

ADVISORY COMMITTEE FOR NOVEL FOODS AND PROCESSES

DRAFT OPINION ON AN APPLICATION UNDER THE NOVEL FOODS REGULATION FOR ICE STRUCTURING PREPARATION DERIVED FROM FERMENTED GENETICALLY MODIFIED BAKER'S YEAST *Saccharomyces cerevisiae* AS A FOOD INGREDIENT

Applicant: Unilever PLC
Responsible Person: Dr Nigel Lindner
EC Classification: 5.1

Introduction

1. An application was submitted by Unilever PLC on 15 June 2006 for the authorisation of an ice structuring protein Type III HPLC 12 preparation derived from a fermented genetically modified baker's yeast as a novel food ingredient. A copy of the application dossier was placed on the FSA website for public consultation.
2. Ice structuring proteins (ISP) are naturally occurring proteins and peptides, which are found in a variety of living organisms such as fish. ISP protect them from damage to tissues in very cold conditions by lowering the temperature at which ice crystals grow and by modifying the size and shape of ice crystals. ISP found in ocean pout¹ are defined as Type I, II, III or IV. Twelve different ISP type III have been identified in the serum of ocean pout using high performance liquid chromatography (ISP Type III HPLC 1-12).
3. Sourcing ISP Type III from ocean pout is not sustainable or economically feasible. The applicant has therefore developed a fermentation system using a genetically modified baker's yeast (*Saccharomyces cerevisiae*) carrying a synthetic gene encoding for the ISP Type III HPLC 12.
4. Unilever seeks approval to market its ISP Type III HPLC 12 preparation in edible ices at level not exceeding 0.2%. The presence of the ISP Type III HPLC 12 during the manufacture of frozen products, at the freezing stage, causes ice crystals to form in a particular way so that there are a large number of very small crystals. Normally, in these products, there are a small number of relatively large ice crystals. The continuing presence of the ISP is not necessary for the maintenance of the small crystal size once the product is frozen. Physical interactions between the very small ice crystals provide a structure that differs from conventionally frozen iced products. This effect allows, for example, the production of ice cream with a low fat content.

¹Cold water fish found off the North East American coast (*Macrozoarces americanus*)

5. The applicant's ISP Type III HPLC 12 preparation has already been authorised in Australia, New Zealand, Chile, Indonesia, Mexico, the United States and the Philippines under their local regulatory procedures². In the EU, the proposed ISP Type III HPLC 12 preparation is considered to be a food ingredient with no significant history of consumption in the EU prior to 15 May 1997. It therefore falls under the scope of the novel food regulation (EC) 258/97 (Article 1(2)(d)). This was confirmed at the Standing Committee on Food Chain and Animal Health meeting of 14 December 2006³.
6. The application for authorisation of this preparation was prepared pursuant to Commission Recommendation 97/618/EC of 29 July 1997 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. This preparation has been classified as a product of a GM microorganism, the host microorganism used for the genetic modification having a history of use as food or as a source of food in the Community under comparable conditions of preparation and intake (class 5.1).
7. EFSA have recently published their guidance document on the risk assessment of genetically modified microorganisms⁴. This guidance document describes three distinct groups of genetically modified microorganisms (GMMs). The ISP Type III HPLC 12 preparation would be classed as a group 2 GMM "*Complex products derived from GMMs but not containing viable GMMs nor unit length of any cloned (foreign) open reading frames (e.g. lysed cell extracts, some feed enzymes, wine, some beers, etc.)*" as, although it has been partially purified, the composition of the preparation has not been fully defined. A summary of the information required of applications for the placing of food/feed products derived from GMMs on the market is provided in Annex 1 below, with an indication of the corresponding sections of the application dossier.

I Specifications of the novel ingredient (NI)

Information on this aspect is provided on p. 10-12 of the application dossier

8. The novel food ingredient (NI) is a yeast-derived preparation containing a particular type of ISP known as ISP type III HPLC 12. Isoform HPLC 12 (ISP type III HPLC) is the most functionally active form of type III ISP *in vitro* and is composed of 66 amino acids.
9. The NI is a light brown liquid and consists of ISP type III HPLC protein, glycosylated ISP type III HPLC 12, and other components derived from the

² See Food Standards Australia New Zealand initial assessment report (October 2004) http://www.foodstandards.gov.au/srcfiles/A544_ISP_IAR.pdf and a response from the US FDA concerning the manufacturer's determination that the ISP preparation is Generally Regarded as Safe (April 2003) <http://www.cfsan.fda.gov/~rdb/opa-g117.html>

³ See under Item 7 at:

http://ec.europa.eu/food/committees/regulatory/scfcah/toxic/summary23_en.pdf

⁴ A summary of the information required of applications for the placing of food/feed products derived from GMMs on the market is provided in Section E Table 1 of the guidance document (pp52-

58):http://www.efsa.europa.eu/etc/medialib/efsa/science/gmo/gmo_guidance/gmo_guidance_ej374.Par.0001.File.tmp/gmo_guidance_ej374_gmm.pdf

fermentation (proteins and peptides from yeast, sugars, acids and salt). The concentrate is stabilised with 10mM citric acid buffer.

