10. TOXICOLOGICAL INFORMATION ON THE NOVEL FOOD INGREDIENT

10.1 General Approach to the Safety Evaluation of ISP Preparation

Ice structuring protein Type III preparation, expressed by genetically modified food-grade baker's yeast, is a mixture of ISP Type III HPLC 12, glyco-ISP, proteins and peptides from the yeast, and sugars, acids, and salts commonly found in food. A subchronic rat toxicity study and a battery of genotoxicity tests were undertaken to assess the safety of the material and provide assurance that nothing unexpected would occur. This also led to the establishment of a no observed adverse effect level (NOAEL) and safe level of intake. Furthermore, when evaluating the constituents of the ISP preparation, a major issue was the possibility that ISP Type III HPLC 12 could cause allergic reactions. Potential allergenicity generally has been identified as a key safety issue with proteins in the diet and must be assessed when proteins are introduced into foods where they have not been found before, such as in the present application involving a protein for use in edible ices that is identical to a protein found in nature in fish. An extensive programme of testing based upon the most current expert consensus was undertaken to make sure that neither individuals already sensitised to fish would react to the ingredient nor that sensitisation would be likely to occur.

10.2 Assessment of General Toxicity in Rats

Ice structuring protein Type III preparation, concentrated approximately 5-fold by ultrafiltration, was administered to rats by daily oral gavage for 13 weeks. The study was designed to meet the guidelines in the FDA's *Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food* (1993), *Redbook 2000: Toxicological Principles for the Safety of Food Ingredients* (2001), and the Organisation for Economic Co-operation and Development's (OECD) guideline for repeated dose oral toxicity studies in rodents (1998a). The complete study report (Study KF010105) and supporting analytical studies (Studies AH000168 & AC000169) are attached as Appendices 5, 9 & 10, a summary of these follows below.

Study details
The study was performed at Covance Laboratories, Harrogate, United Kingdom. The date of first treatment was 4 June 2001 and the study terminated on 7 September 2001. The study was performed in compliance with Good Laboratory Practice regulations (OECD, 1998b).

Ice structuring protein Type III preparation was concentrated by ultra filtration to the greatest extent possible without altering its properties compared to the commercial preparation. This concentrated material, Batch 201008, was characterised, and stability and homogeneity measured (Studies AH000168 & AC000169 attached as Appendices 6 & 10). Please also refer to section 3.3, table 2, for compositional data for batch 201008 alongside commercial batch data. The test and control articles were supplied to the testing facility frozen and individually packaged for use each day. Each container was kept frozen until thawed just prior to dosing.

The test material was administered at a dose volume of 20 ml/kg once a day. This provided a top dose of 4000 mg total solids/kg bodyweight/day, which is 580 mg ISP Type III HPLC 12/kg body weight/day. Lower doses were one-half and one-tenth this dose, by dilution.

One control group received ultra-purified water. Another group received a citric acid solution (citric acid is present in high concentration in Batch 201008), in order to control for acidity by administering a solution with a pH equivalent to that of ISP Type III preparation.

A comparison of what each group received is given in Table 8, below.

Table 8. Dosing information for groups in the 13-week rat study.

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

The rats were of the strain Crl:WI(Glx/BRL/Han)BR. There were 20 rats per sex per group. The animals were approximately 28 days of age on arrival at Covance and six weeks old at the start of dosing. The animals were individually housed during the study.

Experimental observations

Clinical signs	Animals were observed daily for signs of ill health or overt toxicity. Additional observations were conducted daily during Week 1 immediately post dosing, and 30 minutes, 1, 2, and 4 hours after dosing. Post dosing observations were made once weekly after Week 1.
Physical examination	Performed at weekly intervals.
Mortality/morbidity	All animals were observed at the beginning and end of the working day.
Functional observation battery	Ten males and ten females were subjected to a battery of behavioural tests and observations before treatment and once weekly afterwards, including observations, open field and motor activity.
Body weights	Individual body weights were recorded before treatment on the first day of dosing, at weekly intervals, and before necropsy.
Food consumption	The amount of food consumed by each animal was determined weekly.
Opthalmoscopy	Investigations were performed on all rats before treatment and on control and high dose animals during week 12.
Clinical pathology	Blood samples were taken from ten male and ten female animals during weeks 4 and 8 and from all surviving animals at the end of the study. Urine samples were taken when possible from ten male and ten female rats from each group during week 12.
Terminal procedures	All animals were subjected to a necropsy. A full macroscopic examination was carried out and all lesions recorded. A full complement of tissues from all animals was retained in the appropriate preservatives.

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

Results

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

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

The highest dose that could be tested, 580 mg ISP Type III HPLC 12/kg body weight/day is the NOAEL in this study.

10.3 Assessment of Genotoxicity

The potential genotoxic activity of ISP Type III Batch 201008 FD was assessed using four different assays. These were the bacterial mutation assay, the *in vitro* chromosome aberration assay in human peripheral blood lymphocytes, the gene mutation assay in mouse lymphoma L5178Y cells and the *in vivo* rat bone marrow micronucleus assay. All assays were performed in compliance with the United Kingdom Statutory Instrument 1999 No. 3106, The Good Laboratory Practice Regulations 1999 and the OECD Principles of Good Laboratory Practice (1998b). For the purposes of the mutagenicity studies, the sample was freeze-dried prior to testing and the concentrations are stated in terms of total weight of sample per unit volume, not as concentrations of ISP Type III per unit volume. The complete study reports for all the Genotoxicity studies are attached as Appendices 11-15. Summaries of each study are given in the following text.

Batch 201008 of ISP Type III preparation, described in the previous section 10.2, was still too dilute for use in genotoxicity (and *in vitro* allergenicity studies described in following sections) studies. Therefore a portion of Batch 201008 solution as used to make a freeze-dried sample, identified as Batch 201008 FD, which was used in these studies. Analyses were carried out to characterise Batch 201008 and derived materials (Study AC000169 attached as Appendix 5). No significant difference, after accounting for water removal, was found between Batch 201008 and Batch 201008 FD.

Bacterial Reverse Mutation Assay (Studies KA010138 and KA010276)
The bacterial reverse mutation assay was performed using *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100, and TA102 and was compliant with OECD Guideline 471 (1997a) and ICH Tripartite Harmonised Guideline on Genotoxicity: Specific Aspects of Regulatory Tests (FDA, 1997). Three independent assays were performed in the presence and absence of rat liver derived S9 fraction (10%) and both plate-incorporation (Experiments 1, 2, and 3) and pre-incubation methods (Experiments 2 and 3) were used. For all three of these experiments, ISP Type III Batch 201008 FD was dissolved in water. The test was negative with strains TA1537, TA98, TA100, and TA102, both in the presence and absence of rat liver S9 fraction. A small but statistically significant increase in the number of revertant colonies was observed in Experiments 1 and 2 with strain TA1535. However, these results were equivocal as in Experiment 1 they occurred only in the absence of rat liver S9, while in Experiment 2 they occurred only in its presence (using plate incorporation method). These findings therefore required further investigation, which was done in Experiment 3.

In this experiment, the maximum concentration of ISP Type III preparation was increased to 8,000 µg/plate, above the conventional maximum concentration for this assay of 5,000 µg/plate. This increase in concentration revealed that the test material preparation was slightly contaminated, resulting in colonies that were not

Salmonella typhimurium TA1535, the test organism. The microbiology of the plates with the highest degree of contamination was assessed using the Oxoid Salmonella Latex kit, designed for the detection of Salmonella organisms in food. Positive identification of the true Salmonella organisms permitted the re-calculation of the number of revertant colonies. This showed no statistically or biologically significant differences between the numbers of colonies on plates exposed to the test material and those exposed to the control solvent.

This experiment demonstrated that the increases in revertants observed in all three experiments were highly likely to be due to a low-level contamination of the test material. This was only apparent in the assays with *Salmonella typhimurium* TA1535 because the strain has a very low background of spontaneous reversion.

As these results revealed no mutagenic activity for Batch 201008 FD, the bacterial reverse mutation assay was not repeated using this same test sample. However, to verify that the issues encountered were specific to the material and freeze-drying operation described, another batch (production batch 200034 - composition details of Batch 200034 are provided in Table 2 in section 3.3.) of ISP Type III preparation was freeze-dried (taking particular care to prevent contamination) and assayed in the same five histidine-requiring strains of *Salmonella typhimurium* (strains TA1535, TA1537, TA98, TA100, and TA102). The test was negative with all strains, both in the presence and absence of rat liver S9 fraction.

Based on these assessments, it was concluded that ISP Type III preparation possesses no mutagenic activity, as measured by the bacterial reverse mutation assay.

In Vitro Chromosome Aberration Assay in Human Peripheral Blood Lymphocytes (Study KC010135)

The *in vitro* chromosome aberration assay was performed using whole blood cultures of human peripheral blood lymphocytes and was compliant with OECD Guideline 473 (1997b) and the ICH Tripartite Harmonised Guideline on Genotoxicity: Specific Aspects of Regulatory Tests (FDA, 1997). Ice structuring protein Type III Batch 201008 FD was dissolved in water and assessed at concentration up to, and including, 5000 µg total solids/ml or the limit of toxicity. The assay was performed on two independent occasions in the presence and absence of rat liver derived S9 fraction (2%). The whole blood cultures were exposed to Batch 201008 FD for either 3 h (with and without metabolic activation) or 20 h (without metabolic activation only). Cultures were harvested 20 hours after the initiation of treatment. A total of 200 cells were assessed for chromosome aberrations per concentration. There was no evidence of either a biologically or statistically significant increase in the percentage of cells with aberrations observed in any of the treated cultures when compared to the solvent control cultures. In addition, the incidence of polyploid and endoreduplicated cells was assessed in 2000 mitotic cells per treatment. No numerical aberrations

were observed in any of the treated cultures in comparison with the solvent control cultures.

Under the conditions of this study, ISP Type III Batch 201008 FD showed no evidence of genotoxic potential.

Gene Mutation Assay in Mouse Lymphoma L5178Y Cells (Study KM010136)
Gene mutation was assessed using the *thymidine kinase (tk)* locus in mouse lymphoma L5178Y cells and was compliant with OECD guideline 476 (1997d) and the ICH Tripartite Harmonised Guideline on Genotoxicity: Specific Aspects of Regulatory Tests (FDA, 1997). Batch 201008 FD was dissolved in water and assessed at concentration up to, and including, 5000 µg total solids/ml or the limit of toxicity. The assay was performed on two independent occasions in the presence and absence of rat liver derived S9 fraction (2%). The mouse lymphoma L5178Y cells were exposed to Batch 201008 FD for either 3 hours (with and without metabolic activation) or 24 hours (without metabolic activation only). There was no evidence of either a biologically significant or a statistically significant increase in mutation frequency in treated cultures in comparison with the solvent control cultures.

Under the conditions of this study, ISP Type III Batch 201008 FD showed no evidence of mutagenic potential.

In Vivo Rat Bone Marrow Micronucleus Assay (Study KC010137)
The rat bone marrow micronucleus assay was performed using groups of seven male Crl:HanWist (Glx:BRL) BR rats of approximately 7 weeks age and was compliant with OECD Guideline 474 (1997c) and the ICH Tripartite Harmonised Guideline on Genotoxicity: Specific Aspects of Regulatory Tests (FDA, 1997). Induction of micronuclei is used as an indicator of chromosome damage in immature erythrocytes. A preliminary dose-range finding assay had shown no significant difference in the toxicity observed in male and female rats and thus only males were used for this study. Batch 201008 FD was suspended in water and dosed once daily on two consecutive days via gavage at 500, 1000, and 2000 mg total solids/kg. The animals were sacrificed 24 hours after final dosing and slides were prepared from the bone marrow. The ratio of polychromatic erythrocytes to normochromatic erythrocytes was assessed in 1000 cells per animal. A change in this ratio is indicative of bone marrow toxicity but there was no evidence of a change in ratio in this study. The incidence of micronuclei was assessed in a total of 2000 polychromatic erythrocytes per animal. There was no evidence of an increase in micronucleated polychromatic erythrocytes in any of the treatment groups compared to the solvent control treated group. Chemical analysis of dosing solutions confirmed the doses administered.

