GR

Original Submission

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October 18, 2002

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRAS Notification for Ice Structuring Protein

To Whom It May Concern:

Enclosed please find for filing an original and two copies of a GRAS notification submitted by Good Humor-Breyers, a Unilever company, for ice structuring protein.

Also enclosed is an additional copy of the notification letter to be stamped as received and returned to us by messenger.

Thank you for your assistance with this matter.

Sincerely,

Daniel R. Dwyer

enclosures

RECEIVED
OCT 23 2002
FOOD ADDITIVE SAFETY

Unilever

October 18, 2002

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: Notice of a GRAS Exemption Claim
for Ice Structuring Protein

To Whom It May Concern:

Good Humor-Breyers, a Unilever company, hereby provides the following information pursuant to proposed 21 CFR 170.36(c)(1) (62 Fed. Reg. 18938, 18961; April 17, 1997):

GRAS Exemption Claim: Unilever/Good Humor-Breyers hereby claims that a preparation consisting of Ice Structuring Protein Type III HPLC 12 (described in detail in the accompanying comprehensive summary of safety information, and referred to by the common or usual name "ice structuring protein" (ISP)), intended for use as a texturizer and for related uses in frozen novelty products in amounts not to exceed 0.01% by weight, is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because Unilever/Good Humor-Breyers has determined that it is generally recognized as safe (GRAS) for such use, using scientific procedures. The ISP covered by this GRAS notification is intended to be identified on the label of frozen novelties only by the common or usual name (e.g., ice structuring protein) declared in the designation of ingredients pursuant to 21 CFR 101.4.

(i) ***Name and Address of Notifier***: Nancy L. Schnell
Deputy General Counsel –
Marketing and Regulatory
Good Humor-Breyers, a Unilever Company
390 Park Avenue
New York, NY 10036
Tel. 212-906-4573
Fax 212-318-3680

(ii) ***Common or Usual Name of the Substance***: Ice Structuring Protein.

Unilever United States, Inc.
Lever House 390 Park Avenue New York, New York 10022-4698
Telephone (212) 888-1260

(iii) ***Applicable Conditions of Use*:** As a texturizer and for related uses in frozen novelty products in amounts not to exceed 0.01% by weight.

(iv) ***Basis for the GRAS Determination*:** Scientific procedures.

(v) ***Statement of Availability*:** The data and information that are the basis for Unilever/ Good Humor-Breyers' GRAS determination are available for FDA review and copying at reasonable times at the offices of Kleinfeld, Kaplan and Becker, 1140 Nineteenth Street, N.W. Washington, D.C. 20036 (Daniel Dwyer, 202-223-5120), and such data and information will be sent to FDA upon request.

Additional required information, including a comprehensive summary of the data relied on to establish safety, is enclosed.

Please contact the undersigned if you have any questions regarding this notification.

Sincerely,

Nancy L. Schnell
Deputy General Counsel –
Marketing and Regulatory

enclosure

GRAS NOTIFICATION

for

Ice Structuring Protein
used in Frozen Novelty Desserts

Submitted by:

Good Humor-Breyers, a Unilever company

Unilever United States, Inc.
Lever House
390 Park Avenue
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Contact:
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October 18, 2002

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ABBREVIATIONS, ACRONYMS, AND TERMS

ADI – Acceptable Daily Intake
AU – Absorbance Unit
CFR – Code of Federal Regulations
cpm – counts per minute
CSFII – USDA's Continuing Survey of Food Intakes by Individuals
EDI – Estimated Daily Intake
FAO/WHO – Food and Agricultural Organization/World Health Organization
FCS – Fetal Calf Serum
FDA – Food and Drug Administration
GFC – Gel Filtration Chromatography
Glyco-ISP – ISP type III HPLC 12 with mannose(s) glycoconjugated by the yeast
GRAS – Generally Recognized as Safe
GU – Glycine units
HPLC – High Performance Liquid Chromatography
IC50 – Concentration producing a 50% inhibition
ISGP – Ice-structuring glycoprotein from fish such as cod
ISP – Ice-Structuring Protein(s), a general term for any and all forms
ISP type III – a specific type of ISP, but not otherwise specified.
ISP type III HPLC 12 – the specific, functionally active molecule in the commercial preparation
ISP type III preparation – the commercial material to be used by Unilever/Good Humor-Breyers containing ISP type III HPLC 12, other proteins, and sugars, acids, and salts
JECFA – Joint FAO/WHO Expert Committee on Food Additives
kDa – kiloDalton
LC – Liquid Chromatography
MF – Microfiltration
MS – Mass Spectrometry
NHANES – National Health and Nutrition Examination Survey
NOAEL – No Observed Adverse Effect Level
OD – Optical Density
OECD – Organisation for Economic Co-operation and Development
ppm – parts per million
RAST – RadioAllergoSorbent Test
SDS-PAGE – Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SPT – Skin prick test
Total ISP type III – ISP type III HPLC 12 and glycoconjugated ISP type III HPLC 12.
U – Unit
UF – Ultrafiltration
USDA – United States Department of Agriculture
YNB – Yeast Nutrient Broth
+ – positive
- – negative

I. INTRODUCTION

A. Regulatory Background

Pursuant to 62 Fed. Reg. 18938, 18960 (April 17, 1997) (proposed 21 C.F.R. §170.36), Good Humor-Breyers, a Unilever company, hereby claims that a preparation consisting of Ice Structuring Protein Type III HPLC 12 (described in detail in this document, and referred to by the common or usual name "ice structuring protein" (ISP)), intended for use as a texturizer and for related uses in frozen novelty products in amounts not to exceed 0.01% by weight, is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because Unilever/Good Humor-Breyers has determined that it is generally recognized as safe (GRAS) for such use, using scientific procedures. The ISP covered by this GRAS notification is intended to be identified on the label of frozen novelties only by the common or usual name (e.g., ice structuring protein) declared in the designation of ingredients pursuant to 21 CFR 101.4.

This document accompanies the GRAS exemption claim required by proposed 21 CFR §170.36(c)(1) and provides the detailed information required by proposed 21 C.F.R. §170.36(c)(2), (3), and (4). The following table correlates the requirements of the proposed regulation with the sections in this report where the information may be found:

Requirements of the proposed rule	*Chapter No(s).*
§170.36(c)(2): Detailed information about the identity of the notified substance, composition, method of manufacture, characteristic properties, and specifications	I, II, III
§170.36(c)(3): Information on any self-limiting levels of use	VI.E
§170.36(c)(4)(i)(A): Comprehensive discussion of, and citations to, generally available and accepted scientific data and information, including consideration of probable consumption	IV, V, VI, VII
§170.36(c)(4)(i)(B): Comprehensive discussion of any reports that may appear to be inconsistent with the GRAS determination	IV, V, VI, VII
§170.36(c)(4)(i)(C): The basis for concluding that there is a consensus of qualified experts that there is reasonable certainty that the substance is not harmful under the intended conditions of use	VII, VIII

This GRAS determination is based on generally available and accepted scientific data, information, methods, and principles, corroborated by unpublished scientific data and information. The ISP covered by this GRAS determination is derived from baker's yeast (*Saccharomyces cerevisiae*). It is identical in structure to a protein found in certain fish, therefore this GRAS determination has carefully evaluated the potential allergenicity of ISP and

concluded that it is safe both for fish-allergic individuals and the population as a whole. This conclusion is supported by the views of a panel of independent experts, including experts on food allergy (Wesley Burks, M.D., Hugh A. Sampson, M.D., and Steve L. Taylor, Ph.D.) and food safety (Joseph F. Borzelleca, Ph.D., Walter H. Glinsmann, M.D., and Michael W. Pariza, Ph.D.). Accordingly, this GRAS determination meets the requirements of §201(s) of the Federal Food, Drug, and Cosmetic Act; 21 C.F.R. §§170.3 and 170.30; and the amendments to these rules proposed at 62 Fed. Reg. 18960.

B. Use of Ice-Structuring Proteins to Modify Ice Structure in Frozen Novelty Desserts

Ice cream is a complex, frozen, aerated emulsion, made up of four phases: air, ice, matrix (the unfrozen part containing sugar, protein, stabilizers, flavors, and emulsifiers), and fat. Water ice has a simpler structure, generally comprising only ice and matrix, although air is sometimes added. For both ice cream and water ice, the ice phase is the largest part of the products, and as such has a major effect on their physical and sensory properties. The size and structure of the ice crystals affect temperature stability, which is an important factor in maintaining good product quality in extended supply chains. It also affects the sensory attributes of the product such as hardness, creaminess, and flavor delivery, and therefore is important in determining consumer acceptance and appeal.

Until recently, the ability to influence the properties of the ice phase in frozen novelty desserts (individually wrapped, single-serve ice creams and water ices) has been fairly limited. However, the discovery over the last four decades of proteins in nature able to control the growth, size, and structure of ice crystals has opened up a host of opportunities for the ice cream and water ice industry. These proteins were originally termed "antifreeze proteins," but more recently have been termed "ice-structuring proteins" (ISPs) since they do not actually stop ice formation but instead bind to and directly influence the growth and structure of ice crystals (Clarke et al., 2002). This binding modifies the resulting ice structure and therefore the physical properties of the product. The primary interest in producing ISPs is to create novel ice cream product textures. However, ISPs also improve the quality of frozen novelty products by increasing their resistance to temperature abuse.

Unilever/Good Humor-Breyers has been evaluating the use of ISP type III, originally identified in ocean pout, *Macrozoarces americanus*, an eel-like fish found in cold water from Labrador to Delaware, as an ingredient in frozen novelty desserts. This ingredient, when added at low levels (approximately 0.005%), alters the ice structure of the products, resulting in products that provide totally novel eating experiences and are more resistant to temperature abuse.

C. Literature Searches

As an integral part of its assessment of the safety of ice-structuring proteins, Unilever has performed literature searches in different scientific databases in an on-going basis over the years.

Additional searches were performed in preparing the articles accepted for publication. Finally, to be certain that nothing new had been published in the few months before the Expert Panel met, a series of searches was performed on publicly available scientific literature databases in late April and September, 2002.

Other databases used for evaluating protein structures, comparing the amino acid sequence of ISP type III HPLC 12 to known allergens, and the cleavage of proteins are cited in the relevant sections.

Unilever will continue to monitor the literature in this area.

D. Background on Ice-Structuring Proteins

Function and mechanism of action

Ice-structuring proteins protect organisms against ice damage both by lowering the point at which ice crystals grow and by modifying the size and shape of the ice crystals that are formed. Unlike chemical compounds such as ethylene glycol and salt, ISPs exert their freezing point-depression activity by a non-colligative mechanism, thereby minimizing their effect on the osmotic pressure of cells or plasma. On a molar basis, they are estimated to be 200- to 500-times more effective than sodium chloride (Avanov, 1990). Ice crystal growth can be inhibited by 3 to 25 μM ISP type III (Li and Hew, 1991).

Ice-structuring proteins exert their effect by binding directly to the growing ice crystal and thereby modifying its size and morphology, with different ISPs showing preference for different crystal planes (Barrett, 2001). Mechanisms of interaction of different ISPs with ice crystals are thought to include hydrogen bonding (DeVries and Lin, 1977), hydrophobic interactions (Harding et al., 1999; Zhang and Laursen, 1998) and hydrophilic interactions (Li and Hew, 1991), depending on the type of ISP.

Occurrence of ISPs in Nature

Ice-structuring proteins are naturally occurring proteins and peptides that were first identified over thirty years ago in the blood of fish living in areas where the sea freezes, such as cod and herring (DeVries and Wohlschlag, 1969; Fletcher et al., 1999). Since then, ISPs have been found in a wide variety of organisms that need to protect themselves against freeze damage, including many plants, insects, fungi, and bacteria (Griffith and Ewart, 1995). Duman and Olsen (1993) noted that ISPs had been found in at least 23 species of angiosperms, including a number of edible ones. Plants in which ISPs have been found include such common food sources as oats, rye, barley, wheat, carrot, and potato (Griffith and Ewart, 1995). In many plants, they are found in the edible parts, such as the carrot tap root, potato tuber, or leaves of Brussels sprouts (Urrutia et al., 1992; Smallwood et al., 1999).

