

Effects of Industrial Processing on the Immunogenicity of Commonly Ingested Fish Species

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Key Words

Fish protein • Parvalbumin • IgE binding • Immunogenicity • Food processing

Abstract

Background: Food-processing techniques may induce changes in fish protein immunogenicity. Allergens from >100 fish species have been identified, but little is known on the effects of processing on fish protein immunogenicity. **Methods:** IgE binding of sera of patients allergic to fresh and processed (smoked, salted/sugar-cured, canned, lye-treated and fermented) cod, haddock, salmon, trout, tuna, mackerel and herring and of hydrolysates based on salmon and whiting was investigated using immunoblot and inhibition ELISA. **Results:** Parvalbumin oligomers were identified using monoclonal and polyclonal antibodies. IgE binding was seen in most sera at 12–14 kDa (parvalbumin), and at 17–60 kDa for all fish except tuna. Changes in IgE binding appeared to reflect altered parvalbumin monomers and oligomers. Smoked haddock, salmon and mackerel had increased IgE binding and novel bands at 30 kDa. Chemically processed cod, salmon, trout and pickled herring had reduced or abolished IgE binding. The serum of 1 subject, however, had increased IgE binding to these products and also inhibition of

binding by both fish hydrolysates to their constituent fish species. **Conclusion:** Process-induced changes in fish protein immunogenicity were more dependent on process rather than species, although individual responses varied. Changes in the allergenicity of a product may depend on the net effect of processing on parvalbumin oligomerization patterns, which may also vary in different species. Chemical processes generally caused loss in IgE-binding activity, though sensitization may occur to modified or degraded rather than intact peptides as shown by increased binding by chemically processed fish and hydrolysates in 1 subject. The clinical significance of these findings remains to be established.

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Introduction

Fish is a frequent elicitor of immunoglobulin (Ig) E-mediated type I food allergy and is also known to cause severe food reactions like anaphylaxis. Approximately 0.1% of the Norwegian population is thought to have IgE-mediated fish allergy [1], which is in agreement with the estimated prevalence of fish allergy of 0.1% in children and 0.4% in adults in the US [2]. Reactions to cod, trout

and salmon appear to be frequent in Norway [1]. However, changes in patterns of fish consumption, both for nutritional benefits and for gastronomic reasons, may lead to an altered risk of allergic sensitization and reactions.

IgE antibodies frequently demonstrate cross-reactivity in that they bind to other proteins sharing structural features [3]. Parvalbumins (12–13 kDa), calcium-binding muscle proteins identified as the major fish allergens [4, 5], are typical in this respect. Parvalbumins from various fish species are recognized by IgE in more than 95% of fish-allergic patients [6]. Cod parvalbumin, Gad c1, prevents fish-specific IgE from binding to extracts of other fish species, demonstrating cross-sensitization between Gad c1-homologous parvalbumins from different fish species: salmon, perch, carp, eel and tuna [7], herring, plaice and mackerel [5], catfish and snapper [8] and pollock, wolffish, halibut and flounder [9]. Tuna, however, is poorly recognized in some studies [8, 9]. Additional IgE-binding proteins between 18 and 130 kDa have been described in the Baltic cod (*Gadus callarias*) [10, 11] and in the Atlantic cod (*G. morhua*) [12]. Several of these protein bands were also specifically recognized by monoclonal anti-parvalbumin antibodies [10–12], and it has been suggested that, in particular, bands at 24, 38 and 51 kDa in *G. morhua* may be parvalbumin (Gad m1) dimers, trimers and tetramers, respectively [12], which may in part explain IgE binding observed at molecular weights higher than 12–13 kDa. In rarer cases of monospecificity, IgE usually binds to protein bands other than the 12- to 13-kDa band. Unique IgE-binding bands have been reported at 46 kDa [13] and at 40 kDa in tuna [8] and at 25 kDa in swordfish [14]. Reactions to different fish species vary in fish-allergic patients, and allergenicity differs between species [15]. Furthermore, sensitization does not necessarily cause clinical symptoms [16]. However, fish-allergic consumers are in general considered likely to react clinically to cross-reactive proteins in other fish species and are often advised to avoid ingestion of any fish species [5, 15, 17].

Conventional food-processing techniques may induce changes in protein conformation [18], which may thus affect allergenicity if the allergen epitopes are modified by the process [19]. Fish is subjected to a wide variety of processes such as thermal (boiling, frying, grilling), freezing, canning, salting, drying and fermentation, any of which might reduce or increase allergenicity. Fish is also included as an ingredient in surimi, sauces, soups, and so on, and fish proteins, e.g. gelatin, ice-structuring protein, are widely used in food-processing and pharmaceutical

industries. Furthermore, fish hydrolysates are used as flavorings and nutritional additives in food.

Although allergens from over 100 fish species (<http://www.allergome.org>) have been identified, there have been few studies on the effects of food processing on these fish proteins and their allergenicity. The major fish allergens, in common with many other food allergens, have been reported to be stable proteins that resist the effects of heating, although allergenicity to certain proteins in tuna and salmon [20] and in pomfret and hilsa [21] was reported to be reduced by heat processing. Resistance to digestive hydrolysis is also attributed to typical food allergens, and early studies found that Gad c 1 was relatively digestion resistant [22]. However, recent reports show that parvalbumin is rapidly degraded under simulated gastric conditions, but that the extent of hydrolysis, and thus allergenic potency, is influenced by marginal alterations in pH [23].

In the present study, fresh and cured fish (smoked, salted/sugar-cured, canned, lye-treated and fermented products of cod, haddock, salmon, tuna, mackerel and trout) have been investigated. In addition, commercially produced hydrolyzed extracts from wild fish, intended as substitute flavorings for beef, chicken and pork, have also been studied. The effects of processing on the immunogenicity of these products have been investigated by measuring IgE binding in sera from fish-allergic patients, using Western blot and inhibition ELISA.

Materials and Methods

Study Population

Twelve children (4 girls, 8 boys, median age 9.3 years, range 3.5–17 years), with confirmed fish allergy, referred to Voksentoppen Children's Center for Asthma and Allergy, Rikshospitalet University Hospital, Norway, were included in the study on the basis of IgE-binding activity to extracts of 7 fresh commonly consumed fish species, described below. Clinical data and laboratory findings are summarized in table 1. Two of the children had a positive open oral food challenge with either cod or trout. The others had a history of one or more clinical reactions to fish, combined with specific IgE to fish, diagnosed by skin prick tests (SPTs) or serum analyses. Symptoms ranged from urticaria, angioedema and exacerbation of atopic dermatitis to gastrointestinal and respiratory symptoms. Seven adult subjects with confirmed fish allergy (3 females, 4 males, median age 26 years, range 18–53 years), referred to the Section for Clinical Allergology, Department of Occupational Medicine, Haukeland University Hospital, Norway were similarly recruited to the study on the basis of IgE screening. These patients had histories of repeated generalized or anaphylactic reactions to fish ingestion, together with fish-specific serum IgE and/or positive SPTs (table 1). Six nonallergic, fish-consuming adults (5 females and 1 male, median age

46 years) were used as control subjects. Fish-specific IgE serum levels were measured using the ImmunoCAP 100® Specific IgE FEIA system (Phadia AB, Uppsala, Sweden), with the following antigens: cod (f3), salmon (f41), yellowfin tuna (f40), herring (f205) and mackerel (f206). The cut-off value for a positive result was set at 0.35 kU_A/l.