Under the conditions of this study, ISP Type III Batch 201008 FD showed no evidence of genotoxic potential.

10.4 Human Studies

Information on human exposure to ISP has come from a number of sources:

- Intake of ISP from food (section 7)
- A randomised, placebo-controlled trial to evaluate a single ingestion of ISP (see Appendix 16, Study KQ 990234)
- A study to investigate antibody response following ingestion of ISP (see section 10.5.3)

There is no indication of toxicity or allergenicity from human consumption studies.

10.5 Allergenicity

10.5.1 Overview of Allergenicity Testing

Assessment of the potential allergenicity of a protein must consider two issues: (i) whether the protein is likely to sensitise potentially susceptible individuals and thereby risk provoking a reaction on subsequent exposure to that protein, and (ii) whether the protein is likely to provoke a reaction in individuals allergic to the source from which the protein originated (or to structurally related proteins). No single predictive method currently exists which can give complete assurance that a protein lacks the ability to induce reactions or sensitise. The approach adopted by the Unilever team in this evaluation sought to generate a body of evidence, which in totality permits a judgment to be made of the protein's ability to induce or provoke allergic reactions. This approach is consistent with recent international consensus documents, including the recommendations of the FAO/WHO (2001) consultation and those of the Codex Alimentarius Commission *Ad Hoc* Task Force on Foods Derived from Biotechnology (2002). The information provided by each test is summarised in Table 9 and described in further detail in the following text (sections 10.5.2-10.5.6). Individual study reports are provided in Appendices 17-20 and relevant publications as Appendices 21 & 22.

Table 9. Tests conducted to assess the allergenic potential of ISP Preparation.

TEST	**INFORMATION PROVIDED WITH RESPECT TO**	
	Potential to sensitise	*Potential to elicit reactions in sensitised individuals*
Sequence analysis	Identifies similarity to known allergens and classes of proteins containing known allergens	Identifies short sequences in common with known allergens (possible epitopes). Can provide information for additional serum screening
IgE binding *in vitro* – RAST and RAST inhibition		Indicates whether protein can bind specific IgE that might provoke reactions in individuals with a specific allergy
IgE binding *in vitro* – Immunoblotting		Indicates whether protein can bind specific IgE and might provoke reactions in individuals with a specific allergy and visualizes implicated proteins
IgE binding *in vitro* – Basophil histamine release		Indicates whether protein can bind specific IgE and might provoke reactions in individuals with a specific allergy and shows whether binding is biologically meaningful
Skin prick testing		Indicates whether protein could provoke reactions in individuals with a specific allergy

Antibody response to ingestion	Provides information on immunogenicity of protein	
Pepsin resistance	Ready hydrolysis by pepsin suggests lower probability of sensitisation via GI tract	Ready hydrolysis by pepsin may indicate low probability of reactions in GI tract

The FAO/WHO (2001) decision tree includes the possible use of animal models, as does the Codex Alimentarius Commission *Ad Hoc* Task Force on Foods Derived from Biotechnology (2002), and both recognized that such models were at too early a stage of development to generate data useful for risk assessment. Therefore an animal study was not included in this part of the investigation.

10.5.2 Amino Acid Sequence Analysis

Amino acid sequence analysis can identify regions in the linear sequence of a protein that resemble the sequence of known allergens. The presence of such regions suggests that the protein in question may be able to bind specific IgE to those allergens and provoke allergic reactions in sensitised individuals. Conversely, the absence of any similarity suggests that a protein does not possess any sequential epitopes resembling those present in known allergens. This increases the confidence that the protein will not provoke reactions in individuals sensitised to known allergens, the amino acid sequence of which has been reported. Sequence analysis can also indicate whether the protein shares any structural similarity with classes of proteins containing known allergens and thus provide guidance for subsequent serum screening.

Sequence similarity between proteins can be established for the whole protein (global alignment) or for sequences within the proteins (local alignment). Since recognition of proteins by T-cell receptors or antibody binding sites only involves the relatively small sequences that form the epitopes, local alignment is the most relevant. However, it is also useful to know whether an unknown protein shares a significant proportion of its sequence with an allergen, since individual epitopes are not defined for most proteins. Moreover, this information can indicate whether the protein of interest belongs to a family which contains known allergens. Several algorithms have been proposed, but the most frequently used are FASTA and BLAST, from which computer programmes of the same name have been generated. Both methods rely on assessing the probability that an alignment between a query sequence (the unknown protein) and a sequence in the database occurs by chance.

The FASTA algorithm of Pearson and Lipman (1988) is the most frequently used method for global alignments. The current version offered by databases and search interfaces is FASTA3, which is one of a family of related programmes which differ in the sort of sequence they are designed to compare. The principle used to determine the degree of global similarity is to compare the protein of interest with those in the database, using pairwise comparison of amino acids. These comparisons are done for segments of specified word lengths (i.e.,

number of amino acids) and generate segments with several matches along the protein. The segments are given scores, which are a function of the number of successful matches, with negative scores for gaps and non-matching amino acids. The scoring uses a substitution matrix – a table of scores for mismatched amino acids at particular points in the sequence. This type of matrix allows a conservative substitution to attract a lower penalty than one in which there is a complete change in the type of amino acid. The initial segments are then further combined and scored. Finally, the programme finds an optimized gapped alignment around the initial segment which gave the best score. The results include an estimate of the likelihood of particular alignments arising by chance.

The programme automatically searches for and eliminates regions of low complexity, for example multiple repeats of one or two amino acids, which would otherwise result in apparently significant similarity, but without necessarily having any biological significance.

Some of the limitations of global alignment include the fact that the statistical basis for the procedure is not fully established, since the shape of the distribution of alignments within the database is not known. As a result any probability estimate is approximate. Another limitation is that the scoring matrices and therefore the scores given mismatched amino acids and gaps are arbitrary, although different matrices can be used. No matrices exist which address the effects of specific amino acid substitutions on protein binding to antibodies or T-cell receptors.

BLAST (Basic Local Alignment Search Tool) is the most commonly used algorithm for establishing local alignments between protein sequences. Unlike FASTA, it has a firm statistical basis, using the methods of Karlin and Altschul (1990). The BLAST programme works on the basis of finding High-Scoring Segment Pairs (HSPs), which are pairs of sequences of equal length (one in the query protein, the other in the database protein) whose scores cannot be improved by extension or trimming. The current version (2.2) allows gaps in local alignments and imperfect matches, using a substitution matrix to score non-matching amino acids. The expected number (E) of HSPs with a score of at least S is calculated for each match and is a measure of the probability of such a match occurring at random in the searched database. The E-value is a selectable threshold for reporting matches, so that distant similarities can be identified, if appropriate. As for the FASTA programme, low complexity regions, which would be expected to give very high alignment scores without biological significance are screened out. The limitations of FASTA with respect to the substitution matrices apply equally to BLAST analyses.

Sequence analysis of ISP Type III was performed in line with the suggested procedure formulated by the 2001 FAO/WHO consultation on assessment of the allergenicity of genetically modified foods, although with some differences described below. It consisted of three main steps:

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

2. Identification of local alignments also using the programme BLAST 2.2.1. The database examined was the subset of the nr database described above. The initial search for alignments was performed with the default settings (Matrix BLOSUM62, gap existence score 11, extension 1, Word size 3, Expect value 10). As those default parameters are known not to be sensitive enough to identify short alignments, even where they are good matches (Gendel, 1998), the search was also performed with settings optimized for finding short, nearly exact alignments (Matrices: PAM30 and BLOSUM80, gap existence penalty 9 or 10, extension 1, Word Size 3 and 2 and Expect value 1000). Each of the alignments produced was then examined to find the highest number of contiguous amino acids in each match.

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

A search for similarity to sequences contained in the whole NCBI nr (non-redundant) as well as the PIR-NREF database, using BLAST 2.2.1 with default parameters, produced 61 matches. All but four of the matches in the NCBI database and all but six of those in the PIR-NREF database were with ice structuring protein sequences. None of the non-ISP matches was with known allergens or related proteins (Table 10). The FASTA 3.2 search in PIR-NREF also did not reveal any matches with known allergens, nor did a search of the FARRP allergen database, using the same programme. A BLAST search against the "allergen database" produced a single hit against allergen Asp f6 *Aspergillus fumigatus* (Crameri *et al*., 1996). The match only occurred over a very short part of the sequences and was therefore not significant.

Table 10. Identification of the Six Non-ISP Sequence Matches Returned by BLAST 2.2.1

Matching sequence	Protein	Accession numbers	
		PIR	SwissProt/Trembl
GIPAEDI	Sensor Histidine kinase	B75466	Q9RW09
IPAEDIP	Sensor Histidine kinase	B75466	Q9RW09
IPRLVSM	PILT protein	Q9ZIU8	8896033
NRAVPLG	ADP-glucose pyrophosphorylase	Q9AT46	13487709
AVPLGTT	Succinate dehydrogenase cyt B560 subunit Integral membrane protein	I48085	C560 HUMAN C560 BOVIN P70097
VPLGTTL	α-amylase (P furiosis) NADP reducing hydrogenase	A49512 F69073	AMYPYRFU 027592

A BLAST search of the "allergen database," using parameters optimized to detect short alignments, produced 355 alignments at the most sensitive settings (Matrix BLOSUM 80, Expect value 1000, gap creation penalty 10, gap extension penalty 1). The longest contiguous sequence in any alignment was five amino acids, and all but one alignment possessed four or fewer contiguous amino acids.

The number of exact matches obtained with octamers, heptamers, and hexamers was 1674, 1771, and 2442, respectively. All the matches obtained with the octamers and most of the exact matches of seven contiguous amino acids identified by the programme "Peptide Match" in the PIR database were with sequences within ISPs. Matches with sequences in six unrelated proteins were also found. Of those proteins, none has been associated with the induction or elicitation of allergic reactions. However, a match with an α -amylase from a bacterium, *Pyrococcus furiosis*, was investigated further as certain members of the bacterial α-amylase family are known allergens in man (Bernstein *et al.*, 1994). This protein was therefore compared with an α -amylase which is widely used in industrial applications, the α-amylase from *Bacillus licheniformis* (PIR ref ABSL). This analysis showed that the seven-amino acid contiguous sequence shared by the *P. furiosis* enzyme and ISP Type III HPLC 12 was not present in the *B. licheniformis* enzyme. Exact matching of the hexamers generated 515

matches with unrelated proteins. Careful examination of these proteins also did not reveal any known allergens.

Thus, the structure of ISP Type III HPLC 12 is highly characteristic of that of fish ice structuring proteins and shows little similarity with that of any other proteins. In particular, sequence analysis clearly showed no primary sequence similarity between ISP Type III HPLC 12 and the sequence of any known allergens, including fish allergens. Furthermore, the searches did not produce any matches against classes of proteins commonly known to be allergens, such as chitinases or lipid transfer proteins. Significantly, when analyzed using an eight-amino acid reading frame, the only matches were with other ISPs. Narrowing the reading frame to seven or six amino acids increased the number of matches with unrelated proteins, but still did not produce any matches with known allergens.

Parts of this work have been published by Baderschneider *et al*. (2002), and Bindslev-Jensen *et al*. (2003) and presented by Basketter *et al*. (2002). Baderschneider *et al*. (2002) and Bindslev-Jensen *et al*. (2003) are provided as Appendices 21 & 22 respectively.

The original amino acid sequence analysis was performed in 2002 and as there have been many new proteins added to the sequence database since then, an updated analysis was carried out in 2005. This updated sequence analysis did not reveal any new significant sequence alignment with known allergenic proteins and supports the findings of the original analysis.

10.5.3 Investigations in Individuals with Established Allergy to Fish

Fish allergy occurs from sensitisation to a codfish muscle protein, Gad c1, which is extremely stable to heat and acid (Bindslev-Jensen and Poulsen, 1997) and partially resistant to proteases (Metcalfe, 1997). Gad c1, a parvalbumin that controls calcium flow across cell membranes, has a high degree of sequence homology with parvalbumins from other fish species, and individuals allergic to Gad c1 will react upon ingestion of other fish (Hansen *et al*., 1996, 1997).

