



Lack of immunogenicity of ice structuring protein type III HPLC12 preparation administered by the oral route to human volunteers

R.W.R. Crevel ^{a,*}, K.J. Cooper ^a, L.K. Poulsen ^b, L. Hummelshoj ^b,
C. Bindsvlev-Jensen ^c, A.W. Burks ^d, H.A. Sampson ^e

^a Safety and Environmental Assurance Centre, Sharnbrook, Unilever Colworth, Bedford MK44 1LQ, UK

^b Allergy Center, Odense University Hospital, Denmark

^c Allergy Department 7542, National University Hospital, Copenhagen, Denmark

^d Duke University Medical Center, Durham, NC, USA

^e Mount Sinai School of Medicine, New York, USA

Received 28 July 2005; accepted 18 July 2006

Abstract

Before a novel protein can be used in foods, its potential allergenicity must be assessed. In this study, healthy volunteers consumed ice structuring protein (ISP) Type III preparation or a control material 5 days a week for a total of 8 weeks. General measures of health were recorded during the study, and the immunogenicity of the protein was assessed by monitoring the levels of IgG and IgE antibodies specific for ISP Type III. The participants remained in good health throughout the study and during the 4 week follow-up period. No IgG or IgE antibodies specific for ISP Type III were detected in the blood of the participants. Investigations of immunogenicity in man have not been previously applied in the context of safety evaluation and they do not form part of the regimens proposed for the evaluation of protein allergenicity. Consequently no standardised protocols exist for such studies, nor any background against which to interpret the results. Nevertheless, the absence of an immune response using a protocol which could have been expected to result in a response with a strongly immunogenic protein, confirms the conclusions of earlier published work, and attests to the lack of allergenicity of ISP Type III preparation.

© 2006 Elsevier Ltd. All rights reserved.

Keywords: Novel protein; Safety assessment; Allergenicity prediction

1. Introduction

Ice structuring proteins (ISPs) are naturally occurring proteins and peptides that bind to ice and are found widely in nature, for example, in cold water fish, vegetables, grains, lichens and bacteria. These proteins help organisms cope with very cold environments by both lowering the temperature at which ice crystals grow and by modifying the size and shape of the ice crystals that are formed so that the ice is less damaging to tissues. ISP Type III HPLC12 was one of 12 isoforms originally isolated from the ocean pout (*Macrozoarces americanus*), a cold-water fish found off the northeast coast of North America. Incorporation

Abbreviations: BSA, bovine serum albumin; EAACI, European academy of allergy and clinical immunology; ELISA, enzyme-linked immunosorbent assay; HRP, horseradish peroxidase; ISP, ice structuring protein; KLH, keyhole limpet haemocyanin; OPD, *O*-phenylenediamine; PBS, phosphate-buffered saline; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); PVDF, polyvinylidene fluoride; RAST, radio-allergosorbent test; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

* Corresponding author. Tel.: +44 01234 264757; fax: +44 01234 264744.

E-mail address: rene.crevel@unilever.com (R.W.R. Crevel).

of ISP Type III into edible ices offers benefits both in terms of nutritional and organoleptic profile and greater temperature stability.

Before a novel protein can be incorporated into foods, its safety must be assessed. According to internationally recognized standards for products of biotechnology (Codex Alimentarius Commission, 2002), this assessment must include an evaluation of potential allergenicity. This allergenicity evaluation must consider both the potential of the protein to provoke reactions in people already sensitized to similar (e.g. cross-reactive) proteins, and the potential to induce a *de novo* immune response in susceptible individuals. We evaluated the general safety and allergenicity of ice structuring protein (ISP) Type III preparation using the recommended methodologies, and showed it to be safe and lacking in allergenicity (Bindslev-Jensen et al., 2003; Baderschneider et al., 2002; Hall-Manning et al., 2004). However, it is recognized that no current test, or combination of tests, is fully predictive of the potential to sensitise. We therefore undertook an additional study in which volunteers ingested ISP Type III preparation over a period of two months and monitored both their general health, and the immune response to the protein.

Ingested proteins, including dietary proteins, cross the intestinal mucosa and generate an immune response in both experimental animals and man (Husby et al., 1985; Husby, 2000; Heyman, 2001). In man, ingestion of soluble proteins generally, although not invariably, results in very low specific serum antibody levels (Korenblatt et al., 1968; Husby et al., 1994), although mucosal antibody responses can be more vigorous. This immune response has been extensively studied and it is now generally recognized to be a normal, physiological response to protein ingestion, rather than pathological one. Investigations of antibody production resulting from ingestion of proteins in man have thus been undertaken in a research context, and could be difficult to interpret in the context of the safety assessment of a protein. However, we reasoned that a study of the antibody response to ISP Type III could provide valuable additional evidence for very limited allergenic potential, and thus greater safety.

The purpose of this investigation was thus twofold

1. To determine whether exposure to ISP Type III preparation daily for up to 8 weeks would induce the production of specific IgG or IgE antibodies to the protein.
2. To observe the effect of daily exposure to ISP Type III preparation for up to 8 weeks on physiological parameters in normal healthy individuals.

