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."

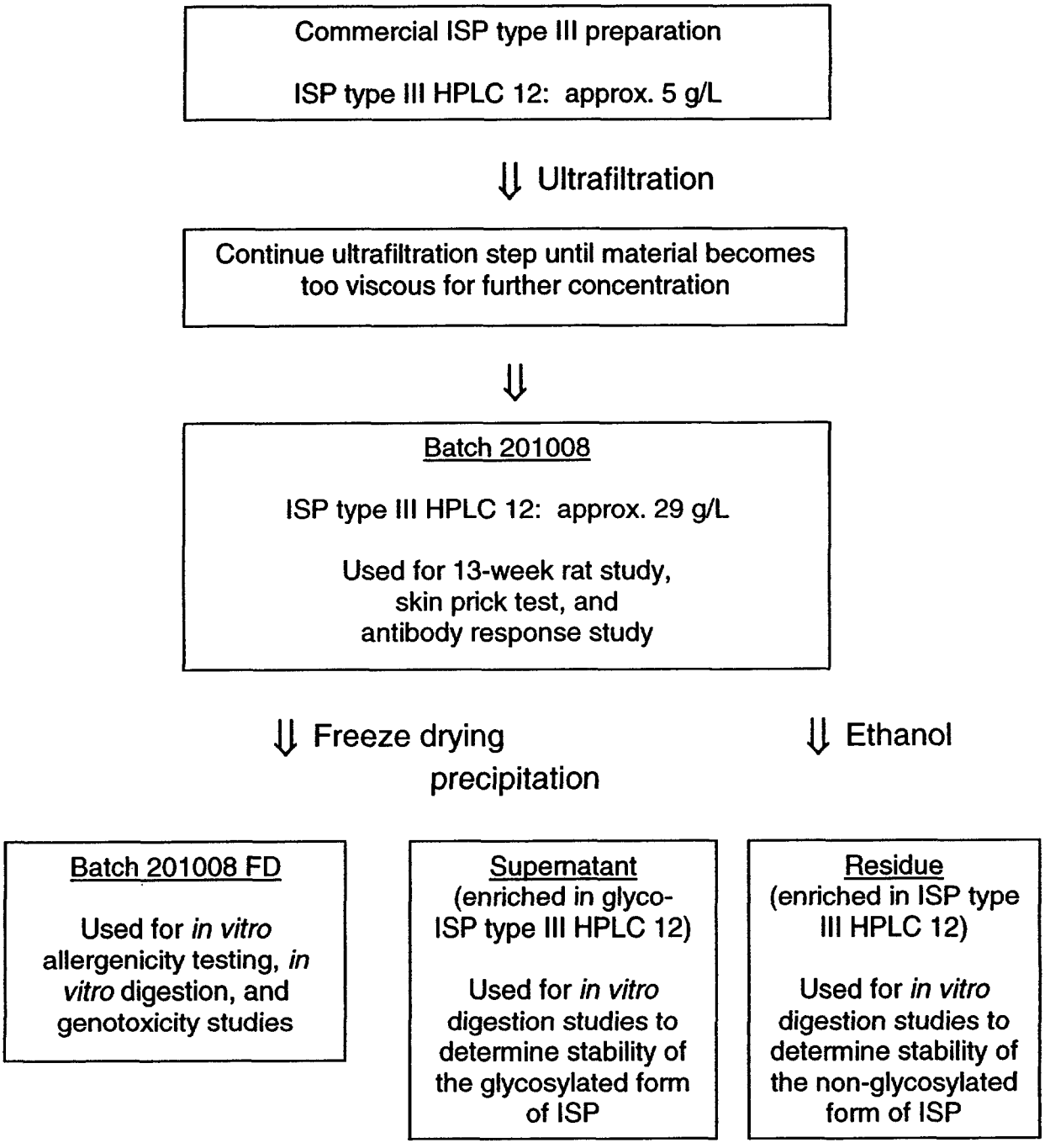
This concentrated material was still too dilute for use in the *in vitro* allergenicity and genotoxicity studies, so a portion of Batch 201008 solution was used to make a freeze-dried sample, identified as "Batch 201008 FD," which was used in those studies. For use in skin-prick testing, aliquots of 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).

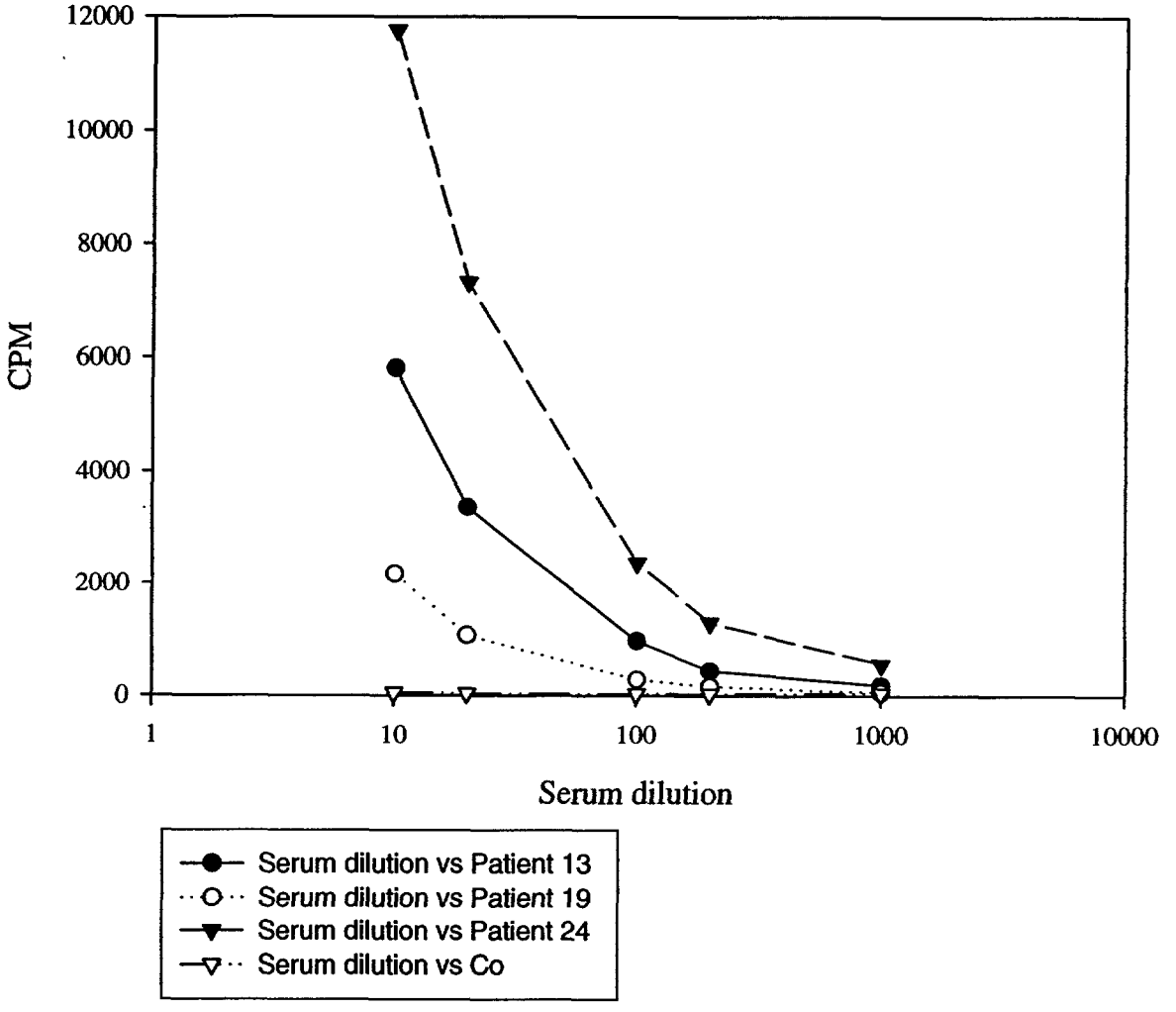
Subject	SPT	Direct MaxiSorp RAST*			Inhibition MaxiSorp RAST (Ocean pout extract as solid phase)			Histamine Release**	
					Inhibitor				
	Ocean pout	Ocean pout	ISP type III prepara-tion	Blank (RPMI-FCS)	Ocean pout	IC50 (ng/ml)	ISP type III	Ocean pout (µg/ml)	ISP type III
1	+	340 ± 21	32 ± 18	24 ± 4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

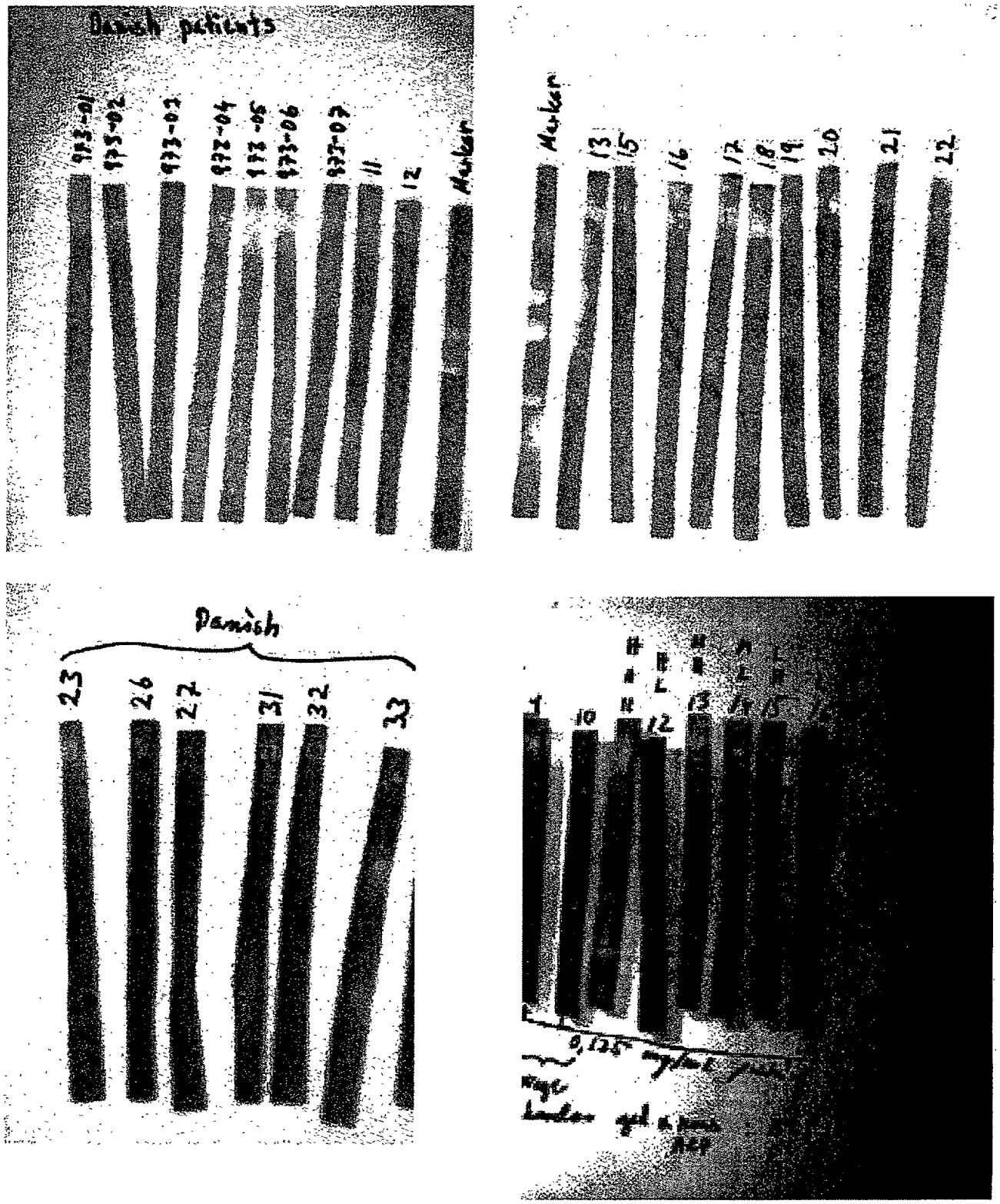
Table 11: Skin prick test responses to ISP type III HPLC 12 standard in the four individuals who reacted to ISP type III preparation and yeast fermentation supernatant.

	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.

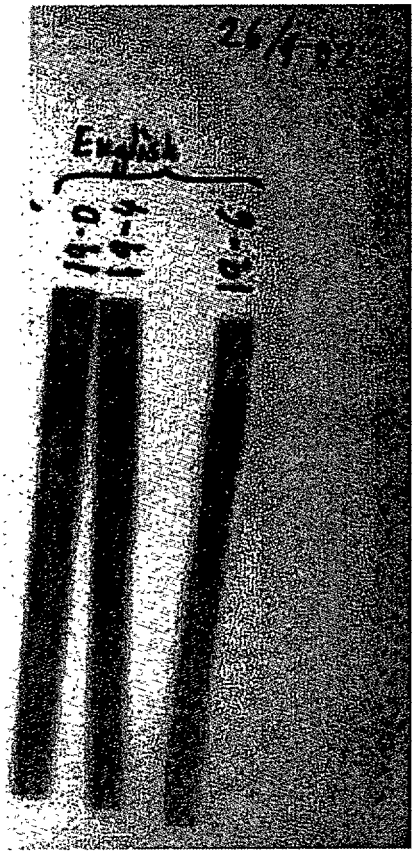


Table 15. Summary of allergenicity screen by skin prick testing on Subject 19.

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.

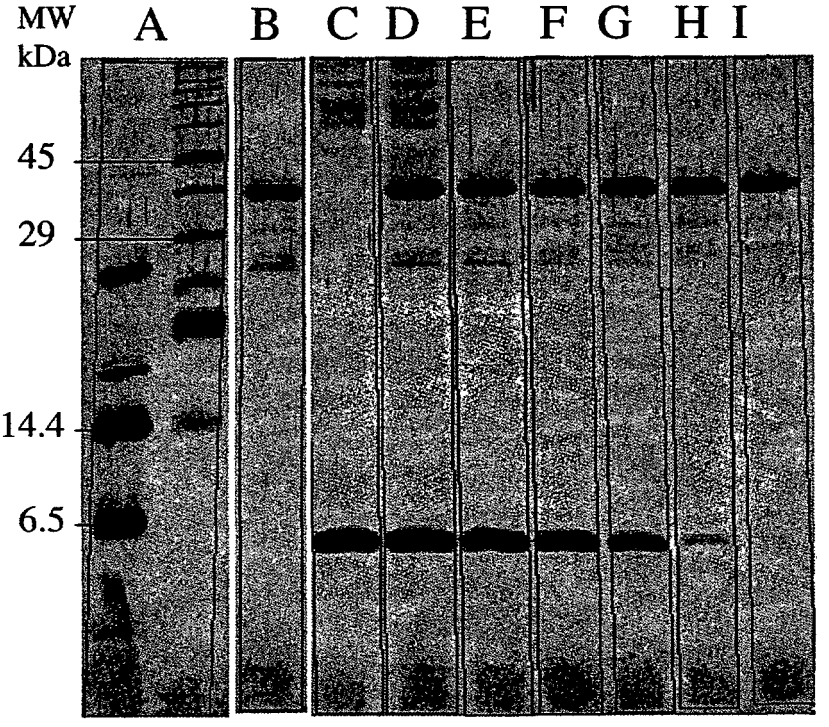


Figure 7. Proteolytic degradation of ISP type III HPLC 12 by pepsin under different pH conditions estimated by densitometric analysis of SDS-PAGE gels.

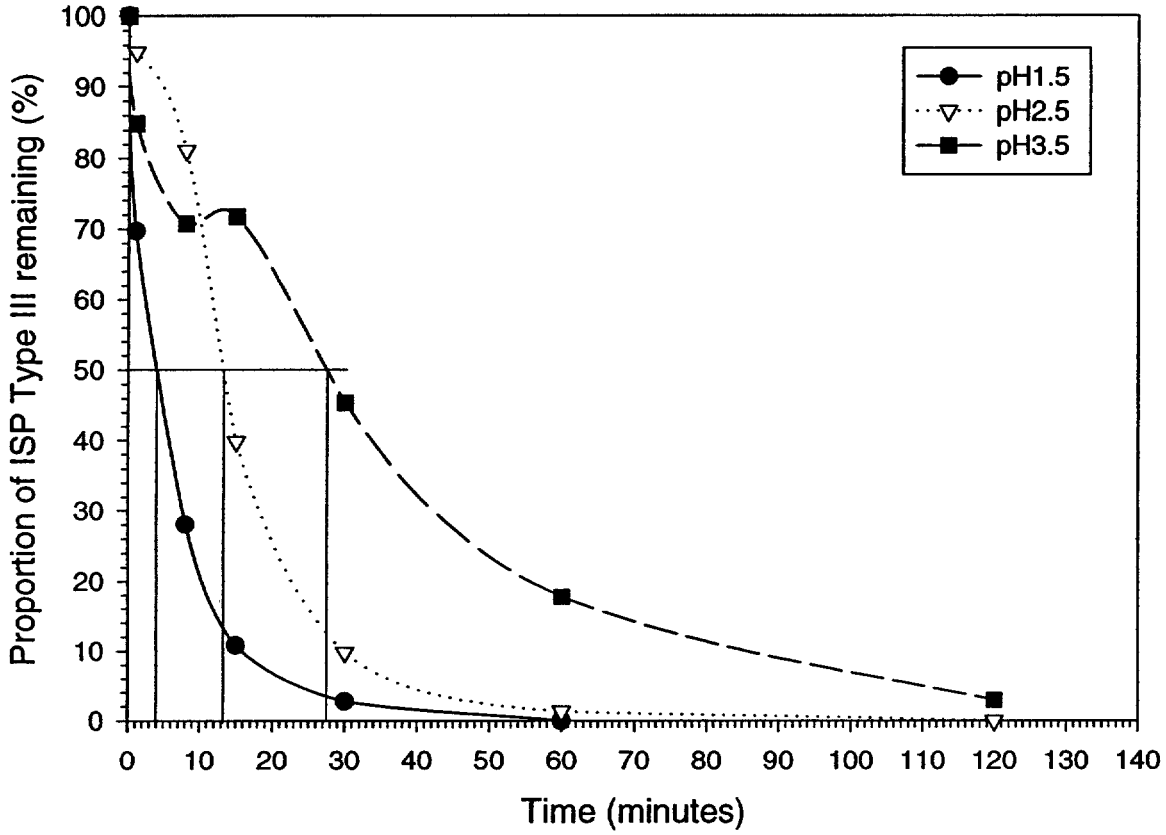
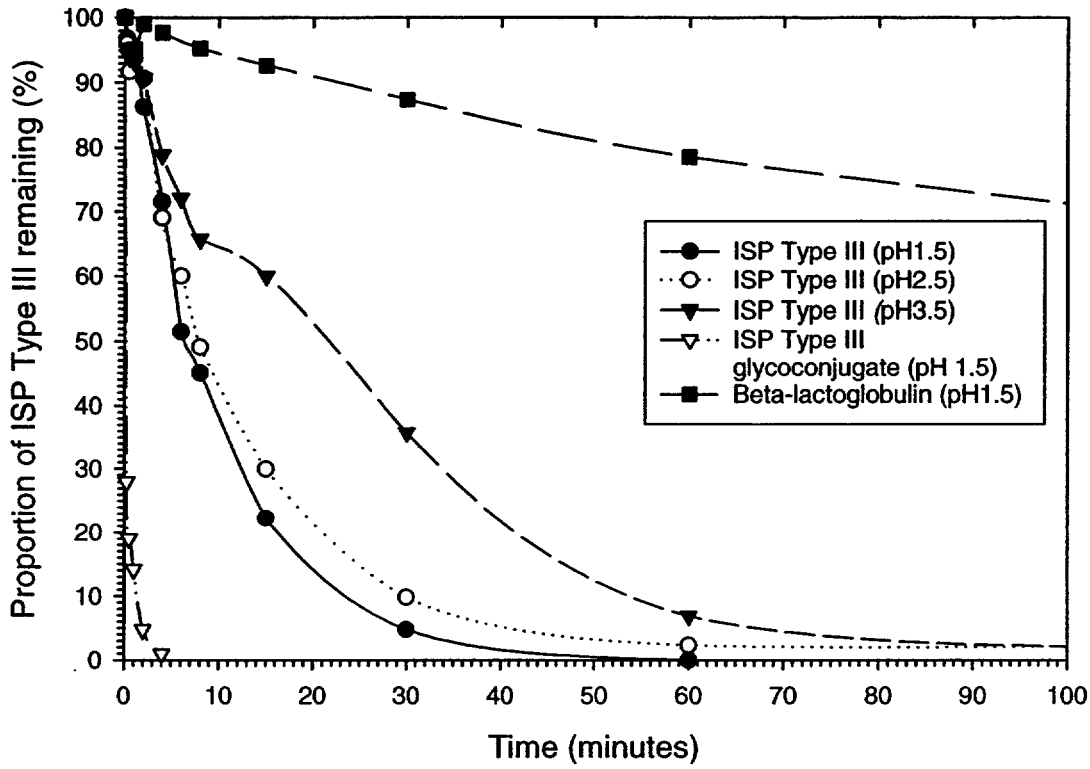


Figure 8. Proteolytic degradation of ISP type III HPLC 12 by pepsin under different pH conditions estimated by HPLC analysis (native ISP) and gel filtration chromatography (glycoconjugate). Degradation of the positive control β-lactoglobulin is shown for comparison.



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.

Acknowledgements: Unilever/Good Humor-Breyers gratefully acknowledges the work of the members of the Expert Panel, Richard Lane, Nigel Lindner, René Crevel, Mary Spurgeon, Anja Lalljie, and Alison Wolfreys in the preparation of this document.

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End Submission

M

Unilever

FAX COVER SHEET

Date: June 5, 2003

To: Linda S. Kahl, Ph.D.

Location: Food and Drug Administration

cc:

Telephone No.:

Fax No.: 202-418-3131

Subject:

Comments/Special Instructions:

We are transmitting 3 pages, plus this cover sheet.