SPTs were performed on the children with cod (Allergopharma, Reinbek, Germany), and in the adults with cod (ALK-Abello Laboratories, Copenhagen, Denmark) and salmon (Allergy Research Laboratory, Haukeland University Hospital). Testing was performed in duplicate on the volar side of the forearm, according to international guidelines [24]. A positive SPT result was defined as a mean wheal diameter ≥ 3 mm larger than that of the negative control. Histamine dihydrochloride 10 mg/ml (ALK-Abello) served as positive control and the Allergopharma diluent as negative control. The study was approved by the Regional Committee for Medical Research Ethics, Southern Norway. Enrolment was carried out after written informed consent was obtained from the subjects, or in the case of the children, from their parents.

Preparation of Fish Extracts and Purified Cod Parvalbumin

Seven commonly consumed fish species (Atlantic cod, haddock, Atlantic salmon, yellowfin tuna, rainbow trout, Atlantic mackerel, and Atlantic herring), both fresh and processed (table 2), were purchased from local stores in Norway. Traditional Norwegian cod products included *tørrfisk*, which is dried naturally by the wind and sun for up to 3 months, and *lutefisk*, dried cod soaked in a lye solution (pH 11–12) and subsequently in cold water. Salted cod is dried after salting, whereas salted herring is simply soaked in a salt brine. Salmon is usually cold-smoked at 20–30°C, after being cured in a salt- and sugar-brine for a day. Hot smoking at around 80–100°C is more commonly used for mackerel, haddock and trout. *Gravlaks* is salmon cured in a mixture of sugar, spices and up to 3–6% salt, causing autolytic protein degradation by the fish's own enzymes. *Rakørret*, however, is salted trout that undergoes controlled fermentation by enzymes from microorganisms. Pickled herring products, including rollmops, *bismarckherring* and jellied herring, are all prepared in an acetic acid-salt brine, and *brathering* is, in addition, fried. Canning involves processing under high temperatures (116–121°C) and pressure, for up to 14 h.

Fresh blue whiting and two hydrolyzed fish protein extracts, SG-3 and SG-5 (a kind gift from Seagarden, Avaldsnes, Norway), were also obtained. These products are made, respectively, from blue whiting and Atlantic salmon, enzymatically treated with Neutrase® (Novozymes, Bagsværd, Denmark). Skeletal muscle samples from fresh and processed fish were homogenized with the exception of blue whiting, which was homogenized whole. Homogenized samples were extracted 1:5 w/v (lye-treated cod 1:2) in 10 ml 0.1 M Tris/0.5 M glycine extraction buffer (both Amersham Biosciences, Uppsala, Sweden), pH 8.7, overnight at 45°C in a shaking water bath. Total protein concentrations of the supernatants following centrifugation (39,200 g, 25 min, 4°C) were determined using the DC Protein assay (Bio-Rad, Hercules, Calif., USA). Parvalbumin was extracted from muscle tissue from fresh Atlantic cod, as described previously [25]. The resulting parvalbumin-enriched fraction was separated by gel filtration chromatography on Sephadex G-50 fine column (Amersham), eluted with 50 mM Tris/1 mM dithiothreitol buffer, pH 8.0. Fractions demonstrating a 12-kDa protein band, parvalbumin, were pooled

and concentrated using Vivaspinn 6 centrifugal concentrators, 10,000 MWCO (Vivascience, Hannover, Germany) in sterile-filtered phosphate-buffered saline (PBS; Oxoid, Basingstoke, UK), and total protein content determined. All extracts were stored at –20°C until needed.

IgE Screening ELISA

The sera from all fish-allergic patients and healthy controls were screened for IgE-binding activity as described elsewhere [26]. Briefly, ELISA plates were coated with 10 µg/ml fish extract/negative control (equine myoglobin; Sigma-Aldrich), or 40 µg/ml of fish hydrolysate. The plates blocked with 150 µl PBS-Tween containing 1% human serum albumin (Calbiochem, La Jolla, Calif., USA). PBS/Tween/human serum albumin with the addition of 0.5 mM Ca²⁺ was used thereafter as assay buffer, as the presence of Ca²⁺ has been shown to enhance IgE binding to parvalbumin [7, 25]. Patient sera (50 µl, diluted 1:20) were added in duplicate to all experimental and control wells. Bound IgE was detected using rabbit antihuman IgE (1:7,500) and goat-antirabbit IgG-HRP conjugate (1:10,000) and wells were developed with tetramethyl benzidine substrate. The absorbance at 450 nm in negative control wells was subtracted from the absorbance in fish-extract-coated wells to correct for nonspecific binding. Where optical densities (ODs) in excess of the working range of the plate reader (OD_{450nm} ≥ 2.0) were observed, further screening was performed as necessary, to establish suitable serum dilutions for inhibition ELISA and immunoblotting studies.

IgE Inhibition ELISA

Fresh and processed fish extracts were studied (table 2) using sera demonstrated to have IgE reactivity to the fresh fish extract in the screening ELISA. For experiments using mackerel and tuna, a pool was prepared from the sera of 8 patients with OD_{450nm} ≥ 1.0 in the screening ELISA for mackerel and/or tuna extracts. Appropriately diluted serum aliquots were preincubated overnight at 4°C with 10-fold serially diluted fish extracts or hydrolysates in assay buffer. ELISA plates were coated and blocked as described previously, the coat concentrations were selected on the basis of preliminary experiments at 5, 10, 20 or 40 µg/ml. After washing, 75-µl triplicates of the inhibited sera (containing 5–0.0005 µg fish extract/ml) were added to coated wells and incubated for 1 h at room temperature under gentle shaking. For experiments using fish hydrolysates, the inhibitor concentrations ranged from 10 mg/ml to 3.2 µg/ml. Subsequent steps were carried out as previously described. Percentage inhibition was calculated using the following formula: % inhibition = 100 – (OD_{450nm} with inhibitor $\times$ 100/OD_{450nm} without inhibitor). Inhibition curves were plotted for each fish extract, and the concentration required for 50% inhibition of IgE binding (IC₅₀) was calculated from the graphs.

SDS-PAGE and Immunoblotting of Fish Extracts

Electrophoretic separation of the fish product extracts and myoglobin (10 µg/lane), hydrolysates (40–200 µg/lane), purified cod parvalbumin (5 µg/lane) and SeeBlue® Plus2 standard was carried out under reducing conditions using the NuPAGE Gel system (Invitrogen, Carlsbad, Calif., USA) in accordance with the manufacturer's instructions. Separation was performed on 4–12% Bis-Tris precast gels and gels were stained using Simply Blue Coomassie (Invitrogen) or electrophoretically transferred onto