10. The NI is produced according to Good Manufacturing Practice and the applicant has confirmed the following specification:
 - Assay – Not less than 5g/l active ISP type III HPLC 12
 - pH – 3.0 +/- 0.5
 - Ash – Not more than 2%
 - Heavy metals – Not more than 2mg/l
11. The applicant has provided compositional data on five commercial batches of the NI and data on one batch of concentrated NI in table 2 of the application dossier. Some key parameters from the commercial batches are summarised in the attached table A:
12. The applicant has stated that any variations observed between the batches are due to the concentration step employed during the production. The differences between the quantifiable and total solids are reflective of the cumulative variability inherent in the large number of analytical techniques used for characterisation. The analysis demonstrated a minimum mass balance of 97.9% (w/w) and all batches of the NI were found to be homogenous.
13. The applicant has stated that the NI is stable at -20°C for extended periods without preservatives. The final commercial material will be shipped in frozen sealed containers and the recommended storage time will be 6 months.
14. The applicant was asked to provide further information on the variability between batches of the ISP preparation, including the extent and pattern of glycosylation. The applicant indicated that the GM yeast strain used to produce the NI has a limited ability to glycosylate proteins, resulting in the majority of ISP III-12 proteins in the NI being unglycosylated. The amino acid sequence of the ISP III-12 has 8 theoretical sites for O-glycosylation. The applicant provided liquid chromatographic-mass spectroscopic analytical results on commercial batches of the NI showing that the pattern of glycosylation is constant between batches and has been shown to be unaffected by either process or media changes. The applicant has also provided results from gel filtration chromatography on commercial batches of the NI indicating that 40% of the total ISP III-12 is glycosylated, of which 75% in glycoform I and 25% in glycoform II. The applicant highlighted that the glycosylated ISP III-12 is inactive and only the non-glycosylated ISP III-12 can bind to ice crystals. The applicant was of the view that the presence of glycosylated ISP III-12 in the NI will not affect its binding properties. The applicant also provided additional information on the purpose of the glycosylated ISP III-12 in the NI and indicated that the glycosylated ISP III-12 is inactive and only the non-glycosylated ISP III-12 can bind to ice crystals. The applicant was of the view that the presence of glycosylated ISP III-12 in the NI will not affect its binding properties. In response to a request by the ACNFP, the applicant has also confirmed that the inactive glycosylated form of ISP protein has no function in the preparation. The application draws a parallel with the manufacture of food enzyme preparations, which are generally subjected to minimal processing in order to maintain high functional activity, resulting in varying degrees of purity. The applicant also points out that an

extensive test regime has been carried out on the complete ISP preparation to ensure that it is safe for human consumption.

Discussion: After questioning the applicant about the reason for glycosylated proteins being present in the NI, the Committee was content that these were inactive and that the purification process is designed to retain the maximum functional activity. The Committee therefore accepted the applicant's proposed specification for the NI. The Committee also noted that the complete ISP preparation has been submitted to toxicological tests to verify that it is safe for human consumption (See section XIII).

II. Effect of the production process applied to the novel food

Information on this aspect is provided on p. 13-14 of the application dossier

15. The production process involves fermentation with a genetically modified food grade yeast (*S. cerevisiae*) in 15,000 litre batches in sealed fermentation vessels (i.e. under contained use conditions). The applicant states that all steps in the production process are commonly used throughout the food industry. The 3 main steps are as follows:

- Fermentation – the volume is scaled up in stages to 15,000 litres and protein production is then induced. ISP is secreted into the medium during a controlled phase of slow growth;
- Cell removal – after fermentation the medium is filtered by microfiltration or filter press leaving a yeast cell free liquid. The yield and purity of the protein is increased by washing the remaining biomass with water;
- Concentration – cell removal is followed by an ultrafiltration step which retains all material above 1kDa, including ISP which is 6kDa, but removes small molecules. The product is then packaged and stored in frozen sealed containers.

Discussion: The Committee was satisfied with the applicant's proposed production process for the NI.

III. History of the organism used as a source of the novel food

Information on this aspect is provided on p. 16 and Appendix 7 of the application dossier

16. The parent organism *Saccharomyces cerevisiae* has been widely used in the food industry for fermentation purposes for a very long period. The specific yeast strain used for production of ISP, a derivative of strain CEN.PK, has been classified in the Netherlands, under Council Directive 90/219/EC⁵, as belonging to Group 1 AB. The applicant also noted that commercial production of ISP for markets outside Europe commenced in the second quarter of 2003.

Discussion: The Committee was content with the information provided on the history of the GM *Saccharomyces cerevisiae* strain used by the applicant as a source of the NI.

⁵ Council Directive 90/219/EC of 23 April 1990 on the contained use of genetically modified micro-organisms

