



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

C. Bindslev-Jensen^{a,*}, E. Sten^b, L.K. Earl^c, R.W.R. Crevel^c, U. Bindslev-Jensen^a,
T.K. Hansen^a, P. Stahl Skov^{b,d}, L.K. Poulsen^b

^aAllergy Center, Department of Dermatology, Odense University Hospital, Sdr Boulevard 29, DK 5000 Odense C, Denmark

^bLaboratory of Allergy Clinic, National University Hospital, Copenhagen, Denmark

^cSafety and Environmental Assurance Centre, Unilever, Colworth House, Sharnbrook, Bedford, UK

^dThe Reference Laboratory, National University Hospital, Copenhagen, Denmark

Accepted 19 July 2002

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.

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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.

* Corresponding author. Tel.: +45 65413620; fax: +45 63121507.

E-mail address: carsten.bindslev-jensen@allergi.sdu.dk (C. Bindslev-Jensen).

produced by genetic modification (Metcalf 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).

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 anti-freeze 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; Bruinjeel-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

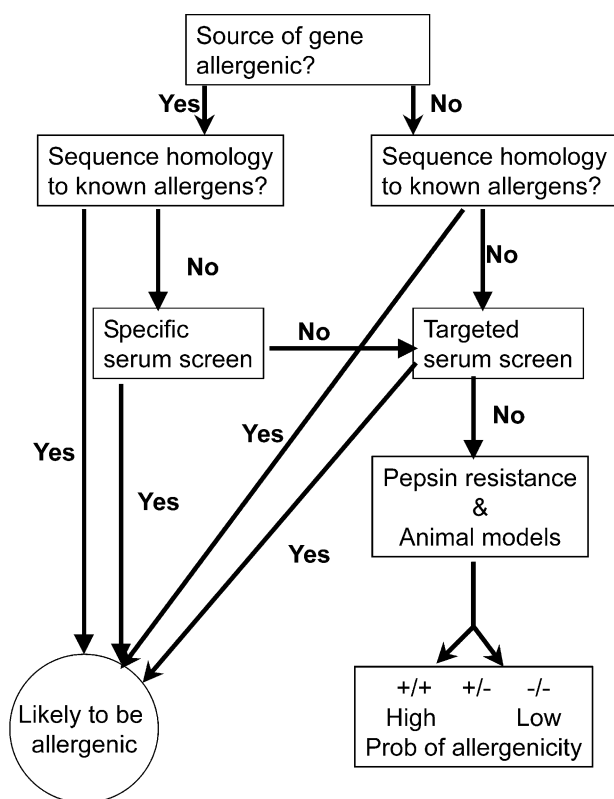


Fig. 1. The FAO/WHO (2001) decision tree.

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 H₂O. 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 I¹²⁵ 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 Videnskabetiske 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.

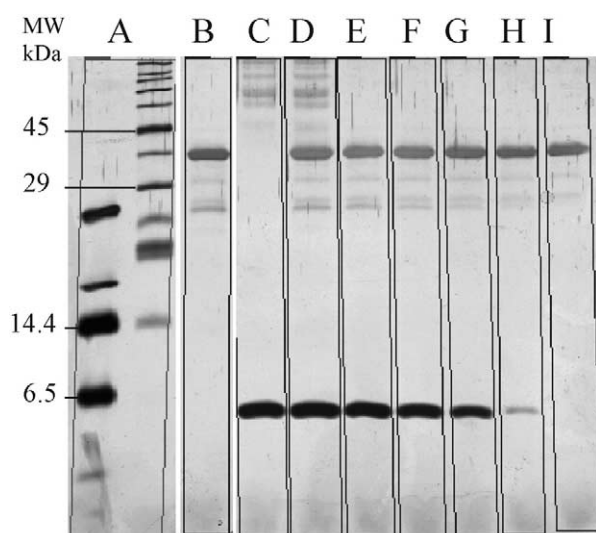


Fig. 2. SDS-PAGE gel illustrating pepsin hydrolysis of ISP Type III at pH 1.5. (A) Molecular weight markers, (B) pepsin control, (C) ISP control, (D) 0 min, (E) 1 min, (F) 6 min, (G) 15 min, (H) 30 min, (I) 60 min.