Table 1. Clinical features of patients

No.	Age	Sex	Clinical history	Total IgE kU/l	Specific IgE, kU/l (ImmunoCAP)				
					C	S	H	M	T
1	12.5	F	AD, reactions to fish, egg, fruit	3,342	10.6				
2	8.5	M	asthma, AD, contact reaction to fish, allergic to fruit, vegetables, pollen	1,500	13.9				
3	13	M	AD, asthma, urticaria on ingestion of fish	707	9	2.0	11.3	3.1	1.57
4	3.5	F	AD, allergic reaction to fish	81					
5	7	M	AD, urticaria, allergic reactions to fish, milk, egg	>5,000	>100	>100	>100	85	91
6	5.5	M	AD, allergic reactions to milk, egg, fish	1,300	41				
7	10	M	urticaria, AD exacerbated by fish, egg, milk	571	36	58	36	19.4	15.7
8	14	M	history of serious reaction to fish, reacts to steam	340	23				
9	13.5	M	asthma, AD, multiple food and inhalant allergies incl. fish	1,316	33	6.2	21	8.3	4.9
10	4.5	M	asthma, AD, multiple food and inhalant allergies incl. fish; labial itching and swelling to fish	>5,000	13.7	26	6.6	3.0	1.9
11	8	F	asthma, AD, multiple food and inhalant allergies incl. fish; reaction to fish steam: facial swelling and itching	1,530	56	17	48	24	8.6
12	17	F	asthma, AD, multiple food and inhalant allergies incl. fish	1,956	25	31	24	11.3	8.9
13	25	M	anaphylaxis to fish	134	0.95	0.79	1.6	<0.35	0.45
14	26	M	anaphylaxis multiple food and inhalant allergies incl. fish	5,620	43	20	46	21	7.9
15	43	F	anaphylaxis reaction to fish	56	10.3	12.9	20	2.9	4.4
16	27	M	throat swelling vomiting, breathlessness, on inhalation/ ingestion of fish	50	5.9	3.9	6.1	3.3	1.95
17	23	F	throat itching, asthma, breathlessness, vomiting on inhalation/ ingestion of fish	700	11.6	11.7	11.1	6.4	3.7
18	52	M	oral itching/swelling, dyspnea, breathlessness on inhalation/ ingestion of fish	49	11.5	8.6	1.6	0.44	12.0
19	18	F	vomiting, abdominal pain, breathlessness, asthma on inhalation/ ingestion of fish	265	23	10.1	18.9	8.6	4.3

Sera were screened (diluted 1:20) for IgE-binding reactivity to extracts of fresh fish and fish hydrolysates using indirect ELISA (OD 0.20–0.49: +; OD 0.5–0.99: ++; OD $\geq$ 1.0: +++).

C = Cod; S = salmon; H = herring; M = mackerel; T = tuna; Ha = haddock; R = rainbow trout; W = whiting; SG = fish hydrolysates; AD = atopic dermatitis.

Protran BS83 nitrocellulose membranes (Schleicher and Schuell). Membranes were incubated overnight with individual patient sera (diluted 1:5–1:100), selected on the basis of IgE-binding activity in the indirect screening ELISA (hydrolysates incubated with serum pool, 1:20). Blots were further developed as described elsewhere [26]. Tris-buffered saline/0.05% Tween-20 containing 5% horse serum (Gibco, Paisley, UK) was used as blocking buffer, and the same buffer with 0.5 mM Ca^{2+} was used as assay buffer. Gels and blots were scanned and the images analyzed using the Gel-Pro® Analyzer-program (MediaCybernetics, Silver Spring, Md., USA).

In order to confirm the presence of parvalbumin epitopes in the immunoblotted proteins, additional membranes were incubated as described above, using a monoclonal antibody to frog parvalbumin, PARV-19 (1:5,000; Sigma-Aldrich, St. Louis, Mo., USA) detected using a rabbit antimouse-HRP conjugate (1:5,000; Sigma-Aldrich). In addition, a rabbit polyclonal anti-cod parvalbumin was used, diluted 1:15,000 [25] and detected using goat anti-rabbit-HRP (1:5,000; Zymed).

Results

Screening for IgE-Binding Activity to Fresh Fish Extracts

IgE-binding activity to extracts of fresh cod, haddock, salmon, trout, herring, mackerel, tuna, blue whiting and the 2 fish hydrolysates was screened in 19 patient sera (12 children, 7 adults) using ELISA at a serum dilution of 1:20 (table 1). Serological cross-reactivity ($\text{OD}_{450\text{nm}} \geq 0.2$) to more than 4 fish species was observed in all patients. Only 4 of the 17 patients who were positive for both whiting and salmon bound to both hydrolysates. No IgE binding to any fish extract was observed in the controls, neither was there any binding to the negative control myoglobin. Several sera had IgE levels in excess of the working range of the plate reader ($\text{OD}_{450\text{nm}} \geq 2.0$) at 1:20, and further screening

SPT+	IgE-binding reactivity in ELISA								
	C	Ha	W	H	S	R	M	T	SG
	+++	+++	++	+++	+++	+++	+	+++	+++
	+++	+++	+++	+++	++	+++	+++		
	+	+	++	+		+			
C	+++	++		++	+++	+++	+		
C	+++	+++	+++	+++	+++	+++	+++	+++	+++
C	+++	+++	+++	++	+	+++			
C	+++	+++	+++	+++	+++	+++	+++		
C	+++	+++	+++	+++	+++	+++	+	+	
C	+++	++	+	+++	+		+++	++	
C, S	+++	++	+++	+++	+		+++	++	
C, S, W	+++	+++	+++	+++	+++	+	+++	+++	
C, S	+++	+++	+++	+++	++	++	+++	+++	++
C, S, H	+++	+	+	+	+		++	+	
	+++	++	+++	+++	++	+++	+		
C, S, T, H, M	+++	+	+++	++	++	++	++	+	
C, S, W	+	+	++	++	+++	+++	++	+	
C, S, W	++	++	+++	+++	++	++	+++	+	
C, S, W, SG	+++	+++	+++	+++	+++	+++	++	+++	+++
C, S, W, SG	+++	++	+++	+++	++	++	+++	++	

was performed to establish appropriate serum dilutions for inhibition ELISA and immunoblotting studies.

Effects of Processing on Total IgE-Binding Activity

Inhibition ELISA was performed using selected individual sera with high reactivity to cod ($n = 12$), haddock ($n = 6$), salmon ($n = 10$), trout ($n = 11$) or herring ($n = 8$), or in the case of mackerel and tuna products, a high IgE-binding serum pool. Inhibition of IgE binding to each type of fresh fish extract was compared in fresh and processed products from the same species (fig. 1a–g), and the alteration in IC_{50} values for processed, relative to fresh, fish extracts was calculated (fig. 2).

An increase in IC_{50} of up to 167 times for all 12 cod-reactive sera showed that lye-treated cod (*lutefisk*) exhibited reduced IgE binding when compared with fresh cod.

The IC_{50} for salt cod also increased in 7 of these sera, but was decreased in 4. Increased IgE-binding by dried cod, shown by decreased IC_{50} values, was observed in all 12 sera. Smoked haddock and smoked salmon also showed increased IgE binding in the majority of sera (5/6 and 8/10 sera, respectively). IgE binding was also increased by smoked mackerel: the IC_{50} for cold-smoked mackerel had decreased to 8% of fresh mackerel, and warm-smoked mackerel to 21%. The IC_{50} for canned tuna, however, had increased 5.8 times relative to fresh tuna, showing reduced IgE binding.

