

Recognition of an extensive range of IgE-reactive proteins in cod extract

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Allergy to fish is one of the most common food allergies. Gad c 1 is the only fish allergen which has been purified and characterized. Other allergens have been detected by Western blot in cod extracts. We have now improved the Western-blot procedure in order to characterize fish IgE-reactive proteins from extracts prepared under different conditions: pre-rigor mortis and post-rigor mortis, EDTA addition or not, and DEAE ion-exchange chromatography. Several IgE-reactive protein bands have been identified over a wide molecular-weight range. In particular, the 104- and 130-kDa IgE-reactive protein bands were detected. These new bands may correspond to aggregates, as EDTA increased the relative amount of the 60-, 67-, 104-, and 130-kDa IgE-reactive protein bands in Western blot. All these bands were also detected by an antiparvalbumin monoclonal antibody, specific to the first calcium-binding site. The longer period of storage increased the relative amounts of the 41-, 80-, 104-, and 130-kDa IgE-reactive protein bands. The 18-kDa band was detected only in fish stored for several days. In conclusion, we have described IgE-reactive protein bands over a wide molecular-weight range (12–130 kDa) in Western blot of cod extract, and shown that EDTA and storage conditions may influence the relative distribution of IgE-reactive protein bands.

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Food allergy affects 1–5% of the population (1–5). Children are especially affected, as 1–10% of them are food sensitive (4, 6, 7). These percentages increase in the case of certain diseases (e.g., atopic dermatitis [1, 8–10], urticaria [11, 12], asthma [12], and pollen allergy [13]). Severe and even fatal anaphylactic shocks have been reported in the literature (14–16).

Fish is one of the most commonly allergenic foods (1, 12, 17–22). Aas has shown that 88% of children hypersensitive to fish have specific IgE against cod (12). Other studies have shown that cod often causes allergy (21, 22).

Until now, only the major cod allergen, Gad c 1, has been purified and characterized. Aas separated the cod proteins by ion-exchange chromatography on DEAE cellulose (23), gel filtration (24), and isoelectrofocalization (25). Gad c 1 is a 12.3-kDa parvalbumin (26) with an isoelectric point of 4.75

(25). Five allergenic sequences have been described (27–31). Other cod allergens were shown in extracts from the post-rigor mortis state by Western blotting with sera of patients allergic to salmon (21) or cod (32). Our group also found a 63-kDa allergen in cod-surimi (33). None of these allergens had been purified. In addition, the effects of fish storage conditions on allergenicity have not been studied until now, particularly as far as the consequences of postmortem degradation of the fish is concerned; e.g., the pre-rigor mortis and post-rigor mortis states.

Therefore, the present study aimed to characterize cod IgE-reactive protein bands by Western blotting of crude extract, in the pre-rigor mortis state, of semipurified fractions collected from ion-exchange chromatography, and to evaluate the effect of postmortem degradation on the allergenic proteins.

Material and methods

Sera

The patients involved in this study had had an immediate adverse reaction to cod. Three patients were followed up in the allergology medical unit of the Nancy university hospital center and had clinical symptoms of cod allergy and positive prick tests. Nine other patients had had an immediate adverse reaction to cod and were investigated for the detection of specific IgE against cod in a medical laboratory specialized in allergy (CERBA Laboratory, Paris, France). Of the 12 patients included in this study, 11 had positive cod (f3) CAP-RAST (Pharmacia, Uppsala, Sweden). The blood was collected in dry tubes and centrifuged at 1000 *g* for 5 min. The serum was aliquoted and kept at -80°C.

Nine control sera were collected from healthy subjects without fish allergy, with a total IgE serum level lower than 30 kUI/l.

Preparation of cod extracts

Three cod extracts were prepared for this study:

- 1) Pre-rigor mortis cod: a fillet was cut before the postmortem rigidity and immediately placed in liquid nitrogen.
- 2) Post-rigor mortis cod was obtained by a standardized procedure: the fillet was cut just after postmortem rigidity and placed in liquid nitrogen. These fillets were provided by IFREMER, the French Marine Research Institute.
- 3) Commercial cod: a fresh fillet kept at 4°C was bought in a fish shop; proteins were extracted less than 1 h after the fillet was bought.

