

**ICE STRUCTURING PROTEIN AS A PROCESSING AID  
IN ICE CREAM AND EDIBLE ICES**

**A SAFETY ASSESSMENT**

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**FOOD STANDARDS AUSTRALIA NEW ZEALAND**

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## **SUMMARY AND CONCLUSIONS**

Ice Structuring Protein type III HPLC 12 (ISP), derived from a northern hemisphere fish species, has been assessed for 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. ISP type III HPLC 12 is to be used as a processing aid in the manufacture of products such as ice cream and water ices.

As natural fish sources are limited, commercial quantities of ISP are produced 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 factors 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 in 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, which 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.

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

### **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, ISP type III 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**

A number of studies were provided in relation to the potential toxicity of ISP and to determine whether ISP is likely to be allergenic in humans.

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 provided, 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 GM baker's yeast can be considered safe for human consumption.

## **INTRODUCTION**

A safety assessment has been conducted on Ice Structuring Protein Type III HPLC 12 (ISP) to be used 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.

### **History of use**

In order to generate commercial quantities of frozen dessert products, hundreds of kilograms of ISP would be required each year. 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.

### **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.

## **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.

## **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 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 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 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.

## **DESCRIPTION OF THE GENETIC MODIFICATION**

### **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)

### *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.

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 of 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 bacterial DNA.

### **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.

#### *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.

## CHARACTERISATION OF THE ISP PROTEIN

### 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 anti-parallel sheets and one double-stranded anti-parallel 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.

### *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.

### 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.

This paper presents data from a set of *in vitro* and *in vivo* genotoxicity assays (bacterial mutation, chromosome aberration, mammalian cell gene mutation and rat bone marrow micronucleus) and a 3-month repeat-dose gavage study in the rat using high levels of ISP type III HPLC 12 preparation produced by recombinant baker's yeast. No evidence was seen of a genotoxic potential (using levels accepted as limit concentrations for the assays used) or notable subchronic toxicity following oral administration for 3 months in the rat at up to 580 mg ISP type III HPLC 12/kg/day, the highest dose tested (which was considered to be a NOAEL).

### *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.

A sub-chronic (13 weeks) oral toxicity study in rats to support the safety of ISP was considered. The study was performed at Covance Laboratories (UK) according to

FDA guidelines<sup>1</sup> and OECD guidelines<sup>2</sup> for repeated dose oral toxicity studies in rodents, and in compliance with international regulations for Good Laboratory Practice<sup>3</sup>.

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 preparation.

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

<b>Group</b>	<b>ISP type III HPLC 12</b>	<b>Total ISP</b>	<b>Total Solids</b>
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, ophthalmoscopic 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

<sup>1</sup> 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.

<sup>2</sup> 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.

<sup>3</sup> Series on Principles of Good Laboratory Practice and Compliance Monitoring, OECD, 1998b.

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.
- Ophthalmoscopy: 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, lumbar 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.

### *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.

#### *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 published studies in humans evaluating the long-term safety of the ISP preparation from yeast, details of a randomised, placebo-controlled clinical trial<sup>4</sup> to evaluate any possible adverse effects of a single ingestion of ISP was made available to us. 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.

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 week 1, 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

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<sup>4</sup> 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.

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.

### **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 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.

Recent scientific research suggests that food protein allergens belong to a limited number of protein families<sup>5</sup> and the ISP does not appear to belong to any of these protein families that are allergens.

**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

### *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.

<sup>5</sup> Breitender, H. and Mills, ENC (2005) Molecular properties of food allergens. *J. Allergy and Clinical Immunology*, **115(1)**:14-23.

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 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<sup>6</sup>.
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.

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<sup>6</sup> University of Nebraska, Food Allergy Research and Resource Program allergen database: <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* (Cramer 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 ice structuring proteins. 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 on the protein 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.

## *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 the fish muscle protein, parvalbumin, which is extremely stable to heat and acid (Bindslev-Jensen and Poulsen, 1997) and partially resistant to proteases (Metcalfe, 1997). Parvalbumin, which controls calcium flow across cell membranes, has a high degree of sequence homology in a number of fish species. Individuals allergic to one type of fish may also react to other types (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). Therefore, it was important to demonstrate that fish-allergic individuals, who may be expected to react to ocean pout flesh 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.

