APPENDIX 1. GENETIC MODIFICATION INFORMATION

(1) EFFECT OF THE GENETIC MODIFICATION ON THE PROPERTIES OF THE HOST MICROORGANISM

The parent microorganism used for the production of the ISP is a strain of baker's yeast, *Saccharomyces cerevisiae*, which has a long tradition of use in food fermentation in the EU.

The host strain is a derivative of the *Saccharomyces cerevisia*e strain CEN.PK (Entian KD, Koetter P (1998) Yeast Mutant and Plasmid Collections. Methods in Microbiology 26:431-449). This strain was developed specifically for use in metabolic analysis studies by breeding of laboratory yeast to generate an isogenic series of strains. Although this strain was not that used in the systematic sequencing project, it shares ancestry with this strain (S288C) but some genes are absent (P. Daran-Lapujade et al, 2003. Comparative genotyping of the *Saccharomyces cerevisiae* laboratory strains S288C and CEN.PK113-7D using oligonucleotide microarrays FEMS Yeast Research, 4: 259). A recently published more detailed study of one chromosome of CEN.PK shows that the large areas of the sequence are identical to the reference strain (D. Gresham et al, 2003. Genome-wide Detection of Polymorphisms at Nucleotide Resolution with a Single DNA Microarray, Published online 9 March 2006; 10.1126/science.1123726). The host strain has also been the subject of extensive functional analysis in the context of the European Union programme EUROFAN details of which are available at http://mips.gsf.de/proj/eurofan/eurofan_1.html.

To prepare the production strain a synthetic gene coding for ISP type III HPLC 12 originating from ocean pout was created for insertion into the yeast using the known amino acid sequence of the ISP type III HPLC 12 (Hew CL, Wang NC, Joshi S, Fletcher GL, Scott GK, Hayes PH, Buettner B, Davies PL. 1988). Multiple genes provide the basis for antifreeze protein diversity and dosage in the ocean pout, *Macrozoarces americanus.* J Biol Chem 263(24):12049-55) 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 efficiently. The use of a synthetic gene based on the actual amino acid sequence ensures efficient production of a nature-identical (in terms of amino acid sequence) protein and also ensures that no other genes from the host can be accidentally incorporated into the production organism, as no fish genetic material is actually transferred.

The synthetic gene coding for the amino acid sequence of ISP Type III HPLC 12 originating from ocean pout was inserted into a *Saccharomyces cerevisiae* CEN.PK strain (van Dijken *et al*, (2000) An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. Enzyme Microb Technol.26(9-10):706-714.) This was done by transforming the

yeast with a multi copy rDNA integration vector carrying an expression cassette for the *Macrozoarces americanus* HPLC12 ice structuring protein (Figure 1).

The main components of the expression cassette are: (1) a *S. cerevisiae GAL7* promoter (galactose inducible) which allows production to be turned on, (2) a *S. cerevisiae TDH3* leader sequence to improve protein synthesis, (3) a *S. cerevisiae* invertase (*SUC2*) signal sequence to ensure secretion of the protein into the medium, and (4) the synthetic ISP Type III HPLC 12 gene which was designed to ensure production of the correct amino acid sequence from the yeast. This marker-free expression cassette was introduced in to the host strain by the lithium chloride transformation method as described by Gietz and Woods (Yeast Transformation by the LiAc/SS Carrier DNA/PEG Method. *Methods in Molecular Biology*:313:107-120 (2005)). The final designation of the yeast production strain is strain CENPK338 containing multi-copy integration fragment of pUR3993 integrated at the rDNA locus.

The copy number of the expression cassette and the integration site was determined by Southern blotting and the integration site and the absence of the ampicillin selectable marker were confirmed further by polymerase chain reaction (PCR).

The resulting modified yeast contains no antibiotic resistance markers, no bacterial or fish DNA and the expression cassette (Figure 1 overleaf) is integrated directly into the yeast genome to form a stable multi-copy transformant.

The yeast strain used for the production of ISP type III preparation has been classified as 1 AB (Council Directive 90/219/EEC of 23 April 1990 on the contained use of genetically modified micro-organisms) by the Netherlands ministry of the environment in 2002 with permission granted for large scale fermentation (Appendix 7).The host yeast strain has also been used since 2003 for commercial production (60m^3 fermentation scale) of the ISP for use in our non-European ice cream business.

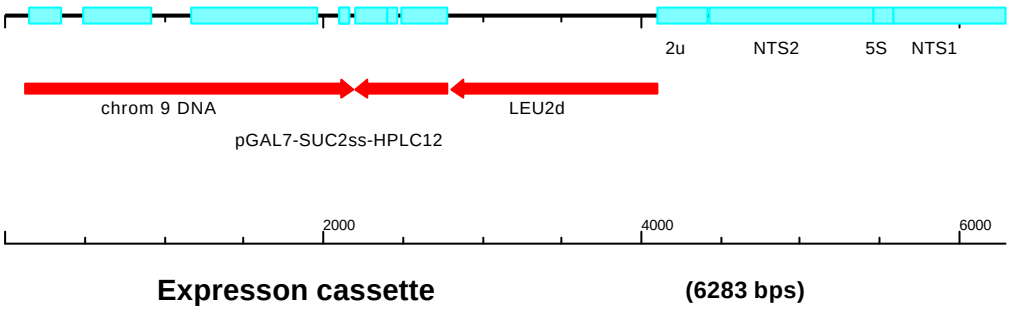


Figure 1: Integrated marker-free expression cassette

Key:
1-126 NTS1 – *Saccharomyces cerevisiae* rDNA non transcribed spacer
127-2186 *Saccharomyces cerevisiae* chromosme IX DNA
- 144-348 partial ORF= hypothetical protein
- 485-916 RNAse P subunit
- 1165-1959 weak similarity. to glucosidase, exo sialidase, mucins
- 2103-2165 questionable ORF
- 2165-2096 transcriptional activator of sulphur a.a. metabolism

2397-2197- Synthetic gene encoding ice structuring protein HPLC12
2457-2398 ISS - *Saccharomyces cerevisiae SUC2* I(Invertase) signal sequence
2775-2486 Pgal7 - *Saccharomyces cerevisiae GAL7* promoter (synthetic)
4009-2801 LEU2d - *Saccharomyces cerevisiae LEU2d*
4100-4413 2u - *Saccharomyces cerevisiae* 2 plasmid fragment (non functional)
4420-5460 NTS2 - *Saccharomyces cerevisiae* rDNA non transcribed spacer
55461--5581 5S - *Saccharomyces cerevisiae* rDNA 5S RNA
5582-6238 NTS1 - *Saccharomyces cerevisiae* rDNA non transcribed spacer

(2) GENETIC STABILITY OF THE GM SACCHAROMYCES CEREVISIAE

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

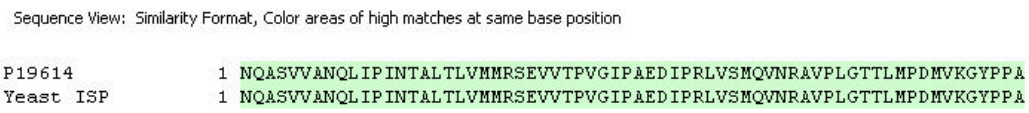
- Plating cells on selective and non-selective media revealed the same amount of viable cells.
- Inductive growth (after 70 generations) showed identical expression levels of ISP Type III HPLC 12 when tested in shake flasks.
- PCR analysis on whole yeast cells (chromosomal DNA as template) demonstrated 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.

(3) SPECIFICITY OF EXPRESSION OF NOVEL GENETIC MATERIAL

(i) The ISP expression cassette includes a pGAL7 promoter (galactose induction) which ensures that expression of the gene is repressed during growth on media containing more than 0.5 % glucose and is only highly expressed when the yeast is grown in the presence of galactose.

(ii) Sequence comparison of Ocean pout (*Macrozoarces americanus*) ice structuring protein HPLC12 from Genbank file P19614 with the amino acid sequence of ISP derived from yeast transformed with the ISP expression cassette shows that the two proteins are identical.



(iii) The ISP expression cassette includes only yeast DNA. It contains no antibiotic markers

(4) TRANSFER OF GENETIC MATERIAL FROM GM MICRORGANISMS

The ISP preparation contains no detectable DNA contamination as evidenced by PCR analysis:

The evidence for ISP DNA contamination analysis was performed using iCycler realtime PCR analysis for the presence of a 184 bp region of the ISP Type III synthetic gene encoding the HPLC-12 variant linked to the invertase signal sequence. Specificity of the PCR product was quantitated by melt curve analysis.

A dilution series of 1 nanogram to 0.01 femtograms of the ISP plasmid was spiked in duplicate into a solution of the ISP protein (1 mg/ml concentration). This indicated a linear detection range down to 1 femtogram of the ISP plasmid. Analysis of lyophylised ISP extract was performed in duplicate and at various extract concentrations. Based on this analysis no detectable ISP DNA contamination above the detection limit of 2×10^{-10} g ISP plasmid DNA /g of lyophylised ISP protein preparation was observed.

(ii) The ISP expression cassette is integrated directly into the yeast genome at the rDNA locus to form a multi-copy stable transformant. In addition to the HPLC coding sequence, the cassette contains only yeast DNA and contains no antibiotic markers.

(5) ABILITY TO SURVIVE IN AND COLONISE THE HUMAN GUT

The production process removes all yeast from the final preparation and the final specification states that GM yeast must be absent. Therefore, the resulting ISP preparation is GM free as are the products made from this preparation.

The yeast host strain well characterised and has been used for metabolic studies for many years. The production strain has been used at commercial scale (60 m^3) since 2003 with no issues.

APPENDIX 2. CONFIDENTIAL INFORMATION RELATING TO DOSSIER SECTIONS 2.4 AND 4.2

APPENDIX 7. YEAST CERTIFICATES FROM THE NETHERLANDS MINISTRY OF THE ENVIRONMENT

Netherlands Ministry of Environment strain classification.

Ministerie van Volkshuisvesting,
Ruimtelijke Ordening en Milieubeheer

p/a Postbus 1
3720 BA Bilthoven
Telefoon : 030 - 2744197
Fax : 030 - 2744461
E-mail : csr.ggo@rivm.nl
URL : www.rivm.nl/csr/bggo_nl.html

Directoraat-Generaal Milieubeheer
Directie Stoffen, Afvalstoffen en Straling
Afdeling SNB

Unilever
t.a.v. de directie,
Postbus 114
3130 AC Vlaardingen

Uw kenmerk	Uw brief	Kenmerk	Datum
.	.3mei 2002	. GGO 02g03.bes	. 27. JUNI 2002

Onderwerp

IAB status

Geachte Directie,

Naar aanleiding van de brief met bovenvermelde datum en de aanvullende informatie, ontvangen op 12 juni 2002, bericht ik u het volgende.

De *Saccharomyces cerevisiae* stam VWK 18 *gal1 pmt1 leu2* is geschikt bevonden als genetisch gemodificeerde organisme dat behoort tot groep I waarmee activiteiten van zowel categorie A als B mogen worden uitgevoerd.

Tegen dit besluit kunnen belanghebbenden binnen zes weken na verzending van de beschikking op grond van de Algemene wet bestuursrecht een gemotiveerd bezwaarschrift indienen bij de Minister van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer. Het bezwaarschrift moet gezonden worden aan: De Minister van VROM, t.a.v DGM/SAS/SNB IPC 645, Postbus 30945, 2500 GX, Den Haag. Een bezwaarschrift moet van een datum en een naam en adres voorzien zijn. Er moet duidelijk worden aangegeven waarom tegen het besluit bezwaar wordt aangetekend en zo mogelijk wordt een kopie van de beschikking meegezonden.

Den Haag,
De Minister van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer,
voor deze, de directeur-generaal Milieubeheer,
o.l.,

directeur Stoffen, Afvalstoffen, Straling,

mr. A.B. Holtkamp

c.c.: - dr. J.W. Chapman
- P. de Lang
- COGEM

Bijlagen: - VROM Inspectie Noord-West

Verzoeke bij beantwoording onderwerp, datum en kenmerk van deze brief te vermelden.
In verband met het regeringsbeleid om het aantal auto-kilometers terug te dringen wordt het zeer op prijs gesteld dat u bij bezoek aan een VROM-kantoor gebruik maakt van het openbaar vervoer.

Netherlands ministry of environment large scale fermentation licence

Ministerie van Volkshuisvesting,
Ruimtelijke Ordening en Milieubeheer

Beschikking
DGM/SAS GGO 02-237

Gelezen de kennisgeving van Unilever Nederland B.V., te Vlaardingen, van 25 juli 2002, kenmerk: AM/2049/lk, met de titel "Productie van peptide." en de aanvullende informatie van 9 september 2002, kenmerk: AM/2057/am en van 6 november 2002, kenmerk: AM/2088/pdl,

De Staatssecretaris van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer,

Overwegende,
het bepaalde in de Regeling genetisch gemodificeerde organismen (Staatscourant 1998, 108), artikel 8, en in het bijzonder het bepaalde in bijlage 6, onder 6.9.1 van die regeling,

Besluit:
Unilever Nederland B.V., te Vlaardingen, vergunning te verlenen als bedoeld in paragraaf 2 van het Besluit genetisch gemodificeerde organismen Wet milieugevaarlijke stoffen (Staatsblad 1993, 435). Aan de vergunning, waarvan de op 25 juli 2002 ingediende vergunningaanvraag en de daarbij behorende stukken deel uitmaken, worden de hierna volgende voorschriften verbonden:

Artikel 1: begrippen
In deze vergunning wordt verstaan onder:
a. Regeling: Regeling genetisch gemodificeerde organismen;
b. GGO's: genetisch gemodificeerde organismen.