Only limited data are available on the ISP content of different organisms. For plants, the only quantification is up to 0.307 mg/g fresh weight in the leaves of winter rye (Griffith, 1999). More

data are available on the ISP content of fish. The ISP type III content of ocean pout is estimated to be about 30 mg/mL in blood, and for Atlantic cod ice-structuring glycoprotein (ISGP) concentration ranges from around 7 mg/mL in adult fish to 14 mg/mL in juveniles, which spend more time in icy waters (Kao and Fletcher, 1988). Assuming that the blood volume of teleost (modern bony) fish is about 30-70 mL/kg (Olson, 1992), the ISP content of an ocean pout will be around 900-2100 mg/kg, while that of a cod will range from 210-490 mg/kg in adults and from 420-980 mg/kg in juveniles.

A more detailed review of the occurrence of ISPs in nature can be found in Crevel et al. (2002).

Characteristics of ISPs

As would be expected from the diversity of organisms that produce ISPs, the characteristics of ISPs are extremely diverse also. Amino acid composition of ISPs varies considerably both between species and sometimes within species (see, for instance, Hon et al., 1994). Molecular weight range is also extremely wide, from 2.5 kDa in some fish ISGPs to 36 kDa for one of the ISPs of winter rye. Primary structure ranges from the repeating amino acid subunits (Ala-Ala-Thr) of cod ISGP to the general structure of fish type III, carrot ISP, and rye ISPs, in which no amino acid predominates. The various ISPs are compared in Table 1.

Table 1. Comparison of various and diverse ISPs.

Ice-Structuring Proteins							
Characteristic	**Fish ISGP**	**Fish type I**	**Fish type II**	**Fish type III**	**Fish type IV**	**Carrot**	**Winter rye**
Mass, kDa	2.6 - 33	3.3 – 4.5	11 - 24	6.5 - 14	12.3	36	11 - 36
Primary structure	Repeated (alanine-alanine-threonine) disaccharide	Alanine-rich multiple of 11-amino acid repeats	Cysteine-rich, disulfide linked	General[1]	Glutamine- and glutamate-rich (26%)	General[1]	General[1]
Glycoprotein	Yes	No	No, except that smelts have <3% carbo-hydrate	No	No	Yes	No
Secondary structure	Expanded	α -Helical amphiphilic	β-Sheet	β-Sandwich	α -Helix	ND[2]	ND[2]
Tertiary structure	ND[2]	100% Helical	Globular c-type lectin fold	Globular	Four-helix bundle	ND[2]	ND[2]; includes disulfide bonds
Protein components	8	7	2-6	12	1	1	7
Gene copies	ND[2]	80-100	15	30-150	ND[2]	1	ND[2]
Natural source	Antarctic notothe-nioids, northern cods (Atlantic cod, Greenland cod)	Right-eyed flounders (winter flounder), shorthorn sculpin	Sea raven, smelt, herring	Ocean pout, eelpout, wolffish	Longhorn sculpin	Carrot	Winter rye

[1] General structure with no predominant amino acids or repeat units
[2] Not determined
Adapted from Fletcher et al. (1999) and Barrett (2001).

The evolutionary origin of ISPs is also diverse. For instance, ice-structuring glycoprotein from some Antarctic fishes was found to have evolved from a trypsinogen gene (Chen et al., 1997), while in winter rye and a wheat variety, ISPs were encoded by genes for a class of pathogenesis-related proteins, chitinases (Yeh et al., 2000).

Intake of ISPs from food

One of the main sources of ISP in the human diet is cold-water fish. A 200 g portion of ocean pout would result in an intake of between 180 mg and 420 mg of ISP type III, while the same weight of cod could contain between 42 and 196 mg of ISGP. These figures represent acute exposure, but chronic exposure is also relevant when considering the safety of a protein. Long-term exposure was estimated for the USA and Iceland from data on fish catches and consumption published by the U.S. National Marine Fisheries Service and U.S. Department of Agriculture's Economic Research Service. The USA was chosen as a country where fish consumption is moderate, in contrast to Iceland, where it is not only high, but where most, if not all of the fish will be from cold waters. Using the extremes of ISP content, and depending on the assumptions used, total "available ISP" from fish was estimated to be from 0.25 to 12 mg/day for the USA and from 52 to 520 mg/day for Iceland. These figures do not represent an upper and lower limit of consumption, but a "best guess" estimate from available data. In particular, they do not take into account the fact that only a proportion of the population eats fish, and therefore, for any single estimate, some people will have been exposed to considerably higher amounts. They also do not take into account the proportion of fish discarded during preparation for eating (Crevel et al., 2002).

The ocean pout lives in the waters of the western Atlantic. Commercial interest in ocean pout in the U.S. has fluctuated widely (Wigley, 2000). They were marketed as a food fish during World War II, and landings peaked at 2,000 tons in 1944. However, an outbreak of a protozoan parasite that caused lesions eliminated consumer demand. From 1964 to 1974 a commercial fishery developed, and nominal annual catches by the U.S. fleet averaged 4,700 tons (Wigley, 2000). United States landings declined to an average of 600 tons annually during 1974 to 1983, but increased in 1984 and 1985 to 1,300 tons and 1,500 tons, respectively, due to the development of a small fishery in Cape Cod Bay supplying the fresh fillet market (Wigley, 2000). Landings have declined since 1987 in spite of market demand. Currently, the ocean pout is overfished and overfishing occurred in the last year reported, 1999 (Wigley, 2000).

Characteristics of ISP type III

The first report of ISPs in ocean pout was by Hew et al. (1984), and ISPs from the serum of Newfoundland ocean pout were resolved into at least 12 components (Li, et al., 1985). The amino acid sequence of ISP type III HPLC 12 was published in 1988 (Hew et al.):

1	Asn	Gln	Ala	Ser	Val	Val	Ala	Asn	Gln	Leu	Ile	Pro	Ile	Asn	Thr
16	Ala	Leu	Thr	Leu	Val	Met	Met	Arg	Ser	Glu	Val	Val	Thr	Pro	Val
31	Gly	Ile	Pro	Ala	Glu	Asp	Ile	Pro	Arg	Leu	Val	Ser	Met	Gln	Val
46	Asn	Arg	Ala	Val	Pro	Leu	Gly	Thr	Thr	Leu	Met	Pro	Asp	Met	Val
61	Lys	Gly	Tyr	Pro	Pro	Ala									

These amino acids are all commonly found in proteins in the diet. It was noticed that ISPs from ocean pout have a protein structure different from other ISPs (Li, et al., 1985; Hew et al., 1988), a finding confirmed by two-dimensional nuclear magnetic resonance spectroscopy (Sonnichsen et al., 1993). The protein has an unusual fold in which eight beta strands form two triple-stranded antiparallel sheets and one double-stranded antiparallel sheet, with the two triple-stranded sheets arranged as an orthogonal beta-sandwich (Sonnichsen et al., 1993; Chao et al., 1994). Fletcher et al. (1985) reported that the ISPs are present in plasma of ocean pout all year, so consumption of this fish will always entail consumption of ISP type III.

Safety of ISPs

No data on the safety of ISPs *per se* exist, but some general observations about their safety can be made. First, ISPs are composed only of common amino acids and sugars. There are no unusual amino acids or other constituents. Second, they are components of edible foods, and are therefore constituents of a normal diet. The history of consumption of plants and animals in which ISPs are found suggests that the proteins do not cause effects due to their functional characteristics (i.e., freezing point depression and ice structuring). Thus, there is no reason to believe ISPs should cause adverse effects when ingested, nor is there evidence of such effects.

Beyond these general points, the extreme diversity of ISPs both among plants and fish makes it difficult to draw any firm conclusions about the potential adverse effects of individual ISPs. Protein toxicity is generally manifested by acute toxicity, endocrine modulation, and/or allergenicity. Information about the consumption of foods containing ISPs provides no evidence for any of those manifestations. The evidence is circumstantial (i.e., absence of evidence, rather than evidence of absence). However, for allergenicity it might be considered somewhat stronger because fish allergy is a known adverse effect and the allergenic proteins in fish have been thoroughly investigated (Pascual et al., 1992; Poulsen et al., 2001). Ice-structuring proteins are not among those proteins. It is possible, of course, that ISP is not present in the materials that are used for diagnostic identification of fish allergy or that sensitization to ISP always occurs concurrently with sensitization to better known fish allergens. However, there is no evidence to support these positions.

E. Summary

Ice-structuring proteins control the size and morphology of ice crystals and are found throughout nature where low temperatures threaten survival. They are routinely consumed as part of the food supply. The amino acids in ISPs are commonly found in food proteins. Their structures have been known for years. There is no evidence in the literature to suggest that these proteins have any safety issues associated with them. The same conclusions apply to ISP type III HPLC 12: ocean pout have been safely eaten for decades, the amino acids are commonly consumed, and the structure is completely known. Based on the preliminary assessment that ISPs are nontoxic, and along with their low use level, they could be useful ingredients in frozen novelty foods. It was in this context that the work described in this document was undertaken.

II. COMMERCIAL SOURCING OF ICE-STRUCTURING PROTEIN TYPE III PREPARATION

A. Background and Overview

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

- ISPs only occur at relatively low levels in the species that contain them and complex, lengthy, expensive extraction procedures are required to isolate them
- the ISP must come from a sustainable source – current fish stocks could not supply the amount of ISP type III that is expected to be required
- the material needs to be available at the appropriate specification and not be prone to seasonal variation
- the material must be cleared for food use by the appropriate authorities
- the material must be available at an acceptable cost
- the material must be available in the necessary quantities.

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

The production process consists of fermentation with a genetically modified food-grade yeast *Saccharomyces cerevisiae* VW transformant, which carries a multi-copy insert containing a synthetic gene encoding ISP type III HPLC 12 linked to the invertase signal sequence. It is under the control of the GAL7 promoter, integrated at the rDNA locus. Galactose is required to induce production of the ISP.

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

As discussed further below, although the yeast-produced ISP Type III protein is identical to a protein found in certain fish, neither ISP Type III preparation nor the genetic material from which it is derived has ever had any contact with fish, and the ingredient has no sensory or other food characteristics associated with fish. It is a product of yeast.

Note: The information in subchapters B and C below provide a summary of the production process; confidential commercial information and trade secrets have been omitted.

B. Production Organism

A synthetic gene coding for ISP type III HPLC 12 was created for insertion into yeast (the production organism) using the known amino acid sequence of the ISP type III HPLC 12 (Hew et al., 1988) as a template. In this case, the order of the nucleic acids was put together in the laboratory as exactly required for yeast to make this protein. If the actual gene from the fish were inserted, the yeast would almost certainly make a slightly different protein because codon usage is different between fish and yeast. That is, the codon for amino acid "X" in fish may actually code for amino acid "Y" in yeast, resulting in a different protein. Use of a synthetic gene based on the actual amino acid sequence to be produced overcomes this problem, ensuring a nature-identical (in terms of amino acid sequence) protein. It also ensures that no other genes from the host can be accidentally incorporated into the production organism.

The synthetic gene coding for the amino acid sequence of ISP type III HPLC 12 originating from ocean pout was inserted into baker's yeast, *Saccharomyces cerevisiae* (VWk18 *gal1 leu2*), using osmotic shock. The gene was inserted using an expression cassette that integrates directly into the yeast genome to form a stable multi-copy insert. The modified yeast contains no antibiotic resistance markers, and no bacterial or fish DNA.

Genetic stability of *Saccharomyces cerevisiae* VWk18 gal1, leu2, pmt1 pUR3993

Strain stability was measured after more than 70 generations of growth under non-selective conditions.

- Plating cells on selective and non-selective media revealed the same amount of viable cells.
- Inductive growth (after 70 generations) showed identical expression levels of ISP type III HPLC 12 when tested in shakeflasks.
- PCR analysis on whole yeast cells (chromosomal DNA as template) demonstrated that the ISP-gene is present.
- Southern analysis showed that the strain's identity with regard to integration site was identical to initial engineered strain.

These results demonstrate the stability of the engineered strain containing the ISP type III HPLC 12 gene.