Sugar-cured salmon (*gravlaks*) exhibited reduced IgE binding in 8/10 sera, but increased by up to 65 times in 2 subjects. Similarly, fermented trout (*rakørret*) demonstrated decreased IgE binding in most sera (9/11) but an increase of up to 30 times for the same 2 sera (subjects 1

Table 2. Biological classification of species and summary of fresh and processed fish products tested

Common name	Latin name	Family	Order	Product
Atlantic cod	<i>Gadus morhua</i>	Gadidae (cods haddocks)	Gadiformes	fresh cod fillet
Atlantic cod	<i>Gadus morhua</i>	Gadidae (cods haddocks)	Gadiformes	<i>lutefisk</i> : dried, lye-treated cod
Atlantic cod	<i>Gadus morhua</i>	Gadidae (cods haddocks)	Gadiformes	salted cod
Atlantic cod	<i>Gadus morhua</i>	Gadidae (cods haddocks)	Gadiformes	<i>tørrfisk</i> : dried cod
Haddock	<i>Melanogrammus aeglefinus</i>	Gadidae (cods haddocks)	Gadiformes	fresh haddock fillet
Haddock	<i>Melanogrammus aeglefinus</i>	Gadidae (cods haddocks)	Gadiformes	smoked haddock
Blue whiting	<i>Micromesistius poutassou</i>	Gadidae	Gadiformes	fresh blue whiting
Atlantic salmon	<i>Salmo salar</i>	Salmonidae	Salmoniformes	fresh salmon fillet
Atlantic salmon	<i>Salmo salar</i>	Salmonidae	Salmoniformes	cold-smoked salmon fillet
Atlantic salmon	<i>Salmo salar</i>	Salmonidae	Salmoniformes	<i>gravlaks</i> : sugar-cured salmon
Rainbow trout	<i>Oncorhynchus mykiss</i>	Salmonidae	Salmoniformes	fresh rainbow trout
Rainbow trout	<i>Oncorhynchus mykiss</i>	Salmonidae	Salmoniformes	<i>rakørret</i> : fermented rainbow trout
Atlantic mackerel	<i>Scomber scombrus</i>	Scombridae	Perciformes	fresh mackerel
Atlantic mackerel	<i>Scomber scombrus</i>	Scombridae	Perciformes	cold-smoked mackerel
Atlantic mackerel	<i>Scomber scombrus</i>	Scombridae	Perciformes	warm-smoked mackerel
Yellowfin tuna	<i>Thunnus albacores</i>	Scombridae	Perciformes	fresh yellowfin tuna
Yellowfin tuna	<i>Thunnus albacores</i>	Scombridae	Perciformes	canned tuna
Atlantic herring	<i>Clupea harengus harengus</i>	Clupeidae	Clupeiformes	fresh herring
Atlantic herring	<i>Clupea harengus harengus</i>	Clupeidae	Clupeiformes	brine-salted herring
Atlantic herring	<i>Clupea harengus harengus</i>	Clupeidae	Clupeiformes	<i>sursild</i> : salted, acetic acid pickled herring
Atlantic herring	<i>Clupea harengus harengus</i>	Clupeidae	Clupeiformes	rollmops: salted, acetic acid pickled herring (bottled)
Atlantic herring	<i>Clupea harengus harengus</i>	Clupeidae	Clupeiformes	<i>bismarckherring</i> : salted, acetic acid pickled herring (bottled)
Atlantic herring	<i>Clupea harengus harengus</i>	Clupeidae	Clupeiformes	<i>brathering</i> : salted, cooked, acetic acid pickled herring
Atlantic herring	<i>Clupea harengus harengus</i>	Clupeidae	Clupeiformes	jellied acetic acid pickled herring
Atlantic herring	<i>Clupea harengus harengus</i>	Clupeidae	Clupeiformes	rollmops: salted, acetic acid pickled herring
Fish protein extract	<i>Micromesistius poutassou</i> ¹	Gadidae	Gadiformes	hydrolyzed blue whiting
Atlantic salmon extract	<i>Salmo salar</i> ¹	Salmonidae	Salmoniformes	hydrolyzed salmon

Processes employed in fish products include drying, salting, smoking (warm and cold), lye-treating, fermenting/curing, acidification (vinegar), heating and enzymatic hydrolysis.

¹ Principal ingredient (warm and cold), lye-treating, fermenting/curing, acidification (vinegar), heating and enzymatic hydrolysis.

and 18). In general, a decrease in IgE binding was seen in herring products: salted and pickled herring (*sursild*) both showed reduced IgE binding in 7/8 sera. German vinegar-pickled herring products (rollmops, *bismarckherring*, *brathering* and jellied herring) showed decreased IgE binding in 3/5 of the sera. Increased IgE binding was seen in 4 products for serum No. 18.

Preincubation of sera from subjects 5, 12 and 18 with the salmon hydrolysate SG-5, with an inhibitor concentration of 10 mg/ml, inhibited binding to salmon extract by 18, 41 and 67%, respectively. No inhibition of binding to blue whiting was seen in sera No. 5 or 12 after preincubation with SG-3 at an inhibitor concentration of 10 mg/ml, but it inhibited binding of serum 18 to blue whiting by 80% (fig. 1h).

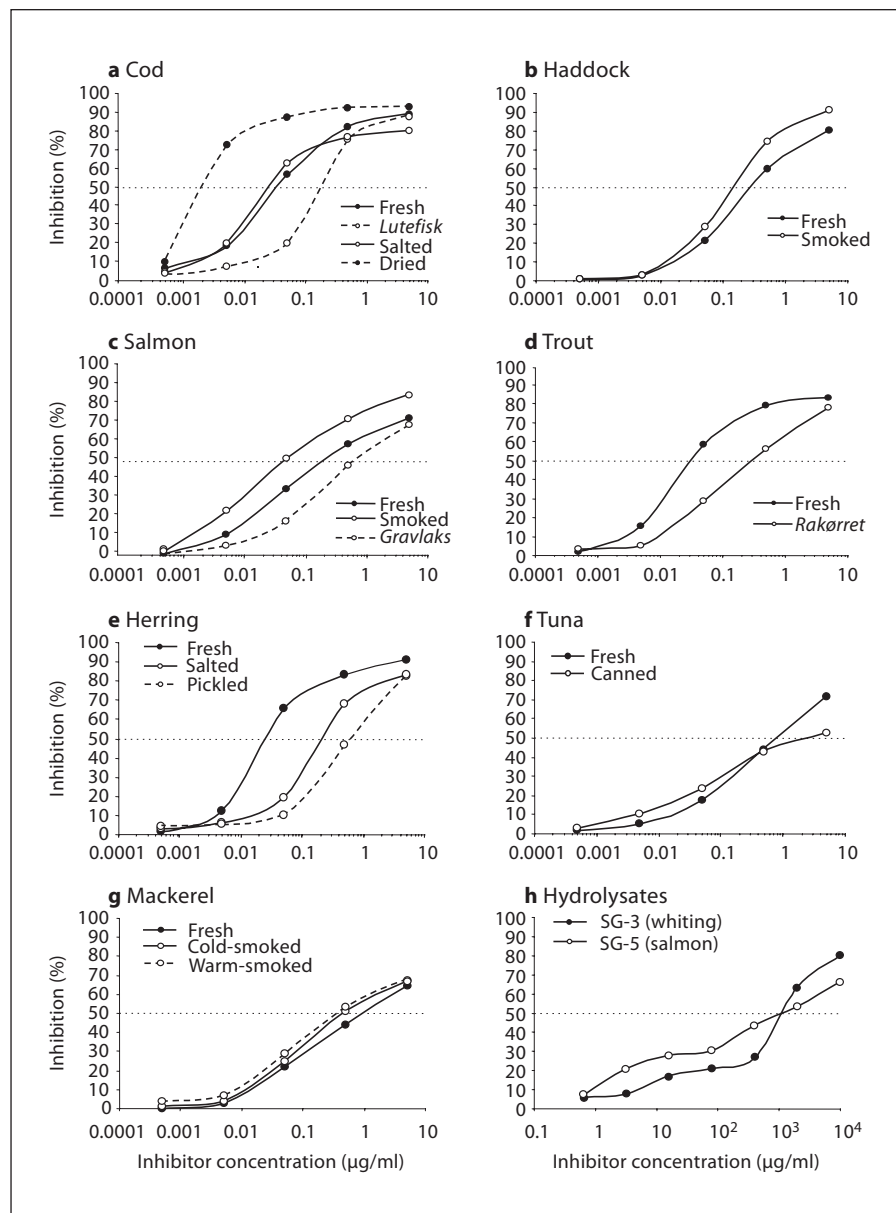
Effects of Processing on Fish Proteins (SDS-PAGE)

Electrophoretic separation of the fish extracts (fig. 3) showed strongly-stained single bands, corresponding to cod parvalbumin (Gad m1) at 12–14 kDa in fresh cod,

haddock, salmon, trout and herring (table 3), and a barely visible band in mackerel. These were equally prominent in dried cod, smoked haddock and smoked salmon. The parvalbumin band intensities, measured using the Gel-Pro software, had decreased by 48% in *lutefisk*, 34% in *gravlaks* and 40% in *rakørret*, compared with fresh. The 12-kDa band was also less strongly stained in salted cod, but a second, weakly stained band at approximately 10 kDa was observed. The band intensities increased by 188 and 124%, respectively, in the cold- and warm-smoked mackerels. This band was more weakly stained in salted herring and *brathering*, but not observed in the other herring products. No bands were visible between 12–14 kDa in fresh tuna.