<p><b>Phase I (20 subjects):</b></p> <p>Tests:</p> <ul style="list-style-type: none"> <li>▪ Confirmatory skin prick test (eel, eel pout, and ocean pout)</li> <li>▪ MaxiSorp radioallergosorbent test (RAST) using ocean pout and ISP</li> <li>▪ MaxiSorp inhibition RAST, using ISP and ocean pout to inhibit ocean pout RAST</li> <li>▪ Basophil histamine release</li> </ul>
<p><b>Phase II (22 subjects, 17 from Phase I):</b></p> <p>Tests:</p> <ul style="list-style-type: none"> <li>▪ 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).</li> <li>▪ MaxiSorp RAST using ISP type III preparation and, for selected samples, yeast fermentation supernatant.</li> <li>▪ Immunoblotting</li> <li>▪ Basophil histamine release (selected samples)</li> </ul>

### 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.

Experiments were also conducted 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. 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) <sup>1</sup>		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	0.008 7	Phase I <sup>2</sup>	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 <sup>3</sup>	7.5	4.5	4	2.5	5	2.5	2	1	33	<b>1239</b>
12	Negative				Negative				83	<b>137</b>
13	Negative				Negative				279	<b>591</b>
15	Negative				Negative				42	35
16	Negative				Negative				162	<b>141</b>
17	Negative				Negative				47	<b>225</b>
18	Negative				Negative				52	41
19 <sup>3</sup>	4	0	0	0	5	3	0	0	76	<b>243</b>
20	Negative				Negative				884	73
21	Negative				Negative				75	41
22	Negative				Negative				136	60
23	Negative				Negative				33	<b>140</b>
26	Negative				Negative				N.D.	29
27	4.5	3	0	0	4.5	2.5	0	0	N.D.	70
31 <sup>3</sup>	7	6	6	4	6	4.5	0	0	N.D.	<b>1908</b>
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. 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.

#### *Additional assessment of potential allergenicity of ISP preparation*

Additional investigations were undertaken 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 90<sup>th</sup> 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. Given the negative results in the other investigations, particularly the skin prick tests, together with the very marginal response to ISP preparation by this subject, this RAST inhibition result was considered a false positive.

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

#### *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  $\beta$ -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  $\beta$ -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.

### 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 would be extensively degraded to small peptides in the gastrointestinal tract.

### *Summary and conclusion of potential allergenicity assessment*

A range of studies were considered to determine the likely allergenic potential of ISP 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 in humans.

This conclusion is based on ISP related data and observations that can be summarised as follows:

- 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.

## **RISK CHARACTERISATION**

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 very 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.

Based on the dietary exposure assessment the 95<sup>th</sup> percentile exposure for the highest consumers (Australian toddlers aged 2-4 years, being 1.3 mg/kg bw/day) is substantially below the highest dose level tested in animals (being 580 mg/kg bw/day), which showed no adverse effects.

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

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## DIETARY EXPOSURE ASSESSMENT REPORT

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 including frozen yoghurts and frozen fruit and/or vegetable juices and drinks.

### 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 95<sup>th</sup> 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 aged 2-4 years;
- 34 mg/day for primary school children aged 5-12 years; and
- 49 mg/day for teenagers aged 13-19 years.

The 95<sup>th</sup> 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 bodyweight (bw)/day, Australian toddlers aged 2-4 years have the highest dietary exposures to ISP.

## 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.

$$\text{Dietary exposure} = \text{food chemical concentration} \times \text{food consumption}$$

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. The Application did not contain additional consumption data to refine the dietary modelling.

### *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. Available information suggests that the typical level of ISP in food products would be 0.005%, with a maximum concentration of 0.01%. 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. Data on concentrations of ISP in foods as a percentage was converted to mg/kg concentrations<sup>7</sup> 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 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**

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

### *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 95<sup>th</sup> percentile exposures, are then derived from the individuals’ ranked exposures.