No data exist on allergy to ocean pout. Allergy to a closely related species, eel, has been described (Bruijnzeel-Koomen *et al*., 1995), so one could expect fish-allergic individuals to react to ocean pout. Therefore, it was essential to demonstrate that individuals allergic to fish do not react to ISP Type III preparation even though they may react to ocean pout flesh containing Gad c1 or its analogues.

Allergy to fish is relatively common in Scandinavian countries (Hansen and Bindslev-Jensen, 1992), so allergy experts in Denmark were used to carry out studies with fish-allergic volunteers. Calculations based on binomial theory were used to determine the statistical power of tests with different numbers of subjects (Armitage *et al*, 2001). These results are shown in Table 11 (overleaf). They can also be interpreted as the degree of confidence in a negative result. For

instance, if 29 fish-allergic people were tested with ISP and none reacted, it can be inferred with 95% confidence that fewer than 10% of the population of interest are reactive to the ISP.

Table 11. Likelihood of observing at least one reaction to an allergen (%) according to number of subjects tested and putative prevalence of reactivity to the allergen of interest in the test population.

Number of subjects tested	**Putative prevalence of reactivity to the allergen of interest in test population**			
	50%	*20%*	*10%*	*2%*
22	100.00	99.26	90.25	35.88
25	100.00	99.62	92.82	39.65
29	100.00	99.85	95.29	44.34

In order to ensure that the study participants were not placed at any risk from the investigation, a step-wise process was used. Investigations started with serological studies on the sera of fish-allergic patients. Once data were available to attest to the toxicological safety of the ISP Type III preparation, the testing was extended to skin prick testing and ingestion. The design of the allergological testing programme is summarised in Table 12 below.

Table 12. Approach to the allergological assessment of ISP Type III using human subjects with documented allergy to fish.

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

10.5.3.1 Phase I Studies in Fish-allergic Individuals

Subjects. Twenty subjects with allergy to codfish, diagnosed according to the guidelines published by the European Academy of Allergy and Clinical Immunology (Hansen and Bindslev-Jensen, 1992; Bruijnzeel-Koomen *et al*., 1995) were recruited. Samples of blood (20 ml) without added anti-coagulant and heparinized blood (10 ml) were drawn and shipped in anonymous form for *in vitro* experiments. Ethical approval for the study was given by the local Institutional Review Board and written informed consent was obtained from all participating individuals.

Skin prick testing. Each participant was skin prick tested in duplicate with freshly thawed eel, eel pout, and ocean pout, using the prick-prick method recommended by the European Academy of Allergy and Clinical Immunology

(Hansen and Bindslev-Jensen, 1992; Hansen *et al*., 1996). Drugs interfering with skin prick testing were discontinued prior to testing. All patients demonstrated positive skin prick test reactions to eel, eel pout, and ocean pout. Average diameters of the wheals were 16.7 ± 10.1 mm, 17.3 ± 7.1 mm and 16.1 ± 6.7 mm respectively.

MaxiSorp™ RAST. Since the binding between an allergen and IgE is central to eliciting a response, a test system that measures this binding is very useful. The RadioAllergoSorbent Test (RAST) and modifications of it play an important role in allergen determination and standardization, as well as measurement of specific IgE levels. The RAST using the direct MaxiSorp™ tube method was performed as previously described (Poulsen *et al*., 1989). The MaxiSorp™ tubes were coated with an extract from ocean pout at 2 mg protein/ml. The Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) was used for determining the protein content of the extract which was produced by homogenizing 1 g ocean pout/ml H_2O. After incubation overnight, the tubes were blocked with RPMI 1640 including 5% fetal calf serum (FCS) for 2 h and thereafter incubated with sera diluted 10- to1000-fold. The next day anti-IgE coupled to ^{125}I was added and after incubation overnight IgE binding was measured as bound counts per minute. All measurements were performed in duplicate. For inhibition assays the sera diluted 10-times were preincubated overnight at 4°C together with ocean pout extract or freeze-dried ISP preparation, at concentrations from 20 ng/ml to 200 µg/ml and centrifuged at 1650 g for 15 min, before use. The degree of inhibition was calculated and expressed as the percentage of the counts-per-minute value for the inhibited sample divided by the value of the uninhibited. The background value determined with pooled sera from non-allergic donors was subtracted from all values.

None of the patients' sera demonstrated binding of IgE to the freeze-dried ISP preparation as determined by Maxisorp™ RAST. The binding seen with some of the sera was non-specific as the counts per minute values without coating were in the same range. These values probably reflect IgE binding to the blocking agent (RPMI 1640 with 5 % FCS) (Table 13). Eighteen of 20 patients had IgE against ocean pout as determined by Maxisorp™ RAST (Table 13). By preincubating these sera with extracts from ocean pout, an increasing inhibition up to at least 92% was observed using extract dilutions from 20 ng/ml to 200 µg/ml (Figure 4). The IC_{50} values for 16 out of 18 sera were <0.2 µg/ml. The specific IgE binding to ocean pout could in no case be inhibited by the freeze-dried ISP preparation when protein concentrations up to 200 µg/ml were used.

Basophil histamine release. IgE binding *in vitro* can sometimes occur without translating into any biologically meaning event, such as mast cell degranulation (Taylor and Hefle, 2001). This possibility was ruled out by prospective inclusion of investigations on basophils to ascertain the potential biological significance of any IgE-binding. Washed, heparinized blood samples from the patients were used for histamine release as described elsewhere (Hansen *et al*., 1996).

Duplicate samples of 25 µl washed blood were incubated with 25 µl of nine different concentrations (3.5-fold dilutions) of ocean pout extract or freeze-dried ISP preparation. The highest concentrations of eel pout extract and freeze-dried ISP preparation used were 0.2 mg/ml and 10 mg/ml respectively. A release of >15 ng histamine/ml blood was considered positive. The results were expressed as the highest dilution titer inducing a positive reaction.

None of the patients' basophils released histamine when exposed *in vitro* to the freeze-dried ISP preparation, whereas the test was positive with eel, eel pout, and ocean pout extracts in all patients (Table 13).

This work has been presented by Basketter *et al*. (2002) and published by Bindslev-Jensen *et al*. (2003).

Table 13. Summary of the Phase I experiments on IgE binding (MaxiSorp™ and histamine release).

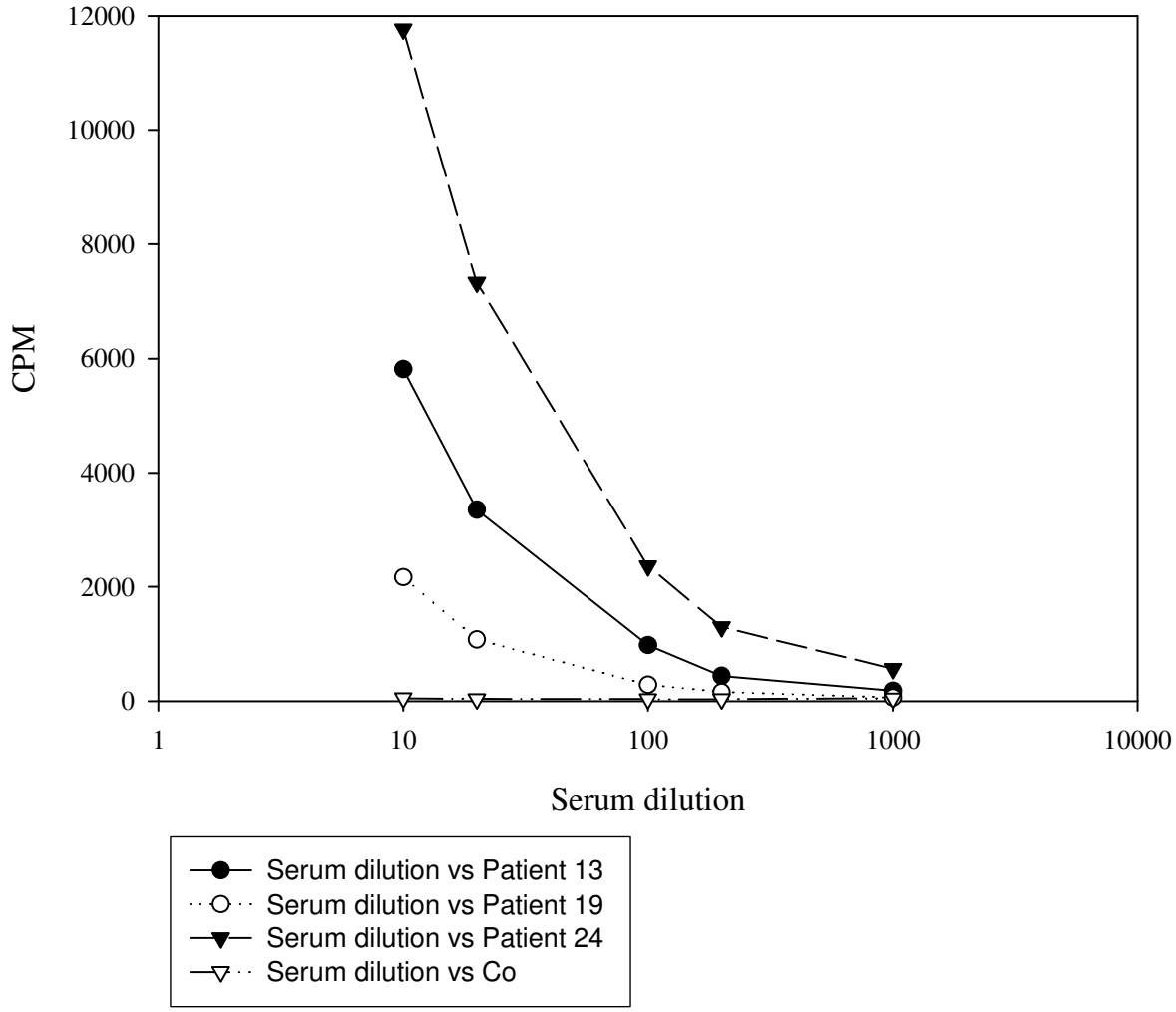
Subject	SPT	Direct MaxiSorp™ RAST*			Inhibition MaxiSorp™ RAST (Ocean pout extract as solid phase)			Histamine Release**	
	Ocean pout	Ocean pout	ISP Type III prepara-tion	Blank (RPMI-FCS)	Inhibitor			Ocean pout (µg/ml)	ISP Type III
					Ocean pout	IC50 (ng/ml)	ISP Type III		
1	+	340 ± 21	32 ± 18	24 ± 4	+	<200	-	0.06	-
2	+	373 ± 34	26 ± 7	25 ± 12	+	36	-	6	-
3	+	327 ± 6	29 ± 6	23 ± 9	+	44	-	6	-
4	+	11935 ± 472	46 ± 1	28 ± 8	+	591	-	inconcl	inconcl
5	+	266 ± 17	15 ± 5	13 ± 1	+	40	-	20	-
6	+	4551 ± 1112	691 ± 69	886 ± 19	+	<200	-	0.6	-
7	+	2200 ± 161	81 ± 2	94 ± 6	+	<200	-	0.2	-
9	+	5814 ± 325	375 ± 68	214 ± 6	+	<200	-	0.6	-
11	+	336 ± 42	33 ± 3	30 ± 16	+	<200	-	6	-
12	+	12104 ± 683	83 ± 4	76 ± 5	+	349	-	0.02	-
13	+	2705 ± 18	279 ± 37	135 ± 3	+	<200	-	0.2	-
15	+	657 ± 18	42 ± 4	33 ± 9	+	<200	-	20	-
16	+	2167 ± 122	162 ± 21	131 ± 37	+	<200	-	20	-
17	+	2858 ± 243	47 ± 11	60 ± 1	+	<200	-	2	-
18	+	7539 ± 570	52 ± 10	65 ± 11	+	<200	-	0.2	-
19	+	1369 ± 28	76 ± 1	101 ± 14	+	<200	-	0.6	-
20	+	11765 ± 368	884 ± 34	1104 ± 239	+	<200	-	60	-
21	+	21 ± 5	75 ± 5	22 ± 0	nd	nd	nd	inconcl	inconcl
22	+	1486 ± 32	136 ± 10	75 ± 1	+	<200	-	2	-
23	+	44 ± 1	33 ± 2	11 ± 8	nd	nd	nd	200	-

*CPM values for sera diluted 10-fold (mean ± interval between mean and individual replicates)
** Lowest concentration of test material giving a positive histamine release

Abbreviations: SPT : skin prick test; CPM : counts per minute; Nd : not determined; Inconcl : inconclusive; + : positive; - : negative; IC50 : concentration producing 50% inhibition

Data from Bindslev-Jensen *et al* (2003)

Figure 4. Demonstration of the specific IgE binding to ocean pout for three representative serum samples using Maxisorp™ RAST. "Co" is a pool of sera from non-allergic individuals.



