Safety & Environmental Assurance Centre

Application for the Approval of Ice Structuring Protein Type III HPLC 12 Preparation for use in Edible Ices.

Regulation (EC) N° 258/97 of the European Parliament and of the Council of 27th January 1997 Concerning Novel Foods and Novel Food Ingredients

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APPLICATION FOR THE APPROVAL OF ICE STRUCTURING PROTEIN TYPE III HPLC 12 PREPARATION FOR USE IN EDIBLE ICES

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EXECUTIVE SUMMARY

Unilever is seeking approval in Europe under Regulation (EC) N^{o} 258/97 of the European Parliament and of the Council of 27th January 1997 concerning novel foods and novel food ingredients, for the use of a particular type of Ice structuring protein (ISP), Ice Structuring Protein Type III HPLC 12 preparation (ISP Type III HPLC 12 preparation) as a novel food ingredient in the production of edible ices. The term 'edible ices' encompasses ice cream, including dairy ice cream, milk ice, water ice, fruit ice, sorbets, frozen desserts and any similar products such as iced smoothies and products of which edible ice is a component. The ice structuring protein will be used in edible ice at a level not exceeding 0.01% by weight.

Regulatory approval for the use of ISP Type III HPLC 12 preparation has previously been obtained in Australia and New Zealand, Chile, Indonesia, Mexico and the Philippines based on local regulatory procedures. ISP Type III HPLC 12 preparation has been determined to be generally recognized as safe (GRAS) in the United States, and this determination was reviewed and accepted by the US Food and Drug Administration.

Using ISP during the manufacture of edible ices results in a variety of benefits to consumers including improved nutrition profiles (such as the amount of calories, fat, saturated fat, sugars and fruit), organoleptic properties (such as hardness, brittleness or creaminess and enhanced flavour delivery) and also greater temperature stability (an important factor in maintaining good quality products throughout the supply chain.) All of the product properties and benefits are a consequence of the increased connectivity of ice produced in the presence of ISP Type III HPLC 12 preparation.

Ice structuring proteins (ISPs) are naturally occurring proteins and peptides that bind to ice and are found widely in nature, for example, in cold water fish, vegetables, grains, lichens and bacteria. ISPs function to help organisms 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 tissues.

ISP Type III was originally isolated from the ocean pout (*Macrozoarces americanus*), a cold-water fish found off the northeast coast of North America. This type of ISP consists of 12 isoforms that can be separated by high performance liquid chromatography (HPLC). Isoform HPLC 12 (ISP Type III HPLC 12), composed of 66 common amino acids, was selected for commercial development.

Sourcing ISPs from nature is currently not sustainable or economically feasible. Therefore Unilever has developed a contained-use production system which produces ISP Type III HPLC 12. The production system is based on fermentation using genetically modified baker's yeast which is an established approach common in the production of vitamins and enzymes e.g. chymosin used in making vegetarian cheese. The process includes a purification stage where the yeast cells are removed which yields an ISP Type III HPLC 12 preparation to a stringent specification and means that the ISP Type III HPLC 12 preparation does not contain any residual modified yeast cells.

As indicated, ISPs occur naturally in many foods consumed by man. Based on ISP concentrations in cold-water fish and the landings of such fish, the average consumption of fish ISP in the diet is estimated to be 1-10 mg/day in the USA and 50-500 mg/day in Iceland. Specifically, exposure resulting from consumption of the proposed products would be well within the estimated range of current population exposures to ISPs.

An extensive safety testing programme was undertaken for ISP Type III HPLC 12 preparation. Firstly, the scientific and medical literatures were reviewed to find whether any specific adverse effects had been attributed to ISPs in general, and to gauge population exposure to these proteins. Secondly, as allergenicity is a potential hazard of exposure to proteins, a detailed assessment of the allergenicity of ISP Type III was undertaken. The third element of the safety assessment was an evaluation of the general toxicity and genotoxicity of the ISP Type III HPLC 12 preparation, using standard toxicological methods which complied with OECD guidelines.

Amino acid sequence analysis and susceptibility to proteolytic breakdown were evaluated and neither indicated allergenic potential. The potential of ISP Type III to provoke a reaction in fish-allergic individuals was also tested and ISP Type III did not provoke skin prick test reactions, nor did it bind IgE from fish-allergic individuals. In addition, daily ingestion by volunteers of ISP Type III HPLC 12 preparation for eight weeks failed to generate a detectable immune response. These results demonstrate the safety of ISP Type III to persons allergic to fish, as well as to individuals potentially susceptible to producing IgE responses to proteins (atopic individuals). Based on data and observations outlined, it is concluded that ISP Type III HPLC 12 preparation presents no allergenic risk to fish-allergic individuals or the population at large.

The genotoxic potential of ISP Type III HPLC 12 preparation was assessed by a bacterial mutation assay, an *in vitro* chromosome aberration assay in human peripheral blood lymphocytes, a gene mutation assay in mouse lymphoma L5178Y cells, and an *in vivo* rat bone marrow micronucleus assay. There was no evidence of genotoxic activity in any of these tests.

A 13-week gavage study in rats was conducted to assess the potential for toxicity of ISP Type III HPLC 12 preparation, with the top dose of ISP Type III HPLC 12 being 580 mg/kg body weight/day. Lower doses were one-half and one-tenth the highest dose, by dilution. The results showed no differences between control and test groups in clinical signs, body weights, haematological parameters, clotting potential, in the biochemical composition of the blood, or in organ weights. There were also no macroscopic or microscopic findings due to the effects of the test material.

The highest dose tested in the 13-week rat study, 580 mg ISP Type III HPLC 12/kg body weight/day, was selected as the no observed adverse effect level (NOAEL) because of the lack of toxicity established by detailed observations. This was then used to derive a safe level of intake. A safety factor of 100 was used, based on the totality of analytical, animal, human, and *in vitro* data summarised in this document, general knowledge of proteins, and from the approaches to estimating a safety factor described in published articles. Therefore, a safe level of intake for the preparation was determined as the NOAEL divided by the safety factor, and calculated to be 5.8 mg ISP Type III HPLC 12/kg body weight /day.