Cod muscles were crushed and homogenized in phosphate buffer 0.07 M, pH 7.0, or phosphate buffer 0.07 M, pH 7.0, 0.6 M NaCl at 4°C in a 2-ml solution per gram of tissue. The mixture was centrifuged at 20 000 *g* for 20 min at 4°C. The supernatants were lyophilized and stored at 4°C. Less than 1 month later, the pre-rigor mortis cod extract was used for the DEAE chromatography, and less than 4 months later for the other experiments. Only soluble proteins, mainly sarcoplasmic proteins, were extracted at low ionic strength. Myofibrillar proteins, such as actin and myosin complex, were also solubilized in 0.6 M NaCl.

Protein concentration

The protein concentrations of the extracts and of the fractions (the latter obtained by ion-exchange chromatography) were determined by the Bradford method (34), the Coomassie[®] Plus Protein Assay Rea-

gent (Pierce, Rockford, IL, USA) being used according to the manufacturer's instructions. Bovine serum albumin (BSA) was used as a protein standard.

IgE radioimmunoassay (RIA)

The pre-rigor mortis cod proteins were coupled to nitrocellulose disks by ALLERBIO (Varennese-Argonne, France). The IgE radioimmunoassay was performed as follows (33, 35–37). A volume of 50 µl of serum was incubated with a cod nitrocellulose disk for 3 h at 20°C in duplicate. The nitrocellulose disk was washed three times with 2.0 ml of 20 mM Tris-HCl, pH 7.4, 0.02% (w/v) BSA, and 0.5 M NaCl. The disk was incubated overnight at 20°C with 50 µl of ¹²⁵I-labeled antihuman IgE (Immunotech, Marseille, France) (30 000 cpm). The excess of labeled anti-IgE was eliminated by washing as described above. The gamma-radioactivity was counted. The results were expressed as a mean percentage of gamma-radioactivity bound onto the cod disk, and as the ratio of the mean percentage of total gamma-radioactivity bound to the cod disk for the patient to the mean percentage of radioactivity bound for nonatopic subjects: a ratio > 3 indicates the presence of specific IgE (35, 37).

Inhibition of the IgE-RIA was performed by the same protocol except that the patient's serum was incubated in duplicate for 1 h at 20°C with 50 µl of cod protein solution (33, 36, 37) before incubation with the reactive phase. The inhibition percentage (IP) was estimated from gamma-counting of the solid phase with or without preincubation of the patient's serum with the protein solution:

$$IP = [(cpm_i - cpm_0) / cpm_0] \times 100$$

where cpm_0 represented the radioactivity adsorbed to the solid phase without incubation with the cod protein solution, and cpm_i represented the radioactivity adsorbed with incubation with the cod protein solution.

DEAE chromatography

The cod proteins were separated by ion-exchange chromatography on DEAE-Sepharose[®] CL-6B (Pharmacia, Uppsala, Sweden) using a two-pump gradient system P-50 (Pharmacia). The column was 16 mm across and 200 mm high. An amount of 40 mg of cod extract was applied to the column. The proteins were eluted with a NaCl gradient of 0.07 M phosphate buffer, pH 7.0, as buffer A and 0.07 M phosphate buffer, pH 7.0, and 1 M NaCl as buffer B. The flow rate was constant: 1.0 ml/min. The elution of the proteins was analyzed by absorbance measurement at 280 nm. The proteins were collected in 5-ml fractions. The fractions were dialyzed against

distilled water. These fractions were studied by dot-blotting and Western blotting. The relative allergenicity for each collected fraction was determined by the dot-blotting analysis with a pool of the 12 cod-allergic sera and was estimated as the ratio of the radioactivity bound in the protein fraction to the radioactivity bound in the crude extract.

SDS-PAGE

Polyacrylamide gel electrophoresis (PAGE) of cod proteins (pre-rigor mortis with or without incubation with 5 mM EDTA, post-rigor mortis, DEAE protein fractions, commercial cod) and in the presence of sodium dodecyl sulfate (SDS) was performed with a 10–20% polyacrylamide gel gradient in 20 mM Tris-HCl (pH 8.3), 0.18 M glycine, and 0.1% SDS, using a vertical Mini Protean® II apparatus (Bio-Rad, Richmond, CA, USA). A buffer containing 30 mM Tris-HCl, pH 8.0, 3 mM EDTA, 3% SDS, 120 mM DTT, 30% glycerol, and 0.05% bromophenol blue was used for the preparation of samples submitted to electrophoresis. The mixture was heated at 100°C for 3 min before loading. An amount of 2 µg of proteins was loaded and separated for 90 min at 200 V when the gel was silver stained. An amount of 10 µg of proteins was loaded for the Western blot. Two molecular mass markers were used. The first one was provided by Pharmacia (Uppsala, Sweden): phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and lactalbumin (14.4 kDa). The second was provided by Bio-Rad (Richmond, CA, USA): myosin (208 kDa), β -galactosidase (144 kDa), BSA (87 kDa), carbonic anhydrase (44.1 kDa), soybean trypsin inhibitor (32.7 kDa), lysozyme (17.7 kDa), and aprotinin (7.1 kDa).

