

## **Safety of ‘Ice Structuring Protein (ISP)’<sup>1</sup>**

### **Scientific Opinion of the Panel on Dietetic Products, Nutrition and Allergies and of the Panel on Genetically Modified Organisms**

(Question No EFSA-Q-2008-073)

**Adopted on 02 July 2008 by the GMO Panel  
and on 09 July 2008 by the NDA Panel**

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#### **SUMMARY**

Following a request from the European Commission, the Panel on Dietetic Products, Nutrition and Allergies and the Panel on Genetically Modified Organisms were asked to deliver a scientific opinion on the safety of ‘Ice Structuring Protein (ISP)’ as food ingredient.

Ice structuring proteins (ISPs) are widely distributed in nature, for example in cold water fish, vegetables, grains, lichens and bacteria. ISPs bind to ice and help organisms cope with very cold environments by both lowering the temperature at which ice crystals form and by modifying the size and shape of the ice crystals so that the ice is less damaging to tissues. The applicant wishes to take advantage of these properties by adding an ISP to edible ice at a level not exceeding 0.01 % by weight.

ISP type III was originally isolated from the ocean pout (*Macrozoarces americanus*) a cold-water fish found off the northeast coast of North America. There were 12 isoforms of the protein that could be separated by HPLC and ISP type III HPLC 12, a protein of 66 amino acids was selected for commercial application. The production system is based on the fermentation

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fermentation of a genetically-modified strain of baker's yeast in which multiple copies of a synthetic gene encoding ISP type III HPLC 12 has been inserted into the yeast genome. The production process yields an ISP type III HPLC 12 preparation that does not contain any residual modified yeast cells or detectable recombinant DNA. A specification for the preparation stipulates not less than 5 g/l active ISP type III HPLC 12.

The estimated daily intake (EDI) for the population group that had the highest edible ice intake in the UK (males aged 11-14 years) is 0.21 mg ISP type III HPLC 12/kg body weight (bw) at the 97.5 percentile. The population group in the UK with the highest estimated intake of ISP/kg bw is females aged 1.5-4.5 years at 0.53 mg/kg bw/day at the 97.5 percentile. These calculations assume that all the ice cream eaten contains ISP at the highest proposed level of use *i.e.* 0.01 % by weight. Based on ISP concentrations in cold water fish the average consumption of fish ISP in the diet was estimated at 1-10 mg/day in the USA and 50-500 mg/day in Iceland. Thus exposure from consumption of the edible ices would be well within the estimated range of population exposures to ISPs.

The applicant supplied the details of a sub-chronic (13-week) gavage study in rats using a maximum dose of ISP type III HPLC 12 of 580 mg/kg bw/day. No relevant differences were recorded between the control and test groups and this dose was taken as the no observed adverse effect level (NOAEL).

Thus there is a sufficient margin of safety of approximately 1100 – 2800 taking into account the 97.5 percentile intake estimate in the UK (0.53 mg/kg bw/day) for young females aged 1.5-4.5 years and for males (0.21 mg/kg bw/day) aged 14-17 years, and the NOAEL derived from the sub-chronic gavage study in rats.

The genotoxic potential of the ISP type III HPLC 12 preparation was tested in a bacterial gene mutation assay, a gene mutation assay using mammalian cells, an *in vitro* chromosome aberration assay and a rat bone marrow micronucleus assay. There was no evidence of genotoxic activity.

ISP type III HPLC 12 is not a major allergen from fish and bioinformatic studies did not evidence similarity with known allergens. Furthermore, the protein was degraded by pepsin with a measured half-life of 4 minutes (at pH 1.5). No adverse reactions were reported in countries where the ISP is authorised. Human studies were performed and the ISP preparation did not provoke a skin prick test reaction in, or bind IgE from, individuals allergic to fish. On the basis of these results the risk of an allergenic reaction in fish-allergic individuals or the population at large is very unlikely.

From 2003 to 2007 more than 470 million ISP-containing edible ice products have been sold in the USA and 47 thousand litres of ISP containing ice cream has been sold in Australia/New Zealand. There have been no reported safety issues.

With regards to the potential of adverse allergic reactions against yeast allergens, the Panel considers it is unlikely that such reactions would occur after ingestion of the ISP-containing products.

The Panel concludes that the use of the ISP type III HPLC 12 preparation at a maximum level equivalent to 0.01 % ISP type III HPLC 12 in edible ices is safe subject to adherence to the specification and production practices described by the applicant.