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

Information on this aspect is provided in Appendix 1 of the application dossier

17. The expression vector contains a synthetic gene coding for ISP type III HPLC 12 originating from ocean pout. This ISP has the same amino acid sequence as the ocean pout ice protein, but the nucleotide sequence has been engineered to reflect optimal codon usage in yeast, thus maximising expression in this host.
18. The vector used to introduce the ISP expression cassette was designed to integrate the expression cassette into the ribosomal DNA (rDNA) of the yeast genome. The resulting yeast strain, CENPK338, contains a multicopy expression cassette inserted at the rDNA locus with no antibiotic resistance markers and no bacterial or fish DNA.
19. The copy number of the inserted expression cassette and the integration site have been determined by Southern blotting. The absence of the ampicillin selectable marker and the location of the integration site were also confirmed by PCR.
20. The applicant was asked to provide additional data on the molecular characterisation of the insert in the GM yeast, or comparative protein analysis of the GM yeast and its conventional counterpart. The additional molecular data provided included a more detailed description of the vector, the insertion event and its characterisation. Unlike most eukaryotes, insertion of transformed DNA into the yeast genome occurs through homologous recombination. In addition, the efficiency of targeting is increased in direct proportion to number of copies of the target gene. The integration system used by the applicant exploits these two features of homologous recombination in yeast by targeting the vector to the multicopy ribosomal DNA locus. After targeted integration into the yeast genome the copy number of the expression cassette was increased by selection under growth conditions (prototrophic growth) that require multiple copies of the defective *Leu2* gene. Southern blotting revealed two bands of the expected size, with a high intensity band of 6.2Kb representing the multicopy expression cassette and a faint band of 2.2Kb representing the chromosomal *Leu2* fragment. The copy number of the 6.2Kb fragment was estimated at 30-50 copies. The absence of any other bands was interpreted as indicating that the expression cassette was integrated exclusively as tandem repeats. PCR of the flanking sequences using appropriate 5' and 3' primer pairs for the rDNA locus and the insert revealed bands of roughly the expected size for the 2 flanks; their size and identity was confirmed by cloning and sequencing.
21. Following the Committee's consideration of the above information, the applicant was asked to provide further data to demonstrate the absence of secondary integration sites in the genome of the host organism and on the sequence analysis of the flanking regions of the insertion site(s) to check whether this revealed the creation of any potential open reading frames in these regions. The applicant has not found any secondary integration sites in the genome of the *S. cerevisiae* used for producing the ISP preparation. The applicant provided a figure showing restriction maps of the DNA structure generated on integration of the cassette and the fragments detected. Results obtained using five different restriction enzyme digests did not demonstrate the

presence of a secondary integration site and the applicant was of the view that it is unlikely that this site would be masked, following this digestion. The applicant concluded that the rDNA locus was the sole location for integration of the expression cassette. Finally, the applicant also highlighted that the mechanism of integration regenerates the existing NTS1 sequence in rDNA, as confirmed by sequencing of boundary fragments. Integration therefore did not lead to generation of any additional open reading frames.

Discussion: The Committee agreed that the tests carried out by the applicant had confirmed that the inserted DNA had been integrated at the expected site. The Committee was reassured by the further information provided by the applicant which showed that there was only one integration site and that no additional open reading frames were generated.

V. Genetic stability of the GMO

Information on this aspect is provided in Appendix 1 of the application dossier

22. Strain stability was measured after more than 70 generations of growth under non-selective conditions. The following parameters were compared:

- Cell viability;
- Presence of the ISP gene (as detected by PCR);
- Structure of the integration site (as revealed by Southern blotting);
- Protein expression levels (under inductive growth conditions).

23. The applicant states that no differences were found for any of the parameters measured after the period of growth used for comparison.

Discussion: The Committee was content with the information provided by the applicant on the genetic stability of the genetically modified yeast used for the production of the NI.

VI. Specificity of expression of novel genetic material

Information on this aspect is provided in Appendix 1 of the application dossier

24. Expression of the ISP is under the control of an inducible pGAL7 promoter that only permits high levels of expression of the protein in the presence of galactose. Expression is repressed during growth in the presence of more than 0.5% glucose.

Discussion: The Committee noted the above information and did not raise any concerns.

VII. Transfer of genetic material from GM microorganisms

Information on this aspect is provided in Appendix 1 of the application dossier

25. The applicant has tested the ISP preparation for contamination with DNA derived from the inserted ice structuring protein gene using an ISP gene specific PCR assay. No DNA contamination was detectable using this

approach. The detection limit was estimated at 2×10^{-10} g ISP plasmid DNA/g of lyophilised ISP protein preparation.

Discussion: The Committee was satisfied that no DNA derived from the ISP gene inserted in the GM baker's yeast had been detected in the NI, at the limit of detection of the PCR method used.

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

Information on this aspect is provided in Appendix 1 of the application dossier

26. The production process is designed to remove all yeast from the ISP preparation and the final product should not contain any GM microorganism that could survive in or colonise the human gut.

Discussion: The Committee was content that there will be a filtration step within the production process to remove the GM yeast cells. The GMM will therefore not be present in the NI. The Committee noted that yeast proteins will however be present (See section XIII).

IX. Anticipated intake/extent of use of the novel ingredient

Information on this aspect is provided on p. 16-19 of the application dossier

27. The applicant intends to use the NI in edible ice products to improve their nutrition profiles, organoleptic properties (taste and mouthfeel) and stability. The term "edible ices" encompasses ice cream, including dairy ice cream, milk ice, water ice, fruit ice, sorbets, frozen desserts and products such as iced smoothies. The level of ISP will not exceed 0.01% by weight and will more commonly be less than 0.005% in the final product. As the ISP comprises 5-8% of the commercial product, the level of addition of the ISP preparation or the NI will be up to 0.2%.

28. The anticipated intake of ISP Type III HPLC 12 from its use in edible ices has been calculated using the latest UK NDNS data for children, young children and adults. Estimates are for consumers only, which means that only those who have consumed ice cream at some point during the survey period are included.

29. Results are given as daily edible intakes estimated as grams per day. Based on the information given the applicant estimates that boys aged 11-14 have the highest potential intake of edible ice per day with a high level (97.5th percentile) intake of 99 g/day. Using the maximum proposed level of inclusion of the NI and the average recorded body weight for this group of 47 kg, the estimated daily intake is 0.21mg of ISP type III HPLC 12 /kg body weight. The applicant's estimates for each age group are presented in the table B:

30. The NDNS surveys were carried out in 4 waves, covering January to March, April to June, July to September and October to December and the applicant has taken seasonal differences into consideration by providing estimates for each of the waves. This has found that, at the 97.5th percentile, for adults and children, there was only a small difference between the highest and the lowest consumption estimates, suggesting there is little change amongst those who consume edible ices in each season. However, there is a larger difference

between the January to March wave and the June to September wave in the survey of young people (ages 4-16), where high level consumption (97.5th percentile) increases from 58 to 80 grams/day.