UNILEVER UNITED STATES, INC.
Law Department

By: Nancy L. Schnell

Telephone No.:

Telecopier No.: (212) 318-3680

000302

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Unilever

June 5, 2003

Via Facsimile: (202) 418-3131
and Overnight Mail

Linda S. Kahl, Ph.D.
Division of Biotechnology and GRAS Notice Review (HFS-255)
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: Website listing for GRN No. 117, Ice Structuring Protein

Dear Dr. Kahl:

Unilever submitted a GRAS notification for ice structuring protein, GRN No. 117, which was the subject of an FDA response dated April 17, 2003. On May 22, 2003 we noticed that the listing of GRN No. 117 on FDA's website had changed. The listing previously had described our ingredient as "Ice structuring protein" (consistent with the description in our GRAS notification). Now, the website describes the ingredient as: "Ice structuring protein preparation from *Saccharomyces cerevisiae* carrying a gene encoding an ice structuring protein derived from ocean pout."

We believe that it would be more accurate, and more consistent with past FDA practice, to return the designation of our ingredient on FDA's website to its original form, i.e., "Ice structuring protein." Our reasons are as follows:

1. ***The revised description of our ingredient is misleading because it implies that the ingredient is made from genetic material taken from fish.*** As our GRAS notification explains in detail, the genetic material from which our ingredient is made is created in a laboratory. This material is based on certain genetic coding of the ocean pout, but no substances taken or derived from fish are used. Indeed, our product has never had any contact with fish and has no sensory or other food characteristics associated with fish. It is critical that persons reading about our ingredient understand this fact, so that they will not mistakenly think that our ingredient may contain potentially allergenic materials from fish.

   The revised wording on FDA's website suggests that materials from ocean pout are used to produce our ingredient. Although we realize that FDA has used the phrase "derived from" (rather than simply "from") in an effort to indicate that the

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genetic material is not taken directly from fish, this wording is not adequate to convey accurately the nature of our ingredient. Because this wording could reasonably be interpreted as meaning that fish components are used to produce our ingredient, it is misleading.

2. ***The revised description of our ingredient is inconsistent with past FDA practice for designating GRAS substances.*** The revised wording on FDA's website describes our ingredient by referring to the source organism and genetic material used in the manufacturing process. FDA has used this type of description in the past primarily with enzymes – presumably because this provides the best means of describing the enzyme with specificity, and because it reflects commonly accepted scientific nomenclature and practice.

   Our ingredient, however, is not an enzyme. It is a preparation with ice structuring protein as a characterizing component. It may be described generically without any need to refer to the source organism or genetic material. In this regard, it is similar to other ingredients that have been described on FDA's website using generic terms. For example:

   - *Pullulan* (GRN No. 099). Pullulan is a polysaccharide derived from *Aureobasidium pullulans* but is described on FDA's website as simply "Pullulan."

   - *Mycoprotein* (GRN No. 091). Mycoprotein is a protein-containing substance obtained from *Fusarium venenatum* strain PTA-2684 but is described on FDA's website as simply "Mycoprotein."

   - *Nisin* (GRN No. 065). Nisin is a peptide derived from *Lactococcus lactis* subsp. *lactis* but is described on FDA's website as simply "Nisin."

   Our ingredient is similar to these types of ingredients in that it can be described on FDA's website in generic terms. Like these types of ingredients, the term "Ice structuring protein" is adequate to inform readers of the nature of the GRAS substance, and readers may refer to more detailed information if they wish to learn the source and method of manufacture of the substance.

For the reasons discussed above, we respectfully request that FDA reconsider its decision to revise its website, and return the designation of our ingredient to its original form, i.e., "Ice structuring protein."

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Thank you for your consideration of this request. Please let me know if you have any questions or need further information.

Sincerely,

Nancy L. Schnell

Deputy General Counsel –
Marketing and Regulatory

000305

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Unilever

AM

February 5, 2003

Via Overnight Mail

Jeremiah M. Fasano, Ph.D.
Division of Biotechnology and GRAS Notice Review (HFS-255)
Office of Food Additive Safety
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 (GRN No. 117)
Information responding to FDA requests

Dear Dr. Fasano:

This follows up on FDA's request for additional information regarding our GRAS notification for ice structuring protein (ISP), GRN No. 117. As you know, on December 11, 2002, you conveyed to us orally five questions about which FDA requested additional information. We then prepared a written version of the questions and agreed with you on the text (a copy of which is attached). On January 14, 15, and 21, 2003, we sent to you by e-mail electronic copies of our responses to these questions. At a meeting between FDA and Unilever on January 23rd we discussed these responses with the members of our GRAS Expert Panel who are experts in the area of food allergy (Wesley Burks, M.D., Hugh Sampson, M.D. and Steve Taylor, Ph.D.), and also with the principal investigator in our studies, who is an expert in food allergy (Carsten Bindslev-Jensen, M.D., Ph.D.).

At this time, we are submitting to the GRAS notification file paper copies of the documents that were previously sent to you by e-mail. These versions are identical to the electronic copies, except that we have removed "confidential" designations that inappropriately appeared on the electronic copies.

Because FDA wished to obtain the views of our Expert Panel on the response to Question 2 (having to do with the issue of a food challenge study), the response to that question includes a signature page for the Panel members. The enclosed response reflects the views of the Panel members; however, at this time we are still in the process of gathering the Panel members' signatures. A copy of the signed version will be provided for your files when it is completed.

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[redacted]

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In addition, to provide you with documentation of the expertise of the members of our Expert Panel and principal investigator with whom you spoke on January 23rd, we are providing copies of their curricula vitae. These CVs have been redacted to remove personal information and may be publicly released.

Finally, we would like to make a correction to the GRAS notification: on page 43 of the notification we refer to a pepsin activity value of 2.87 units/mg protein. The correct value is 2,870 units/mg protein. This corrected information is also provided in the enclosed response to FDA Question 1.

Thank you for your kind attention to this matter.

Sincerely,

Nancy L. Schnell
Deputy General Counsel –
Marketing and Regulatory

Enclosures
1. FDA questions
2. Five issue papers
3. CVs of experts

000159

[redacted]

The following are requests conveyed by FDA/CFSAN/Office of Food Additive Safety (OFAS), in response to a meeting of December 4, 2002 between OFAS and Unilever regarding the GRAS notification for ice structuring protein (GRN 117). Responses to these requests should be provided in writing.

FDA stated that they are particularly interested in points #2 and #3 below, but all of the points are of interest.

1. Discuss the pepsin digestion experiment and whether there is any discrepancy between the FAO/WHO guidelines for this experiment and the analysis used in the GRAS notification.

2. Provide an extended, formal discussion of the reasoning and evidence to support a conclusion that all relevant safety issues for the fish allergic population have been addressed without doing a food challenge study in a fish allergic population (in particular, in the individuals who reacted positively in RAST and skin prick testing in Phase I and Phase II testing).

3. Provide an extended, formal discussion of the reasoning and evidence to support a conclusion that the various ISP proteins evaluated were immunologically equivalent. Note that functional equivalence may provide benchmark evidence to evaluate whether or not the shape of the protein has changed. In addition, discuss whether proteins from the yeast supernatant might have an adjuvant or "boosting" effect on the immunogenicity of ISP.

4. Provide a description of the administration of the Phase III food challenge study. In particular, describe how monitoring was done for symptoms of allergic response (e.g., the nature and frequency of any questionnaires used, or other methods), and whether any symptoms were reported. In addition, the GRAS notification mentioned that these individuals had not consumed ISP previously. Would the fact that these individuals probably had consumed the same or similar proteins from fish have any effect on the interpretation of the results of this study?

5. Provide an extended, formal discussion of the reasoning and evidence to support a conclusion that the positive reactions from yeast do not present a significant food allergy risk.

[redacted]

Unilever

ISSUE PAPER 1: PEPSIN RESISTANCE EXPERIMENTS

January 15, 2003

Issue: How close is the pepsin resistance protocol used by Unilever to that recommended by the Joint FAO/WHO (2001) Expert Consultation? How do any differences affect the interpretation of the results?

FDA Question: Discuss the pepsin digestion experiment and whether there is any discrepancy between the FAO/WHO guidelines for this experiment and the analysis used in the GRAS notification.

Summary of Response:

In our GRAS notification, we explained that the protocol for our pepsin hydrolysis experiment was "based on the recommendations published by FAO/WHO (2001)." (GRAS notification, pp. 43.) Although based on the recommendations of the FAO/WHO report, our protocol varied from those recommendations in some cases because:

- The report of the FAO/WHO consultation had not been published at the time work on the pepsin resistance of ISP Type III was begun. However, after publication of the report, we aligned our methodology with the recommendations, where possible and desirable.

- The recommendations of the FAO/WHO consultation report are not intended to be, and cannot be, used as a complete protocol for any specific pepsin-resistance study. Rather, they are intended to be interpreted and varied as necessary to ensure that an experiment is conducted accurately and efficiently. In short, the FAO/WHO report provides *guidelines*, not requirements.

- The variations we elected to use were scientifically equivalent to, or preferable to, those in the recommendations of the FAO/WHO consultation report.

- Similarly, the conclusions drawn from our study are based on, and consistent with, the recommendations of the FAO/WHO consultation report.

The FAO/WHO report provides guidance on when study results "suggest" that a protein might be a potential allergen, and they emphasize that these results must be evaluated in combination with other data. Our conclusions are consistent with this guidance and contribute to the weight of the evidence which demonstrates that ISP Type III preparation presents no significant allergenic risk.

[redacted]

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In the paragraphs below, we discuss each of the recommendations of the FAO/WHO consultation report ("The Report") and how we interpreted them in our study.

Response

Comparison of conditions recommended in the FAO/WHO consultation report and conditions used in our experiments.

The procedures used in our pepsin resistance investigations were based on the recommendations of the FAO/WHO (2001) consultation report (an extract from which is provided in the appendix). (These recommendations were based on a preparatory paper by Ricki Helm (http://www.who.int/fsf/GMfood/bi_01_07.pdf).) The Report of the FAO/WHO consultation had not been published at the time work began on assessing the pepsin resistance of ISP Type III, so adaptations to our protocol were made after the publication of the Report, to the extent that they were possible and desirable from a scientific point of view. The recommendations in the Report are intended to provide guidelines, not requirements; they do not outline a complete protocol, but are intended to be used by investigators who have sufficient expertise to interpret and apply these guidelines correctly in specific cases. In a few respects, the recommendations set forth specifications that may be inconsistent or unclear, and these also require expert interpretation. Finally, the recommendations provide for the use of equivalent analytical methods and appropriate alternative protocols. Thus, these recommendations are intended not to be prescriptive, but to present generally accepted principles in a useful and flexible way.

Below we address each element of the recommendations in the order in which they appear in the FAO/WHO document and explain how each recommendation was addressed by our study:

1. The Report recommends testing the expressed protein in its edible form and in its purified or enriched form. This was done. The protein portion of the preparation contained greater than 60% total ISP type III, which is the same content as the ingredient to be used in our products. Furthermore, we investigated separately the proteolytic degradation of both the glycosylated and unglycosylated ISP Type III species.

2. The Report recommends testing with comparator proteins. This was done using bovine serum albumin, a protein known to be broken down rapidly by simulated gastric juices (Astwood et al., 1996), and beta-lactoglobulin.

3. The Report recommends a colorimetric assay to determine protein concentration, such as bicinchoninic acid (BCA), Bradford Protein Assay, or equivalent. We determined total protein content of the test material by the Kjeldahl method, which is considered a superior assay in terms of accuracy. Kjeldahl is the standard method in our laboratory; it is validated and we have extensive experience with it. In addition, we determined the concentration of specific proteins using a variety of analytical methodologies (reverse phase HPLC, GFC, LC-MS) with the appropriate purified proteins as standards.

4. The Report recommends that the pepsin specific activity should be assessed using the Ryle method. Fresh pepsin preparation of known specific activity determined by the Ryle method was purchased from Sigma (2,870 units/mg protein).

5. The Report includes various specifications for hydrolysis conditions which, as discussed above, were first published in April 2001. Our development of the study protocol preceded that publication and was based mainly on the work of Astwood et al. (1996). This work heavily influenced subsequent work in this field including the recommendations of the FAO/WHO consultation report. Upon publication of the recommendations, we revised our protocol to be consistent with those recommendations to the extent that this could be done without detriment to the science.

   The volume of incubation used in our study was 1.0 ml rather than the 200 µL specified by the Report. This larger volume was of the same order as that used by others (Astwood, personal communication) and was more efficient to work with. This difference from the Report is insignificant, however, because the ratio (w/w) of substrate protein to pepsin was the same as in the FAO/WHO protocol (1:1.28).

   A range of pHs was used (1.5, 2.5, 3.5) in our study, rather than the single pH of 2.0 specified by the Report, in order to provide a better overall estimate of the resistance of ISP Type III to pepsin hydrolysis. This also permitted comparison with other published studies, which had used more stringent pH conditions than those recommended by the Report.

   Other assay parameters were as specified by the Report (i.e., time exposed in water bath, exposure of aliquots for specified time periods, etc.). In addition, our assay parameters are also within the range used by other protocols, where it has been possible to compare them. In particular, the specific activity per unit of total substrate protein was calculated as 5.4 units/µg in our assays, which compares with the 10 units/µg on which the Astwood protocol is standardized. (Surprisingly, the Report does not specify a value for this important parameter). The difference between our value and that used in the Astwood protocol is not material to the outcome, given that a wide range of substrate protein to enzyme ratios has been shown to have little influence on assay kinetics (Astwood et al., 1996).

6. The Report recommends the use of SDS-PAGE 10-20% gradient Tricine, or an equivalent gel system for measurement of protein hydrolysis. We used a system that similarly provides excellent resolution, i.e., the method of Schägger and von Jagow (1987) using SDS-PAGE on Tricine buffered gel with a 4% stacking gel, 10% spacer gel, and 16.5% resolving gel. Protein loading on the gel was 500 ng/lane (the same as that used by Astwood et al. (1996); we note that the specification of 5 µg/cm cited in the Report is unclear as it is not a measure of concentration). Bands were visualized by silver staining, as recommended by the Report. The SDS-PAGE was run under reducing conditions, as recommended by the Report. Running under non-reducing conditions was not relevant to this study, as ISP Type III contains no disulfide bonds.

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   In addition, we measured the hydrolysis of ISP Type III by reverse phase HPLC

[redacted]

with UV detection at 214 nm. This allowed us to more accurately follow the hydrolysis of our specific protein and more precisely quantify the rate of disappearance.

In summary, our analytical conditions were tailored to our specific study and test material, and provided accuracy at least qualitatively equivalent to, and quantitatively better than, the conditions recommended by the FAO/WHO consultation report.

7. The Report recommends immunoblotting for detection of protein in an edible food source (where expressed protein levels are often low). Immunoblotting was not considered necessary in our study for two reasons. First, the large amount of protein being used meant that unequivocal identification and quantification of intact protein was possible by the use of various other methods employed. Second, we could use a method as accurate and sensitive which would identify small peptide fragments as well (LC-MS).

8. The Report does not provide detailed guidance on how to interpret the assay. It states that "Evidence of intact expressed protein and/or intact fragments > 3.5 kDa would suggest a potential allergenic protein," but it does not specify at which time point and how sensitive the detection methodology should be. Nonetheless, in evaluating our study, we can draw two firm conclusions.

   - First, our results clearly show that ISP Type III is hydrolyzed. The half-life is estimated at between 4 and 10 minutes at pH 1.5, with the ISP Type III glycoconjugates being degraded even faster. Based on SDS-PAGE densitometry, less than 7% of the original amount of ISP Type III remained after 30 minutes incubation, and none was detectable at 60 minutes. In this context, our Expert Panel interpreted these results to indicate that ISP Type III was "readily hydrolyzed" by pepsin, a statement which was not questioned during the peer review process preceding publication in *Food and Chemical Toxicology* (Baderschneider et al., 2002).

   - Second, our results clearly show the absence of any potentially immunogenic fragments (i.e., peptides >3.5 kDa, as specified by the Report). This point is particularly important because it indicates that the focus of our allergenicity analysis should be on intact ISP Type III protein, not on any degradation products – and in fact all of our data show that this protein is not allergenic.

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There is currently no consensus on the interpretation of the results of pepsin resistance experiments, particularly when the protein is neither hydrolyzed immediately nor totally resistant to digestion. For instance, Fu et al. (2002) found no clear relationship between pepsin resistance and allergenicity over a wide range of different protein types. This lack of consensus was also recognized in the value attributed to the test by the recent meeting of a Codex Task Force (Codex Alimentarius Commission, 2002). In our view, this assay provides useful data which support the view that ISP Type III is unlikely to sensitize susceptible individuals. However, this information only has value within the wider context of the ***weight of evidence approach***, which we adopted. This use of the results is entirely consistent with the FAO/WHO Consultation Report which states that "there is no absolute certainty that pepsin

resistance or complete degradation of a protein will predict the allergenicity of novel proteins and *must be taken into consideration with other decision tree criteria"* (emphasis added).

Conclusion

In conclusion, we believe that the conduct and interpretation of our pepsin resistance experiment was consistent with the Report, recognizing that:

- The Report provides *guidelines*, not requirements; and
- In conducting and reaching conclusions about our study, we interpreted the recommendations in the Report, and in some cases varied from them, only in ways that were scientifically appropriate and consistent with the overall intent of the recommendations.

As such, our study contributes meaningfully to the weight of the evidence demonstrating that ISP Type III preparation presents no significant allergenic risk.

References

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Appendix: Section on pepsin resistance from FAO/WHO consultation report

6.4. Pepsin Resistance

Purified or enriched expressed protein (non-heated and non-processed) should be subjected to pepsin degradation conditions using Standard Operating Procedures and Good Laboratory Practices (SOP/GLP). In addition, the expressed protein should be assessed in its principal edible form under identical pepsin degradation conditions to those used to examine the expressed protein. Both known non-allergenic (soybean lipoxygenase, potato acid phosphatase or equivalent) and allergenic (milk beta lactoglobulin, soybean trypsin inhibitor or equivalent) food proteins should be included as comparators to determine the relative degree of the expressed proteins pepsin resistance. The protein concentrations should be assessed using a colorimetric assay (e.g., Bicinchoninic acid assay (BCA), Bradford Protein Assay, or equivalent protein assay) with bovine serum albumin (BSA) as a standard. Pepsin proteolytic activity should be assessed (Ryle). Enzyme/protein mixtures should be prepared using 500 μg of protein in 200 μL of 0.32% pepsin (w/v) in 30 mM/L NaCl, pH 2.0, and maintained in a shaking 37 C water bath for 60 minutes. Individual 500 microgram aliquots of pepsin/protein solution should be exposed for periods of 0, 15, 30 seconds and 1, 2, 4, 8, 15, and 60 minutes, at which time each aliquot should be neutralised with an appropriate buffer. Neutralised protein solutions should be mixed with SDS-PAGE sample loading buffer with and without reducing agent (DTT or 2-ME) and heated for 5 minutes at 90°C. Samples containing 5μg/cm gel of protein should be evaluated using 10-20% gradient Tricine SDS-PAGE gels or equivalent gel system under both non-reducing and reducing electrophoretic conditions. Protein in the gels should be visualised by silver or colloidal gold staining procedures. Evidence of intact expressed protein and/or intact fragments greater than 3.5 kDa would suggest a potential allergenic protein. Evidence of protein fragments less than 3.5 kDa would not necessarily raise issues of protein allergenicity and the data should be taken into consideration with other decision tree criteria. For detection of expressed protein in an edible food source, a polyclonal IgG immunoblot analysis should be performed according to the laboratory procedures. The immunoblot analysis should be compared to the silver or colloidal gold stained SDS-PAGE gel and reflect the stained pattern of the expressed protein run under identical conditions.

The investigator should be aware of and consider the following precautions. Edible food sources may contain protease inhibitors or other substances that may promote or reduce protein degradation. Resulting fragments may not be reactive with the polyclonal IgG antibody source. Finally, there is no absolute certainty that pepsin resistance or complete degradation of a protein will predict the allergenicity of novel proteins and must be taken into consideration with other decision tree criteria. Although the present pepsin resistance protocol is strongly recommended, it is recognized that other enzyme susceptibility protocols exist. Alternative protocols may be used for which adequate justification is provided. The producer is expected to take these results into consideration in combination with other decision tree criteria.

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ISSUE PAPER 2: Determination of the Reactivity of a Population of Highly Fish-Allergic Individuals to ISP Type III: The Issue of Food Challenges

January 13, 2003

Issue: Is a food challenge in fish-allergic individuals necessary to ascertain that ISP Type III preparation will not constitute a risk to the fish-allergic population?

FDA Question: Provide an extended, formal discussion of the reasoning and evidence to support a conclusion that all relevant safety issues for the fish allergic population have been addressed without doing a food challenge study in a fish allergic population (in particular, in the individuals who reacted positively in RAST and skin prick testing in Phase I and Phase II testing).

Summary of Response:

- As a general principle, it is well accepted that, in evaluating fish allergy, skin prick tests and RASTs have excellent negative predictive values, but poor positive predictive values. If a substance containing a protein originating in fish produces a negative skin prick test (or RAST) response in a test group of individuals highly allergic to fish, this demonstrates with a very high degree of certainty that the substance will not provoke allergic reactions in the fish-allergic population.

- Given the negative skin prick test and RAST responses to ISP type III in our study, a food challenge is not necessary. It is not likely to provide additional information necessary to reach the conclusion that a substance containing a protein originating in fish will not provoke allergic reactions in the fish-allergic population.

- Our Expert Panel carefully reviewed all of the available data on ISP Type III preparation, the yeast control, and pure ISP Type III (i.e., the ice structuring protein separate from the preparation), and concluded that ISP Type III preparation does not present a risk of provoking reactions in the fish allergic population.

    - The Expert Panel noted that (1) no subject reacted to pure ISP Type III, and (2) all subjects who reacted to ISP Type III preparation also reacted to yeast protein.

    - The Expert Panel concluded that the positive skin prick test and RAST responses observed in some subjects were due to the yeast protein in the ISP

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Type III preparation and not to the ISP Type III protein. As discussed in a separate paper, responses to yeast protein are not clinically significant.

- Our Expert Panel and also Prof. Carsten Bindslev-Jensen, a pre-eminent expert on fish allergy and food challenges, have unanimously advised us that, in light of this conclusion, it is not scientifically necessary to conduct a food challenge study in a fish allergic population or in any subjects who responded positively to ISP Type III preparation. (As noted above, all positive responses in our experiments are attributable to yeast protein.)