**Key words:**

Novel food ingredient, Ice Structuring Protein, ISP, genetically modified micro-organism, yeast, ocean pout

**TABLE OF CONTENTS**

NDA Panel Members .....	1
GMO Panel Members.....	1
Summary .....	1
Table of Contents .....	4
Background as provided by EC.....	5
Terms of reference as provided by EC .....	5
Acknowledgements .....	5
Assessment .....	6
I. Specification of the novel food (NF) .....	6
II. Effect of the production process applied to the NF .....	8
III. History of the organism used as the source of the NF .....	8
IV. Effect of the genetic modification on the properties of the host organism .....	8
V. Genetic stability of the GMO used as NF source.....	9
VI. Specificity of expression of novel genetic material .....	9
VII. Transfer of genetic material from GMO .....	9
VIII. Ability of the GMM to survive in and colonize the human gut.....	9
IX. Anticipated intake/extent of use of the NF .....	9
X. Information from previous human exposure to the NF or its source .....	10
XI. Nutritional information on the NF .....	10
XII. Microbiological information on the NF .....	11
XIII. Toxicological information on the NF .....	11
Discussion .....	14
Conclusions and Recommendations.....	16
Documentation provided to EFSA .....	16
References .....	17

## BACKGROUND AS PROVIDED BY EC

On 15 June 2006, Unilever PLC submitted a request under Article 4 of the Novel Food Regulation (EC) N° 258/97 to the competent authorities of the United Kingdom for placing on the market 'Ice Structuring Protein (ISP)' as food ingredient.

On 31 July 2007 the competent authorities of the United Kingdom forwarded to the Commission their initial assessment report, which came to the conclusion that the product was acceptable for the proposed food uses.

On 1 August 2007, the Commission forwarded the initial assessment report to the other Member States. In accordance with Article 6 (4) of Regulation (EC) No 258/97 objections were raised against the placing on the market of ISP.

In consequence, a Community Decision is required under Article 7, paragraph 1 of Regulation (EC) No 258/97.

The concerns of a scientific nature raised by the Competent Authorities of Member States can be summarised as follows:

- In the antibody response to ingestion of ISP preparation experiments the ISP preparation was provided in a water ice product in the absence of milk components. Are these results sufficiently representative for the estimation of an overall potential allergenic response?
- What is the effect of milk lipid components on the resistance of ISP to pepsin hydrolysis? What is the possibility that a hydrolysis resistant ISP could elicit an allergic reaction?
- Other concerns but of a non-scientific nature were raised by some MS *e.g.* whether ISP should be approved as an additive rather than a novel food, and labelling issues. These are not issues for consideration by EFSA.

## TERMS OF REFERENCE AS PROVIDED BY EC

In accordance with Article 29 (1) (a) of Regulation (EC) No 178/2002, the European Food Safety Authority is asked to carry out the additional assessment for 'Ice Structuring Protein (ISP)' as food ingredient in the context of Regulation (EC) N° 258/97.

EFSA is asked to carry out the additional assessment, in particular, to consider the elements of a scientific nature in the comments/objections raised by the other Member States.

## ACKNOWLEDGEMENTS

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## ASSESSMENT

Ice-structuring proteins (ISPs) are naturally-occurring proteins and peptides that bind to ice, and are found widely in nature *e.g.* in cold water fish, vegetables, grains, lichens and bacteria. ISPs help organisms to cope with very cold environments by both lowering the temperature at which ice crystals grow and by modifying the size and shape of the ice crystals that are formed so that the ice is less damaging to the tissues.

The incorporation of an ISP found in the North Atlantic fish, the ocean pout, in the product enables the manufacturer to manipulate the composition of edible ices to have less fat and sugar and to improve their organoleptic quality.

Regulatory approval for the use of ISP Type III HPLC12 preparation has been granted in Australia and New Zealand, Chile, Indonesia, Mexico and the Philippines and has been generally recognised as safe (GRAS) in the United States and accepted by the FDA.

The application for authorisation of the ISP type III HPLC 12 preparation was submitted under the Novel Foods Regulation 258/97/EC (European Commission, 1997a) and according to Commission Recommendation 97/618/EC concerning the presentation of information necessary to support an application for the placing on the market of novel foods and novel food ingredients (European Commission, 1997b). This preparation falls into Class 5.1 "a novel food produced using a GM micro-organism, the host for the genetic modification having a history of use as a food or as a source of food in the EU under comparable conditions of preparation and intake". This would have required information under sections I to XIII of the Commission Recommendation.