2. Study design, participants, materials and methods

2.1. Study design

The study was a single-blind, placebo-controlled study consisting of an 8 week treatment phase, followed by a 4 week follow-up period. Parameters recorded included indices of general health, including an interview

with a medical practitioner, blood pressure, heart rate, general clinical chemistry parameters, as well as specific measures of immune responsiveness, such as specific serum IgG and IgE antibody levels. These measures were repeated during the study at 4, 6, 8 and 13 weeks. Participants were also asked to report any adverse effects that they may have experienced during the course of the study, regardless of whether they considered them to be treatment-related. The study design and informed consent form were approved by the local Research Ethics Committee.

2.2. Participants

Forty-two normal, apparently healthy adults (age range 22–58 years) without a history of previous consumption of ISP type III were recruited for the study. Participants were selected on the results of a pre-study medical in which they were questioned on medications, allergy, hay fever, asthma and eczema status and their general health. Pulse rate and blood pressure readings also were taken, and a non-fasting pre-screen blood sample was taken for clinical chemistry/haematological analysis. Thirty-seven volunteers (8 male, 29 female) met the eligibility criteria for inclusion. Twenty-eight of these individuals were then allocated randomly to the test group and nine to the control group. Based on the pre-study questionnaire, seven members of the test group and four of the control group had an atopic predisposition. All participants provided written informed consent prior to the start of the study.

2.3. Test materials

The preparation produced by yeast fermentation is a cell-free brown liquid consisting of functionally active ISP Type III HPLC 12 (one of 12 isoforms of ISP Type III), inactive mannose-conjugated (glyco-)ISP Type III, proteins and peptides from the yeast fermentation, and sugars, acids and salts commonly found in food. Lack of toxicity and allergenic reactivity was established prior to the study (Hall-Manning et al., 2004; Bindslev-Jensen et al., 2003; Baderschneider et al., 2002). The material was diluted with ultrapure water, filter-sterilised, aliquoted in amounts sufficient for a daily dose and stored frozen until use. Immediately prior to use, it was mixed into a flavoured drink. Flavoured ultrapure water served as the placebo.

Additional test materials were used in the laboratory phases of the study. These included pure ISP Type III, a freeze-dried form of the ISP preparation and a fermentation supernatant from the wild type of the *Saccharomyces cerevisiae* strain used to produce the ISP preparation.

2.4. Treatment

Individuals in the test group received filter-sterilised ISP type III preparation providing 16.3 mg ISP type III HPLC 12 in a flavoured drink daily for 5 days a week for 8 weeks. The selected dose corresponds to an estimate of ISP type III HPLC 12 intake for the 90th percentile of ice-cream consumers in the USA, assuming that the product contained the highest envisaged concentration of ISP Type III (0.1 mg/ml). No correction was made for body weights. The control group received the flavoured drink alone. Blood samples (20 ml) were obtained immediately prior to the start of the test, and at 4, 6, 8 and 13 weeks for the measurement of serum concentrations of IgG and IgE antibodies specific to ISP type III, as well as for haematological and clinical chemistry indices.

2.5. Haematology and clinical chemistry

The following routine haematological indices were measured on a Beckman Coulter GEN.F haematology analyser: erythrocyte count, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration, platelet count, reticulocyte count, leukocyte count and differential count to include neutrophils, lymphocytes, monocytes and eosinophils. The following routine clinical chemistry parameters were analysed on a Roche Cobas Integra 700 clinical chemistry analyser using standard Roche methods.

logy: sodium, potassium, creatinine, urea, total protein, albumin, cholesterol, C reactive protein, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, γ -glutamyl transferase and bilirubin.

3. Measurement of specific antibody to ISP Type III HPLC12

3.1. Specific IgG

Specific IgG to ISP type III HPLC 12 was measured by enzyme-linked immunosorbent assay (ELISA). The solid phase was prepared by coating the wells of a microtitre plate (Greiner, UK) with an anti-ISP monoclonal antibody in carbonate buffer, followed by ISP type III preparation in phosphate-buffered saline containing Tween 20 and 1% normal rabbit serum. Test or control sera, diluted 1:50 in the same buffer, were then added, followed by anti-human IgG (Sigma) conjugated to alkaline phosphatase. In the absence of human serum containing IgG antibodies to ISP Type III, sera from individuals with specific antibodies to a bacterial protease or fungal lipase served as positive controls, the plates being coated with the relevant antigen instead of ISP Type III. Absorbance at 490 nm was measured on a Spectramax 190 plate reader (Molecular Devices, Sunnyvale, CA, USA). The inhibition ELISA was performed as above except that the test sera were replaced by inhibition mixtures consisting of different ratios of test serum and potential antibody binding material, incubated overnight at 1–8 °C.

3.2. Specific IgE

Specific IgE to ISP type III preparation was measured using the direct MaxiSorp™ RAST tube method as previously described (Poulsen et al., 1989). Maxisorp polystyrene test tubes (Nunc, Roskilde, Denmark) were coated with the appropriate antigen diluted in PBS (pH 7.4) and incubated overnight at 37 °C. Antigens included ISP Type III preparation (freeze-dried) (2 μ g/ml), yeast fermentation supernatant (2 μ g/ml) or pure ISP Type III (1 μ g/ml). Binding of ISP Type III was verified by detecting with the mouse monoclonal antibody to ISP Type III also used in the IgG detection experiments. Free binding sites on the tubes were then blocked by incubation for 2 h with RPMI 1640 containing 10% foetal calf serum. The serum samples were diluted in PBS (pH 7.4) with 0.1% Tween 20, and 250 μ l aliquots were added to each tube. After a further overnight incubation, ^{125} I labelled anti-IgE (Pharmacia, Uppsala, Sweden), diluted 1:12 in PBS with 0.1% Tween 20, was added and the mixture was incubated on a vibrating shaker overnight at room temperature. Activity (cpm) was counted for 2 min in a gamma-scintillation counter. Between each step, the test tubes were washed three times with PBS containing 0.1% Tween 20. One tube, for which the antigen coating step was omitted, but the remaining steps were identical to those described above, served as a negative control for the secondary antibody. Serum from