Artikel 2: toegestane werkzaamheden

1. Betreft: Productie van een peptide met behulp van *Saccharomyces cerevisiae* tot een volume van 15000 liter, gevolgd door separatie en concentratie van het peptide;
   gastheer soort: *Saccharomyces cerevisiae;*
   stammen: CENPK338-pUR3993-7;
   vectoren: pUR3393;
   donorsequenties: synthetisch peptide.
   De in dit lid bedoelde werkzaamheden moeten volgens de bepalingen van bijlage 4 van de Regeling, onder 4.2.1 (GILSP), worden uitgevoerd.
   Aanvullende voorschriften:
   - de biomassa wordt afgedood met behulp van een gevalideerde methode.

2. Plaats van uitvoering van de werkzaamheden is:
   - BAC, Huizerstraatweg 28, te Naarden

APPENDIX 8. ANTIFREEZE PROTEINS: CHARACTERISTICS, OCCURRENCE AND HUMAN EXPOSURE.

PERGAMON

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Review

Antifreeze proteins: characteristics, occurrence and human exposure

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Accepted 17 January 2002

Summary

Antifreeze proteins (AFPs), also known as ice structuring proteins, bind to and influence the growth of ice crystals. Proteins with these characteristics have been identified in fish living in areas susceptible to ice formation and in numerous plants and insects. This review considers the occurrence of AFPs and relates it to the likely intake by human populations, with a view to forming a judgment about their safety in foods. Intake of AFPs in the diet is likely to be substantial 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. Inadequate data exist to estimate intakes from plants but estimates of intake of AFP from fish are presented for two countries with very different fish consumption, the USA and Iceland. Typical short-term exposure, for instance a portion of cod may contain up to 196 mg AFGP, while the AFP content of the same weight of ocean pout would be up to 420 mg. Average available fish AFP in the diet is calculated to be around 1–10 mg/day in the USA and 50–500 mg/day in Iceland, but these estimates are subject to considerable uncertainty. As far as can be ascertained, AFPs are consumed with no evidence of adverse health effects, either short- or long-term. Given the structural diversity of AFPs, one firm general conclusion that can be drawn from the history of consumption of AFPs is that their functional characteristics do not impart any toxicologically significant effect, in a way that, for instance, a property such as cholinesterase inhibition would. Furthermore, specifically in the case of fish AFPs where some consumption data are available, it is reasonable to infer a lack of allergenicity from the absence of reports of this effect.

Keywords: Antifreeze protein; Ice structuring protein; Fish; Plants; Abundance; Exposure

1. Introduction

Antifreeze proteins (AFPs) were first identified by De Vries in 1969 in the blood of fish living in areas where the sea froze (De Vries and Wohlschlag, 1969). They apparently served to lower the freezing point (the temperature at which ice crystals grow) of the fish's blood to below the freezing point of seawater, without increasing significantly the osmotic pressure of the plasma. Since the discovery of the first AFP, proteins with these characteristics have been identified not only in a wider range of fish living in areas susceptible to ice formation, but also in numerous plants and insects (Griffith and Ewart, 1995). The ability of AFPs to influence ice crystal growth has led to proposals for the use of the proteins in a wide variety of applications, such as improving the properties of frozen foods, cryopreservation of transplant organs and cells, cryosurgery and aquaculture (reviewed by Fletcher et al., 1999).

The purpose of this review is to consider the occurrence of AFPs and relate this to the likely intake by human populations, with a view to forming a judgment about their safety in foods.

2. Nomenclature

Antifreeze proteins are also often termed thermal hysteresis proteins. Other names have been proposed, for instance "ice growth control compounds" (Harding et al., 1999) or "ice crystal growth control proteins" (Griffith, 1999). This confusion arises from the fact that these proteins have a number of functions in nature. The term "antifreeze" protein was coined because they

Abbreviations: AFP, antifreeze protein; AFGP, antifreeze glycoprotein; ISP, ice-structuring protein; NMFS, National Maritime Fisheries Service; USDA–ERS, US Department of Agriculture Economic Research Service.

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PII: S0278-6915(02)00042-X

lowered the freezing point of the blood of the fishes in which they were first identified. However, with the discovery of proteins able to influence ice formation in plants, it became clear that depression of freezing point was only one of their effects (and possibly not the most common). They also alter the way ice crystals form and develop, the exact change in crystallisation being dependent on the type of protein and other conditions. More recently, the term "Ice Structuring Proteins (ISPs)" has been proposed as a more suitable generic name, based on the fact that all AFPs, regardless of their source and conditions of use, bind to and influence the growth of ice crystals (C.J. Clarke, N.M. Lindner, S.L. Buckley, unpublished).

3. Characteristics

Antifreeze proteins have been found in a wide variety of organisms, including bacteria, fungi, insects, animals and plants (Griffith and Ewart, 1995; Fletcher et al., 1999). Although they perform similar functions, the structures of AFPs are extremely diverse. Amino acid composition varies considerably both between species and sometimes within species (see for example Hon et al., 1994). Molecular weight range is also extremely wide, ranging from 2.5 KD in some fish AFPs (AFGP) to 36 KD for one of the AFPs of winter rye. Consistent with this structural and compositional diversity, the evolutionary origin of AFPs is also diverse. Thus the 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 AFPs were encoded by genes for a class of pathogenesis-related proteins, chitinases (Yeh et al., 2000). Interestingly the chitinase genes in rye are also regulated by cold, as are the AFPs from carrot (Smallwood et al., 1999). Table 1 lists some of the characteristics of AFPs that occur, or could occur, in the food supply and illustrates their great diversity.

4. Function and mechanism of action

AFPs protect against ice by several mechanisms. These include lowering the point at which ice crystals grow (lowering the freezing point but not the melting point, the so-called thermal hysteresis effect), but also modification of ice crystallisation, such that smaller crystals and crystals of different shape are formed. Unlike traditional chemical antifreeze compounds such as ethylene glycol and salt, AFPs exert their freezing point depression activity by a non-colligative mechanism, thereby minimising their effect on the osmotic pressure of the fish plasma. On a molar basis they are estimated to be 200–500 times more effective than NaCl

Table 1
Diversity of AFPS

Characteristic	AFP						
	Fish AFGP	Fish type I	Fish type II	Fish type III	Fish type IV	Carrot	Winter rye
Molecular mass, Da	2600–33,000	3300–4500	11,000–24,000	6500–14,000	12,300	36,000	11,000–36,000
Primary structure	$(Alanine\text{-}alanine\text{-}threonine)_n$ disaccharide	Alanine-rich multiple of 11 amino acid repeats	Cystine rich, disulfide linked	General[a]	Glutamine and glutamate-rich (26%)	General[a]	General[a]
Glycoprotein	Yes	No	No (exception: smelts have <3% carbohydrate)	No		Yes	No
Secondary structure	Expanded	α-Helical amphiphilic	β-Sheet	β-Sandwich	α-Helix	ND[b]	ND[b]
Tertiary structure	ND[2]	100% Helical	Globular c-type lectin fold	Globular	Four-helix bundle	ND[2]	ND[b] includes disulphide bonds
Protein components	8	7	2–6	12	1	1	7
Gene copies	ND[b]	80–100	15	30–150	ND[2]	1	ND[b]
Natural source	Antarctic notothenioids, 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

Table adapted and modified from Fletcher et al. (1999) and Barrett (2001).
[a] General: no dominant aminoacid(s) or repeat units (e.g. as in AFGP).
[b] ND: not determined.

(Avanov, 1990), with the effect being manifest at very low concentrations. Thus 0.003–0.025 mM AFP Type III is sufficient to inhibit ice crystal growth (Li et al., 1991).

AFPs appear to exert their effect by accumulating at the water–ice interface and thereby modifying crystal growth, with different AFPs apparently showing preference for different crystal planes (Barrett, 2001). However, at the molecular level the mechanism of interaction of different AFPs may be different. De Vries and Lin (1977) initially proposed that hydrogen bonding was involved in the activity of Type I AFPs, but recently Harding et al. (1999) and Zhang and Laursen (1998) have suggested that this may only be the first step in the interaction. They have proposed that hydrogen bonding is succeeded by hydrophobic interactions. For Type III fish AFPs, Li and Hew (1991) have proposed that hydrophilic interactions are key to ice-binding activity. For Type III AFPs, flatness of the protein surface which interacts with ice also seems critical. Activity is lost if point mutations are introduced that affect this property (DeLuca et al., 1998). Sidebottom et al. (2000) noted a lack of correlation between the size of the thermal hysteresis gap and ice recrystallisation inhibition activity, with *Lolium perenne* (rye grass) AFP demonstrating very low thermal hysteresis but potent recrystallisation inhibition capacity compared to the Type III fish AFP.

5. Abundance of AFPs

As previously mentioned, AFPs have been found in a wide variety of organisms in nature that need to protect themselves against freezing damage. Teleost fish were among the first organisms in which such proteins were identified and include a range of species such as cod, herring and ocean pout (Fletcher et al., 1999). In 1993, Duman and Olsen noted that AFPs had been found in at least 23 species of angiosperms, including a number of edible ones. They also reported their presence in fungi and bacteria. Edible plants in which AFPs have been found include such common food sources as oats (*Avena sativa*), rye (*Secale cereale*), barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), carrot (*Daucus carota*) and potato (*Solanum tuberosum*) (Griffith and Ewart, 1995). While in some cases the AFPs have only been found in parts of the plant which are not eaten (e.g. leaves of wheat, rye) (Hon et al., 1994), in many others 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 AFP content in different organisms. Griffith (1999) found up to 0.307 mg/g fresh weight in the leaves of winter rye. More data are available on the AFP content of fish. For example, the Type III AFP content in the blood of ocean pout is estimated to be about 30 mg/ml, while for Atlantic cod AFGP it 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 teleosts is about 30–70 ml/kg (Olson, 1992), the AFP content of an ocean pout will be around 900–2100 mg/kg, while that of a cod will range from 210 to 490 mg/kg in adults and from 420 to 980 mg/kg in juveniles.

5.1. Intake of AFPs

One of the main sources of AFP in the human diet is cold-water fish. Intakes can be estimated either on the basis of acute or long-term exposure. One measure of acute exposure is the amount of AFP ingested when a typical (200-g) portion of fish is consumed. A portion of ocean pout would thus give an intake of between 180 and 420 mg of AFP Type III, while the same weight of cod could contain between 42 and 196 mg of AFGP. Long-term exposure can be estimated from data on fish catches and consumption. For this review, data published by the US National Maritime Fisheries Service (NMFS) and the US Department of Agriculture Economic Research Service (USDA–ERS, 2001) on the USA, a country with moderate fish consumption, and Iceland, a country where fish are consumed in large amounts, have been used. Overall population exposure for the USA has been estimated by two different approaches. In the first, data for the overall catch were scrutinised and the species that have been reported to contain AFPs (e.g. herring, Atlantic cod, eelpout) were identified. The total catch for these species was then calculated. Based on 1999 figures, this amounted to 118,390 metric tons. Taking the extremes of AFP content, calculated in the previous paragraph, this gives a total "available AFP" from those fish species of 25–248 million grams approximately. Using a US population of 274 million people (US Census Bureau, 2000), this amounts to an annual per capita available AFP of 91–899 mg; that is, 0.25–2.46 mg/day. The second approach was to look at the fish landings in Alaska, on the assumption that the catches will be of fish likely to be living in cold waters. Total catch landed in Alaskan ports was 585,085 metric tonnes in 1998. Assuming that all these fish produce and contain some sort of AFP and that all the fish are consumed in the USA, and again using the figures for AFP content previously cited, this gives "available AFP" amounts of 123–1228 million grams. This amounts to an annual per capita intake for the USA ranging from 449 to 4483 mg; that is, 1.23–12.28 mg/day. Iceland is a country where fish consumption is very high and where most of the fish landed probably originates from cold waters. It would be reasonable to assume that such fish belong to species producing AFP. Data are available for average fish consumption in the USA, but were not used since they did not provide any information about the

distribution of this intake among different fish species. The 1993–1995 estimate of gross per capita fish intake for Iceland was approximately 91 kg live weight (cited by USDA–ERS, 2000). Using the previously cited figures for AFP content, the "available AFP" ranges from 19,110 to 191,100 mg; that is, 52–520 mg/day. In evaluating these data two biases, operating in opposite directions should be noted. Firstly the figures for "available AFP"do not take into account the fact that a portion of the fish is not consumed. Secondly, the average per capita intakes ignores the distribution of fish intake in the population and while overestimating some intakes, will also underestimate the intakes of individuals who consume a lot of fish. In this respect, the figure calculated for acute exposure may be more representative of true intake. The available data indicate that the intake of AFPs through the diet is likely to be substantial 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. Producing numerical estimates of intake is problematic, given that it is likely that not all important sources have been identified, let alone quantified and that consumption figures for individual species are not available. However, for fish AFPs, consideration of the "available AFP" and overall fish consumption indicates that mean intake from that source could reach up to 500 mg per day among populations which consume a lot of fish. In Western European and US populations, a plausible figure is more likely to be around 1–10 mg per day, although these figures are subject to considerable uncertainty.

No data on safety of AFPs per se exist, but the history of consumption suggests several conclusions:

- The proteins in question are not acutely toxic.
- The functional characteristics of AFPs do not impart upon the proteins any obvious adverse toxicological properties.
- AFPs have not been associated with any known toxic effect of fish consumption, either in the short or long term.

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

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APPENDIX 21. SEQUENCE ANALYSIS AND RESISTANCE TO PEPSIN HYDROLYSIS AS PART OF AN ASSESSMENT OF THE POTENTIAL ALLERGENICITY OF ICE STRUCTURING PROTEIN TYPE III HPLC 12.