31. In order to complement the information provided in the original dossier, which gave seasonal data intake estimates for ISP from its use in edible ices only for the combined 4-18 age group, the UK Competent Authority asked the applicant to provide a breakdown of these estimates for the age bands 4-6 years, 7-10, 11-14 and 15-18, using the latest UK NDNS data (see below). The applicant provided the estimates of the daily consumption of edible ices by young people, broken down by both season and age (see Table C):
32. Although these estimates of high level consumption are less robust due to the relatively small number of consumers in each sub-group, the data show that the highest estimated intake, on a body weight basis, is in 4-6 year old children during the summer months (equivalent to 0.38 mg of ISP III-12 per kg bodyweight per day). Although this exceeds the highest estimates mentioned in the application dossier, the applicant points out that there is still a factor of 1500 between this and the NOEL of 5.8 g/kg bw/day observed in the animal feeding studies (expressed as ISP III-12).
33. The applicant has also estimated daily intakes of ice cream for the Netherlands using the 1997-98 Dutch National Food Consumption Survey and for France using CREDOC, Enquête individuelle et nationale sur les consommations alimentaires (INCA, 1999). In the Netherlands, the highest consumption of ice cream is found in adults, where high level consumption (95th centile) is 100g/day⁶. Ice cream consumption recorded in the French survey is lower, with an average value of less than 10 grams/day in all age groups.

Discussion: The Committee considered that the consumption of the NI at the proposed levels of incorporation on edible ices did not raise any specific concerns.

X. Information from previous human exposure to the novel ingredient or its source

Information on this aspect is provided on p.16 of the application dossier

34. ISP occur naturally in the blood of fish living in areas where the sea freezes, such as cod and herring, and so are normally consumed in the diet. They are also found in edible plants such as oats, rye, barley, wheat, carrot, potato, taproot and leaves of Brussels sprouts. However, despite their similar functionality, ISP have a range of different structures and it is not possible to draw any meaningful comparisons with the NI.
35. 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 is consumed in North Eastern USA. The applicant suggests that eating a 200g portion of ocean pout would result in an intake between 120 and 420 mg of ISP type III. This is higher than the estimated daily intake from edible ices (see above).

⁶ Note: The Dutch values are averaged over only 2 days, compared with 7 days in the British surveys (or 4 days for pre-school children). On statistical grounds it is to be expected that the observed high level consumption of most foods will decrease as the survey period increases.

36. In addition to the ISP and its glycosylated counterpart, the NI contains other components derived from the fermentation. *Saccharomyces cerevisiae* has a very long history of use in food production and there is therefore a long history of consumption of the yeast itself and its fermentation products.
37. The applicant states that edible ices containing the NI have been on the market in the USA since the second quarter of 2003 with no reported consumer issues. Similar products have also been on sale in other countries such as the Philippines since 2004.

Discussion: The Committee was content with the information provided on previous human exposure to the NI and its yeast source.

XI Nutritional information on the NF

Information on this aspect is provided on p.21 of the application dossier

38. As the NI will be used in edible ices at a level not exceeding 0.2% by weight (equivalent to 0.01% of the ISP component), the applicant has stated that no nutritional implications are expected. The NI's protein sequence is comprised of amino acids which are commonly found in the human diet and for this reason it would be digested as a protein according to normal metabolic processes and will not have any significant effect on total protein intake. The NI would not displace existing ingredients, although its use might facilitate the manufacture of ice cream products with as reduced fat content.

Discussion: The Committee was satisfied with the nutritional information provided for the NI.

XII. Microbiological information on the novel food

Information on this aspect is provided on p.21-22 of the application dossier

39. The microbiological specification for the NI is as follows:

| | |
|------------------------------|----------------------------------|
| Total microbial count | <3000/g |
| Coliforms | <10/g |
| <i>Listeria</i> spp. | Absent in 25g |
| <i>Salmonella</i> spp. | Absent in 25g |
| Yeast and mould count | <100/g (GM yeast absent by test) |
| <i>Staphylococcus aureus</i> | <10/g |
| <i>Bacillus cereus</i> | <100/g |

40. Table 7 in the dossier summarises the microbiological analysis of 10 commercial batches of the NI.
41. The microbiological safety of the edible ices containing the NI will be ensured by using the accepted principles of good manufacturing practice and conditions for processing and distribution currently applied to edible ices. The applicant considers that no additional controls will be necessary.

Discussion: The Committee was of the view that the microbiological safety of the NI had been demonstrated.

XIII. Toxicological information on the novel food

Information on this aspect is provided on p.23-71 of the application dossier

42. The applicant has carried out an evaluation of the general toxicity and genotoxicity of the NI. The potential allergenicity of the NI was also assessed.