The estimated daily intake (EDI) for the group that had the highest estimated edible ice intake in the UK (males aged 11-14 years old at the 97.5th percentile), is 0.21 mg of ISP Type III HPLC 12/kg body weight. This conservatively assumes that all the ice-cream eaten contains ISP at the highest proposed level of use i.e. 0.01% by weight and uses the average bodyweight recorded for this group (NDNS anthropometric data) of 47 kg. This EDI is 28-times less than a conservatively established safe level of intake.

Use of ISP preparation in products is not expected to significantly change population consumption of edible ices, but rather to influence product choice within that market.

In conclusion, available published data, experimental findings and calculations of projected consumption indicate that ISP Type III HPLC 12 preparation is safe for consumers under the intended conditions of use in edible ices.

1. PURPOSE OF THIS APPLICATION

Unilever is seeking approval under Regulation (EC) N^{o} 258/97 of the European Parliament and of the Council of 27th January 1997 concerning novel foods and novel food ingredients, for the use of an Ice Structuring Protein Type III HPLC 12 preparation (ISP Type III HPLC 12 preparation) as a novel food ingredient in the production of edible ices. The ice structuring protein will be used at a level not exceeding 0.01% by weight.

According to the general basic definition in the Euroglaces (Association of the Ice Cream Industries of the EU) 'Code for Edible Ices', edible ices are food products; the solid or pasty texture of which is obtained by freezing and which are stored, transported, sold and consumed in a frozen state. The term edible ices therefore encompasses ice cream, including dairy ice cream, milk ice, water ice, fruit ice, sorbets, frozen desserts and any similar products such as iced smoothies and products of which edible ice is a component.

Article 1(2) of EC 258/97 states that 'this regulation shall apply to the placing on the market within the Community of foods and food ingredients which have not hitherto been used for human consumption to a significant degree within the Community…' and which fall under one of four (formerly six) categories of novel foods and novel ingredients. ISP Type III HPLC 12 preparation has no significant history of consumption within the European Community and is isolated from genetically modified yeast *Saccharomyces cerevisiae* [baker's yeast]. The ISP preparation does not contain any residual modified yeast cells.

ISP preparation is considered to be a novel food ingredient that falls into category (d) of Article 1 (2) of Regulation 258/97. This application has been prepared taking into account Commission Recommendation 97/618/EC concerning the scientific aspects and presentation of information necessary to support applications for the placing on the market of novel foods and novel food ingredients, and the preparation of initial assessment reports (hereafter referred to as the Commission Recommendation).

In the light of the revision of Regulation 258/97 – i.e. the removal of novel food categories (a) and (b)- Unilever considers that of the still valid classes of the Commission Recommendation and based on Table 1 of the Recommendation, all novel foods and novel food ingredients produced with micro-organisms whether GM or not, including ISP preparation, now fall into Class 2. Class 2 should accordingly be read as extended in scope. Unilever is of the opinion that information about the genetic modification of Saccharomyces *cerevisiae* is essential for the safety assessment of ISP preparation. Unilever has therefore included and structured the information in Appendix 1 along the lines of the former Class 5 (GM microorganisms and their products).

2. GENERAL INTRODUCTION

2.1 What are Ice Structuring Proteins?

Unilever has been exploring novel functional ingredients known variously as Ice Structuring Proteins (ISPs), Anti-Freeze Proteins (AFPs), Thermal Hysteresis Proteins (THPs) to control ice formation and structure.

ISPs are naturally occurring proteins and peptides that bind to ice and are found widely in nature, for example, in cold water fish, vegetables, grains, lichens and bacteria. ISPs function to help organisms 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 tissues.

Unlike chemical compounds such as salt and ethylene glycol, ice structuring proteins exert their freezing point-depression activity by a non-colligative mechanism, thereby minimizing their effect on the osmotic pressure of cells or plasma. On a molar basis, they are estimated to be 200- to 500-times more effective than sodium chloride (Avanov, 1990). Ice structuring proteins exert their effect by binding directly to the growing ice crystal and thereby modifying its size and morphology (Barrett, 2001). Mechanisms of interaction of different ice structuring proteins with ice crystals are thought to include hydrogen bonding (DeVries and Lin, 1977), hydrophobic interactions (Harding *et al*., 1999; Zhang and Laursen, 1998) and hydrophilic interactions (Li and Hew, 1991).

Various ice structuring proteins have been identified in different organisms and are classified into groups with similar protein structures and properties. The ISP considered in this application was originally identified in ocean pout (*Macrozoarces americanus*). Fish ISPs are termed Type I, II, III or IV (Crevel *et al* 2002). The ISP considered in this application is a Type III. At least 12 different ISPs have been found in the serum of ocean pout and separated by high performance liquid chromatography (HPLC); in this application one of these ISPs, ISP Type III HPLC 12, is considered. Please note that in some of the documentation provided as appendices with this application, the original but now superseded nomenclature for ISP Type III HPLC 12 i.e. AFP III HPLC 12 is used. AFP III HPLC 12 and ISP Type III HPLC 12 are the same protein (Accession number P19614 in the Swiss-Prot protein database).

2.2 Sourcing ISPs

Sourcing ISPs from nature is currently not sustainable or economically feasible, therefore Unilever has developed a contained-use production system based on fermentation using genetically modified baker's yeast and can now produce an ISP preparation containing ISP Type III HPLC 12. As described in more detail in Appendix 1 (confidential material), a synthetic gene has been inserted into baker's yeast to produce the ISP during fermentation. At no time during the process was the extraction of DNA or any material from fish carried out. This production system uses an established approach common in the production of vitamins and enzymes e.g. chymosin used in making vegetarian cheese. The process includes a purification stage where the yeast cells are removed which yields an ISP preparation to a stringent specification that means that the ISP preparation does not contain any residual modified yeast cells. In addition, the preparation does not contain residual modified DNA.