a birch pollen-allergic individual (RAST Class 4) was used as the positive control, the tubes being coated with birch pollen antigen instead of ISP Type III preparation. The background value, determined with a pool of sera from non-allergic donors, was subtracted from all values. An inhibition MaxiSorp™ RAST was performed as above, except that the diluted sera were pre-incubated overnight at 4 °C together with the materials above at a range of concentrations (0, 1:125, 1:25 and 1:5). They were then centrifuged at 1650 $\times$ g for 15 min before use. The degree of inhibition was calculated and expressed as the percentage of the CPM value for the inhibited sample divided by the value of the uninhibited.

4. SDS-PAGE and immunoblotting

The SDS-PAGE and Western blots were performed broadly in accordance with the protocols described by Laemmli (1970) and Hansen et al. (1997). Slightly different protocols were used for IgG and IgE immunoblotting.

SDS-PAGE and IgG immunoblotting. The ISP Type III preparation was electrophoresed in a 4–20% linear gradient Ready Gel (Biorad). The samples and molecular weight markers were first diluted in a reducing buffer with SDS, and boiled for 5 min. They were then loaded at the volume recommended by the manufacturer, and electrophoresed at a constant voltage of 200 V until the dye front had nearly run off the gel. The proteins were blotted on to a PVDF membrane (Biorad) at a constant voltage of 100 V for 4 h. The membranes were then blocked with phosphate buffered saline containing 0.05% Tween 20 (PBST) and 1% fish gelatin for at least 1 h at room temperature and cut into strips before incubating for at least 1 h with serum samples diluted 1:50 in PBST. After another wash the membranes were incubated with monoclonal mouse anti-human IgG conjugated to alkaline phosphatase (Sigma). A similar process was used to provide a positive control, except that the serum dilutions were replaced by a monoclonal anti-ISP Type III antibody, and the anti-human IgG antibody was replaced by a biotinylated goat anti-mouse IgG (Sigma), followed by streptavidin conjugated to alkaline phosphatase. Binding was visualised using the BCIP/NBT substrate.

SDS-PAGE and IgE immunoblotting. The ISP Type III preparation was electrophoresed in a 1 mm gel with a 16% separation gel and a 3.6% stacking gel. The samples were first diluted in a reducing buffer with SDS, but not boiled. A 200 μ l sample with a final protein concentration of 0.125 mg/ml was added to a 53 mm long well. Rainbow markers (Low range, Amersham Pharmacia Biotech), diluted 1:10, were added to one well on each gel. The proteins were blotted on to a PVDF membrane (Millipore, Bedford, MA) for 1.5 h ($I = 1.0$ mA/cm²). The membranes were then blocked with Tris buffered saline (TBS) containing 5.0% BSA for 2 h at room temperature and cut into strips before incubating overnight at 4 °C with the sera (diluted 1:10 in TBS). After another wash the membranes

were incubated with rabbit anti-human IgE-Horseradish peroxidase (HRP) conjugate (Dako A/S, Glostrup, Denmark) at a concentration of 1.3 µg/ml for 2 h and washed with PBS. ECL Western blotting detection reagent (Amersham Pharmacia Biotech) was used as substrate. The blots were then exposed to X-ray film for approximately 5–10 s to visualise antibody binding. A similar process was used to optimise ISP concentrations and demonstrate binding to ISP, except that the anti-human IgE antibody was replaced by a goat anti-mouse IgG HRP conjugate.

4.1. Skin prick testing

Skin prick testing was performed in accordance with EAACI guidelines (Hansen and Bindslev-Jensen, 1992; Hansen et al., 1996). Drugs interfering with skin prick testing were discontinued prior to testing. The following test materials were used: ISP Type III preparation and pure ISP Type III (both at 5 mg/ml, 1 mg/ml, 100 µg/ml and 10 µg/ml, expressed as ISP), Yeast Fermentation Supernatant (3 mg/ml, 0.87 mg/ml, 87 µg/ml and 8.7 µg/ml). Dilutions were prepared in skin prick test buffer (ALK-Abello, Horsholm, Denmark), consisting of 50% glycerol in 0.9% saline. In addition to the above solutions, the panellist was also skin prick tested with a range of common food and inhalant allergens to determine allergological status.

4.2. Basophil histamine release

Basophil histamine release was performed by the glass fibre technique, in accordance with the manufacturer's instructions (RefLab, Copenhagen, Denmark). Each test material was diluted in PIPES buffer and a passive sensitisation technique was used. In brief, buffy coat leukocytes were enriched on a Lymphoprep gradient and sensitised for 1 h with 250 µl of test or control serum. Cells were then challenged with dilutions of the extracts, and the released histamine trapped on glass-fibre coated microtitre plates. Remaining cellular histamine was measured by the OPD-method and percentage histamine release calculated on the basis of total histamine content in non-challenged cells.