Response:

In evaluating this issue, Unilever has relied on the advice of a panel of independent experts. As explained in our GRAS notification, this panel consists of experts on food allergy (Wesley Burks, M.D., Hugh A. Sampson, M.D., and Steve L. Taylor, Ph.D.) and experts on food safety (Joseph F. Borzelleca, Ph.D., Walter H. Glinsmann, M.D., and Michael W. Pariza, Ph.D.). These individuals are recognized worldwide as pre-eminent in their fields.

We have also relied on the advice of other experts on food allergy, particularly the investigator who conducted our allergenicity testing, Prof. Carsten Bindslev-Jensen of Odense University Hospital in Denmark. Prof. Bindslev-Jensen is one of the world's foremost experts on fish allergy and on food challenges. Although Prof. Bindslev-Jensen is not a member of our Expert Panel, we have consulted with him on the issues discussed in this paper.

The signatures of the Expert Panel members and of Prof. Bindslev-Jensen at the end of this paper indicate that they have reviewed and agree with the views expressed in this paper.

In the paragraphs below we will first discuss the general principles that are used in assessing whether a substance is likely to provoke reactions in a specific allergic population, as elucidated in the scientific literature and by our Expert Panel. We will then discuss the application of those general principles to ISP Type III preparation and the conclusions drawn. In each of these discussions, we will address the potential need for a food challenge study.

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(a) General Principles in Assessing whether a protein is Likely to be Allergenic in a Population allergic to the organism in which the protein is normally found.

When a protein is expressed by an organism (such as yeast) that is different from the organism in which the protein is found in nature (such as fish), then it is necessary to assess the risk that the protein poses to individuals who are allergic to the organism that is the natural source of the protein. For this purpose, generally accepted international guidelines specify the use of serological tests (RAST, basophil histamine release, immunoblotting) and skin prick tests (FAO-WHO 2001, Codex Alimentarius Commission 2002). These tests are conducted in a sufficient number of individuals sensitized to the organism of interest (such as fish) to provide the desired statistical degree of assurance of lack of reactivity in the relevant allergic

population. It is accepted that, in diagnosing fish allergy, skin prick tests, and to a lesser extent RASTs, have excellent negative predictive value, but poor positive predictive value. This means that when a substance containing a protein(s) originating from fish produces a negative skin prick test (or RAST) response, then this demonstrates with a very high degree of certainty that the substance will not provoke reactions in a fish-allergic population. Indeed, the skin prick test and RAST with fish allergens are likely to be positive in a proportion of individuals who are not allergic, so they provide a particularly rigorous assessment of a substance's ability to provoke reactions (Bock, 1996; May, 1976; Bock et al., 1978; Sampson, 1988; Sampson, 2001).

The purpose of our study was to determine the risk that a fish allergic individual would react to ISP Type III. While some of the methodology is the same as that used for diagnosis of food allergy, the manner in which the data are interpreted is different. The purpose of diagnosis in the field of food allergy is to enable the physician to provide guidance to the patient regarding which foods are safe to eat. As discussed above, skin prick tests and RASTs often over-predict clinical reactivity; therefore a positive result is not definitive in diagnosis and, in most instances, food challenges are necessary for a definitive diagnosis. In contrast, when assessing the risk that a substance will provoke a reaction in an allergic population (for instance, the risk that ISP Type III might provoke a reaction in individuals allergic to fish), negative skin prick tests and RAST are essentially comparable to food challenges and provide all the information needed to make a judgement.

In short, in assessing the risk that a substance will provoke a reaction in an allergic population, the occurrence of consistent negative skin prick and RAST results is conclusive, so long as other available data, such as immunoblotting and basophil histamine release, are also consistent. Given all of these data, it is not necessary to conduct a food challenge study because such a study would be unlikely to provide useful new data.

(b) Applying these General Principles to ISP Type III Preparation Leads to a Conclusion that the Preparation Does Not Present a Risk of Provoking Reactions in Fish-allergic Individuals

In evaluating ISP Type III preparation, one of the primary safety issues is the risk to the fish-allergic population (because ISP Type III is identical to a protein found in fish). This association with fish has permitted an investigation of the allergenic risk of ISP Type III preparation to be undertaken with a high degree of confidence, because (1) the extensive experience of allergy experts with fish allergy is directly relevant to the analysis of ISP Type III preparation, (2) the wealth of information available on the types of allergenic proteins in fish allergy can be used to assess whether ISP Type III protein is likely to be an allergen, and (3) an adequate number of subjects with documented severe fish allergy was available to enable a statistically meaningful study to be designed. As a result, we can be confident that any conclusions drawn about ISP Type III preparation are robust and have appropriate statistical power.

In evaluating the risk to fish-allergic individuals from ISP Type III preparation, our Expert Panel first analyzed our "Phase I" serological data (RAST and RAST inhibition, and basophil histamine release). The Panel agreed with a conclusion that the substance is unlikely to pose a risk to fish-allergic individuals, but recommended confirmatory "Phase II" testing consisting of skin prick tests, as well as additional RAST and immunoblotting studies.[1]

In analyzing the data on ISP Type III preparation, our Expert Panel considered the following to be critical points:

- ISP Type III preparation (the product to be marketed) contains ISP Type III in solution with salts and yeast proteins. Accordingly, skin prick tests and RASTs were conducted on ISP Type III preparation (which includes yeast protein) and on pure ISP Type III (which does not include yeast protein).[2] These same tests were also conducted on a yeast control that consists of the yeast protein component of ISP Type III preparation.[3]

- In skin prick tests, four (of 22) subjects reacted to ISP Type III preparation (which includes yeast protein) and also reacted to the yeast control (which includes no ISP Type III protein).[4] (Table 1.)

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

These tests indicated that the positive reactions were attributable solely to yeast protein. The RAST results were similar:

- Positive RAST results to ISP Type III preparation (which includes yeast protein) and to the yeast control (which includes no ISP Type III protein) were obtained in eight (of 22) subjects[5] (Table 2). These eight subjects included three of the four subjects who reacted positively in skin prick tests. A summary of all subjects with positive reactions is provided in Table 3.

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

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[1] The data discussed in this paragraph are described in detail in section IV.A of our GRAS notification. The Expert Panel considered all of the tests to be methodologically sound.

[2] The ISP Type III proteins in these different test substances are immunochemically equivalent, as discussed in an accompanying paper.

[3] The yeast control was produced by growing the untransformed yeast strain and then processing it in exactly the same way as the yeast producing the ISP Type III. The yeast control therefore contained only the components originating from the yeast, and no components associated with the expression of the ISP Type III gene.

[4] In the skin prick tests, the magnitude of the response with the yeast control was always sufficient to account for the observed response with ISP Type III preparation.

[5] In the RAST tests, the amount of IgE binding to the yeast control was always sufficient to account for the binding observed with ISP Type III preparation.

Based on these results, the Expert Panel concluded that the reactions to ISP Type III preparation that were observed are attributable solely to yeast protein, because:

(1) skin prick test and RAST results consistently show that all subjects who reacted to ISP Type III preparation also reacted to the yeast control, and

(2) none of the subjects who reacted to ISP Type III preparation reacted to pure ISP Type III.

The Expert Panel was confident that the observed reactions to ISP Type III preparation do not indicate reactivity to ISP Type III protein, because:

(1) skin prick test and RAST results consistently show that all subjects reacted negatively to pure ISP Type III, and

(2) other data, namely basophil histamine release and immunoblotting, corroborate the skin prick test and RAST data.

As a result, the Expert Panel concluded that the scientific evidence presented, including consistent skin prick test and RAST results, as well as extensive and consistent corroborative data, demonstrates with a very high degree of certainty that ISP Type III preparation does not present a risk of provoking reactions in the fish allergic population.

- The Expert Panel noted, in particular, that no subjects reacted to pure ISP Type III.
- The Expert Panel concluded that the positive skin prick test and RAST responses observed in some subjects were due to the yeast protein in the ISP Type III preparation and not to the ISP Type III. As discussed in a separate paper, responses to yeast protein are not clinically significant.

Because all reactions to pure ISP Type III were negative, and because all reactions to ISP Type III preparation were well understood to be reactions to yeast protein, the Expert Panel did not recommend (or even raise the issue of) a food challenge study in a fish allergic population or in any subjects who responded positively to ISP Type III preparation.

In response to FDA's inquiry, our Expert Panel and Prof. Bindslev-Jensen have fully re-examined this issue. They have unanimously advised us that no further testing is necessary to demonstrate the safety of ISP Type III in fish-allergic individuals. They have also reiterated that a food challenge study in fish-allergic individuals would not be necessary because, as discussed above, negative skin prick test and RAST results are excellent indices of lack of an allergic response to the protein.

Accordingly, based on the advice of our Expert Panel, Unilever concludes that a food challenge study is not necessary to assess the risk of fish allergenicity from ISP Type III preparation.

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Table 1. Summary data for subjects with positive skin prick reactions, showing that positive reactions are attributable to the yeast protein and not to ISP Type III protein.

Subject	ISP Type III preparation (mg/ml)				Yeast fermentation control (mg/ml)				ISP Type III pure (mg/ml)			
	5	1	0.1	0.01	3	0.87	0.087	0.0087	5.0	1.0	0.1	0.01
1												
2												
4												
7												
9												
11	7.5	4.5	4	2.5	5	2.5	2	1	0	0	0	0
12												
13												
15												
16												
17												
18												
19	4	0	0	0	5	3	0	0	0	0	0	0
20												
21												
22												
23												
26												
27	4.5	3	0	0	4.5	2.5	0	0	0	0	0	0
31	7	6	6	4	6	4.5	0	0	0	0	0	0
32												
33												

Table 2. Summary data for subjects with positive RAST reactions, showing that positive reactions are attributable to the yeast protein and not to ISP Type III protein.

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

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[redacted]

Quality control parameters for RAST assays

* Negative control: 33cpm; total activity added: 21,000

**Negative control: 71cpm; total activity added: 21,893

Criteria for positivity:

- >100 cpm or 2 x Blank (66 cpm for *assay; 142 for **assay) (whichever is greatest)
- >100 cpm or mean of blank + 2SDs (whichever is greatest)

Table 3. Summary of all subjects with positive reactions, showing that such reactions are attributable to the yeast protein and not to ISP Type III protein.

Subject	Skin prick tests			RAST results		
	ISP preparation	Yeast control	Pure ISP	ISP preparation	Yeast control	Pure ISP
11	+	+	-	+	+	-
12	-	-		-	-	-
13	-	-		+	+	-
16	-	-		+	+	-
17	-	-		+	+	-
19	+	+	-	+	+	-
23	-	-		-	-	-
27	+	+	-	-		
31	+	+	-	+	+	-

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ISSUE PAPER 3: IMMUNOCHEMICAL IDENTITY OF ISP TYPE III IN DIFFERENT TEST SAMPLES AND ISP TYPE III IN ISP PREPARATION

January 15, 2003

Issue: A sample of pure ISP Type III was used for skin-prick testing individuals who were positive in the skin-prick test with ISP Type III preparation. Is the pure ISP Type III immunochemically identical to the ISP Type III in the preparation?

FDA Question: Provide an extended, formal discussion of the reasoning and evidence to support a conclusion that the various ISP proteins evaluated were immunologically equivalent. Note that functional equivalence may provide benchmark evidence to evaluate whether or not the shape of the protein has changed. In addition, discuss whether proteins from the yeast supernatant might have an adjuvant or "boosting" effect on the immunogenicity of ISP.

Summary of Response:

Immunochemical similarity of ISP Type III in the preparation and pure ISP Type III has been demonstrated by two independent methodologies: functional activity of the purified ISP Type III and specific antibody binding.

- Functional interaction of ISP Type III with ice molecules depends on the integrity of the protein's three-dimensional structure. Recognition of conformational epitopes by antibodies is also dependent on retention of three-dimensional structure. We have demonstrated that purified ISP Type III has the same ice structuring activity as two different batches of ISP Type III preparation.

- Purified ISP Type III, separated by SDS-PAGE, and then electro-transferred to a suitable membrane, was fully recognized by a monoclonal antibody specific for ISP Type III.

Data based on the functionality of the protein, as well as on antibody binding both confirm that the ISP Type III in all test materials retained its immunochemical activity.

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- There is no evidence from our own studies that the yeast proteins in the ISP Type III preparation are acting as adjuvants. Also the published literature on *Saccharomyces cervisiae* does not provide any evidence that such proteins act as adjuvants.

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Introduction

The immunochemical identity of ISP Type III in different test samples was demonstrated by functionality and recognition by a monoclonal antibody against ISP Type III HPLC12.

Functionality

The activity of ice structuring protein Type III depends critically on the maintenance of an unchanged three-dimensional structure. Indeed it has been shown that even single amino acid mutations at certain sites can entirely eliminate ice-structuring activity (DeLuca et al., 1998). Retention of functionality is therefore a sensitive indicator of immunochemical identity, as it indicates an unchanged three-dimensional structure.

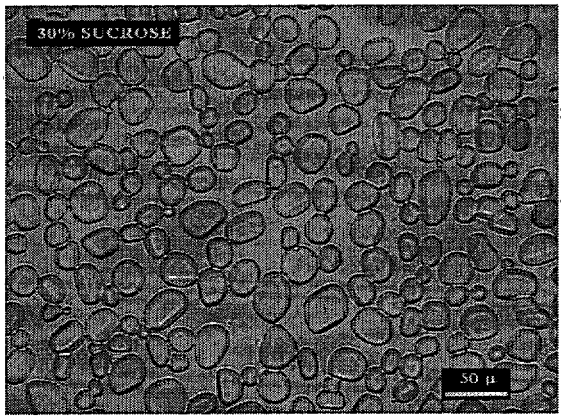
Functionality experiments

The biological function of ice structuring proteins is to protect organisms from the damaging effects of freezing. Ice structuring proteins do this by binding to growing ice crystals and influencing their size and shape. Two tests have been used to demonstrate that the various ISP type III preparations/test samples have retained their functionality:

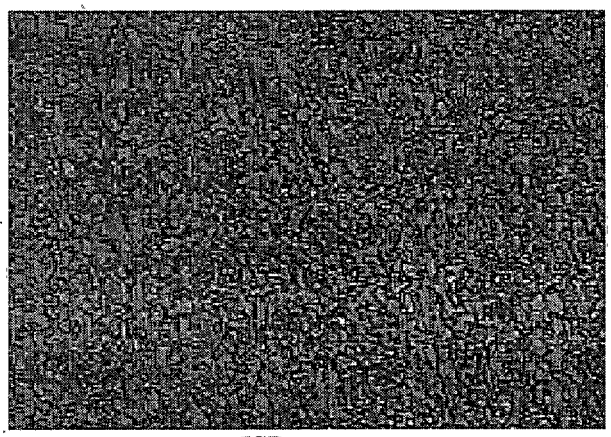
(i) Re-crystallization inhibition test

Re-crystallization is the process whereby large crystals grow at the expense of smaller ones, at constant ice phase volume, as the system lowers its total free energy by reducing the ice-water surface area. In the presence of ISP, this process is inhibited and the ice crystals remain small.

The effect of ISP on re-crystallization is shown below. A drop of 30% sucrose solution, with and without ISP, was placed between two coverslips. This was cooled rapidly to -40°C to produce a large population of small ice crystals. The sample was then warmed to -6°C and held for 60 minutes, during which re-crystallization was observed. The ISP inhibits re-crystallization, (i.e., preserves the small crystals) compared to the control. In this experiment, the activity of a sample of purified ISP Type III, identical to the one used for skin prick testing, was compared with samples of ISP Type III preparation.



Control



+ ISP

There was no difference between the re-crystallization inhibition activities of purified ISP Type III and ISP type III preparation.

(ii) Brittleness test

The conferment of "brittleness" to frozen sucrose solutions is unique to ISP Type III. This mechanical property of frozen water ice bars can be determined by measuring the work of fracture using an Instron universal texture tester. The brittleness of frozen water ice bars prepared with a sample of purified ISP Type III and samples of ISP Type III preparation was compared in this test.

There was no difference between the brittleness of water ice bars prepared with the purified ISP Type III and the ISP type III preparations.

The re-crystallization inhibition and brittleness tests therefore demonstrate conclusively that the purification process does not affect the functional activity of ISP Type III. These results, on their own, provide strong evidence of immunochemical identity between purified ISP Type III and ISP Type III in ISP preparation.

Immunochemical experiments

The ability of the monoclonal antibody to ISP Type III to recognize the molecule in different test samples was evaluated by immunoblotting (Western blotting), using a monoclonal antibody against ISP Type III, following separation by SDS-PAGE.

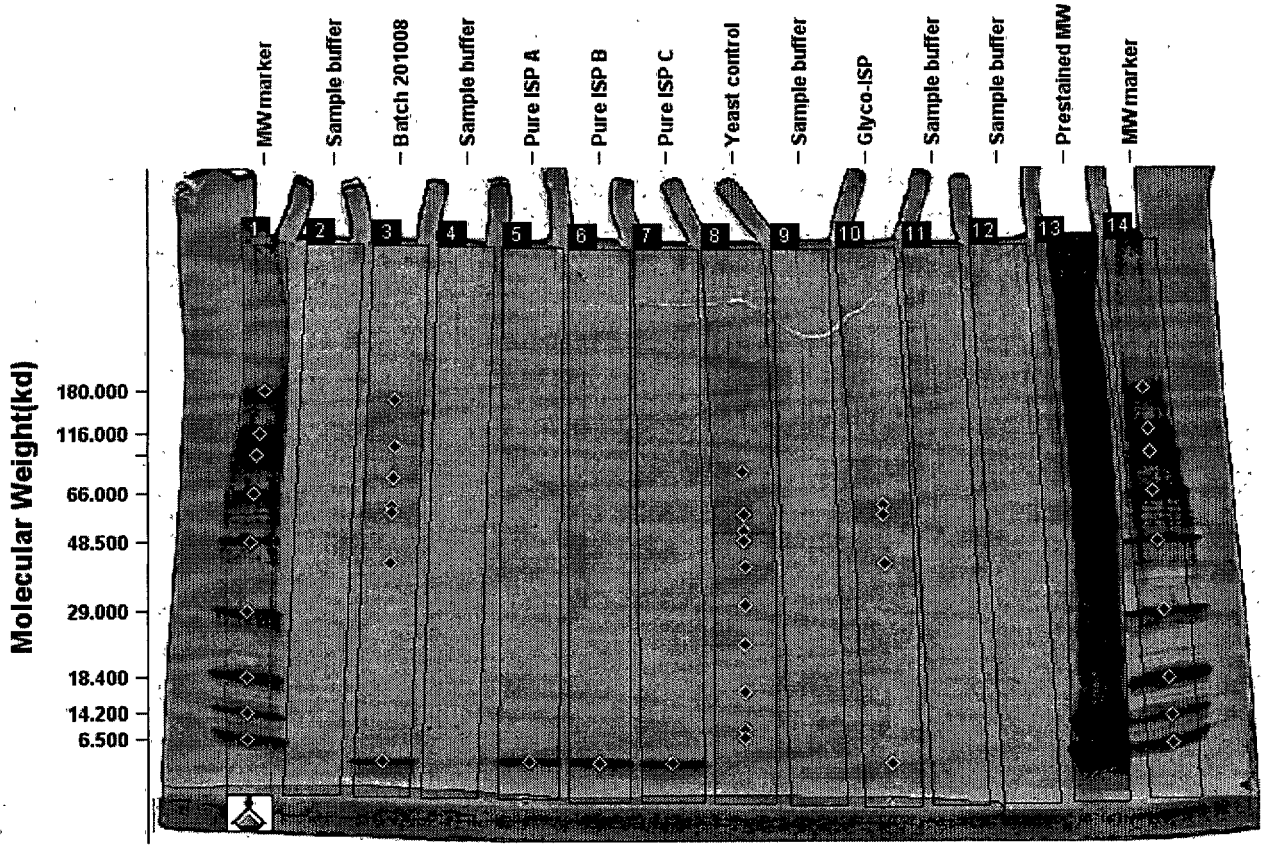
The different test materials described below were separated on 4-20% linear gradient SDS-PAGE gels. One gel was stained, while another was used for electro-transfer of proteins onto a PVDF membrane for immunostaining, using the monoclonal anti-ISP Type III for detection. The presence of ISP Type III was visualized by treatment with a biotinylated anti-mouse IgG antibody, followed by alkaline phosphatase conjugated to streptavidin, and substrate.

Test materials were:

- ISP Type III preparation (Batch 201008)
- ISP Type III glycoconjugate preparation (enriched in glycoconjugates and containing only very small amounts of unglycosylated ISP – approximately 99% of ISP as glycoconjugated ISP Type III).(Batch 201008 EtOH)
- ISP Type III standard (purified ISP Type III) – sample 1 (P-ISP A)
- ISP Type III standard (purified ISP Type III) – sample 2 (P-ISP B)
- ISP Type III standard (purified ISP Type III) – sample 3 (P-ISP C)
- Yeast control (YFS)

ISP Type III standard (samples 1, 2 and 3) were aliquots of the same material and were from the same batch used in the functional studies.

ISP Type III was detected in all preparations containing the ice structuring protein, but not in the yeast control.