2.3 The Novel Use of ISP Preparation in Edible Ice Manufacture

Edible ices are food products, the solid or pasty texture of which is obtained by freezing and which are stored, transported, sold and consumed in a frozen state. All contain large amounts of ice. The discovery of the ability of ice structuring proteins has provided an opportunity to influence the formation of this ice structure during manufacture. Taking, by way of example, two such edible ice products –

- Ice cream is a complex, frozen, aerated emulsion, made up of four phases: air, ice, matrix (matrix is the unfrozen part containing sugar, protein, stabilisers, flavours, and emulsifiers), and fat.

- Water ice has a simpler structure, generally comprising only ice and matrix, although air is sometimes added. The size and structure of the ice crystals affect temperature stability, which is an important factor in maintaining good product quality in extended supply chains. It also affects the sensory attributes of the product such as hardness, creaminess, and flavour delivery.

When the ISP preparation described in this application is added to an ice cream or water ice mix, it has no effect until ice forms. ISP has no effect on the quantity of ice present at a given final temperature, but as previously mentioned it does affect the formation (size and shape) of the ice crystals present. In the presence of ISP, the crystals formed in the freezer are rod shaped rather than round. The effect of ISP is to control the growth of the ice crystals in the freezer such that they remain very small and elongated (Figure 1 overleaf).

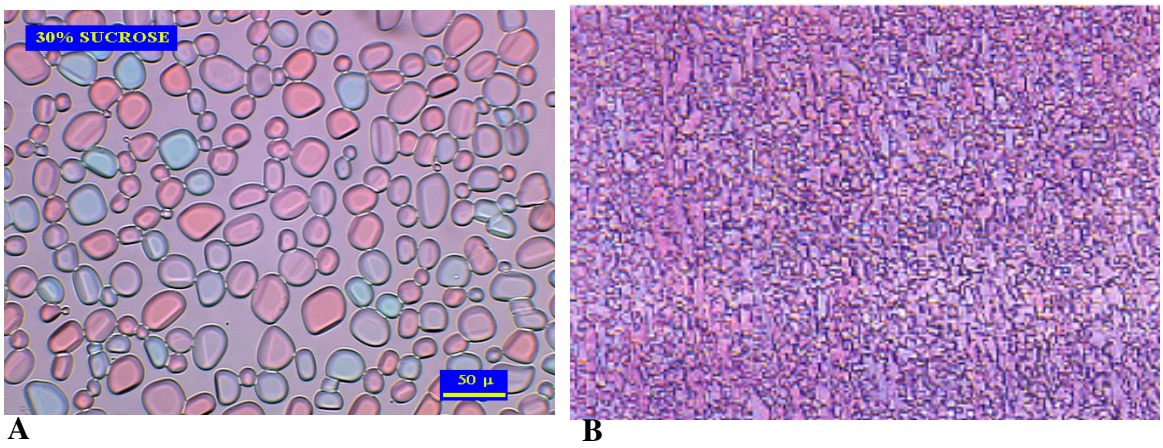


Figure 1. Ice structure formed without ISP preparation present during freezing (A) and with ISP preparation present (B).

As ice particles are “interacting” at a high density of small particles, aggregation is promoted leading to highly connected ice structures giving an ice cream at extrusion that is firmer and has a higher viscosity. The ice crystals formed in the presence of ISP aggregate to form a network that is so highly connected that the resulting ice structure is ice-continuous rather than matrix-continuous (Figure 2). ISP itself, unlike stabilisers or thickeners, provides no structuring to the product; this is exclusively provided by the ice.

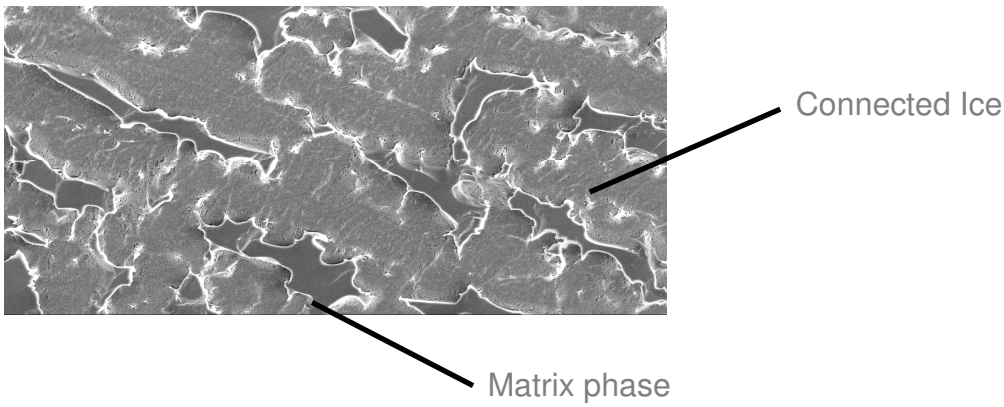


Figure 2. An electron micrograph showing the continuous ice phase of an ISP-produced water ice.

2.4 What are the Benefits to Consumers of ISP Preparation-containing Products?

Unilever has been making edible ices for over 70 years. It currently produces a range of edible ices (including ice creams, milk ice, frozen desserts, fruit sorbets fruit ice and water ices) in diverse formats such as individual portions, e.g. ice cream cones through to scoopable ice cream.

Until recently, the ability to influence the properties of the ice phase during manufacture of edible ices has been limited. Using ISPs, it is now possible for the first time to influence the ice phase of the product resulting in a wider range and variety of products including healthier products. It is the intention to include ISP preparation into some of our current product portfolio as well as to extend its use into new products.