(a) Toxicological and genotoxicological assessments

Information on this aspect is provided on p.23-30, Appendices 6 and 10-15 of the application dossier

43. The applicant has provided details of a number of toxicological and genotoxicological studies carried out on the NI. The results of these studies are presented in the attached Table D. To increase their sensitivity these tests were conducted on a specially prepared batch of the NI, designated 201008, which was subjected to an additional concentration stage using ultrafiltration to remove excess water and low molecular weight components. The applicant was asked to provide additional information on batch 201008 of the NI regarding the way it was prepared and how its composition compares with the commercial product. The applicant explained that the final stage of the production process of the NI involves ultrafiltration with Synder spiral wound membrane modules (1 kDa). Batch 201008 is obtained by continuing the ultrafiltration for longer to obtain 30g/L of ISP III-12. Compositional comparison of batch 201008 with other batches of the NI is provided in table 2 of the dossier. The applicant has confirmed that the additional ultrafiltration does not modify the NI, as shown in study report AC000082 (Appendix 4 of dossier).

44. The applicant concludes that the NI does not present any toxicological or genotoxicological potential.

(b) Allergenicity assessment

Information on this aspect is provided on p.30-66, Appendices 17-20 (study reports), Appendices 20 and 22 (publications)

45. A summary of the tests assessing the potential allergenicity of the NI is given in table 9 (Annex 1, p.31). The results of these tests are summarised in the attached Table E.

46. The applicant concludes that the NI is safe for both fish-allergic individuals and other consumers.

47. The applicant was asked to provide further details on the amino acid sequence analysis of ISP III -12. The applicant has therefore explained that the original amino acid sequence analysis of ISP III-12 against public protein databases was carried out in 2001/02. This analysis generated some false positives and was not repeated. In 2005, the amino acid sequence of ISP III-12 analysis was analysed again using a customised allergen database (FARRP AllergenOnline database) and a general protein database (NCBI non-redundant database). This analysis did not reveal any significant sequence alignment with known allergenic proteins. The applicant concluded that ISP III-12 is unlikely to be a food allergen.

48. The applicant was also asked whether any information exists on the potential for the GM *Saccharomyces cerevisiae* proteins present in the preparation to induce allergic reactions in individuals sensitised to *Candida* 'yeast' or other fungi. The applicant indicated that sensitisation to yeast proteins most occurs

via the respiratory tract and via the skin, and there is no evidence to indicate that it arises from the consumption of foods and drinks containing *S. cerevisiae*. This conclusion is supported by the fact that the three allergens in *S. cerevisiae* namely enolase, manganese super-oxide dismutase and cyclophin have only been associated with inhalant and/or skin allergies. It is recognised that people with atopic dermatitis which is associated with allergic reaction to yeasts such as *Candida albicans*, *Pytirisporum ovale* and *Malassezia furfur* are likely to cross-react to *S. cerevisiae* proteins when challenged in skin prick tests or RASTs. The applicant however referred to conclusions from Kortekangas-Savolainen et al (1994) that "the IGE-mediated allergy to baker's yeast should not lead to the denial of bakery, brewery and wine products".

(c) Potential yeast (*Saccharomyces cerevisiae*) allergenicity assessment

Information on this aspect is provided on p.48-51 and table 16 of the application dossier

49. The applicant notes that three proteins identified in *Saccharomyces cerevisiae* are associated with inhalant and/or skin allergies and adds that "*all the fish allergic subjects who were skin prick test positive to yeast in the above studies are able to consume foods containing yeast without adverse reaction*" (see table 16, p.51). The applicant is therefore of the opinion that the yeast component of the NI does not pose a clinically significant allergic risk.

Discussion: *The Committee was satisfied with the toxicological assessment carried out by the applicant on the NI which showed that it is safe for human consumption at the proposed level of use. The Committee particularly discussed the following points:*

- **Inflammatory potential of the NI** – *during our public consultation on this application, a member of the public suggested a need to conduct studies to test long-term inflammation in both young and older animals. The Committee asked for expert advice on this point from specialists in animal pathology and immunology of the UK Committee on Toxicity of chemicals in food, consumer product and the environment. They were of the view that the applicant had carried out all the appropriate studies needed to assess potential immunogenicity. The 90-day study did not show any indication of any effects on the immune system, whether inhibitory or stimulatory, and there were no clinical signs of inflammatory responses that might justify further investigations in this area. The Committee therefore concluded that the NI did not have any inflammatory potential.*
- **Animal models of sensitisation** - *The Committee was of the opinion that animal models of sensitisation had improved in recent years and that the quote in the dossier from the 2001 WHO Rome conference to the effect that, "such models were at too early a stage of development to generate data for risk assessment", was no longer valid. The Committee discussed the value of having a study of sensitisation using the ISP preparation on an appropriate animal model and concluded that, in this case, this additional information was not necessary.*

- **Amino acid sequence homology of ISP Type III -12 with A.niger superoxide dismutase** - The Committee noted that reference to Baderschneider et al (2002) findings in the application that the match over a very short part of the amino acid sequences between a superoxide dismutase (allergen Asp f6) from *Aspergillus fumigatus* and ISP Type III was not significant and asked for an external expert view on that point. The expert's view was that the similarity between ISP type III and AspF6 was very low and it is very unlikely that *Aspergillus* allergic individuals will react to ISP. The Committee therefore concluded that the NI will not induce a reaction in *Aspergillus* allergic individuals
- **Fish allergy** – The Committee queried whether the results from tests on cod-allergic people was sufficiently representative of fish allergy in general. The Food Standards Agency's allergy experts indicated that sera from cod allergic subjects are considered to be a relatively good candidate for assessment of whether the NI is likely to bind IgE of fish allergies. This is because of the high homology of parvalbumin (the major fish allergen) across fish species. Fish allergic individuals can be mono-sensitised to other non-parvalbumin allergens in fish, such as collagen, but this is relatively rare and it seems unlikely, although not impossible, that the non-parvalbumin allergens would cross-react with the ISP. Further, as cod allergic subjects, many of which also showed positive SPTs to ocean pout, eel pout and eel (indicating cross-reactivity among these species), did not react to the ISP preparation it seems unnecessary to extend the tests to subjects with other fish allergies besides cod. The Committee concluded that using the cod allergic individuals in Phase I of the assessment of the allergic potential of the ISP preparation is representative of the fish allergic population.
- **Yeast allergy** - The Committee did not agree with the applicant that the yeast proteins present in the ISP preparation did not present any potential allergenic risk and recommended that the labels of products containing the ISP preparation should indicate that it is derived from a yeast source.