5. Results

5.1. Clinical measures and participants' reports

Vital signs recorded on each blood sampling occasion showed all panellists to be in a good and generally consistent state of health throughout the study. Several comments were recorded, e.g. sore throat, grumbling stomach. However, on the basis of their nature and pattern of occurrence, these were judged by the study physician to be unrelated to ingestion of the test material. When asked about their allergic status at the pre-medical, 12 of the 28 panellists in the test group and six of the nine in the control group considered themselves to be allergic/intolerant (Table 1). Analysis of the pre-study questionnaire sug-

gested that seven members of the test group, and four of the control group were atopic.

All haematological and clinical chemistry indices remained within their reference ranges (results not shown), except in subject 36 (treatment group), who experienced a very high C-reactive protein level at week 4. This was directly attributable to a cold she was suffering at the time.

Subject 7, assigned to the test group, reported mild itching on the upper body from the outset of drinking the test material. The sensation started after ingestion of the material on the Monday of weeks 1–8, with a gradual subsidence in intensity throughout the course of each week. There was no accompanying rash or any other independently observable sign, nor any antibody response of any type. To elucidate the reason for her reaction, treatment was extended for this panellist only, by an extra two weeks, during which the panellist received the control material flavoured with the same flavour cordial (blackcurrant), as selected throughout the study. To avoid bias, the panellist was unaware of the change in treatment. The panellist did not experience itching during the two weeks she received the control material. Analysis for IgG antibodies in the serum from this subject also showed elevated optical densities in the pre-test samples, suggesting either a recall response to a non-ISP component of the preparation or non-specific binding. As discussed later, a recall response would likely have been stimulated by continued ingestion. The kinetics of the response and its disappearance on ingestion of the control material therefore suggest that it is most likely a non-immune mediated intolerance to one of the constituents of the ISP preparation, most likely the yeast products.

5.2. IgG response to ISP Type III HPLC12

Sera from subjects 7, 18, 21, 25, and 28 in the treatment group, and subject 16 in the control group produced optical densities above the cut-off point of the assay throughout the study (Table 2). As these optical densities were elevated in the pre-test sera and did not increase as the study progressed, these results indicate that ingestion of the test material did not induce production of specific IgG antibody, nor did it stimulate any potential pre-existing response. The binding of the sera from the test group was further investigated in inhibition experiments with the test material (ISP type III preparation) or mannose (the sugar residue found on glycosylated ISP). Neither material produced any meaningful inhibition, indicating that they were not responsible for the binding of IgG (results not shown). Since these values were elevated prior to any exposure to the test material, they therefore seem most likely to be due to a higher level of non-specific binding of the individuals' IgG antibodies. Immunoblotting analysis of the same samples showed a weak band at the same position as ISP Type III, but this band, as well as other bands corresponding to the yeast proteins, also

Table 1
Allergological medical history of study participants

Subject	Allocation	Relevant medical history	Atopic (by history)
1	Test	Mild asthma (no treatment)	Yes
2	Test	Eczema in auditory canals (no treatment)	
3	Test		
4	Control		
6	Test		
7	Test	Caffeine intolerance	
8	Test	Mild hayfever (no treatment), mild eczema (occasional hydrocortisone, but none at present), some metal allergy to jewellery	Yes
9	Control	Childhood asthma (now OK)	
10	Test	Allergy to nickel	
11	Test		
12	Test	Allergy to penicillin and primulas	
13	Control	Hayfever (allergic rhinitis—no treatment), mild eczema (no treatment)	Yes
14	Test	Allergy to nickel	
15	Test		
16	Control	Mild hayfever (no treatment)	Yes
17	Control	Mild hayfever (no treatment), allergy to nickel	Yes
18	Test		
19	Test	Occasional hayfever (no treatment)	Yes
20	Test		
21	Test		
22	Test		
23	Test		
24	Test		
25	Test		
27	Test		
28	Test		
30	Test	Hayfever (mild allergic rhinitis—no treatment)	Yes
31	Test		
32	Control		
34	Control	Possible nickel allergy	
35	Test	Hayfever	Yes
36	Test	Allergies to nickel, palladium and elastoplast	
38	Test	Hayfever	Yes
39	Control	Hayfever (no treatment), childhood asthma (now OK)	Yes
40	Test	Mild hayfever (no treatment), mild asthma (no treatment)	Yes
41	Control		
42	Test		

stained with a negative control serum sample (non-participant in study).

5.3. IgE response to ISP Type III

The test revealed weak specific IgE binding to ISP Type III preparation with one serum (subject 19), peaking at week 4. It was not accompanied by an IgG response, casting some doubt on whether it was a true positive finding, and the subject did not report any symptoms on ingestion of ISP Type III preparation. Nonetheless, this response was further investigated using RAST inhibition, basophil histamine release, and immunoblots to identify the IgE binding components, as well as skin prick testing to confirm the result.

5.4. Additional investigations in subject 19

Subject 19 showed a marginally positive skin prick test to ISP Type III preparation and yeast fermentation supernatant, but not to pure ISP Type III. This subject also did not respond when skin prick tested with ocean pout, which contains a higher concentration of ISP Type III than the skin prick test preparation. Immunoblots and basophil histamine release experiments were similarly negative (Fig. 1). Yeast fermentation supernatant inhibited the RAST response to ISP Type III preparation, as did pure ISP Type III, but to a much lower extent, raising doubt on its relevance. However, the biological significance of this result is doubtful given the initial very low activity of the sample.