The ISP preparation produces a variety of benefits to consumers including nutrition profiles (such as the amount of calories, fat, saturated fat, sugars and fruit), organoleptic properties (such as hardness, brittleness or creaminess and enhanced flavour delivery) and also greater temperature stability (an important factor in maintaining good quality products throughout the supply chain). All of the product properties and benefits are a consequence of the increased connectivity of ice produced in the presence of ISP preparation. The exact nature of the product and the benefits are dependent on the formulation, the amount of ISP preparation and the processing step used. However, by varying these three factors, a variety of product types with different properties and benefits can be achieved for the consumer.

Improved Nutritional Profile:
The inclusion of ISP preparation in edible ices can improve the nutrition profile and final "healthiness" of the products for the consumer. Using ISP preparation it is possible to produce high quality better tasting products that, compared to the standard, are:

- lower in fat, and/or
- lower in added sugar, and/or
- with increased fruit content.

The use of ISP preparation will provide real nutrition benefits to the consumer. See Table 1 (as this is confidential information it is provided in Appendix 2) for examples.

Enhanced/New organoleptic properties:
ISP preparation allows Unilever to produce products with more intense flavour delivery, a wider range of novel textures and more intricate shapes (e.g. thin layers of different fruit tastes, chocolate-type texture flakes). Due to longer lasting product experiences in combination with the increased connectivity of ice, smaller portions are also possible.

2.5 Current Status of Regulatory Approval Worldwide for ISP Preparation

Regulatory approval for the use of ISP preparation has previously been obtained in Australia and New Zealand, Chile, Indonesia, Mexico and the Philippines based on local regulatory procedures. ISP preparation has been determined to be Generally Recognized as Safe (GRAS) in the United States, and this determination was reviewed and accepted

by the US Food and Drug Administration. ISP preparation is suitable for vegetarians and has been given Halal and Kosher approval in various countries.

3. SPECIFICATION OF THE NOVEL INGREDIENT

3.1 Description of the Novel Food Ingredient

As briefly described, Ice structuring protein Type III was originally isolated from the blood of the ocean pout, *Macrozoarces americanus*. The ISP from this fish consists of 12 isoforms that can be separated by high performance liquid chromatography (HPLC). Isoform HPLC 12 is the largest peak and is the most functionally active in *in vitro* ice structuring studies. It was this form, known as "ice structuring protein Type III HPLC 12," that was selected for commercial development. Ice structuring protein Type III HPLC 12 is specifically identified by accession number P19614 in the Swiss-Prot protein database. The native protein has the following properties:

- 66 amino acids:

Asn	Gln	Ala	Ser	Val	Val	Ala	Asn	Gln	Leu	Ile	Pro	Ile	Asn	Thr
Ala	Leu	Thr	Leu	Val	Met	Met	Arg	Ser	Glu	Val	Val	Thr	Pro	Val
Gly	Ile	Pro	Ala	Glu	Asp	Ile	Pro	Arg	Leu	Val	Ser	Met	Gln	Val
Asn	Arg	Ala	Val	Pro	Leu	Gly	Thr	Thr	Leu	Met	Pro	Asp	Met	Val
Lys	Gly	Tyr	Pro	Pro	Ala									

- Molecular weight of 7.027 kDa
- Contains three flat surfaces, with a series of "bound" water molecules
- Globular protein with short β strands (50%) and hydrophobic patches
- Not glycoconjugated
- Isoelectric point: 6 – 10
- pH range for stability: 2 – 12
- Heat stability: Heat tolerant

The Ice structuring protein Type III HPLC 12 preparation (ISP preparation) is a light-brown liquid produced by submerged fermentation of a genetically modified strain of food-grade bakers yeast *Saccharomyces cerevisiae* (VW strain), in which a gene for ISP Type III HPLC 12 has been inserted into the yeast's genome. The protein is expressed and secreted into the growth medium, which allows the ISP Type III to be removed from the genetically modified yeast cells at the first stage of down-stream processing. The yeast cells are removed during processing. Ice structuring protein Type III preparation consists of ISP Type III HPLC 12 protein, glyco-ISP Type III HPLC 12, proteins and peptides from the yeast, and sugars, acids, and salts commonly found in food. The concentrate is stabilised with 10 mM citric acid buffer.

3.2 Specification of the Novel Food Ingredient

The ISP preparation is produced in accordance with good manufacturing practices, and is free from foreign material and contamination. The specifications for the preparation are as follows

Assay	Not less than 5 g/l active ISP Type III HPLC 12	
pH	3.0 +/- 0.5	
Ash	Not more than 2%	
Heavy metals	Not more than 2 mg/l	
Microbiology	Total microbial count:	< 3000 per g
	Coliforms:	< 10 per g
	Yeast and Mould count:	<100 per g
	(Production yeast	absent by test)
	Listeria spp:	absent in 25 g
	Salmonella spp:	absent in 25 g
	Staphylococcus aureus:	< 10 per g
	Bacillus cereus:	<100 per g

These requirements are based upon those for enzymes in *Food Chemicals Codex* (2001) due to the similarity of production processes and use levels in food.

3.2.1 Packaging and Storage

The commercial material will be shipped frozen in clean, sealed containers. Efficacy data has shown that Ice structuring protein Type III preparation is stable at -20°C for extended periods without preservatives. The recommended storage time, frozen, is six months.

3.2.2 Proposed Labelling

Consistent with existing EU Novel Food authorisations, Unilever proposes to include the words 'Ice Structuring Protein' in the list of ingredients. This approach is also consistent with non-EU countries where ISP is currently in products (section 2.5).

3.3 Characterisation of Commercial ISP Preparation

Analyses were carried out to characterise the commercial material. Various production batches were characterised (Study AC020025 (Appendix 3) and associated studies AC000082, AC000169 & CA010209 (Appendices 4-6)) and the data for 5 typical production batches, along with a concentrated batch of ISP used in toxicological testing (Batch 201008) are presented in Table 2 (overleaf). A minimum mass balance of 97.9% (w/w) could be achieved and all batches were found to be homogenous. The variation between batches is due to the concentration step during production. The difference between quantifiable and total solids is reflective of the cumulative variability inherent in the large number of analytical techniques used for characterisation.