C. Production Process

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

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

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

As the source of peptone cannot always be known, an estimation of the amount that could be in the final preparation was made. Conservatively assuming that all the peptone is from one source, that there is no usage of the material by the yeast, and that no degradation occurs during processing, it is estimated that the production process would yield a final maximum concentration of 90 ppm peptone. Considering that there will be further dilution of more than 100-fold to make the commercial product, the amount to which consumers would be exposed under these assumptions is negligible. As it stands, based on experience it is known that in the absence of the peptone the yeast biomass level is significantly reduced, demonstrating that the bulk of the peptone is being utilized. It is concluded that essentially all non-ISP proteins in the preparation are from yeast.

An outline of the production process is given in Figure 1.

Figure 1. Schematic diagram of the fermentation and downstream processing of ISP type III preparation.

III. CHEMICAL IDENTITY AND COMPOSITION

A. Chemical Identity

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

- 66 amino acids:

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

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

B. Specifications

Description

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

Ice-structuring protein type III preparation is produced in accordance with good manufacturing practices, and is free from foreign material and contamination.

Function

Stabilizer; structuring agent.

Requirements

Assay	4.5 – 8.5 g ISP type III HPLC 12/L
pH	2.5 - 3.5
Appearance	Light-brown liquid (may be cloudy after thawing)
Lead	< 2 mg/L
Microbial Limits:	
Total viable count	< 3000 per g
Coliforms	< 10 per g
Listeria	Negative in 25 g
Salmonella sp.	Negative in 25 g
Yeast and molds	< 100 per g

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

Common or usual name

The proposed name for this ISP type III preparation is *ice structuring protein.*

Packaging and storage

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

A preservative, either sodium benzoate or potassium sorbate, may be added up to 0.1%, in order to maintain activity while the preparation is being used in the factory after thawing. The package will be labeled accordingly.

C. Characterization of Commercial ISP Type III Preparation

Analyses were carried out to characterize the commercial material. An ISP type III HPLC 12 standard was prepared, characterized, and used to standardize Batch 20000068, which became a secondary standard for ISP type III HPLC 12. Methods were then developed to characterize the rest of the production material. Five production batches were characterized. A minimum mass balance of 97.9% (w/w) could be achieved. All batches were found to be homogenous. The data are presented in Table 2.

Table 2. Composition data of five commercial batches of ISP type III preparation.

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

Mass balance (% w/w) = Volatiles + Quantified solid compounds
Quantified solid compounds = Total Kjeldahl protein + mannose + citric acid + minerals (Na, K, Mg, Ca, PO_4)

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D. Preparation and Characterization of Samples for the Safety Testing Program

Commercial ISP type III is a solution containing 4.5-8.5 g ISP type III HPLC 12/L, which is too dilute to provide sufficient material to adequately test for its potential toxicity in a subchronic study in rats. In addition, it is too dilute to allow the genotoxicity testing program to reach the doses defined in test guidelines and is not suitable for various aspects of allergenicity testing.

Therefore, a batch of commercial material was concentrated by continuing the ultrafiltration step in the down-stream processing to remove water until a concentration of 29 g/L was reached. Beyond that concentration the material became too viscous for further concentration by ultrafiltration and there was a risk of losing comparability with the commercial material. This concentrated material was used for the 13-week rat study and identified as "Batch 201008."

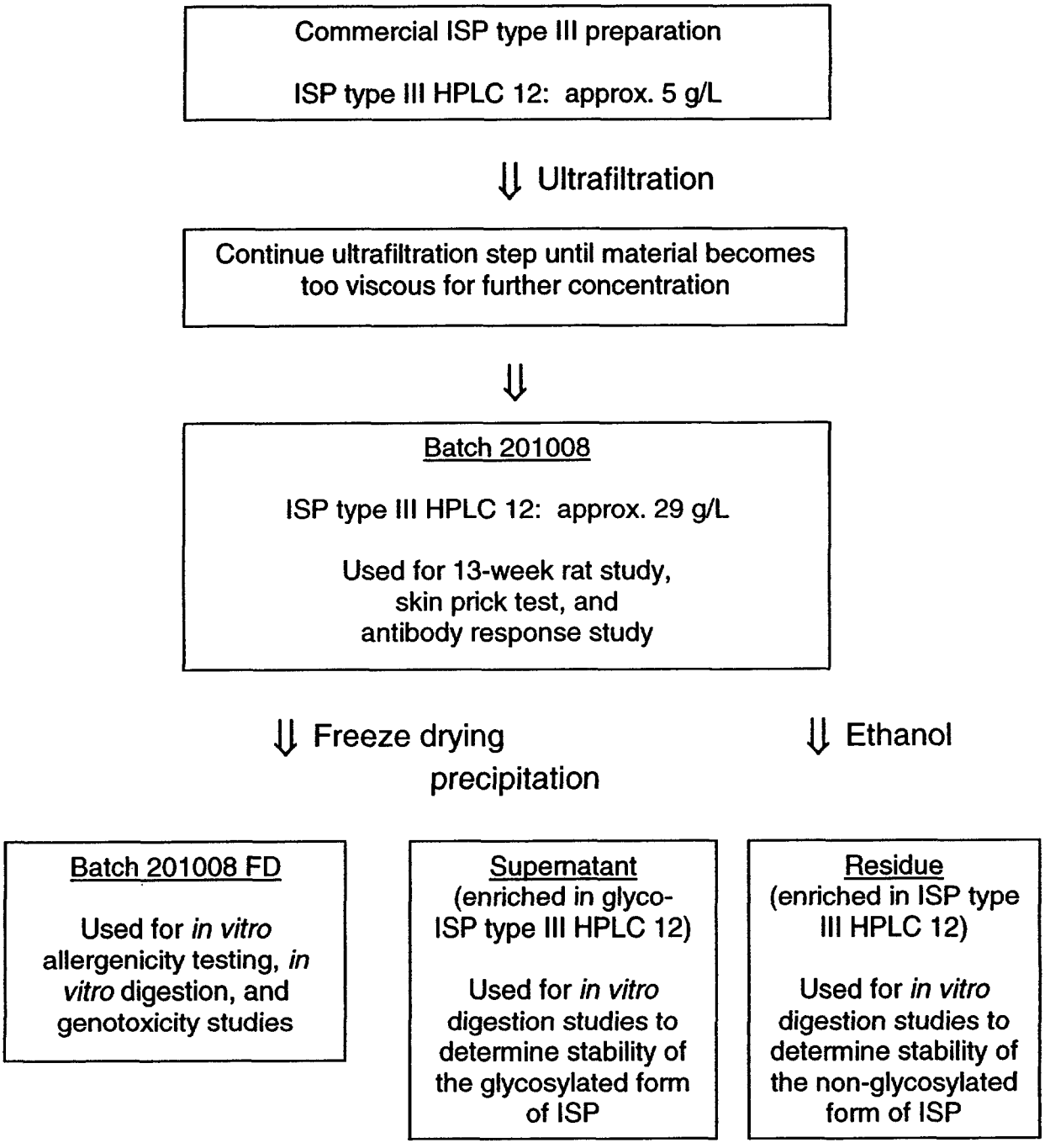
This concentrated material was still too dilute for use in the *in vitro* allergenicity and genotoxicity studies, so a portion of Batch 201008 solution was used to make a freeze-dried sample, identified as "Batch 201008 FD," which was used in those studies. For use in skin-prick testing, aliquots of Batch 201008 solution were diluted with a buffer (50% glycerin in 0.9% saline), filter sterilized, and dispensed into individual vials. A solution of fermentation supernatant from the parent yeast strain (i.e., not genetically modified), processed in exactly the same way as the test batch, was also similarly diluted for use as a skin prick test control. It is referred to as "yeast fermentation supernatant" later in the document.

The purified ISP type III HPLC 12 analytical standard, mentioned in Section C, was also used in certain allergenicity tests.

In order to measure the individual resistance of ISP type III HPLC 12 and glyco-ISP type III HPLC 12 to pepsin, separation of the two forms was required. This was accomplished by precipitating the concentrated ISP type III HPLC 12 with ethanol. "Batch 201008 EtOH residue" was an enriched fraction of ISP type III HPLC 12. "Batch 201008 EtOH supernatant" was an enriched fraction of glyco-ISP type III HPLC 12.

The relationship between these batches is shown in Figure 2 on the next page. Table 3 (following page) contains analytical information on these two fractions.

Figure 2. Process by which the samples for the safety testing program were produced.



Batch 201008 characterization, homogeneity, and stability

Analyses were carried out to characterize Batch 201008 and derived materials (Batch 201008 FD, Batch 201008 ethanol residue, and Batch 201008 ethanol supernatant), and citric acid (citric acid monohydrate) in support of toxicological studies. No significant difference, after accounting for water removal, was found between Batch 201008 and Batch 201008 FD.

Gel filtration chromatography methodology was developed to allow the measurement of glyco-ISP type III HPLC 12 and yeast protein/peptide content. This was necessary to achieve the separation of ISP type III HPLC 12 and its glycoconjugated form. This methodology was also used to monitor glyco-ISP type III HPLC 12 in the *in vitro* digestion experiments.

The protein profiles of the four materials used for the safety tests are shown in Table 3 below.

Table 3. Protein profiles of Batch 201008 and derived materials.

	Batch 201008	Batch 201008 FD	Batch 201008 EtOH residue	Batch 201008 EtOH supernatant
ISP type III HPLC 12 content (% w/w)	2.73 (29.0 g/L)	13.7	14.6	0.17
Total Kjeldahl protein (% w/w)	7.71 (81.7 g/L)	41.1	37.0	42.9
ISP III HPLC 12/Total Kjeldahl protein (%)	35.4	33.3	39.5	0.40
Glyco-ISP III HPLC 12 content (% w/w)[1]	~1.85 (~19 g/L)	~9.9	~7.0	~18
Glyco-ISP III HPLC 12/Total Kjeldahl protein (%)[1]	24	24	19	41
Yeast proteins/Total Kjeldahl protein (%)[1]	31	31	43	20
Yeast peptides/Total Kjeldahl protein (%)[1]	9	12	< det. limit	39

[1] Estimated by GFC

The next table shows the detailed analysis of concentrated Batch 201008, with analyses of commercial batches for comparison.

Table 4. Analysis of Batch 201008 with analyses of five commercial batches for comparison.

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

Stability of Batch 201008 dosing solutions used in the 13-week rat study

A thawing regimen was developed and validated for Batch 201008 dosing solutions used in the 13-week study. Homogeneity and stability data were collected in support of toxicological studies.

The homogeneity of the dosing solutions was not considered to be of concern as they were true solutions. Replicate analysis demonstrated this homogeneity.

Stability was assessed after thawing at ambient, refrigerated, and emergency (1 hour at 37°C) conditions for both neat and diluted test material. The dosing solutions for Batch 201008 were deemed to be stable for 21 hours when thawed at ambient temperatures and for up to 1 hour at ambient temperature when preceded by emergency thawing (1 hour at 37°C). The effect of the different thawing regimes on the stability of the test material was assessed by monitoring the ISP type III content by reverse phase liquid chromatography (Table 5).

Batch 201008 was shown to be stable when thawed at ambient temperature over a 21-hour period (Table 5). Both Batch 201008 and Batch 201008 FD were demonstrated to be stable over six months under frozen conditions (data not shown).

Table 5. Stability of Batch 201008 thawed under ambient conditions.

	ISP type III HPLC 12 Content					
	16 hr[1]		21 hr[1]		24 hr[1]	
Sample	(g/L)	%[2]	(g/L)	%[2]	(g/L)	%[2]
Batch 201008 (neat) A	28.0	92.8	26.7	88.6	24.6	81.8
Batch 201008 (neat) B	27.7	91.9	25.4	84.4	25.9	85.8
Mean	N/A	92.4	N/A	86.5	N/A	83.8
Batch 201008 1→2 dilution A	13.8	91.9	13.2	88.0	12.2	81.6
Batch 201008 1→2 dilution B	13.6	91.3	12.4	83.6	12.7	85.1
Mean	N/A	91.6	N/A	85.8	N/A	83.4
Batch 201008 1→10 dilution A	2.71	91.6	2.75	93.1	2.62	88.7
Batch 201008 1→10 dilution B	2.75	92.5	2.74	92.1	2.66	89.5
Mean	N/A	92.1	N/A	92.6	N/A	89.1

[1] Samples were removed from the freezer, left at ambient temperature, and analyzed 16, 21, and 24 hr later.
[2] Percent of nominal concentration. The nominal concentration was the concentration determined after thawing at refrigerated (16 hr) and ambient (4 hr) conditions.