Silver staining of the gel

The proteins were fixed for 1 h in 40% methanol and 10% acetic acid. The gel was washed three times in 30% ethanol and incubated in 0.02% sodium thiosulfate for 1 min. The gel was washed three times in deionized water and incubated for 20 min in 0.2% silver nitrate and 0.02% formaldehyde. The gel was again washed three times in deionized water and incubated in 3% sodium carbonate, 0.05% formaldehyde, and 0.0005% sodium thiosulfate until the protein bands were sufficiently stained. The gel was washed with deionized water.

Western blotting

After separation by electrophoresis, the proteins were transferred for 16 h at 20 V onto a nitrocel-

lulose membrane (0.2 µm Trans-Blot® Transfert Medium, Bio-Rad, Richmond, CA, USA) with a Trans-Blot® Cell (Bio-Rad). The following buffer was used for the transfer: 25 mM Tris-HCl, pH 8.3, 192 mM glycine, and 20% methanol.

Detection of the blotted proteins with a pool of cod-allergic sera. The membrane was blocked and incubated with the allergic sera as described by Mout  t   et al. (37), except that gelatin was replaced by Tween 20 (38). Unoccupied protein-binding sites were blocked by incubation with 0.3% (v/v) Tween 20, 20 mM Tris-HCl, pH 7.4, and 0.15 M NaCl for 1 h. After washing three times with 0.05% (v/v) Tween 20, 20 mM Tris-HCl, pH 7.4, and 0.15 M NaCl, the nitrocellulose was incubated for 3 h with 1 ml of a pool of 12 sera with specific IgE against cod IgE-reactive protein bands per protein extract tested (the pool of sera was diluted 1:10 in 0.1% (v/v) Tween 20, 20 mM Tris-HCl, pH 7.4, and 0.15 M NaCl) for 3 h at room temperature. The nitrocellulose was washed three times and incubated overnight with 1 ml of ¹²⁵I-labeled anti-IgE (diluted 1:10 in 0.3% (v/v) Tween 20, 20 mM Tris-HCl, pH 7.4, and 0.15 M NaCl) (60 000 cpm). IgE binding was detected by exposure on a Phosphor screen (PhosphorImager, Molecular Dynamics). The screen was scanned with the PhosphorImager 445 SI apparatus. Relative allergenicity was determined for each IgE-reactive protein band in each cod extract. For each cod extract, the intensity of each IgE-reactive protein band was added. The relative allergenicity was expressed as the ratio of the band intensity to the sum of the band intensities $\times 100$.

Detection of the blotted proteins with antiparvalbumin (Sigma, St Louis, MO, USA). The membrane was blocked as described above. It was incubated with monoclonal antiparvalbumin (39) (clone PA-235, Sigma) diluted 1:1000 in 0.1% (v/v) Tween 20, 20 mM Tris HCl, pH 7.4, and NaCl 0.15 M for 3 h at 20°C. The membrane was washed three times and was incubated with anti-mouse IgG (whole molecule) alkaline phosphatase conjugate (Sigma) diluted 1:10 000 in 0.3% (v/v) Tween 20, 20 mM Tris HCl, pH 7.4, and NaCl 0.15 M for 3 h. The membrane was again washed three times and incubated with alkaline phosphatase substrate, 5-bromo-4-chloro-3-indolyl phosphate, and nitroblue tetrazolium, the BCIP/NBT Buffered Substrate Tablet (Sigma) being used. The nitrocellulose membrane was removed when sufficient color had developed and was then rinsed with distilled water.