Table 2
Specific IgG and IgE antibody responses to ISP type III and ISP Type III preparation respectively

Subject no.	Specific IgG (OD units) ^a					Specific IgE ^b (cpm)				
	Week 0	Week 4	Week 6	Week 8	Week 13	Week 0	Week 4	Week 6	Week 8	Week 13
1	0.047	0.047	0.034	0.038	0.073	30.7	38.5	54.8	45.8	56.1
2	0.061	0.062	0.044	0.044	0.058	62.1	56.8	52.1	48.3	45.6
3	0.035	0.036	0.036	0.039	0.050	65.8	37.1	55.5	46.4	64.3
6	0.047	0.047	0.042	0.031	0.057	41.3	45.0	41.1	48.8	65.7
7	0.640	0.648	0.648	0.640	0.590	25.2	28.8	44.2	65.2	50.4
8	0.058	0.051	0.049	0.056	0.037	56.8	44.7	51.1	63.7	59.8
10	0.039	0.036	0.031	0.038	0.032	33.5	62.4	45.4	43.4	44.3
11	0.035	0.032	0.031	0.039	0.024	54.1	33.7	32.0	32.5	43.2
12	0.059	0.034	0.036	0.035	0.036	42.6	44.0	38.3	65.0	57.6
14	0.025	0.032	0.022	0.022	0.023	90.8	38.8	33.8	40.8	40.7
15	0.041	0.044	0.038	0.032	0.074	65.7	41.6	41.9	46.2	37.7
18	0.474	0.535	0.477	0.520	0.502	64.8	48.9	41.5	75.3	52.8
19	0.038	0.042	0.041	0.045	0.025	<i>66.3</i>	141.1	100.0	168.7	137.1
20	0.025	0.028	0.031	0.025	0.018	66.7	51.0	38.8	45.4	27.8
21	0.125	0.125	0.133	0.124	0.139	42.9	42.8	43.7	89.3	78.1
22	0.021	0.021	0.021	0.025	0.026	44.3	45.9	26.2	48.8	41.4
23	0.033	0.035	0.037	0.042	0.048	48.1	33.3	36.3	39.4	33.5
24	0.055	0.051	0.044	0.042	0.035	42.3	42.2	34.6	42.4	39.6
25	0.146	0.158	0.163	0.144	0.117	62.2	55	36.9	37.5	50.9
27	0.061	0.059	0.053	0.063	0.033	58.1	44.7	50.0	34.4	52.3
28	0.143	0.148	0.158	0.148	0.235	55.3	48.9	36.9	43.2	46.8
30	0.077	0.078	0.087	0.076	0.055	61.3	42.9	58.5	50.9	41.8
31	0.064	0.062	0.073	0.059	0.030	52.6	41.5	36.8	55.2	48.1
35	0.066	0.059	0.053	0.055	0.057	57.1	38.4	56.7	58.5	52.7
36	0.036	0.039	0.046	0.042	0.024	34.5	63.7	49.7	54.4	64.3
38	0.064	0.054	0.053	0.064	0.039	45.9	65.7	55.9	67.1	49.4
40	0.117	0.091	0.089	0.089	0.067	49.2	37.2	57.2	63.4	59.6
42	0.041	0.042	0.044	0.043	0.028	48.1	47.8	60.4	53.3	79.2
<i>Controls</i>										
4	0.030	0.025	0.029	0.032	0.032	64.6	44.5	43.5	53.3	50.9
9	0.025	0.019	0.018	0.027	0.023	61.5	34.6	29.7	55.3	41.3
13	0.072	0.032	0.036	0.030	0.050	45.6	55.8	39.7	42.3	36.8
16	0.111	0.120	0.109	0.086	0.105	70.1	46.3	49.7	42.2	49.8
17	0.041	0.060	0.044	0.047	0.061	63.7	29.4	35.6	41.5	46.2
32	0.061	0.054	0.073	0.090	0.048	53.1	42.4	57.8	75.5	42.5
34	0.038	0.061	0.048	0.060	0.052	68.5	46.9	46.4	38.4	47.8
39	0.051	0.041	0.044	0.045	0.027	47.6	58.5	58.6	52.6	49.5
41	0.035	0.032	0.035	0.034	0.025	49.5	40.8	67.3	68.2	64.8

Note: A positive result was defined as mean negative control + three standard deviations, or an O.D. of 0.1, or radioactivity of 100 cpm, if greater. Values classed as positive by those criteria are highlighted in bold.

^a Specific IgG to ISP type III HPLC 12.

^b Specific IgE to ISP type III preparation.

An additional MaxiSorp RAST using yeast fermentation supernatant as a solid phase was positive.

Additional screening for common allergens and a mean total serum IgE level of 290 KU/l indicated that the subject was clearly atopic and sensitised to a multiplicity of common food and inhalant allergens. Inhalant allergens included birch, grass, horse, dog, cat, house dust mite and both the moulds *Alternaria* and *Cladosporium*. Skin prick tests to food were positive to hazelnut, soy, celery root, melon, apricot, and brazil nut. They were negative to wheat, milk, peanut, peach, plum, cashew, walnut, sesame, poppy seed, shrimp, codfish and all species of fish tested including herring, plaice, tuna, mackerel, salmon, trout and ocean pout. Given the negative results in the other investigations, including particularly the skin prick

tests, together with the very marginal response to ISP type III preparation by this subject, this RAST inhibition result with pure ISP type III is considered to be a false positive (Bernstein et al., 1994). Taken as a whole, these additional investigations demonstrated that subject 19 did not respond to ISP type III HPLC 12, but to the yeast proteins in the preparation (Table 3).