Additional protein bands common to most of these fish species were seen at 17, 28, 41, 47, 49 and 60 kDa (table 3). In general, band intensities <62 kDa were reduced or unchanged by drying, salting, smoking and chemical processing. However, novel bands were observed at 30 kDa in smoked salmon and *gravlaks*, at 65 kDa in smoked

Fig. 1. Inhibition ELISA of IgE binding. Representative inhibition curves using fish-allergic patient sera or a serum pool at various dilutions are shown for cod (**a**; patient 6, serum dilution 1:50), haddock (**b**; patient 5, serum dilution 1:200), salmon (**c**; patient 2, serum dilution 1:20), trout (**d**; patient 6, serum dilution 1:20), herring (**e**; patient 7, serum dilution 1:40), tuna (**f**; serum pool, dilution 1:20), mackerel (**g**; serum pool, dilution 1:20) and hydrolysates SG-3 and SG-5 (**h**; wells coated with whiting and salmon, respectively; patient 18, serum dilution 1:20).



haddock, and at 38 kDa in *rakørret* and several of the vinegar-treated herring products. In addition, a five-to-six-fold increase in band intensity was seen in smoked salmon and *gravlaks* (17 kDa), and warm-smoked mackerel (45 kDa), compared with fresh fish. Cod, haddock, salmon and trout also had several bands of varying intensity from 70 to >188 kDa. When compared with fresh fish, these bands, in general, had increased staining intensity in salted and dried cod and smoked haddock, but decreased staining intensity in *lutefisk* and *rakørret*. Mackerel and herring products and fresh tuna showed little or

no staining >62 kDa, whereas canned tuna was uniformly stained, having no definable bands at all.

Blue whiting resembled cod and haddock, with a prominent 12-kDa parvalbumin band and additional bands between 17 and 62 kDa. However, the staining patterns obtained on electrophoretic separation of the blue whiting- and salmon-based hydrolysates, SG-3 and SG-5, resembled more closely that seen in canned tuna, with the absence of discernible bands and weak, relatively uniform staining up to approximately 50 kDa (data not shown).

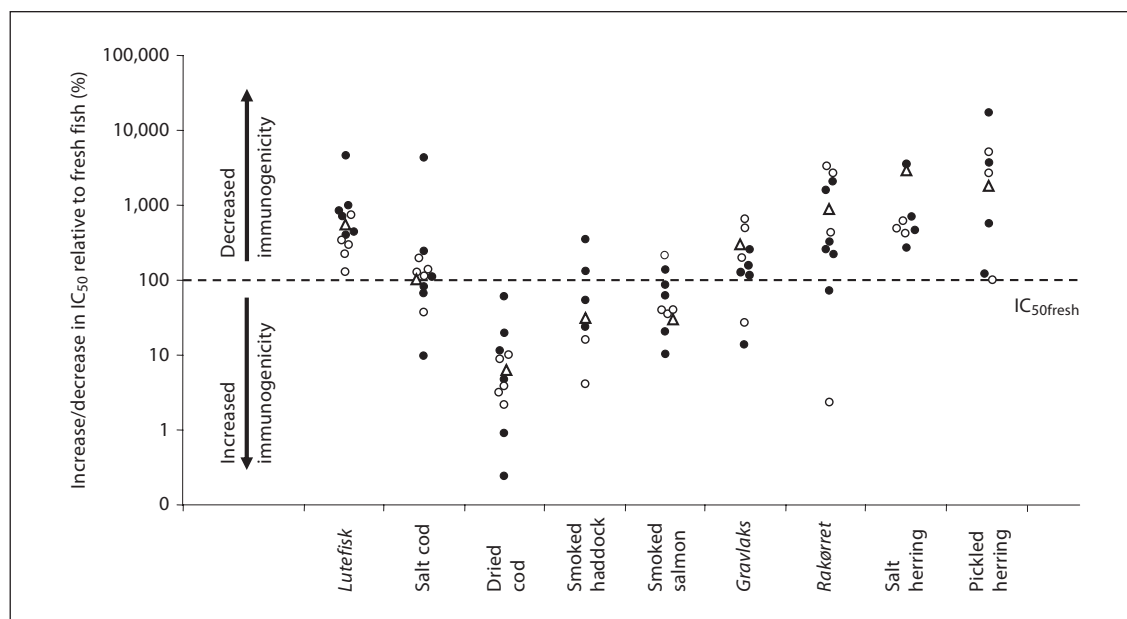


Fig. 2. Altered immunogenicity of processed fish products. The IC_{50} for individual patient sera are shown as percent increase or decrease relative to the IC_{50} for fresh fish of the same species ($IC_{50\text{fresh}} = 100\%$). Values for children (●) and adults (○) are shown. △ = Representative sera from figure 1.

Table 3. Protein band distribution in extracts of fresh cod, haddock, salmon, rainbow trout, mackerel, yellowfin tuna and herring

MW kDa	Cod				Haddock				Salmon				Rainbow trout				Mackerel				Yellowfin tuna				Herring			
	SDS-PAGE	IgE	PARV-19	K991	SDS-PAGE	IgE	PARV-19	K991	SDS-PAGE	IgE	PARV-19	K991	SDS-PAGE	IgE	PARV-19	K991	SDS-PAGE	IgE	PARV-19	K991	SDS-PAGE	IgE	PARV-19	K991	SDS-PAGE	IgE	PARV-19	K991
12–14	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	(×)	×			(×)	×	×	×	×	×	×	×	×
17	×	×	(×)	×				×	×	×	(×)	×	×	×	×	×	×	×	×		×	×	×		×	×	×	×
21	×	×		×	×	×		×				×	×		×		×				×	×			×			
28	×	×			×	×			×	×		×					×	×		×	×	×						×
38								(×)	×												×				×	×		
41	×	×	(×)	×	×	×	(×)	×	×	×	×	×	×	×			×	×	(×)	×			(×)	×				
47	×	×	(×)	×	×	×	(×)	×	×	×	×	×	×	×	×		×	×	×	×	×	×	×	(×)	×	×		×
49	×	×	(×)	×	×	×	(×)	×	×	×	×	×	×	×	×	×	×	×	×	×	×	×	(×)	×	×	×		×
60	×	×		×	×	×		×	×	×	(×)	×	×	×	(×)	×	×	×	×	×	×	×	×	×	(×)	×		
>60	×	×			×	×		×	×			×	×			×				(×)	×							

Bands were identified using SDS-PAGE and immunoblotting with fish-allergic patient sera, monoclonal anti-frog parvalbumin (PARV-19) and polyclonal rabbit anti-cod parvalbumin K991. (×) = Weakly-stained bands; ×* = double bands.