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

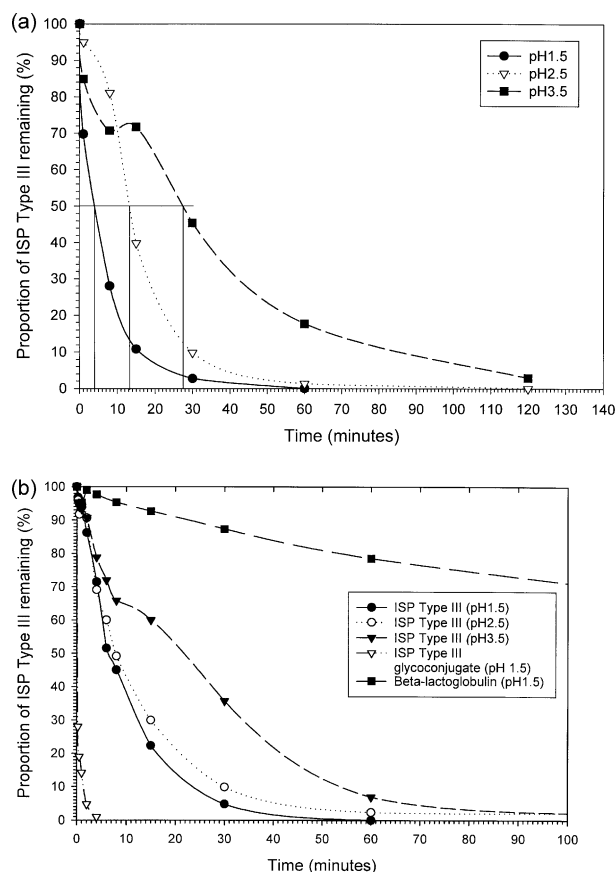


Fig. 3. (a) Proteolytic degradation of ISP Type III by pepsin under different pH conditions estimated by densitometric analysis of SDS-PAGE gels. (b) Proteolytic degradation of ISP Type III by pepsin under different pH conditions estimated by HPLC analysis (native ISP) and gel filtration chromatography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

µg/ml. The IC₅₀ 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).

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 (Metcalf

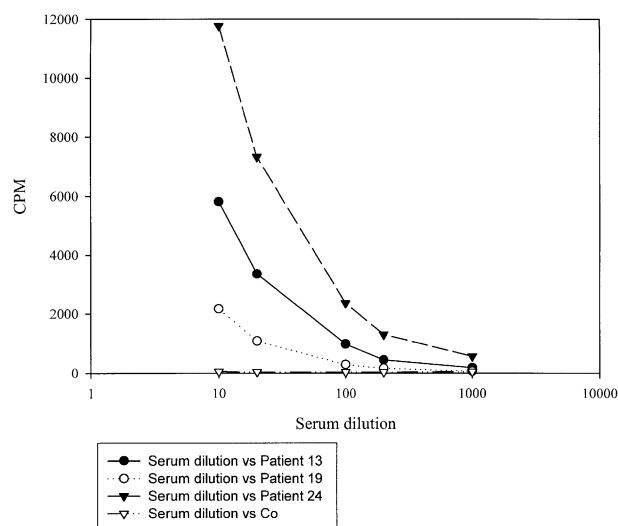


Fig. 4. Demonstration of the specific IgE binding to ocean pout for three representative serum samples using Maxisorp RAST. Control (Co) is a pool of sera from non allergic patients.

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 ^o	
		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. IC₅₀=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

Sources of support: The serological part of the study was funded by the Danish Medical Research Council (The Bio-Risk Study), Grant No 9801279). Patient costs for transportation were covered by Unilever plc.

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