Table 1. Cod (f3) CAP-RAST results and adsorption to cod nitrocellulose disk of serum IgE from patients who had allergic reaction to cod

Patient	Age (years)	f3 (cod) CAP-RAST RIA results (Pharmacia)		Adsorption of cod nitrocellulose disks	
		Specific IgE level (kU/l)	Class	% Radioactivity bound	Ratio patient/control
C.J.	13	5.3	3	42	18.3
M.M.	5	8.3	3	63	27.4
D.W.	55	92.9	4	52	22.6
C.J.2	13	3.3	2	37	16.1
D.M.	4	3.7	3	36	15.6
S.S.	44	1.9	2	8	3.5
R.Y.	18	63.2	4	73	31.7
L.D.	17	Not determined		11	4.8
S.C.	5	3.9	3	35	15.2
B.J.	1	9.8	3	45	19.6
B.M.	13	4.4	3	37	16.1
E.C.	7	4.3	3	44	19.1
Nine negative control sera					
Minimum				1.2	
Maximum				3.1	
Mean value				2.3 ± 0.7	

Dot-blotting

An amount of 10 µg of each DEAE protein fraction or crude pre-rigor mortis cod extract was applied to a nitrocellulose membrane (0.2 µm Trans-Blot® Transfert Medium, Bio-Rad, Richmond, CA, USA) with the Bio-Dot® Microfiltration Apparatus (Bio-Rad, Richmond, CA, USA). The nitrocellulose membrane was blocked and incubated with a pool of the 12 sera with specific IgE against cod diluted 10 times, as described for the Western-blotting protocol, except that only 250 µl of diluted sera was used per cod extract tested.

Relative allergenicity was determined for each DEAE protein fraction. An amount of 10 µg of the crude pre-rigor mortis cod extract served as the allergenicity reference. The relative allergenicity for each DEAE fraction was estimated as the ratio of the fraction allergenicity to the crude extract allergenicity.

Results

The IgE radioimmunoassay (RIA) was performed with 12 cod-allergic sera, cod protein nitrocellulose disks prepared by Allerbio (Varennnes-en-Argonne, France) being used with our cod pre-rigor mortis protein extract. The 12 sera had a ratio of the percentage of ¹²⁵I-labeled anti-human IgE bound to the percentage of radioactivity bound in the case of nonatopic subjects higher than 3 (Table 1). This indicated the presence of specific IgE against cod proteins. The ratios were between 3.5 and 31.7. The mean value of the nine negative sera was 2.3 ± 0.7%.

Pre-rigor mortis cod proteins were extracted in 0.07 M phosphate buffer, pH 7.0, with or without 0.6 M NaCl. The two extracts were analyzed by IgE-RIA inhibition using C.J.'s serum. The proteins obtained by both extraction conditions gave typical inhibition curves (data not shown). The curves were closely related. The dose that inhibited 50% RAST was 1 mg/l in both cases. The subsequent fractionation of protein in DEAE chromatography was performed only with the NaCl-free protein extract.

In preliminary studies, different Western-blot protocols were tested in which the blocking agent was different: gelatin, milk, BSA, and Tween 20. No allergen was detected with C.J.'s serum when gelatin, milk, or BSA was used. IgE-RIA inhibition to cod proteins was observed with gelatin, milk, and BSA, showing the presence of nonspecific interaction between the IgE against cod proteins and these blocking agents (data not shown). When Tween 20 was used as a saturating agent of the Western blot, six IgE-reactive protein bands were found (data not shown): 12.5, 19, 29, 39, 62, and 104 kDa, with C.J.'s serum. The highest band intensity for this patient was the 104-kDa IgE-reactive band.

Separation of the cod proteins by DEAE chromatography and study of their allergenicity

The soluble pre-rigor mortis cod proteins were separated onto DEAE sepharose® CL-6B (Pharmacia, Uppsala, Sweden). The best separation was obtained with the NaCl gradient shown in Fig. 1.

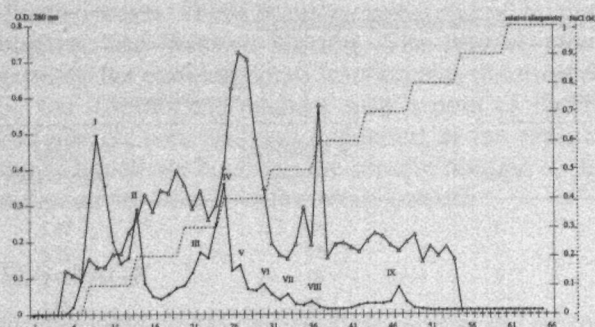


Fig. 1. DEAE chromatography and relative allergenicity of pre-rigor mortis cod proteins. Elution was performed in 0.07 M phosphate buffer, pH 7.0, with NaCl gradient and flow rate of 1 ml/min. Elution of proteins was analyzed by absorbance measurement at 280 nm. Relative allergenicity was determined in dot-blot analysis. ♦ profile elution, △ relative allergenicity, ... : NaCl concentration.