EFSA has recently published a guidance document on the risk assessment of products derived from genetically modified micro-organisms (GMMs) (EFSA, 2006). The guidance document describes three distinct groups of GMM. The ISP type III HPLC 12 preparation is classified as a group 2 GMM product 'Complex products derived from GMMs but not containing viable GMMs, nor any unit length of any cloned (foreign) open reading frames'. The information for this assessment is in sections I, II and III with additional information in sections IX to XIII. This assessment covers the information required under both the Commission Recommendation for Novel Foods and Novel Food Ingredients and EFSA guidance on GMMs. The NDA and GMO Panels have considered the application dossier, the initial assessment report by the UK competent authority, the concerns/objections from Member States and the responses from the applicant to these concerns and also to the questions posed by the UK.

### I. Specification of the novel food (NF)

Ice structuring protein (ISP) Type III was originally isolated from the blood of *Macrozoarces americanus*, the ocean pout. The ISP from this fish consists of 12 isoforms that can be separated by HPLC. Isoform HPLC 12 gives the largest peak and is the most functionally active in *in vitro* studies on ice structuring. This form, known as "ice structuring protein Type III HPLC 12" is the subject of this application and will simply be referred to as ISP in the following assessment. This protein is specifically identified by accession number P19614 in the Swiss Prot database. It consists of a sequence of 66 amino acids described by the applicant, has a molecular weight of 7.027 kDa, is not glycosylated, is heat stable and is stable over a pH range of 2-12. The ISP produced in the recombinant yeast strain is, however, a mixture of unglycosylated (60 %) and glycosylated (40 %) protein. This pattern of glycosylation is constant between batches. However only the unglycosylated ISP is able to bind to ice crystals

and alter the ice structure. The glycosylated ISP is inactive and therefore has no function in the preparation but the partial purification process is designed to maximise the functional activity of the preparation rather than to separate the glycosylated and unglycosylated forms. All the reported toxicology and allergenicity tests have been carried out on the complete ISP preparation containing both the unglycosylated and glycosylated forms.

The ISP preparation is a light-brown liquid produced by submerged fermentation of a genetically-modified strain of food-grade baker's yeast *Saccharomyces cerevisiae* in which a synthetic gene for the ISP has been inserted into the yeast's genome. The protein is expressed and secreted into the growth medium where it is separated from the yeast cells by microfiltration and concentrated by ultrafiltration. The ISP preparation consists of native ISP, glycosylated ISP and proteins and peptides from the yeast and sugars as well as acids and salts commonly found in food. The concentrate is stabilised with 10 mM citric acid buffer.

The ISP preparation is produced in accordance with good manufacturing practices (GMP).

The specification for the preparation is as follows

Assay	Not less than 5 g/l active ISP
pH	3.0 ± 0.5
Ash	Not more than 2 %
Heavy metals	Not more than 2 mg/l

Microbiology

Total microbial count	< 3000/g
Coliforms	< 10/g
Yeast and moulds	< 100/g
Production yeast	absent
<i>Listeria</i> spp	absent in 25 g
<i>Salmonella</i> spp	absent in 25 g
<i>Staphylococcus aureus</i>	< 10/g
<i>Bacillus cereus</i>	< 100/g

These requirements are based upon those for enzymes (Committee on Food Chemicals Codex, 2001) because of the similarity of the production processes and use levels in food.

The commercial material is to be shipped frozen in clean, sealed containers. The recommended storage time, frozen, is six months.

The applicant submitted the results of compositional data from analyses carried out on five commercial production batches and a concentrated batch of ISP used in toxicological testing. There were no causes for concern.

## II. Effect of the production process applied to the NF

The ISP was selected for commercial application because it has the functionality required, together with temperature and pH stability. Since it was not economical, practical or sustainable to obtain ISP from the ocean pout, an alternative supply was developed already used in the production of other food ingredients such as amylase, pectinase, xylanase, chymosin and vitamins, *viz.* fermentation of a genetically modified producer organism.

The production process consists of fermentation with a GM food grade yeast that carries a multicopy insert containing a synthetic gene encoding the ISP linked to the invertase signal sequence under the control of the GAL7 promoter. The addition of galactose to the yeast's growth medium is required to induce production of ISP.

The process is carried out under contained use conditions i.e. in an enclosed fermenter. Food grade materials are used throughout.

During fermentation, the yeast is grown in a 15,000 l reactor and then induced to produce the ISP by addition of galactose. At the end of the fermentation the yeast cells are removed by microfiltration or filter press. The resulting liquor is subjected to an ultrafiltration step which filters the liquor at a molecular scale (1 kDa) to remove small molecules and concentrate the ISP.

The applicant produced the HACCP plan used by the manufacturers of the ISP preparation (Martek, USA).