SDS-PAGE gel of ISP type III preparation, purified ISP Type III samples and yeast control

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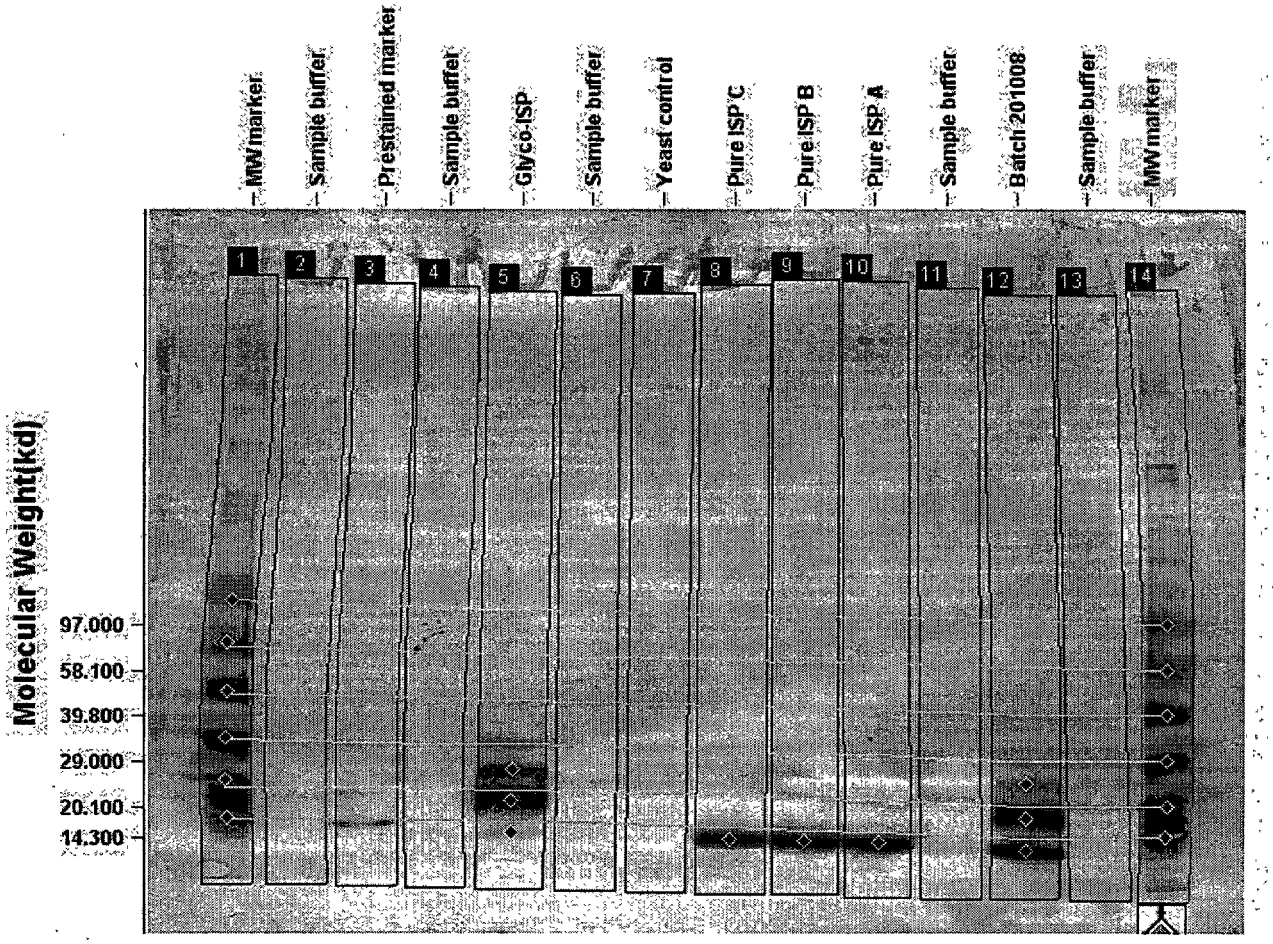
[redacted]



Immunoblot of ISP type III preparation, purified ISP Type III samples and yeast control

Conclusion

Data on the functionality of the protein, together with that from antibody binding studies, both confirm that the ISP Type III in all test materials retained its immunochemical activity. Absence of reactivity to these materials when used in the skin prick test and RAST is therefore a true indication of the absence of reactivity of the tested individuals.

Adjuvanticity of yeast proteins

A search in PubMed did not reveal any reports of adjuvant effects from proteins produced by *Saccharomyces cerevisiae*, although a number of proteins and peptides from a variety of organisms have shown immunomodulatory effects.

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Our ingestion study, which was the only one looking at potential immunogenicity, did not produce any evidence that ISP Type III was immunogenic under the circumstances of the test.

[redacted]

Consequently, the results do not support the proposition that yeast proteins boost the immunogenicity of ISP Type III.

Our study in the fish-allergic population considered only the reactivity to ISP Type III, i.e. the ability of preformed specific IgE to bind the protein and produce a response. Immunological adjuvants act at the sensitization stage of the immune response, rather than at the antibody recognition stage and would therefore not influence any responses we observed.

References

DeLuca CI, Davies PL, Ye Q, Jia Z (1998). The effect of steric mutations on the structure of type III antifreeze protein and its interaction with ice. J. Mol. Biol. 275(3), 515-525.

IC020421-6 Immunostained blot

[redacted]

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ISSUE PAPER 4: INGESTION STUDY DESIGN

January 15, 2003

Issue: Was the ingestion study designed in such a way that, should symptoms occur, they would be noticed and recorded? What would be the (theoretical) consequences on the interpretation of the study of prior exposure to proteins like ISP Type III?

FDA Question: In the interest of clarity we have split the original question into two parts:

Part I: Provide a description of the administration of the Phase III food challenge study. In particular, describe how monitoring was done for symptoms of allergic response (e.g., the nature and frequency of any questionnaires used, or other methods), and whether any symptoms were reported.

Part II: In addition, the GRAS notification mentioned that these individuals had not consumed ISP previously. Would the fact that these individuals probably had consumed the same or similar proteins from fish have any effect on the interpretation of the results of this study?

Summary of Response

In evaluating our ingestion study, it is important to note two points: First, the application of this type of study to a safety evaluation has not previously been done, and therefore there exists only a limited body of knowledge with which to interpret the study. Second, in recruiting for our study, we purposely sought subjects naïve to ISP Type III and similar proteins, as this was necessary in order to accurately evaluate the IgG response of subjects to ingested ISP Type III, and hence the potential immunogenicity of the protein by this route. In this context, then, the following responds to FDA's questions:

Part I

- The ingestion study involved medical monitoring both during and after the study, with both subjective and objective assessments of subject health.

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- No clinical symptoms or biochemical changes suggestive of food allergy were observed. As is almost invariably the case in a study of this duration, some ordinary health conditions were reported by both test subjects and controls. These are not considered to be related to ingestion of ISP Type III preparation.

[redacted]

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Part II

- Exposure to proteins by the oral route can lead to several outcomes in terms of the immune system (e.g., no response, tolerance, or sensitization). However, because these outcomes depend on a number of variables (e.g. protein type, dose, pattern of administration, etc.), there is no basis for predicting with any degree of certainty the effect of prior exposure on the outcome of the study.

- As ISP Type III occurs naturally in only a few species of arctic fish not commonly consumed in the location where the study was conducted (the UK), the likelihood that any participant (let alone all 28 of them) would have been exposed through the diet prior to the study is extremely low.

- No biochemical indications of an immune response to ISP type III were observed. One subject (#19) showed a weak IgE response to ISP type III *preparation* which upon further investigation was found to be due to sensitization to yeast proteins.

The results from this study are consistent with our overall assessment that ISP Type III has little if any immunogenicity via the oral route and presents no significant allergenic risk.

Response

PART I

1. The ingestion study involved medical monitoring both during and after the study, with both subjective and objective assessments of subject health.

The primary purpose of the ingestion study was to find out whether daily ingestion of ISP Type III preparation at a high level of consumption could lead to IgG or IgE antibody production. Data were also collected on other serum and hematological parameters as measures of general health.

a. Design

The study design was double-blind: neither the participants nor the examining physician knew who was in the test and control groups. Volunteers (29 females, 8 males) were randomly allocated to the test group (22 females, 6 males) and control group (7 females, 2 males). Each volunteer underwent a pre-study medical examination and was asked to complete a questionnaire to elicit information about relevant medical conditions and allergological status. Test and control substances were administered for a total of 8 weeks in all

b. Administration

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Individuals in the test group received 3.26 mL ISP type III preparation providing 16.3 mg ISP type III HPLC 12 in 50-100 mL of a flavored drink daily for 5 days a week over 8 consecutive weeks. The control group received 50-100 mL of the

[redacted]

flavored drink alone. Participants were required to drink the test or control substance in the presence of a human studies operative.

c. Monitoring

There were two forms of monitoring to assess subject health:

- Medical examinations were administered at 4, 6, 8, and 13 weeks. At each examination, the physician asked the volunteer about, and recorded any symptoms, whether or not they were thought to be attributable to ingestion of the test material. Copies of the record forms are in appendices 1-3.

- Clinical measures included specific IgG and IgE antibodies to ISP Type III as well as routine clinical chemistry and hematology screens. Each of those measures was assessed pre-study and at 4, 6, 8 and 13 weeks. All blood samples were taken under non-fasting conditions and no lifestyle restrictions were placed on the participants as a condition of inclusion.

2. **No clinical symptoms or biochemical changes suggestive of food allergy were observed. As is almost invariably the case in a study of this duration, some ordinary health conditions were reported by both test subjects and controls. These are not considered to be related to ingestion of ISP Type III preparation.**

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

Antibody production

The antibody response to ingestion of ISP Type III preparation is discussed in detail in our GRAS notification. Briefly:

- Sera from subjects 7, 18, 21, 25, and 28 displayed optical densities above the cut-off point of the study, defined as the greater of either 0.1 Absorption Units (AU) or the AU of the mean of the negative control sera plus two standard deviations throughout the study. These optical densities were elevated in the pre-test sera and remained at the same level as the study progressed. This indicated that ingestion of the test material did not induce production of specific IgG antibody, nor did it stimulate any recall response.

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- 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, dose-dependent inhibition of the type that would be expected in the case of specific binding. This indicates that the materials tested were not responsible for the binding of IgG. The elevated values in the IgG ELISA most likely reflect a higher level of non-specific binding of the individuals' IgG, compared to controls.

- One panelist (19) showed a weak IgE response at week 4, continuing throughout the study. Further investigations, including skin prick testing with ISP Type III preparation, pure ISP Type III, yeast control and ocean pout, as well as RAST and inhibition RAST, showed that this person was reactive to the yeast protein component of the ISP Type III preparation.

Clinical signs reported

Clinical signs and their frequency are summarized in the following table (T = test subjects, C = control subjects).

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

Subject 7 reported mild itching in the upper body from the start of the study, decreasing in intensity from the beginning to the end of each week. This subject did not display any objective symptoms and clinical measures were all within normal ranges. The only other finding in this person was an elevated AU value at each time point, including pre- and post-study, in the IgG ELISA to ISP Type III, which most likely reflected non-specific binding, as already discussed above. The timing of the symptoms experienced by subject 7 (i.e. from the beginning of the study), together with the absence of any changes in specific antibody production indicate that they are not due to sensitization to a component of the ISP preparation.

PART II

000184

In evaluating our ingestion study, it is important to note two points: First, the application of this type of study to a safety evaluation has not previously been done, and therefore there exists only a limited body of knowledge with which to interpret

[redacted]

this type of study. Second, in recruiting for our study, we purposely sought subjects naïve to ISP Type III and similar proteins, as this was necessary in order to accurately evaluate the IgG response of subjects to ingested ISP Type III, and hence the potential immunogenicity of the protein by this route. In this context, then, the following responds to FDA's questions:

1. **Exposure to proteins by the oral route can lead to several outcomes in terms of the immune system (e.g., no response, tolerance, or sensitization). However, because these outcomes depend on a number of variables (e.g. protein type, dose, pattern of administration, etc.), there is no basis for predicting with any degree of certainty the effect of prior exposure on the outcome of the study.**

Exposure to proteins in general by a dietary route can lead to several outcomes in terms of the immune system (e.g., no response, tolerance, or sensitization). In the event that participants had encountered ISP Type III or a similar protein through eating fish, three possible scenarios would therefore have to be considered. Firstly, their immune system might have been insufficiently stimulated to make a detectable response, or secondly they might have developed tolerance. In both those cases, the expected observations would be the same as seen in this study. The third scenario would be that the subjects had developed the common, low-level physiological IgG response which is an established feature of exposure to food proteins in general. In this case, administration of daily doses of ISP Type III would constitute repeated stimulation of the already primed immune system and might be expected to produce rising IgG levels specific for ISP Type III. Such elevated IgG levels are not, however, indicative of any pathological phenomenon and are not associated with clinically significant responses.

It should be stressed that while these scenarios can be described, there is no basis for predicting their relative likelihood. Only a limited number of studies looking at the effect of the immune response to dietary proteins by ingestion has been performed, and none was done as a safety assessment (as is the case with our study). Such studies provide only a limited basis for interpreting our findings, and therefore there is no basis for predicting with any degree of certainty the effect of prior exposure on the outcome of the study.

2. **As ISP Type III occurs naturally in only a few species of arctic fish not commonly consumed in the UK, the likelihood that any participant (let alone all 28 of them) would have been exposed through the diet prior to the study is extremely low.**

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The participants in this study were selected on the basis that they had not been exposed to ISP Type III (e.g., through taste trials), principally to avoid difficulties of interpretation that might arise from recall responses. ISP Type III is only known to occur in a small number of species of arctic fish, not in fish commonly consumed in the United Kingdom. The probability that even one subject, let alone all 28, had been exposed to this protein in the diet is therefore extremely low.

2. **No biochemical indications of an immune response to ISP type III were observed. One subject (#19) showed a weak IgE response to ISP type III preparation which upon further investigation was found to be due to sensitization to yeast proteins.**

As noted in the response to Part I above, there was no IgG or IgE response to pure ISP Type III in any subject. The response of subject 19 to the presence of yeast proteins in the preparation demonstrates the ability of the protocol to detect subtle changes in immune status. This gives us some reassurance that we would have detected a specific response to ISP Type III had it occurred. The results from this study are therefore consistent with our overall assessment that ISP Type III has little if any immunogenicity via the oral route and presents no significant allergenic risk.

CONCLUSION

In response to Part 1 of FDA's specific question:

- The ingestion study involved medical monitoring both during and after the study, with both subjective and objective assessments of subject health.

- No symptoms suggestive of food allergy were observed. Some ordinary health conditions were reported by both test subjects and controls, and these are not considered to be related to ingestion of ISP Type III preparation.

In response to Part 2 of FDA's question:

- There is no basis for predicting how prior exposure to ISP Type III might affect the results of the study, although several scenarios can be considered based on what is known of the immunological response to dietary proteins. However, the likelihood that the test population could have been exposed to any meaningful amount of ISP Type III prior to the study is extremely low.

- The fact that we were able to detect a weak response to yeast proteins indicates that had any subject been exposed and become sensitized to ISP Type III, this would have been evident.

000186

As discussed in our GRAS notification, the ingestion study demonstrated that ISP Type III was not immunogenic by the oral route when given 5 days a week for 8 weeks at a daily dose corresponding to a high level of consumption. No specific IgE to ISP Type III was detected in any subject. (The fact that specific IgE was detected in one subject, attributable to yeast protein, indicates that the study was sensitive enough to detect the endpoints of interest.) This study therefore supports the other evidence demonstrating that ISP Type III preparation poses no significant allergenic risk, and in addition it also provides further evidence of the general safety of ISP Type III preparation.

SUBJECT ALLOCATION AND ALLERGIC BACKGROUND

Panelist No.	Test/Control (T/C)	Panelist sensitivities (from pre-study medical and consent forms)
1	T	Mild asthma (no treatment)
2	T	Eczema in auditory canals (no treatment)
3	T	
4	C	
6	T	
7	T	Caffeine intolerance
8	T	Mild hay fever (no treatment), mild eczema (occasional hydrocortisone, but none at present), some metal allergy to jewelry
9	C	Childhood asthma (now OK)
10	T	Allergy to nickel
11	T	
12	T	Allergy to penicillin and primulas
13	C	Hay fever (allergic rhinitis – no treatment), mild eczema (no treatment)
14	T	Allergy to nickel
15	T	
16	C	Mild hay fever (no treatment)
17	C	Mild hay fever (no treatment), allergy to nickel
18	T	
19	T	Occasional hay fever (no treatment)
20	T	
21	T	
22	T	
23	T	
24	T	
25	T	
27	T	
28	T	
30	T	Hay fever (mild allergic rhinitis – no treatment)
31	T	
32	C	
34	C	Possible nickel allergy
35	T	Hay fever
36	T	Allergies to nickel, palladium and elastoplast
38	T	Hay fever
39	C	Hay fever (no treatment), childhood asthma (now OK)
40	T	Mild hay fever (no treatment), mild asthma (no treatment)
41	C	
42	T	

000187

[redacted]

Appendix 1: Pre-study medical record form

Study XH020169

HUMAN STUDIES RAW DATA
(ASSESSMENT OF ANTIBODY REPSONSE FOLLOWING EXPOSURE TO ISP TYPE III PREPARATION)

PRE - STUDY MEDICAL

Panellist Name:	

1. Are you taking any medication (including inhalers / nosedrops / sprays) YES / NO

If yes, include details below:

_____
_____
_____
_____
_____
_____

If a panellist is on antidepressants – is it a monoamine oxidase inhibitor? YES / NO
(if yes, panellist cannot be included in trial)

2. Do you suffer with any of the following:

Hayfever YES / NO
.....

Eczema YES / NO
.....

Asthma YES / NO
.....

Food allergies YES / NO
.....

Any other allergies YES / NO
.....

3. Is your general health currently good? YES / NO

_____
_____
_____

4. Blood pressure / pulse readings:
Systolic .....
Diastolic .....

000188

Pulse .....

5. Any other comments:

_____

_____

_____

This panellist **is fit / is not fit** *to participate in this study*
(delete as appropriate)

Dr D J Rhodes: _____ Date: _____
(Medical Practitioner)

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[redacted]

Appendix 2: Weeks 4, 6, 8 medical record form

HUMAN STUDIES RAW DATA
(ASSESSMENT OF ANTIBODY REPSONSE FOLLOWING EXPOSURE TO ISP TYPE III PREPARATION)

WEEK FOUR MEDICAL QUESTIONNAIRE

Panellists name: **Panel number:**

Have you experienced any change to your general state of health since the start the trial? Yes/No*

*If yes, please detail how:

_____
_____
_____
_____
_____
_____

Have you any comments about the trial?

_____
_____
_____
_____
_____
_____

Pulse rate: _____ within normal parameters Yes/No

Blood pressure _____ within normal parameters Yes/No

This panellist **is fit / is not fit** *to continue participating in this study*
(delete as appropriate)

Dr D J Rhodes: _____ Date: _____
(Medical Practitioner)

000190

[redacted]

Appendix 3: Post study medical record form

HUMAN STUDIES RAW DATA
(ASSESSMENT OF ANTIBODY REPSONSE FOLLOWING EXPOSURE TO ISP TYPE III PREPARATION)

POST MEDICAL QUESTIONNAIRE

Panellists name: **Panel number:**

Did you experience any change to your general state of health during the trial? Yes/No*

*If yes, please detail how:

_____
_____
_____
_____
_____
_____

Have you any comments about the trial?

_____
_____
_____
_____
_____
_____

Pulse rate: _____ within normal parameters Yes/No

Blood pressure _____ within normal parameters Yes/No

This panellist **is fit / is not fit**
(delete as appropriate)

Dr D J Rhodes: _____ Date: _____
(Medical Practitioner)

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[redacted]

Unilever

ISSUE PAPER 5: RESPONSES TO YEAST

January 15, 2003

Issue: What is the clinical significance of the positive skin prick test and RAST responses to yeast?

FDA Question 5: Provide an extended, formal discussion of the reasoning and evidence to support a conclusion that the positive reactions from yeast do not present a significant food allergy risk.

Summary of Response

As described in our GRAS notification (p. 48), our Expert Panel concluded that the occurrence of skin prick test reactions and specific IgE to the yeast protein component of ISP Type III preparation is of little clinical significance. This conclusion was based on the following key points:

- Sensitization to yeast appears to be relatively common, particularly in persons who have experienced inhalation exposure, in persons who have experienced a yeast infection, or in persons with atopic dermatitis. This sensitization can be measured by highly sensitive tests such as skin prick tests and RASTs.

- Allergic responses to yeast are extremely rare, and serious allergic reactions to yeast are almost unknown. This is despite the fact that yeast is ubiquitous in the food supply. Indeed, yeast has been safely used as a food ingredient throughout recorded history.

- In our studies, all of the persons who experienced positive skin prick tests and RAST responses also have atopic dermatitis or related conditions. Therefore, it is not surprising to observe yeast sensitization in these individuals. Significantly, all of these individuals are able to eat yeast-containing foods (such as bread, wine, and beer) without adverse reaction.

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In response to FDA's inquiry, our Expert Panel and Prof. Carsten Bindslev-Jensen have fully re-examined the issue of the clinical significance of yeast sensitization. They have unanimously advised us that, based on (1) the lack of significant reported allergic reactions to yeast in the U.S. and worldwide and (2) the long history of safe use of yeast and yeast based products in foods, the yeast component of ISP Type III preparation does not pose a clinically significant allergenic risk.

[redacted]

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Introduction

In evaluating this issue, Unilever has relied on the advice of a panel of independent experts. As explained in our GRAS notification, this panel consists of experts on food allergy (Wesley Burks, M.D., Hugh A. Sampson, M.D., and Steve L. Taylor, Ph.D.) and experts on food safety (Joseph F. Borzelleca, Ph.D., Walter H. Glinsmann, M.D., and Michael W. Pariza, Ph.D.). These individuals are recognized worldwide as pre-eminent in their fields.

We have also relied on the advice of other experts on food allergy, particularly the investigator who conducted our allergenicity testing, Prof. Carsten Bindslev-Jensen of Odense University Hospital in Denmark. Prof. Bindslev-Jensen is one of the world's foremost experts on fish allergy and on food challenges. Although Prof. Bindslev-Jensen is not a member of our Expert Panel, we have consulted with him on the issues discussed in this paper.

Like many other fermentation products used in foods (e.g., enzymes), ISP Type III preparation contains residual yeast proteins. As explained in detail in our GRAS notification and in the accompanying document entitled "Issue Paper 2: Determination of the Reactivity of a Population of Highly Fish-Allergic Individuals to ISP Type III: the Issue of Food Challenges," the positive skin prick test and/or RAST results occurring in some subjects tested with ISP Type III preparation are attributable solely to a response to yeast. None of these subjects responded to pure ISP Type III protein, and all of them responded to a yeast control; therefore their responses could only be caused by exposure to yeast or yeast protein.[1] The question arises, then, whether the presence of yeast protein in ISP Type III preparation constitutes any hazard to consumers sensitized to yeast. This paper outlines why yeast, despite producing positive results in highly sensitive assays (skin prick tests and/or RAST) in some subjects, is not a clinically significant food allergy risk.