Three main peaks were cleaved (I, II, and IV) concurrently with six minor peaks. The peaks were analyzed by SDS-PAGE (Fig. 2). Each collected fraction was analyzed by dot-blot with 250 µl of a 10-fold diluted pool of the 12 sera indicated in Table 1 with specific IgE against cod. The highest positivity of dot-blot was observed at retention times of 90, 130, and 185 min, corresponding, respectively, to peaks III, V, and VIII (Fig. 1).

Western blotting was performed with samples corresponding to the peaks eluted from DEAE chromatography and also with the crude extracts. The same pool of the 12 sera with specific IgE against cod as for the dot-blot was used (1 ml of 10-fold diluted sera per extract). The results are presented in Fig. 3. The distribution of the IgE-reactive protein bands is also given in Table 2,

taking into account that 16 molecular sizes ranging from 12 to 130 kDa were observed in the Western blot. The crude extract was composed of eight IgE-reactive protein bands: 12, 22, 30, 45, 60, 67, 104, and 130 kDa. The 12-, 30-, and 67-kDa bands gave the most intensive radioactivity bands. The 12-kDa IgE-reactive protein band was found in the nine analyzed fractions. The 130- and the 104-kDa bands were more intensively observed in eluted fractions IV and V than in the crude extract. Bands of 13, 23, 25, 41, 49, 55 and 80 kDa were observed only in the DEAE fractions.

Detection of a common epitope to parvalbumins

The blotted proteins were detected by antiparvalbumin (Sigma, clone PA-235) (Fig. 4, Table 2). This antibody is directed against an epitope in the first parvalbumin Ca^{2+} -binding site and specifically stains the Ca^{2+} -bound parvalbumin form (39). All the IgE-reactive protein bands in the crude extract were recognized by antiparvalbumins. The 25-, 50-, and 80-kDa IgE-reactive protein bands which were found only in the DEAE peaks were also recognized by antiparvalbumins in Western blot.

To study the influence of Ca^{2+} , we incubated the pre-rigor mortis cod extract with and without 5 mM EDTA overnight. The extract was analyzed by Western blot with the same pool of the 12 cod-allergic sera as for the dot-blot and Western blot described above. The same IgE-reactive protein bands were detected in both experiments, but the 30-kDa band relative amount was lower, and the 60-, 67-, 104-, and 130-kDa band relative amount was increased, in the presence of EDTA (Figs. 3 and 5).

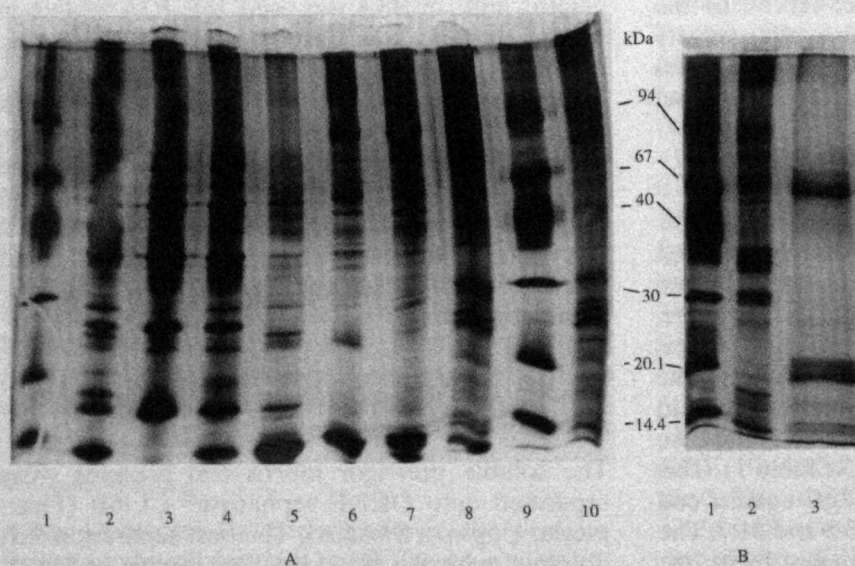


Fig. 2. SDS-PAGE of pre-rigor mortis cod DEAE peaks. Proteins were separated on polyacrylamide gel gradients of 10–20%. Amount of 2 µg of each protein fraction was applied and separated for 90 min (200 V). Proteins were silver-stained. A) Lane 1: molecular weight markers; lane 2: total pre-rigor mortis cod extract; lane 3: DEAE peak I; lane 4: DEAE peak II; lane 5: DEAE peak III; lane 6: DEAE peak IV; lane 7: DEAE peak V; lane 8: DEAE peak VI; lane 9: molecular weight markers; lane 10: DEAE peak VII. B) Lane 1: molecular weight markers; lane 2: DEAE peak VIII; lane 3: DEAE peak IX.