IV. SAFETY TESTS

Ice-structuring protein type III preparation, expressed by genetically modified food-grade bakers' 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. When evaluating these constituents, the main issue was the possibility that ISP type III HPLC 12 could cause allergic reactions. Potential allergenicity 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 frozen novelties that is identical to a protein found in nature in fish. An extensive program of testing based upon the most current expert consensus was undertaken to make sure that neither individuals already sensitized to fish would react to the ingredient nor that sensitization would be likely to occur. Additionally, a subchronic rat toxicity study and a battery of genotoxicity tests were undertaken to further assess the safety of the material and to provide assurance that nothing unexpected would occur.

A. Allergenicity

Assessment of the potential allergenicity of a protein must consider two issues: (i) whether the protein is likely to sensitize 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 sensitize. The approach adopted by the Unilever team in this evaluation seeks 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 summarized in Table 6.

Table 6. Tests conducted to assess the allergenic potential of ISP type III preparation.

TEST	INFORMATION PROVIDED WITH RESPECT TO	
	Potential to sensitize	*Potential to elicit reactions in sensitized individuals*
Sequence analysis	Identifies similarity to known allergens and classes of proteins containing known allergens	Identifies short sequences in common with known allergens (possible epitopes) Can provide information for additional serum screening
IgE binding *in vitro* – RAST and RAST inhibition		Indicates whether protein can bind specific IgE that might provoke reactions in individuals with a specific allergy
IgE binding *in vitro* – Immunoblotting		Indicates whether protein can bind specific IgE and might provoke reactions in individuals with a specific allergy and visualizes implicated proteins
IgE binding *in vitro* – Basophil histamine release		Indicates whether protein can bind specific IgE and might provoke reactions in individuals with a specific allergy and shows whether binding is biologically meaningful
Skin prick testing		Indicates whether protein could provoke reactions in individuals with a specific allergy
Antibody response to ingestion	Provides information on immunogenicity of protein	
Pepsin resistance	Ready hydrolysis by pepsin suggests lower probability of sensitization through GI tract	Ready hydrolysis by pepsin may indicate low probability of reactions in GI tract

The FAO/WHO (2001) decision tree includes the possible use of animal models, as does the Codex *ad hoc* task force (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.

Amino acid sequence analysis

Introduction. 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 sensitized 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 sensitized 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 programs of the same name have been generated. Both methods rely on assessing the probability that an alignment between a query sequence (the unknown protein) and a sequence in the database occurs by chance.

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

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 program 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 program, 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.

Amino acid sequence analysis of ISP type III HPLC 12. 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 programs BLAST (version 2.2.1, 13 April, 2001) and FASTA (version 3.2, 1998). Databases examined were the nr database of NCBI (all non-redundant GenBank CDS translations + PDB + Swiss-Prot + PIR + PRF) and PIR-NREF, a non-redundant protein database compiled from PIR, Swiss-Prot, TrEMBL, RefSeq, GenPept and PDB. A subset of the nr database was searched with the terms "allergen [ALL]" NOT "immunoglobulin [ALL]" to restrict the search space to entries relevant to allergens ("ALL" specifies the fields where the terms occur). The subset of the nr database served as the allergen database, although it is acknowledged that it has limitations compared to a dedicated allergen database prepared for the purpose. However, these limitations are balanced by the advantage that the databases used are the most up to date. In addition, ISP 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 program 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 program "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. The FASTA 3.2 search in PIR-NREF also did not reveal any matches with known allergens, nor did a search of the FARRP allergen database, using the same program. A BLAST search against the "allergen database" produced a single hit against allergen Asp f6 *Aspergillus fumigatus* (Crameri et al., 1996). The match only occurred over a very short part of the sequences and was therefore not significant.

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 program "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), presented by Basketter et al. (2002). Other parts have been accepted for publication (Bindslev-Jensen et al. 2002).

Investigations in individuals with established allergy to fish

Fish allergy occurs from sensitization 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 7, below. 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 7. 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

Approach to allergological testing. 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 program is summarized in Table 8.

Table 8. 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)

Phase I studies in fish-allergic individuals

Subjects. Twenty subjects had 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). 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 was 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 9). Eighteen of 20 patients had IgE against ocean pout as determined by Maxisorp RAST (Table 9). 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 3). 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 9).

This work has been published by Basketter et al. (2002) and has been accepted for publication by Bindslev-Jensen et al. (2002).

Table 9. Summary of the Phase I experiments on IgE binding (MaxiSorp and histamine release).

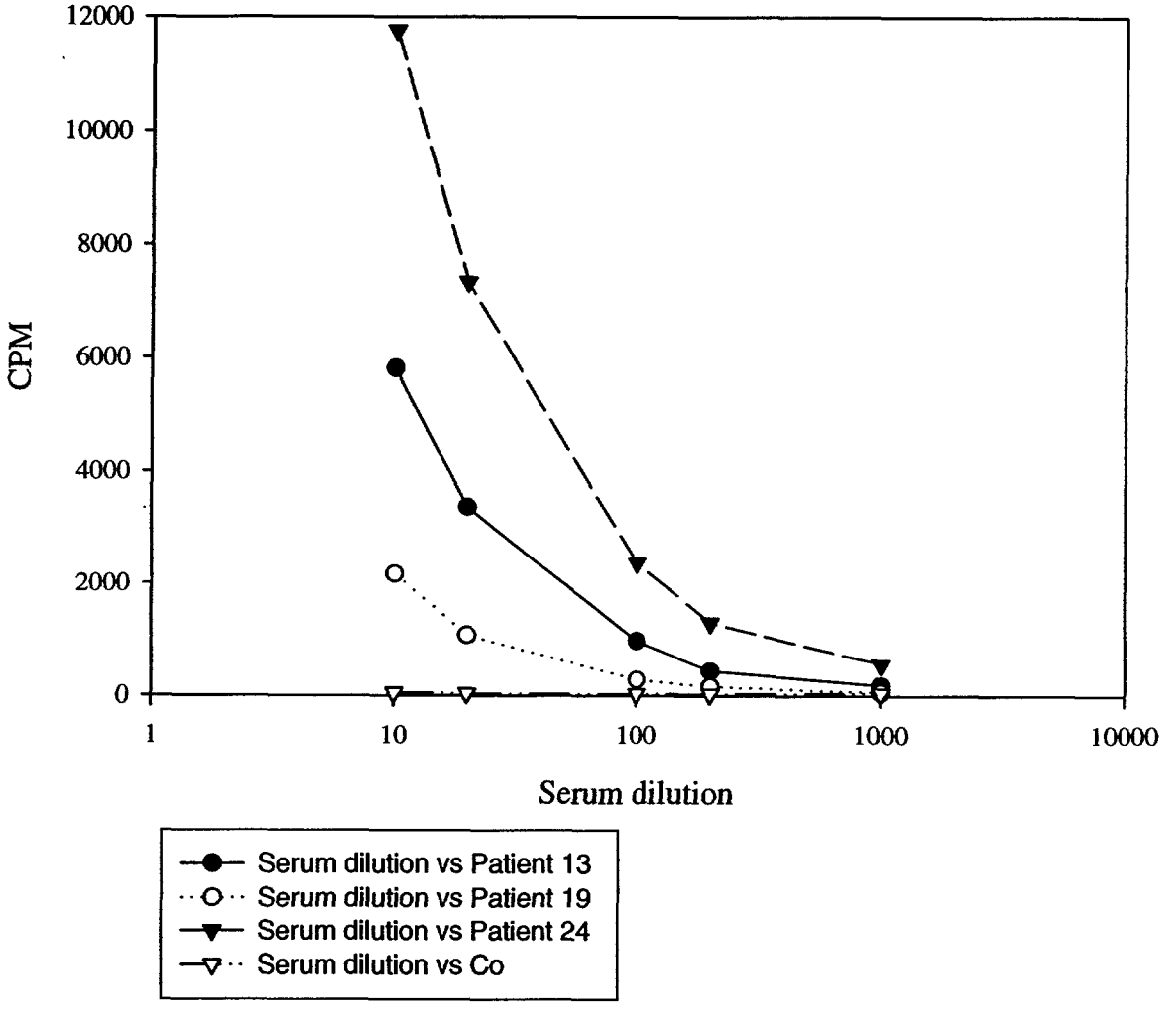
Subject	SPT	Direct MaxiSorp RAST*			Inhibition MaxiSorp RAST (Ocean pout extract as solid phase)			Histamine Release**	
					Inhibitor				
	Ocean pout	Ocean pout	ISP type III prepara-tion	Blank (RPMI-FCS)	Ocean pout	IC50 (ng/ml)	ISP type III	Ocean pout (µg/ml)	ISP type III
1	+	340 ± 21	32 ± 18	24 ± 4	+	<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 (2002)

Figure 3. 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.



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 (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 are presented in Table 10. Four individuals reacted to both the ISP preparation and the yeast fermentation supernatant and were further investigated using the ISP type III HPLC 12 standard, at the same concentrations of ISP as in the preparation. They did not react to the pure ISP type III HPLC 12 (Table 11), revealing that they were sensitized to other proteins in the preparation.

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 fetal 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. Based on a cut-off point of 100 cpm, eight sera were judged to demonstrate specific binding of IgE to the freeze-dried ISP preparation (Table 10). Significant binding was largely confined to the samples from individuals who had positive skin prick tests to the whole ISP preparation and yeast fermentation supernatant. However, in the light of the skin prick test results, these findings almost certainly reflect either sensitization to the yeast protein component of the preparation or non-specific binding, as evidenced by the increase in some counts compared to their Phase I values (see for instance Subjects 12 and 13; Table 10). As skin prick tests are considered more sensitive than RAST in detecting marginal sensitization (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. Sensitization 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 10: Skin prick test responses to ISP type III preparation and yeast fermentation supernatant, and RAST responses to ISP type III preparation (Phase II).

Subject	Skin prick tests responses (mm) [1]								RAST responses to ISP type III preparation (cpm)	
	ISP type III preparation (mg/mL)				Yeast fermentation supernatant (mg/mL)					
	5	*1*	*0.1*	*0.01*	*3*	*0.87*	*0.087*	*0.0087*	*Phase I* [2]	*Phase II*
1	Negative				Negative				32	34
2	Negative				Negative				26	32
4	Negative				Negative				46	63
7	Negative				Negative				81	33
9	Negative				Negative				375	46
11[3]	7.5	4.5	4	2.5	5	2.5	2	1	33	1239
12	Negative				Negative				83	137
13	Negative				Negative				279	591
15	Negative				Negative				42	35
16	Negative				Negative				162	141
17	Negative				Negative				47	225
18	Negative				Negative				52	41
19[3]	4	0	0	0	5	3	0	0	76	243
20	Negative				Negative				884	73
21	Negative				Negative				75	41
22	Negative				Negative				136	60
23	Negative				Negative				33	140
26	Negative				Negative				N.D.	29
27	4.5	3	0	0	4.5	2.5	0	0	N.D.	70
31[3]	7	6	6	4	6	4.5	0	0	N.D.	1908
32	Negative				Negative				N.D.	49
33	Negative				Negative				N.D.	95

[1] Skin prick test values are the mean of largest perpendicular diameters, in mm.
[2] RAST values obtained with the same sera in Phase I are reproduced for comparison.
[3] Subjects determined to be sensitive to *S. cerevisiae* by CAP RAST method: Subject 11, Class 3; Subject 19, Class 4; Subject 31, Class 2.