1. Sensitization to yeast appears to be relatively common, particularly in persons who have experienced inhalation exposure, in persons who have experienced a yeast infection, or in persons with atopic dermatitis. This sensitization can be measured by highly sensitive tests such as skin prick tests and RASTs.

IgE-mediated sensitization to yeast appears to be relatively common, arising mainly from inhalation exposure, from exposure due to saprophytic growth on mucous membranes (e.g., *Candida albicans*), or in cases of atopic dermatitis. It does not appear to arise from the consumption of foods containing yeast.

IgE-mediated sensitization to airborne yeast has been reported among people exposed occupationally (Bataille et al., 1995). This is accompanied by respiratory symptoms such as bronchial asthma. In addition to IgE antibodies, some of these individuals also manifest

[1] The yeast control was produced by growing the untransformed yeast strain and then processing it in exactly the same way as the yeast producing the ISP Type III. The yeast control therefore contained only the components originating from the yeast, and no components associated with the expression of the ISP Type III gene.

symptoms of allergic extrinsic alveolitis and the production of IgG antibodies to the yeast, which form immune complexes *in vivo*.

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

Finally, in one Japanese study (Arai et al., 1998), 3102 asthmatics were tested in series of skin scratch tests. Roughly one-fifth of these, 625, showed a positive skin scratch test reaction to one or more foods. Of those 625, 24% (or about 5% of the total asthmatic sample) showed a positive result with yeast. The prevalence of yeast sensitization in the general population is not known, but can safely be assumed to be lower. The scratch test is a non-standard procedure, however, and results are therefore difficult to compare with those of other studies.

2. **Allergic responses to yeast are extremely rare, and serious allergic reactions to yeast are almost unknown. This is despite the fact that yeast is ubiquitous in the food supply. Indeed, yeast has been safely used as a food ingredient throughout recorded history.**

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

A study by Kortekangas-Savolainen et al.,(1994) investigated the association between the presence of specific IgE antibodies to baker's yeast, measured by skin prick test and RAST, and tolerance of foods containing yeast and yeast proteins (wine, bread, beer). The test population consisted of 13 individuals (all skin prick test-positive to baker's yeast and of which six reported symptoms of allergy upon ingestion of yeast-containing foods) and 24 healthy subjects. The investigators found that skin prick test reactions could be elicited by extracts of yeast-derived foods in all of the test population plus two of the control group. However, they went on to show that the symptoms of food allergy reported by some of the test population were not associated with the yeast component of the food. The authors concluded that "the IgE mediated allergy to Baker's yeast alone should not lead to the denial of bakery, brewery and wine products." These

results are entirely consistent with our findings, in that the fish allergic subjects who were skin prick test positive to yeast in our studies are able to ingest yeast-containing foods without adverse reaction.

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

The only report of a *possible* severe reaction to yeast following ingestion is an anecdotal account of an infant suffering angioedema of the lips after eating bread with Marmite, a yeast extract (Higson, 1989).

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

3. **In our studies, all of the persons who experienced positive skin prick tests and RAST responses also have atopic dermatitis or related conditions. Therefore, it is not surprising to observe yeast sensitization in these individuals. Significantly, all of these individuals are able to eat yeast-containing foods (such as bread, wine, and beer) without adverse reaction.**

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

Conclusions

In response to FDA's inquiry, our Expert Panel and Prof. Carsten Bindslev-Jensen have fully re-examined the issue of the clinical significance of yeast sensitization. They have unanimously advised us that, based on (1) the lack of significant reported allergic reactions to yeast in the U.S. and worldwide and (2) the long history of safe use of yeast and yeast based products in foods, the yeast component of ISP Type III preparation does not pose a clinically significant allergenic risk.

Accordingly, based on this advice, Unilever concludes that the presence of yeast in ISP Type III preparation does not present a significant food allergy risk. In light of this conclusion, no food challenge study is necessary to assess the risk of reaction to the yeast component of ISP Type III preparation, and there is no need to label ISP Type III preparation as being derived from yeast.[2]

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[2] This conclusion is consistent with the historical use of *S. cerevisiae* in the United States. This yeast is an approved food additive and GRAS-affirmed for a number of different uses. To our knowledge, FDA has never identified yeast as a food allergen and has never required that its presence be declared on the food label as a condition of safe use.

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Appendix: Medical background of patients reacting to yeast. ***Part 1***: SPT positive

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

Skin prick test reactions are given as two largest diameters in mm.

Appendix: Medical background of patients reacting to yeast. ***Part 2***: RAST positive

	Patient 12	Patient 13	Patient 16	Patient 17	Patient 23
Medical history	Severe atopic eczema treated with topical steroids, UVB therapy, cyclosporin. Hay fever (due to grass pollen and mugwort); sneezing on contact with dog	Moderate to severe atopic eczema treated with topical steroids Hay fever (due to grass and birch pollen); sneezing on contact with animals	Moderate to severe atopic eczema treated with topical steroids. Bronchial asthma treated with inhalant steroids. Hay fever (due to grass and dust mite)	Very severe atopic dermatitis treated with azathioprine, prednisolone, cyclosporin, tar and UVB, as well as topical steroids. Moderate to severe asthma treated with inhalant steroids and beta-2 agonists.Perrenial rhino-conjunctivitis	Moderate to severe atopic eczema treated with topical steroids. Bronchial asthma treated with inhalant steroids and beta-2 agonists
Positive control	5.0-5.5	7.5-8.0	8.5-7.5	6.5-7.0	5.5
Negative control	0	1.0-1.0	0	0	0
Birch	0	6.5	1.5	7.5	4
Grass	9.5	11	6.5	9	11
Mugwort	1.5	4.5	0	10.5	6
Horse	0	14	3	11	6.5
Cat	8	12	3	9.5	5
Dog	11	13.5	3	11	5.5
House dust mite (Der p)	8.5	11	7.5	8.5	8
Storage mite (Der f)	8.5	10.5	5	7.5	8.5
Alternaria	3.5	4.5	5	0	7
Cladosporium	0	5.5	0	6.5	0
Pityrosporium ovale		7.5		0	0
Milk					5
Egg					6
Hazelnut				9	
Soy				5	
Celery root				8	
Brazil nut				3.5	
Latex 100				16	0
Cod	17.5-16.0	16.5-16.5	17.0-12.0	22.0-30.0	14.0-10.5
Plaice	14.0-15.5	14.5-12.5	16.0-9.0	23.5-25.0	16.0-8.5
Tuna	3.5-5.5	7.0-5.5	8.0-8.5	7.0-8.0	7.0-6.0
Mackerel	10.0-9.5	14.5-12.0	14.5-8.5	22.0-20.5	6.0-10.
Salmon	12.0-10.5	9.5-19.5	19.5-13.5	23.0-21.0	10.0-10.0
Trout	16.0-10.5	26.0-23.5	15.5-15.0	19.5-30.0	9.0-10.0
Eel	13.5-10.5	16-18.5	25.0-14.0	36.5-51.5	17.5-15.0
Eel pout	13.5-18.5	13-16.5	13.5-22.5	22.0-39.0	16.0-13.5
Ocean pout	17.5-16.0	16.5-11.5	20.5-22.0	16.5-25.0	16.5-18.0

Skin prick test reactions are given as two largest diameters in mm.

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Chair, Food Chemicals Codex Committee, Food and Nutrition Board, National Academy of Sciences (1990-2000)
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Member, Subcommittee on Upper Reference Levels, Food & Nutrition Board, National Academy of Sciences (1996-2000)
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Ad Hoc Advisory Committee on Hypersensitivity to Food Constituents- FDA, member (1985-87)
American Council on Science & Health, Board of Scientific Advisors (1986-present)
Editor, Food Science and Technology Series, Academic Press, Inc.
Editor, Advances in Food and Nutrition Research, Academic Press, Inc.
Editorial Board, Journal of Natural Toxins
Editorial Board, Journal of Food Composition and Analysis
Editorial Board, Food and Chemical Toxicology
Editorial Board, Lebensmittel-Wissenschaft Technologie
Scientific Editor, Food Chemistry and Toxicology Section, Journal of Food Science (1998-2001)
International Food Biotechnology Council, Ad Hoc Committee on Allergenicity of Genetically Engineered Foods (1993-96)
Snack Food Association, Scientific Review Committee (1994-present)
American Gastroenterological Association Foundation, Consensus Conference on E. coli 0157:H7 Infections, panelist (1994)
Member, Board of Directors, Food Allergy Network (1994-05; Chair: 2003-05)
Member, Medical Advisory Board, Food Allergy Network (1991-present)
Board of Directors, Nebraska Industrial Competitiveness Alliance (1994-present)
Board of Directors, Food Update Conference (1994-00); Program Chairman (1998); Chairman (1999)
Chair, FAO/WHO Expert Consultation, Safety of Genetically Modified Foods, Rome (1996)
Member, FAO/WHO Expert Consultation, Biotechnology and Food Safety, Geneva (2000)
Member, FAO Expert Consultation, Labeling of Allergenic Foods (1995)
Member, WHO Expert Consultation, Labeling of Allergenic Foods (1999)
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Invited Advisor on Health Effects of Genetically Modified Foods: Mexico (2002), Brazil (2002)
Advisory Board, Joint Institute of Food Safety & Applied Nutrition (JIFSAN), University of Maryland and Food & Drug Administration (1999-present)

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195. Hefle, S. L. and S. L. Taylor. 2002. How much food is too much? Threshold doses for allergenic foods. Curr. Allergy Asthma Rpts. 2:63-66.

196. Taylor, S. L. 2002. Protein allergenicity assessment of foods produced through agricultural biotechnology. Ann. Rev. Pharmacol. Toxicol. 42:99-112.

197. Taylor, S. L., S. L. Hefle, C. Bindslev-Jensen, S. A. Bock, A. W. Burks, L. Christie, D. J. Hill, A. Host, J. O'B. Hourihane, G. Lack, D. D. Metcalfe, D. A. Moneret-Vautrin, P. A. Vadas, F. Rance, D. J. Skrypec, T. A. Trautman, I. Malmeheden Yman, and R. S. Zeiger. 2002. Factors affecting the determination of threshold doses for allergenic foods: how much is too much? J. Allergy Clin. Immunol. 109:24-30..

198. Taylor, S. L. and S. L. Hefle. 2002. Food Allergy Issues Alliance and food allergen labeling guidelines. Food Allergy Intolerance 3:37-43.

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200. Taylor, S. L. and S. L. Hefle. 2002. Genetically engineered foods: implications for food allergy. Curr. Opinion Allergy Clin. Immunol. 2:249-252.

201. Taylor, S. L., S. L. Hefle, G. Soylemez, and J. Kabourek. 2002. Sesame seed allergy. Food Allergy Intolerance 3:115-122.

202. Taylor, S. L. and S. L. Hefle. 2002. Food allergy assessment for products derived through plant biotechnology. In: Biotechnology and Safety Assessment, 3rd ed., ed. J. A. Thomas and R. L. Fuchs, Academic Press, San Diego, pp. 325-345.

203. Soni, M. G., S. L. Taylor, N. A. Greenberg, and G. A. Burdock. 2002. Evaulation of the health aspects of methyl paraben: a review of the published literature. Food Chem. Toxicol. 40:1335-1373.

204. Taylor, S. L. 2002. Assessment of the allergenicity of foods produced through agricultural biotechnology. In: Crop Biotechnology, ed. K. Rajasekaran, T. J. Jacks, and J. W. Finley, American Chemical Society, Washington, D.C., pp. 205-216.

205. Taylor, S. L. and S. L. Hefle. 2002. Foods as allergens. In: Food Allergy and Intolerance, ed. J. Brostoff and S. J. Challacombe, Saunders, London, pp. 403-412.

206. Taylor, S. L. 2002. Disease processes in foodborne illness. In: Foodborne Diseases, 2nd ed., ed. D. O. Cliver and H. P. Riemann, Academic Press, London, pp. 3-29.

207. Taylor, S. L. 2002. Chemical intoxications. In: Foodborne Diseases, 2nd ed., ed. D. O. Cliver and H. P. Riemann, Acadmic Press, London, pp. 305-316.

208. Taylor, S. L. and S. L. Hefle. 2002. Naturally occurring toxicants in foods. In: Foodborne Diseases, 2nd ed., ed. D. O. Cliver and H. P. Riemann, Academic Press, London, pp. 193-210.

209. Sulaeman, A., K. B. Tan, S. L. Taylor, and J. A. Driskell. 2002. Sensory acceptability of a deep-fried carrot chip product as evaluated by American and Southeast Asian consumer panels. J. Food Qual. 25:453-467.

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213. Taylor, S. L. and S. L. Hefle. 2003. Foods and ingredients that cause food allergies and intolerances. In: Food Safety, ed. J. Rodricks, M. Taylor, and K. L. Hulebak, Chapman & Hall, New York. (In Press).

214. Taylor, S. L. 1999. Assessment of the allergenicity of genetically modified foods. Proc. 16th. Int. Congress Nutrition, Montreal, Canada, August, 1997. (In Press).

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215. Taylor, S. L. 2003. Nutrition labeling. In: McGraw-Hill Encyclopedia of Science & Technology, ed. S. P. Parker, McGraw-Hill Publishers, New York. (In Press).

216. Bush, R. K. and S. L Taylor. 2003. Histamine. In: Encyclopedia of Food Sciences and Nutrition, ed. B. Caballero, L. Trugo, and P. Finglas, Academic Press, London (submitted).

217. Taylor, S. L. and J. Kabourek. 2003. Food intolerance (c) milk allergy. In: Encyclopedia of Food Sciences and Nutrition, ed. B. Caballero, L. Trugo, and P. Finglas, Academic Press, London (submitted).

218. Taylor, S. L. 2003. Toxins in foods – naturally occurring. In: Encyclopedia of Food Sciences and Nutrition, ed. B. Caballero, L. Trugo, and P. Finglas, Academic Press, London (submitted).

219. Taylor, S. L. 2003. Approaches to the safety assessment of novel foods and novel gene products. Comments Toxicol. (submitted).

220. Sulaeman, A., L. Keeler, D. W. Giraud, S. L. Taylor, and J. A. Driskell. 2002. Changes in carotenoid, physicochemical, and sensory values of deep-fried carrot chips during storage. Int. J. Food Technol. (submitted).

221. Taylor, S. L. 2003. Safety assessment of genetically modified foods. In: Molecular Nutrition, ed. H. Daniel and J. Zempleni, CABI Publishing, Wallingford, U.K. (submitted).

222. Taylor, S. L. 2003. Comment on use of digestive stability as a criterion in the assessment of the potential allergenicity of genetically modified foods. J. Agr. Food Chem. (submitted).

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CURRICULUM VITAE

Name: A. WESLEY BURKS, M.D.

Primary Academic Appointment: Department of Pediatrics

Citizenship: USA

Present Academic Rank: Professor of Pediatrics

Education:

College	University of Central Arkansas	1976	B.S.
Medical School	University of Arkansas Medical School	1980	M.D.

Professional Training and Academic Career:

Internship	Arkansas Children's Hospital	1980-1981 Pediatric
	University of Arkansas for Medical Sciences	1981-1983 Pediatric Residency
	Arkansas Children's Hospital	1983 Chief Resident
	Duke University Medical Center (Allergy/Immunology)	1983-1985 Fellowship

Date and Rank of Faculty Appointments:

Assistant Professor of Pediatrics University of Arkansas for Medical Sciences Arkansas Children's Hospital	1985 - 1991
Associate Professor of Pediatrics (with tenure) University of Arkansas for Medical Sciences Arkansas Children's Hospital	1991 - 1996
Professor of Pediatrics University of Arkansas for Medical Sciences Arkansas Children's Hospital	1996 - present
Chief, Pediatric Allergy and Immunology University of Arkansas for Medical Sciences Arkansas Children's Hospital	1987 - present
Director, HLA Tissue Typing Laboratory University of Arkansas for Medical Sciences Arkansas Children's Hospital	1991 - present
Co-Director, Pediatric Clinical Research Unit Arkansas Children's Hospital	1991- 1996
Faculty Member of the Interdisciplinary Program of Cellular and Molecular Immunology and Immunopathology University of Arkansas for Medical Sciences	1995 - present
Vice Chairman for Research Department of Pediatrics	

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	University of Arkansas for Medical Sciences	1996 – present
	President and CEO of Arkansas Children's Hospital Research Institute	1999-present
Board Certification:	American Board of Pediatrics	1985
	American Board of Allergy and Immunology	1985
Professional Societies:	American Academy of Pediatrics	
	American Academy of Allergy, Asthma and Immunology	
	American Association of Immunologists	
	Clinical Immunology Society	
	Society for Pediatric Research	
	American Federation for Clinical Research	
	Southern Society of Pediatric Research	
	Southeastern Allergy Society	
	Alan Cazort Allergy Society	
	Arkansas Academy of Pediatrics	
Volunteer Activities:	Soccer, Baseball, Basketball - youth coach	1991 - present
	Kanakuk - camp physician	1993 - present
Honors:	President - Alpha Chi (National Honors Organization)	1975 - 1976
	Presidential Scholar - University of Central Arkansas	1976
	Outstanding Graduate - University of Central Arkansas	1976
	Most Honored Faculty (Red Sash Award) University of Arkansas for Medical Sciences Classes	1989, 1990, 1991, 1995, 1999, 2001
	"Best Doctors in America", Woodward and White	1993, 1996, 1997
	Distinguished Faculty Award, University of Arkansas for Medical Sciences	2000
	Alpha Omega Alpha	2000 – present
	University of Arkansas for Medical Sciences College of Medicine Distinguished Faculty Scholar	2001
	American Academy of Allergy Asthma & Immunology C. Warren Bierman Lectureship	2001
Editorial Board:	Journal of Allergy and Clinical Immunology	1992 - 1996
	International Archives of Allergy and Immunology	1997 - present
Ad Hoc Reviewer:	Journal of Immunology	
	Pediatrics	
	Journal of Pediatrics	
	Journal of Allergy and Clinical Immunology	
	Pediatric Allergy and Immunology	
	Annals of Allergy and Immunology	
	New England Journal of Medicine	
	International Archives of Allergy and Immunology	
National Office:	Chairman, Adverse Reactions to Foods Committee, American Academy of Allergy and Immunology	1992 - 1995
	Board of Directors, Food Allergy and Anaphylaxis Network	1992 - present
	NIH - NHLBI, ad hoc study section	1992
	NIH - NIAID, ad hoc study section, Immunological Sciences	1993, 1999
	Southern Society for Pediatric Research, Research Councilor	1989 - 1991

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President, Southern Society for Pediatric Research	1995
Chairman, Allergen Standardization Committee, American Academy of Asthma, Allergy, and Immunology	1995-1996
American Board of Allergy and Immunology	1996 - present
American Academy of Allergy Asthma & Immunology, Task Force on Allergic Disorders: Promoting Best Practice	1998 - present
American Academy of Allergy Asthma & Immunology, Professional Education Council Component Committee Member	1998 - present
American Academy of Pediatrics, Executive Committee Member, Section on Allergy and Immunology	1998 - present
American Academy of Allergy & Immunology, Adverse Reactions To Foods Committee Member	2000 – present
American Academy of Allergy & Immunology, Food & Drug Reactions & Anaphylaxis, Secretary	2000 – present
American Academy of Allergy & Immunology, Liason to Lay Charitable Organizations Committee Member	2000 – present
Accreditation Council for Medical Education, Residency Review Committee	2000 – present
American College of Allergy, Asthma & Immunology, Dermatological Allergy Committee Member	2002

Clinical & Committee Appointments:

Young Faculty Advisory Committee - Department of Pediatrics	1986-1990
Promotion and Tenure Committee, Department of Pediatrics, University of Arkansas for Medical Sciences and Arkansas Children's Hospital	1987-1989, 1990-present
Molecular Biology Committee - part of six year planning committees at UAMS	1988
Day Medicine Committee, ACH	1988-1991
Research Council, University of Arkansas for Medical Sciences	1989 - present,
Chairman, Out-Patient Care Committee, Arkansas Children's Hospital	1989 - 1993
Research Advisory Committee, Department of Pediatrics, University of Arkansas for Medical Sciences	1990 - 1995
Pilot Study Committee (Research Council-UAMS)	1991 - 1998
Admissions Committee, University of Arkansas for Medical Sciences	1992 - 1995
Board of Directors, Arkansas Regional Organ Recovery Agency	1992 - 1999
Dean's Research Advisory Committee, University of Arkansas for Medical Sciences	1992 - 1994
Alan Cozart Allergy Society, Program Chairman	1992 - 1995
Arkansas Children's Hospital Executive Committee	1992-93, 1995-97
Inpatient Advisory Committee ACH/UAMS	1992 - 1994
Chairman, Student Research Day Subcommittee	1992, 1993
Transplantation Committee, Arkansas Children's Hospital	1992 - 1993
Committee on Latex, ACH	1993 - 1994
Member, Arkansas Cancer Research Center	1994 - present
Patient Care Committee, Arkansas Children's Hospital	1994 - 1995
Society of Pediatric Research, Educational Committee	1994 - 1995
Special Committee for Scientific Misconduct, UAMS	1994 - 1995
Vice-Chief of Pediatric Staff, Arkansas Children's Hospital	1996 – 1997
Oversight Committee for Act I Tobacco Settlement Committee	2001 – present

GRANTS AND AWARDS (Selected ones)

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Areas of research focus include the broad area of the interaction of nutrition and the immune system. Much of the work has been in the specific area of adverse immunologic reactions to foods in childhood. The funding for these studies has come from a combination of national, regional, and local funding.

Ross Laboratories, "Allergenicity of soy formula", 1987 - 1991, PI - Wesley Burks, M.D., $23,000.

Arkansas Science & Technology Authority, "Allergenicity of major component proteins of soybean", March 1988 - February 1990, PI - Wesley Burks, M.D., $23,075.

National Institutes of Health FIRST Award, "Allergenicity of major component proteins of soybean", April 1989 - June 1994, $450,000, PI - Wesley Burks, M.D.

Ross Laboratories, "Protein antigenicity modeling", February 1990 - March 1993, PI $69,600, PI - Wesley Burks, M.D.