10.5.3.2 Phase II Studies in Fish-allergic Individuals

Thirty subjects were asked to participate in this phase of study to supply supportive information about the allergenic potential of ISP Type III preparation. Twenty-five accepted. Of these, 22 agreed to participate in the skin prick testing.

Skin prick testing. Twenty-two participants were skin prick tested in duplicate with solutions of sterile ISP Type III preparation (5.0, 1.0, 0.1, and 0.01 mg ISP Type III HPLC 12/ml), as well as with solutions of the parent yeast strain fermentation supernatant (yeast control) (3.0, 0.87, 0.087, and 0.0087 mg yeast protein/ml). Tests were performed exactly as in Phase I. Dilutions of test materials were prepared in 0.9% saline in 50% glycerol (ALK, Denmark). Results, presented in Table 14, were as follows:

- Four (of 22) subjects reacted to ISP Type III preparation (which includes yeast protein) and also reacted to the yeast control (which includes no ISP Type III protein)

- None of these four subjects reacted to pure ISP Type III (which includes only the ice structuring protein with no yeast protein) in skin prick tests, and none had positive RAST results to pure ISP Type III.

MaxiSorp™ RAST. The RAST was performed as described for Phase I, except that the buffer used to dilute the serum was phosphate buffered saline with 0.1% Tween 20 instead of RPMI 1640 with 5 % FCS. The change in buffer was introduced in an attempt to overcome the binding to the foetal calf proteins observed with some samples in Phase I. The serum used was the same as that used in Phase I, with the additional five patients recruited as part of Phase II. Results, presented in Table 15, were as follows:

- Positive RAST results to ISP Type III preparation (which includes yeast protein) and to the yeast control (which includes no ISP Type III protein) were obtained in eight (of 22) subjects. These eight subjects included three of the four subjects who reacted positively in skin prick tests.

- All eight subjects had negative RAST results to pure ISP Type III (which includes only the ice structuring protein with no yeast protein).

SDS PAGE and Western Blotting. SDS-PAGE and Western blots were performed in accordance with the protocols described by Laemmli (1970) and Hansen *et al* (1997). Briefly, test samples were dissolved in a reducing buffer containing SDS and boiled for 5 minutes. They were then separated by electrophoresis on a polyacrylamide gel optimized for separation of the proteins of interest. After separation, the proteins were electro-transferred (blotted) on to

a polyvinylidene fluoride membrane (Millipore, Bedford, MA) (1.5 h using a current of 1.0 mA/cm^2). Vacant protein binding sites on the membranes were blocked with Tris-buffered saline with 0.5% Tween and 3% skimmed milk powder for 2 h at room temperature before incubation overnight at 4^{o}C with the test sera. The membranes were then washed and incubated with anti-IgE-horseradish peroxidase (Dako A/S, Glostrup, Denmark) for 2 h and washed again. Enhanced chemoluminescence Western blotting detection reagent (Amersham Pharmacia Biotech) was used as substrate. No IgE binding to the ISP Type III protein was demonstrated in these experiments (Figure 5).

Basophil Histamine Release Test. In Phase II, the basophil histamine release test was used only to investigate positive skin prick test results to the ISP preparation. Two of the patients (19 & 27) who had a positive skin prick test showed a positive basophil histamine release when the ISP preparation was used as the antigen. Their basophils also responded similarly when the yeast supernatant skin prick test reagent was used as the antigen. In contrast, no histamine release was observed when their basophils were exposed to pure ISP standard as the antigen or when cord blood basophils were sensitised with their serum and subsequently exposed to pure ISP standard. The other two individuals (11 & 13) with positive skin prick tests produced results in the basophil histamine release test that were not interpretable (data not shown). However, as indicated on page 44, these patients did not react to pure ISP Type III (which includes only the ice structuring protein with no yeast protein) but did react to the yeast control in skin prick tests.

In the light of the skin prick test results, some of these findings almost certainly reflect non-specific binding, as evidenced by the increase in some counts compared to their Phase I values (see for instance Subjects 12 and 13; Table 15). As skin prick tests are considered more sensitive than RAST in detecting marginal sensitisation (Bernstein *et al*., 1994), a positive result in the RAST in the presence of a negative skin prick test is almost certainly a false positive. Sensitisation to *Saccharomyces cerevisiae* was also confirmed in three of the subjects (11, 19, and 31, RAST classes 3, 4, and 2, respectively) by the commercial CAP RAST method (Pharmacia, Sweden).

Table 14. Summary data for subjects with positive skin prick reactions, showing that positive reactions are attributable to the yeast protein and not to ISP Type III protein.

Subject	Skin prick tests responses (mm) [1]							
	ISP Type III preparation (mg/ml)				Yeast fermentation supernatant (mg/ml)			
	5	*1*	*0.1*	*0.01*	*3*	*0.87*	*0.087*	*0.0087*
1								
2								
4								
7								
9								
11[2]	7.5	4.5	4	2.5	5	2.5	2	1
12								
13								
15								
16								
17								
18								
19[2]	4	0	0	0	5	3	0	0
20								
21								
22								
23								
26								
27	4.5	3	0	0	4.5	2.5	0	0
31[2]	7	6	6	4	6	4.5	0	0
32								
33								

Shaded rows indicate negative skin prick test results

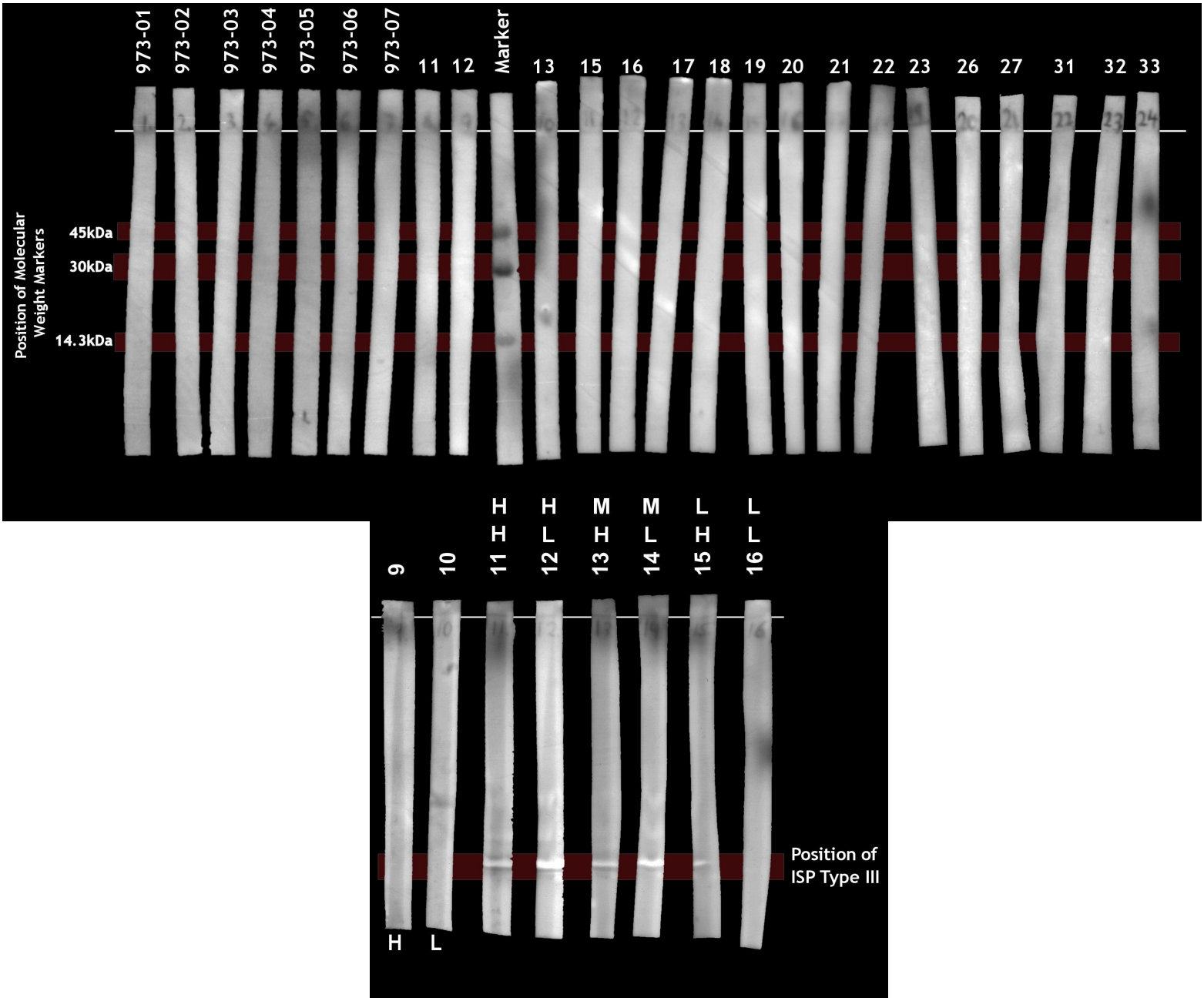
[1] Skin prick test values are the mean of largest perpendicular diameters, in mm.

[2] Subjects determined to be sensitive to *S. cerevisiae* by CAP RAST method: Subject 11, Class 3; Subject 19, Class 4; Subject 31, Class 2.

Table 15. Summary data for subjects with positive RAST responses to ISP Type III preparation.

Subject	RAST responses (cpm)				
	ISP Type III preparation		Yeast Control	Pure ISP	Blocking only
	Phase 1	Phase 2			
11	33	1239	1564	99	61
12	83	137	114	71	40
13	279	591	664	105	104
16	162	141	185	53	49
17	47	225	246	88	54
19	76	243	234	94	57
23	33	140	90	94	80
27	N.D.	70			
31	N.D.	1908	2068	120	86

Figure 5. Immunoblots (Western blots) of ISP Type III preparation using sera from fish-allergic individuals.In the positive control (lower picture), the ISP band (approx. 6.5Kda) is clearly visible at the bottom of the blot (the only band present). It was stained with the anti-ISP monoclonal antibody.



10.5.3.2.1 Yeast Allergy

Yeast is a very broad term for single-celled fungi that reproduce by budding and encompasses a very large group of organisms. For purposes of clarity, only proteins found in baker's yeast (*Saccharomyces cerevisiae*) that are known to bind IgE antibodies in humans are discussed here. *Saccharomyces cerevisiae* is the organism used in ISP production and the major yeast that is consumed as food stuff.

No *Saccharomyces cerevisiae* proteins have been identified as food allergens.

However, three proteins identified in *S. cerevisiae* are associated with inhalant and/ or skin allergies. These are:

1. Enolase (Baldo and Baker 1988).
2. Manganese super-oxide dismutase (Fluckiger *et al* 2002b).
3. Cyclophin (Fluckiger *et al* 2002a).

There is no evidence to indicate that sensitisation to yeast arises from the consumption of foods or drinks containing yeast. IgE-mediated sensitization to yeast appears to be relatively common, arising mainly from inhalation exposure, from exposure due to saprophytic growth on mucous membranes (e.g., *Candida albicans*), or in cases of atopic dermatitis.