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Research Section

Sequence analysis and resistance to pepsin hydrolysis as part of an assessment of the potential allergenicity of ice structuring protein type III HPLC 12

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Abstract

The recently published WHO/FAO guidelines on the assessment of allergenicity of novel food proteins provide a strategy with which to approach the determination of the potential of novel proteins in foods to be allergens. Key to this strategy are the assessment of sequence similarity to known allergens and the assessment of the resistance to pepsin hydrolysis. Ice structuring proteins (also commonly referred to as anti-freeze or thermal hysteresis proteins) are a group of naturally occurring proteins that bind to ice and structure ice crystal formation. The amino acid sequence of the ice structuring protein (ISP) type III HPLC 12 (ISP type III) was compared in silico with the sequences of known allergens. Secondly, the resistance to pepsin hydrolysis of ISP type III and its glycoconjugates (produced in recombinant baker's yeast) was assessed. The results indicate that ISP type III has no sequence similarity with known allergenic proteins. Both ISP type III and ISP type III glycoconjugates contained within the fermentation product were hydrolysed readily by pepsin (50% loss in <10 min at pH 1.5) to give peptide fragments that were too small to be allergenic or to trigger cross-linking to IgE. In an accompanying study, we demonstrated that IgE from fish-allergic individuals did not bind ISP Type III. Therefore, in accordance with the WHO/FAO strategy, the assessment of ISP type III and ISP type III glycoconjugates by sequence analysis together with lack of resistance to pepsin hydrolysis and the absence of IgE binding supports the conclusion that both are unlikely to present a potential sensitisation hazard. © 2002 Published by Elsevier Science Ltd.

Keywords: Allergen; Antifreeze protein; Sequence analysis; Pepsin digestion; Ice structuring protein

1. Introduction

Food allergy is an adverse reaction, mediated by the immune system, to an otherwise harmless food component, usually a protein. Food allergy, defined as an IgE-mediated reaction, is estimated to affect 1–2% of the total population and 5–8% of young children in the industrialised world (Bock, 1987; Sampson, 1990; European Commission, 1998). The development of a food allergy in an individual depends on the interplay between the way their immune system responds to a protein and the characteristics of that protein. The introduction into the food supply of proteins, which have not previously been widely consumed, requires an assessment of their potential to be food allergens. This need derives from the general requirement of the food industry to market safe products, as well as from specific legislation (e.g. EU Novel Foods Regulation EC 258/97). Unfortunately, the potential of a protein to be an allergen, when ingested, cannot be categorically predicted experimentally or on the basis of its physical and chemical characteristics. Every novel protein must therefore be considered as a potential food allergen. However, the possession by a protein of certain characteristics can increase the confidence that it is unlikely to prove a food allergen. These characteristics, based on experimental observations made on known food allergens include sensitivity to pepsin digestion, lack of

Abbreviations: GFC, gel filtration chromatography; ISP, ice structuring protein; LC–MS, liquid chromatography–mass spectrometry; MALDI–TOF, matrix-assisted laser desorption ionisation–time of flight; NCBI, National Center for Biotechnology Information; RP–HPLC, reversed phase high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TICs, total ion chromatograms.

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amino acid sequence similarity with known allergens (foods and non-foods) and absence of binding of IgE from patients allergic to the source of the protein or related proteins. Several decision trees incorporating these elements have been devised to guide assessment of the allergenicity of novel proteins. The best known is that formulated by ILSI/IFBC (Metcalfe et al., 1996), which was effectively adopted by the Food and Agriculture Organisation and the World Health Organization (FAO/WHO) (WHO, 2000) and then revised in a recent consultation (WHO, 2001).

The decision-tree is shown in Fig. 1. Central to the recommended approach are the following elements: the source of the protein, identification of sequence similarity between the peptide and known allergenic proteins and resistance of the protein to hydrolysis by pepsin. Here, we describe a practical example in which the key steps of the FAO/WHO recommendations have been interpreted and used as guidance as part of an overall assessment of the likely allergenicity of a novel protein (Bindslev-Jensen et al., 2002). It is hoped that the experiences gained from this work will be helpful to others in ensuring the safety of new foods prior to their introduction to the consuming public.

Ice structuring proteins (ISP) are a group of naturally occurring proteins that bind to ice and structure ice crystal formation. They are also commonly referred to as anti-freeze or thermal hysteresis proteins (Clarke et al., in press). Their function in nature is to help protect

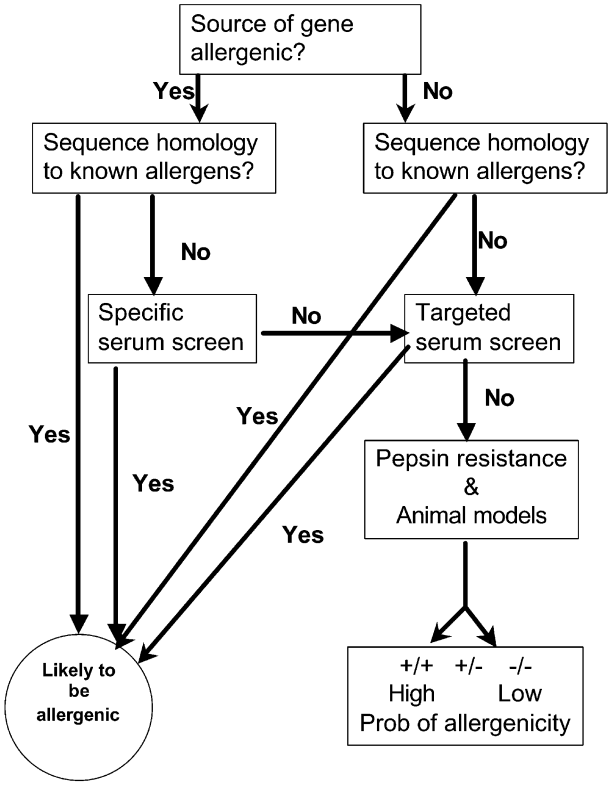

Fig. 1. FAO/WHO 2001 decision tree.

organisms in cold habitats from ice crystal damage (Madura et al., 2000). The ISPs have been reported to have a number of potential commercial applications, including in the food industry (Griffith and Ewart, 1995: Fletcher et al., 1999; Muldrew et al., 2001). The ISP type III HPLC 12 in this investigation (hereafter referred to as ISP type III) was originally identified in the Ocean Pout (*Macrozoarces americanus*), a fish living in or near Arctic waters. It has been produced using recombinant baker's yeast (*Scerevisiae*) and harvested by filtration as a cell-free product containing both ISP type III and ISP type III glycoconjugates. In this paper we have used in silico analysis to investigate the degree of similarity between the ISP type III amino acid sequence and those of known allergenic proteins. We have also investigated the pepsin resistance of ISP Type III.

2. Materials and methods

2.1. Test materials

The primary test material was a freeze-dried fermentation product containing 41.1% (w/w) total protein determined by Kjeldahl nitrogen methodology, of which 33.3% (w/w) was ISP type III, 24% (w/w) was ISP type III glycoconjugates and 43% (w/w) was other yeast proteins and peptide fragments. The protein has an amino acid sequence identical to the Ocean Pout ISP type III and is registered under accession number P19614 in Swiss Prot. Protein characterisation was carried out using RP–HPLC, GFC, MALDI–TOF MS, LC–MS and SDS–PAGE. In addition, an ISP type III glycoconjugate enriched test material, of which 40% (w/w) of the total protein was ISP type III glycoconjugates (containing $<0.5\%$ ISP type III), and a purified ISP type III test material, of which all protein was ISP type III protein, were both prepared in the Colworth laboratory from the fermentation product.

2.2. Sequence analysis of ISP type III

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

- 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 respectively the nr database of NCBI (All non-redundant GenBank CDS translations + PDB + SwissProt + 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]" used 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.

- 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 optimised 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.
- Finally, all 6-, 7- and 8-amino acid peptides (61 hexamers, 60 heptamers and 59 octamers) that could be produced from 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.

2.3. Incubations with pepsin

2.3.1. Materials

Pepsin from porcine stomach with an activity of 2.87 U/mg protein, β-lactoglobulin from bovine milk and bovine serum albumin were all purchased as freee-dried powders from Sigma (Poole, UK). Other reagents were laboratory grade and obtained from standard commercial suppliers.

2.3.2. Pepsin hydrolysis

The protocol described is based on the recommendations published by WHO (2001). 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 min in a shaking water-bath at 37 °C. The pepsin activity was stopped by the addition of 0.8 M sodium carbonate at 0.3 ml per 1 ml of incubation solution. The solution was then neutralised to approximately pH 7.0 for chromatography with concentrated hydrochloric acid at 30 μl per ml of incubation solution. Reagent, substrate and pepsin protein blanks and test material control samples (without pepsin) were prepared and incubated as indicated.

Table 1 summarises the way in which five different analytical techniques have been used to complement each other in the detection and quantification of the proteins and fragments present in the fermentation product, the purified fractions and the pepsin hydrolysate solutions.

2.4. SDS–PAGE

2.4.1. Materials

Acrylamide, ammonium persufate, methanol, acetic acid, hydrochloric acid, glycerol, sodium dodecyl sulfate, *N,N,N′,N′*-tetramethylethylenediamine, Tris, 25% (w/v) thiodiglycol and dithiothreitol, were all purchased from standard commercial suppliers. Wide range molecular weight markers were purchased from Sigma (Poole, UK). Tricine sample buffer, Tris–tricine–SDS, silver stain kit, polypeptide molecular weight markers and Tris–tricine 10–20% linear gradient gels were all purchased from Bio-Rad Laboratories (Hemel Hempstead, UK).

2.4.2. Test material characterisation

Tris–tricine 10–20% acrylamide linear gradient gels were supplied by Bio-Rad. Prior to each run the samples were initially prepared in ultra-pure water, diluted 1 in 40 in sample buffer containing 350 mM dithiothreitol and heated for 5 min at 100 °C.

Table 1
Analytical techniques used to detect or quantify proteins in the pepsin digestion experiments

Technique	ISP type III	ISP type III glycoconjugates	Hydrolysis products	Yeast protein	β-LG	BSA	Pepsin
SDS–PAGE	SQ			D			SQ
HPLC–MS		Q	SQ				
MALDI–TOF	D	D					
HPLC–RP	Q				Q	Q	D
GFC		Q	D	SQ			Q

D, detection only; Q, quantitative analysis; SQ, semiquantitative estimates based on changes in peak height or band intensity.

2.4.3. Pepsin hydrolysis

Tris–tricine gels consisting of 16.5% acrylamide resolving, 10% spacer and 4% stacking were prepared according to Schagger and von Jagow (1987). Prior to each run the digestion samples and controls were diluted 1 in 4 and standards prepared at 30, 10 and 1 μg protein/ml in sample buffer containing 350 mM dithiothreitol and heated for 5 min at 100 °C.

All samples were then centrifuged and 10 μl of each solution were loaded onto the gel. Molecular weight markers (wide range markers and polypeptide markers) were loaded with the digestion samples. Immediately prior to electrophoresis antioxidant (thiodiglycol) was added to buffer in the inner (cathodic) tank of the Mini Protean 3 Bio-Rad Laboratories, Hemel Hempstead, UK) giving a final concentration of ~0.1 mM. Electrophoretic separation was performed using a Mini Protean 3 mini gel system at a constant of 100 V.

After electrophoresis, protein bands on the gels were visualised using the silver staining kit. The stained gels were then scanned and quantification and molecular weight estimation of the digested ISP type III performed using Imagemaster 1D Elite (Amersham Pharmacia Biotech, Amersham, UK).

2.5. HPLC–MS

2.5.1. Materials

Acetonitrile (HPLC grade) and formic acid (analytical grade) were obtained from Fisher Scientific (Loughborough, UK). For preparation of the HPLC eluents, high-purity water (Milli-Q system, Millipore, Bedford, MA, USA) was used. Digestion samples were filtered prior to analysis and directly injected onto the HPLC system.

2.5.2. Chromatography

A Waters Alliance HPLC system (Model 2690) (Micromass, Manchester, UK) was used, consisting of a low-pressure pump, degasser, column oven and a LabPro rheodyne 3 way column switcher.

Separations were perfomed on a C18 Jupiter column (150×2 mm ID, 5 u, 300 Å) (Phenomenex, Macclesfield, Cheshire, UK) with a flow rate of 200 l/min using the following gradient: mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. $t=0$ min 20% B and 80% A; $t=5$ min 20% B and 80% A; $t=25$ min 50% B and 50% A; $t=27$ min 90% B and 10% A; $t=30$ min 90% B and 10% A; $t=32$ min 20% B and 80% A.

2.5.3. Electrospray mass spectrometry

ESI–MS was perfomed on a Waters ZMD single quadrupole mass spectrometer (Micromass) equipped with the electrospray ion source. The eluent was transferred without splitting into the mass spectrometer via a flow line. The following instrument settings were used:

Tune page settings	Capillary	3.2 kV
	Cone	programmed as part of the method
	Extractor	5 V
	RF Lens	0.6 V
	Source block temperature	150 °C
	Desolvation temperature	150 °C
	LM Resolution	15.0
	SM Resolution	15.0
	Ion Energy	1.0
	Multiplier	650
	Desolvation gas flow	320–340 l/h
	Cone gas flow	190–210 l/h
MS method (SIR)	Data were collected between 10 and 35 min Single ion monitoring (SIR) was performed for the following ions at cone voltage 30 V: m/z 1406.4, 1438.7, 1601.6, 1666.4, 1731.0, 1828.2 (5+ charge state of ISP type III and ISP type III glycoconjugates) *For all SIR functions the acquisition was set as follows:* Inter channel delay: 0.05 s Span: 2 Da Dwell time: 0.4 s	
MS method (TIC)	Data were collected between 10 and 35 min Full scan function: m/z 500–2000 with cone voltage of 30V: Scan time: 10 s Inter scan time: 0.10 s SIR function: m/z 284 with cone voltage of 70 V	

Mass chromatograms and mass spectra were recorded with the Mass Lynx software (version 3.4).

2.6. MALDI–TOF MS

Matrix assisted laser desorption ionisation time of flight mass spectrometry (MALDI–TOF MS) was performed using a PerSeptive Biosystems Voyager STR Biospectrometry Research Station Laser-desorption mass spectrometer (Applied Biosystems, Foster City, CA, USA) coupled with Delayed Extraction. Aliquots of 1 μl of the prepared samples were analysed using a

matrix of sinapinic acid over an initial protein screening range of 1–100 kDa. An additional mass range of 400–4000 Da. was scanned for the pepsin digest samples using a α-cyano, 4-hydroxycinnamic acid. Myoglobin and adrenocorticotropic hormone Clip 1–16 were used as external molecular weight calibrants.