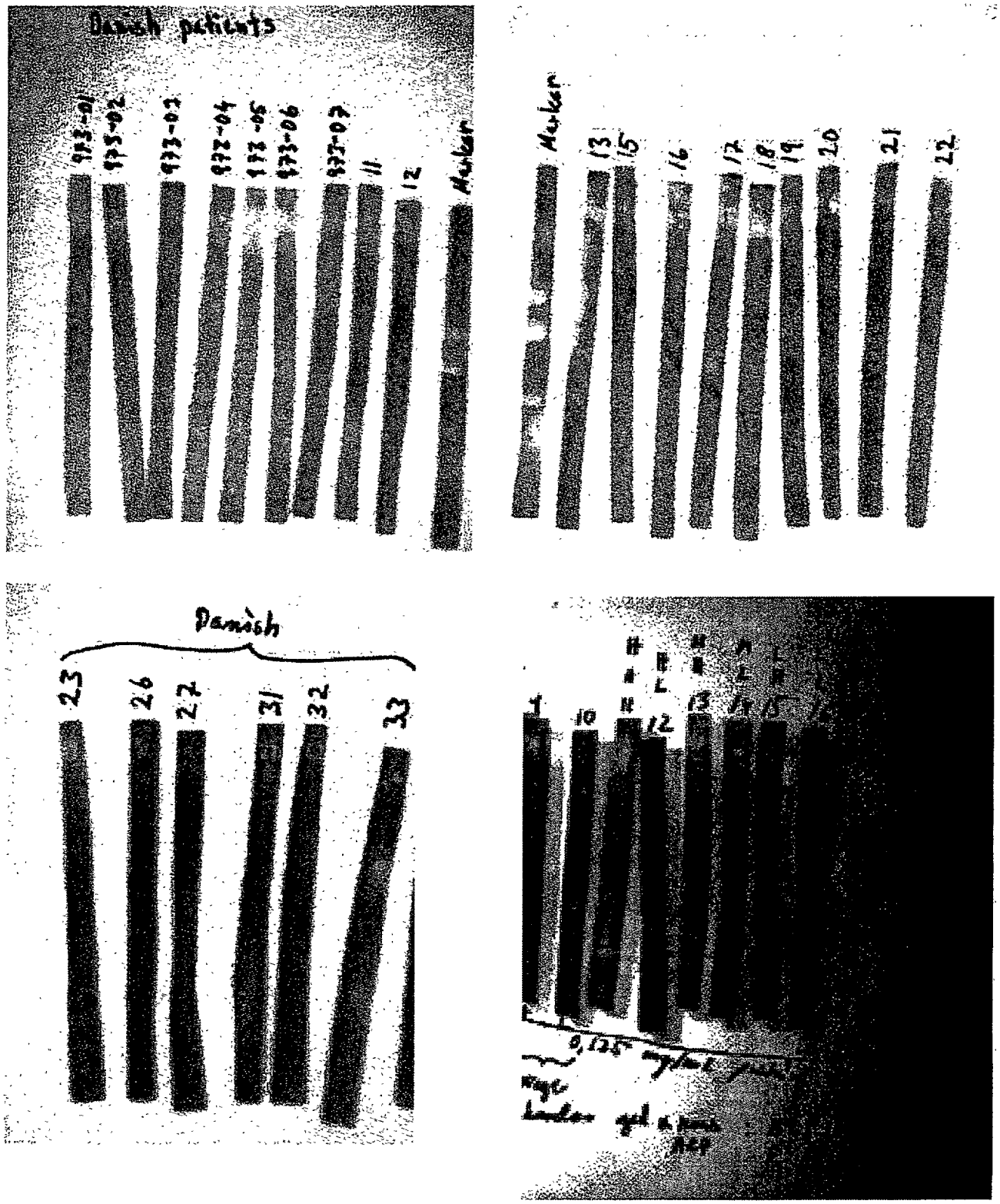
Table 11: Skin prick test responses to ISP type III HPLC 12 standard in the four individuals who reacted to ISP type III preparation and yeast fermentation supernatant.

	ISP type III standard (mg/mL)			
Subject	*5*	*1*	*0.1*	*0.01*
11	0	0	0	0
19	0	0	0	0
27	0	0	0	0
31	0	0	0	0

Figures are mean of two largest perpendicular diameters in mm

SDS-PAGE, protein transfer, and immunoblotting. 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°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 4).

Figure 4. Immunoblots (Western blots) of ISP type III preparation using sera from fish-allergic individuals. The positive control is in the lower right, where the ISP band is clearly visible at the bottom of the blot (the only band present). It was stained with the anti-ISP monoclonal antibody.



In Phase II, the basophil histamine release test was used only to investigate positive skin prick test results. Two of the patients 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 nor when cord blood basophils were sensitized with their serum and subsequently exposed to pure ISP standard. The other two individuals with positive skin prick tests produced inconclusive results in the basophil histamine release test with ISP preparation and yeast fermentation supernatant (data not shown).

Antibody response to ingestion of ISP type III 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. Twenty-eight of these individuals were allocated randomly to the test group and nine to the control group. Individuals in the test group received ISP type III preparation providing 16.3 mg ISP type III HPLC 12 in a flavored 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 flavored drink alone. Based on a pre-study questionnaire, seven members of the test group and four of the control group had an atopic predisposition. Blood samples (20 mL) were obtained immediately prior to the start of the test and at 4 and 6 weeks for the measurement of serum concentrations of IgG and IgE specific to ISP type III.

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 12) 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 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 13 and 14, and Figure 5). 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 sensitization (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 RAST using yeast fermentation supernatant as a solid phase was positive. Additional screening for common allergens (Table 15) indicates that the subject is sensitized 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 demonstrates that ISP type III HPLC 12 does not possess any significant immunogenicity. Table 12 summarizes all the individual results. Tables 13, 14, and 15 give the results of the additional investigations on Subject 19.

Table 12: 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 13. 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 14. Basophil histamine release (passive sensitization) (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 5. Western blot of serum from Subject 19 against ISP preparation.

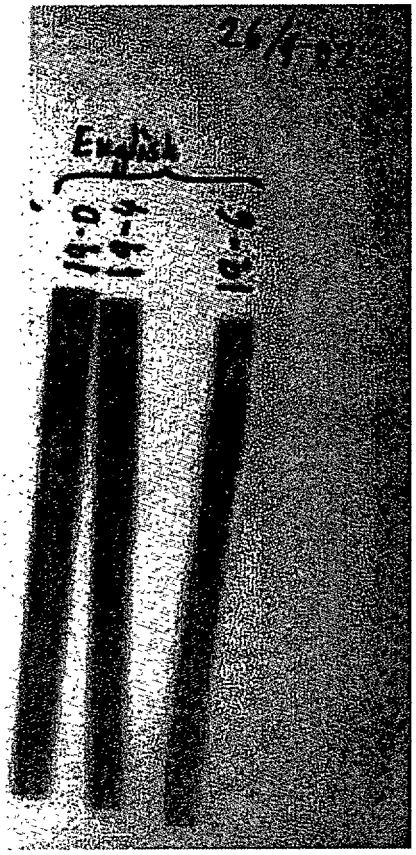


Table 15. Summary of allergenicity screen by skin prick testing on Subject 19.

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
Molds	
Alternaria	2.5
Cladosporium	2.5

Resistance to pepsin hydrolysis

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 6). 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 7). 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 7). 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.

Other analyses. The breakdown of ISP type III HPLC 12 was also quantified by HPLC (Figure 8), 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 6. 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.

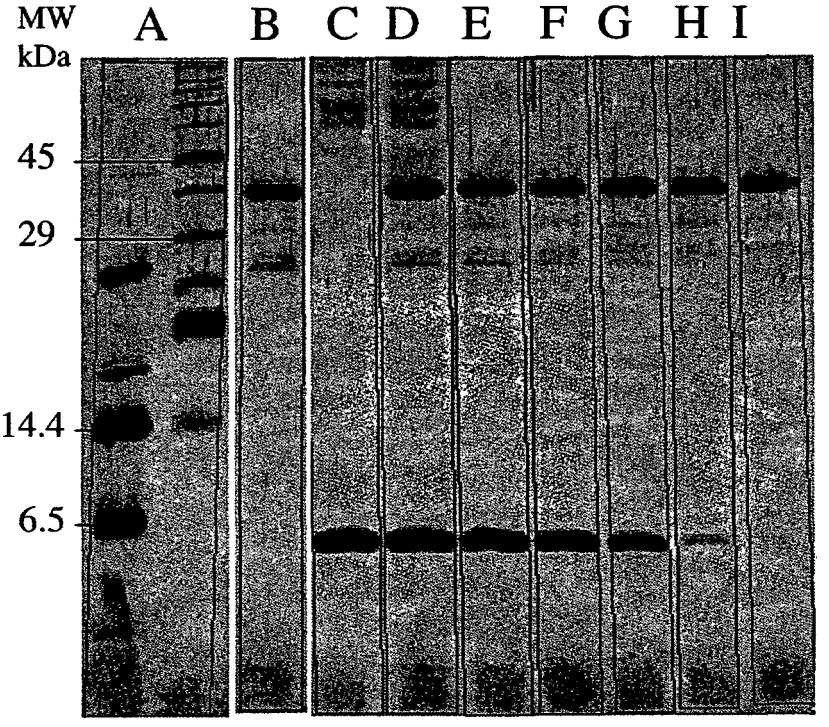


Figure 7. Proteolytic degradation of ISP type III HPLC 12 by pepsin under different pH conditions estimated by densitometric analysis of SDS-PAGE gels.

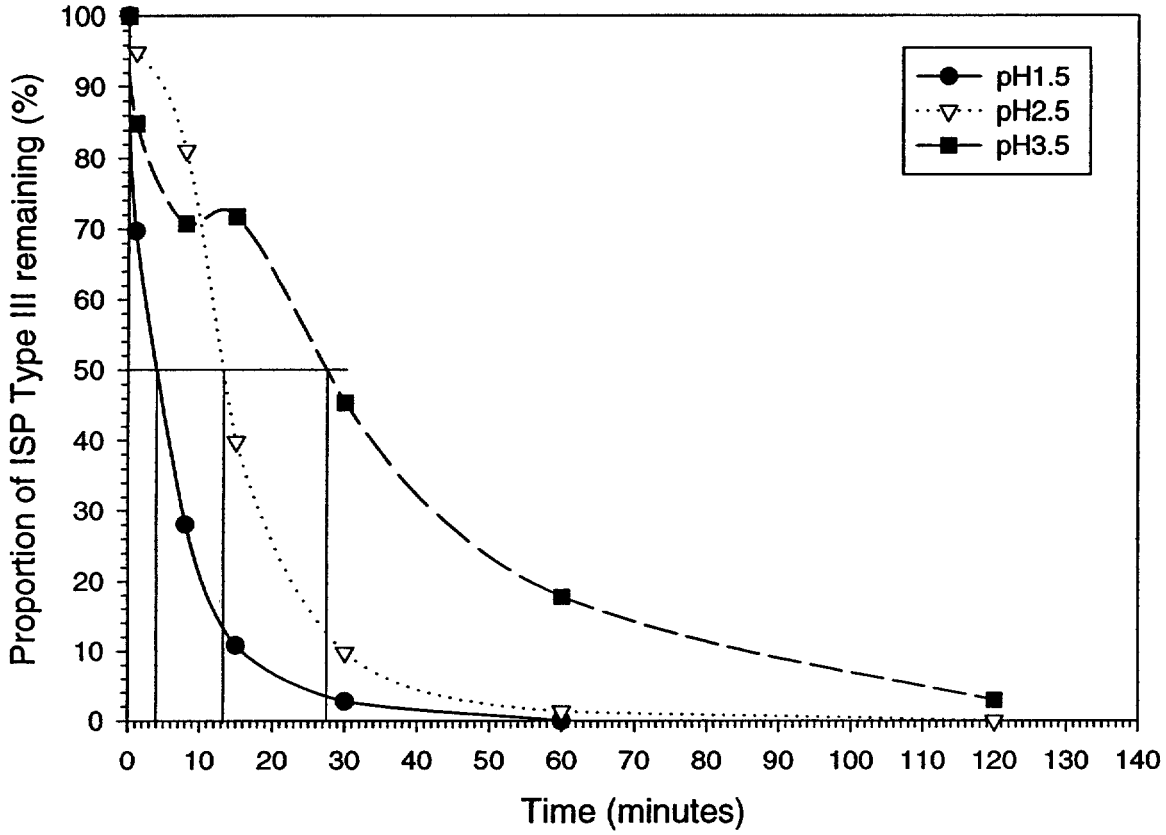
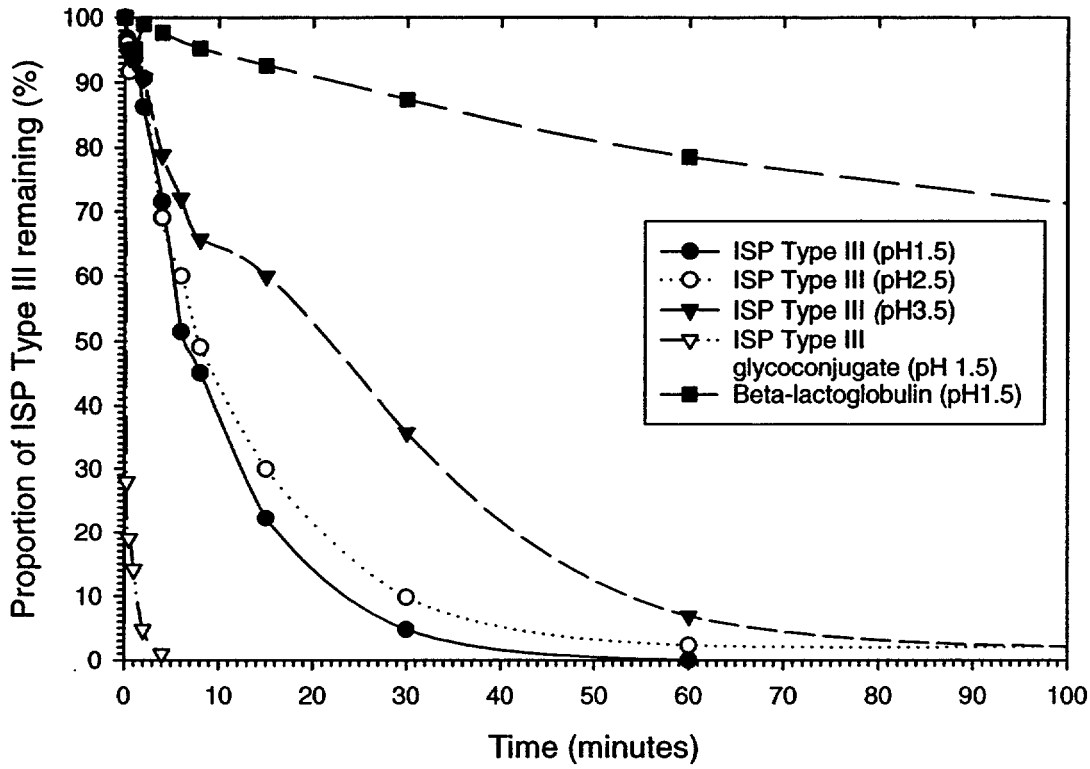