IgE-mediated sensitization to airborne yeast has been reported among people exposed occupationally (Bataille et al., 1995). This can be accompanied by respiratory symptoms such as bronchial asthma. In addition to IgE antibodies, some of these individuals also manifest symptoms of allergic extrinsic alveolitis and the production of IgG antibodies to the yeast, which form immune complexes *in vivo*.

Several studies have indicated that persons with atopic dermatitis are particularly likely to be sensitised and have IgE antibodies to various types of yeast. Atopic dermatitis is a chronic, itching, inflammatory skin disease associated with IgE-mediated allergic disease, although its precise aetiology is unclear. Dry skin and a weakened barrier may allow microorganisms such as yeast to come into contact with the immune system. Additionally, the yeast may be an aggravating factor in some cases. Thus, atopic dermatitis, especially of the head and neck region, is frequently associated with IgE antibodies to various yeasts, including *Candida albicans*, *Pytirisporum ovale,* and *Malassezia furfur* (Faergemann, 2002). Additionally some of the proteins in these yeasts are cross-reactive with equivalent ones in *Saccharomyces cerevisiae* proteins (Morita et al., 1999; Mayser et al., 2000; Savolainen et al., 2001). Thus, people with atopic dermatitis are likely to show positive skin prick tests or RASTs with *Saccharomyces cerevisiae* proteins, as reported by Kortekangas-Savolainen et al., (1993).

Yeast and yeast products are extensively consumed in most societies, yet yeast is not an important food allergen. It is barely mentioned in textbooks (only with respect to a suspected link to some cases of chronic urticaria, along with other food additives), and no authoritative or regulatory bodies have listed yeast as a food allergen. Although epidemiological and clinical data on sensitisation and allergic symptoms following exposure to yeast do not provide a basis for estimating the prevalence of yeast allergy, the lack of reported allergic reactions (particularly in light of ubiquitous exposure) indicates that the prevalence of allergy is extremely low. The rarity of reported reactions to ingested yeast products probably explains why it is not a priority for investigation in food allergy.

A study by Kortekangas-Savolainen et al (1994) investigated the association between the presence of specific IgE antibodies to baker's yeast, measured by skin prick test and RAST, and tolerance of foods containing yeast and yeast proteins (wine, bread, beer). The test population consisted of 13 individuals (all skin prick test-positive to baker's yeast and of which six reported symptoms of allergy upon ingestion of yeast-containing foods) and 24 healthy subjects. The investigators found that skin prick test reactions could be elicited by extracts of yeast-derived foods in all of the test population plus two of the control group. However, they went on to show that the symptoms of food allergy reported by some of the test population were not associated with the yeast component of the food. The authors concluded that "the IgE mediated allergy to baker's yeast alone should not lead to the denial of bakery, brewery and wine products." These results are entirely consistent with our findings, in that all the fish-allergic subjects who were skin prick test positive to yeast in our studies are able to consume foods containing yeast without adverse reaction.

Allergic reactions to yeast by ingestion were investigated in one double-blind placebo controlled challenge study (Parker et al., 1990). Forty-five patients with classic signs of food allergy and/or subjective food-related complaints not traditionally associated with food allergy underwent evaluation. The authors found that reports of reactions to yeast were associated with patients having non-specific and subjective symptoms (e.g., headache, malaise, etc.), rather than the typical signs of classical food allergy (e.g., wheezing, breathlessness, swelling of throat, etc.). Double-blind challenge of a person reporting generalized itching on eating yeast failed to reproduce the symptoms described in her clinical history.

The only report of an objective reaction that might be attributable to yeast following ingestion is an anecdotal account of an infant who experienced angioedema of the lips after eating bread with Marmite, a yeast extract (Higson, 1989). However, this product also contains biogenic amines, which are the endogenous mediators of allergic reactions, so the report does not prove that the reaction was due to allergy to yeast.

Taken together, these studies indicate that significant allergic responses to yeast are extremely rare. Indeed, it is debatable whether yeast induces any significant allergic response at all, as reported symptoms often cannot be verified as true food allergy to yeast.

The fish-allergic subjects in our studies belonged to an exceptionally sensitive population in terms of allergic susceptibility. All of the subjects who experienced a positive reaction to yeast in the skin prick test and had a positive RAST response had atopic dermatitis or other IgE mediated conditions such as bronchial asthma and hay fever (Table 16). They thus belong to a subset of the allergic population that is particularly likely to be sensitised to yeast (Kortekangas-Savolainen *et al*., 1993c). However, despite their sensitivity they are able to tolerate foods containing yeast. In short, the reactions of these subjects in our studies are expected, and are not suggestive of any clinically significant reaction to yeast. Indeed, the allergological specialist treating them indicated that they were all able to tolerate foods containing yeast.

In conclusion, based on (1) the lack of significant reported allergic reactions to yeast and (2) the long history of safe use of yeast and yeast based products in foods, the yeast component of ISP Type III preparation does not pose a clinically significant allergenic risk.

Table 16. Medical background of patients reacting to yeast.

	Patient 11	Patient 19	Patient 27	Patient 31
Medical history	Severe atopic dermatitis treated with tacrolimus, group 3 steroids, UVB therapy, emollients azathioprine and prednisolone previously. Severe asthma treated with Inhalant steroids, methyl xanthines and beta-2 agonists Hay fever (due to grass pollen)	Severe atopic eczema since childhood currently treated with group 2 and 3 steroids Prednisolone, antibiotics, tar, azathioprine used in the past. Bronchial asthma (horse) treated with local steroids, beta-2 agonists.	Moderate atopic eczema controlled with group 2 and 3 steroids. Hay fever in grass pollen season and in contact with cat and dog.	Bronchial asthma treated with inhalant steroids and beta-2 agonists Hay fever to birch and grass pollen, and in contact with dog and horse. Allergic to parsley, garlic and tomato (in addition to fish)
Positive control	4.5	8	6.0	6.5
Negative control	0	0	0	0
Birch	4	0	0	12
Grass	5	8	3.0	14
Mugwort	5	0	0	3
Horse	6.5	6.5	0	2
Cat	4.5	8.5	13.0	6
Dog	8	7.5	7.0	9
House dust mite (Der p)	4	2	0	2
Storage mite (Der f)	5	0	0	0
Alternaria	3.6	0	0	7
Cladosporium	3	0	0	4
Hazelnut	5.5			
Soy	0			
Celery root	4			
Brazil nut	3			
Sesame	6			
Cod	9-8.5	18.5-19	16.0-10.0	11-9.5
Herring	13.5			
Plaice	17	19.5-10.5		
Tuna		10-6.5		
Mackerel	12.5	14.5-13.5		
Salmon	12.5-9.5	9.0-9.0	10.5-11.5	11-9
Trout	9.5-15	12.5-13.0	13.0-15.5	19-16.5
Eel	23-18	12.5-16.00	12.0-15.5	12-14
Eel pout	15.5-16.5	19.5-17.0	11.5-8.5	14-17.5
Ocean pout	20.5-17.5	17.5-15.5	11.0-7.5	11-18.5

Skin prick test reaction are given as 2 largest diameters in mm.

10.5.4 *Antibody Response to Ingestion of ISP Preparation*

Investigations of antibody production resulting from ingestion of proteins in man have been undertaken in a research context (reviewed by Husby, 2000). However, they have not been previously applied in the context of safety evaluation and are not part of the regimens of the FAO/WHO consultation (2001) or the Codex Alimentarius *ad hoc* task force (2002). Interpretation therefore must be done cautiously because there is no background knowledge about the meaning of the data as there is for other procedures described in this document.

Forty-two normal, healthy adults without a history of previous consumption of ISP Type III were recruited for the study, to supply further supportive information about the allergenic potential of ISP Type III preparation. Thirty-seven met the eligibility criteria for inclusion. The study design was double-blind: neither the participants nor the examining physician knew who was in the test and control groups. Twenty-eight of these individuals were allocated randomly to the test group and nine to the control group. Each volunteer underwent a pre-study medical examination and was asked to complete a questionnaire to elicit information about relevant medical conditions and allergological status. Individuals in the test group received ISP Type III preparation providing 16.3 mg ISP Type III HPLC 12 in 50-100ml of a flavoured drink daily for 5 days a week for 8 weeks. The selected dose corresponds to an estimate of ISP Type III HPLC 12 intake for 90th percentile consumers in USA. No correction was made for body weights. The control group received the flavoured drink alone. Based on the pre-study questionnaire, seven members of the test group and four of the control group had an atopic predisposition. The health of the subjects was monitored in two ways:

- Medical examinations were administered at 0, 4, 6, 8, and 13 weeks. At each examination, the physician asked the volunteer about, and recorded any symptoms, whether or not they were thought to be attributable to ingestion of the test material.
- Clinical measures included specific IgG and IgE antibodies to ISP Type III as well as routine clinical chemistry and haematology screens. Each of those measures was assessed pre-study and at 4, 6, 8 and 13 weeks. All blood samples (13ml) were taken under non-fasting conditions and no lifestyle restrictions were placed on the participants as a condition of inclusion.

No clinical symptoms or biochemical changes suggestive of food allergy were observed (Table 17). As is almost invariably the case in a study of this duration, some ordinary health conditions were reported by both test subjects and controls. These are not considered to be related to ingestion of ISP Type III preparation. Subject 7 reported mild itching in the upper body from the start of the study, decreasing in intensity from the beginning to the end of each week. This subject did not display any objective symptoms and clinical measures were all within normal ranges. The only other finding in this person was an elevated AU value at each time point, including pre- and post-study, in the IgG ELISA to ISP Type III, which most likely reflected non-specific binding, as already discussed above. The timing of the symptoms experienced by subject 7 (i.e. from the beginning of the study), together with the absence of any changes in specific antibody production indicate that they are not due to sensitisation to a component of the ISP preparation.

Table 17. Clinical signs recorded during the ingestion study.

Clinical sign	Week 4		Week 6		Week 8		Week 13	
	T	C	T	C	T	C	T	C
Itching (upper body)	1/28		1/28		1/28			
Upper respiratory tract infection				1/9	2/28		1/28	
Sore throat	1/28				1/28			
Sickness and diarrhoea	1/28	1/9						
Grumbling stomach			1/28					
Gastritis				1/9				
Palmar eczema (flare of)	1/28							
Seborrheic dermatitis								1/9
Vesicular rash							1/28	
Exercise-induced bronchial asthma			1/28					
Achilles tendonitis						1/9		
Lumbar pain							1/28	
Suspected infection (urine dipstick)	1/28							
Irregular menstrual cycle					1/28			

Fourteen panellists (including 3 controls) were considered to have an atopic status, based on the pre-study questionnaire. There were no significant changes in clinical chemistry or haematology attributable to ingestion of ISP Type III preparation.

Specific IgG to ISP Type III HPLC 12 was measured by enzyme-linked immunosorbent assay (ELISA). The solid phase was prepared by coating the wells of a microtitre plate (Greiner, UK) with an anti-ISP monoclonal antibody, followed by ISP Type III preparation. Test or control sera, diluted 1:50, were then added followed by anti-human IgG (Sigma) conjugated to alkaline phosphatase. Absorbance at 490 nm was measured on a Spectramax 190 plate reader (Molecular Devices, Sunnyvale, CA, USA).

Sera from subjects 7, 18, 21, 25, and 28 displayed optical densities above the cut-off point of the assay, defined as the greater of either 0.1 AU or the AU of the mean of the negative control sera plus two standard deviations (Table 18) throughout the study. As these optical densities were elevated in the pre-test sera and did not increase as the study progressed, that ingestion of the test material did not induce production of specific IgG antibody, nor did it stimulate any potential pre-existing response. The binding of the sera showing the two strongest responses were further investigated in inhibition experiments with the test material (ISP Type III preparation) or mannose (the sugar residue found on glycosylated ISP). Neither material produced any meaningful inhibition, indicating that they were not responsible for the binding of IgG (data not shown). Since these values were elevated prior to any exposure to the test material, they therefore seem most likely to be due to a higher level of non-specific binding of the individuals' IgG.