2.7. Reversed phase high-performance liquid chromatography (RP–HPLC)

2.7.1. Materials

Acetonitrile (Far UV), isopropanol and trifluoroacetic acid (TFA), solvents and reagents were of HPLC or analytical grade and purchased from Fisher Scientific (Loughborough, UK). All ultrapure water was generated by the Milli-Q system (Millipore, Bedford, MA, USA)

2.7.2. Chromatography

The ISP type III content was measured, using RP–HPLC and UV detection at 214 nm by referring to an external ISP type III standard range. The test material was dissolved into 0.05% aqueous TFA at a concentration of 0.75 μg/ml and filtered through 0.2-μm Acrodisc filters into HPLC vials.

The RP–HPLC analysis was performed using a C_{18} Protein & Peptide 218 TP 54 (4.6×250 mm) column, supplied either by Vydac (Hesperia, CA, USA) or Phenomenex (Macclesfield, Cheshire, UK), on an AKTA explorer XT chromatography system, Amersham Pharmacia Biotech (Uppsala, Sweden). The samples were separated using gradient elution at 1 ml/min with an injection volume of 50 μl. Mobile phase A was 0.05% (v/v) aqueous TFA and mobile phase B was 0.05% TFA (v/v) in acetonitrile. The following gradient was used; $t=0$ min 100% A; $t=5$ min 100% A; $t=35$ min 42% A and 58% B; $t=36$ min 100% B; $t=40$ min 100% B; $t=41.5$ min 100% A; $t=44$ min 100% A. The column temperature was ambient. The working range of the external ISP type III standard was 40–200 μg/ml. Quantification was achieved by linear regression. The correlation coefficient was 0.9999.

2.8. Gel filtration chromatography (GFC)

2.8.1. Materials

Sodium chloride, sodium dihydrogen phosphate dihydrate and citric acid monohydrate were all of analytical reagent or HPLC grade and obtained from Fisher Scientific. A range of 12 peptide and protein standards (mol. wt 75–2000,000 Da) was obtained from commercial suppliers.

2.8.2. Chromatography

The test material was dissolved in 0.12% citric acid at a concentration of 8 mg/ml and filtered through 0.2 μm Acrodisc filters into HPLC vials. The ISP type III glycoconjugates, yeast protein and peptides content[1] of the primary test material was estimated through separation by GFC. The GFC analysis was performed on an AKTA explorer XT chromatography system (Amersham Pharmacia Biotech, Uppsala, Sweden). The Superdex 75 HR and Superdex Peptide HR (both 24 ml bed volume, 10×300 mm) (Amersham Pharmacia Biotech), coupled in series, were used for separation. The column temperature was ambient (approximately 22 °C). The samples were analysed using linear elution at 0.45 ml/min with 0.01 M phosphate buffer containing 0.15 M sodium chloride at pH 7.5. The injection volume was 50 μl. The UV responses of the ISP type III glycoconjugates and yeast protein and peptide peaks were quantified as a relative ratio to the total UV response. The area of the respective peaks was divided by the total protein area at 214 nm.

2.9. Data analysis

The rates of hydrolysis of β-LG, BSA, ISP type III and ISP type III glycoconjugates were calculated by estimating the time at which 50% of the original protein chromatogram peak (determined by peak area) had disappeared. Where it was not possible to determine an accurate half-life ($t1/2$) because hydrolysis was too rapid or too slow, the nearest sample point was used to express the limits of estimation, for example $t1/2 < 15$ s.

3. Results

3.1. ISP type III sequence similarity

3.1.1. Global alignment

A search for similarity to sequences contained in the whole NCBI nr database, using BLAST 2.2.1 with default parameters, produced 61 hits, all but four of which were with ice structuring protein sequences. Those four matches were with a *N*-acetyl neuraminic acid phosphate synthase from *Homo sapiens* and the related protein from mouse. The lowest (most significant) E value for these unrelated proteins was 0.02. The longest matching sequence in all the four alignments was four contiguous amino acids. The same search in PIR–NREF (which is compiled from the same databases) produced the same result, although a slightly larger *E* value. None of the matches was against proteins known to be allergenic. The FASTA search in PIR–NREF produced essentially the same results as the BLAST search in the NCBI nr database.

[1] The various peak identities were confirmed by fractionation followed by SDS–PAGE, MALDI–TOF MS, HPLC–RP and GFC (for correct peak fractionation confirmation) (Fig. 2).

A BLAST search against the nr database, but limited to entries containing the term "allergen" produced a single hit against a superoxide dismutase from *Aspergillus fumigatus* [allergen Asp f6 identified by Crameri et al. (1996)]. The match found was between amino acids 34 and 44 of the ISP type III and 54 to 64 of the superoxide dismutase. However, over that length the longest sequence of contiguous amino acids was 3 (see below).

```
Query:   34 AEDIPRLVSMQ 44
           A D+P+LVS+Q
Sbjct:   54 ATDVPKLVSVQ 64
```

The alignment showed 63% similarity in the area of the matching sequences. However, a comparison of the full sequence of ISP type III with the superoxide dismutase sequence showed no significant similarity.

3.1.2. Local alignment

A BLAST search of the nr database limited to the entries containing the term "allergen" and optimised to detect short alignments produced 355 alignments with the most sensitive settings (Matrix BLOSUM 80, Expect value 1000, gap creation penalty 10, gap extension penalty 1). Distribution of these alignments in terms of numbers of contiguous amino acids was as follows:

Greatest number of contiguous amino acids	Number of alignments
5	1
4	50
3	84
2	219

3.1.3. Exact matches

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" were with sequences within ice structuring proteins. Matches with sequences in six unrelated proteins were also found. In contrast, exact matching of the hexamers generated 515 matches with unrelated proteins. Careful examination of these proteins did not reveal any known allergens.

Matches with sequences of seven contiguous amino-acid in other proteins are listed in Table 2.

Of the proteins listed in Table 2, which shared 7-amino acid contiguous sequences, none has been associated with the induction or elicitation of allergic reactions. However, certain members of the bacterial α-amylase family are known allergens in man (Bernstein et al., 1994). The α-amylase from *Pyrococcus furiosis* was therefore compared further with a member of that family which is widely used in industrial applications, the α-amylase from *Bacillus licheniformis* (PIR ref ABSL). A pairwise alignment between the two proteins using the ClustallW program indicated only 19% identity. More importantly, the 7-amino acid contiguous sequence found in the *P. furiosis* enzyme was not present in the *B. licheniformis* enzyme. It could not therefore belong to any structure recognised by IgE antibodies to *B. licheniformis*. A pairwise alignment using ClustallW between ISP Type III and the *B. licheniformis* enzyme confirmed the absence of similarity.

3.2. Protein separation and identification

The proteins contained within the primary test material were separated and identified by a combination of analytical techniques. Figure 2 shows the primary test material eluted by GFC and the constituent peaks 1–4. The identity of the peaks has been confirmed by SDS–PAGE RP-HPLC, HPLC–MS and/or MALDI–TOF techniques (see legend for Fig. 2). The good resolution between the peaks of interest and the major pepsin peak by RP–HPLC is shown in Fig. 3 (see legend for Fig. 3). This has enabled quantification by RP–HPLC of the ISP type III protein.

Table 2
Sequence matches with ISP type III

Matching sequence	Protein	Accession numbers		
		PIR	SwissProt/Trembl	RefSeq/GenPept
GIPAEDI	Sensor histidine kinase	B75466	Q9RW09	
IPAEDIP	Sensor histidine kinase	B75466	Q9RW09	
IPRLVSM	PILT protein	Q9ZIU8	8896033	
NRAVPLG	ADP-glucose pyrophosphorylase	Q9AT46	13487709	
AVPLGTT	Succinate dehydrogenase cyt B560 subunit	I48085	C560 HUMAN	NP002992
			C560 BOVIN	786511
	Integral membrane protein		P70097	1518874
VPLGTTL	α-Amylase (*P. furiosis*)	A49512	AMYPYRFU	347940
	NADP reducing hydrogenase	F69073	027592	2622670

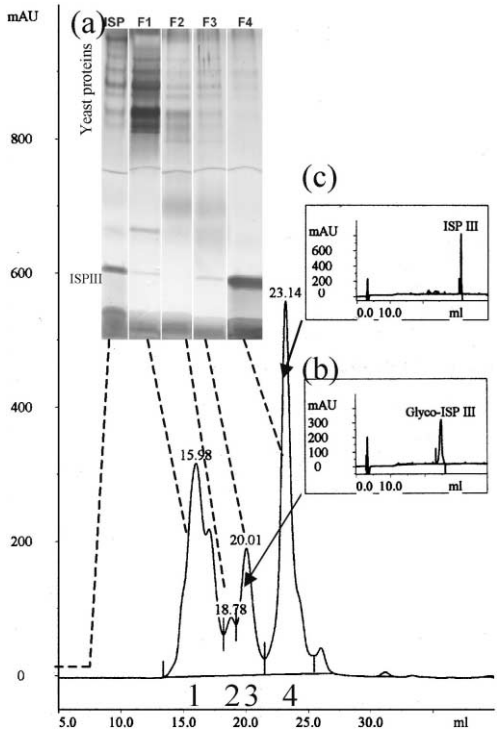

Fig. 2. Gel filtration chromatography and peak fractionation of the primary test material. The chromatogram shows the GFC elution profile of the primary test material with peak fractionation as indicated. The respective collected peaks were analysed by GFC (to demonstrate correct peak fractionation): SDS–PAGE (a), RP–HPLC (b) and (c) HPLC–MS and MALDI–TOF MS (not shown). (a) SDS–PAGE of the primary test material (ISP) and the GFC fractions 1 (F1), 2 (F2), 3 (F3) and 4 (F4). GFC peak 1 (RT = 16.0 ml) was confirmed to be yeast proteins by SDS–PAGE [(a) F1]. GFC peak 2 (RT = 18.8 ml) was identified as ISP type III glycoconjugates by MALDI–TOF MS. GFC peak 3 (RT = 20.0 ml) was also identified as ISP type III glycoconjugates by MALDI–TOF MS and HPLC–MS. Analysis of GFC fractions 2 and 3 by RP–HPLC revealed a major peak at 24.7 min (b). Analysis of GFC fraction 4 by MALDI–TOF MS, HPLC–MS, SDS–PAGE [(a) F4] and RP–HPLC [(c) RT = 1.6 min] revealed the fraction to be mainly ISP type III and lower molecular weight peptides.

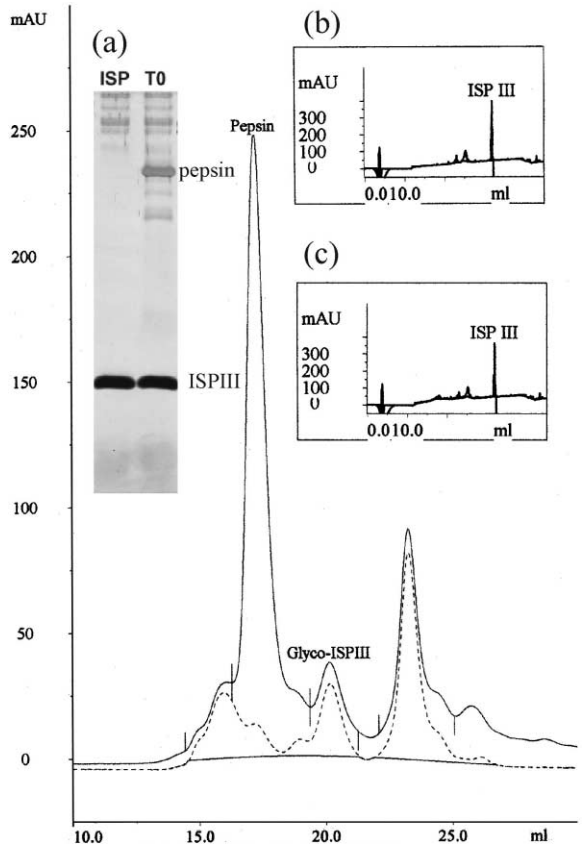

Fig. 3. Gel filtration chromatography elution profile of the primary test material showing the degree of separation between the peaks of interest. Chromatograms of the GFC elution profile of the ISP test material with (solid line) and without (dashed line) pepsin, showing separation between pepsin and the ISP type III glycoconjugates peak. The SDS–PAGE lanes (a) of the primary test material with (T0) and without (ISP) pepsin illustrate that pepsin does not interfere with the detection of ISP type III and the yeast proteins. The RP–HPLC chromatograms of the primary test material in the absence of (b) and presence of (c) of pepsin show that pepsin does not interfere with the detection of ISP type III.

3.3. Hydrolysis by pepsin

3.3.1. SDS–PAGE analysis

At pH 1.5 the pepsin and ISP type III controls comprised four and five silver staining bands, respectively (Fig. 4). These bands were found to match all of the bands observed in the digest sample at t0, with the exception of one band at 62–64 kDa that was not visible after t0. ISP type III, at an estimated molecular weight of approximately 5.8 kDa in SDS–PAGE, was visible as a strongly staining band at *t*0 to *t*15 min, as a faintly staining band at t30 and was not detectable at t60. At this time point only two high molecular weight bands were still visible, matching bands observed in the pepsin control. Volume estimation using purified ISP type III as a volume standard at 300, 100 and 10 ng protein/ml with the results normalised against *t*0 gave a hydrolysis half-life (*t*1/2) value of approximately 4 min (Table 3). At pH 2.5 the pepsin and ISP type III controls com-

Table 3
Estimated *t*1/2 values for proteins hydrolysed by pepsin in the primary test material at pH 1.5

Technique	ISP type III	ISP type III glycoconjugates	Yeast protein
SDS–PAGE	4 min	ND	NA
HPLC–MS	ND	<1 min	ND
RP–HPLC	6 min	ND	ND
HPLC–GFC	ND	<1 min	ND

The appearance and disappearance rates of the hydrolysis products could not be determined quantitatively.