## III. History of the organism used as the source of the NF

The host organism that expresses the synthetic gene that encodes ISP is the baker's yeast *Saccharomyces cerevisiae*. The specific yeast strain used for the production of the ISP has been classified as 1 AB (Council Directive 90/219/EEC of 23 April 1990 on the contained use of genetically modified micro organisms) by the Netherlands' Ministry of the Environment in 2002 with permission granted for large-scale fermentation. This strain has been used since 2003 for commercial production of the ISP for use in non European ice cream production.

## IV. Effect of the genetic modification on the properties of the host organism

The expression cassette contains a synthetic gene coding for ISP. The ISP expressed by the yeast has the same amino acid sequence as that from the ocean pout but the nucleotide sequence was constructed to favour codon usage in yeast to maximise expression. The cassette was integrated into the ribosomal DNA (rDNA) locus of the yeast genome by homologous recombination. The resulting strain, CENPK338, contains a multi-copy expression cassette inserted at the rDNA locus. No gene encoding antibiotic resistance and no bacterial DNA were introduced. After targeted integration the number of the expression cassette was increased to 30-50 copies by selection under growth conditions that favoured yeast cells with multiple copies of the recombinant DNA, as demonstrated by restriction endonuclease analysis using five restriction endonucleases. Full details of the molecular structure of the cassette and of the integration event have been provided by the applicant. Southern transfer and hybridisation experiments were used to estimate the copy number. Evidence was also provided to show that no new open reading frames were created from the integration event that could result in the generation of novel proteins.

## V. Genetic stability of the GMO used as NF source

Cell viability, the presence of the ISP gene, the structure of the integration site and protein expression levels were compared in the producer strain before and after 70 generations of growth under non selective conditions. No differences were found for any of the parameters measured.

## VI. Specificity of expression of novel genetic material

Expression of the ISP is under the control of an inducible GAL7 promoter that only permits expression of the protein in the presence of galactose. Expression is repressed during microbial growth in the presence of more than 0.5 % glucose.

## VII. Transfer of genetic material from GMO

The applicant demonstrated the absence of DNA derived from the ISP gene using a specific PCR assay. The detection limit was estimated at  $2 \times 10^{-10}$  g ISP plasmid DNA/g lyophilised ISP preparation.

## VIII. Ability of the GMM to survive in and colonize the human gut

The production process is designed to remove all yeast cells from the ISP preparation by microfiltration and the final product should be yeast free. This was confirmed by analysis of all production batches. Yeast proteins will, however, be present.

## IX. Anticipated intake/extent of use of the NF

ISP will be used in the production of edible ice products *e.g.* ice cream, milk ice, water ice, fruit ice, sorbets, frozen desserts and any similar products such as iced smoothies. ISP is proposed to be used in products at levels not exceeding 0.01 % by weight and more commonly less than 0.005 %. This would be equivalent to 0.20 and 0.10 % of ISP preparation with 5 % content of ISP.

The anticipated intake of ISP from its use in edible ices was calculated for the UK using the UK NDNS data for children, young people and adults. The data were collected from July 1992 to June 1993 for children, January to December 1997 for young people and during July 2000-June 2003 for adults. The estimates are for consumers only. The child survey was a 4-day survey; the young people and adult survey were 7-day surveys. For children aged 1.5-4.5 years the mean intake was 16 and 15.25 g/day for males and females respectively and at the 97.5<sup>th</sup> percentile 62 and 63.5 g/day. For young people aged 4-6 years the corresponding values were 16.7 and 13.7 g/day (mean) and 59 and 73 g/day (97.5<sup>th</sup> percentile); for young people aged 7-10 years, 17.9 and 17.4, and 63 and 64 g/day; for young people aged 11-14 years, 22.1 and 17.7, and 99 and 76 g/day; for young people aged 15-18; 16.4 and 12.9, and 83 and 71 g/day; and for adults aged 19-64 years, 17.1 and 16.5, and 78 and 72 g/day.

The data indicate that 11-14 year old UK males have the highest intake at 99 g/day at the 97.5<sup>th</sup> percentile. If all this edible ice were to contain ISP at the maximum proposed level of 0.01 % by weight this would equate to a daily intake of 9.9 mg of ISP. Using the average bodyweight for this group of 47 kg this would be an estimated daily intake (EDI) of 0.21 mg ISP/kg bw.

The UK population having the highest intake per kg bw is females aged 1.5-4.5 years with an estimated intake of ISP of 6.36 ml/day at the 97.5 percentile equivalent to 0.53/kg bw/day for a body weight of 11.9 kg.

The applicant also considered the seasonal intake values for January-March, April-June, July-September and October-December. For children aged 1.5-4.5 years and adults aged 19-64 there was little variation. At the 97.5<sup>th</sup> percentile the variation was less than 10 % between the minimum and maximum. There was a larger difference for young people aged 4-16 years of 37.7 % (22 g) at the 97.5<sup>th</sup> percentile.