Arkansas Science & Technology Authority, "Allergenicity of rice protein", March 1990 - May 1992, $29,180, PI - Wesley Burks, M.D.

UAMS Equipment Grant, "Pharmacia Liquid Chromatography System", March 1990 - May 1992, $29,180.94, PI - Wesley Burks, M.D.

Ross Laboratories, "The gastrointestinal and immune systems: The formula-fed neonatal piglet model, 1992 - 1995, $225,000, PI - Gary Wheeler, M.D., Co-PI - Wesley Burks, M.D.

National Yogurt Association, "The impact of yogurt on laboratory and clinical immune responses", May 1993 - April 1995, $160,000, PI - Ricki Helm, Ph.D., Co - PI - Wesley Burks, M.D., Gary Wheeler, M.D.

Asthma & Allergy Foundation of America, "Molecular and immunologic studies of peanut allergens", June 1993 - June 1995, $40,000, PI - Wesley Burks, M.D.

Arkansas Science & Technology Authority, "Molecular cloning of cockroach allergens", October 1992 - September 1993, $29, 863, PI - Ricki M. Helm, Ph.D., Co-PI - Wesley Burks, M.D.

United States Department of Agriculture, "Nutrition and development: From community to gene", July 1994 - April 1995, Project Manager - Thomas Badger, Ph.D., PI - Project #7, Wesley Burks, M.D., "Detection of specific allergens in manufactured food products" $56,114; PI - Project #2, Wesley Burks, M.D., "Genetically engineered allergen-free peanut and soy proteins", $43,074, Co-PI Project #6, Wesley Burks, M.D., "Nutritional augmentation of neonatal immunity", $55,841.

Environmental Protection Agency, "The Arkansas Clinical and Developmental Toxicology Program", Project Manager - Thomas Badger, Ph.D., July 1994 - June 1995, July 1995 - June 1996, $579,949, Co-PI - Project C "Respiratory function in farm workers and families living on farms using pesticides", $40,923; Co-PI - Project D "Evaluation of the immunotoxic properties of the herbicide, Propanil, in families", $57,750.

National Institutes of Health, R01"Molecular and immunologic studies of peanut allergens", December 1995 - November 1998, $613,987, PI - Wesley Burks, M.D.

United States Department of Agriculture, "Nutritional Impact on Immune Development", November 1997 – September 1998, $220,000, PI – Wesley Burks, M.D., (Co-PI, Gary Bannon).

Burroughs Wellcome Research Travel Grant, "Molecular and immunologic mechanisms in peanut allergen recognition and immunotherapy", June 1998 – July 1998, $6,000, PI – Wesley Burks, M.D.

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Panacea Pharmaceuticals, LLC, "Peanut Hypersensitivity", Co-PI Wesley Burks, M.D., Gary Bannon, Ph.D., (Co-PI, Hugh Sampson, M.D. – Mt. Sinai), 1999-2003, $1,500,000.

Rued Allergy Research Foundation, "Evaluation of immunomodulation of walnut allergy", July 1999 – June 2004, $150,000/year , Co-PI, Wesley Burks, M.D., Gary Bannon, Ph.D., Hugh Sampson, M.D.

United States Department of Agriculture, "Nutritional Impact on Immune Development", October 1998 – September 2000, $220,000, PI – Wesley Burks, M.D., (Co-PI, Gary Bannon).

National Institutes of Health, "Immunotherapy for Patients with Peanut Anaphylaxis", July 1999 – June 2004, $100,000/year, PI – Wesley Burks, M.D.

Wallace Research Foundation, "The Molecular Elimination of Peanut Allergens:, 1998-2003, $40,000/year, PI - Wesley Burks, M.D.

Monsanto, "Allergenicity of Soy Extracts", 2000-2001, $29,400, Co-PIs - Wesley Burks, M.D., Gary Bannon, Ph.D.

Food Allergy Initiative, "Identification and Characterization of Soybean Allergens", 2001-2003, $304,980, Co-PIs - Wesley Burks, M.D., Gary Bannon, Ph.D.

Wyeth-Ayerst International Inc., "Effects of Hydrolyzed Soy and Milk Formula on an Allergic Mouse Model", 2001-2002, $127,450, Co-PIs - Wesley Burks, M.D., Gary Bannon, Ph.D.

The Peanut Foundation, "Peanut Allergen Immunotherapy", 4-1-99/9-30-03, $150,000, Co-PIs - Wesley Burks, M.D., Gary Bannon, Ph.D.

Hittman Family Foundation, "Peanut Allergen Immunotherapy II", 1-1-01/12-31-03, $90,000, Co-PIs - Wesley Burks, M.D., Gary Bannon, Ph.D.

National Institutes of Health, "Extramural Research Facilities Construction Project", 9-30-01/9-29-03, $1,036,008, PI - Wesley Burks, M.D.

SELECTED OTHER GRANTS

Baxter Healthcare Corporation, Hyland Division, "Gammagard in children undergoing renal transplant and bone marrow transplant", April 1987 - June 1992, $51,000, PI - Eileen Ellis, M.D., Co-PI - Wesley Burks, M.D.

Sandoz Pharmaceutical, 1987, "Role of IVIG in neonatal sepsis", $2,000/patient, PI - Wesley Burks, M.D.

Mead Johnson Nutritional Group, "Comparison of infant formula proteins in development of atopic disease from birth to age 18 months for formula-fed children with family history of atopic disease", October 1988 - March 1991, $236,700, PI -Wesley Burks, M.D.

Mead Johnson Nutritional Group, "Immunoglobulin studies in rice protein allergy", September 1989 - September 1990, $39,560, PI - Wesley Burks, M.D.

Cutter Biological, "A controlled randomized study of IGIV, pH 4.25 in children with IgG subclass deficiency and/or impaired antibody response". June 1989 - September 1990, $26,000, PI - Wesley Burks, M.D.

Ellis Pharmaceutical/Biotest Institute, "A Phase III clinical study to compare the efficacy and safety of Intraglobin F® vs Sandoglobulin® or Gamimmune® (or other IGIV products) in primary humoral immunodeficiency disease", January 1990 - December 1992, $55,000, PI - Wesley Burks, M.D.

Ross Laboratories, "Double-blind, placebo-controlled oral challenges of Alimentum in patients with documented IgE mediated cow's milk hypersensitivity", April 1990 - January 1991, $18,000, PI - Wesley Burks, M.D.

Ross Laboratories, "Double-blind placebo-controlled oral challenge of soy formula in patients with documented IgE mediated cow's milk sensitivity", September 1990 - July 1991, $31,644, PI - Wesley Burks, M.D.

Mead-Johnson Nutritional Group, "Double-blind placebo controlled trial of Nutramigen vs a modified case in hydrolysate formula in children with milk hypersensitivity", October 1991 - December 1992, $42,000, PI - Wesley Burks, M.D.

Ross Nutrition Center , Core Center for Infant Formula Studies, 1992 - 1994, $450,000, PI - Wesley Burks, M.D., Co-PI - Thomas Badger, Ph.D.

Ross Laboratories, "Effect of powdered and liquid casein hydrolysate formula on the growth of healthy, term infants", January 1992 - May 1993, $28,023, PI - Wesley Burks, M.D.

R. Clifton Brooks, Jr., Sons of Confederate Veterans, Medical Research Grant, $12,000, 1995 - 1996, PI - Wesley Burks, M.D.

Ross Nutrition Center, Core Center for Infant Studies, 1995-1997 $360,000/year, Co- PI, Wesley Burks, M.D., Margaret Bogle, Ph.D.

Ross Nutrition Center, Core Center for Infant Studies, 1997-1999 $677,907,
PI – Wesley Burks, M.D.

Ross Nutrition Center, Core Center for Infant Studies, 2001-2003, $157,990, PI - Wesley Burks, M.D.

PATENTS

15 – related to peanut allergen and immunotherapy work

PANACEA PHARMACEUTICALS, LLC

4% stockholder

PUBLICATIONS:

Peer Review

1. Burks AW, Sampson HA, Buckley RH. Anaphylactic reactions following gammaglobulin administration in patients with hypogammaglubulinemia: detection of IgE antibodies to IgA. N Engl J Med 1986;314:560-564.

2. Burks AW. Manifestations of milk allergy in infancy. J Pediatr 1987;100:500.

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3. Burks AW, Sampson HA. Double-blind placebo-controlled trial of oral cromolyn sodium in children with egg hypersensitivity. J Allergy Clin Immunol 1988; 81:417-423.

4. Burks AW, Brooks JR, Sampson HA. Allergenicity of major component proteins of soybean determined by enzyme linked immunosorbent assay (ELISA) and immunoblotting in children with atopic dermatitis and positive soy challenges. J Allergy Clin Immunol 1988;81:1135-1142.

5. Burks AW, Mallory SB, Williams LW, Shirrell MA. Atopic dermatitis: Clinical relevance of food hypersensitivity reactions. J Pediatr 1988;113:447-451.

6. Keepen LD, Fasules JW, Burks AW, Gollin SM, Miller CH. Confirmation of autosomal dominant transmission of the DiGeorge malformation complex. J Pediatr 1988;113:506-508.

7. Burks AW, Williams LW, Steele RW. Chronic erythematous skin rash and thrush in a 16 month-old child. Ann Allergy 1988;60(1):25,61-63.

8. Beck SA, Burks AW, Woody RC. Auriculotemporal syndrome seen clinically as food allergy. Pediatr 1989;83(4):601-603.

9. Burks AW, Casteel HB, Brooks JR, Hardin J, Williams LW, Connaughton C: Enzyme linked immunosorbent assay and immunoblotting determination of antibody response to major component proteins of soybeans in patients with soy protein intolerance. J Pediatr Gastro Nutr 1989;8:195-203.

10. Jacobs RF, Tabor DR, Lary CH, Burks AW, Campbell GD. Elevated interleukin-1 release by human alveolar macrophages during the adult respiratory distress syndrome. Am Rev Respir Dis 1989;140:1686-1692.

11. Burks AW, Charlton R, Casey P, Poindexter A, Steele RW. Immune function in patients treated with phenytoin. J Child Neurol 1989;4:25-29.

12. Burks AW, Williams LW, Mallory SB, Shirrell MA, Williams C. Peanut protein as a major cause of adverse food reactions in patients with atopic dermatitis. Allergy Proc 1989;10:265-269.

13. Burks AW, Williams LW, Casteel HB, Fiedorek SC, Connaughton CA. Antibody response to milk proteins in patients with milk protein intolerance documented by challenge. J Allergy Clin Immunol 1990;85:921-927.

14. Sitz KV, Burks AW, Williams LW, Kemp SF, Steele RW. Confimation of X-linked hypogammaglobulinemia with isolated growth hormone deficiency as a disease entity. J Pediatr 1990;116:292-294.

15. Conley ME, Burks AW, Herrod HG, Puck JM. Molecular analysis of X-linked agammaglobulinemia with growth hormone deficiency. J Pediatr 1991;119:393-397.

16. Burks AW, Williams LW, Helm RM, Connaughton C, Cockrell G, O'Brien T. Identification of a major peanut allergen Ara h I, in patients with atopic dermatitis and positive peanut challenge. J Allergy Clin Immunol 1991;88:172-179.

17. Beck SA, Williams LW, Shirrell MA, Burks AW. Egg hypersensitivity and measles, mumps, rubella vaccine administration. Pediatr 1991;88:913-917.

18. Kletzel M, Beck SA, Elser J, Shock N, Burks W. Trimethoprim sulfamethoxasole (RMP/SMX) oral desensitization in HIV infected hemophiliacs with a previous history of hypersensitivity reactions. Am J Dis Child 1991;145:1128-1129.

000222

19. Bock SA, Burks AW, Sampson HA. Documentation of food hypersensitivity [letter], J Pediatr 1991;118:655-656.

20. Burks AW, Williams LW, Helm RM, Thresher W, Brooks JR, Sampson HA. Identification of soy protein allergens in patients with atopic dermatitis and positive soy challenges; determination of change in allergenicity after heating or enzyme digestion. Adv Exp Med Biol 1991;289-:295-307.

21. Keller K, Fiedorek SC, Casteel HB, Williams LW, Burks AW. X-lined hypogammaglobuinemia and Crohn's disease. Pediatr Asthma Allergy Immunol 1992;6:105-107.

22. Burks AW, Williams LW, Connaughton C, Cockrell G, O'Brien TJ, Helm RM. Identification and characterization of a second major peanut allergen, Ara h II, utilizing the sera of patients with atopic dermatitis and positive peanut challenge. J Allergy Clin Immunol 1992;90:962-969.

23. Burks AW, Williams LW, Thresher W, Connaughton C, Cockrell G, Helm RM. Allergenicity of peanut and soybean extracts altered by chemical or thermal denaturation in patients with atopic dermatitis and positive food challenges. J Allergy Clin Immunol 1992;90:889-897.

24. Burks AW, Williams LW, Wheeler JW, Wilson G. Atopic dermatitis and food hypersensitivity in children. Allergy Proc 1992;13:285-288.

25. Hankins CC, Noland PR, Burks AW, Connaughton C, Cockrell G, Metz CL. Effect of soy protein ingestion on total and specific IgG concentrations in neonatal porcine serum measured by enzyme-linked immunosorbent assay. J Anim Sci 1992;70:3096-3101.

26. Burks AW, Sampson HA. Diagnostic approaches to the patient with suspected food allergies. J Pediatr 1992;121(5pt.2):S64-71.

27. Helm RM, Burks AW, Williams LW, Brenner RJ. Identification of cockroach aeroallergens from living cultures of German or American cockroaches. Int Arch Allergy Appl Immunol 1993;101:359-363.

28. Helm RM, Cockrell G, Sharkey P, Brenner RJ, Burks AW. In vitro translation of RNA from the german cockroach (*Blattella germanica*). Mol Immunol 1993;30:1685-1688.

29. Burks AW, Cockrell G, Connaughton C, Helm RM. Epitope specificity and immunoaffinity purification of a major peanut allergen, Ara h I. J Allergy Clin Immunol 1994;93:743-750.

30. Burks AW, Cockrell G, Connaughton C, Guin J, Allen W, Helm Rm. The identification of peanut agglutinin and soybean trypsin inhibitor as minor legume allergens. Int. Arch Appl Allergy Immunol 1994;105:143-149.

31. Dorion BJ, Burks AW, Harbeck R, Williams LW, Trumble A, Helm RM, Leung DYM. The production of interferon-γ in response to a major penaut allergen, Ara h II, correlates with serum levels of IgE anti-Ara h II. J Allergy Clin Immunol 1994:93:93-99.

32. Helm RM, Brenner RJ, Williams LW, Burks AW. Isolation of the 36 kD German (*Blattella germanica*) cockroach allergen using fast protein liquid chromatography. Int Arch Allergy Immunol 1994;103:59-66.

33. Burks AW, Castell HB, Fiedorek SC, Williams LW, Pumphrey CL. Prospective oral food challenge study of two soybean protein isolates in patients with possible milk or soy protein entercolitis. Pediatr Allergy Immunol 1994;5:40-45.

34. Crespo JF, Pascual C, Ferrer A, Burks AW, Pena JMD, Esteban MM. Egg white specific IgE levels as a tolerance marker in the follow-up of egg allergy. Allergy Proc 1994;15:73-76.

35. Crespo JF, Pascual C, Burks AW, Helm RM, Esteban MM. Frequency of food allergy in a pediatric population from Spain. Pediatr Allergy Immunol 1995;6:39-43.

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118.Pomes A, Lewis K, Burks W, Bannon GA, Chapman MD, Helm RM. Allergen-Specific Monoclonal Antibodies Directed Against the Major Peanut Allergens Ara h 1 and Ara h 2. J Allergy Clin Immunol 2002;109:874, S286.

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121.Palmer GW, Dibbern DA, Burks W, Bannon GA, Bock SA, Dreskin SC. Relative Potency of the Major Peanut Allergens, Ara h 1 and Ara h 2, in a Functional Assay. J Allergy Clin Immunol 2002;109:918, S300.

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124.Scurlock AJ, Althage K, Christie L, Burks W, Jones S. Anaphylaxis Following Ingestion of Gummi Bears. J Allergy Clin Immunol 2002;109:952, S310.

INVITED PRESENTATIONS

National and International – (Selected)

European Society for Dermatological Research, "Food Allergy in Atopic Dermatitis of Children and Adults", Utrecht, The Netherlands, March 1990.
American Academy of Asthma, Allergy and Immunology Seminars, "Dietary Management of Infant Food Allergens"; "IGIV therapy in immunodeficiency disorders", 1990-1995.
International Life Sciences Food Allergy and the Lung Symposium, Orlando, Florida, "Food Additives and Respiratory Reactions", March 1992.
Adverse Reactions to Food in Infancy and Childhood Symposium, Madrid, Spain, "Diagnostics Approaches To The Patient With Suspected Food Allergies", April 1992.
Eastern Allergy Society, New Orleans, Louisiana, "Food Allergy And Atopic Dermatitis", April, 1992.
American Academy of Pediatrics Visiting Professor, Madigan Army Hospital, Tacoma, Washington, "Cornucopia Of Pediatric Allergy; Childhood Immunodeficiency Diseases; Food Allergies", September 1992.
University of Florida at Gainesville Visiting Professor, Gainesville, Florida, October 1992.
American College of Allergy and Immunology Workshop, American College of Allergy and Immunology Annual Meeting, "Adverse Food Reactions", November 1992.
Allergic Diseases Symposium, Vancouver, British Columbia, "Food Allergies", December, 1992.
Tulane University Visiting Professor, New Orleans, Louisiana, "Food Allergies", January 1993.
Food and Drug Administration Conference, Washington, D.C., "Diagnosis Of Food Allergies", January, 1993.
Ohio Society of Allergy & Immunology, Cleveland, Ohio, "Food Allergy - Natural History, Treatment And Diagnosis", April 1993.
Swineford Conference, Charlottesville, Virginia, "Legumes as Food Allergens", April 1993.
16th Annual Review Selected Topics in Pediatrics, New York, New York, "Diagnostic Approaches to the Child With Suspected Food Allergies", June 1993.
VIII International Food Allergy Symposium, Denver, Colorado, "Characterization of Food Allergens", July 1993.
Louisiana Allergy Society, New Orleans, Louisiana, "Atopic Dermatitis and Food Hypersensitivity", July 1993.
University of Alabama at Birmingham Grand Rounds, Birmingham, Alabama, "Trigger Factors of Atopic Dermatitis", August 1993.
Lloyd Crawford Symposium, Memphis, Tennessee, "Food Allergy, Diagnosis & Management of Adverse Food Reactions", September 1993.
Quebec Association Of Allergists & Immunologists, Montreal, Canada,"Office Management of Double-Blind Placebo-Controlled Food Challenges", November 1993.
American College of Allergy and Immunology Annual Meeting, Workshops "Meet the Professor", "Adverse Reactions to Foods - The Advanced Course, Treatment Approaches", November 1993.

International Food and Information Council, Anaheim, California, "Understanding Food Allergy", March 1994.
Florida Allergy Society, Orlando, Florida, "Anaphylactic Reactions to Foods", May 1994.
Royal Society of Canadian Physicians, Canadian Society of Allergy & Clinical Immunology, Toronto, Canada, "Atopic Dermatitis & Diet", September 1994.
Wisconsin Allergy Society, Madison, Wisconsin, "Update on Food Allergy". September 1994.
Collegium Internationale Allergologicum Symposium, Nantucket, Massachusetts "Isolation, Identification, and Characterization of Clones Encoding Antigens Responsible for Peanut Hypersensitivity", September 1994.
American Dietetic Association, Orlando, Florida, "Adverse Food Reactions with Special Emphasis on Food Allergy", October 1994.
Susan Dees Symposium, Duke University Medical School, Durham, NC, "Update on Food Allergen Identification", October 1994.
Advances in Pediatric Nutrition, Baltimore, MD, "Food Allergy: An Overview", November 1994.
Allergy Care in the "High Tech" Era, Squaw Valley, CA "Update on Food Allergy in the Biotechnology Era" January 1995.
American Academy of Asthma, Allergy and Immunology Seminar, New York, NY, "Immunologically-Mediated Adverse Food Reactions in the Gastrointestinal Tract", 1995.
Literature Review for American College of Allergy and Immunology Board Review Examination Course, Chicago, IL, "Mucosal Immunity, Immunologic GI Diseases, Food Allergy" April 1995.
Southwest Allergy Forum, New Orleans, LA, "Introduction to Food Allergy", April 1995.
European Academy of Allergy and Immunology, Madrid, Spain, "New Treatments in Food Allergy", June 1995.
American Academy of Pediatrics, San Francisco, CA, "Food Allergy", October 1995.
American College of Allergy, Asthma and Immunology, Dallas, Texas, "What's New in Food Allergy", November 1995.
The University of Vermont, Burlington, Vermont, "Asthma Update", February 1996.
Ehrlich Symposium, Bethesda, Maryland, "The Role of Major Allergens in Allergenic Products Standardization", March 1996.
American Academy of Allergy, Asthma and Immunology, New Orleans, Louisiana, "Dietary Management of Infant Food Allergens"; "Food Allergens for the Practicing Allergist", "Molecular Characterization of Peanut and Other Food Allergens", March 1996.
Asthma and Allergy Symposium, Cape Girardeau, Missouri, "Food Allergies", April 1996.
Central Pediatric Society of Mississippi, Jackson, Mississippi, "The Role of Food Allergy in Atopic Dermatitis and Anaphylaxis", August 1996.
AACC Rice Utilization Workshop, New Orleans, Louisiana, "Hypoallergenicity of Rice Protein", August 1996.
American College of Allergy, Asthma and Immunology. Boston, Massachusetts. "Adverse Reactions to Foods: State of the Art", November 1996.
American Academy of Allergy Asthma and Immunology, San Francisco, California, "Food Allergy in Infants: Dietary Management", "Practice Parameters for the Diagnosis and Management of Humoral Immunodeficiency", February 1997.
Milk Allergy Meeting. Chicago, Illinois, "Mechanism of Milk Hypersensitivity/Nature of Milk Allergens", February 1997.
Practical Pediatrics Symposium, Winston-Salem, North Carolina, "Asthma: An Update", "Anaphylaxis: A Changing Perspective", April 1997.
Grand Rounds, Birmingham, Alabama, "Diagnosis and Management of Food Allergens", April 1997.
Academic Societies' 1997 Annual Meeting, Washington, D.C., "Non-NIH Sources of Funding-Basic and Clinical Research", May 1997.
19th Annual Symposium, An Update in Allergy and Immunology, Louisville, Kentucky, "Current Approach to the Food Allergy Patients", May 1997.
Duke Children's Nutrition Symposium. Durham, North Carolina, "The Clinical Spectrum of Food Allergies in Infancy", June 1997.
Pennsylvania Allergy and Asthma Association, Harrisburg Pennsylvania, "Clinical Presentations of Food Allergic Disease and their Evaluation", June 1997.
106th Ross Conference on Pediatric Research, Palm Beach, FL, "Diagnosis and Natural History of Food Allergies", September 1997.