Table 2. Composition data of five commercial batches of ISP Type III preparation and the concentrated Batch 201008. (Study AC020025 (Appendix 3)).

	ISP Type III Batch					
	201008	200030	200034	200046	201024	201083
ISP III HPLC 12 content (% w/w)	2.73	0.54	0.48	0.49	0.62	0.83
(g/L)	29.0	5.5	4.8	5.0	6.2	8.4
Total Kjeldahl protein (% w/w)	7.71	1.49	1.41	1.62	2.33	3.09
(g/L)	81.7	15.0	14.3	16.4	23.7	31.5
Ratio ISP III HPLC 12/Total Kjeldahl protein (% w/w)	35.4	36.2	34.1	30.5	26.6	26.8
Estimation by Gel Filtration Chromatography (% of total protein)	24 (~19 g/L)	22 (~3.3 g/L)	18 (~2.6 g/L)	20 (~3.3 g/L)	23 (~5.5 g/L)	25 (~7.9 g/L)
glyco-ISP III HPLC 12	31	23	24	22	29	32
Yeast proteins	9	20	24	28	22	17
Peptides						
Mannose (% w/w)	9.9	1.44	1.52	1.53	2.6	3.6
Glucose (% w/w)	<1.8	<0.25	<0.25	<0.25	<0.46	<0.46
Citric acid (g/L)	1.1	2.0	1.5	1.7	1.3	0.46
Pyruvic acid (g/L)	<0.2	<0.15	<0.15	<0.15	<0.5	<0.5
Succinic acid (g/L)	<0.2	<0.15	<0.15	<0.15	<0.5	<0.5
Lactic acid (g/L)	<0.2	<0.15	<0.15	<0.15	<0.5	<0.5
Acetic acid (g/L)	<0.2	<0.03	<0.03	<0.03	<0.5	<0.5
Ethanol (g/L)	<0.2	<0.15	<0.15	<0.15	<0.5	<0.5
Glycerol (g/L)	<0.2	<0.15	<0.15	<0.15	<0.5	<0.5
Acetaldehyde (g/L)	<0.2	<0.15	<0.15	<0.15	<0.5	<0.5
Sodium (ppm)	28	51	33	39	40	29
Potassium (ppm)	11	50	26	28	38	20
Magnesium (ppm)	7.2	4.9	4.9	6.1	6.1	5.8
Calcium (ppm)	85	58	47	54	61	72
Phosphate % (w/w)	0.40	0.14	0.10	0.13	0.17	0.18
Lead (ppm)	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Arsenic (ppm)	<0.5	<0.5	<0.5	<0.5	<0.5	<0.5
Ash (% w/w)	0.06	0.08	0.05	0.08	0.06	0.05
Ammonia (% w/w)	0.24	<0.04	<0.04	<0.04	<0.005	<0.005
pH	3.2	3.2	3.2	3.2	2.9	3.2
Protease activity (GU/mg)	1.9	0.51	0.47	0.53	0.70	0.83
Volatiles (% w/w)	81.0	96.6	95.9	96.0	92.7	92.2
Total solids (% w/w)	19.0	3.45	4.10	3.97	7.30	7.77
Quantified solids compounds (% w/w)	18.1	3.15	3.10	3.33	5.24	6.93
Mass balance (% w/w)	99.1	99.7	99.0	99.3	97.9	99.1

Mass balance (% w/w) = Volatiles + Quantified solid compounds

Quantified solid compounds = Total Kjeldahl protein + mannose + citric acid + minerals (Na, K, Mg, Ca, PO_4)

4. EFFECT OF THE PRODUCTION PROCESS APPLIED TO THE NOVEL FOOD INGREDIENT

4.1 Background & Overview

Ice structuring protein Type III HPLC 12 was selected for commercial application because it has appropriate functionality together with pH and temperature stability. As effective as it is, nevertheless hundreds of kilograms would be needed each year to commercialise edible ice products. Obtaining this amount from fish would be expensive, unreliable in quantity and quality, and seriously deplete fish stocks. As it is not economical, practical, or sustainable to obtain ISP directly from nature, it was necessary to develop an alternative source. The following were considered in selecting the commercial route of production:

- Availability in appropriate quantities and at acceptable cost: ISPs only occur at relatively low levels in the species that contain them and complex, lengthy, expensive extraction procedures are required to isolate them
- Sustainability: current fish stocks could not supply the amount of ISP Type III that is expected to be required without risk to the survival of the relevant fish species
- Specification: the material needs to be available at the appropriate specification, must be stable and should not be prone to seasonal variation
- Acceptability for food use: the material must be approved for food use by the appropriate authorities

To ensure a consistent, reproducible supply, the most sensible option was to produce the material by fermentation using a genetically modified microorganism. This approach is identical to that which has been taken for the production of many other food ingredients (e g., amylase, pectinase, xylanase, chymosin, vitamins), and is thus based on well proven technology.

The production process consists of fermentation with a genetically modified food-grade yeast *Saccharomyces cerevisiae* VW transformant, which carries a multi-copy insert containing a synthetic gene encoding ISP Type III HPLC 12 linked to the invertase signal sequence. It is under the control of the GAL7 promoter, integrated at the rDNA locus. Galactose is required to induce production of the ISP (Appendix 1 – confidential material). As previously mentioned, although the yeast-produced ISP Type III protein is identical to a protein found in certain fish, neither ISP Type III preparation nor the genetic material from which it is derived has ever had any contact with fish, and the ingredient has no sensory or other food characteristics associated with fish. It is a product of yeast.

The process is contained use, i.e. the yeast is used in sealed equipment that can be operated over a range of scales. Food-grade materials are used throughout, and the unit operations which make up the overall process are all commonly used in the food industry.