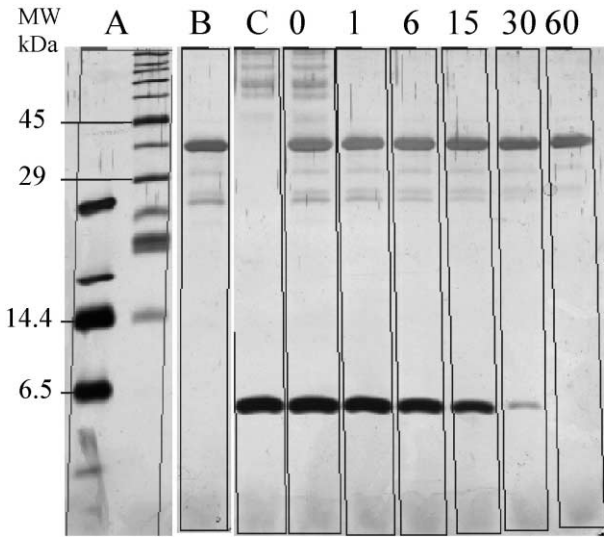

Fig. 4. Pepsin digestion of ISP type iii at pH 1.5. (A) Molecular weight markers, (B) pepsin control, (C) ISP type III control, (0) ISP type III digestion at 0 min, (1) ISP type III digestion at 1 min, (6) ISP type II digestion at 6 min, (15) ISP type III digestion at 15 min, (30) ISP type III digestion at 30 min, (60) ISP type III digestion at 60 min. Peaks 1–4 are products of pepsin hydrolysis after 120 min. Peak 5 is residual ISP type III after 120 min of incubation.

prised seven bands and five bands, respectively (data not shown). These bands were found to match all of the bands observed in the digest sample at *t*0. The ISP type III was visible as a strongly staining band at *t*0 to *t*30, as a faintly staining band at *t*60 and was not detectable at *t*120. At *t*120 a total of seven, high molecular weight bands were also still present, matching bands observed in the pepsin control. ISP type III was estimated to have a half-life of 13 min at pH 2.5. At pH 3.5 the pepsin and ISP type III controls consisted of seven bands each (data not shown). These bands were found to match all of the bands observed in the digest sample at t0. The ISP type III was visible as a strongly staining band at t0 to t60 and as a very faintly staining band at t120. At t120 a total of eleven other high molecular weight bands were still visible, matching bands observed in the pepsin and ISP type III controls. ISP type III was estimated to have a half-life of <28 min at pH 3.5. Samples of pepsin alone at pH 1.5 incubated over 0, 1 and 2 h were analysed. Seven bands were observed at 0 and 1 h and 6 at 2 h, with the main pepsin component being unchanged at approximately 40 kDa. Neither ISP type III glycoconjugates nor the ISP type III hydrolysis products were visible as discrete bands upon silver staining.

3.3.2. HPLC–MS analysis

In order to monitor the degradation of ISP type III and ISP type III glycoconjugates during the course of pepsin digestion, single ion chromatograms for the $[M+5H]^{5+}$ ions of ISP type III and eight representative ISP type III glycoconjugates were recorded. The analysis of samples from different incubation time points confirmed that ISP type III as well as its glycoconjugates are rapidly degraded with half-life times of less than 10 min for the ISP type III and less than 1 min for ISP type III glycoconjugates.

To investigate the formation of degradation products, HPLC total ion chromatograms (TICs) were recorded. Additionally, as an indicator ion for the presence of ISP type III related peptides, an ion *m*/*z* 284 was monitored. This fragment ion, which represents the last three C-terminal amino acids of the sequence of ISP type III, can be formed at increased cone voltage through collisional-induced dissociation. Figure 5 shows the chromatograms recorded for the original test material after 0 and 2 h of incubation. The compositional changes taking place in the course of pepsin digestion are obvious. While only traces of ISP type III are present at the end of the digestion experiment (cf. Fig. 5, peak 5), four new peaks can be detected. Combined mass spectra were extracted from these peaks in order to gain more information about the nature of the respective digestion products. A range of ions was detected between 500 and 2000 Da (cf. Fig. 6a–d) with one predominant signal at *m*/*z* 639 (cf. Fig. 6c). Based on this m/z values and the *m*/*z* value of the related monosodium adduct ion a molecular weight of 1276 Da could be calculated for this main digestion product. The molecular masses of other detected species, which gave about 100 times weaker mass spectral response, were determined to be below 4000 Da. This has been confirmed by MALDI–TOF MS. The molecular weights of some of the identified peptide species match with partial sequences of ISP type III; however, further investigation of the identity of those species was not carried out.

In order to study the time course of formation of the major digestion product, single ion chromatograms for *m*/*z* 639 were extracted from TICs of samples from different digestion time points. The respective fragment appears to accumulate during incubation with a maximum around 30 min but then gradually disappears, which demonstrates that it is not totally resistant to pepsin digestion.

3.3.3. RP–HPLC analysis

The disappearance of ISP type III as measured by RP–HPLC is shown in Fig. 7 at three different pHs. The rate of disappearance has been calculated and is presented in Table 3. Table 4 shows that purified ISP type III is hydrolysed at a similar rate compared with the primary test material. The ISP type III protein when incubated at 37 °C alone does degrade gradually with time. However, this rate is relatively slow ($t1/2>60$ min) and has not been subtracted from the pepsin catalysed rate, since the pepsin catalysed rate reflects the total loss of ISP type III by both enzymic and non-enzymic hydrolysis. The major pepsin peak remained essentially unchanged throughout the incubations up to 2 h.

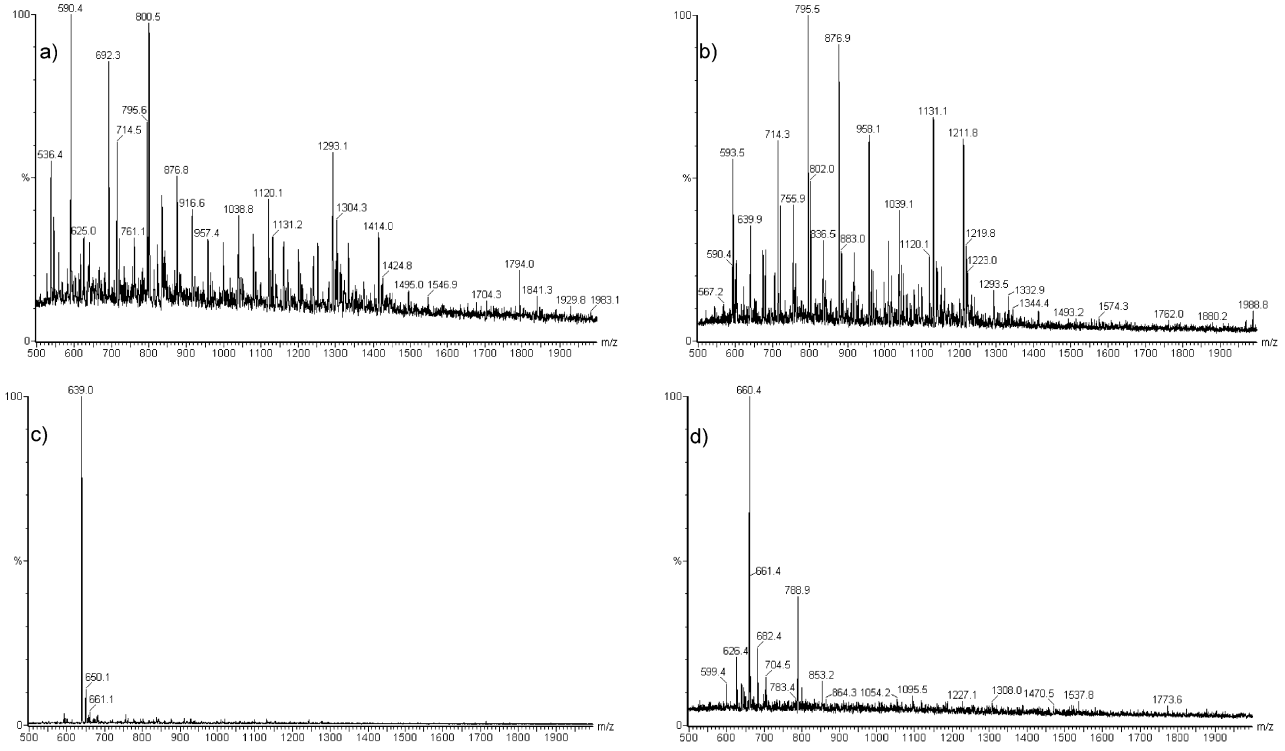

Fig. 5. TICS and SIR of m/z 284 of primary test material at 0 and 120 min of pepsin hydrolysis.

The reference proteins β-LG and BSA were incubated with pepsin and the disappearance measured by RP–HPLC. The disappearance of β-LG was monitored over 2 h incubation and the $t1/2$ was found to be greater than 2 h, even in the presence of pepsin (Table 4). Conversely, BSA, when incubated with pepsin could not be measured at the first incubation sample point at 15 s (Table 4).

3.3.4. GFC analysis

The rate of disappearance of the ISP type III glycoconjugates was determined from the GFC data. The ISP type III glycoconjugates were hydrolysed even more rapidly than the ISP type III (Fig. 8). The rates of hydrolysis in the fermentation product are summarised in Table 3. Table 4 shows that the rate of hydrolysis of the purified ISP type III glycoconjugates is equivalent to that in the fermentation product.

Table 4
Estimated $t1/2$ values for purified proteins hydrolysed by pepsin at pH 1.5

Technique	ISP type III	ISP type III glycoconjugates	β-LG	BSA
SDS–PAGE	4 min	ND	ND	ND
HPLC–MS	ND	<1 min	ND	ND
HPLC–RP	6 min	ND	>2 h	<15 s
HPLC–GFC	ND	<1 min	ND	ND

4. Discussion

The allergenicity of food proteins is well documented and is a hazard that must be guarded against when introducing any novel protein into the food chain. Currently no fully validated models exist to predict the allergenic potential of a novel protein (WHO, 2001). In order to provide assurance that a novel protein is not a potential allergen, approaches have been proposed which are based on decision trees (Metcalfe et al., 1996; WHO, 2000, 2001). The key feature of such decision trees is that they drive consideration of multiple features of the protein in question which, taken together, enable a judgement to be made on the probability of a protein proving allergenic. In this report, a novel protein and its glycoconjugates have been assessed using the most recent FAO/WHO decision tree for guidance (Fig. 1). Here, we have focused on three key elements, namely the source of the protein, sequence similarity with known allergens and resistance to pepsin hydrolysis as part of an overall safety assessment.

As a first stage, the original source of the protein was considered in relation to known food allergens. The protein was originally identified in the Ocean Pout (*Macrozoarces americanus*), which is a bony fish of the family *Zoarcidae* (Eelpouts), although such proteins can occur in other species. Initial consideration of the original source of the protein determined that the Ocean Pout is a fish living in the temperate waters of the

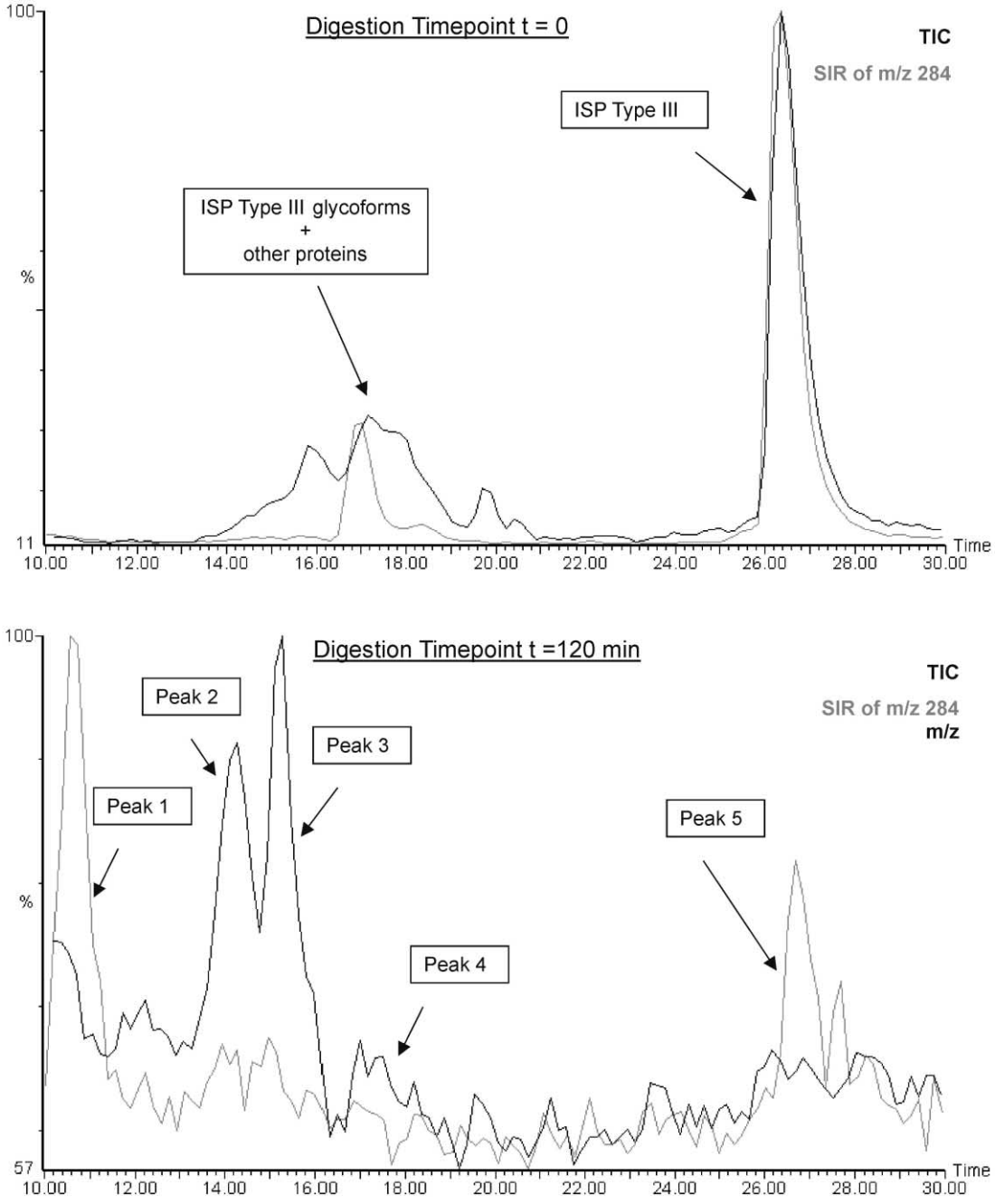

Fig. 6. Combined mass spectra extracted tic after 120 min of digestion.