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The American Dietetic Association, Boston, MA, " Food Allergy: Diagnosis, Management Strategies and Future Trends", October 1997.
American College of Allergy, Asthma and Immunology, San Diego, CA, "Adverse Reactions to Foods and Food Additives", "Food Allergen-Specific T-cell Lines", November 1997.
American Academy of Allergy Asthma & Immunology, Postgraduate Symposium, Washington, D.C., "Food Induced Anaphylaxis", March 1998.
Texas Medical Association, Austin, TX, "Food Allergy Into the Next Century", April 1998.
Pediatric Academic Society, New Orleans, LA,"Anaphylaxis", May 1998.
San Diego Allergy Society, San Diego, CA, "Peanut Allergy", May 1998.
The Canadian Society of Allergy and Clinical Immunology, Toronto, Canada, "New Developments in Peanut Allergy, Can We Make Hypoallergenic Food?", September 1998.
American Academy of Pediatrics, San Francisco, CA, "Clinical Presentations of Anaphylaxis", October 1998.
Children's Hospital, Boston, MA, "Diagnosis and Management of Food Allergy", December 1999.
Presbyterian Hospital of Dallas Internal Medicine Grand Rounds, Dallas, TX "Food Allergy: Fact or Fiction", February 1999.
American Academy of Allergy, Asthma & Immunology, Allergy Press Conference, National Press Club, Washington, D.C., February 1999.
American Academy of Allergy, Asthma & Immunology 55th Annual Meeting, Orlando, FL, "Current Research of Food Antigens – How Does It Appear to Allergy Practice" and "Gastrointestinal Disease in Food Allergy", Feb./Mar. 1999.
Protein Technologies International, St. Louis, MO, "Soy Hypersensitivity", March 1999.
American Academy of Pediatrics, The Complete Course for Primary Care Providers, Orlando, FL, "Specific Anaphylaxis Syndromes", "Introduction to Allergic Diseases" and "Anti-Inflammatory Medication for Asthma (Steroids)", March 1999.
Visiting Professor, Tulane - LSU Grand Rounds, New Orleans, LA, "Peanut Anaphylaxis – Can We Prevent It?" and "Prospects for Peanut Allergy Immunotherapy", April 1999.
Sixth Annual Allergy Fellows Symposium, American Thoracic Society, San Diego, CA, "Food Allergies", April 1999.
Louisiana State University Medical Center, Shreveport, LA, "anaphylaxis in Children", and "Update: Food Allergy in Children", May 1999.
Alfred I. DuPont Hospital for Children, Philadelphia, PA, "Food Allergies", June 1999.
American Peanut Research and Education Society, Savannah, GA, "Peanut Allergies", July 1999.
Pan Am Congress of Digestive Diseases, Toronto, Canada,"The Molecular Basis of Food Antigenicity", and "Food Anaphylaxis", August 1999.
Pediatric Grand Rounds, LeBonheur Children's Medical Center, Memphis, TN, "Food-Induced Anaphylaxis – Can We Prevent It?", September 1999.
Wisconsin Allergy Society, Madison, WI, "Peanut Anaphylaxis: Prospects of Immunotherapy", October 1999.
Pittsburgh Allergy Society, Pittsburgh, PA, "Update on Diagnosis and Management of Food Allergy", October 1999.
Children's Hospital, Pittsburgh, PA, "Gastrointestinal Disease in Food Allergy" and "Prospects for Peanut Allergy Immunotherapy", October 1999.
American College of Allergy, Asthma, & Immunology, Chicago, IL, "Peanut Allergenicity", November 1999.
Anaphylaxis Foundation of Canada, Toronto, Canada, " Food Anaphylaxis", November 1999.
Anaphylaxis Network of Canada, Toronto, Canada, "Anaphylaxis: A Synopsis of Current Research", November 1999.
American Academy of Allergy, Asthma & Immunology 56th Annual Meeting, San Diego, CA, "Immunotherapy for the Next Millenium" and "What Makes Food Allergenic", March 2000.
Universidad Central Del Caribe, Bayamon, Puerto Rico, "Update on Diagnosis and Management of Food Allergy", April 2000.
University of Vermont/Fletcher Allen Health Care, Burlington, Vermont, "Peanut Anaphylaxis: Prospects of Immunotherapy" and "Atopic Dermatits", April 2000.
7th Annual Allergy Fellows Symposium, Toronto, Canada, "Food Allergies", May 2000.
Aspen Allergy Conference, Aspen, Colorado, "Epidemiology – Important Unanswered Questions", July 2000.

Pediatric Grand Rounds, Children's Hospital Medical Center, Cincinnati, OH, "Novel Immunotherapuetic Strategies for Allergic Disease", October 2000.

Cincinnati Allergy Society, Cincinnati, OH, "Update on Peanut Allergy", October 2000.

University of Iowa Hospitals and Clinics, Iowa City, Iowa, "Food Allergy", October 2000.

American Academy of Pediatrics Annual Meeting, Chicago, Illinois, "Asthma: Practice Guidelines'", October 2000.

American Academy of Pediatrics, Chicago, Illinois, The Allergy Generation-Caring for Children in an Allergenic World, "The Impact of Childhood Allergies: What Pediatricians Need to Know", October 2000.

National Center for Food Safety and Technology, Summit-Argo, Illinois, "Current Understanding of Food Allergy", December 2000.

Pediatric Allergy and Asthma: The Complete Course for Primary Care Providers, Orlando, Florida, "Introduction to Allergic Diseases/Allergy Testing", "Anaphylaxis", and "Asthma-Controller Medications/Steroids", March 2001.

8th International Symposium on Immunological, Chemical and Clinical Problems of Food Allergy, Venice, Italy, "Classic Specific Immunotherapy and New Perspectives in Specific Immunotherapy For Food Allergy", March 2001.

American Academy of Allergy, Asthma & Immunology 57th Annual Meeting, New Orleans, LA, "Food Allergy: Cause of Upper Airways Symptoms", "Case Studies of IgE-Mediated Diseases", and "Genetically-Modified Foods and Allergy: Benefits and Risks", March 2001.

8th Brazilian Congress of Allergy & Immunology in Pediatrics and 3rd Congress of SLAAIP, Rio de Janeiro, Brazil, "Food Allergy", and "Anaphylaxis", April 2001.

William E. Pierson Visitation Program, University of South Alabama, Mobile, Alabama, "Anaphylaxis: Can We Prevent It?", May 2001.

8th Annual Fellows Symposium, American Thoracic Society, San Francisco, California, "Food Allergies", May 2001.

British Society of Allergy & Clinical Immunology Annual Meeting, Nottingham, United Kingdom, "Peanut Allergy: Clinical Management, Prospects for Future Treatment", July 2001.

Anaphylaxis Campaign, Nottingham, United Kingdom, "Food Allergy – The American View", July 2001.

Grand Rounds, Children's Hospital of the King's Daughters, Norfolk, Virginia, "Novel Immunotherapeutic Strategies for Allergic Disease, and Resident Conference, "Update on Food Allergy", October 2001.

American Academy of Pediatrics, San Francisco, CA, "Food Allergy", October 2001.

Visiting Professor, Wright State University School of Medicine, Dayton, Ohio, Grand Rounds and Core Conference, "Food Allergy", and "Anaphylaxis", October 2001.

Western Ohio Pediatric Society, Dayton, Ohio, "Update on Peanut Allergy", October 2001.

Duke University, Durham, NC, "Anaphylaxis – Can We Prevent It?", December 2001.

5th Annual Workshop on Rhino-sinusitis, Quebec City, Canada, "Use of *Invitro* Testing in the Diagnosis and Management of Food Allergy", February 2002.

American Academy of Allergy, Asthma & Immunology 58th Annual Meeting, New York City, NY, "Allergens and the Allergic Individual", "The Evaluation of Food Allergy in Atopic Dermatitis", "Breast Feeding and Food Allergy", "Gastrointestinal Disease in Food Allergy", and "Genetically Modified Foods from the Allergist's Perspective: Benefits vs. Risks", March 2002.

Food Allergy and Anaphylaxis Network, Food Allergy: A Comprehensive Update, New York City, NY, "Skin Manifestations of Food Allergy", April, 2002.

Food Allergy and Anaphylaxis Network, 9th Annual Food Allergy Conference, Better Living with Food Allergy, Rosemont, IL, "Food Allergy Definitions and Diagnosis" and "The Year in Review: Current Issues in Food Allergies", April 2002.

9th Annual Allergy Fellows Symposium, Atlanta, GA, "Food Allergies", May 2002.

Food Allergy Research & Resource Program, University of Nebraska-Lincoln, 2nd International Roundtable workshop Thresholds for Allergenic Foods: How Much is Too Much?, West Palm Beach, FL, "Reproducibility of Reactions - Should Mild Reactions to Low Doses be Repeated for Confirmation?", May 2002.

Johns Hopkins Division of Clinical Immunology Weekly Clinical Conference, Baltimore, MD, "Peanut Allergens", May 2002.

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Indiana Allergy Society, Indianapolis, IN, "Update on Food Allergy for the Allergist", October 2002.
UT Southwestern Medical Center, Dallas, TX, "Life Threatening Allergic Reactions· Can They be Prevented?", October 2002.
Dallas/Fort Worth Allergy Society, Dallas, TX, "Food Allergies for the Practicing Allergist", October, 2002.
American College of Allergy, Asthma & Immunology Annual Meeting, San Antonio, TX, "What has Allergy Got to do With Atopic Dermatitis?", November 2002.

THE MOUNT SINAI SCHOOL OF MEDICINE
CURRICULUM VITAE

Name: HUGH A. SAMPSON, M.D.

Primary academic appointment: Department of Pediatrics

Citizenship: USA

Present academic rank: Professor of Pediatrics

Education:	High School	Hamburg Senior High School	1967 N.Y. Regents
	College	Hamilton College	1971 B.A.
	Medical School	State Univ. of NY at Buffalo	1975 M.D.
Professional Training and Academic Career:	Center for Immunology, SUNY at Buffalo 1973		Summer Grant
	Children's Memorial Hospital, Chicago		1975-78 Pediatric Residency
	Duke University Medical Center (Allergy Immunology-Pulmonary)		1978-80 Fellowship
Date and rank of faculty appointments:	Assistant Professor of Pediatrics Division of Allergy/Immunology Duke University		7/80-1/86
	Associate Professor of Pediatrics Duke University		2/86-7/86
	Associate Professor of Pediatrics Division of Allergy/Immunology Johns Hopkins University		8/86 -11/91
	Professor of Pediatrics		11/91 - 8/97
	Professor of Cellular & Molecular Medicine Johns Hopkins University		3/97 – 8/97
	Professor of Pediatrics Mount Sinai School of Medicine		8/97-present
Professional Appointments:	Assoc. Director - Duke University Clinical Research Unit		5/83-7/86
	Director - Johns Hopkins Pediatric Clinical Research Unit		7/88 – 8/97
	Immunologic Sciences Study Section		1/94-6/97
	American Board of Allergy & Immunology		2000-2005

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Hugh A. Sampson, M.D.
Curriculum Vitae

Board Certification:	American Board of Pediatrics	#24855	1980
	American Board of Allergy and Immunology (CBABIMABP)	#2243	1981

Medical licensure:	National Board of Medical Examiners	7/76 No. 151134
	Maryland	8/86 No. 034137
	New York	9/97 No. 208277

Honors/Awards:	Alpha Omega Alpha	1974
	Sigma Xi	1980
	Allergic Disease Academic Award, NIAID KO7-AI 00830	1986

Honorary Named Lectureships:	Julius Stone Amer. Dermatologic Society for Allergy & Immunology	9/85
	Jerome A. Glaser Amer. Academy Allergy & Immunology	2/87
	Richard Farr Lectureship Colorado Allergy Society	7/94
	William P. Buffum Oration Rhode Island Allergy Society	4/97
	William Pierson Lectureship Washington State Allergy Soc	9/98
	Warren Bierman Lectureship AAAAI	3/99

Professional Affiliations:

Societies:	Society for Pediatric Research	1985 - present
	American Academy of Allergy Asthma & Immunology	1981 - present
	Chairman - Cutaneous Allergy Committee	1985-1988
	Chairman - Scientific Exhibits & Workshop Committee	1985-1988
	Chairman - Interest Section on Dermatologic Diseases	1988-1990
	Vice-Chair – Interest Section of FDRA	1997-2002
	International Committee	1992-1999
	Chairman	1994-1996
	Adverse Reactions to Foods Committee	1987-1998
	Chairman	1997-1999
	Anaphylaxis Committee	1993-1998
	Eczema Committee	1992-1998
	Ethics Committee	1992-1996
	Research Grant Awards	1994-1996, '99-2003
	Executive Board	1997-2000
	Program Committee	2001-2002
	Research Subcommittee	2000-2002
	American Academy of Pediatrics	1988 – present
	Executive Committee - Sec on Allergy & Immunology	1992-2001
	Chairman	1994-1996
	American Association of Immunologists	1987- present

Editorial Appointments:	Journal Allergy and Clinical Immunology	1987-1992; 1998-
	Clinical and Experimental Allergy	1988 – 2000
	Clinical Reviews in Allergy	1989 - present
	Co-Editor - Journal Pediatric Allergy & Immunology	1989 – present
	Internat Arch Allergy Immunology	2001-2004

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Hugh A. Sampson, M.D.
Curriculum Vitae

Boards of Directors:

Allergy & Asthma Network/Mothers of Asthmatics	1988 - present
Eczema Association for Science and Education	1989 - present
International Life Sciences Institute; Board of Trustees	1990 - 2000
Medical Advisor - Allergy & Immunology Institute	1990 - 2000
Food Allergy & Anaphylaxis Network	1991- present
The Inflammatory Skin Disease Institute	2000- present

Major Academic Services:

Director, Pediatric Clinical Research Center Johns Hopkins Hospital	1988-1997
Child Health Research Center Advisory Committee	1991-1997
LCME Subcommittee on Research - Co-Chair	1990-1991
Advisory Committee for Training Program in Clinical Investigation	1992-1997
Professorial Promotions Committee	1996-1997
Chief, Division of Pediatric Allergy/Immunology	1997-present
Appointments & Promotions Committee	1998-present
Director, General Clinical Research Center Mount Sinai School of Medicine	1999-present
Dean's Research Council	2000 -present

Research Support:

NIH RO1-AI19116 "Role of Food Hypersensitivity in Atopic Dermatitis" Salary 50%
Principal Investigator - Hugh A. Sampson 8/82-7/86

NIH K07-AI00830 "Allergic Diseases Academic Award"
Principal Investigator - Hugh A. Sampson 9/86-7/88

Internat. Life Sci. Institute "Role of Food Hypersensitivity in Airway Hyperreactivity Salary 10%
Principal Investigator - Hugh A. Sampson 7/91-8/94

NIH DRR M01-RR-00052 "General Research Center, Pediatric Clinical Research Unit" Salary 50%
Principal Investigator - Michael Johns [$1,200,000/yr] 7/88-8/97

NIH R01-AI24439 "Mechanisms of Food Hypersensitivity in Atopic Dermatitis" Salary 40%
Principal Investigator - Hugh A. Sampson [$196,948/yr] 9/86-12/00

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Clarissa Sosin Allergy Research Foundation - "Immunomodulation of Peanut Hypersensitivity" Salary 15%
Principal Investigator - Hugh A. Sampson [$150,000/yr] 7/95-6/00

Tomich Family Fund - "Immunomodulation of Peanut Hypersensitivity" Salary 10%
Principal Investigator - Hugh A. Sampson [$100,000/yr] 7/95-6/98

NIH RO1 AI43668 "Immunomodulation of Food Allergy by DNA-Based Therapy" Salary 10%
Principal Investigator: Hugh A. Sampson [$189,482] 5/98-4/03

NIH DRR M01-RR-00071 "General Research Center,
General Clinical Research Unit" Salary 50%
Principal Investigator – Arthur Rubenstein [$4,500,000/yr] 3/99-2/05

NIH PO1 AI44236 "Immunologic Basis of Cow Milk-induced
Hypersensitivity." Salary 25%
Principal Investigator: Hugh A Sampson [$472,179] 9/99- 8/03

NIH RO1 AI 49570 "Mechanistic Analysis of Anti-IgE Therapy
of Asthma" Salary 5%
Principle Investigator: Rebecca Gruchalla [$250,000] 9/01-9/03

NIH R21 AT 00194 "Effects of Chinese Herbal Medicines on
Allergic Asthma" Salary 0%
Principle Investigator: Xiumin Li [$125,000] 6/01-6/03

N01-AI-25496 "Inner-city Asthma Consortium"
Salary 20% 1/03-12/07
Principle Investigator: William Busse [$1.2 million]

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Hugh A. Sampson, M.D.
Curriculum Vitae
Publications

Peer Reviewed Articles:

1. Rose NR, Milisauskas V and **Sampson HA**: Species-specific tissue antigens. III. Immunological relationships of enzymic antigens in various species. Clin Exp Immunol 20:359-70, 1975.

2. **Sampson HA** and Buckley RH: Human IgE synthesis in vitro: a reassessment. J Immunol 127:829-34, 1981.

3. Rich KC, **Sampson HA**, Edwards NL and Fox IH: Familial hypogammaglobulinemia with variable serum immunoglobulins: concordance with lymphocyte ecto-5'-nucleotidase deficiency. Am J Dis Child 135:795-98, 1981.

4. **Sampson HA**, Walchner A and Baker P: Recurrent pyogenic infections in individuals with absence of the second component of complement. J Clin Immunol 2:39-45, 1981.

5. LoGalbo PR, **Sampson HA** and Buckley RH: Symptomatic giardiasis in three patients with X-linked agammaglobulinemia. J Pediatr 101:78-80, 1982.

6. **Sampson HA**: Role of immediate food hypersensitivity in the pathogenesis of atopic dermatitis. J Allergy Clin Immunol 71:473-80, 1983.

7. **Sampson HA** and Albergo R: Comparison of results of skin tests, RAST, and double-blind placebo-controlled food challenges in children with atopic dermatitis. J Allergy Clin Immunol 74:26-33, 1984.

8. **Sampson HA** and Jolie PL: Increased plasma histamine concentrations after food challenges in children with atopic dermatitis. New Eng J Med 311:372-76, 1984.

9. Smego RA, DeVoe PW, **Sampson HA**, Perfect JR, Wilfert CM, Buckley RH: Candida meningitis in two children with severe combined immunodeficiency. J Pediatr 104:902-04, 1984.

10. Leiferman KM, Ackerman SJ, **Sampson HA**, Haugen HS, Venencie PY, Gleich GJ: Dermal deposition of eosinophil granule major basic protein in atopic dermatitis. New Eng J Med 313:282-85, 1985.

11. **Sampson HA** and McCaskill CM: Food hypersensitivity and atopic dermatitis: evaluation of 113 patients. J of Peds 107:669-75, 1985.

12. Burks AW, **Sampson HA**, Buckley RH: Anaphylactic reactions after gammaglobulin administration in patients with hypogammaglobulinemia. New Engl J Med 314:560-64, 1986.

13. Buckley RH, Schiff SE, **Sampson HA**, Schiff RI, Markert ML, Knutsen AP, Hershfield MS, Huang AT, Mickey GH, Ward FE: Development of immunity in human severe primary T cell deficiency follow haploidentical bone marrow stem cell transplantation. J Immunology 136:2398-2407, 1986.

14. **Sampson HA**: Differential diagnosis in adverse reactions to foods. J Allergy Clin Immunol 78:212-19, 1986.

15. Leinhas JL, McCaskill C, **Sampson HA**: Food Allergy Challenges: guidelines and implications. J Am Dietetic Assoc. 87:604-608, 1987.

16. Ruff ME, Pincus LG, **Sampson HA**: Phenytoin-induced immunoglobulin depression. Am J Dis Child 141:858-61, 1987.

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17. Schiffman SS, Buckley CE, **Sampson HA**, Massey EW, Baraniuk JN, Follett JV, Warwick ZS: Aspartame and Headache Susceptibility: A focused clinical study. New Engl J Med. 317:1181-85, 1987.

18. Gil KM, Keefe FJ, **Sampson HA**, McCaskill CM, Rodin J, Crisson JE: The relation of stress and family environment in atopic dermatitis symptoms in children. J Psychosomatic Res. 31:673-84, 1987.

19. Burks AW, **Sampson HA**: Double-blind placebo-controlled trial of oral cromolyn sodium in children with documented food hypersensitivity. J Allergy Clin Immunol. 81:417-23, 1988.

20. **Sampson HA**: IgE mediated food intolerance. J Allergy Clin Immunol. 81:495-504, 1988.

21. **Sampson HA**: Immunologically mediated food allergy: The importance of food challenge procedures. Ann Allergy 60:262-69, 1988.

22. **Sampson HA**: The role of food allergy and mediator release in atopic dermatitis. J Allergy Clin Immunol 81:635-45, 1988.

23. Gil KM, Keefe FJ, **Sampson HA**, McCaskill CM, Rodin J, Crisson JE: Direct observations of scratching behavior in children with atopic dermatitis. Behavior Therapy 19:213-27, 1988.

24. Burks AW, Brooks JR, **Sampson HA**: Allergenicity of major component proteins of soybean determined by enzyme linked immunosorbent assay and immunoblotting. J Allergy Clin Immunol. 81:1135-42, 1988.

25. **Sampson HA**: Comparative study of commercial food antigen extracts for the diagnosis of food hypersensitivity. J Allergy Clin Immunol 82:718-26, 1988.

