 mg ISP/kg bw/day. However, neither the Joint FAO/WHO Expert Committee on Food Additives (JECFA) nor FSANZ have set an ADI for ISP. Consequently, the estimated dietary exposures to ISP have not been compared to a reference health standard such as an ADI.

The Applicant has estimated, using the highest ice cream consumption figure for males aged 16-18 years, the ISP concentration in ice creams of 0.01% and a body weight of 60 kg, that the dietary exposure to ISP is 0.52 mg/kg body weight/day. The estimated dietary exposure figure is approximately 11 times lower than the ADI proposed by the Applicant (of 5.8 mg/kg bw/day).

The dietary exposure assessment provided by the Applicant was not comprehensive enough to allow FSANZ to determine a firm conclusion about the likely exposure to ISP for the following reasons:

- the Applicant focussed on male teenagers aged 16-18 years only;
- the Applicant provided dietary exposure information for Australia only; and
- the Applicant only provided estimated mean exposure.

For the estimated dietary exposure assessment to be comprehensive, modelling needed to be conducted for the whole population and for vulnerable sub-groups (females and males) in the Australian and New Zealand populations. High consumer (95^{th} percentile) exposure also needed to be assessed. Therefore, FSANZ conducted a dietary exposure assessment to supplement that provided by the Applicant.

Dietary modelling

The dietary exposure assessment was conducted using dietary modelling techniques that combine food consumption data with food chemical concentration data to estimate the exposure to the food chemical from the diet. The dietary exposure assessment was conducted using FSANZ's dietary modelling computer program, DIAMOND.

Dietary exposure = food chemical concentration x food consumption

The exposure was estimated by combining usual patterns of food consumption, as derived from national nutrition survey (NNS) data, with proposed levels of use of ISP in foods.

Dietary survey data

DIAMOND contains dietary survey data for both Australia and New Zealand; the 1995 NNS from Australia that surveyed 13 858 people aged 2 years and above, and the 1997 New Zealand NNS that surveyed 4 636 people aged 15 years and above. Both of the NNS's used a 24-hour food recall methodology.

Additional food consumption data or other relevant data

No further information was required or identified for the purpose of refining the dietary exposure estimates for this application.

Population groups assessed

The dietary exposure assessment was conducted for both Australian and New Zealand populations. An assessment was conducted for the whole population (aged 2 years and above for Australia; 15 years and above for New Zealand), toddlers (2-4 years for Australia), primary school aged children (5-12 years for Australia), and teenagers (13-19 years for Australia; 15-19 years for New Zealand). Dietary exposure assessments were conducted for the whole population as a proxy for lifetime exposure. Children were examined separately because they generally have higher exposures due to their smaller body weight, and they consume more food per kilogram of body weight compared to adults. They also consume a significant proportion of the food types that can contain ISP, such as ice cream and thick shakes. For children aged 5-12 years, 41% of those surveyed in the 1995 NNS consumed ice cream or edible ice products on the day of the survey. This was the highest proportion of consumers to respondents for all of the population groups examined. For further details see Table A1.1 of Appendix 1. It is important to note that, while children aged 2-4 years, 5-12 years, 13-19 years in Australia and 15-19 years in New Zealand have been assessed as separate groups, these groups have also been included in the whole population's dietary exposure assessment.

The dietary exposure assessment for toddlers and school children was only conducted for the Australian population, as the New Zealand NNS does not include consumption data for people aged less than 15 years.

ISP concentration levels

The levels of ISP in foods that were used in the dietary exposure assessment were derived from the Application. Information provided by the Applicant stated the typical level of ISP in food products would be 0.005%, with a maximum concentration of 0.01%. Where the Applicant provided a range of possible concentrations, the highest level in the range was used for calculating the estimated exposures in order to assume a worst-case scenario. Therefore, for this dietary exposure assessment a concentration of 0.01% was used. Since the Applicant provided concentrations of ISP in foods as a percentage, it was converted to mg/kg concentrations[13] for use in the DIAMOND program. The foods and proposed levels of use are shown below in Table 1.