Average daily ice cream intakes for consumers only have also been estimated for the Netherlands using the Dutch National Food Consumption Survey (DNFCS – 3, 1997-98). Using these data it is adults who have the highest potential ice cream intake of 100 g/day at the 95<sup>th</sup> percentile. If all this ice cream were to contain ISP at the maximum proposed level of 0.01 % by weight this would equate to 10 mg ISP/day.

Ice cream intake in France appears to be lower with an estimated average intake for adults of 6 g/day rising to 8 g/day for younger females and 9 g/day for younger males. This would equate to 0.9 mg of ISP/day at 0.01 % ISP by weight.

The intake estimates are based on conservative assumptions.

## **X. Information from previous human exposure to the NF or its source**

Ice structuring proteins are already consumed as part of the human diet. They were first identified over thirty years ago in the blood of fish *e.g.* cod and herring living in areas where the sea freezes (DeVries and Wohlschlag, 1969). Since then they have been found in a wide variety of organisms that need protection from freeze damage including many plants, insects, fungi and bacteria (Griffith and Ewart, 1995). Plants in which ISPs have been found include such common food sources as oats, rye, barley, wheat, carrot and potato.

Although ocean pout has no history of consumption in Europe, it has been eaten in the North Eastern USA. The applicant estimates the ISP content of ocean pout at about 30 mg/ml in blood and assuming the blood volume to be 30-70 ml the ISP content of an ocean pout would be 900-2100 mg/kg. Thus a 200 g portion of fish would contain between 180 and 420 mg of ISP.

The exposure from consumption of edible ices would be well within estimated current population exposures. The estimated daily intake of ISP for the group with the highest estimated edible ice intake in the UK (males aged 11-14) is 9 mg, equivalent to 0.21 mg/kg bw.

Edible ices containing ISP have been on sale in the USA since the second quarter of 2003 with 470 million products sold in the period 2003/07 with no consumer issues reported through company contact centres.

## **XI. Nutritional information on the NF**

At the levels of proposed use, less than 0.01 % by weight, there are no nutritional implications from the ISP preparation as such. The ISP would be digested as protein according to normal metabolic processes and the very small amounts involved would not make a significant contribution to protein consumption patterns. The use of ISP preparation in products is not

expected to change the level of consumption of edible ices but rather to influence product choice.

## **XII. Microbiological information on the NF**

The formulation and process rules and distribution practices currently used to ensure the safety of edible ices are equally applicable to products containing ISP. The microbiological specifications are set out in Section I. A summary of the microbial analysis of 10 ISP commercial runs shows the level of total counts to be < 10/g (spec. < 3000/g), those of coliforms, yeast and moulds, *Staphylococcus aureus* and *Bacillus cereus* to be < 1/g (spec. < 10, < 100, < 10 and < 100/g respectively), and *Listeria* spp. and *Salmonella* spp. to be absent from 25 g. Thus the microbiological status of the preparations is well within specification.

## **XIII. Toxicological information on the NF**

### **Sub-chronic toxicity**

An ISP preparation, concentrated approximately 5-fold by ultrafiltration was administered to rats by daily oral gavage for 13 weeks. The study was compliance with OECD guideline 408 for repeated dose 90-day oral toxicity studies in rodents (OECD, 1998a). The applicant has supplied the complete study report and supporting analytical studies.

The test material was administered at a dose level of 20 ml/kg/ bw/day. This provided a top dose of 4000 mg total solids/kg bw/day equivalent to 580 mg ISP/kg bw/day. Lower doses were one-half and one-tenth of this dose by dilution, *viz.* 290 and 58 mg/kg bw/day respectively. One control group received ultra-purified water. A second group received a citric acid solution in order to control for acidity. There were 20 rats per sex per group. Animals were observed daily for signs of ill health or overt toxicity. Individual body weights and food intake were recorded weekly. Blood samples were taken from 10 male and 10 female rats during weeks 4 and 8 and from all animals at the end of the study. Urine samples were taken from 10 male and 10 female rats during week 12. All animals were subjected to a necropsy. Organ weights before fixation were recorded and extensive histopathology conducted. In addition 10 male and 10 female rats were subjected to a battery of behavioural tests and observation before and once weekly afterwards.

Animals given 290 or 580 mg/kg bw/day gained slightly more bodyweight than the 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 biochemical composition of the blood. There were no differences between the groups in organ weights related to treatment and no macro- or microscopic findings due to effects of the test material.

The highest dose that could be tested, 580 mg ISP/kg bw/day is the NOAEL in this study.