26. Casale TB, **Sampson HA**, Hanifin J, Kaplan AP, et al: Guide to Physical Urticarias. J Allergy Clin Immunol. 82:758-63, 1988.

27. Bock SA, **Sampson HA**, Atkins FM, Zeiger RS, Lehrer S, Sachs M, Bush RK, Metcalfe DD: Double-blind placebo-controlled food challenge (DBPCFC) as an office procedure: A manual. J Allergy Clin Immunol 82:986-997, 1988.

28. Broadbent JB, **Sampson HA**: Cross-allergenicity in the legume botanical family in children with food hypersensitivity. J Allergy Clin Immunol 83:435-440, 1989.

29. Turner CR, Darowski MJ, **Sampson HA**, Spannhake EW, Hirshman CA: Dermal mast cell releasibility and end-organ responsiveness in atopic and non-atopic dogs. J Allergy Clin Immunol. 83:643-48, 1989.

30. Singer KH, Tuck DT, **Sampson HA**, Hall RP. Epidermal keratinocytes express the adhesion molecule intercellular adhesion molecule-1 (ICAM-1) in inflammatory dermatoses. J Invest Dermatol 92:746-750, 1989.

31. **Sampson HA**, Scanlon S: Natural history of food hypersensitivity in children with atopic dermatitis. J. Pediatr 115:23-27,1989.

32. **Sampson HA**, Broadbent KR, Bernhisel-Broadbent J: "Spontaneous" basophil histamine release and histamine releasing factor in patients with atopic dermatitis and food hypersensitivity. New Engl J Med. 321:228-232, 1989.

33. Bernhisel-Broadbent J, Taylor SL, **Sampson HA**: Cross-allergenicity in the Legume botanical family in children with food hypersensitivity. II. Laboratory correlates. J Allergy Clin Immunol. 84:701-709, 1989.

34. **Sampson HA**: Food Allergy. J Allergy Clin Immunol 84:1062-1067, 1989. [redacted]

35. Wood RA, Schuberth KC, and **Sampson HA**: Value of a multi-antigen radioallergosorbent test in diagnosing atopic disease in children. J. Pediatr. 117:882-885, 1990.

36. Bernhisel-Broadbent J, Yolken RH, **Sampson HA**: Allergenicity of orally administered immunoglobulin preparations in food allergic children. Pediatrics 87:208-214, 1991.

37. Gern JA, Yang E, Evrard HM, **Sampson HA**: Allergic reactions to milk-contaminated "Non-Dairy" products. New Engl J Med. 324:976-979, 1991.

38. **Sampson HA**, Bernhisel-Broadbent J, Yang E, Scanlon SM: Safety of casein hydrolysate formula in children with cow milk allergy. J Pediatr. 118:520-525, 1991.

39. James JM, Cooke SK, Barnett A, **Sampson HA**: Anaphylactic reactions to a psyllium-containing cereal. J Allergy Clin Immunol. 88:402-408, 1991.

40. Kleinman RE, Bahna S, Powell GF, **Sampson HA**: Use of infant formulas in infants with cow milk allergy: A review and recommendations. Pediatr Allergy and Immunol 2:146-155,1991.

41. Bernhisel-Broadbent J, Scanlon SM, **Sampson HA**: Fish hypersensitivity I: in vitro and oral challenge results in fish allergic patients. J Allergy Clin Immunol 89:730-737,1992.

42. Fasano MB, Wood RA, Cooke SK, **Sampson HA**: Egg hypersensitivity and adverse reactions to measles, mumps, and rubella vaccine (MMR) J of Pediatr 120: 878-881, 1992.

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44. **Sampson HA**, James JM, Bernhisel-Broadbent J: Safety of amino-acid derived infant formula in cow's milk allergic children. Pediatr 90:463-465, 1992.

45. James JM, **Sampson HA**: Immunologic changes associated with the development of tolerance in children with cow's milk allergy. J Pediatr 121:371-377, 1992.

46. Bernhisel-Broadbent J, Strousse D, **Sampson HA**: Fish hypersensitivity II: clinical relevance of altered fish allergenicity secondary to various preparation methods. J Allergy Clin Immunol 90:622-629, 1992.

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allergens. J Clin Invest 92:1374-1380, 1993.

52. Kelso JM, **Sampson HA**: Food protein-induced enterocolitis to casein hydrolysate formulas. J Allergy Clin Immunol 92:909-910, 1993.

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57. Abernathy-Carver KJ, **Sampson HA**, Picker LJ, Leung DYM: Milk-induced eczema is associated with the expansion of T cells expressing cutaneous lymphocyte antigen. J Clin Invest 95:913-918, 1995.

58. James JM, Burks AW, **Sampson HA**: Safe administration of measles vaccines in egg-allergic patients: an update. New Engl J Med 332:1262-1266, 1995.

59. Jones SM, Magnolfi CF, Cooke SK, **Sampson HA**: Immunologic cross-reactivity among cereal grains and grasses in children with food hypersensitivity. J Allergy Clin Immun 96:341-351, 1995.

60. Dahl RE, Bernhisel-Broadbent J, Scanlon-Holdford S, **Sampson HA**, Lupo M: Sleep disturbances in children with atopic dermatitis. Arch Pediatr & Adolesc 149:856-860, 1995.

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62. James JM, Eggleston PA, **Sampson HA**: Airway reactivity changes in food-allergic, asthmatic children undergoing double-blind placebo-controlled food challenges. Am J Respir Crit Care Med 153:597-603, 1996.

63. Sicherer SH, **Sampson HA**: Auriculotemporal syndrome: a masquerader of food allergy. J Allergy Clin Immunol 97: 851-852, 1996.

64. Eigenmann PA, Huang SK, **Sampson HA**: Characterization of ovomucoid-specific T-cell lines and clones from egg allergic subjects. Pediatr Allergy & Immunol 7:12-21, 1996.

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Immunol 99:293-300, 1997.

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69. Cooke SK, **Sampson HA**: Allergenic properties of ovomucoid in man. J Immunol 159:2026-2032, 1997.

70. Groeben H, Linderman KS, Davis FM, Cheng WI, **Sampson HA**, Hirshman CA: Dermal and airway responses to monoclonal antibodies specific for canine IgE. Vet Immunol Immunopathol 58:209-218, 1997.

71. **Sampson HA**, Ho DG: Relationship between food-specific IgE concentrations and the risk of positive food challenges in children & adolescents. J Aller Clin Immunol 100:444-451,1997.

72. Eigenmann PA, Sicherer SH, Borkowski TA, Cohen BA, **Sampson HA**: Prevalence of IgE-mediated food allergy among children with atopic dermatitis. Pediatr 101:e8, 1998.

73. Hoffman KM, **Sampson HA**: Serum specific-IgE antibodies to peptides detected in a casein hydrolysate formula. Pediatr Allergy Immunol 8:185-189, 1998.

74. Sicherer SH, Bollinger ME, Hershfield MS, **Sampson HA**, Lederman HM: Food allergy in a patient with adenosine deaminase deficiency (ADA) undergoing enzyme replacement with polyethylene glycol(PEG)-modified ADA. J Allergy Clin Immunol 101:61-62, 1998.

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76. Sicherer SH, Burks AW, **Sampson HA**: Clinical features of acute allergic reactions to peanut and tree nuts in children. Pediatrics 102:e6, 1998.

77. Sicherer SH, Eigenmann PA, **Sampson HA**: Features of food protein-induced enterocolitis. J Pediatr 133:214-219, 1998.

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79. James JM, Zeiger R, Lester MR, Fasano MB, Gern JE, Mansfield L, Schwartz H, **Sampson HA**, Windom H, Machtinger S, Lensing S: Safe administration of influenza vaccine to patients with egg allergy. J Pediatr 133:624-628, 1998.

80. Eigenmann PA, **Sampson HA**: Interpreting skin prick tests in the evaluation of food allergy in children. Pediatr Allergy Immunol 9:186-191, 1998.

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83. Rabjohn P, Helm EM, Stanley JS, West CM, **Sampson HA**, Burks AW, Bannon GA: Molecular cloning and

epitope analysis of the peanut allergen, Ara h3. J Clin Invest 103:535-542 1999.

84. Sicherer SH, **Sampson HA**: Cow's milk protein-specific IgE concentrations in two age groups of milk-allergic children and in children achieving clinical tolerance. Clin & Exper. Allergy 29:507-512, 1999.

85. Sicherer SH, Munoz-Furlong A, Burks AW, **Sampson HA**. Prevalence of peanut and tree nut allergy in the United States of America. J Allergy Clin Immunol 103:559-562, 1999.

86. Zeiger RS, **Sampson HA**, Bock SA, Burks AW, Harden K, Noone S, Martin D, Leung S, Wilson G: Soy allergy in infants and children with IgE-mediated cow milk allergy. J Pediatr 134:614-622, 1999.

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Re: GRAS notification for Ice Structuring Protein (GRN No. 117)
Information responding to FDA requests

Dear Dr. Fasano:

This follows up on a letter dated February 5, 2003 from Nancy Schnell of Unilever/Good Humor-Breyers, providing responses to certain FDA questions relating to the above-referenced GRAS notification. In her letter, Ms. Schnell noted that, because FDA wished to obtain the views of the Expert Panel on the response to Question 2 (having to do with the issue of a food challenge study), the response to that question includes a signature page for the Panel members. Ms. Schnell indicated that a signed copy of the response would subsequently be provided to FDA.

At this time, we have obtained the signatures of the Expert Panel members, and also of the Principal Investigator (Dr. Bindslev-Jensen), on the response to Question 2. A signed copy (original and two copies) is enclosed.

Sincerely,

Daniel R. Dwyer

enclosures

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ISSUE PAPER 2: Determination of the Reactivity of a Population of Highly Fish-Allergic Individuals to ISP Type III: The Issue of Food Challenges

January 13, 2003

Issue: Is a food challenge in fish-allergic individuals necessary to ascertain that ISP Type III preparation will not constitute a risk to the fish-allergic population?

FDA Question: Provide an extended, formal discussion of the reasoning and evidence to support a conclusion that all relevant safety issues for the fish allergic population have been addressed without doing a food challenge study in a fish allergic population (in particular, in the individuals who reacted positively in RAST and skin prick testing in Phase I and Phase II testing).

Summary of Response:

- As a general principle, it is well accepted that, in evaluating fish allergy, skin prick tests and RASTs have excellent negative predictive values, but poor positive predictive values. If a substance containing a protein originating in fish produces a negative skin prick test (or RAST) response in a test group of individuals highly allergic to fish, this demonstrates with a very high degree of certainty that the substance will not provoke allergic reactions in the fish-allergic population.

- Given the negative skin prick test and RAST responses to ISP type III in our study, a food challenge is not necessary. It is not likely to provide additional information necessary to reach the conclusion that a substance containing a protein originating in fish will not provoke allergic reactions in the fish-allergic population.

- Our Expert Panel carefully reviewed all of the available data on ISP Type III preparation, the yeast control, and pure ISP Type III (i.e., the ice structuring protein separate from the preparation), and concluded that ISP Type III preparation does not present a risk of provoking reactions in the fish allergic population.

    - The Expert Panel noted that (1) no subject reacted to pure ISP Type III, and (2) all subjects who reacted to ISP Type III preparation also reacted to yeast protein.

    - The Expert Panel concluded that the positive skin prick test and RAST responses observed in some subjects were due to the yeast protein in the ISP Type III preparation and not to the ISP Type III protein. As discussed in a separate paper, responses to yeast protein are not clinically significant.

[redacted]

- Our Expert Panel and also Prof. Carsten Bindslev-Jensen, a pre-eminent expert on fish allergy and food challenges, have unanimously advised us that, in light of this conclusion, it is not scientifically necessary to conduct a food challenge study in a fish allergic population or in any subjects who responded positively to ISP Type III preparation. (As noted above, all positive responses in our experiments are attributable to yeast protein.)

Response:

In evaluating this issue, Unilever has relied on the advice of a panel of independent experts. As explained in our GRAS notification, this panel consists of experts on food allergy (Wesley Burks, M.D., Hugh A. Sampson, M.D., and Steve L. Taylor, Ph.D.) and experts on food safety (Joseph F. Borzelleca, Ph.D., Walter H. Glinsmann, M.D., and Michael W. Pariza, Ph.D.). These individuals are recognized worldwide as pre-eminent in their fields.

We have also relied on the advice of other experts on food allergy, particularly the investigator who conducted our allergenicity testing, Prof. Carsten Bindslev-Jensen of Odense University Hospital in Denmark. Prof. Bindslev-Jensen is one of the world's foremost experts on fish allergy and on food challenges. Although Prof. Bindslev-Jensen is not a member of our Expert Panel, we have consulted with him on the issues discussed in this paper.

The signatures of the Expert Panel members and of Prof. Bindslev-Jensen at the end of this paper indicate that they have reviewed and agree with the views expressed in this paper.

In the paragraphs below we will first discuss the general principles that are used in assessing whether a substance is likely to provoke reactions in a specific allergic population, as elucidated in the scientific literature and by our Expert Panel. We will then discuss the application of those general principles to ISP Type III preparation and the conclusions drawn. In each of these discussions, we will address the potential need for a food challenge study.

(a) General Principles in Assessing whether a protein is Likely to be Allergenic in a Population allergic to the organism in which the protein is normally found.

When a protein is expressed by an organism (such as yeast) that is different from the organism in which the protein is found in nature (such as fish), then it is necessary to assess the risk that the protein poses to individuals who are allergic to the organism that is the natural source of the protein. For this purpose, generally accepted international guidelines specify the use of serological tests (RAST, basophil histamine release, immunoblotting) and skin prick tests (FAO-WHO 2001, Codex Alimentarius Commission 2002). These tests are conducted in a sufficient number of individuals sensitized to the organism of interest (such as fish) to provide the desired statistical degree of assurance of lack of reactivity in the relevant allergic population. It is accepted that, in diagnosing fish allergy, skin prick tests, and to a lesser extent RASTs, have excellent negative predictive value, but poor positive predictive value. This means that when a substance containing a protein(s) originating from fish produces a

negative skin prick test (or RAST) response, then this demonstrates with a very high degree of certainty that the substance will not provoke reactions in a fish-allergic population. Indeed, the skin prick test and RAST with fish allergens are likely to be positive in a proportion of individuals who are not allergic, so they provide a particularly rigorous assessment of a substance's ability to provoke reactions (Bock, 1996; May, 1976; Bock et al., 1978; Sampson, 1988; Sampson, 2001).

The purpose of our study was to determine the risk that a fish allergic individual would react to ISP Type III. While some of the methodology is the same as that used for diagnosis of food allergy, the manner in which the data are interpreted is different. The purpose of diagnosis in the field of food allergy is to enable the physician to provide guidance to the patient regarding which foods are safe to eat. As discussed above, skin prick tests and RASTs often over-predict clinical reactivity; therefore a positive result is not definitive in diagnosis and, in most instances, food challenges are necessary for a definitive diagnosis. In contrast, when assessing the risk that a substance will provoke a reaction in an allergic population (for instance, the risk that ISP Type III might provoke a reaction in individuals allergic to fish), negative skin prick tests and RAST are essentially comparable to food challenges and provide all the information needed to make a judgement.

In short, in assessing the risk that a substance will provoke a reaction in an allergic population, the occurrence of consistent negative skin prick and RAST results is conclusive, so long as other available data, such as immunoblotting and basophil histamine release, are also consistent. Given all of these data, it is not necessary to conduct a food challenge study because such a study would be unlikely to provide useful new data.

(b) Applying these General Principles to ISP Type III Preparation Leads to a Conclusion that the Preparation Does Not Present a Risk of Provoking Reactions in Fish-allergic Individuals

In evaluating ISP Type III preparation, one of the primary safety issues is the risk to the fish-allergic population (because ISP Type III is identical to a protein found in fish). This association with fish has permitted an investigation of the allergenic risk of ISP Type III preparation to be undertaken with a high degree of confidence, because (1) the extensive experience of allergy experts with fish allergy is directly relevant to the analysis of ISP Type III preparation, (2) the wealth of information available on the types of allergenic proteins in fish allergy can be used to assess whether ISP Type III protein is likely to be an allergen, and (3) an adequate number of subjects with documented severe fish allergy was available to enable a statistically meaningful study to be designed. As a result, we can be confident that any conclusions drawn about ISP Type III preparation are robust and have appropriate statistical power.

In evaluating the risk to fish-allergic individuals from ISP Type III preparation, our Expert Panel first analyzed our "Phase I" serological data (RAST and RAST inhibition, and basophil histamine release). The Panel agreed with a conclusion that the substance is unlikely to pose

a risk to fish-allergic individuals, but recommended confirmatory "Phase II" testing consisting of skin prick tests, as well as additional RAST and immunoblotting studies.[1]

In analyzing the data on ISP Type III preparation, our Expert Panel considered the following to be critical points:

- ISP Type III preparation (the product to be marketed) contains ISP Type III in solution with salts and yeast proteins. Accordingly, skin prick tests and RASTs were conducted on ISP Type III preparation (which includes yeast protein) and on pure ISP Type III (which does not include yeast protein).[2] These same tests were also conducted on a yeast control that consists of the yeast protein component of ISP Type III preparation.[3]

- In skin prick tests, four (of 22) subjects reacted to ISP Type III preparation (which includes yeast protein) and also reacted to the yeast control (which includes no ISP Type III protein).[4] (Table 1.)

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

These tests indicated that the positive reactions were attributable solely to yeast protein. The RAST results were similar:

- Positive RAST results to ISP Type III preparation (which includes yeast protein) and to the yeast control (which includes no ISP Type III protein) were obtained in eight (of 22) subjects[5] (Table 2). These eight subjects included three of the four subjects who reacted positively in skin prick tests. A summary of all subjects with positive reactions is provided in Table 3.

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

Based on these results, the Expert Panel concluded that the reactions to ISP Type III preparation that were observed are attributable solely to yeast protein, because:

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[1] The data discussed in this paragraph are described in detail in section IV.A of our GRAS notification. The Expert Panel considered all of the tests to be methodologically sound.

[2] The ISP Type III proteins in these different test substances are immunochemically equivalent, as discussed in an accompanying paper.

[3] The yeast control was produced by growing the untransformed yeast strain and then processing it in exactly the same way as the yeast producing the ISP Type III. The yeast control therefore contained only the components originating from the yeast, and no components associated with the expression of the ISP Type III gene.

[4] In the skin prick tests, the magnitude of the response with the yeast control was always sufficient to account for the observed response with ISP Type III preparation.

[5] In the RAST tests, the amount of IgE binding to the yeast control was always sufficient to account for the binding observed with ISP Type III preparation.

(1) skin prick test and RAST results consistently show that all subjects who reacted to ISP Type III preparation also reacted to the yeast control, and

(2) none of the subjects who reacted to ISP Type III preparation reacted to pure ISP Type III.

The Expert Panel was confident that the observed reactions to ISP Type III preparation do not indicate reactivity to ISP Type III protein, because:

(1) skin prick test and RAST results consistently show that all subjects reacted negatively to pure ISP Type III, and

(2) other data, namely basophil histamine release and immunoblotting, corroborate the skin prick test and RAST data.

As a result, the Expert Panel concluded that the scientific evidence presented, including consistent skin prick test and RAST results, as well as extensive and consistent corroborative data, demonstrates with a very high degree of certainty that ISP Type III preparation does not present a risk of provoking reactions in the fish allergic population.

- The Expert Panel noted, in particular, that no subjects reacted to pure ISP Type III.
- The Expert Panel concluded that the positive skin prick test and RAST responses observed in some subjects were due to the yeast protein in the ISP Type III preparation and not to the ISP Type III. As discussed in a separate paper, responses to yeast protein are not clinically significant.

Because all reactions to pure ISP Type III were negative, and because all reactions to ISP Type III preparation were well understood to be reactions to yeast protein, the Expert Panel did not recommend (or even raise the issue of) a food challenge study in a fish allergic population or in any subjects who responded positively to ISP Type III preparation.

In response to FDA's inquiry, our Expert Panel and Prof. Bindslev-Jensen have fully re-examined this issue. They have unanimously advised us that no further testing is necessary to demonstrate the safety of ISP Type III in fish-allergic individuals. They have also reiterated that a food challenge study in fish-allergic individuals would not be necessary because, as discussed above, negative skin prick test and RAST results are excellent indices of lack of an allergic response to the protein.

[redacted]

Accordingly, based on the advice of our Expert Panel, Unilever concludes that a food challenge study is not necessary to assess the risk of fish allergenicity from ISP Type III preparation.

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Table 1. Summary data for subjects with positive skin prick reactions, showing that positive reactions are attributable to the yeast protein and not to ISP Type III protein.

Subject	ISP Type III preparation (mg/ml)				Yeast fermentation control (mg/ml)				ISP Type III pure (mg/ml)			
	5	1	0.1	0.01	3	0.87	0.087	0.0087	5.0	1.0	0.1	0.01
1												
2												
4												
7												
9												
11	7.5	4.5	4	2.5	5	2.5	2	1	0	0	0	0
12												
13												
15												
16												
17												
18												
19	4	0	0	0	5	3	0	0	0	0	0	0
20												
21												
22												
23												
26												
27	4.5	3	0	0	4.5	2.5	0	0	0	0	0	0
31	7	6	6	4	6	4.5	0	0	0	0	0	0
32												
33												

Table 2. Summary data for subjects with positive RAST reactions, showing that positive reactions are attributable to the yeast protein and not to ISP Type III protein.

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

Quality control parameters for RAST assays

* Negative control: 33cpm; total activity added: 21,000

**Negative control: 71cpm; total activity added: 21,893

Criteria for positivity:

- >100 cpm or 2 x Blank (66 cpm for *assay; 142 for **assay) (whichever is greatest)
- >100 cpm or mean of blank + 2SDs (whichever is greatest)

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Table 3. Summary of all subjects with positive reactions, showing that such reactions are attributable to the yeast protein and not to ISP Type III protein.

Subject	Skin prick tests			RAST results		
	ISP preparation	Yeast control	Pure ISP	ISP preparation	Yeast control	Pure ISP
11	+	+	-	+	+	-
12	-	-		-	-	-
13	-	-		+	+	-
16	-	-		+	+	-
17	-	-		+	+	-
19	+	+	-	+	+	-
23	-	-		-	-	-
27	+	+	-	-		
31	+	+	-	+	+	-

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