6. Discussion

In this study, volunteers ingested a comparatively high dose of ISP Type III preparation over a two-month period and did not show or report any significant adverse events. None showed any detectable antibody response to ISP Type III, although in the specific IgG assay, optical density

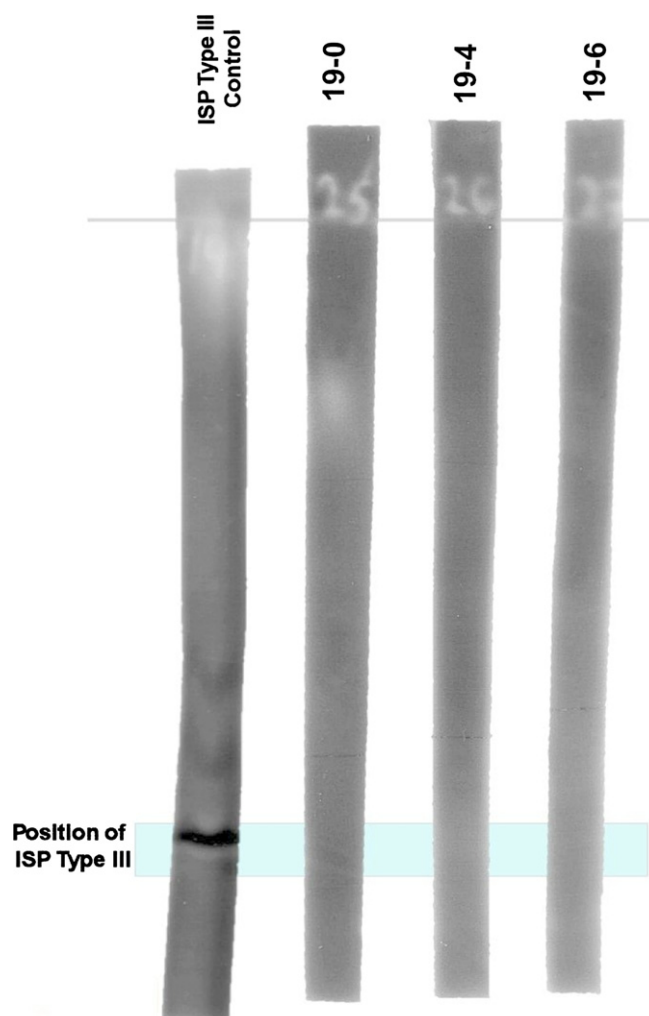


Fig. 1. Immunoblot with ISP preparation using serum from subject 19. The positive control, to show the position of ISP Type III, was detected using a monoclonal anti-ISP Type III antibody followed by a goat anti-mouse IgG HRP conjugate.

values above the cut-off point were observed in sera at all time points in one control and five test subjects. No obvious explanation exists for this observation. Prior consumption of ocean pout is almost impossible in so many subjects, as the species has never been commonly available in the United Kingdom and, in any event, has been overfished in its region of origin (Crevel et al., 2002). Cross-reactivity with ISP Type III from another source also appears highly unlikely, as one might then have expected to see a gradual increase in the response as exposure continued. One participant showed a weak IgE response to the test material, which was found to be due to the yeast component. Overall, the results confirm the general safety, and lack of allergenicity demonstrated in earlier studies.

This study is the first investigation of the immunogenicity of proteins by the oral route in man in the context of safety evaluation. The protocol was therefore based on published reports of studies designed for different purposes, modified to take into account the expected mode of use of,

and exposure to ISP Type III. It would therefore have been desirable to include a positive control of well-documented immunogenicity, instead of the diluent for the test material. An optimal choice would have been a conventional food protein of documented allergenicity. However, there are currently no accepted procedures for ranking proteins in order of allergenicity, so this option was not open to us. Furthermore, a conventional food protein would probably have been consumed as part of the diet by most, if not all of the participants. Any immune response to such a protein would have been a recall response, and therefore qualitatively different from the possible response to ISP Type III.

The dose of ISP Type III (16.3 mg/day, 652 mg in total) may be judged rather low. However, it was based on the amount of ISP Type III that would be consumed by the 90th percentile of the highest-consuming group of ice cream consumers in the USA on any single eating occasion, assuming all the product consumed contained 100 ppm ISP Type III. Products are actually expected to be formulated with a maximum of 50 ppm ISP, and the highest daily consumption would not be repeated throughout every day of the year. The dose corresponds to the ISP type III present in at least 13 kg of ice cream, or more than 325 g ice cream per working day every day for the 8 week duration of the study. The dose thus represents a considerably exaggerated exposure. Regardless of the arguments for setting the dose by considering possible exposure, a test of possible immunogenicity should use amounts of antigen that would be expected to generate a response, based on responses to other antigens. Clinical and experimental data indicate that such a dose could produce a response, provided the protein was a sufficiently potent immunogen. Thus Farr et al. (1960) found that 6.25 mg/day of bovine serum albumin (BSA) produced an antibody response in rabbits after 30 days. While Korenblatt et al. (1968) used large doses of BSA in humans to study the recall response, they found that the principal factor determining whether a response occurred at all was the presence of anti-BSA antibodies at the start of the experiment. They also observed a poor correlation between the estimated exposure to BSA in the diet and the antibody levels. In an experiment which arguably is more relevant to the present study, Tacket et al. (1998) demonstrated a vigorous serum IgG response to the cholera heat-labile enterotoxin (LT-B), engineered into potatoes, and given as three doses over 21 days. The mean dose on each occasion was 0.75 mg (0.4–1.1 mg), amounting to a total dose ranging from 1.2 to 3.3 mg. Other investigators have used daily doses of the same order as in the present study. Thus Husby et al. (1994) fed 50 mg of Key-hole Limpet Haemocyanin (KLH) per day for two periods of 5 days, separated by 10 days, to volunteers and examined both antibody and T-cell responses. They found significant proliferative responses, but no detectable serum antibodies. However, their study differs in significant respects from ours. Apart from the shorter exposure, the antigen was administered in gelatin capsules. These would have had to be digested to release the antigen, and it is