Labelling

Information on this aspect is provided on p.11 of the application dossier

50. The applicant proposes to describe the NI as "Ice Structuring Protein" in the list of ingredients of edible ices, consistent with other ISP-containing products on the market outside the EU.

Discussion:

The Committee considered whether the NI should be labelled as derived from a GM source. It was stated in a recent Commission report⁷ that ingredients produced by fermentation using genetically modified micro-organisms not present

⁷ Section 10 of the Report from the Commission to the Council and the European Parliament on the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council on genetically modified food and feed, COM(2006) 626, October 2006.
http://eurex.europa.eu/LexUriServ/site/en/com/2006/com2006_0626en01.pdf

in the final product do not fall under the scope of legislation on GM food and therefore do not need to be labelled as GM under this specific piece of legislation. This conclusion applied to the NI as the yeast cells are removed from the final product.

The Committee was aware that other food ingredients derived from GM micro-organisms, such as enzymes used as processing aids and some highly-refined vitamins and amino acids, are not labelled to indicate their source. Nevertheless, the use of a synthetic gene sequence and the presence in the NI of a significant proportion of cellular by-products from the fermentation process such as yeast proteins (as noted in Section VIII above) made this a special case. In particular, the committee felt that the omission of this information through the absence of labelling could be potentially misleading to consumers.

The Committee therefore recommended that information should be provided to consumers indicating that the ingredient is manufactured using a GM yeast. This could be achieved either through information provided on food packaging or via other easily-accessible routes.

OVERALL DISCUSSION

51. The information supplied by the applicant offers sufficient reassurance that the consumption of the NI in edible ices does not give rise to any toxicological or allergenic concerns.
52. Regarding the labelling of the product, the applicant needs to comply with the Food Labelling Regulations 1996 (as amended). They should ensure that the labelling and presentation of the products does adequately inform the consumer, particularly in relation to its consumption by yeast allergic individuals.

CONCLUSION

53. The Advisory Committee on Novel Foods and Processes is satisfied by the evidence provided by Unilever that the range of uses for its ice structuring protein preparation is acceptable, subject to the applicant's adherence to the proposed specification and the production parameters described above. In order that consumers should be adequately informed and are not misled, the Committee also recommends that information should be provided in an easily-accessible format to consumers indicating that the ingredient is manufactured using a GM yeast.

Annex 1**Comparison of requirements described in EFSA guidance on the risk assessment of genetically modified microorganisms (May 2006) against the data provided in the application dossier**

A summary of the information required of applications for the placing of food/feed products derived from GMMs on the market is provided in Table 1 of the guidance document (pp52-58). The main categories are:

| Category described in the EFSA guidance document | Corresponding section of the application dossier (see ACNFP/78/2) |
|--|---|
| Characteristics of the recipient or parental microorganism | <u>Section III</u> : History of the organism used as the source of the NF |
| Characteristics of the donor organism | <u>Section X</u> : Information from previous human exposure to the NF or its source <i>(Note: the ISP gene introduced into the production organism is synthetic, designed to code for the same protein that is found in fish)</i> |
| Description of the genetic modification process | <u>Section IV</u> : Effect of the genetic modification on the properties of the host organism |
| Information relating to the GMM and comparison of the GMM with its conventional counterpart | <u>Section IV</u> : Effect of the genetic modification on the properties of the host organism <u>Section V</u> : Genetic stability of the GMO <u>Section VI</u> : Specificity of expression of novel genetic material <i>(comparison between the GMM and its conventional counterpart is not applicable as there is no equivalent product from non-GM yeast)</i> |
| Information relating to the production process | <u>Section II</u> : Effect of the production process applied to the NF |
| Information relating to the production purification process | <u>Section II</u> : Effect of the production process applied to the NF |
| Description of the product | <u>Section I</u> : Specification of the NF <u>Section XI</u> : Microbiological information on the NF |
| Assessment of the presence of recombinant DNA and of the potential risk of gene transfer | <u>Section VII</u> : Transfer of genetic material from GM microorganisms <u>Section VIII</u> : Ability to survive in and colonise the human gut |
| Comparison of the GM product with its conventional counterpart | <i>(not applicable as there is no conventional counterpart)</i> |
| Considerations for human health and animal health of the GM product (including toxicity and allergenicity) | <u>Section IX</u> : Anticipated intake/extent of use of the NF <u>Section XI</u> : Nutritional information on the NF <u>Section XIII</u> : Toxicological information on the NF |