Concentrations of ISP were assigned to food groups using DIAMOND food classification codes. These codes are based on the Australian New Zealand Food Classification System (ANZFCS) used in Standard 1.3.1 - Food Additives (for example, classification code 3 represents "Ice cream and edible ices"). The foods proposed by the Applicant to contain ISP, were matched to the most appropriate DIAMOND code(s) for dietary modelling purposes.

Table 1: Proposed use of ISP in foods and levels of use

DIAMOND Code	Food Name	ISP concentration used in the dietary modelling (mg/kg)
1.2.2.3	Frozen fermented & rennet milk products	100
3	Ice cream and edible ices	100

[13] 0.01% = 100 mg/kg

How were the estimated dietary exposures calculated?

The DIAMOND program allows ISP concentrations to be assigned to food groups. Individual foods reported as consumed in the NNS were assigned to the relevant DIAMOND codes in Table 1 for Australia and New Zealand. All foods in each DIAMOND code were then assigned the ISP concentration specified for the group.

Each individual's dietary exposure to ISP was calculated using his or her individual food records from the NNS. The DIAMOND program multiplies the specified concentration of ISP by the amount of each food that an individual consumed from that group in order to estimate the exposure to ISP from each food. Once this has been completed for all of the foods specified to contain ISP, the total amount of ISP consumed from all foods is summed for each individual. Population statistics such as mean, and 95th percentile exposures, are then derived from the individuals' ranked exposures.

Where estimated dietary exposures are expressed per kilogram of body weight, each individuals' total dietary exposure is divided by their own body weight, the results ranked, and population statistics derived. A small number of NNS respondents did not provide a body weight. These respondents are not included in calculations of estimated dietary intakes that are expressed per kilogram of body weight.

Food consumption amounts for each individual take into account where each food in a classification code is consumed alone and as an ingredient in mixed foods. For example, ice cream eaten on its own or ice cream used to make a thick shake, are all included in the consumption of ice cream. In DIAMOND, all mixed foods in classification codes 20 and 21 have a recipe. Recipes are used to break down mixed foods into component ingredients that are in classification codes 1-14. The data for consumption of the ingredients from the recipe are then used in the exposure assessment and multiplied by ISP concentrations for each of the ingredients.

Dietary exposure assessments usually compare the estimated dietary exposure to a food chemical to a reference health standard, such as an Acceptable Daily Intake (ADI). The Applicant provided an Acceptable Daily Intake for ISP of 5.8 mg ISP/kg bw/day. However, neither the Joint FAO/WHO Expert Committee on Food Additives (JECFA) nor FSANZ have set an ADI for ISP. Consequently, the estimated dietary exposures to ISP have not been compared to a reference health standard such as an ADI and the dietary exposures are simply expressed in mg/day and mg/kg bw/day only.

Assumptions in the dietary modelling

The aim of the dietary exposure assessment was to make as realistic an estimate of dietary exposure as possible. However, where significant uncertainties in the data existed, conservative assumptions were generally used to ensure that the dietary exposure assessment did not underestimate exposure.

Assumptions made in the dietary modelling include:

- where a permission is given to a food classification, all foods in that group contain ISP;

- all the foods within the group contain ISP at the proposed levels specified in Table 1. Unless otherwise stated, the maximum concentration of ISP in each food category has been used;
- consumption of foods as recorded in the NNS's represent current food consumption patterns;
- consumers always select the products containing ISP;
- consumers do not alter their food consumption habits to substitute non-ISP containing products with ISP containing products;
- consumers do not increase their consumption of foods/food groups upon foods/food groups containing ISP becoming available;
- all ISP present in food is absorbed by the body;
- naturally occurring sources of ISP have not been included in the dietary exposure assessment;
- where a food was not included in the exposure assessment, it was assumed to contain a zero concentration of ISP; and
- where a food has a specified ISP concentration, this concentration is carried over to mixed foods where the food has been used as an ingredient e.g. ice cream used in thick shakes.