Table 3
Results of further investigations of the IgE response of subject 19

Concentration (mg/ml)				
<i>Skin prick testing</i> (mean of perpendicular diameters, in mm)				
	5.0	1.0	0.1	0.01
ISP preparation ^a	3.6	2.6	0	0
Yeast fermentation supernatant	3.5	0	0	Not tested
ISP standard	0	0	0	Not tested
Serum dilution				
<i>RAST (ISP preparation)</i> (cpm)				
Time point	1:2	1:5		1:10
Week 0	88	78		66
Week 4	165	251		133
Week 6	163	182		124
Control	53	41		37
Inhibitor	Concentration of inhibitor			
<i>RAST inhibition</i> using week 4 serum (% inhibition)				
Yeast fermentation supernatant	0	1:125	1:25	1:5
Cpm	140	50	52	39
% Inhibition	–	64	63	79
ISP standard	0	4	20	100
Cpm	140	159	110	75
% Inhibition	–	–14	21	46

^a Subject 19 also had a negative skin prick test to ocean pout, which contains high levels of ISP type III HPLC 12.

plausible that the antigen was therefore released more distally in the intestine than where the exposure to ISP would have taken place, with possible effects on the kinetics of the observed response. Matsui et al. (1996) fed 30 mg of KLH to volunteers every other day for at least 6 weeks, and observed a decrease in KLH-reactive cells over that period. While the investigations of Husby et al. and Matsui et al. differ from our own in detail, they do demonstrate that both the dose and the duration that we used are adequate for influencing the immune status of an individual with respect to an antigen they have not previously encountered. The study by Tacket et al. furthermore demonstrates that a serum antibody response can be observed with a strong immunogen when it is given over a similar time course. Experiments in animals also attest to the efficacy of a dose of this order, when given as a bolus, rather than ad libitum in the diet or drinking water (Knippels et al., 1998). Our study therefore arguably maximised the opportunity for ISP Type III to generate a serum antibody response; the fact that it did not do so is a good indication of a lack of significant immunogenicity, and consequently allergenicity. Other investigators also looked at T-cell responses; these could indeed have provided direct evidence of response to ISP. However, not only would the logistics have been difficult because of the number of volunteers in our study, interpretation would have been even more fraught with difficulty. In the circumstances, investigations of antibody responses provide a more direct read-out of the endpoint of concern with regard to safety.

The generation of an immune response to an ingested protein requires that some of that protein, or at least significant fragments of that protein reach the gastrointestinal immune system intact, although absorption of allergeni-

cally active protein through the buccal mucosa has also been demonstrated (Dirks et al., 2005). Impaired digestion therefore conceivably increases the probability of sensitisation to any protein. Recent experimental and clinical data support this contention (Untersmayr et al., 2005), showing more frequent formation of IgE to dietary proteins in patients using anti-acid medication. However, these observations pertain to dietary proteins that would be consumed in significant quantities. Without information on exposure, those data are difficult to interpret in the context of this study where the total exposure over the whole study was less than the amount of a common dietary protein that would be consumed on a single occasion. ISP Type III is readily broken down by pepsin, but has a half-life under conditions of simulated gastric digestion of approximately 10 min. As our volunteers consumed the material as a drink, rather than a meal, it is likely that some intact ISP would have reached the Peyer's patches in the intestine, as well as following absorption through the buccal mucosa. The absence of a response is thus not due to failure to the ISP to reach the immune system, but to its low immunogenicity. Caution thus needs to be exercised in applying this methodology to other proteins without due consideration of dose and other factors.

Investigations of immunogenicity in man have not been previously applied in the context of safety evaluation and they do not form part of the regimens proposed by the FAO/WHO consultation (2001) or the Codex Alimentarius ad hoc task force (2002) for the evaluation of protein allergenicity. The lack of context and historical data could have made interpretation of results difficult, had any significant responses been observed. In the event, the absence of a response using a protocol which could have been expected

to result in a response with a strongly immunogenic protein, confirms the conclusions of earlier published work, and attest to the lack of allergenicity of ISP Type III preparation.

Acknowledgements

The authors wish to thank Dr. David Rhodes for performing the medical examinations and the blood sampling, and Professor Per Stahl Skov (RefLab, Copenhagen) for performing the basophil histamine release assays.