Figure 8. Proteolytic degradation of ISP type III HPLC 12 by pepsin under different pH conditions estimated by HPLC analysis (native ISP) and gel filtration chromatography (glycoconjugate). Degradation of the positive control β-lactoglobulin is shown for comparison.



Bioinformatics. Tools are available to predict potential protease cleavage sites in a given protein sequence. The table below 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 sensitize 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 sensitize or elicit reactions.

Table 16. 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 presented by Basketter et al. (2002). Other parts have been accepted for publication (Bindslev-Jensen et al., 2002).

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,

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

Manufacturers of hypoallergenic infant formulae are required to demonstrate with 90% certainty that their product will not produce reactions in more than 10% of the at risk population. Taking the "at risk" population for ISP type III to be the fish allergic population, the studies performed exceed this criterion.

Studies on the allergenicity of ISP revealed the occurrence of several positive skin prick tests to yeast proteins, confirmed in three cases (out of four) by positive RAST. Sensitization to yeast as measured by specific IgE or skin prick testing is common, according to the fairly limited literature (Kortekangas-Savolainen et al., 1994; Savolainen et al., 1998, 2001). Clinical symptoms appear to be principally respiratory and cutaneous, while classical symptoms of food allergy are rare (Parker et al., 1990). Severe reactions to yeast following ingestion appear to be extremely rare, despite extensive exposure to common foods containing yeast. Most individuals allergic to yeast appear able to tolerate foods containing yeast (Kortekangas-Savolainen et al., 1994). The occurrence of reactions to the yeast protein component of the ISP preparation is therefore of little clinical significance.

B. 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).

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 ultrafiltration to the extent possible without altering its properties compared to the commercial preparation. This concentrated material, Batch 201008, was characterized, and stability and homogeneity measured as described in Section III. 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, see Table 4), 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 17, below.

Table 17. Dosing information for groups in the 13-week rat study.

Group	ISP type III HPLC 12	Total ISP	Total Solids
Water control	0 mg/kg/day	0 mg/kg/day	0 mg/kg/day
Citric acid control	0 mg/kg/day	0 mg/kg/day	100 mg/kg/day
Low dose	58 mg/kg/day	100 mg/kg/day	400 mg/kg/day
Middle dose	290 mg/kg/day	480 mg/kg/day	2000 mg/kg/day
High dose	580 mg/kg/day	960 mg/kg/day	4000 mg/kg/day

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 behavioral 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, cecum, colon, duodenum, eyes, femur, heart, ileum, jejunum, kidney, liver, lungs with bronchi, mammary gland, mandibular lymph nodes, mesenteric lymph nodes, muscle, esophagus, optic nerve, ovaries,

pancreas, Peyers patches, pituitary, prostate, rectum, salivary glands, sciatic nerve, seminal vesicles, skin, spinal cord (cervical, 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 his 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 hematological 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.

C. 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.

Bacterial Reverse Mutation Assay

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 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 was 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. Based on this assessment, 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

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 was 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 was 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 using Mouse Lymphoma L5178Y Cells

Gene mutation was assessed using the *thymidine kinase (tk)* locus in mouse lymphoma L5178Y cells and was compliant with OECD guideline 476 (1997d) and the ICH Tripartite Harmonised Guideline on Genotoxicity: Specific Aspects of Regulatory Tests (FDA, 1997). 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

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.

D. 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 sensitized to fish or sensitize susceptible individuals. Assessing the potential allergenicity of a protein requires systematically assembling a body of evidence to show that neither individuals already sensitized to fish would

react to the preparation nor that sensitization would occur. The Unilever team devised a program of testing 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, indicating that ISP type III is no more immunogenic than the majority of dietary proteins. In addition, amino acid sequence analysis and susceptibility to proteolytic breakdown both indicated an extremely low possibility of inducing sensitization.

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 sensitized to fish is not likely. The same information also suggests that sensitization 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 sensitized to fish, and is highly unlikely to sensitize 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.

V. POTENTIAL DIETARY EXPOSURES TO ICE-STRUCTURING PROTEIN TYPE III PREPARATION IN FROZEN NOVELTY DESSERTS

Ice-structuring protein type III preparation will be used as an ingredient in novelty ice creams and water ices. These products are single-serve, individually wrapped, frozen desserts either purchased in boxes at the supermarket or purchased individually at locations where ice cream is sold. This type of product includes those that are sometimes referred to as "impulse" products because people purchase them when they are seen, for immediate consumption. The portion size is generally not more than 60 g. Excluded from frozen novelty desserts are ice cream and sherbets in pint containers or larger.

A. Estimating Consumption of Frozen Novelty Desserts

Estimating intake of this type of dessert is difficult because consumption tends to be sporadic. Some days might have one or a few eating occasions, but these days would likely be interspersed with long periods of no consumption. Given that a toxicologic endpoint of concern has not been identified, even at the highest doses tested, a screening-level risk characterization can be based on "worst-case" exposure estimates.

Unilever/Good Humor-Breyers employed Novigen Sciences Inc. (Washington, DC) to estimate the intake of frozen novelty desserts. Because the use of ISP type III preparation will be phased into new products as experience increases, Novigen analyzed consumption using three different scenarios. The intake from the initial, or "short-term," products to be manufactured was modeled on data from frozen novelty desserts that are similar to ones that will be rolled out in the first couple of years. A second analysis was done on products that might be manufactured further in the future, called the "longer-term" phase. This assessment uses data from all other frozen novelty desserts in the publicly available databases. A third analysis evaluated consumption from the entire group of frozen novelty desserts.

Novigen estimated the weekly and monthly dietary intake based on how much of the frozen novelty product – in grams – was consumed each time the respondent reported eating the product (i.e., eating occasion). This was done for the overall U.S. population, infants, children 1 to 6, children 7 to 12, and the following age groups, separated by gender: 13 to 20, 21 to 35, 36 to 60; and 61 and older. Analyses were conducted for all population groups described for "short-term," "long-term," and both groups combined.

Dietary intake of frozen novelties on a per-eating-occasion basis was estimated using Novigen's Foods and Residue Evaluation Program (FARE™) software, which incorporates food consumption data from the USDA's Continuing Survey of Food Intakes by Individuals (CSFII). Consumption frequency information from the National Health and Nutrition Examination Survey (NHANES), was used to determine how often ice cream products were consumed in a month.

Because the frozen novelties are only a portion of total ice cream products reported in NHANES, a ratio was calculated based on reported use in the CSFII to adjust the consumption frequency to represent the amount of frozen novelties consumed in relation to ice cream.

Monthly intake was calculated as the amount consumed per eating occasion multiplied by the mean monthly frequency and multiplied again by the ratio of frozen novelties to all ice creams. To calculate the weekly intake, it was assumed that consumption occurred evenly throughout the month. Therefore, the monthly intake was divided by four to estimate weekly intake.

"Short-term" frozen novelty desserts. The mean *per capita* monthly dietary intake for the U.S. population is 143.2 g/month, and the weekly intake is 35.8 g/week. Of all subgroups considered in the analyses, the highest mean *per capita* monthly and weekly intakes is by males aged 13 to 20, at 425.0 g/month and 106.3 g/week. At the 90th percentile of intake, this group consumed 1191 g/month (298 g/week). Among "users," that is, persons who reported consuming the specified frozen novelty foods in the CSFII, mean monthly "short-term" frozen novelty intake for the general U.S. population is 193.1 g/month and 48.3 g/week. The highest per-user intakes are for males aged 13 to 20, with mean monthly and weekly intakes of 492.2 g/month and 123.1 g/week. At the 90th percentile of intake, this group consumed 1380 g/month (345 g/week).

"Long-term" frozen novelty desserts. The mean *per capita* monthly dietary intake for the U.S. population is 71.2 g/month, while the weekly intake is 17.8 g/week. These intake values are substantially less than the intake estimates for the "short-term" foods. Of the subgroups examined, the highest mean *per capita* monthly and weekly "long-term" frozen novelty intakes are by children aged 7 to 12, at 104.8 g/month and 26.2 g/week. At the 90th percentile of intake children consumed 199.9 g/month. Among "users," mean monthly intake for "long-term" frozen novelty foods for the general U.S. population is 96.0 g/month, and 24.0 g/week. The highest mean intake per user of "long-term" frozen novelty foods is again for children 7 to 12, at 142.3 g/month and 35.6 g/week. At the 90th percentile of intake children 7 to 12 consumed 271 g/month (67.8 g/week).

Total frozen novelty desserts. The mean *per capita* monthly and weekly dietary intakes for the U.S. population are 218.4 g/month and 54.6 g/week. The per-user values for all frozen novelties for the U.S. population is 294.4 g/month (73.6 g/week). At the 90th percentile, users consumed 584 g/month (146 g/week). Males aged 13 to 20 have the highest mean *per capita* and per user intakes for all frozen novelty foods: *per capita* estimates were 567.6 g/month (141.9 g/week), and per-user estimates were 657.4 g/month and 164.4 g/week. At the 90th percentile of intake adolescent males consumed 1871 g/month (468 g/week).

These data are summarized in Table 18 on the next page.

Table 18. Estimated monthly and weekly intakes of frozen novelty desserts under various scenarios.

		Mean intake (g/month)	90th %-ile intake (g/month)	Mean intake (g/week)	90th %-ile intake (g/week)
"Short term"					
Per capita	U.S. Population	143.2	297.5	35.8	74.4
	Max[1]: males 13-20	425.0	1191.0	106.2	297.8
Users only	U.S. Population	193.1	401.1	48.3	100.3
	Max[1]: males 13-20	492.2	1379.4	123.0	344.8
"Long term"					
Per capita	U.S. Population	71.2	138.0	17.8	34.5
	Max[1]: children 7-12	104.8	199.9	26.2	50.0
Users only	U.S. Population	96.0	186.0	24.0	46.5
	Max[1]: children 7-12	142.3	271.4	35.6	67.8
Total					
Per capita	U.S. Population	218.4	433.1	54.6	108.3
	Max[1]: males 13-20	567.6	1615.7	141.9	403.9
Users only	U.S. Population	294.4	583.8	73.6	146.0
	Max[1]: males 13-20	657.4	1871.3	164.4	467.8

[1] Maximum intake for all groups for that category.