### **Genotoxicity**

The potential genotoxicity of the ISP preparation was assessed using four different assays, *viz.* the bacterial reverse mutation assay, the gene mutation assay in mouse lymphoma cells, the *in vitro* chromosome aberration assay in human peripheral blood lymphocytes, and the *in vivo* rat bone marrow micronucleus assay. All assays were performed in compliance with the OECD guidelines (OECD, 1998b). The complete study reports were supplied by the applicant.

The concentrated ISP preparation used in the subchronic toxicity experiment was still too dilute for use in the genotoxicity studies so a portion of it was freeze dried. Analysis showed no significant difference between the starting material and the freeze dried material apart from the removal of water.

#### *The bacterial reverse mutation assay*

The bacterial reverse mutation assay in compliance with OECD Guideline 471 (OECD 1997a) was performed using *Salmonella enterica* var Typhimurium strains TA1535, TA1537, TA98, TA100 and TA102 at a maximum concentration of 5000 µg/ plate in the presence and absence of rat-liver derived S9 fraction. The test was negative with strains TA1537, TA98, TA100 and TA102 in the presence and absence of S9. However there was a small but statistically significant increase in the number of revertant colonies with strain TA1535. A further experiment using a maximum concentration of 8000 µg/plate revealed that the test material was slightly contaminated, resulting in bacterial colonies that were not revertants of strain TA1535 growing on the test plates. Each colony was tested for identity as *Salmonella* or contaminant and the numbers of non-*Salmonella* deducted from the total count. The recalculated values showed no significant differences between the number of revertants on the test and control plates. Of course, the contaminants would have grown on all the test plates but were only significant on TA1535 because of this strain's very low background of spontaneous reversion.

A further sample of ISP preparation was freeze dried and assayed in the same five strains with and without the S9 fraction. The test was negative in all five strains.

Based on these assessments it was concluded that ISP preparation possesses no mutagenic activity in the Ames bacterial mutation assay.

#### *Gene mutation assay in mouse lymphoma cells*

Gene mutation was assessed using the *tk* locus in mouse lymphoma cells and was compliant with OECD Guideline 476 (OECD, 1997b). The freeze dried preparation was dissolved in water and tested up to 5000 µg total solids or the limit of toxicity. The assay was performed on two occasions in the presence and absence of S9 fraction. Exposure to the test material was for 3 hours, or 20 hours (without metabolic activation only). There was no evidence of either a biologically significant or statistically significant increase in mutation frequency in treated cultures compared with controls. The ISP preparation showed no evidence of mutagenic potential in this test.

#### *In vitro chromosome aberration assay*

This assay was performed in accordance with OECD Guideline 471 (OECD, 1997c). The freeze dried ISP preparation was dissolved in water and tested at concentrations of up to 5000 µg total solids or the limit of toxicity. The assay was performed on two separate occasions in the presence and absence of an S9 fraction. The blood cultures were exposed to the test material for 3 hours, or 20 hours (without metabolic activation only). Cultures were harvested 20 hours after start of the treatment. 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 with controls. Nor was there any increase in polyploid or endoreduplicated cells in tested versus control cultures. The ISP preparation tested showed no evidence of genotoxic potential in this test.

*In vivo rat bone marrow micronucleus assay*

This assay was performed using groups of seven male rats of approximately 7 weeks of age and was compliant with OECD Guideline 475 (OECD, 1997d). The ISP freeze dried preparation was dissolved in water and dosed once daily on two consecutive days by gavage at 500, 1000 and 2000 µg total solids/kg bw. Twenty four hours after the final dose slides were prepared from the bone marrow. There was no evidence of a change in the ratio of polychromatic to normochromatic erythrocytes nor an increase in micronucleated polychromatic erythrocytes in any of the treatment groups compared with controls. Under the conditions of this study ISP preparation showed no evidence of genotoxic potential.

**Allergenicity**

No method currently exists which can give assurance that a protein lacks the ability to induce an allergic reaction or sensitise an individual consumer to subsequent challenge. The applicant describes a number of tests to generate a body of evidence that the ISP is not likely to induce or provoke allergic reactions.

The applicant notes that ISP is not a major allergen from fish and that bioinformatic analysis of sequence homology did not provide evidence of similarity with known allergens. Furthermore, native ISP and glycosylated ISP are degraded by pepsin with measured half-lives of 4 minutes (at pH 1.5).

In addition, the applicant pointed out that no adverse reactions have been reported in countries (*e.g.* USA and Australia/New Zealand) where ISP-containing ice creams are authorized and are currently consumed.

However, the Panel notes that ISP is derived from an allergenic source, *i.e.* fish. The applicant conducted clinical studies on patients allergic to fish to test the eventual allergenic potential of native ISP and of ISP in the presence of the yeast fermentation supernatant.