Tables**Table A: Composition of batches of the commercial the ISP preparation**

| Batch | 200030 | 200034 | 200046 | 201024 | 201083 |
|---|---------------|---------------|---------------|---------------|---------------|
| Total protein (g/litre) | 15.0 | 14.3 | 16.4 | 23.7 | 31.5 |
| ISP III-12 (g/litre) | 5.5 | 4.8 | 5.0 | 6.2 | 8.4 |
| <i>Protein breakdown (% of total)</i> | | | | | |
| <i>ISP III-12</i> | 36% | 34% | 31% | 27% | 27% |
| <i>glycosylated ISP III-12</i> | 22% | 18% | 20% | 23% | 25% |
| <i>yeast protein</i> | 23% | 24% | 22% | 29% | 32% |
| <i>peptides</i> | 20% | 24% | 28% | 22% | 17% |
| Total solids (g/litre) | 34.5 | 41.0 | 39.7 | 73.0 | 77.7 |
| Unquantified solids* (g/litre) | 3.0 | 10.0 | 6.4 | 20.6 | 8.4 |
| Unquantified solids (% of total) | 9% | 24% | 16% | 28% | 11% |

Quantified solids = Total Kjeldahl protein + mannose + citric acid + minerals (Na, K, Mg, Ca, PO₄)

Table B: Consumption of edible ices by British consumers

| Age | Consumption of edible ices recorded in NDNS surveys (grams / day) | | | | | |
|---------------------------------|--|---------|---------|---------|---------|---------|
| | 1.5-4.5 | 4-6 | 7-10 | 11-14 | 15-18 | 19-64 |
| Proportion of consumers | 42.9% | 61.1% | 59.3% | 49.6% | 35.7% | 27.3% |
| Median (M / F) | 16 / 15 | 17 / 14 | 18 / 17 | 22 / 18 | 16 / 13 | 17 / 17 |
| High level (97.5th centile) M/F | 62 / 64 | 59 / 73 | 63 / 64 | 99 / 76 | 83 / 71 | 78 / 73 |

Table C: Consumption of edible ices by British children, by season

| Age groups | Wave | N | Centiles of consumption of edible ices recorded in 1997 NDNS survey (grams / day) | | | | | |
|------------|------|-----|--|------------------|------------------|------------------|------------------|--------------------|
| | | | 5 th | 10 th | 50 th | 90 th | 95 th | 97.5 th |
| 4-6 yrs | 1 | 42 | 2.14 | 6.43 | 14.86 | 31.14 | 32.29 | 39.71 |
| | 2 | 57 | 4.29 | 5.00 | 15.00 | 40.43 | 57.00 | 57.14 |
| | 3 | 61 | 5.43 | 7.57 | 16.57 | 38.43 | 59.43 | 76.29 |
| | 4 | 57 | 5.16 | 7.14 | 16.86 | 36.43 | 45.43 | 45.57 |
| 7-10 yrs | 1 | 45 | 5.00 | 5.43 | 17.14 | 43.00 | 50.43 | 51.71 |
| | 2 | 72 | 6.86 | 8.57 | 20.50 | 42.00 | 60.00 | 63.00 |
| | 3 | 102 | 7.57 | 8.57 | 21.07 | 51.86 | 63.00 | 77.71 |
| | 4 | 68 | 7.00 | 8.00 | 15.86 | 33.90 | 45.43 | 58.43 |
| 11-14 yrs | 1 | 44 | 4.29 | 4.71 | 17.93 | 50.71 | 58.29 | 62.29 |
| | 2 | 70 | 4.86 | 6.93 | 17.71 | 51.71 | 61.71 | 84.57 |
| | 3 | 73 | 7.29 | 9.14 | 21.57 | 58.57 | 80.86 | 83.14 |
| | 4 | 45 | 4.86 | 5.43 | 16.71 | 43.14 | 49.29 | 60.00 |
| 15-18 yrs | 1 | 26 | 5.00 | 6.86 | 12.43 | 33.00 | 35.14 | 60.57 |
| | 2 | 27 | 5.66 | 5.71 | 17.00 | 54.86 | 62.43 | 80.86 |
| | 3 | 55 | 5.71 | 7.14 | 15.43 | 45.86 | 70.86 | 83.00 |
| | 4 | 31 | 6.00 | 7.00 | 15.71 | 39.29 | 77.86 | 85.57 |

Wave 1: Jan – Mar, Wave 2: Apr – Jun, Wave 3: July – Sep And Wave 4: Oct – Dec

Table D: Toxicological and genotoxicological studies on the novel ingredient

| Appendix to application dossier | Test material | Tests | Result |
|---------------------------------|---|--|---|
| Appendix 9 | ISP Type III HPLC 12 preparation Batch 201008 ⁽¹⁾ | 90-day sub-chronic oral toxicity test in rats, at doses equivalent to 58, 290 and 580 mg ISP per kg bodyweight per day | NOAEL is 580mg ISP Type III HPLC 12/kg bw/day, equivalent to 6.9 – 12.1g of the NI/kg bw/day ⁽²⁾ |
| Appendix 11 | ISP Type III HPLC 12 preparation Batch 201008 FD ⁽³⁾ | Bacterial reverse mutation assay using 4 strains of <i>Salmonella typhimurium</i> | Negative on 3 strains False-positive on 1 strain due to contamination with other microorganisms |
| Appendix 12 | ISP Type III HPLC 12 preparation Batch 2010034 | Bacterial reverse mutation assay using 4 strains of <i>Salmonella typhimurium</i> | Negative on all strains |
| Appendix 13 | ISP Type III HPLC 12 preparation Batch 201008 FD ⁽³⁾ | <i>In vitro</i> chromosome aberration assay in human peripheral blood lymphocytes | Negative |
| Appendix 14 | ISP Type III HPLC 12 preparation Batch 201008 FD ⁽³⁾ | Gene mutation assay at the thymidine kinase locus of mouse lymphoma L5178Y cells | Negative |
| Appendix 15 | ISP Type III HPLC 12 preparation Batch 201008 FD ⁽³⁾ | <i>In vivo</i> rat bone marrow micronucleus assay | Negative |
| Appendix 16 | AFP III HPLC 12 preparation ⁽⁴⁾ | Randomised placebo controlled human trial to evaluate single ingestion | No toxicity detected |

⁽¹⁾ Batch 201008 is a concentrated form (~5-fold) of the commercial preparation. Compositional data for batch 201008 and for 5 standard commercial batches of the NI can be found in the application dossier, Table 2, p.12.