western Atlantic. The Ocean Pout is commercially fished but its popularity as a food has fluctuated since the Second World War when it was more widely consumed, its peak commercial landing being 2000 tonnes in 1944 (www.nefsc.nmfs.gov/sos/spsyn/og/pout/). The Ocean Pout is closely related to the smaller Eelpout (*Zoarces viviparous*), which lives in the temperate waters of the northeast Atlantic. There are no published reports of allergic reactions to Ocean Pout (Crevel et al., 2002), but individuals allergic to one fish species frequently react to others (Hansen et al., 1996). In addition, the Codex Committee on Food Labelling lists fish as a common allergen. The source of the protein was therefore interpreted as "allergenic" in accordance with the first stage of the FAO/WHO decision tree. Comparison of the amino acid sequence with those of known allergens was the second stage. Amino acid sequence comparison in silico was proposed as a tool to identify possible allergenic structures in novel proteins in an ILSI/IFBC report in 1996 (Metcalfe et al., 1996). The authors reasoned that if a novel protein possessed a sequence similar to one present in a known allergen and of sufficient size to be recognised by the immune system (B- and T-cell epitopes), it might be recognised by the immune system of individuals sensitised

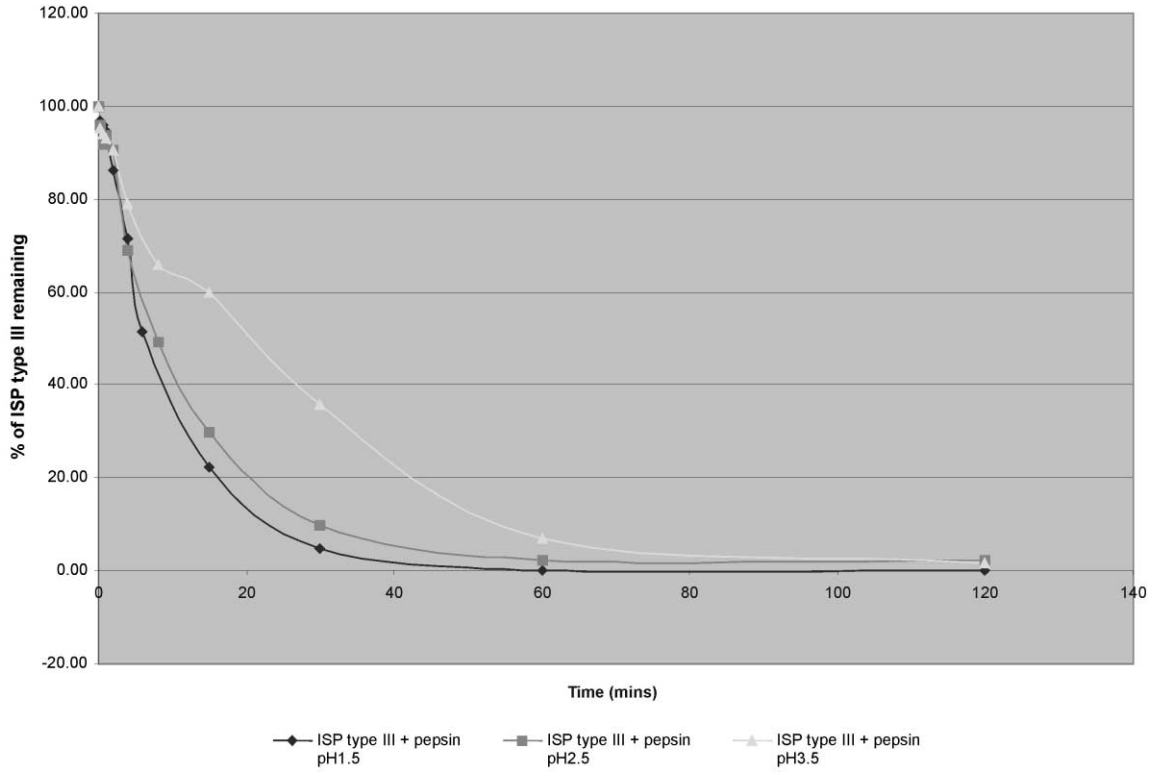

Fig. 7. Loss of ISP type III from the primary test material in the presence of pepsin.

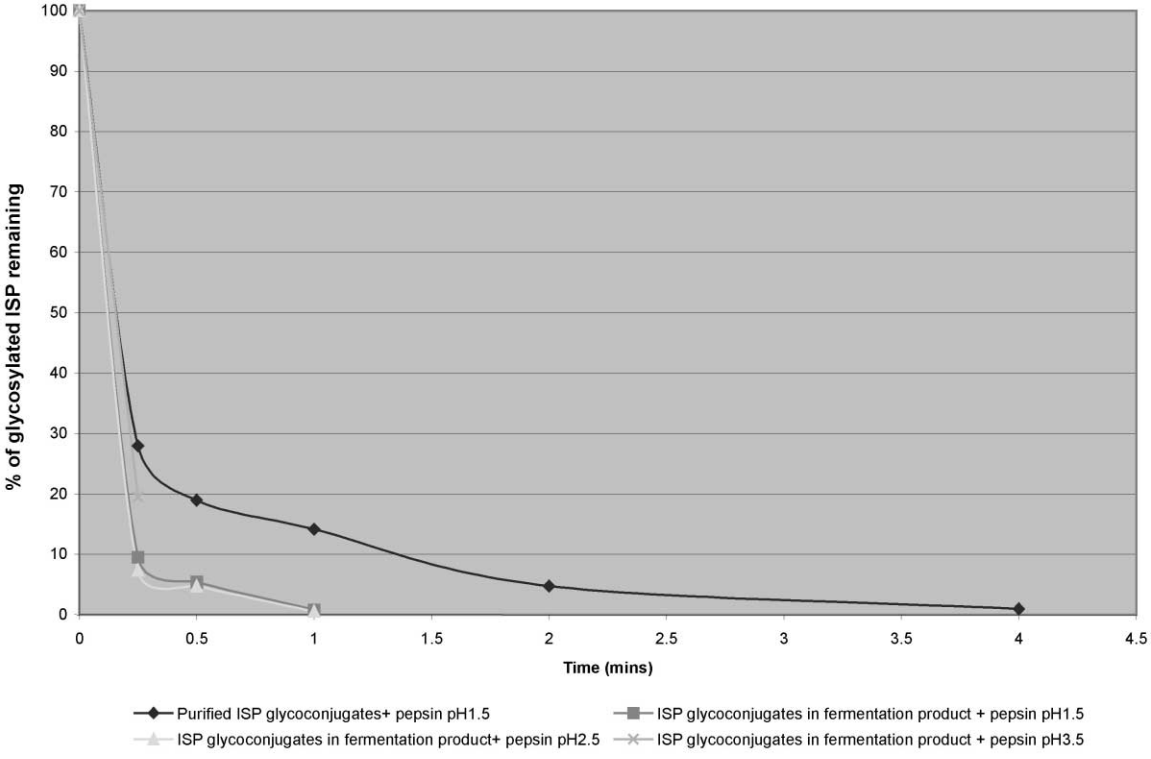

Fig. 8. Loss of ISP type III glycoconjugates in the presence of pepsin.

to that allergen and provoke a response. The significance of any match would therefore need to be verified in a serological screen. The criterion, which served to flag the need for this further investigation, was a match of at least eight contiguous amino acids. This requirement was criticised later for lacking rigour in the light of recent knowledge of B-cell epitopes (Stanley et al., 1997). To address this perceived flaw, the FAO/WHO consultation proposed the following criteria to signal the need for further investigation of possible allergenicity:

> 35% similarity between the novel protein and a known allergen in any 80-amino acid window *OR*
> a six contiguous amino acid match between the novel protein and the allergen.

Sequence analysis of ISP type III against non-redundant protein databases showed that it did not meet either of those criteria. The closest match to a reported allergen when globally aligned was with Asp f6, but involved a maximum of three contiguous amino acids within a 10-amino acid peptide. The overall structure showed no significant similarity between the two proteins. The ISP type III also showed no relevant sequence similarity when analysed against a subset of the NCBI non-redundant database, using parameters for the alignment programs designed to enable short exact matches to be detected. Finally, using the program Peptide Match against the PIR–NREF database ('virtual allergen database'), analysis of all the heptamer peptides that could be generated from ISP type III failed to reveal any matches with known allergens. The choice of heptapeptides as the basis for analysis, rather than the recommended hexapeptides, was based on the earlier negative results and the fact that heptapeptides represented a reasonable compromise between excessive chance hits and the reduced sensitivity afforded by the use of octapeptides. Although none of the matches were with sequences present in known allergens, the match with a bacterial α-amylase from *Pyrococcus furiosis* was scrutinised in more detail because some bacterial α-amylases are known allergens in man (*Bacillus licheniformis* α-amylase) (Bernstein et al., 1994). The relevant amino acid sequence was not actually found in the *B. licheniformis* amylase (Yuuki et al., 1985). The lack of sequence similarity (i.e. $>35\%$ in any 80-amino acid sequence or more than six identical contiguous amino acids) is significant, and indicates that the only serological investigations required are with sera from fish allergic individuals. Matching hexamers using "Peptide match" yielded a considerable number of hits against unrelated proteins, none of which were known allergens.

Other structural information relevant to allergenic potential includes the size of the protein and its degree of glycosylation. The ISP type III is a small protein of approximately 7.0 kDa mol wt. There are no known food allergens with molecular weight less than 7 kDa. This does not preclude the possibility of smaller proteins being allergenic, but the weight of evidence so far suggests that the probability of proteins smaller than 7 kDa being allergenic is low (Taylor et al., 1987). The degree of glycosylation is of interest and relevance to ISP type III since the product of fermentation contains protein post-translationally conjugated with mannose. Sugar residues may either form epitopes which are recognised by IgE (Tretter et al., 1993; Batanero et al., 1994), or they may alter allergenicity by modifying the resistance of the protein to enzymatic attack (Swärd-Nordmo et al., 1988). However, the data taken overall show that no consistent relationship exists between glycosylation and allergenic potential. In addition, carbohydrate epitopes cause extensive serological cross-reactivity between glycoproteins from different species and even kingdoms, but with no clinical significance (van der Veen et al., 1997).

In the evaluation of the resistance of the protein to digestion by pepsin and comparison with reference proteins, the reference proteins behaved as previously reported when exposed to pepsin (Astwood et al., 1996). Bovine serum albumin underwent rapid hydrolysis ($t1/2<15$ s) and the milk allergen, β-lactoglobulin showed pronounced resistance to hydrolysis with a $t1/2>2$ h (Table 4). These results demonstrate consistency with the work of Astwood et al. (1996) and the validity of the protocol. Proteins that are rapidly hydrolysed by pepsin to small fragments are considered less likely to be allergenic. In the case of ISP type III, pepsin hydrolysed the protein readily, resulting in 50% loss of the protein in less than 10 min in the fermentation product, as assessed by both SDS–PAGE and RP–HPLC (Table 3). As expected, pepsin activity decreased with increasing pH, as demonstrated in Fig. 7. However, the hydrolysis of ISP type III continued even at pH 3.5 and the protein had virtually disappeared (below the detection limit) by 60 min. Demonstration of the continued activity of pepsin at higher pH is important because the human stomach can achieve pH values of up to 6 in the presence of a large amount of food. Moreover, the most appropriate pH conditions are still a matter of debate and data on the rates of proteolytic breakdown under different conditions can therefore aid the interpretation of results.

Assessment of the degradation of the glycoconjugates required additional analytical methodology. The techniques of HPLC–MS, MALDI–TOF and GFC were used to detect and quantify the glycoconjugates. With regard to the ISP type III glycoconjugates in the fermentation product, the hydrolysis rate appeared to be even faster than ISP type III with $t1/2$ estimates of <1 min by GFC and HPLC–MS at pH 1.5. The pH had little effect and ISP type III glycoconjugates appeared to hydrolyse rapidly between pH 1.5 and 3.5 (Fig. 7). The rates of pepsin hydrolysis of the purified proteins were measured

to determine if the fermentation product (i.e. other proteins) had a modifying effect on pepsin activity. Interestingly, Table 4 shows that hydrolysis rates for purified ISP type III and ISP type III glycoconjugates were similar to those measured in the fermentation product, showing that the fermentation products had no discernible influence on degradation.

The appearance and size of the fragments produced by pepsin hydrolysis was also investigated. Clearly, incomplete hydrolysis and accumulation of a fragment of protein with a sufficiently large molecular weight to be considered as a cause for concern with regard to potential allergenicity would require further investigation of the identity of the fragment. In the case of the fermentation product, many fragments were detected with a mass of less than 4 kDa; however, a single fragment appeared to accumulate up to a maximum at about 30 min of incubation, followed by a gradual disappearance. The identity of this fragment is unknown, but it has a molecular mass of 1276. A fragment of this size is too small to generate an IgE-mediated response, or to elicit a response in an individual sensitised to the native protein (Senpuku et al., 1997; Takai et al., 1997; Nicholas et al., 1988; Tamura et al., 2000). Continuous (linear) B-cell epitopes are at least eight amino acids long and that as cross-linking of IgE is required to trigger an allergic response, a fragment of 1276 Da, which can only bind to one IgE molecule, cannot trigger a reaction.