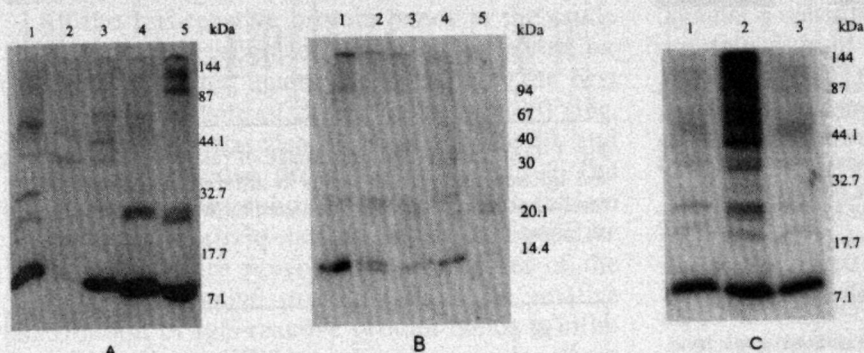


Fig. 3. Western blot of pre-rigor mortis cod DEAE peaks with pool of 12 cod-allergic sera. SDS-PAGE was performed on 10–20% polyacrylamide gel. Amount of 10 µg of each protein fraction was separated (90 min at 200 V). Proteins were transferred onto nitrocellulose membrane. Membrane A) Lane 1: crude pre-rigor mortis cod extract without EDTA; lane 2: DEAE peak I; lane 3: DEAE peak II; lane 4: DEAE peak III; lane 5: DEAE peak IV. Membrane B) Lane 1: DEAE peak V; lane 2: DEAE peak VI; lane 3: DEAE peak VII; lane 4: DEAE peak VIII; lane 5: DEAE peak IX. Membrane C) Lane 1: post-rigor mortis cod obtained just after postmortem rigidity; lane 2: post-rigor mortis cod bought in fish shop; lane 3: pre-rigor mortis cod proteins incubated with 5 mM EDTA overnight.

Influence of freshness on allergen distribution

Two post-rigor mortis cod extracts were used: the first was obtained by a standardized procedure, i.e., just after postmortem rigidity, and the second was bought in a fish shop. Western blot using the same pool of 12 sera from allergic patients with specific IgE as the pool used above showed a different IgE-reactive protein band relative distribution with the commercial fillet from the crude pre-rigor mortis cod extract (Figs. 3 and 5). All the IgE-reactive protein bands found in the crude pre-rigor mortis extract (Fig. 3, Table 2) were found in the commercial fillet. A longer period of storage might have increased the relative amounts of these bands and also of the additional 18-, 41-, and 80-kDa IgE-reactive protein bands in the commercial fish extract. A 41-kDa IgE-reactive protein band was detected in the DEAE peak VIII, and an 80-kDa IgE-reactive protein band was detected in the DEAE peaks I and III. The 18-kDa IgE-reactive protein band was not detected elsewhere. The cod protein extract prepared just after postmortem rigidity did not affect the relative distribution of IgE-reactive protein bands, compared to the pre-rigor mortis one (Figs. 3 and 5). An amount of 1 µg of pre-rigor mortis cod extract inhibited 50% of D.W.'s serum IgE-RIA, whereas only 10 ng of commercial cod extract could inhibit this IgE-RIA at the same level (Fig. 6).

Discussion

The RIA of the specific IgE against pre-rigor mortis cod proteins was positive for all the patients included in this study (Table 1). The IgE-RIA

Table 2. Distribution of proteins detected by specific IgE and antiparvalbumins in different pre-rigor mortis cod DEAE peaks and in crude extract

Protein (kDa)	Crude	DEAE I	DEAE II	DEAE III	DEAE IV	DEAE V	DEAE VI	DEAE VII	DEAE VIII	DEAE IX
160										
150										
130										
104										
97										
92										
87										
80										
72										
67										
60										
57										
55										
49										
45										
41										
35										
30										
25										
23										
22										
13										
12										

Protein detected by antiparvalbumin
IgE-reactive protein band

inhibition test of pre-rigor mortis cod proteins, the Western blot (Fig. 4), and the dot-blot showed the presence of IgE-reactive protein bands in the pre-rigor mortis cod soluble proteins. These results agree with the results obtained by Aas (23).