Specific IgE to ISP Type III preparation was measured using the MaxiSorp^{TM} RAST system as described previously. The test revealed one weak specific IgE response, peaking at week 4, and possibly indicative of a physiological phenomenon. It was not accompanied by an IgG response, casting doubt on whether it was a true positive finding. Nonetheless, this response was further investigated using RAST inhibition, basophil histamine release, and immunoblots to identify the IgE binding components, as well as skin prick testing to confirm the result (Tables 19 and 20, and Figure 6). The test materials used were as described for the study in the fish-allergic patients. Subject 19 showed a positive skin prick test to ISP Type III preparation and yeast fermentation supernatant, but not to the ISP Type III HPLC 12 standard. This subject also did not respond when skin prick tested with ocean pout. Immunoblots and basophil histamine release experiments were similarly negative. ISP Type III HPLC 12 standard inhibited Subject 19's RAST response to ISP Type III preparation, although not as effectively as yeast fermentation supernatant. However, the significance of this result is doubtful given the initial low activity of the sample. As discussed previously, the skin prick test is generally considered more sensitive than *in vitro* methods in detecting low levels of sensitisation (Bernstein *et al*., 1994), implying that a positive response in the RAST in the presence of a negative skin prick test is a false positive. An additional MaxiSorp^{TM} RAST using yeast fermentation supernatant as a solid phase was positive.

Additional screening for common allergens (Table 21) indicates that the subject is sensitised to a multiplicity of common allergens. Given, therefore, the negative results in the other investigations, including particularly the skin prick tests, together with the very marginal response to ISP Type III preparation by this subject, this RAST inhibition result is considered to be a false positive. Taken as a whole, these investigations demonstrate that Subject 19 did not respond to ISP Type III HPLC 12, but to the yeast proteins in the preparation.

It is concluded that this study shows no evidence of ISP Type III HPLC 12 immunogenicity. Table 18 summarises all the individual results. Tables 19, 20 and 21, and Figure 6, give the results of the additional investigations on Subject 19. This work has been submitted for publication by Crevél *et al*. (2006).

Table 18. Specific IgG and IgE antibody response to ISP Type III.

Subject No.	**Group**	**Specific IgG (OD units) [1]**			**Specific IgE (cpm) [2]**		
		Wk 0	*Wk 4*	*Wk 6*	*Wk 0*	*Wk 4*	*Wk 6*
1	T	0.047	0.047	0.034	30.7	38.5	54.8
2	T	0.061	0.062	0.044	62.1	56.8	52.1
3	T	0.035	0.036	0.036	65.8	37.1	55.5
4	C	0.030	0.025	0.029	64.6	44.5	43.5
6	T	0.047	0.047	0.042	41.3	45.0	41.1
7	T	***0.640***	***0.648***	***0.648***	25.2	28.8	44.2
8	T	0.058	0.051	0.049	56.8	44.7	51.1
9	C	0.025	0.019	0.018	61.5	34.6	29.7
10	T	0.039	0.036	0.031	33.5	62.4	45.4
11	T	0.035	0.032	0.031	54.1	33.7	32.0
12	T	0.059	0.034	0.036	42.6	44.0	38.3
13	C	0.072	0.032	0.036	45.6	55.8	39.7
14	T	0.025	0.032	0.022	90.8	38.8	33.8
15	T	0.041	0.044	0.038	65.7	41.6	41.9
16	C	0.111	0.120	0.109	70.1	46.3	49.7
17	C	0.041	0.060	0.044	63.7	29.4	35.6
18	T	***0.474***	***0.535***	***0.477***	64.8	48.9	41.5
19	T	0.038	0.042	0.041	***66.3***	***141.1***	***100.0***
20	T	0.025	0.028	0.031	66.7	51.0	38.8
21	T	***0.125***	***0.125***	***0.133***	42.9	42.8	43.7
22	T	0.021	0.021	0.021	44.3	45.9	26.2
23	T	0.033	0.035	0.037	48.1	33.3	36.3
24	T	0.055	0.051	0.044	42.3	42.2	34.6
25	T	***0.146***	***0.158***	***0.163***	62.2	55	36.9
27	T	0.061	0.059	0.053	58.1	44.7	50.0

28	T	***0.143***	***0.148***	***0.158***	55.3	48.9	36.9
30	T	0.077	0.078	0.087	61.3	42.9	58.5
31	T	0.064	0.062	0.073	52.6	41.5	36.8
32	C	0.061	0.054	0.073	53.1	42.4	57.8
34	C	0.038	0.061	0.048	68.5	46.9	46.4
35	T	0.066	0.059	0.053	57.1	38.4	56.7
36	T	0.036	0.039	0.046	34.5	63.7	49.7
38	T	0.064	0.054	0.053	45.9	65.7	55.9
39	C	0.051	0.041	0.044	47.6	58.5	58.6
40	T	0.117	0.091	0.089	49.2	37.2	57.2
41	C	0.035	0.032	0.035	49.5	40.8	67.3
42	T	0.041	0.042	0.044	48.1	47.8	60.4
Negative control [3]		0.058 (0.014)			46.0		

[1] Specific IgG to ISP Type III HPLC 12.
[2] Specific IgE to ISP Type III preparation
[3] Mean and standard deviation OD of four control sera from people unrelated to the study; mean cpm from a pool of nonallergic sera.

Table 19. Results of further investigations of the IgE response by Subject 19.

SKIN PRICK TESTING (mean of perpendicular diameters, in mm)				
	Concentration (mg/ml)			
	5.0	**1.0**	**0.1**	**0.01**
ISP preparation [1]	3.6	2.6	0	0
Yeast fermentation supernatant [2]	3.5	0	0	Not tested
ISP standard	0	0	0	Not tested
RAST (ISP preparation) (cpm)				
	Serum dilution			
Time point	**1:2**	**1:5**	**1:10**	
Wk 0	88	78	66	
Wk 4	165	251	133	
Wk 6	163	182	124	
Control	53	41	37	
RAST (Yeast fermentation supernatant) (cpm)				
	Serum dilution			
Time point	**1:2**	**1:5**	**1:10**	
Wk 0	Not tested	Not tested	47	
Wk 4	Not tested	Not tested	134	
Wk 6	Not tested	Not tested	112	
Control	Not tested	Not tested	33	
RAST INHIBITION using week 4 serum (% inhibition)				
Inhibitor	*Concentration of inhibitor*			
Yeast fermentation supernatant	**0**	**1:125**	**1:25**	**1:5**
cpm	140	50	52	39
% inhibition	--	64	63	79
ISP standard	0	4	20	100
cpm	140	159	110	75
% inhibition	--	-14	21	46

[1] Subject 19 also had a negative skin prick test to ocean pout, which contains high levels of ISP Type III HPLC 12.
[2] Concentrations of yeast fermentation supernatant were 3.0, 0.87, and 0.087 mg/ml.

Table 20. Basophil histamine release (passive sensitisation) (ng/ml) for Subject 19.

Test material	**ISP standard**			**ISP preparation**			**Yeast fermentation supernatant**			**ISP preparation freeze-dried**		
Reciprocal serum dilution	#19	C	B	#19	C	B	#19	C	B	#19	C	B
1	43	27	0	55	31	7	63	57	62	2	1	1
3.5	8	5	0	30	37	8	33	31	24	1	0	2
12.5	4	1	0	18	24	6	12	11	11	2	0	2
43	2	0	0	6	3	4	5	4	5	3	2	2
150	1	0	0	4	0	3	4	2	4	5	1	0
525	0	1	0	3	0	2	4	1	4	1	0	0
1838	1	1	1	1	1	0	2	0	0	1	0	0
6434	1	0	2	1	0	0	1	1	0	2	0	2
22519	0	0	1	1	0	0	2	2	0	1	0	0
78816	0	0	0	2	0	0	1	1	1	2	0	0

#19: Subject 19
C: Control serum from non-allergic patient
B: Buffer control (no cells, no test material)

Figure 6. Western blot of serum from Subject 19 against ISP preparation.

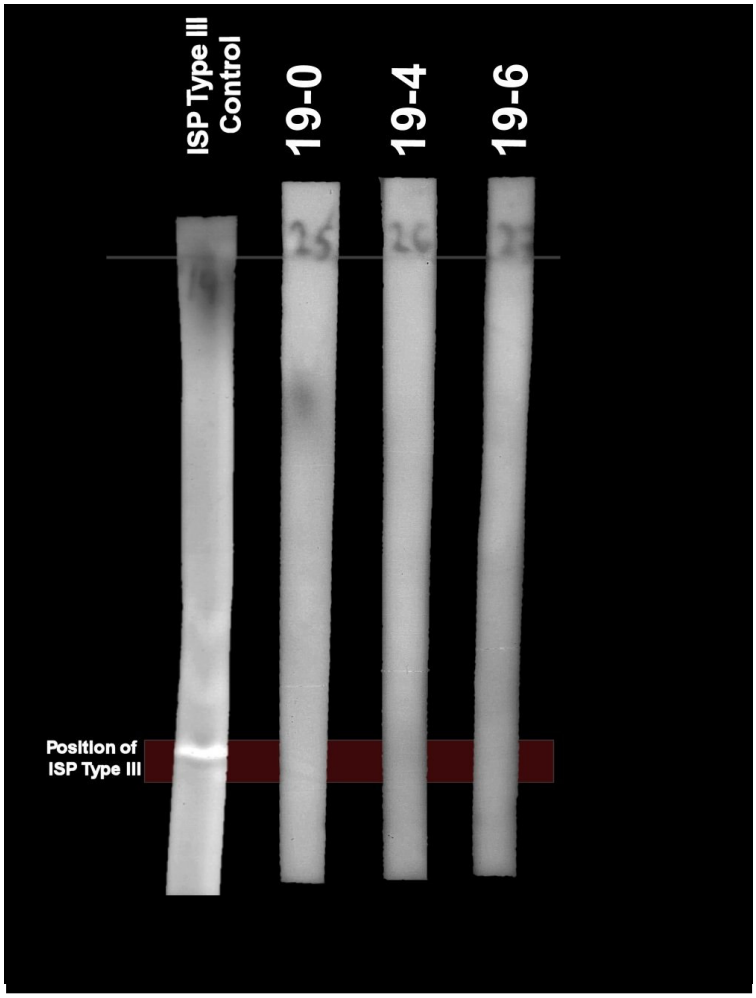


Table 21. Summary of allergenicity screen by skin prick testing on Subject 19.

Test material	**Mean diameter of reaction (mm)**
Histamine (positive control)	5.8
Diluent (negative control)	0
Pollens	
Birch	7.5
Grass	6.0
Mugwort	0
Foods	
Hazelnut	4.0
Soy	8.0
Celery	4.3
Brazil nut	3.0
Sesame seed	2.8
Melon	4.0
Nectarine	4.0
Fish	
Cod	2.0
Herring	0
Plaice	0
Tuna	0
Mackerel	0
Salmon	0
Trout	0
Eel	0
Eel pout	0
Ocean pout	0
Animals	
Horse	7.0
Cat	8.5
Dog	6.0
Mites	
D pteronyssinus	5.0
D farinae	2.5
Moulds	
Alternaria	2.5
Cladosporium	2.5

10.5.5 Resistance to Pepsin Hydrolysis (Study AE010134, Appendix 18)

In general, ingested proteins that are stable to gastric juices are more likely to come in contact with the intestinal mucosa where absorption and recognition by the immune system could occur, increasing the likelihood that they could be allergenic. On the other hand, unstable ingested proteins are less likely to reach the intestine and therefore less likely to be allergenic.

The stability of ISP Type III HPLC 12 and glyco-ISP was determined by incubating each with pepsin and following degradation by taking samples for analysis at various time points. A protein susceptible to digestion, bovine serum albumin, and a protein resistant to digestion, bovine β-lactoglobulin, were also tested in the same system.