*Investigations in individuals having established allergy to fish*

No data exist on allergy to ocean pout although allergy to a closely-related species, eel, has been described (Bruijnzeel-Koomen *et al.*, 1995) so it might be expected that fish-allergic individuals would react to ocean pout. The applicant deemed it essential to demonstrate whether fish-allergic individuals would react to ISP. Investigations started with studies on the sera of fish-allergic patients. Once data showing safety of the ISP preparation were available the testing was extended to skin-prick testing and ingestion.

Twenty subjects with established allergy to codfish were skin-prick tested in duplicate with freshly thawed eel, eel pout and ocean pout. All patients demonstrated positive reactions to the three test materials. The Radio-Allergo Sorbent Test (RAST) was applied to measure the binding between the allergen and IgE in the sera of the 20 subjects. Eighteen of the 20 had IgE against ocean pout whereas none of the patients' sera demonstrated binding of IgE to the freeze-dried ISP preparation.

In a second phase 22 participants (17 from the first phase and 5 new ones) were skin-prick tested in duplicate with solutions of sterile ISP preparation as well as with solutions of the parent yeast strain fermentation supernatant (yeast control). Four of the 22 reacted to both ISP preparation (which includes yeast protein) and the yeast control. The RAST was performed which showed that 8 of the 22 gave positive results to both ISP preparation and the yeast fermentation supernatant (the 8 included the 4 who were positive for skin-prick testing). All

eight subjects had negative RAST results to pure ISP (which includes only the ISP and no yeast proteins).

SDS-PAGE and Western blotting were performed in accordance with the protocols described by Laemmli (1970) and Hansen *et al* (1997). No IgE binding to the ISP was demonstrated.

All of the subjects who experienced a positive reaction to yeast in the skin-prick test and had a positive RAST response had atopic dermatitis or other IgE mediated conditions such as bronchial asthma and hay fever. The applicant argues that despite their sensitivity they are able to tolerate foods containing yeast, and that the yeast component of ISP preparation does therefore not pose a clinically significant allergenic risk. The Panel notes that it is unclear from the information presented by the applicant what types of yeast foods were tolerated well, nor whether these foods included ISP-containing products mentioned in their application; in other words whether a double-blinded placebo controlled food challenge, which is the gold standard for establishing the clinical consequence of ingesting allergenic food in case of positive skin-prick test, has been carried out. Incidental cases of allergy to yeast have been described, and the Panel can not exclude adverse reactions to occur in after ingestion of ISP-containing products. However, as yeast is not a major food allergen, and as the prevalence of adverse allergic reactions to yeast ingestion is rare, the Panel considers the likelihood of such reactions occurring after ingestion of the ISP-containing products to be low.

With regard to the question of whether the ingestion of ISP preparation in water ice gave unrepresentative results because association with lipid molecules could alter its immunogenicity; the applicant argued that the study subjects were given the test or control material in the morning and were not asked to refrain from eating before consuming the test material. Under these circumstances it would be likely that food would be in the stomach and the ISP protein would be able to associate with any lipid material present. Thus any potential modulation of immunogenetic potential by lipid association was covered by the study design.

Additionally recent studies (Garcia-Arribas *et al.*, 2007) showed that the ISP protein readily unfolded above 0°C thereby losing its activity and hence its ability to associate with lipids. These studies also showed that unlike immunogenic proteins such as the caseins, ISP did not form aggregates when it lost its secondary structure.

In addition to the 'weight of evidence' presented the applicant points out that ice creams containing ISP have been on sale in the USA since the second quarter of 2003 and up until 2007 more than 470 million products were sold. In Australia/New Zealand 47,370 litres of ISP containing ice cream have been sold. No safety issues have been reported.

## DISCUSSION

The applicant has selected an ISP from the ocean pout, type III HPLC 12, for commercial development as a food ingredient to be added to edible ices to improve their nutritional profile and organoleptic qualities. Sourcing the ISP from the ocean pout would not be sustainable or economically feasible so the manufacturer has developed a production system based on the fermentation of a genetically-modified baker's yeast in a contained use facility. This is an established approach common in the production of vitamins and enzymes. The genetic modification involved the introduction into the yeast genome of multiple copies of a synthetic gene that encodes the ISP. No gene encoding antibiotic resistance was introduced into the yeast. The yeast culture is grown and then induced to make the ISP, which is secreted from the yeast cells into the growth medium. The process includes a purification stage where the yeast

cells are removed by filtration and the applicant has demonstrated the absence of the production yeast and transgenic DNA from the resulting ISP preparation subject to the limits of detection. Since the recombinant DNA does not contain any sequence that causes concern and as no DNA is detectable in the final product, its use does not raise any environmental safety concern. The ISP preparation consists of native ISP, glycosylated ISP and proteins and peptides from the yeast as well as sugars, acids and salts commonly found in foods. The Panel was satisfied with the applicant's description of the production process for the ISP preparation to be used as a novel food ingredient.