Interestingly, the purified ISP type III and the ISP type III glycoconjugates appear to hydrolyse to the same fragment.

Together with an accompanying paper (Bindslev-Jensen et al., 2002), this study constitutes one of the first to have interpreted the new WHO/FAO recommendations (WHO/FAO, 2001) and used them to assess the likely allergenicity of an example fermentation product as part of an assessment of safety. We have considered the source of the protein, compared the sequence with known allergenic proteins and determined the resistance to pepsin hydrolysis of both the ISP type III and its glycoconjugates. The ISP type III protein showed no sequence similarity with known allergens, nor did any heptamer peptides produced from it. In addition, the ISP type III and the ISP type III glycoconjugates were rapidly hydrolysed by pepsin at pH 1.5–3.5 (50% loss in <10 min), resulting in fragments with molecular weight less than 4 kDa. We therefore conclude that the ISP type III, its glycoconjugates and pepsin hydrolysis products are unlikely to provoke allergic responses in individuals sensitised to known allergens or to induce an IgE-mediated response in unsensitised individuals.

A fourth element of the decision tree consists of investigating whether IgE antibodies in sera from individuals allergic to the source of the protein (in this case fish), or a food of the same origin bind to the novel protein. An accompanying paper (C.B. Bindslev-Jensen et al., unpublished) describes this work and demonstrates that there is no IgE binding. Thus, we conclude that ISP type III is unlikely to be a potential food allergen.

Acknowledgements

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APPENDIX 22. ASSESSMENT OF THE POTENTIAL ALLERGENICITY OF ICE STRUCTURING PROTEIN TYPE III HPLC 12 USING THE FAO/WHO 2001 DECISION TREE FOR NOVE

PERGAMON

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Assessment of the potential allergenicity of ice structuring protein type III HPLC 12 using the FAO/WHO 2001 decision tree for novel foods

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Abstract

The introduction of novel proteins into foods carries a risk of eliciting allergic reactions in individuals sensitive to the introduced protein. Therefore, decision trees for evaluation of the risk have been developed, the latest being proposed by WHO/FAO early in 2001. Proteins developed using modern biotechnology and derived from fish are being considered for use in food and other applications, and since allergy to fish is well established, a potential risk from such proteins to susceptible human beings exists. The overall aim of the study was to investigate the potential allergenicity of an Ice Structuring Protein (ISP) originating from an arctic fish (the ocean pout, *Macrozoarces americanus*) using the newly developed decision tree proposed by FAO/WHO. The methods used were those proposed by FAO/WHO including amino acid sequence analysis for sequence similarity to known allergens, methods for assessing degradability under standardised conditions, assays for detection of specific IgE against the protein (Maxisorb RAST) and histamine release from human basophils. In the present paper we describe the serum screening phase of the study and discuss the overall application of the decision tree to the assessment of the potential allergenicity of ISP Type III. In an accompanying paper [Food Chem. Toxicol. 40 (2002) 965], we detail the specific methodology used for the sequence analysis and assessment of resistance to pepsin-catalysed proteolysis of this protein. The ISP showed no sequence similarity to known allergens nor was it stable to proteolytic degradation using standardised methods. Using sera from 20 patients with a well-documented clinical history of fish allergy, positive in skin prick tests to ocean pout, eel pout and eel were used, positive IgE-binding in vitro to extracts of the same fish was confirmed. The sera also elicited histamine release in vitro in the presence of the same extracts. The ISP was negative in all cases in the same experiments. Using the proposed decision tree, we demonstrated the safety of the ISP to patients already sensitised to fish, as well as to individuals potentially susceptible to producing IgE responses to proteins. Furthermore, the practicability of the new decision tree was confirmed.

Keywords: Fish protein; Biotechnology; Allergy; Risk assessment; Food hypersensitivity; IgE; Sequence analysis; Histamine release; Stability

1. Introduction

The introduction of novel proteins into foods carries a risk of eliciting allergic reactions in individuals sensitive to the introduced protein (Bindslev-Jensen, 1998) or of sensitising susceptible individuals (e.g. atopics). No single predictive test can identify the allergenic potential of an unknown protein, but various schemes have been formulated. The first of these was the IFBC/ILSI decision tree for the assessment of the allergenicity of foods

Abbreviations: AFPs, antifreeze proteins; FCS, fetal calf serum; GFC, gel filtration chromatography; ISP, ice structuring protein; MALDI-TOF, matrix-assisted laser desorption ionisation-time of flight; SDS-PAGE, sodium dodecyl sulphatepolyacrylamide gel electrophoresis.

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produced by genetic modification (Metcalfe et al., 1996). This scheme was subsequently adopted by the World Health Organization (WHO) and the Food and Agriculture Organisation of the United Nations (FAO) and then extensively modified and updated in 2001 (WHO, 2001). Each of these decision trees identifies features of allergens which must be considered and tests which should be performed to give assurance that a novel protein is not likely to elicit reactions. These schemes thus use a weight of evidence approach to assess the likelihood that a novel protein will prove allergenic.

The FAO/WHO (WHO, 2001) decision tree (Fig. 1) starts by analysing the primary amino acid sequence of the novel protein and looking for similarity with known allergens. This is followed by in vitro investigations with sera from patients with an established allergy to the source of the protein, whenever possible. If those tests are negative, and especially when only a few sera from confirmed allergic patients are available, this procedure can be followed by targeted serum screening using sera from patients with allergies to foods of a related type. Since resistance to proteolytic degradation is a characteristic feature of several allergenic proteins, a standardised protocol for investigation of this property is also included in the decision tree.

Allergy to fish is most abundant in Scandinavian countries, reflecting eating habits (Hansen and Bindslev-Jensen, 1992). Sensitisation occurs to the major muscle protein, named Gad c1 in codfish, which is extremely stable both to heat treatment and to pepsin digestion. Allergy to Gad c1, analogues of which are also present in other fish, is reflected clinically, that is, the allergic individual will react on ingestion of all fish species containing Gad c1 (Hansen et al., 1996, 1997).

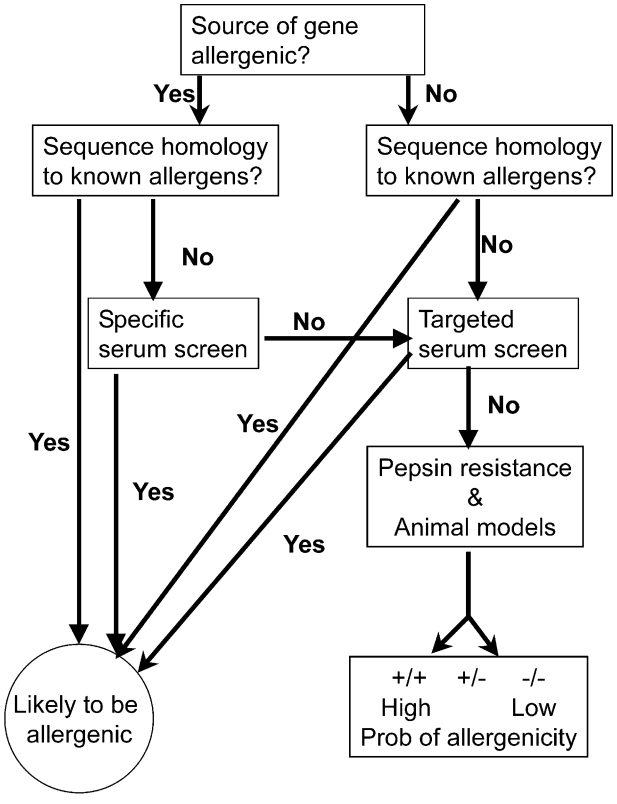

Fig. 1. The FAO/WHO (2001) decision tree.

Many fish species living in Arctic waters contain proteins in their blood that prevent ice crystal growth thus enabling survival at water temperatures below 0 °C. This group of proteins is commonly referred to as antifreeze proteins (AFPs), but more recently the term ice structuring proteins (ISPs) has been proposed as a more appropriate name (Clarke et al., in press). ISPs have a range of potential commercial applications including those in food products (Fletcher et al., 1999). The protein investigated in this study is ISP Type III isoform HPLC 12 (hereafter referred to as ISP Type III) originating from the Arctic fish ocean pout (*Macrozoarces americanus*), where it occurs at high concentrations (30 mg/ml) in the blood (Fletcher et al., 1985). No data exist on allergy to ocean pout itself, although it is reasonable to expect fish-allergic individuals to react to ocean pout and also to eel pout, which lives in Scandinavian waters. However, allergy to the closely related fish species eel has been described (De Martino et al., 1990). Before any food application is considered, it is therefore essential to demonstrate that such individuals do not react to the novel protein even though they may react to ocean pout flesh containing Gad c1 or its analogues.

In the present paper, we report the results of serum screening with IgE from fish-allergic individuals and assess the overall application of the newly-launched FAO/WHO 2001 decision tree to the assessment of the allergenicity of ISP Type III. In the accompanying paper we analyse sequence similarity to known allergens and assess resistance to proteolysis. The serological investigation was performed as part of a large scale scientific program, funded by the Danish Medical Research Council, *The Bio-Risk Study*, aimed at developing appropriate methods for the assessment of the allergenicity of foods produced by biotechnology.

2. Materials and methods

2.1. Patients

Twenty patients (aged 12–44 years, median 30 years) with allergy to codfish, diagnosed according to the guidelines published by The European Academy of Allergy and Clinical Immunology (Hansen and Bindslev-Jensen, 1992; Bruinjzeel-Koomen et al., 1995) were recruited. All had a positive skin prick test to cod, and were positive in double-blind, placebo-controlled food challenges, except where their case history indicated that they had suffered anaphylaxis or a very severe reaction

on ingestion and where challenge would therefore have been unethical. Samples of blood (20 ml) without added anticoagulant and heparinized blood (10 ml) were drawn and shipped in anonymous form for in vitro experiments.

2.2. Test material

The test material, produced by fermentation using recombinant *Saccharomyces cerevisiae,* is ISP Type III isoform HPLC12. The amino acid sequence of the expressed ISP III protein was identical to the sequence found in the native ocean pout protein. The material used in all in vitro experiments was a freeze-dried fermentation product containing 41.1% (w/w) total protein determined by Kjeldahl nitrogen methodology, of which 33.3% (w/w) was ISP Type III isoform HPLC 12 and 24% (w/w) was ISP Type III glycoconjugates, the balance consisting of yeast proteins and peptide fragments. Preparations enriched in glycoconjugates or ISP Type III HPLC 12 were used in some experiments. The native ISP Type III HPLC 12 has a molecular weight of 7.0 kD and is registered under accession number P19614 in SwissProt.

For the skin prick testing, freshly thawed preparations of eel and eel pout bought locally and ocean pout (Aqua Bounty Canada Inc., St John's, NF, Canada) were used. The ocean pout extract was also used in the serological in vitro experiments.

2.3. 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 EAACI (Hansen and Bindslev-Jensen, 1992; Hansen et al., 1996). Drugs interfering with skin prick testing were discontinued prior to testing.

2.4. Methods

Sequence analysis, pepsin digestion and serum screening were conducted in accordance with the recommendations of the FAO/WHO 2001 Consultation, except that HPLC-MS, MALDI-TOF (Chapman, 1996) and GFC (Yau et al., 1979) were used in addition to densitometric analysis of SDS–PAGE gels to quantify pepsin hydrolysis of ISP Type III, as described in the accompanying paper (Baderschneider et al., 2002). Additional investigations were further carried out as described below.

Serological experiments were carried out in a blinded fashion. Investigations on basophil histamine release were added in order to ascertain biological activity of any positive IgE-binding found in the Maxisorp RAST or the RAST inhibition assay.

2.5. Maxisorp RAST

The direct Maxisorp RAST was performed as described previously (Poulsen et al., 1989). The tubes were coated with an extract from ocean pout at 2 μg protein/ml. The Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) 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 10–1000-fold. The next day, anti-IgE coupled to I^{125} were added and after incubation overnight, the gamma radiation was measured as CPM. 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 ISP, at concentrations of 200 μg/ml down to 20 ng/ml, and centrifuged at 1650 *g* for 15 min before use. The inhibition was calculated in percent as the CPM value for the inhibited sample divided with the value of the uninhibited sample. The background value determined with a pool of sera from non-allergic donors was subtracted from all values.

2.6. Basophil histamine release

Washed heparinized blood from the patients was used for histamine release as described elsewhere (Hansen et al., 1996). In brief, 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 ISP. The highest concentrations of eel pout and ISP were 0.2 and 10 mg/ml, respectively. A release of greater than 15 ng histamine/ml blood was considered positive. The results were expressed as the highest dilution titre inducing a positive release.

2.7. Ethics

All patients gave written consent to participate in the study. The study was approved by the local ethics committee (Den Videnskabsetiske Komité for Vejle og Fyns Amter, Denmark, Ref. No. 20010095). The study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983. The serological phase of the study was funded by the Danish Medical Research Council (The Bio-Risk Study, Grant No. 9801279). Patient costs for transportation etc. were covered by Unilever plc.

2.8. Statistics

A power calculation based on binomial probability theory can determine the required number of subjects for the study. This shows that the use of 20 sera gives an

approximate 99% probability that an allergen for which the prevalence in the test population is 20% will be detected, if present (Armitage et al., 2001). Even where the prevalence in the test population is 2%, the likelihood of detection is still approximately 1 in 3.

3. Results

3.1. In vivo investigations

All patients demonstrated positive skin prick test to eel, eel pout and ocean pout. Average wheal size (largest diameter plus perpendicular diameter divided by 2) was 16.7 ± 10.1, 17.3 ± 7.1 and 16.1 ± 6.7 mm, respectively.