The protocol described is based on the recommendations published by FAO/WHO (2001). Pepsin from porcine stomach with an activity of 2.87 U/mg protein, bovine serum albumin, and β-lactoglobulin from bovine milk were all purchased as lyophilized powders from Sigma (Poole, UK). Other reagents were laboratory-grade and obtained from standard commercial suppliers. The test material (0.5 mg/ml Kjeldahl protein) and pepsin (0.64 mg/ml, assuming 100% w/w protein content), at a substrate:enzyme protein ratio of 1:1.28 (by weight), were incubated in 0.03 M sodium chloride adjusted to pH 1.5, 2.5, or 3.5 with hydrochloric acid. Incubations continued for up to 120 minutes in a shaking waterbath at 37˚C. The pepsin activity was stopped by the addition of 0.3 ml of 0.8 M sodium carbonate per 1 ml of incubation solution. The solution was then neutralized to approximately pH 7.0 with 30 µl concentrated hydrochloric acid per 1 ml of incubation solution. Reagent, substrate, pepsin protein blanks, and test material control samples (without pepsin) were prepared and incubated as indicated.

The breakdown of ISP Type III HPLC 12 was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting, as well as by reverse phase HPLC. Gel filtration chromatography (GFC) (Yau *et al*., 1979) was used to monitor hydrolysis of the glyco-ISP, while matrix assisted laser desorption ionization time of flight (MALDI-ToF) mass spectrometry (Chapman, 1996) was used in addition to densitometric analysis of SDS-PAGE gels to identify and quantify fragments generated by pepsin hydrolysis of ISP Type III HPLC 12.

SDS-PAGE analysis. At pH 1.5, ISP Type III HPLC 12 (approximately 5.8 kDa band) was visible as a strongly staining band at 0 to 15 minutes, and as a faintly staining band at 30 minutes (Figure 7). After 60 minutes exposure to pepsin the band had disappeared. Densitometric analysis showed that the half-life of ISP Type III HPLC 12, determined from several experiments, was approximately 4 minutes under these conditions (Figure 8). At pH 2.5 and 3.5, the test material was still detectable at 60 minutes and 120 minutes respectively. The

corresponding half-lives were approximately 13 minutes at pH 2.5 and 28 minutes at pH 3.5 (Figure 8). The control proteins, bovine serum albumin and β-lactoglobulin, behaved as previously reported (Astwood *et al*., 1996). Bovine serum albumin was not detectable after 15 seconds while β-lactoglobulin showed a half-life in excess of 2 hr.

The breakdown of ISP Type III HPLC 12 was also quantified by HPLC (Figure 9), which was more reproducible than scanned densitometric readings. Results were consistent with the SDS-PAGE analysis. The glycoconjugates show poor resolution on SDS-PAGE gels and their breakdown could not therefore be followed by that method. GFC was used to investigate the fate of glyco-ISP, and showed that it was readily broken down.

Figure 7. SDS-PAGE gel illustrating pepsin hydrolysis of ISP Type III HPLC 12 at pH 1.5. (A) Molecular weight markers. (B) Pepsin control. (C) ISP control. (D) 0 minute. (E) 1 minute. (F) 6 minutes. (G) 15 minutes. (H) 30 minutes. (I) 60 minutes.

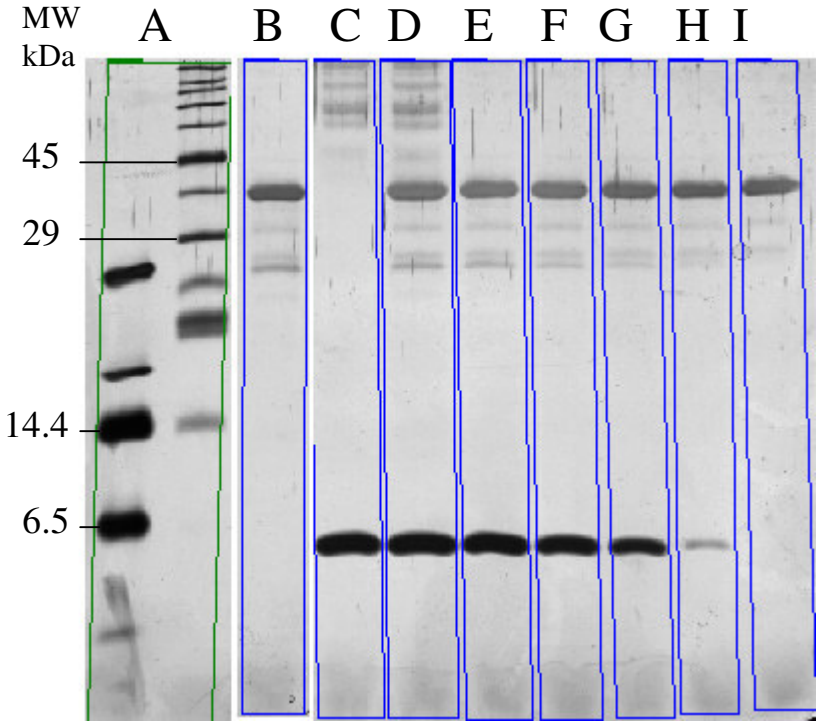


Figure 8. Proteolytic degradation of ISP Type III HPLC 12 by pepsin under different pH conditions estimated by densitometric analysis of SDS-PAGE gels.

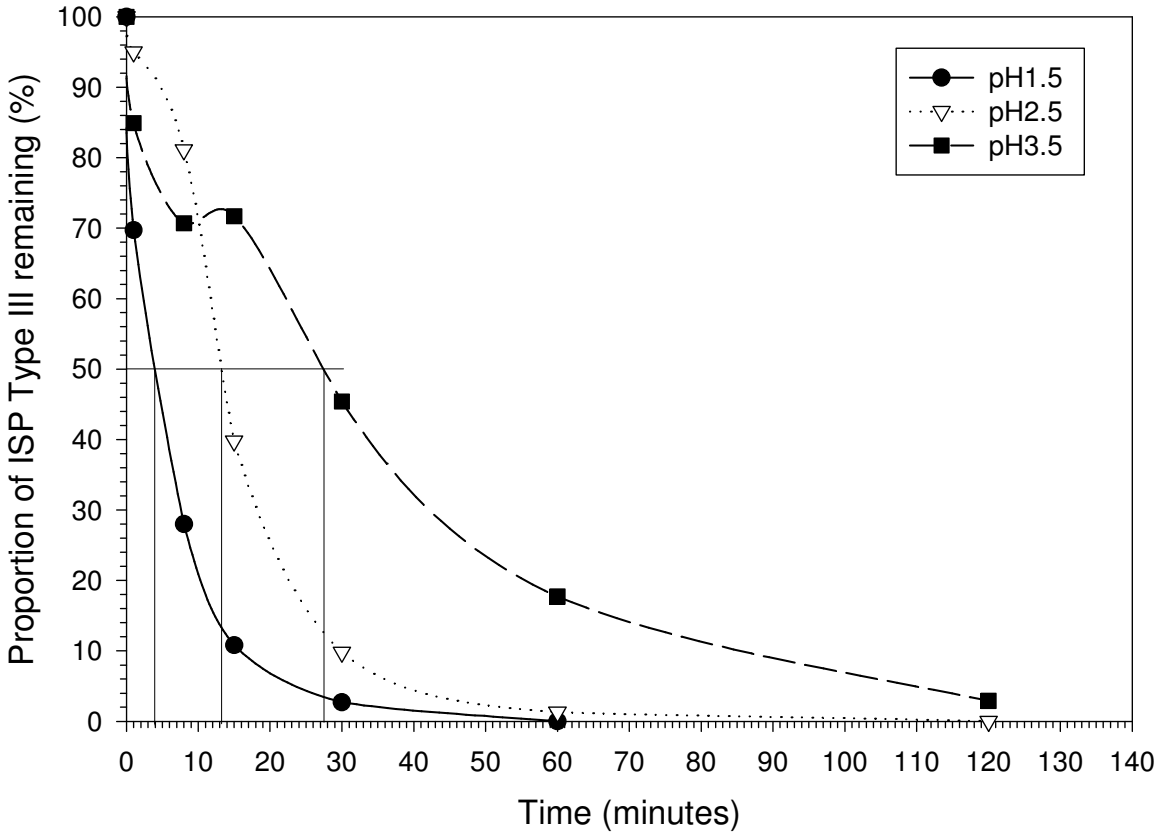
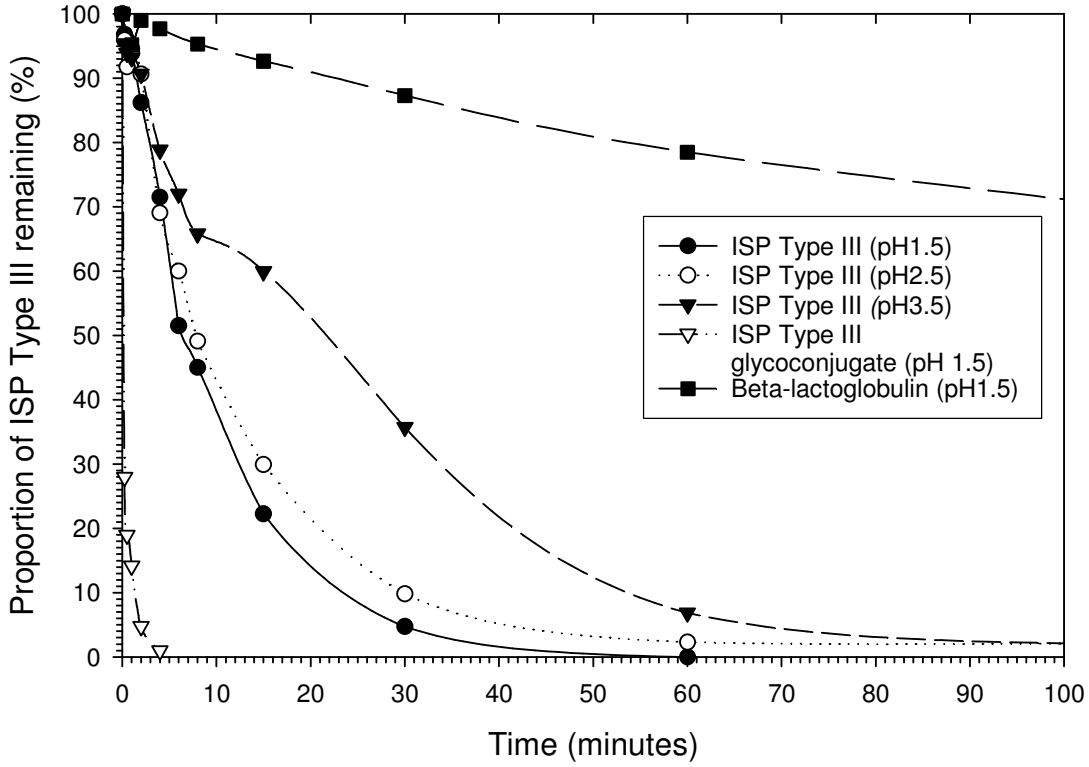


Figure 9. Proteolytic degradation of ISP Type III HPLC 12 by pepsin under different pH conditions estimated by HPLC analysis (native ISP) and gel filtration chromatography (glycoconjugate). Degradation of the positive control β-lactoglobulin is shown for comparison.



Tools are available to predict potential protease cleavage sites in a given protein sequence. The table below (Table 22) shows the peptides predicted to be formed from ISP Type III HPLC 12 by pepsin, based on knowledge of the preferred cleavage sites (PeptideCutter, 2002). Most of the peptides are very small, with the largest having a mass of 2.3 kD. Peptides of this size have a very limited ability to sensitise or elicit reactions. Moreover, a principal breakdown product identified experimentally has a molecular weight of 1.4 kD, further indicating that there is a low probability that ISP Type III HPLC 12 could sensitise or elicit reactions.

Table 22. Cleavage sites of ISP Type III HPLC 12 for pepsin (at pH 1.3 and pH >2), predicted by PeptideCutter.