Detection of Parvalbumin Epitopes

The monoclonal anti-frog parvalbumin antibody, PARV-19, recognised the 12–14-kDa parvalbumin bands in all six extracts, including the double herring bands

(table 3), and also in whiting (not shown). Identification was confirmed by the polyclonal rabbit anti-cod parvalbumin antibody K991, and in addition, a faint staining was seen in tuna (table 3). PARV-19 also recognised a

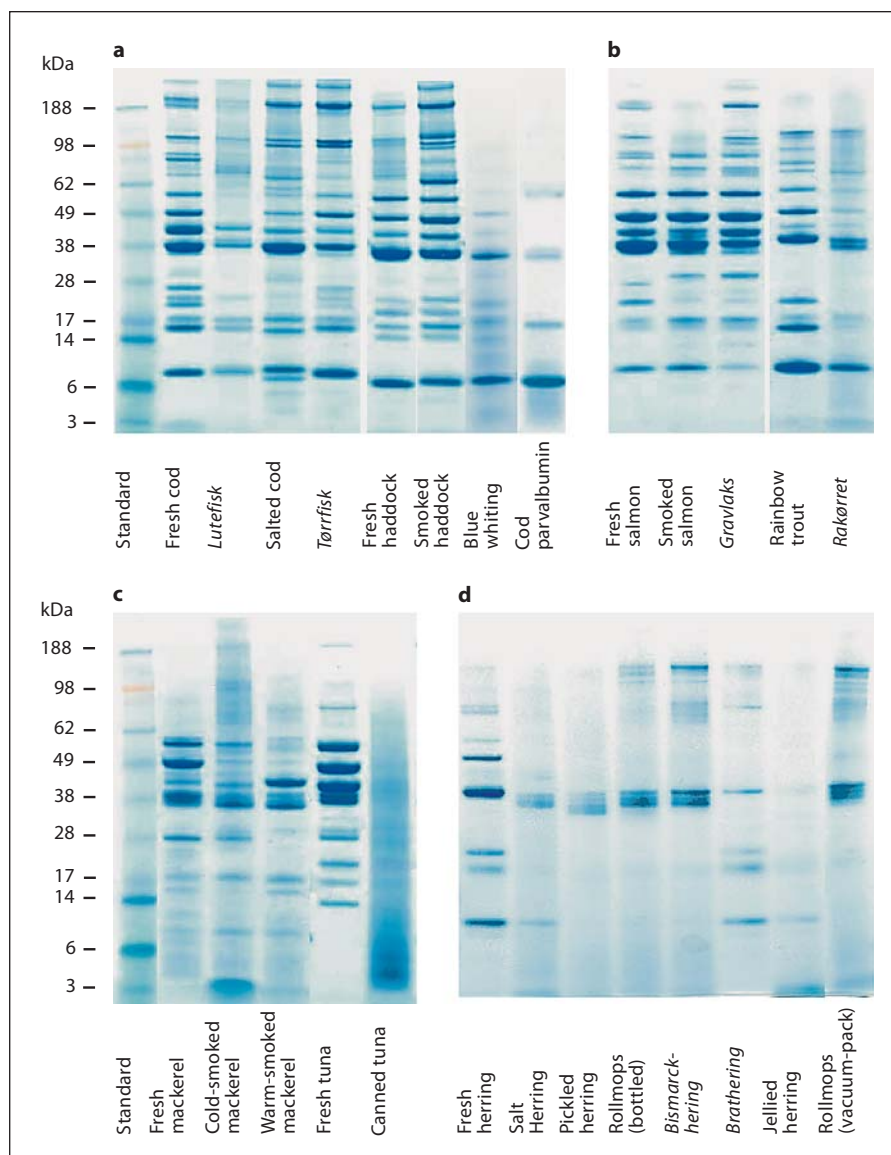


Fig. 3. SDS-PAGE profiles of fish protein extracts. Wells were loaded with 10 μ g protein. **a** Cods and haddocks (family Gadidae, order Gadiformes), wells 2–9. **b** Salmonids (family Salmonidae, order Salmoniformes), wells 10–14. **c** Scombrids (family Scombridae, order Perciformes), wells 16–20. **d** Herrings (family Clupeidae, order Clupeiformes), wells 21–28. Standard: SeeBlue 2™ molecular weight standard, Invitrogen.

number of bands at 17, 41, 47, 49 or 60 kDa in cod, haddock, salmon and, to a lesser extent, trout and tuna, but not in mackerel or herring. Tuna had an intensely stained band at 47 kDa. K991 also recognised additional bands in haddock, salmon, trout, herring, tuna and mackerel (table 3). Neither antibody bound to high MW bands (>62 kDa). Salting and drying (cod) and smoking (haddock, salmon and mackerel) appeared to have only moderate effect on PARV-19 or K991 binding to parvalbumin or to the additional bands from 17–60 kDa, but decreased binding was clearly observed in most of these bands in *lutefisk*, *gravlaks*, *rakørret* and processed herring products.

Identification of IgE-Binding Proteins

Immunoblotting experiments were performed using sera selected for high IgE-binding activity to fish extracts (cod, haddock, mackerel: all $n = 12$; salmon and trout: both $n = 11$; tuna: $n = 13$; herring: $n = 8$) and for availability of serum (fig. 4a, b; table 3). Frequency of binding to parvalbumin and binding patterns between 17 and 60 kDa in all seven fish extracts were similar in children and adults. No binding was observed in the control sera and no patient sera bound to myoglobin. The majority of sera demonstrated prominent bands at 12–14 kDa, corresponding to parvalbumin, in cod, haddock, salmon, trout and purified cod parvalbumin. Weaker bands were seen