4.2 Production Process

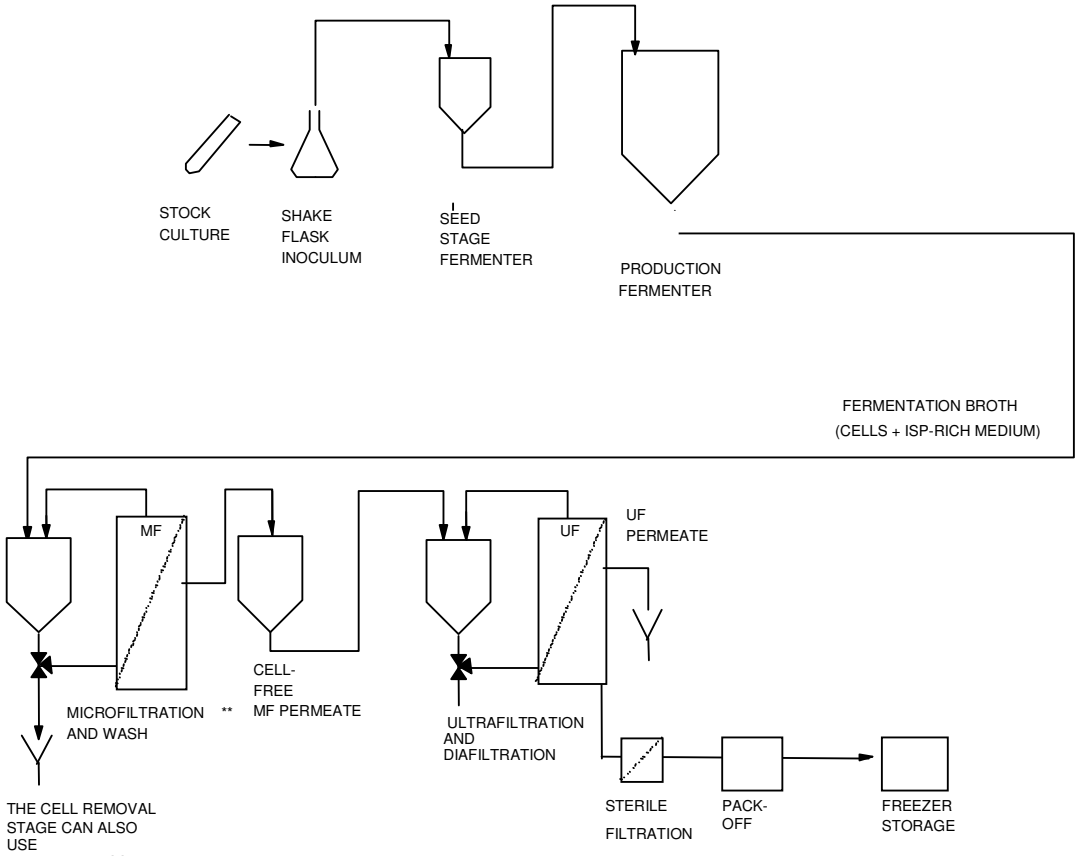
Production of ISP preparation uses standard industrial-scale biotechnology processes and standard food ingredients only. The process consists of the following phases:

- fermentation – the yeast is grown and induced to produce the ISP Type III protein
- cell removal – at the end of the fermentation the broth is filtered leaving a cell-free liquor
- concentration and packaging – the product is concentrated, washed, and packaged.

The process described here is at a 15,000 l scale. As inoculum, cells are grown on yeast nutrient broth (YNB) medium and transferred to yeast peptone dextrose medium. Five litres of this yeast peptone dextrose medium is subsequently transferred to a 300 l fermenter, containing 200 l batch medium. After a batch phase of approximately 22 h, the contents of the 300 l vessel are added to 6,000 l of fresh batch medium in a 15,000 l fermenter. Following a batch fermentation phase to produce biomass, there is a controlled feed phase which allows the yeast cells to continue growing slowly and to produce ISP Type III which is secreted from the cells into the medium. Subsequently, the yeast cells are separated from the broth by filtration (microfiltration or filter press). To increase the yield of ISP, the biomass is washed with water. An ultrafiltration (UF) step, which filters the liquor at a molecular scale (1 kDa), is then used to remove small molecules and to concentrate the ISP.

An outline of the production process is given in Figure 3 (overleaf) and a detailed description of the fermentation process is provided in Appendix 2 - Confidential Information. Please refer to section 3.3, table 2 for compositional data on commercial batches of ISP Type III preparation.

Figure 3. Schematic diagram of the fermentation and downstream processing of ISP Type III preparation.



5. HISTORY OF THE ORGANISM USED AS THE SOURCE OF NOVEL FOOD INGREDIENT

The host organism of the synthetic ISP gene is baker's yeast (*Saccharomyces cerevisiae). Saccharomyces cerevisiae* is the most widely used yeast in the food industry. Baker's yeast is employed for the manufacture of wine, beer and in the leavening of bread and as such has a long history of food use. The specific yeast strain used for the production of 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 (Appendix 7).The host yeast strain has also been used since 2003 for commercial production (60m^3 fermentation scale) of the ISP for use in our non-European ice cream business.

6. ANTICIPATED INTAKE/EXTENT OF USE OF THE NOVEL FOOD INGREDIENT

ISP Type III HPLC12 will be used in the production of edible ice products. The term 'edible ices' encompasses ice cream, including dairy ice cream, milk ice, water ice, fruit ice, sorbets, frozen desserts and any similar products such as iced smoothies and products of which edible ice is a component. ISP is proposed for use in products at levels not exceeding 0.01% by weight, and more commonly less than 0.005%.

The anticipated intake of ISP Type III HPLC 12 from its use in edible ices has been calculated for the UK, as follows:

The average daily edible ice intakes have been estimated using the UK NDNS data for children, young people and adults (Table 3). The UK NSDS data was collected during the period July 1992-June 1993 for children, during January-December 1997 for young people and during July 2000 - June 2001 for adults. The estimates are presented for consumers only. Consumers are those individuals who consume ice cream at some point during the survey period.