The commercially-produced ISP preparation contains, in addition to the native ISP, glycosylated ISP and proteins and peptides from baker's yeast. Since the ISP is identical to that produced by the ocean pout, one major concern is the possibility that the protein added to edible ice might cause allergic reactions in individuals sensitive to fish or sensitise susceptible individuals. The applicant has assembled a body of evidence from *in vitro* studies and from clinical studies on allergic humans to assess the allergenic potential of ISP.

The applicant reported that ocean pout produced positive skin-prick test results and positive results in *in vitro* allergy testing in established fish-allergic individuals. However ISP did not bind IgE from fish-allergic subjects in the RAST and skin-prick testing did not produce any positive reactions to the protein, although four reactions to yeast proteins were observed and confirmed by *in vitro* tests. A skin-prick test with a highly purified ISP (yeast protein < 1%) was negative. Ingestion of the ISP preparation for eight weeks at a high daily dose did not result in specific antibody formation. In addition, amino acid sequence analysis and susceptibility to proteolytic breakdown both indicated a very low probability of inducing sensitisation. The whole sets of results were considered by the applicant to indicate that ISP preparation is highly unlikely to provoke a reaction in consumers already sensitised to fish and is unlikely to sensitise potentially susceptible individuals in the general population. The Panel agrees that the results presented by the applicant support these conclusions. There is also a history of human consumption of fish containing this protein and of consumption of edible ices containing the novel ingredient in the USA and Australia/New Zealand with no indication of adverse effects.

ISP type III HPLC 12 showed no indication of genotoxicity in appropriate studies. In a sub-chronic rat feeding study, which was carried out according to international standard, the highest dose that could be tested was 580 mg ISP/kg bw/day. There was no indication of toxicity in this study and the highest dose level was selected as the no observed adverse effect level (NOAEL). As the ISP 12 is the active constituent in the novel ingredient calculations are based on this component and not on the partially purified preparation.

The applicant calculated intake data for ISP as a novel food ingredient at a maximum use level of 0.01 % in edible ices for UK, Dutch and French populations. The maximum intake for UK population groups was in males aged 11-14 years for whom the intake was 9.9 mg/day at the 97.5 percentile equivalent to 0.21 mg/kg bw/day. The UK population having the highest intake per kg bw is females aged 1.5-4.5 years with an estimated intake of ISP of 6.36 mg/day at the 97.5 percentile equivalent to 0.53/kg bw/day for a bodyweight of 11.9 kg. The values per kg bw/day for other population groups in the UK, the Netherlands and France were smaller.

Thus there is a sufficient margin of safety of approximately 1100 – 2800 taking into account the 97.5 percentile intake estimate in the UK (0.53 mg/kg bw/day) for young females aged 1.5-4.5 years and for males (0.21 mg/kg bw/day) aged 14-17 years and the NOAEL derived from the sub-chronic gavage study in rats.

## CONCLUSIONS AND RECOMMENDATIONS

With regards to the potential of adverse allergic reactions against yeast allergens, the Panel considers it is unlikely that such reactions would occur after ingestion of the ISP-containing products.

The Panel concludes that the use of the ISP type III HPLC 12 preparation at a maximum level equivalent to 0.01 % ISP type III HPLC 12 in edible ices is safe subject to adherence to the specification and production practices described by the applicant.

## DOCUMENTATION PROVIDED TO EFSA

1. Letter from the European Commission to the Chairman of the European Food Safety Authority with the request for an opinion on the safety of 'Ice Structuring Protein' (ISP) as a food ingredient. SANCO E4/AK/bs (2008) D/540054.
2. Initial assessment report by the Advisory Committee on Novel Foods and Processes (UK) concerning the assessment of 'Ice Structuring Protein' preparation derived from fermented genetically modified Baker's yeast *Saccharomyces cerevisiae* as a food ingredient.
3. Letters from Member States with comments on the initial assessment report on 'Ice Structuring Protein' (ISP) as a food ingredient from the Advisory Committee on Novel Foods and Processes (United Kingdom).
4. Response to Member States comments on the UK Advisory Committee on Novel Foods and Processes Opinion for 'Ice Structuring Protein' (ISP) as a food ingredient.
5. Application under regulation No 258-97 for the use of 'Ice Structuring Protein' (ISP) as a food ingredient.

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