Sequence analysis of ISP Type III. Primary amino acid sequence analysis of ISP Type III did not reveal any similarity with known allergens, using either global or local alignment algorithms. None of the possible sequential 6-, 7- and 8-amino acid sequences produced from the ISP Type III sequence matched any sequence from known allergens. For a detailed description of the methodology used and the results, the reader is referred to the accompanying paper (Baderschneider et al., 2002).

3.2. Resistance to pepsin hydrolysis

Assessment of resistance to pepsin hydrolysis by SDS–PAGE analysis (Figs. 2 and 3a), as well as by HPLC (Fig. 3b) showed that ISP Type III (approximately 5.8 kDa band) was broken down with a half-life of approximately 4 min.

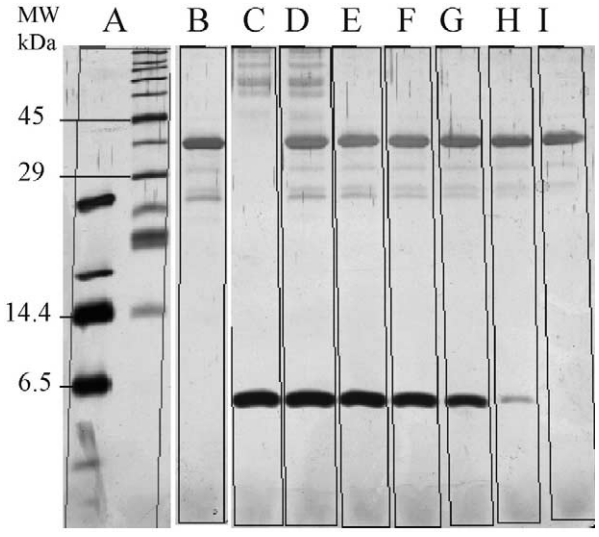

Fig 2. SDS–PAGE gel illustrating pepsin hydrolysis of ISP Type III at pH 1.5. (A) Molecular weight markers, (B) pepsin control, (C) ISP control, (D) 0 min, (E) 1 min, (F) 6 min, (G) 15 min, (H) 30 min, (I) 60 min.

After 60 min of exposure to pepsin, the band had disappeared. The control proteins BSA and β-lactoglobulin behaved as previously reported (Astwood et al., 1996): BSA was not detectable after 15 s, while β-lactoglobulin showed a half-life in excess of 2 h. For a detailed description of the methodology used and the results, the reader is referred to the accompanying paper (Baderschneider et al., 2002).

3.3. Specific IgE binding

None of the patient sera demonstrated binding of IgE to the novel protein as determined by Maxisorp RAST. The binding seen with some of the sera to the protein was non-specific as the CPM values without coating were in the same range. These values reflect IgE binding to the blocking agent (RPMI 1640 with 5% FCS (Table 1). Eighteen out of 20 patients had IgE against ocean pout as determined by Maxisorp RAST (Fig. 4). 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

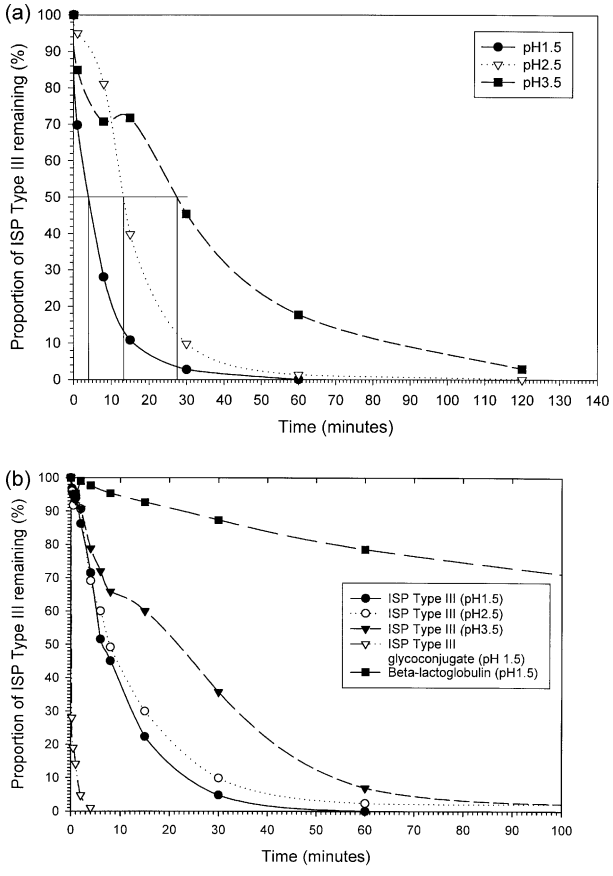

Fig. 3. (a) Proteolytic degradation of ISP Type III by pepsin under different pH conditions estimated by densitometric analysis of SDS–PAGE gels. (b) Proteolytic degradation of ISP Type III by pepsin under different pH conditions estimated by HPLC analysis (native ISP) and gel filtration chromatography (glycoconjugate). Degradation of the positive control β-lactoglobulin is shown for comparison.

μg/ml. The IC_{50} values were for 16/18 sera less than 0.2 μg/ml. The specific IgE binding to ocean pout could in no case be inhibited by the ISP when protein concentrations up to 200 μg/ml were used.

3.4. Basophil histamine release

None of the patients' basophils released histamine when exposed in vitro to the novel protein, whereas the test was positive in all patients with eel, eel pout and ocean pout extracts (Table 1).

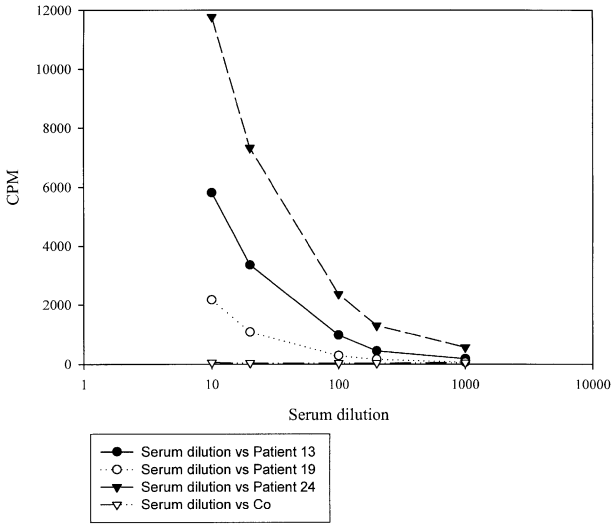

Fig. 4. Demonstration of the specific IgE binding to ocean pout for three representative serum samples using Maxisorp RAST. Control (Co) is a pool of sera from non allergic patients.

4. Discussion

Novel proteins possess two potential hazards with respect to allergenicity, namely they can provoke reactions in individuals who are sensitised to the protein or a structurally-related protein or they can induce sensitisation in susceptible individuals. No predictive methods currently exist that can give complete assurance that a novel protein lacks the ability to induce reactions or to sensitise. However, methods exist which, in combination, can help form a judgment on the safety of a novel protein with respect to those key endpoints. In the present paper we assess the potential allergenicity of an ice-structuring protein (ISP Type III) originating from the ocean pout and expressed in the food-grade bakers' yeast *Saccharomyces cerevisiae*.

In order to assure the safety of foods produced through modern biotechnology, applicants in most developed countries must submit data on their safety to governmental regulatory agencies prior to commercialisation. Regulatory agencies have identified potential allergenicity as a key safety issue with novel proteins (e.g. EU Novel Foods Directive EC97/258). The FAO/WHO recommendations regarding assessment of the allergenicity of novel proteins, published in 2000 and derived from the 1996 IFBC/ILSI scheme (Metcalfe

Table 1
Summary of the in vitro experiments on IgE binding (Maxisorp RAST and histamine release)

Patient	SPT	Direct Maxisorp RAST*			RAST inhibition of the IgE binding to Ocean Pout			HR°	
	Ocean pout	Ocean pout	Ice-structuring protein	RPMI FCS	With ocean pout	IC_{50}	With ice-structuring protein	Ocean pout	Ice-structuring protein
1	+	340±21	32±18	24±4	+	<200 ng/ml	–	60 ng/ml	–
2	+	373±34	26±7	25±12	+	36 ng/ml	–	6 μg/ml	–
3	+	327±6	29±6	23±9	+	44 ng/ml	–	6 μg/ml	–
4	+	11 935±472	46±1	28±8	+	591 ng/ml	–	Inconcl.	Inconcl.
5	+	266±17	15±5	13±1	+	40 ng/ml	–	20 μg/ml	–
6	+	4551±1112	691±169	886±19	+	<200 ng/ml	–	0.6 μg/ml	–
7	+	2200±161	81±2	94±6	+	<200 ng/ml	–	0.2 μg/ml	–
8	+	5814±325	375±68	214±6	+	<200 ng/ml	–	0.6 μg/ml	–
9	+	336±42	33±3	30±16	+	<200 ng/ml	–	6 μg/ml	–
10	+	12 104±623	83±4	76±5	+	349 ng/ml	–	20 ng/ml	–
11	+	2705±18	279±37	135±3	+	<200 ng/ml	–	0.2 μg/ml	–
12	+	657±18	42±4	33±9	+	<200 ng/ml	–	20 μg/ml	–
13	+	2167±122	162±21	131±37	+	<200 ng/ml	–	20 μg/ml	–
14	+	2858±243	47±11	60±1	+	<200 ng/ml	–	2 μg/ml	–
15	+	7539±570	52±10	65±11	+	<200 ng/ml	–	0.2 μg/ml	–
16	+	1369±28	76±1	101±14	+	<200 ng/ml	–	0.6μg/ml	–
17	+	11 765±368	884±34	1104±239	+	<200 ng/ml	–	60 μg/ml	–
18	+	21±5	75±5	22±0	nd	nd	nd	Inconcl.	Inconcl.
19	+	1486±32	136±10	75±1	+	<200 ng/ml	–	2 μg/ml	–
20	+	44±1	33±2	11±8	nd	nd	nd	200 μg/ml	–

*CPM values for sera diluted 10 times. IC_{50} = concentration used for 50% inhibition. Nd = Not determined. The lowest concentration showing a positive HR.

et al., 1996), were themselves updated during a conference in Rome in January 2001 (WHO, 2001). Several significant changes were made to the original decision tree for assessing allergenic potential. First, the requirement for in vivo investigations in man (skin prick test and oral challenges) was removed as a mandatory requirement for ethical reasons. Secondly, detailed guidelines were provided for investigating proteolytic resistance, binding to IgE and amino acid sequence similarity to known allergens. Thirdly, development of suitable animal models for risk assessment was encouraged, although it was acknowledged that available models were not yet sufficiently developed to produce data for use in risk assessments.

We present the results of the assessment of allergenic potential of a novel protein originating from a likely allergenic source using the elements of the new decision tree, proposed by FAO/WHO (WHO, 2001). Assessment of the allergenicity of a protein must consider two issues: first, could the protein provoke reactions in individuals allergic to the source from which it originated and secondly, how likely is the protein to sensitise susceptible individuals? These questions are addressed essentially by the left-hand side and right-hand side of the decision tree (Fig. 1), respectively.

4.1. Risk to fish-allergic individuals

Neither the recommended procedures, nor the additional procedures we used to refine the assessment, produced any indication that the protein would produce adverse effects if ingested by fish-allergic individuals, who were judged to be the principal group potentially at risk. Although originating from a fish which produced positive skin prick test results and positive results in in vitro allergenicity testing in established fish-allergic patients, the protein did not bind IgE from fish-allergic patients, nor did it show any activity in a functional biological assay using basophils from the same fish-allergic individuals. From a statistical point of view, the panel of 20 patient sera used gives approximately a 99% level of confidence that an allergen with a prevalence of 20% in that population would be detected. (Armitage et al., 2001). The existence of biologically insignificant IgE, capable of binding in vitro to allergens but not capable of activating a biological response has been demonstrated (Taylor and Hefle, 2001). This possibility was addressed in the present study by prospective inclusion of investigations on basophil leucocytes to ascertain the potential biological significance of any IgE-binding.

5. Risk of sensitisation in susceptible individuals

No predictive test exists to ascertain the probability that a protein will induce an IgE response in susceptible individuals. However, amino acid sequence analysis and susceptibility to proteolytic breakdown can provide information to form a judgment on this aspect. Sequence analysis clearly showed no primary sequence similarity with any known allergens, including fish allergens. Furthermore, the searches did not produce any hits against classes of proteins known commonly to contain allergens, such as chitinases or lipid transfer proteins. Significantly, when analysed using an 8-amino-acid reading frame, the only hits were with other ISPs. Narrowing the reading frame to 7- or 6-amino acids increased the number of hits with unrelated proteins, but still did not produce any hits with known allergens. In contrast to the major allergen in fish (Gad c1 and analogues), which is heat stable and resistant to proteolytic degradation, ISP Type III and its glycoconjugate were readily broken down by pepsin under the recommended standardised conditions as well as under milder conditions. The size of the protein also shows some relationship to allergenicity, with most food allergens falling within a 10–60 kDa range, in contrast to ISP Type III which has a molecular weight of less than 7.0 kDa. Overall, the weight of evidence indicates that ISP Type III is also very unlikely to sensitise potentially susceptible individuals.

Although the decision tree includes animal models, the Rome Consultation (WHO, 2001) recognised that such models were at too early a stage of development to generate data for risk assessment, but that their development should be encouraged. In these circumstances, the use of animals would arguably have been unethical. We therefore did not include an animal study in this investigation.

In summary, using the proposed decision tree, we demonstrated the safety of the novel protein to patients already sensitised to fish, as well as to a wider population of individuals potentially susceptible to producing IgE responses to proteins. These findings are also consistent with the conclusions that can be drawn from considerations of human exposure to ISPs. These conclusions are that if those proteins were 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).

In addition to these scientific outcomes, this work also demonstrated the practicability of the decision tree approach recommended by the Rome 2001 Consultation.

Acknowledgements

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