⁽²⁾ Calculation of the NOAEL expressed as NI containing between 4.8% and 8.4% of ISP Type III HPLC 12 (application dossier, Table 2, p.12).

⁽³⁾ Batch 201008 was too dilute for use in genotoxicity and was therefore freeze-dried to obtain ISP Type III HPLC 12 preparation Batch 201008 FD. No difference in composition, except for the water content, was observed between these two batches (application dossier, Appendix 5)

⁽⁴⁾ The applicant has explained that "Anti- Freeze Protein (AFP) III HPLC 12" is used in this study report as an alternative name for ISP Type III HPLC 12 (application dossier, p.6)

Table E: Tests on the potential allergenicity of the novel ingredient

| Dossier reference | Test material | Tests | Result |
|--|----------------------------------|--|--|
| Appendix 21 | - | Amino acid sequence analysis using BLAST and FASTA computer programmes | No primary sequence similarity with any known allergens, including fish allergens |
| Appendix 18 Figures 7-9 Table 22 | ISP Type III HPLC 12 preparation | Pepsin hydrolysis resistance | Most of the peptides <2.3kD Low probability that ISP Type III HPLC 12 could elicit reaction |

(a) Phase I Studies in 20 fish allergic individuals (cod)

| | | | |
|----------------------|---|---|--|
| - | Eel, eel pout, ocean pout | Skin prick testing | Positive |
| Table 13 Figure 4 | Ocean pout extract (2 mg protein/mL) Freeze-dried ISP III HPLC 12 preparation (20 ng/mL to 200µg/mL) | IgE binding <i>in vitro</i> – RAST inhibition | 18/20 subjects had IgE against ocean pout No binding of IgE to freeze-dried ISP preparation |
| Table 13 | Nine different concentrations (3.5-fold dilutions) of ocean pout extract (max = 0.2mg/mL) and freeze-dried ISP III HPLC 12 preparation (max = 10 mg/mL) | IgE binding <i>in vitro</i> – <i>Basophil histamine release</i> | Positive for ocean pout extract Negative for freeze-dried ISP III HPLC 12 preparation |

(b) Phase II Studies in 22 fish allergic individuals

| | | | |
|----------------------------------|--|---|---|
| Table 14 | ISP Type III HPLC 12 preparation including yeast protein Yeast fermentation supernatant excluding ISP Type III Pure ISP Type III HPLC 12 preparation (no yeast proteins) | Skin prick testing | 4 subjects reacted to both test materials These 4 subjects did not react to pure ISP Type III HPLC 12 preparation (no yeast proteins) |
| Tables 15 and 16 Figure 5 | ISP Type III HPLC 12 preparation including yeast protein Yeast fermentation supernatant excluding ISP Type III Pure ISP Type III HPLC 12 preparation (no yeast proteins) | IgE binding <i>in vitro</i> – RAST inhibition | 8 subjects were positive to ISP Type III HPLC 12 preparation including yeast protein. These 8 subjects included 3 of the 4 subjects who reacted positive to the skin prick testing. All 8 subjects did not react to pure ISP Type III HPLC 12 preparation (no yeast proteins) No binding of IgE to freeze-dried ISP preparation |
| - | ISP Type III HPLC 12 preparation including yeast protein Yeast fermentation supernatant excluding ISP Type III Pure ISP Type III HPLC 12 preparation (no yeast proteins) | IgE binding <i>in vitro</i> – <i>Basophil histamine release</i> to investigate positive skin prick test results | Positive for both materials Negative on pure ISP Type III HPLC 12 preparation (no yeast proteins) |

(c) General allergy testing on 28 healthy adults

| | | | |
|----------|--|---|--|
| Table 17 | ISP Type III HPLC 12 preparation | Antibody response to ingestion on 28 healthy adults without a history of previous consumption of ISP Type III with 8 controls | No observed clinical symptoms or biochemical changes associated with food allergy |
| Table 18 | ISP Type III HPLC 12 preparation | Enzyme-linked immunosorbent assay (ELISA) | Negative |
| - | ISP Type III HPLC 12 preparation including yeast protein Yeast fermentation supernatant excluding ISP Type III Pure ISP Type III HPLC 12 preparation (no yeast proteins) | Skin prick testing | 1 subject positive to both materials but negative on pure ISP Type III HPLC 12 preparation (no yeast proteins) |
| Table 19 | ISP Type III HPLC 12 preparation including yeast protein Yeast fermentation supernatant excluding ISP Type III | IgE binding <i>in vitro</i> – RAST inhibition | Weak specific IgE response (peaking at week 4) |
| Table 20 | ISP Type III HPLC 12 preparation including yeast protein Yeast fermentation supernatant excluding ISP Type III | IgE binding <i>in vitro</i> – <i>Basophil histamine release</i> | Negative |
| Figure 6 | ISP Type III HPLC 12 preparation including yeast protein Yeast fermentation supernatant excluding ISP Type III | IgE binding <i>in vitro</i> – immunoblotting | Negative |