Based on these monthly and weekly data, the maximum daily intake (90th percentile) of novelty desserts would be by 13-20 year old males, consuming approximately 65 g/day.

A more extreme method to estimate intake is to simply measure consumption on a per-eating-occasion basis. This is the amount that is consumed on a single day without considering any intervening days. Using this approach, the highest intake recorded was of "short-term" desserts by 13-20 year old males. The mean intake by this group was 58 g, or roughly one serving. At the 90th percentile of intake, they consumed 164 g/eating occasion. As expected, this greatly exceeds the estimates based on monthly or weekly intake.

B. Use of ISP Type III HPLC 12 in Frozen Novelty Desserts

Frozen novelty desserts generally weigh not more than 60 g, but not all of the dessert will necessarily contain ISP type III preparation. For the purposes of estimating exposure, it will be assumed that a frozen novelty dessert weighs 60 g and ISP preparation is used throughout the product.

Use levels of ISP type III HPLC 12 will generally be not greater than 0.005% (50 ppm). However, levels up to 0.01% (100 ppm) could be used in some types of products. Thus, in order to be as conservative as possible, a level of 0.01% ISP type III HPLC 12 will be assumed for the following exposure calculations.

C. Estimating Exposure to ISP Type III HPLC 12

Further analysis of the Novigen work was performed to more carefully estimate potential intake of frozen novelty desserts. This work examines the possible intake of novelty desserts on an acute – or daily – basis, on a subacute or subchronic basis, and on a chronic basis. Only data for "short-term" frozen novelty desserts were used as they produced the highest intake levels.

Children are expected to be the main consumers of novelty frozen desserts. As it turns out, the highest level of intake of such products in any of the subgroups was for males 13-20 years when measured on a single day. This allows as conservative a number as possible for exposure estimates: This value is 164 g/eating occasion.

Daily

Exposure estimates were based on the following information: the 90th percentile intake for frozen novelty desserts on a single day by 13-20 year old males, the group with the highest intake; a use level of 0.01% ISP type III HPLC 12 throughout the frozen novelty dessert; and a body weight for this age group of 50 kg, the mean body weight for 13 year old males (Environmental Protection Agency, 1999). The exposure estimate using these data is 0.33 mg ISP type III HPLC 12/kg body weight/day. This represents a "worst-case" exposure and is most appropriately compared to an acute NOAEL from animal or human studies.

Monthly

Using the same information as above, a monthly intake was estimated. The average daily exposure was converted to a monthly basis by:

$$\frac{\text{grams/eating occasion} \times \text{monthly frequency of consumption (times/month)}}{\text{30 days/month}}$$

In this scenario, the exposure estimate for ISP type III HPLC 12 is 0.092 mg/kg body weight/day. These exposure data are best compared to a subacute (30 day) or subchronic (90 day) NOAEL from animal or human studies.

Yearly

Again using the information from above, an average yearly intake was estimated. Realizing that the consumption of novelties is seasonal, one can assume a maximal consumption period of eight

months per year, a level not unreasonable in warm climates like Florida, Arizona, and southern California. The average daily exposure is:

$$\frac{\text{grams consumed over one year (eight high-consumption months)}}{\text{365 days/year}}$$

The exposure under these conditions is 0.061 mg/kg body weight/day. This approach provides the lowest estimate of exposure by looking at the exposure normalized over a year (or a lifetime).

The table below summarizes the daily ISP type III HPLC 12 intake values at the 50th, 75th, and 90th percentile levels based on exposures for a single usage day, monthly consumption, and yearly consumption.

Table 19. Estimated intakes of ISP type III HPLC 12 based on single eating occasion data for males 13-20 years of age, for three periods of consumption.

	Daily ISP Type III HPLC 12 Intake (mg/kg)		
	Daily	Monthly	Yearly
50th percentile	0.058	0.016	0.011
75th percentile	0.12	0.034	0.022
90th percentile	0.33	0.092	0.061

VI. SAFETY ASSESSMENT

A. Determination of the No-Observed Adverse Effect Level

The acceptable daily intake (ADI) of a food additive is usually set on the basis of the highest no-observed adverse effect level (NOAEL) in animal studies (JECFA, 1987). Since there is no basis to assume ISP type III preparation will provoke or induce an allergenic 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.

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.

B. Determination of the Safety Factor

In the traditional assessment of safety of food additives, 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 allergic 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 summarized 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 an ADI.

C. Calculation of the Acceptable Daily Intake

The ADI is calculated as the NOAEL / safety factor (JECFA, 1987). For ISP type III HPLC 12 the ADI is:

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

$$= 5.8 \text{ mg ISP type III HPLC 12/kg body weight /day}$$

D. Determination of the Estimated Daily Intake

The typical level of ISP type III HPLC 12 in consumer products will be 0.005% (50 ppm) The maximum concentration will be 0.01% (100 ppm).

The estimated daily intake (EDI) for the group that will have the highest exposure (13 to 20 year old males), at the 90th percentile of consumption on a single day, is 0.33 mg ISP type III HPLC 12/kg body weight. This conservatively assumes a use level of 100 ppm, that the entire frozen novelty dessert contains ISP, and that the body weight is 50 kg.

E. Safety Assessment

Conservative approaches have been used to establish both the EDI and the ADI. The resulting EDI is 18-times less than the ADI. Thus, there is reasonable certainty that the use of up to 0.01% ISP type III HPLC 12 in novelty frozen desserts will not cause harm.

To demonstrate how large the margin of exposure is between a single serving of a frozen novelty dessert and the ADI, a 60-kg adult could consume 58 servings with the maximal level of 0.01% without exceeding the ADI. If a frozen novelty dessert is consumed every 15 minutes, it would take 14.5 hours to consume the 3.48 kilograms of frozen novelty desserts in 58 servings. To reach the ADI a 20-kg child would have to consume 19 servings per day and a small 10-kg child would have to consume nine servings (nine servings is over half a kilogram of frozen novelty desserts). A 13-20 year old male would need to eat about 48 servings before reaching the ADI. If the more typical level of 0.005% was used in the desserts, then the number of servings that would have to be consumed to reach the ADI would double.

VII. CONCLUSIONS OF THE EXPERT PANEL

The following reflects the determination of a panel of independent experts qualified by scientific training and experience to evaluate the data and information relevant to the safety of ISP Type III preparation:

Ice is the single largest component of ice cream and water ices and therefore has a large effect on their physical and sensory properties. The discovery over thirty years ago of proteins in nature able to control the growth and structure of ice crystals has opened up opportunities for the frozen dessert industry. These proteins were originally termed "antifreeze proteins," but more recently have been renamed "ice-structuring proteins" (ISPs) because they do not stop ice formation but rather bind to and influence the growth and structure of ice crystals. The resulting modified ice structure affects the physical and sensory properties of frozen desserts.

Unilever/Good Humor-Breyers has been evaluating the use of a particular type of ISP, ISP type III, as an ingredient in frozen novelty desserts (single-serving, individually wrapped ice cream and water ices). When added at low levels (approximately 0.005%), this protein alters the structure of the ice phase, resulting in products more resistant to temperature abuse and with textures that provide totally novel eating experiences. Unilever/Good Humor-Breyers has requested a safety assessment for the use of a preparation containing ISP type III to modify the structure of ice in frozen novelty desserts.

Ice-structuring protein type III was originally isolated in the mid-1980s from the blood of the ocean pout (*Macrozoarces americanus*), a cold-water fish found off the northeast coast of North America. This type of ISP consists of 12 isoforms that can be separated by high performance liquid chromatography (HPLC). Isoform HPLC 12 is the largest peak and is the most functionally active species in *in vitro* ice-structuring studies. It was this form, known as ice-structuring protein type III HPLC 12, composed of 66 common amino acids, that was selected for commercial development.

The production process consists of submerged fermentation with a genetically modified food-grade yeast, *Saccharomyces cerevisiae*, which carries a multi-copy insert of a gene encoding ISP type III HPLC 12. The manufacturing methods (fermentation plus protein purification and concentration) used to make ISP type III preparation are widely used for the production of similar protein preparations such as enzymes. Only food-grade materials are used during the fermentation. The commercial material is a light-brown liquid consisting of functionally active ISP type III HPLC 12, inactive mannose-conjugated ISP type III, proteins and peptides from the yeast, and sugars, acids, and salts commonly found in food. It is produced in accordance with good manufacturing practices and is free from foreign material and contamination. Thus, the materials and processes used to make ISP type III preparation are generally considered to be safe and suitable for the production of food ingredients. The resulting preparation meets appropriate specifications.

Ice-structuring proteins occur naturally in many foods consumed by man. Substantial amounts are likely to be consumed in most northerly and temperate regions. Much of this intake is likely to be from edible plants, given their importance in the diet, but in some regions intake from fish will be significant. A portion of cod, for instance, may contain up to 196 mg of ice-structuring glycoprotein, while up to 420 mg ISP type III could be present in the same weight of ocean pout. Based on ISP concentrations in the blood of cold-water fish and the landings of such fish, the average available fish ISP in the diet is estimated to be, subject to considerable uncertainty, approximately 1-10 mg/day in the USA and 50-500 mg/day in Iceland.

Thus, upon preliminary evaluation, ISPs appear to be safe for use in foods. There is background intake through the diet. The amino acids commonly occur in dietary proteins. There is no evidence that the functional characteristic of these proteins (i.e., ice structuring) causes adverse health effects, either short-term or long-term. Specifically in the case of ISP type III HPLC 12, the commercial material is produced according to good manufacturing practices and is comprised of materials already in the food supply. Low levels will be used in frozen novelty desserts. In the case of fish ISPs where some consumption data are available, it is reasonable to infer a lack of allergenicity from the absence of reports of this effect, given the extent to which fish allergy has been studied.

Nevertheless, considering the composition of ISP type III preparation, the main concern was the possibility that a protein originally identified in fish could cause allergic reactions. Thus, an extensive program of testing based on international expert consensus was undertaken to make certain that individuals already sensitized to fish would not react to the protein and to demonstrate that sensitization to ISP type III was unlikely to occur. Amino acid sequence analysis and susceptibility to proteolytic breakdown were evaluated and neither indicated a potential to induce sensitization or elicit a reaction in sensitized individuals. An extract from ocean pout produced positive skin prick test results and positive results in *in vitro* allergenicity testing in individuals with established fish-allergy. However, ISP type III preparation did not bind IgE from fish-allergic individuals, and it did not show any activity in a histamine release assay using basophils from the same fish-allergic individuals. Twenty sera were used in these tests, giving approximately 99% confidence that an allergen with a prevalence of 20% in that population would be detected. These results demonstrate the safety of ISP type III preparation to persons already sensitized to fish, as well as to individuals potentially susceptible to producing IgE responses to proteins. Further assurance about the lack of allergenic potential of ISP type III preparation was gained from the absence of IgE binding noted in immunoblots, lack of skin prick reactivity, and the failure of volunteers ingesting ISP type III preparation for eight weeks to form specific IgG or IgE antibodies to ISP type III HPLC 12. Based on the totality of data and observations , namely no history of allergenicity from human consumption of ocean pout, no structural alerts for allergenicity, no similarity to known allergens, ready hydrolysis by pepsin, lack of histamine release from basophils of fish-allergic individuals in the presence of ISP, lack of binding of ISP type III HPLC 12 to IgE, absence of skin prick test reactivity to ISP itself, and the absence of immunogenicity, measured by the lack of an antibody response in a two-month ingestion study, it is concluded that ISP type III preparation is safe both for fish-allergic individuals and the population at large.