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<sup>7</sup> 0.01% = 100 mg/kg

Where estimated dietary exposures are expressed per kilogram of body weight, each individual's 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). 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 95<sup>th</sup> 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 95<sup>th</sup> percentile exposures for consumers of ISP in Australia were:

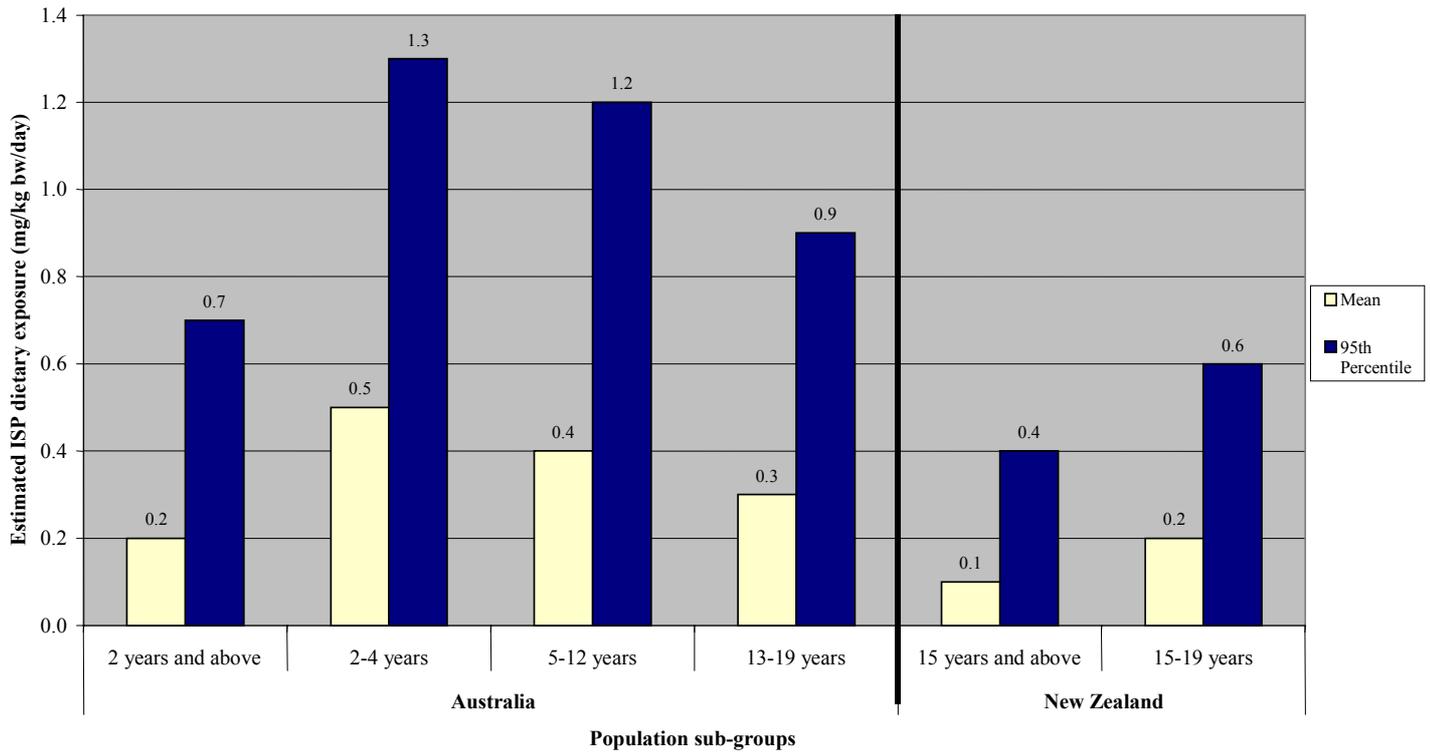
- 33 mg/day (0.7 mg/kg bw/day) for the whole population aged 2 years and above;
- 23 mg/day (1.3 mg/kg bw/day) for toddlers aged 2-4 years;
- 34 mg/day (1.2 mg/kg bw/day) for primary school children aged 5-12 years; and
- 49 mg/day (0.9 mg/kg bw/day) for teenagers aged 13-19 years.