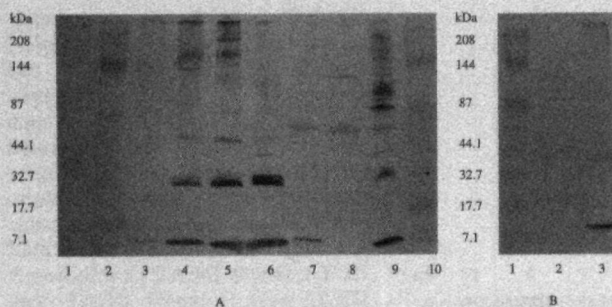


Fig. 4. Western blot of pre-rigor mortis cod DEAE peaks with antiparvalbumin. SDS-PAGE was performed on 10–20% polyacrylamide gel. Amount of 10 μ g of each protein fraction was separated (90 min at 200 V). Proteins were transferred onto nitrocellulose membrane. Membrane A) Lane 1: molecular weight markers; lane 2: crude pre-rigor mortis cod extract; lane 3: DEAE peak I; lane 4: DEAE peak II; lane 5: DEAE peak III; lane 6: DEAE peak IV; lane 7: DEAE peak V; lane 8: DEAE peak VI; lane 9: molecular weight markers; lane 10: DEAE peak VII. Membrane B) Lane 1: molecular weight markers; lane 2: DEAE peak VIII; lane 3: DEAE peak IX.

Optimization of the Western-blotting technique for the detection of interaction between cod IgE-reactive protein bands and IgE from cod-allergic sera was carried out. This optimization was obtained by limiting nonspecific interaction between specific IgE against cod IgE-reactive protein bands and the Western-blotting saturating agent. It allowed us to identify new IgE-reactive protein bands in crude cod extract and in DEAE cod protein fractions. These new bands may correspond to protein aggregates as well as new allergens. The pre-rigor mortis crude extract contained eight IgE-reactive protein bands of 12, 22, 30, 45, 60, 67, 104, and 130 kDa (Fig. 3 and Table 2). They were detected by Western blot using a pool of 12 cod-allergic sera at our disposal with Tween 20 as the saturating agent. This detergent was much more efficient than other proteins in avoiding nonspecific adsorption of IgE to the nitrocellulose membrane. It was hypothesized that hydrophobic interaction between IgE and the blocking agent of the membrane was better eliminated with Tween 20 than with other hydrophobic proteins such as gelatin, milk proteins, and BSA. Hydrophobic interaction between IgE and its target was a phenomenon already observed in drug allergy by our group (35).

The 12-kDa IgE-reactive protein band, which corresponds to the major cod allergen Gad c 1 described in the literature (26), was the major band in Western blot (50% of total radioactivity bound in the crude extract, Figs. 3 and 5). The 30- and 67-kDa IgE-reactive protein bands also showed great activity. Hemmens et al. (32) showed by Western blot with three cod-allergic sera that the semipurified DS22 fraction, which contains Gad c 1 (25),

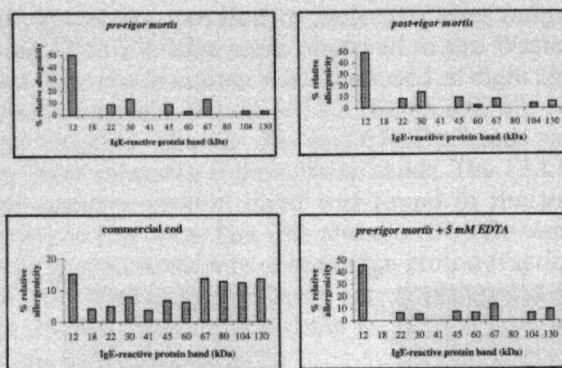


Fig. 5. Distribution and percentage of relative radioactivity of cod IgE-reactive protein bands in different cod extracts. IgE-reactive protein bands were detected by Western blot with pool of 12 cod-allergic sera.

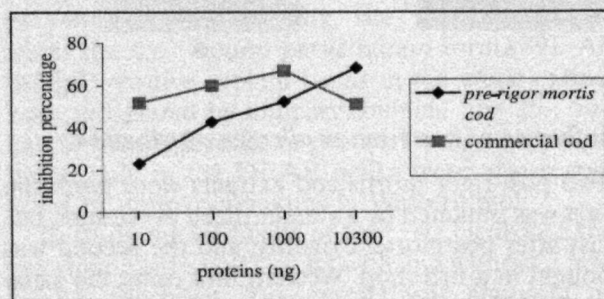


Fig. 6. Inhibition of D.W.'s serum IgE-RIA by pre-rigor mortis and commercial cod extract.

contained six allergenic components. Bernhisel-Broadbent et al. (21) showed, with sera from salmon-allergic patients, that seven cod proteins were recognized by the IgE. The 63-kDa allergen was the only allergen found in cod-surimi (33) by our group. Finally, our study describes the presence of high-molecular-mass IgE-reactive protein bands in Western blot of cod extract, with respective M_r of 104 and 130 kDa. These bands may correspond to protein aggregates rather than to new allergens, as their relative distribution in Western blot is influenced by adding EDTA to the protein sample run in SDS-PAGE.