A 13-week gavage study in rats was conducted to assess the potential for toxicity of ISP type III preparation. The commercial material is too dilute to provide sufficient material to adequately test for its potential toxicity. Therefore, it was concentrated to the maximum extent possible while still remaining representative of the commercial product. This concentrated test material was administered by gavage at a dose volume of 20 mL/kg once a day, providing a top dose of approximately 4000 mg total solids/kg body weight/day. The amount of ISP type III HPLC 12 at this dose was 580 mg /kg body weight/day. Lower doses were one-half and one-tenth the highest dose, by dilution. There were two control groups: one receiving water and one receiving a citric acid solution with a pH similar to that of the concentrated preparation. The results show that there were no differences between groups in clinical signs, body weights, hematological parameters, clotting potential, in the biochemical composition of the blood, or in organ weights. There were no macroscopic or microscopic findings due to the effects of the test material.

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

The highest dose that could be tested in the 13-week rat study, 580 mg ISP type III HPLC 12/kg body weight/day, was selected as the no observed adverse effect level (NOAEL) because of the lack of toxicity established by detailed observations.

A safety factor of 100 was used, based on the totality of analytical, animal, human, and *in vitro* data summarized in this document, general knowledge of proteins, and from the approaches to estimating a safety factor described in published articles.

An acceptable daily intake (ADI) for the preparation was determined as the NOAEL divided by the safety factor, and calculated to be 5.8 mg ISP type III HPLC 12/kg body weight /day.

The estimated daily intake (EDI) was determined from data for the group that has the highest intake of frozen novelty desserts, namely males 13 to 20 years old. At the 90th percentile of consumption on a single day, the calculated intake is 164 g/day of such desserts (just under three servings). As the highest level of ISP type III HPLC 12 in consumer products will be 0.01% (100 ppm), and assuming a body weight of 50 kg for a 13-year-old male, an EDI of 0.33 mg ISP type III HPLC 12/kg body weight was calculated.

The EDI is thus 18-times less than the ADI for the group consuming the most frozen novelty desserts. To put this in perspective, a member of that group would have to eat 48 servings before reaching the ADI, while a 60-kg adult would have to consume 58 servings. At a rate of one frozen novelty dessert every 15 minutes, it would take 12-14.5 hours to consume this much ISP type III HPLC 12. A 20-kg child could consume 19 servings per day before reaching the ADI and a small 10-kg child could consume up to nine servings per day. If a more typical level of ISP type III HPLC 12 is used, 0.005%, then the number of servings that could be consumed without reaching the ADI would double.

The ISP type III preparation covered by this GRAS evaluation may be identified on the label of frozen novelties simply by the common or usual name declared in the designation of ingredients pursuant to 21 CFR 101.4 (e.g., "ice structuring protein"). There is no need for commercial products to be labeled with the word "fish" or any other designation as a condition of safe use.

In conclusion, there is no indication of adverse effects from consuming foods containing ISPs. Ocean pout has been consumed for decades. The amino acids in ISP type III are commonly found in foods. The amino acid sequence and structure of the protein have been known for years. The safety tests conducted with ISP type III preparation, including extensive testing for allergenic potential, produced no evidence of any adverse effects. Given the low levels at which ISP type III preparation will be used in frozen novelty desserts, the ADI will not be exceeded. Using these data and other generally available and accepted scientific data, information, methods, and principles, and corroborated by unpublished information, there is reasonable certainty that ISP type III preparation will be safe under the intended conditions of use in frozen novelty desserts.

Based on a critical evaluation of the information summarized in this report, the Expert Panel concludes that the use of ISP type III preparation, meeting appropriate specifications and produced by current good manufacturing practice, is safe for use as an ingredient in novelty ice creams and water ices in amounts not to exceed 0.01%. Furthermore, it is the Panel's opinion that qualified experts in the field would generally recognize that ISP type III preparation is safe for this use. That is, ISP type III preparation is generally recognized as safe (GRAS) using scientific procedures.

MEMBERS OF THE EXPERT PANEL:

Joseph F. Borzelleca, Ph.D.
Wesley Burks, M.D.
Walter H. Glinsmann, M.D.
Michael W. Pariza, Ph.D.
Hugh A. Sampson, M.D.
Steve L. Taylor, Ph.D.

VIII. BASIS FOR CONCLUDING THAT THERE IS A CONSENSUS AMONG QUALIFIED EXPERTS THAT THERE IS REASONABLE CERTAINTY THAT THE SUBSTANCE WILL NOT BE HARMFUL UNDER THE INTENDED CONDITIONS OF USE

GRAS status is based on common knowledge about the substance throughout the scientific community knowledgeable about the safety of substances added to food. A GRAS evaluation through scientific procedures is based on "generally available and accepted scientific data, information, methods, or principles, which ordinarily are published and may be corroborated by unpublished scientific data, information, or methods." Proposed 21 C.F.R. §170.30(b); 62 Fed. Reg. 18960 (April 17, 1997). *See also* 21 C.F.R. §§170.3(h); 170.30(a), (b). There must be a "consensus among qualified experts about the safety of the substance for its intended use." 62 Fed. Reg. 18940. This section summarizes why there is a basis for concluding that there is a consensus among qualified experts that there is reasonable certainty that ISP will not be harmful under the intended conditions of use.

A. The GRAS determination is based on generally available information, and corroborated by unpublished information

This GRAS notification, particularly Chapters I and IV, sets forth the scientific data and information that is published or otherwise generally available on the safety of ISP Type III preparation and related compounds in humans and animals, and also refers to unpublished corroborative information. The following is a summary of the general availability of this data and information:

1. Assessment of Potential Allergenicity – Chapter IV.A

The data and information used to assess allergenicity include the following:

- Background information on ISP, its appearance in fish, and its history of consumption, indicates that neither allergic reactions by persons already sensitized to fish, nor sensitization to this protein, are likely. This work has been published. Crevel, R.W.R., Fedyk, J.K., and Spurgeon, M.J. Antifreeze proteins: characteristics, occurrence and human exposure. *Food Chem. Toxicol.* 40, 899-903 (April 2002).

- Sequence analysis of the ISP protein shows no similarity with any known allergens. This work has been published. Baderschneider, B., Crevel, R.W.R., Earl, L.K., Lallje, A., Sanders, D.J., and Sanders, I.J. Sequence analysis and resistance to pepsin hydrolysis as part of an assessment of the potential allergenicity of ice structuring protein type III HPLC 12. *Food Chem. Toxicol.* 40, 965-978 (April 2002).

- Assessment of the digestibility of ISP shows that both ISP and its glycoconjugate are readily broken down with half-lives measured in minutes into peptides too small to cause sensitization, resulting in a low likelihood that the intact protein would be allergenic when ingested. This work has been published. Baderschneider, B., Crevel, R.W.R., Earl, L.K., Lallje, A., Sanders, D.J., and Sanders, I.J. Sequence analysis and resistance to pepsin hydrolysis as part of an assessment of the potential allergenicity of ice structuring protein type III HPLC 12. *Food Chem. Toxicol.* 40, 965-978 (April 2002).

- Allergological assessment of ISP using human subjects (including RAST testing for IgE binding and basophil histamine release testing) shows no evidence that ISP presents a risk to fish-allergic individuals. This work has been presented at the Society of Toxicology March 2002 meeting and the abstract has been published. Basketter, D., Bindslev-Jensen, C., Stahl Skov, P., Poulsen, L., and Crevel, R. Assessment of the potential allergenicity of ice structuring protein type III HPLC 12 using the FAO/WHO 2001 decision tree for novel foods. *The Toxicologist.* 66, LB-28 (March 2002). Also considered were the results of additional tests (referred to in Chapter IV.A, Table 8, as "Phase II" tests) consisting of skin prick tests, RAST, immunoblotting, and assessments of development of IgE and IgG in response to ingestion of ISP Type III preparation. These Phase II tests are unpublished at this time; reports of these tests are being prepared for publication.

  Complete data and information on this allergological assessment were made available to and studied in detail by a panel of qualified experts consisting of experts on food allergy (Wesley Burks, M.D., Hugh A. Sampson, M.D., and Steve L. Taylor, Ph.D.) and food safety (Joseph F. Borzelleca, Ph.D., Walter H. Glinsmann, M.D., and Michael W. Pariza, Ph.D.). These individuals are recognized world-wide as pre-eminent in their fields (additional information on the qualifications of these individuals is available on request). Deliberation on these data by these uniquely qualified experts, together with public discussion of these data, establishes both that the data are generally available within the appropriate community of experts in this field and that there is expert consensus on the safety of ISP (as discussed below). Moreover, this allergological assessment is corroborative of the published data on background safety of ISP, sequence analysis, and digestibility, all of which indicate a low risk of allergenicity.

In addition to the information provided above, a comprehensive presentation of the published data and the allergological assessment has been prepared, peer reviewed, and accepted for publication in *Food and Chemical Toxicology.* Bindslev-Jensen, C., Sten, E., Earl, L.K., Crevel, R.W.R., Bindslev-Jensen, U., Hansen, T.K., Stahl Skov, P., and Poulsen, L.K. Assessment of the potential allergenicity of ice structuring protein type III HPLC 12 using the FAO/WHO 2001 decision tree for novel foods. *Food Chem. Toxicol.* 40, (2002) (in press). Publication is expected shortly.

2. Other Safety Data – Chapter IV.B and IV.C

A number of publications, including Crevel et al. (2002) and other references cited in this report, refer generally to the safety of ISP Type III. Further, as discussed in Chapter I.C, the safety of ISP Type III can be inferred from the fact that ocean pout have long been safely consumed, the structure of the ISP is completely known, and its constituent amino acids are common in the diet. This generally available information provides basic assurance of the safety of ISP. As discussed in Chapter IV.B and C, this information is corroborated by an assessment of general toxicity in rats and an assessment of genotoxicity. (These data will be presented at the annual meeting of the American College of Toxicology in November 2002.)

3. Conclusion on General Availability

Unilever/Good Humor-Breyers concludes that its GRAS determination, which is based on the weight of all of the available scientific information, is grounded on generally available scientific data and information, with additional corroborating data.

B. The GRAS determination is based on a consensus among qualified experts

Based on a critical evaluation of the information summarized in this report, an independent Panel of qualified experts convened by Unilever/Good Humor-Breyers unanimously concluded that the use of a preparation consisting of ISP type III, meeting appropriate specifications and produced by current good manufacturing practice, is safe for use as an ingredient in novelty ice creams and water ices in amounts not to exceed 0.01%. It is also the Panel's opinion that qualified experts in the field would generally recognize that ISP type III preparation is safe for this use. Further, the Panel concluded that ISP type III preparation covered by this GRAS evaluation may be identified on the label of frozen novelties simply by the common or usual name declared in the designation of ingredients pursuant to 21 CFR 101.4 (e.g., "ice structuring protein"), and there is no need for commercial products to be labeled with the word "fish" or any other designation as a condition of safe use. In summary, ISP type III preparation is generally recognized as safe (GRAS) using scientific procedures.

As discussed above, the Expert Panel that has provided its advice to Unilever/Good Humor-Breyers consists of experts on food allergy (Wesley Burks, M.D., Hugh A. Sampson, M.D., and Steve L. Taylor, Ph.D.) and food safety (Joseph F. Borzelleca, Ph.D., Walter H. Glinsmann, M.D., and Michael W. Pariza, Ph.D.). These individuals are recognized world-wide as pre-eminent in their fields (additional information on the qualifications of these individuals is available on request). As a result of the unique qualifications of these experts, their individual and collective opinions provide a strong basis for concluding that ISP is generally recognized as safe by experts qualified by scientific training and experience to evaluate its safety, as required by section 201(s) of the Federal Food, Drug, and Cosmetic Act.

Accordingly, Unilever/Good Humor-Breyers concludes that its GRAS determination is based on a consensus among qualified experts that there is reasonable certainty that the substance will not be harmful under the intended conditions of use.

IX. GRAS DETERMINATION

Based on the information summarized in this notification, Unilever/Good Humor-Breyers determines that a preparation consisting of Ice Structuring Protein Type III HPLC 12 (referred to by the common or usual name "ice structuring protein" (ISP)), intended for use as a texturizer and for related uses in frozen novelty products in amounts not to exceed 0.01% by weight, is generally recognized as safe within the meaning of §201(s) of the Federal Food, Drug, and Cosmetic Act; 21 C.F.R. §§170.3 and 170.30; and the proposed rules described at 62 Fed. Reg. 18960. The ISP covered by this GRAS notification is intended to be identified on the label of frozen novelties only by the common or usual name (e.g., ice structuring protein) declared in the designation of ingredients pursuant to 21 CFR 101.4.

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