Position of cleavage site	Resulting peptide sequence	Peptide length [aa]	Peptide mass [Da]
9	NQASVVANQ	9	929
16	LIPINTA	7	740
17	L	1	131
18	T	1	119
19	L	1	131
40	VMMRSEVVTPVGIPAEDIPRL	21	2308
50	VSMQVNRAVP	10	1099
54	LGTT	4	390
66	LMPDMVKGYPPA	12	1318

PeptideCutter also predicts that trypsin and chymotrypsin will hydrolyze ISP Type III HPLC 12, providing more assurance that the protein will be extensively degraded to small peptides in the gastrointestinal tract.

In summary, ISP Type III HPLC 12 and its glycoconjugate are readily broken down by pepsin under the recommended conditions as well as under milder conditions. With half-lives measured in minutes, there is little likelihood that the intact protein or its glycoconjugated form will be allergenic when ingested. This is in contrast to the major allergen in fishes (Gad c1 and analogs) which is heat-stable, acid-stable, and resistant to proteolytic degradation (Bindslev-Jensen and Poulsen, 1997; Metcalfe, 1997).

Parts of the work on pepsin degradation have been published by Baderschneider *et al*. (2002) and Bindslev-Jensen *et al*., (2003), and presented by Basketter *et al*. (2002). Baderschneider *et al*. (2002) and Bindslev-Jensen *et al*. (2003) are provided as Appendices 21 & 22 respectively.

10.5.6 Summary of Allergenicity Evaluation

Based on the data and observations detailed in this section, namely:

- no history of allergenicity from human consumption,
- no structural alerts for allergenicity,
- no similarity to known allergens,
- ready hydrolysis by pepsin,
- lack of binding of ISP Type III HPLC 12 to IgE,
- lack of histamine release from basophils of fish-allergic individuals in the presence of ISP,
- absence of skin prick test reactivity to ISP itself, and
- absence of immunogenicity, measured by the lack of an antibody response in a two-month ingestion study,
- no indication of any clinically significant reactions to yeast and the long history of safe use of yeast and yeast based products in foods

It is concluded that ISP Type III preparation is safe both for fish-allergic individuals and the population at large.

10.6 Summary of Safety Testing

Commercial ISP Type III preparation is a solution of proteins – ISP Type III HPLC 12, glyco-ISP Type III, and proteins and peptides from bakers yeast – and sugars, acids, and salts commonly found in food. When evaluating these components, the main concern was the possibility that the added protein might cause allergic reactions in individuals sensitised to fish or sensitise susceptible individuals. Assessing the potential allergenicity of a protein requires systematically assembling a body of evidence to show that neither individuals already sensitised to fish would react to the preparation nor that sensitisation would occur. A programme of testing was devised, based on the most recent available expert consensus recommendations, in particular the FAO/WHO decision tree (2001), with additional procedures to refine those methods, to thoroughly assess the allergenic potential of ISP Type III.

Ocean pout produced positive skin prick test results and positive results in *in vitro* allergenicity testing in established fish-allergic individuals. However, ISP Type III Batch 201008 FD did not bind IgE from fish-allergic subjects in the RAST, nor did it show any activity in a functional biological assay using basophils from the same fish-allergic individuals. Absence of IgE binding was confirmed visually by immunoblotting. Skin prick testing with ISP Type III did not produce any positive reactions to the protein, although four reactions to yeast proteins were observed and confirmed by *in vitro* tests. A confirmatory skin prick test with a highly purified ISP Type III HPLC 12 (yeast protein content <1% by gel filtration chromatography), was negative. Ingestion of ISP Type III preparation for eight weeks at a high daily dose did not result in specific antibody formation.

In addition, amino acid sequence analysis and susceptibility to proteolytic breakdown both indicated an extremely low possibility of inducing sensitisation.

These findings are consistent with information about the major allergenic proteins in fish and what is known about consumption of fish that contain ISP Type III. This information suggests that reaction by persons already sensitised to fish is not likely. The same information also suggests that sensitisation to this protein is not likely either. The findings thus support the conclusion that if these proteins are important allergens in fish, they would almost certainly have been identified, given the frequency of fish allergy and the intensity with which it has been studied (Crevel *et al*., 2002). Another indication that the protein is unlikely to be allergenic is its size. At 7.0 kDa, it falls outside the range for most common food allergens which is between 10 and 60 kDa (Metcalfe *et al*., 1996). Its proteolytic breakdown products are much smaller.

The weight of evidence obtained through the application of the FAO/WHO decision tree (2001) and the recommendations of the Codex *ad hoc* task force on foods from biotechnology (2002) indicates that ISP Type III preparation is highly unlikely to evoke a reaction in persons already sensitised to fish, and is highly unlikely to sensitise potentially susceptible individuals in the wider population.

An important aspect of the *in vitro* digestion work is the fact that both the protein and glycoprotein are readily degraded. This indicates a minimal likelihood that they will be absorbed intact or accumulate.

Consistent with this ready degradation, there is no evidence of toxicity in a 13-week rat study at the highest dose that could be tested, 4000 mg total solids/kg bodyweight/day, or 580 mg ISP Type III HPLC 12/kg body weight/day.

The genotoxic activity of ISP Type III Batch 201008 FD was assessed using four different assays: the bacterial reverse mutation assay, the *in vitro* chromosome aberration assay in human peripheral blood lymphocytes, the gene mutation assay in mouse lymphoma L5178Y cells, and the *in vivo* rat bone marrow micronucleus assay. There was no evidence of genotoxic activity in any of them.

In conclusion, no adverse effects were observed in any test at any dose of ISP Type III preparation.

10.7 Safety Assessment

10.7.1 Determination of the No-Observed Adverse Effect Level

Since there is no basis to assume ISP Type III preparation will provoke or induce an allergic response, the NOAEL was established from the 13-week rat feeding study. It is the study of longest duration, involved high exposure to the material, and was conducted according to FDA and OECD guidelines that require frequent and thorough examination of the animals. The material tested was representative of commercial batches (please refer to section 3.3., table 2). The ratio of glycosylated to non-glycosylated ISP for batches 201008, 200030, 200034 and 200046 is remarkably constant with a mean of 0.6. The ratio increases slightly to a mean of 0.7 when batches 201024 and 201083 are included, due to loss of the non-glycosylated species upon concentration, although these two batches are still well within specification.

The highest dose that could be tested in the 13-week rat study, 580 mg ISP Type III HPLC 12/kg body weight/day by gavage, showed no adverse effects. This was therefore selected as the NOAEL. As this is the active constituent in ISP Type III preparation, and all commercial calculations will be based on it, this section will make calculations based on the NOAEL for ISP Type III HPLC 12 rather than for total solids.

10.7.2 Determination of the Safety Factor and Safe Level of Intake

In the traditional assessment of safety, an appropriate safety factor is applied to the NOAEL in the study using the most sensitive animal species and/or the study giving the lowest NOAEL. A 100- or 1000-fold safety factor is typically applied to food ingredients to take into account possible differences in susceptibility between humans and the test species, possible individual susceptibilities within the human population, and differences in lengths of exposures. However, higher and lower values may be used depending on the specific material in question (Renwick, 1991). If such substances are free from toxicity when tested in animals at the maximum levels, then smaller safety factors may be appropriate especially if additional data are available to support safety. Such data include chemical structure suggesting low probable toxicity, adequate and reliable human clinical data, a history of safe intake of the ingredient from traditional foods, and studies that indicate that additional exposures are unlikely to result in adverse effects (Rubery *et al.*; 1990; Borzelleca, 1992).

In the case of ISP Type III HPLC 12 there is a history of human consumption of fish containing this protein without any indication of adverse effects. There is an absence of structural alerts from the amino acids present in the protein and from the structure of the protein itself. Both ISP Type III HPLC 12 and its glycoconjugated form have half-lives of minutes in an *in vitro* digestion test system and are therefore unlikely to be absorbed intact or accumulate in the

body. Based on a thorough assessment of allergenic potential, the material has an extremely low probability of being allergenic. There is no indication of toxicity or any toxicological or histopathological changes in the 13-week feeding study in rats. The material is not genotoxic in a series of mutagenicity and cytogenetic studies.

Therefore, based on the totality of analytical, animal, human, and *in vitro* data summarised in this document, and general knowledge of proteins, and using the approaches to estimating a safety factor outlined by Borzelleca (1992) and Renwick (1991), a safety factor of 100 was considered to be suitable for calculating a safe level of intake.

The safe level of intake was calculated as the NOAEL / safety factor and therefore for ISP Type III HPLC 12 the safe level of intake was determined to be:

$$\frac{\text{580 mg ISP Type III HPLC 12/kg body weight/day}}{100}$$

= 5.8 mg ISP Type III HPLC 12/kg body weight /day

10.7.3 Determination of the Estimated Daily Intake

The typical level of ISP Type III HPLC 12 in consumer products will be 0.005% by weight. The maximum concentration will be 0.01% by weight.

The estimated daily intakes (EDIs) have been calculated for:

- The group that has the highest edible ice intake for the UK (males aged 11-14 years old) at the 97.5th percentile
- The group with the highest potential ISP exposure (when bodyweight is considered) in the UK (female children aged 1.5-4.5 years)
- After seasonal shifts in edible ice intake in the UK are taken into account
- The group with the highest exposure for the Netherlands (adults of 22 years+) at the 95th percentile

The EDIs are shown overleaf in Table 23 and these calculations conservatively assume the following:

- That all the edible ice eaten contains ISP Type III HPLC 12 at the highest proposed level of use i.e. 0.01% by weight.
- The bodyweight for UK males aged 11-14 is the mean bodyweight for that age group reported in the UK NSDS anthropometric data.
- The bodyweight for UK females aged 1.5-4.5 years is the mean bodyweight reported for the youngest children in that age group (i.e. females aged 1.5-2.5 years) in the UK NSDS anthropometric data
- A mean bodyweight of 60kg for a Dutch adult

The data taking seasonal shifts into account is even more conservative, as this takes the average edible ice intake for the group of interest over the year and then adds the maximum seasonal shift for that group i.e. the shift from the lowest survey wave to the highest, rather than just the shift from average intake over the year to the maximum. Please refer to section 6 for further information on anticipated intake.

Table 23. Estimated Daily Intake of ISP for Various Population Groups

Group	Edible Ice intake (g)*	Level of ISP in the edible ice	ISP Intake (mg)	Bodyweight (kg)	EDI (mg of ISP Type III HPLC 12/kg bodyweight)
UK males 11-14 years 97.5th percentile	98.71	0.01% by weight	9.871	47	0.21
UK females 1.5-4.5 years 97.5th percentile	63.58	0.01% by weight	6.358	11.9	0.53
UK males 11-14 years 97.5th percentile plus a seasonal shift of 37.7% in edible ice intake	135.92	0.01% by weight	13.592	47	0.29
UK females 1.5-4.5 years 97.5th percentile plus a seasonal shift of 9.25% in edible ice intake	69.46	0.01% by weight	6.946	11.9	0.58
Dutch adults (22 years+) 95th percentile	100	0.01% by weight	10	60	0.17

* Please refer to section 6 for further details

Conservative approaches have been used to establish both the EDIs and the safe level of intake. The resulting EDIs are 28-times (UK young males), 11-times (UK female children aged 1.5-4.5 years) and 34-times (Netherlands adults) less than the safe level of intake. Even with seasonal shifts in edible ice intake in the UK taken into account the resulting EDIs for UK young males and UK female children aged 1.5-4.5 years are 20-times and 10-times less than the safe level of intake.

To illustrate how large the margin of exposure is between a single serving of a product and the safe level of intake, an 11.9 kg pre-school female child could consume 0.7 kg (~1.4 litres of ice-cream) of edible ice per day containing the maximum ISP Type III HPLC 12 level of 0.01% without exceeding the safe level

of intake, a 47 kg young UK male could consume 2.7 kg of edible ice and a 60kg adult from the Netherlands could consume 3.4 kg of edible ice per day. If the more typical level of 0.005% was used in products, then the amounts of product that would have to be consumed to reach the safe level of intake would double.

10.8 Final Conclusion

All 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.