The pre-rigor mortis cod proteins were separated by DEAE chromatography (Fig. 1). Nine peaks with an allergenic activity detected by dot-blot in a pool of 12 cod-allergic sera were obtained. All the peaks were analyzed by Western blot using the same pool of sera (Fig. 3, Table 2). Eight additional IgE-reactive protein bands were found, compared to the crude extract, at 13, 23, 25, 41, 49, 55, and 80 kDa. In the eluted peaks, these proteins may be more concentrated than in the crude extract; this explains why they were detected only after DEAE-chromatography. Gad c 1 is found in all of the peaks. It is the major allergenic component in

peaks II–VIII (Fig. 3). In peaks IV and V, the 104- and 130-kDa IgE-reactive protein bands were detected; the high allergenic activity of peaks IV and V shown in the dot-blot experiment may be explained by the high concentration of these proteins. The 23- and 25-kDa IgE-reactive protein bands may have contributed to the high allergenic activity in peak III.

All the IgE-reactive protein bands in the crude extract were recognized by antiparvalbumins, which are directed against an epitope in the first calcium-binding site of the parvalbumin (39) (Fig. 4, Table 2). This was also the case with the 50- and 80-kDa IgE-reactive protein bands found in the DEAE chromatography. One hypothesis to explain this immunoreactivity is that certain IgE-reactive protein bands are polymers or aggregates of the 12-kDa IgE-reactive protein band. The relative distribution of IgE-reactive protein bands is influenced by adding EDTA to the protein sample run in SDS–PAGE. A second hypothesis is that Gad c 1 is adsorbed to other proteins (26). A third hypothesis is that there is a common antigenic site corresponding to the first calcium-binding site. This last hypothesis is reinforced for the 60-, 67-, 104-, and 130-kDa IgE-reactive protein bands, as their relative amount detected with ¹²⁵I-labeled anti-IgE was higher when the protein extract was prepared with 5 mM EDTA than when it was not incubated with EDTA. These differences were detected by Western blot using a pool of 12 cod-allergic sera (Figs. 3 and 5). It has already been shown that there is a 30% decrease of the radioactivity bound when Gad c 1 is preincubated with EDTA compared to Gad c 1 without preincubation (40).

This study also aimed to compare the IgE-reactive protein band distribution of pre-rigor mortis with post-rigor mortis cod by Western blot. A pool of 12 cod-allergic sera was used for this study. No differences in band distribution between pre-rigor mortis and post-rigor mortis cod were detected just after postmortem rigidity. Moreover, when the fish had been dead for several days, there were three additional IgE-reactive protein bands, compared to the crude pre-rigor mortis cod extract: bands of 18, 41, and 80 kDa (Fig. 3, Table 2). The 41- and 80-kDa IgE-reactive protein bands were also found in DEAE fractions of the pre-rigor mortis cod proteins (Fig. 3, Table 2). The 18-kDa IgE-reactive protein band was not detected elsewhere. In addition, 10 ng of commercial cod protein extract inhibited 50% of DW serum IgE-RIA. In comparison, 1 µg of pre-rigor mortis cod protein extract was needed to inhibit this IgE-RIA at the same level (Fig. 6). Hence, the commercial cod extract was more allergenic than the pre-rigor mortis cod extract.

In conclusion, the Western blots of the cod extracts performed in this study showed IgE-reactive protein bands over a wide molecular-weight range; in particular, the 104- and 130-kDa IgE-reactive protein bands were detected. The IgE-reactive protein bands detected in the crude pre-rigor mortis extract with a pool of 12 cod-allergic sera were detected by antiparvalbumins directed against a calcium-binding epitope. In addition, the relative amount of IgE-reactive protein bands varied when EDTA was added to the cod extract. Freshness played a part in IgE-reactive protein band distribution and in IgE binding, as the relative content in IgE-reactive protein bands was greater after storing the fish for several days.

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