For the children and adults, the intakes are calculated by summing the total daily intakes of the selected edible ice products for each individual and then dividing by the total number of days in the survey. The child survey was a 4-day survey, and the adult survey was a 7-day survey.

A breakdown to daily level is not available for young people; therefore the total amount consumed in the survey period, of all ice cream products, is taken and divided by 7 to give an estimated daily average intake.

Table 3. Average daily edible ice intake estimated using the UK NDNS data for children, young people and adults.

	Average edible ice consumption (g/day)		
Age group	Percentiles	Males	Females
Children Aged 1.5-4.5yrs	5th	4.5	6
	10th	6.75	7.5
	50th	16	15.25
	90th	40	42
	95th	48.75	55.48
	97.5th	62	63.5
Young people Aged 4-6yrs	5th	5.71	4.29
	10th	7.29	4.57
	50th	16.71	13.71
	90th	32.71	40.43
	95th	45.43	45.57
	97.5th	59.43	73.00
Young people Aged 7-10yrs	5th	6.86	6.29
	10th	8.43	7.86
	50th	17.86	17.43
	90th	48.14	45.57
	95th	56.86	61.29
	97.5th	63.00	63.57
Young people Aged 11-14yrs	5th	6.71	4.29
	10th	8.14	5.29
	50th	22.14	17.71
	90th	57.71	42.86
	95th	80.86	61.71
	97.5th	98.71	76.14
Young people Aged 15-18yrs	5th	5.14	6.00
	10th	6.71	7.43
	50th	16.43	12.86
	90th	50.66	33.00
	95th	77.86	67.14
	97.5th	83.0	70.86
Young people Aged 4-18 yrs	5th	5.71	4.57
	10th	7.43	6.43
	50th	17.86	16.93
	90th	50.43	41.66
	95th	60.00	58.43
	97.5th	77.86	70.86
Adults Aged 19-64 yrs	5th	6.43	6.07
	10th	8.00	7.57
	50th	17.14	16.50
	90th	49.14	39.43
	95th	61.00	53.00
	97.5th	78.00	72.57

These intake data indicate that young (11-14 year old) UK males have the highest potential intake of edible ice per day, with an intake of 98.71 g/day for the 97.5th 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 9.871 mg of ISP. Using the average bodyweight recorded for this group (NDNS anthropometric data) of 47 kg, this would be an estimated daily intake (EDI) of 0.21 mg of ISP Type III HPLC 12/kg body weight.

Seasonality of edible ice intake in the UK was also considered and as each UK NDNS was carried out in four waves (Table 4), estimates of the average daily edible ice intakes for children, young people and adults, for each wave have been produced (Table 5). The estimates are presented for consumers only.

For the children and adults, the intakes are calculated by summing the total daily intakes of the selected edible ice products for each individual and then dividing by the total number of days in the survey. The child survey was a 4-day survey, and the adult survey was a 7-day survey.

A breakdown to daily level is not available for young people; therefore the total amount consumed in the survey period, of all edible ice products, is taken and divided by 7 to give an estimated daily average intake.

Table 4. The dates of each UK NDNS wave.

	Children	Young people	Adults
Wave 1	**July – Sept 92**	**Jan – Mar 97**	**July – Sept 00**
Wave 2	**Oct – Dec 92**	**Apr – Jun 97**	**Oct – Dec 00**
Wave 3	**Jan – Mar 93**	**July – Sept 97**	**Jan – Mar 01**
Wave 4	**Apr – Jun 93**	**Oct – Dec 97**	**Apr – Jun 01**

Table 5. Average daily edible ice intake, by survey wave, estimated using the UK NDNS data for children, young people and adults.

		Average Edible Ice consumption (g/day)			
Age group	**Percentiles**	**Wave 1**	**Wave 2**	**Wave 3**	**Wave 4**
Children Aged 1.5-4.5yrs	**5th**	4.33	4.75	5	6.25
	10th	7.5	7.25	7.25	7.5
	50th	17.75	15	15	15
	90th	44.5	36.25	36.58	41.75
	95th	58	46	53.25	49.5
	97.5th	63.5	62.5	60	65.55
Young people Aged 4-16yrs	**5th**	3.86	5.14	6.71	5.29
	10th	5.43	6.71	8.57	7.14
	50th	15.14	17.14	19	16.43
	90th	39.71	49.14	55	39.29
	95th	50.71	59.43	68	48.14
	97.5th	58.29	73	80.29	63.14
Adults Aged 19-64 yrs	**5th**	4.29	5.52	5.86	7.14
	10th	7.14	7.93	7.86	8.57
	50th	17.14	14.93	14.5	18.57
	90th	40.86	47	48.86	45
	95th	55.86	60.5	62.71	54.71
	97.5th	76.57	73.06	72.71	79.14

At the 97.5th percentile for adults and children there is a less than 10% difference in intake between the wave with the highest edible ice intake and the lowest (a difference of 9.25% (5.55g) for children and 8.8% (6.43g) for adults). However, there is a larger difference for young adults between the January-March wave and June-September wave of 37.7% (22g). These data suggest that for adults and children there is little change in edible ice intake at different times of the year, however for young adults there may be a lower intake in winter compared to summer. This information has been taken into account in the safety assessment in section 10.7.

Average daily ice-cream intakes have also been estimated for the Netherlands (Table 6) using the Dutch National Food Consumption Survey (DNFCS-3 1997-1998) data for children, young people and adults (excluding pregnant women). The estimates are presented for consumers only. Consumers are those individuals who consume ice cream at some point during the survey period.

Table 6. Average daily ice cream intake (g/day) estimated using the DNFCS-3 1997-1998 data for children, young people and adults.