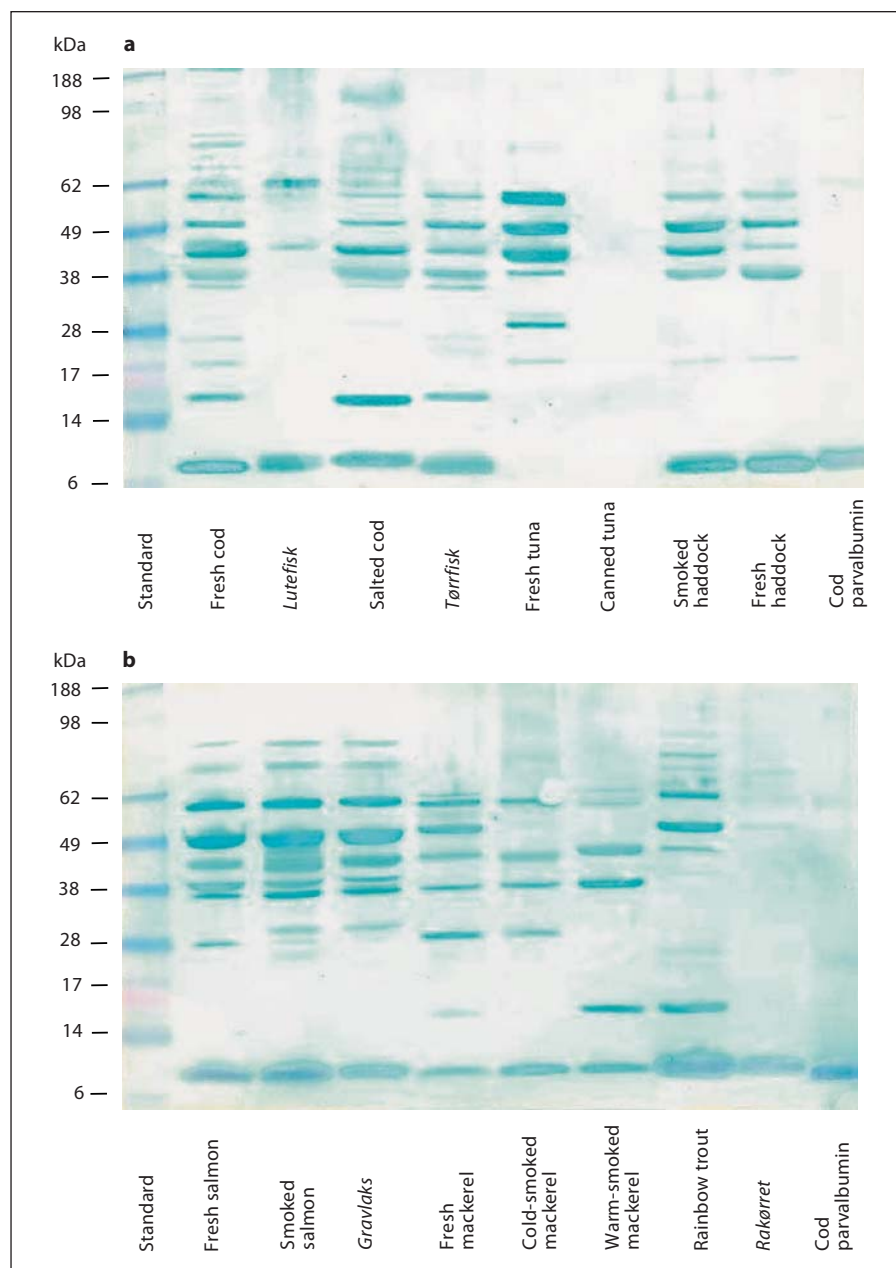


Fig. 4. Immunoblot of fish protein extracts. Blots were probed with appropriately diluted sera from fish-allergic subjects and the IgE reactivity detected. Representative immunoblots are shown using serum from patient 4, diluted 1:5 in Tris-buffered saline-Tween containing 5% horse serum and 0.5 mM Ca^{2+} . Standard: SeeBlue 2™ molecular weight, Invitrogen.

in fresh mackerel extract (13 kDa) and double bands at 12 and 14 kDa in herring. No bands were visible at 12–14 kDa in tuna extracts. Two sera had little or no parvalbumin band staining. IgE binding in blue whiting using the serum pool was similar to cod and haddock, with a prominent 12-kDa parvalbumin band.

Prominently IgE binding bands could also be observed at 17, 28, 41, 47, 49 or 60 kDa (table 3). In general, cod, haddock and salmon had the highest number of bands, and tuna and herring the least. A 21-kDa band was also

seen in some immunoblots of cod, haddock and trout. Discrete IgE-binding bands or diffuse staining were seen at higher molecular weights (>70 kDa) with a few sera in all fish types except mackerel.

Effects of Processing on IgE Binding to Fish Proteins (Immunoblotting)

The intensity of the 12-kDa parvalbumin band was unchanged in salt cod (8/10), but a second parvalbumin band became apparent (2/10). Similarly, parvalbumin in

dried cod (*tørrfisk*) had unchanged (6/10) or even increased band intensity (4/10) and was also generally unchanged. Binding to parvalbumin-like epitopes from 41–60 kDa, recognised using PARV-19 and K991, were also generally unchanged or reduced in both products. However, herring parvalbumin appears to be more susceptible to salting: parvalbumin band intensity decreased (4/5) and the characteristic double band was reduced to a single band.

Smoking also appeared to have relatively little effect on IgE binding to parvalbumin. There was no change in smoked haddock (10/10) and in smoked salmon, binding was unchanged (7/9) or increased (2/9). However, binding in smoked haddock (47 and 49 kDa), and smoked salmon (41 and 47 kDa) was increased in the majority of sera, and a novel 30-kDa band appeared. Binding was also increased in cold-smoked (4/4) and warm-smoked mackerels (3/4), and a second parvalbumin band became visible (1/4). Parvalbumin-related band intensities (17–60 kDa) were in general unchanged or reduced in cold-smoked mackerel, except in subject 18, where they increased. However, in warm-smoked mackerel, these bands had increased staining intensity. Canning appeared to have almost abolished all visible IgE-binding bands in tuna but for a faint diffuse staining.

Lye-treatment in cod (*lutefisk*) caused a decrease in parvalbumin IgE binding in only 2 sera; there was little change in the remainder. Binding to the parvalbumin-related bands at 17–49 kDa was, however, abolished or reduced in all sera except subject 18 (Fi11). Fermentation in trout (*rakørret*) generally caused a decrease or loss (7/10) in 12-kDa parvalbumin band intensity, although in 2 subjects, increased diffuse staining was seen at >38 kDa (subjects 5 and 18). Similarly, the parvalbumin band was unaffected (3/8) or decreased/destroyed (5/8) by curing salmon in sugar-salt brine (*gravlaks*), although several sera showed increased staining at 47–49 kDa, and a novel band at 30 kDa. Serum of subject 18, however, showed increased staining in all bands, with novel bands at 30, 55, and 80–90 kDa.

Of the 6 patients who had IgE to fresh herring parvalbumin, 4 lost or exhibited heavily reduced binding in the herring products, and parvalbumin was reduced to a single band. However, binding to *bismarckherring*, *brathering* and jellied herring was relatively unaffected in 2 subjects, and the double parvalbumin bands were retained. Very faint, diffuse staining at lower molecular weights only (<12 kDa) was seen in the salmon-based hydrolysate SG-5, using the serum pool, but not in the blue whiting-based hydrolysate, SG-3 (data not shown).

Discussion

Protein bands corresponding to parvalbumin (12–14 kDa) were seen on SDS-PAGE in all 7 species except tuna, and additional bands between 17 and 60 kDa. Decreased band intensities suggested that parvalbumin was less stable in the chemically processed fish types, *lutefisk*, *gravlaks*, *rakørret* and pickled herring. Moderate changes in SDS-PAGE band profiles, similar to those seen in the present study, were seen after processing in a study of smoked salmon and trout, and *gravlaks* [27]. In a second study, boiling and frying caused elimination and alteration of some bands in pomfret and hilsa, and the appearance of novel high-molecular-weight bands suggested aggregate formation [21]. Total absence of clearly definable protein bands in canned tuna and salmon as a result of extreme temperature and pressure during canning has been previously reported [20]. However, differences between individual fish or muscle tissue sampling site may also contribute to variation in the protein bands [28].