The estimated 95<sup>th</sup> 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.

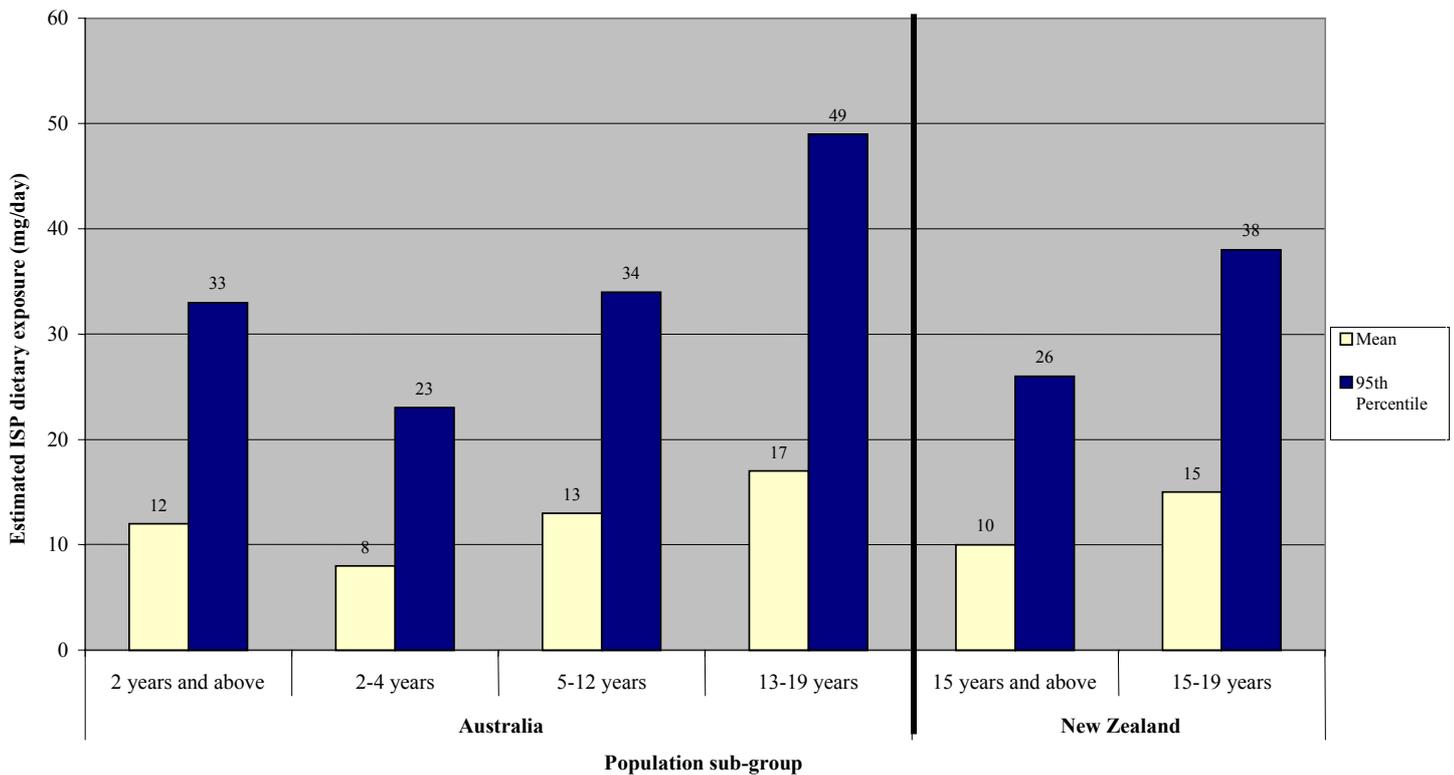
Overall, of the population groups assessed, teenagers had the highest estimated mean dietary ISP exposure (in mg/day) for Australia and New Zealand. This was followed by primary school aged children (5-12 years in Australia), the whole Australian population (2+ years), the whole New Zealand population (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 95<sup>th</sup> percentile dietary exposures for consumers of ISP (mg/kg bw/day) for various Australian and New Zealand population**



groups.

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



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