These assumptions are likely to lead to a conservative estimate for ISP dietary exposure.

Limitations of the dietary modelling

A limitation of estimating dietary exposure over a period of time associated with the dietary modelling is that only 24-hour dietary survey data were available, and these tend to over-estimate habitual food consumption amounts for high consumers. Therefore, predicted high percentile exposures are likely to be higher than actual high percentile exposures over a lifetime.

Food consumption amounts for occasionally consumed foods based on 24 hour food consumption data would be higher than average daily food consumption amounts for those foods based on a longer period of time.

While the results of NNS's can be used to describe the usual intake of groups of people, they cannot be used to describe the usual intake of an individual (Rutishauser, 2000). In particular, they cannot be used to predict how consumers will change their eating patterns as a result of an external influence such as the availability of a new type of food.

FSANZ does not apply statistical population weights to each individual in the NNS's in order to make the data representative of the population. This prevents distortion of actual food consumption amounts that may result in an unrealistic exposure estimate. Maori and Pacific Islanders were over-sampled in the 1997 New Zealand National Nutrition Survey so that statistically valid assessments could be made for these population groups. As a result, there may be bias towards these population groups in the dietary exposure assessment because population weights were not used.

Results

Estimated dietary exposures to ISP

The estimated mean and 95th percentile dietary exposures for consumers of ISP in Australia and New Zealand are shown in Figure 1 (mg/kg bw/day) and Figure 2 (mg/day).

Estimated ISP dietary exposures are presented for consumers of ISP only and not for all respondents (every person in the population group). For details on the number of respondents and consumers in each population group assessed, see Table A1.1 in Appendix 1.

The estimated mean dietary exposures for consumers of ISP in Australia were:

- 12 mg/day (0.2 mg/kg bw/day) for the whole population aged 2 years and above;
- 8 mg/day (0.5 mg/kg bw/day) for toddlers aged 2-4 years;
- 13 mg/day (0.4 mg/kg bw/day) for primary school children aged 5-12 years; and
- 17 mg/day (0.3 mg/kg bw/day) for teenagers aged 13-19 years.

The estimated mean dietary exposures for consumers of ISP in New Zealand were:

- 10 mg/day (0.1 mg/kg bw/day) for the whole population aged 15 years and above; and
- 15 mg/day (0.2 mg/kg bw/day) for teenagers aged 15-19 years.

The estimated 95th percentile exposures for consumers of ISP in Australia were:

- 33 mg/day (0.7 mg/kg bw/day) for the whole population aged 2 years and above;
- 23 mg/day (1.3 mg/kg bw/day) for toddlers aged 2-4 years;
- 34 mg/day (1.2 mg/kg bw/day) for primary school children aged 5-12 years; and
- 49 mg/day (0.9 mg/kg bw/day) for teenagers aged 13-19 years.

The estimated 95th percentile exposures for consumers of ISP in New Zealand were:

- 26 mg/day (0.4 mg/kg bw/day) for the whole population aged 15 years and above; and
- 38 mg/day (0.6 mg/kg bw/day) for teenagers aged 15-19 years.

Overall, of the population groups assessed, teenagers had the highest estimated mean dietary ISP exposure (in mg/day) for Australia and New Zealand. This was followed by primary school aged children (5-12 years in Australia), the whole Australian population (2+ years), the whole New Zealand population (15+ years), and toddlers (2-4 years in Australia). When estimated mean dietary exposures are considered in mg/kg bw/day, toddlers aged 2-4 years have the highest dietary exposures, followed by primary school aged children.

Figure 1. Estimated mean and 95th percentile dietary exposures for consumers of ISP (mg/kg bw/day) for various Australian and New Zealand population groups.