Cross-reactivity among fish species is thought to be mainly due to the presence of parvalbumin [5, 7, 16]. Parvalbumins have been demonstrated to be the major allergens in salmon (Sal s1) [29], mackerel (Sco s1) [30] and herring [9]. In the present study, parvalbumins have been confirmed using a monoclonal anti-parvalbumin antibody, PARV-19, which has previously been used to confirm parvalbumins in carp, catfish, cod and tilapia [31]. The polyclonal anti-parvalbumin antibody K991 was shown to bind specifically to immunoblotted bands of approximately 12–14 kDa in cod, salmon, trout, herring and mackerel 12–13 kDa [25]. Parvalbumins have recently been identified in haddock and whiting using these two antibodies. Faint binding in tuna at 11–12 kDa (K991) may have been due to parvalbumin. Although a 12-kDa IgE-binding protein has been reported in longtail tuna (*Thunnus tonggol*) [28] and yellowfin tuna parvalbumin has been tentatively identified [13], this was not confirmed with PARV-19 [31]. However, PARV-19 binding was observed in white muscle tissue in longtail tuna, not red muscle, and sampling site may affect its identification in other tuna species [28]. Interestingly, bands at 41, 47 and 49 kDa were recognized by both antibodies in cod, haddock and salmon in the present study. Using the anti-parvalbumin monoclonal antibody PA-235, similar bands have previously been identified in *G. callarias*, together with bands at 12 and 28 kDa [10, 11], and it was suggested that this might be due to a common IgE epitope homologous to a site in Gad c I, or to the presence of parvalbumin oligomers [10]. The same antibody was shown to recog-

nize 12-, 24- and 38-kDa bands in *G. morhua*, and the existence of oligomeric forms of parvalbumin has been proposed [12]. This explanation may also apply to other fish species, as bands recognized by monoclonal antibodies correspond approximately to multiples of 12–14 kDa and, with the exception of the 17-kDa band, may be dimeric, trimeric and tetrameric forms. Liquid chromatography/tandem mass spectrometry and sequence alignment studies in our laboratory have supported the identity of the 17-kDa band as troponin C [32], which has a degree of homology with parvalbumin, explaining its recognition by PARV-19.

Reactivity to cod was seen most frequently in sera from both adults and children, followed by haddock and herring, which are widely consumed in Norway. Overall, IgE-binding patterns differed little between adults and children, which agrees with previous observations with respect to the 12-kDa protein in cod, catfish and snapper [8]. However, adults may react more strongly and broadly across fish species [17]. Multiple IgE-binding bands were seen in all fish extracts, typically in cod, at 12, 17, 21, 28, 41, 47, 49 and 60 kDa, which agrees with earlier findings in *G. callarias* [10, 11], and in *G. morhua* [12]. In the present study, the identity of the 12- to 14-kDa IgE-binding bands as parvalbumins was confirmed using PARV-19 and K991, in cod, salmon, trout, mackerel, herring, haddock and whiting. A number of the bands in the present study (28, 41 and 47/49 kDa) were also recognized by PARV-19 and K991, suggesting the presence of parvalbumin dimers and trimers. In tuna, a band at 46 kDa, previously shown to be specific to yellowfin tuna [13], was strongly bound by IgE and both antibodies, which suggested it may also be a parvalbumin trimer. IgE also bound to the 17-kDa troponin C in several extracts, and may play a role as a minor allergen in fish, as well as in several invertebrates (<http://www.allergome.org>).

The appearance of novel bands, as in salted cod (11 kDa) and smoked salmon and *gravlaks* (30 kDa), might be due to changes in oligomer/monomer relationships, and explain variations in band intensities within the same extract. This is supported by moderate changes seen in the bands identified by PARV-19 and K991. The increased IgE binding in smoked salmon (41 and 47 kDa), haddock (41, 47 and 49 kDa) and mackerel (12, 17, 38 and 47 kDa) contributed to the observed increase in total IgE binding. Although only moderate changes in the SDS-PAGE profiles were seen in smoked salmon [present study and ref. 27], novel bands have been found previously at 25, 30 and >70 kDa [33]. Process-induced changes in protein conformation might also expose previously hidden

epitopes, making them more accessible to antibody binding, or proteolytic peptide modification may induce the formation of novel structures [34], both of which events might explain an apparent increase in IgE binding in smoked fish products. However, in dried cod, an apparent increase in total IgE binding was not supported by increased IgE or antibody binding in immunoblot, and might rather be explained by a relative increase in the amount of parvalbumin in the inhibitor extract, compared to other, less stable proteins. Interestingly, fish-allergic subjects responded differently in challenge tests to lyophilized and nonlyophilized fish extracts, but these subtle variations were not detected in vitro [20].

Fish allergens, in particular parvalbumin, are considered relatively heat stable [35]. However, IgE binding may be decreased by boiling or frying [16, 21], or even increased [21]. Inhibition ELISA showed that allergenicity in tuna is decreased after canning [present study and ref. 20]. Canned tuna and salmon were both reported as being less allergenic than fresh fish [13, 20], to the extent that tuna-allergic subjects tolerated up to 180 g tuna [20]. However, diffuse IgE staining from 38–60 kDa in canned tuna (subjects 1, 5, 7 and 18) suggested that residual allergenicity cannot be ruled out, and that some individuals may be sensitized to epitopes on modified proteins. A clinical reaction to canned tuna has been reported, with IgE binding between 52 and 104 kDa [36].

Chemical processes generally resulted in a loss of IgE-binding activity in both immunoblot and ELISA. Adjusting the pH of fish extracts to levels used in *lutefisk* production or pickling caused extensive protein denaturation (data not shown), and parvalbumin was reported to be rapidly degraded in vitro by pepsin at pH 2 whereas it was stable at pH 5 [37]. While IgE binding to monomeric parvalbumin was relatively unchanged in *lutefisk*, PARV-19 and K991 binding decreased, as did binding to the putative oligomeric bands by both IgE and antibodies, suggesting that the oligomeric forms might be more susceptible to process-induced change. Curing and fermentation (*rakørret*, *gravlaks*) are enzymatic processes which may induce changes in parvalbumin oligomers, either by altering the conformation (denaturation) or by direct cleavage of the IgE-binding epitopes [34]. Increased IgE binding to these chemically processed extracts was seen in 2 patients, who may therefore be sensitized to modified rather than intact peptides.

Proteolysis is an efficient way of reducing allergenicity [38], but processing may also expose preexisting epitopes or create neoepitopes by aggregation [19]. Fragments of cod allergen (6.5 and 8.5 kDa) possess allergenic activity

[39], and peptide fragments as small as 3 kDa are sufficient to cross-link IgE molecules on the mast cell membrane and induce IgE-mediated allergic reactions [38]. The frequency of in vitro IgE binding to the hydrolysates SG-3 (15.4%) and SG-5 (17.4%) demonstrated that in a group of subjects sensitized to the constituent fish species, a small number were also sensitized to degraded peptides. IgE binding to salmon was inhibited by SG-3 in 3 of the sera, but SG-5 inhibited binding to whiting only in subject 18, which probably reflects differences in the IgE-binding affinity to peptide fragments in the inhibition ELISA. Interestingly, the unexpected increase in IgE binding to the three chemically processed extracts (*lute-fisk*, *gravlaks*, *rakørret*) was also observed in serum from subject 18, suggesting an increased ability to recognize degraded peptides in several fish species in this subject.

Specific IgE is a prerequisite of clinical reactivity to any protein, but its presence alone need not trigger an allergic response [40], and serological and clinical cross-reactivities may not necessarily be correlated [5, 7, 16]. Although immunochemical methods are useful in comparing the relative immunogenicity of foods before and after processing, the use of functional assays, e.g. the basophil activation test, is essential for assessing the clinical relevance of these types of process-induced changes, and double-blind placebo-controlled food challenge should

ideally be used to compare the eliciting thresholds for processed and unprocessed foods [19]. However, due to practical and ethical considerations, data from such studies are limited.

In conclusion, trends to altered immunogenicity assessed using the sera of fish-allergic subjects were seen in 7 commonly consumed fish species following a variety of traditional methods of processing. However, individual variations in responses emphasized the unpredictable nature of food-allergic responses, and the clinical significance of these findings remains to be established. Highly susceptible fish-allergic subjects may have an increased capacity to recognize peptides in highly processed or partially degraded products.

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