References

- Baderschneider, B., Crevel, R.W.R., Earl, L.K., Lallje, A., Sanders, D.J., Sanders, I.J., 2002. Sequence analysis and resistance to pepsin hydrolysis as part of an assessment of the potential allergenicity of ice structuring protein type III HPLC 12. *Food Chem. Toxicol.* 40, 965–978.
- Bernstein, D.I., Bernstein, I., Gaines, W.G., Stauder, T., Wilson, E.R., 1994. Characterization of skin prick test responses for detecting sensitization to detergent enzymes at extreme dilutions: inability of RAST to detect lightly sensitized individuals. *J. Allergy Clin. Immunol.* 94, 498–507.
- Bindslev-Jensen, C., Sten, E., Earl, L.K., Crevel, R.W.R., Bindslev-Jensen, U., Hansen, T.K., Stahl Skov, P., Poulsen, L.K., 2003. Assessment of the potential allergenicity of ice structuring protein type III HPLC 12 using the FAO/WHO 2001 decision tree for novel foods. *Food Chem. Toxicol.* 41, 81–87.
- Codex Alimentarius Commission, 2002. Report of the third session of the Codex *ad hoc* Intergovernmental Task Force on Foods Derived from Biotechnology. Alinorm 03/34.
- Crevel, R.W.R., Fedyk, J.K., Spurgeon, M.J., 2002. Antifreeze proteins: characteristics, occurrence and human exposure. *Food Chem. Toxicol.* 40, 899–903.
- Dirks, C.G., Pedersen, M.H., Platzter, M.H., Bindslev-Jensen, C., Skov, P.S., Poulsen, L.K., 2005. Does absorption across the buccal mucosa explain early onset of food-induced allergic systemic reactions? *J. Allergy Clin. Immunol.* 115 (6), 1321–1323.
- FAO/WHO, 2001. Evaluation of allergenicity of genetically modified foods. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology, Rome, January 22–25, 2001.
- Farr, R.S., Dickinson, W., Smith, K., 1960. Quantitative aspects of oral immunisation against bovine serum albumin (BSA). *Fed. Proc.* 19, 199.
- Hall-Manning, T., Spurgeon, M.J., Wolfreys, A.M., Baldrick, A.P., 2004. Safety evaluation of ice-structuring protein (ISP) type III HPLC12 preparation. Lack of genotoxicity and sub-chronic toxicity. *Food Chem. Toxicol.* 42, 321–333.
- Hansen, T.K., Bindslev-Jensen, C., 1992. Codfish allergy in adult. Identification and diagnosis. *Allergy* 47, 610–617.
- Hansen, T.K., Bindslev-Jensen, C., Stahl Skov, P., Poulsen, L.K., 1996. Codfish allergy in adults. Specific tests for IgE and histamine release vs double-blind, placebo-controlled challenges. *Clin. Exp. Allergy* 26, 1276–1285.
- Hansen, T.K., Bindslev-Jensen, C., Stahl Skov, P.S., Poulsen, L.K., 1997. Codfish allergy in adults. IgE cross reactivity among fish species. *Ann. Allergy Asthma Immunol.* 78, 187–194.
- Heyman, M., 2001. How dietary antigens access the mucosal immune system? *Proc. Nutr. Soc.* 60, 419–426.
- Husby, S., 2000. Normal immune responses to ingested foods. *J. Pediatr. Gastroenterol. Nutr.* 30 (Suppl), S13–S19.
- Husby, S., Oxelius, V.-A., Teisner, B., Jensenius, J.C., Svehag, S.-E., 1985. Humoral immunity to dietary antigens in healthy adults. Occurrence, isotype, and IgG subclass distribution of serum antibodies to protein antigens. *Int. Archs. Allerg Appl. Immunol.* 77, 416–422.
- Husby, S., Mestecky, J., Moldoveanu, Z., Holland, S., Elson, C.O., 1994. Oral tolerance in humans. T-cell but not B-cell tolerance after antigen feeding. *J. Immunol.* 152, 4663–4670.
- Knippels, L.M., Penninks, A.H., Spanhaak, S., Houben, G.F., 1998. Oral sensitization to food proteins: a Brown Norway rat model. *Clin. Exp. Allergy* 28, 368–375.
- Korenblatt, P.E., Rothberg, R.M., Minden, P., Farr, R.S., 1968. Immune responses of human adults after oral and parenteral exposure to bovine serum albumin. *J. Allergy* 41, 226–235.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Matsui, M., Hafler, D.A., Weiner, H.L., 1996. Pilot study of oral tolerance to keyhole limpet haemocyanin in humans. Down-regulation of KLH-reactive precursor cell frequency. *Ann. New York Acad. Sci.* 778, 398–404.
- Poulsen, L.K., Pedersen, M.F., Malling, H.-J., Søndergaard, I., Weeke, B., 1989. Maxisorp RAST. A sensitive method for detection of absolute quantities of antigen-specific IgE. *Allergy* 44, 178–189.
- Tacket, C.O., Mason, H.S., Losonsky, G., Clements, J.D., Levine, M.M., Arntzen, C.J., 1998. Immunogenicity in humans of a recombinant bacterial antigen delivered in a transgenic potato. *Nat. Med.* 4, 607–609.
- Untersmayr, E., Bakos, N., Schöll, I., Kundi, M., Roth-Walter, F., Szalai, K., Riemer, A.B., Ankersmit, H.J., Scheiner, O., Boltz-Niculescu, G., Jensen-Jarolim, E., 2005. Anti-ulcer drugs promote IgE formation towards dietary antigens in adult patients. *FASEB J.* 19, 632–656.