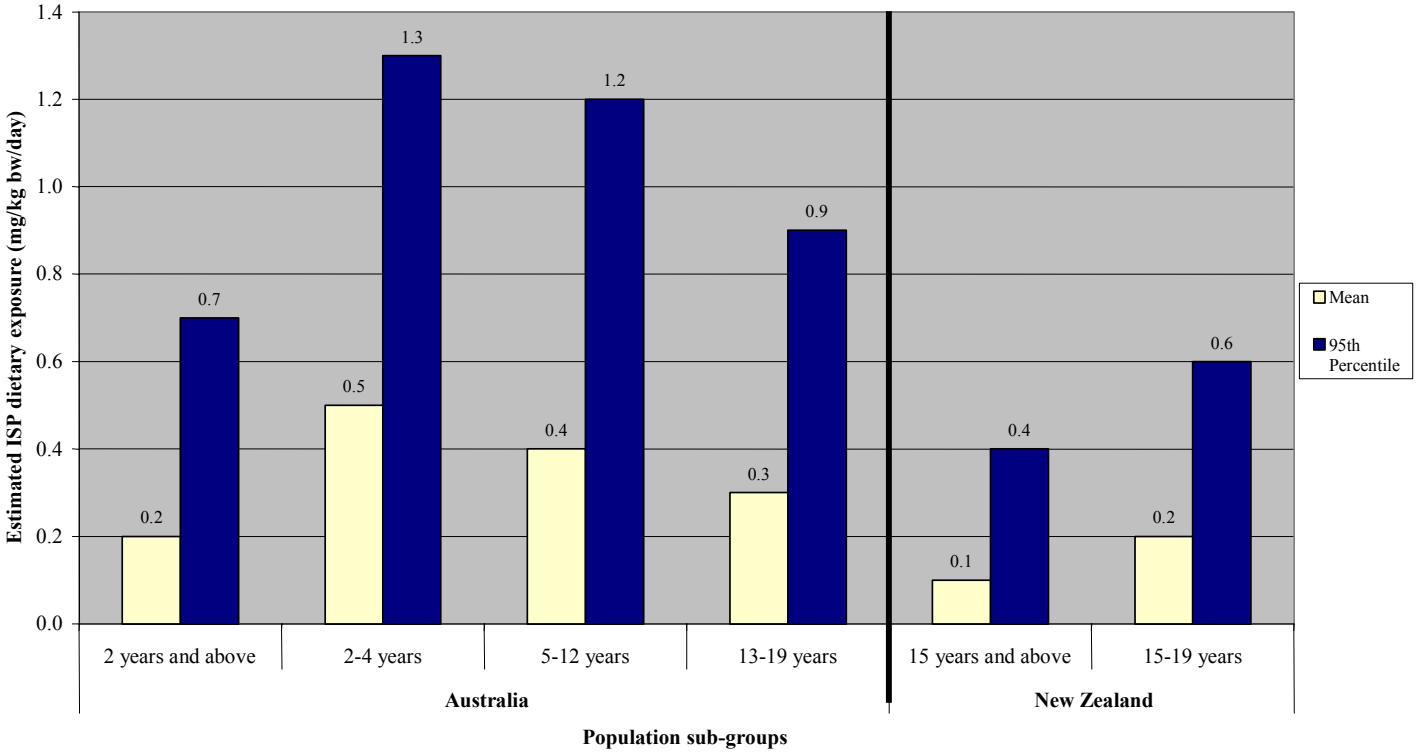
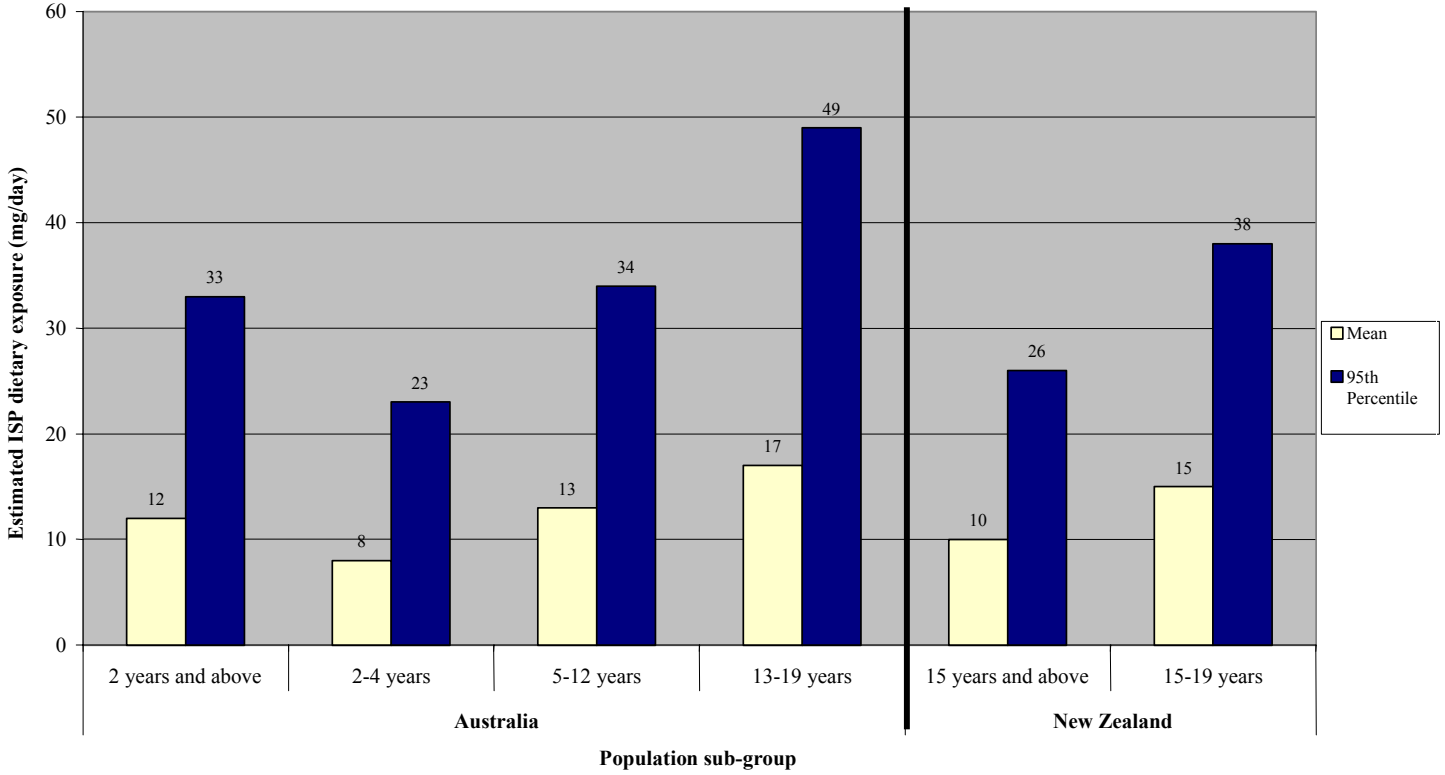


Figure 2. Estimated mean and 95th percentile dietary exposures for consumers of ISP (mg/day) for various Australian and New Zealand population groups.



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Appendix 1

Complete information on dietary exposure assessment results

Table A1.1: Estimated dietary exposures to ISP for Australia and New Zealand

Country	Population group	No. of Respondents #	No. of Consumers of ISP	Consumers* as a % of total respondents#	Mean Consumer Exposure		95th percentile consumer exposure	
					mg/day	mg/kg bw/day	mg/day	mg/kg bw/day
Australia	2+ years	13858	2992	22	12	0.2	33	0.7
	2-4 years	583	183	31	8	0.5	23	1.3
	5-12 years	1496	616	41	13	0.4	34	1.2
	13-19 years	1063	333	31	17	0.3	49	0.9
New Zealand	15+ years	4636	694	15	10	0.1	26	0.4
	15-19 years	297	58	20	15	0.2	38	0.6

* Consumers only – This only includes the people who have consumed a food that contains ISP.
# Respondents include all members of the survey population whether or not they consumed a food that contains ISP.