Age (years)	N	Mean	5th	10th	50th	90th	95th
1-4	4	32	na	na	na	na	na
4-16	51	38.7	16	16	37.3	na	na
16-22	37	46.7	14.7	16	36	na	na
22+	241	49.3	16	22.7	49.3	77.3	100

na – not applicable

Using these data for the Netherlands, it is adults who have the highest potential ice-cream intake, with an intake of 100 g/day at the 95th percentile (due to the air content of ice cream, this equates to approximately 200 ml). If all this ice-cream were to contain ISP at the maximum proposed level of 0.01% by weight, this would equate to a daily intake of 10 mg of ISP.

Ice-cream intake in France appears to be lower, with an estimated average intake of 6 g/per adult/ day (both males and females), rising to 8 g/day for younger females and 9 g/day for younger males (CRÉDOC, Enquête individuelle et nationale sur les consommations alimentaires (INCA) 1999). If the highest potential ice-cream intake of the younger males is considered (9 g/day) and all the ice-cream were to contain ISP at the maximum proposed level of 0.01% by weight, this would equate to a daily intake of 0.9 mg of ISP.

7. INFORMATION FROM PREVIOUS HUMAN EXPOSURE TO THE NOVEL FOOD INGREDIENT OR ITS SOURCE

Ice structuring proteins are already consumed as part of the human diet. They are naturally occurring proteins and peptides that were first identified over thirty years ago in the blood of fish living in areas where the sea freezes, such as cod and herring (DeVries and Wohlschlag, 1969; Fletcher *et al*., 1999). Since then, ISPs have been found in a wide variety of organisms that need to protect themselves against freeze damage, including many plants, insects, fungi, and bacteria (Griffith and Ewart, 1995). Duman and Olsen (1993) noted that ISPs had been found in at least 23 species of angiosperms, including a number of edible ones. Plants in which ISPs have been found include such common food sources as oats, rye, barley, wheat, carrot, and potato (Griffith and Ewart, 1995). In many plants, they 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).

Although ocean pout, the fish from which the ISP that this application refers to was originally isolated, has no history of consumption in the European Community, it has been eaten in the North Eastern USA. More specifically, the ISP Type III content of ocean pout is estimated to be about 30 mg/ml in blood. Assuming that the blood volume of teleost (modern bony) fish is about 30-70 ml/kg (Olson, 1992), the ISP Type III content of an ocean pout will be around 900-2100 mg/kg thus a 200 g portion of ocean pout would result in an intake of between 180 mg and 420 mg of ISP Type III.

The exposure resulting from consumption of the proposed ISP Type III preparation-containing edible ice products would be well within the estimated current population exposures. For example, as explained in detail in section 6, the estimated daily intake of ISP Type III HPLC 12 for the group with the highest estimated edible ice intake in the UK (males aged 11-14 years at the 97.5th percentile) is 9.871 mg of ISP Type III HPLC 12 (0.21mg per kg bodyweight).

A more detailed review of the occurrence of ISPs in nature can be found in Crevel *et al*. (2002), this is also provided as Appendix 8.

ISP Type III HPLC 12 preparation-containing edible ices have been on the market in the USA since the second quarter 2003, with approximately 21.2 million such products sold through 2004 and approximately 128.1 million in 2005, with no reported consumer issues (each product carries details of an address and a website where complaints can be reported). ISP preparation has been used in a range of products within the USA including low/zero fat products in single portions (e.g. bars and sandwiches) and new water ice products.

ISP Type III HPLC 12 preparation-containing products have also been on sale in other countries. For example, over 52.6 million ISP-containing ice cream sticks (approx. 4 million litres of ice cream) have been sold in the Philippines since mid-February 2004.

8. NUTRITIONAL INFORMATION ON THE NOVEL FOOD INGREDIENT

At levels of proposed use (less than 0.01% by weight) there are no nutritional implications of the ISP preparation.

The ISP Type III HPLC 12 protein sequence is comprised of amino acids commonly found in the human diet, and would be digested as protein according to normal metabolic processes. The very small amounts of ISP used in foods would not make any significant contribution to protein consumption patterns. There is no evidence that ISP interferes with or modifies the absorption or metabolism of any other macro- or micronutrient.

No dietary implications are anticipated either. Use of ISP preparation in products is not expected to significantly change population consumption of edible ices, but rather to influence product choice within that market.

9. MICROBIOLOGICAL INFORMATION ON THE NOVEL FOOD INGREDIENT

The stability of all edible ice products is dependent upon several factors such as:

- Composition
- Initial microbiological quality of ingredients and finished product
- Storage & distribution

The microbiological stability of edible ices containing ISP Type III HPLC 12 is determined by the same principles.

Formulation and process rules currently used to ensure the safety of edible ices are equally applicable to product containing ISP Type III HPLC 12. The accepted principles of Good Manufacturing Practice (GMP) will be used in the production of edible ices containing ISP Type III HPLC 12.

The process and distribution conditions used to produce the products containing ISP Type III HPLC 12 will be identical to those currently used for edible ices and no additional controls are considered necessary.

The microbiological specification set for the ISP preparation is in accordance with that for food enzymes (Food Chemicals Codex (2001)) and is as follows:

Total microbial count	< 3000 per g
Coliforms	< 10 per g
Listeria spp.	absent in 25 g
Salmonella spp.	absent in 25 g
Yeast and moulds	< 100 per g (GM yeast absent by test)
Staphylococcus aureus	< 10 per g
Bacillus cereus	<100 per g

The results of microbial analyses for all ISP commercial runs are summarised overleaf in Table 7.

Table 7: Summary of the microbial analyses of all ISP commercial runs.

		Fermentation Run									
		F565	F577	F587	F593	F597	F605	F630	F632	F634	F641
Microbial analysis	**Specification**										
Standard Plate count	<3,000/g	<1	10	1	3	1	<1	3	1	<1	<1
Coliforms	<10/g	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Listeria spp	absent in 25g	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative
Salmonella spp	absent in 25g	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative
Yeast and Mould count	<100/g	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Staph. Aureus	<10/g	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Bacillus Cereus	<100/g	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Foreign materials	Negative by test (<10u/5g)	negative	negative	negative	negative	negative	negative	negative	negative	negative	negative