

IgE-binding and cross-reactivity of a new 41 kDa allergen of codfish

Background: A 41-kDa IgE-reactive protein (p41) was purified from raw cod extract. This protein is homologous to an aldehyde phosphate dehydrogenase (APDH). The present study aims to evaluate the IgE-binding and the cross-reactivity of this protein in 13 patients allergic to codfish.

Methods: IgE binding of sera from 13 patients allergic to codfish was tested by Sepharose RIA and by Western blot.

Results: Among the 13 patients, only 4 had specific IgE to APDH detected by APDH-Sepharose RIA. The two patients who had the highest level of specific IgE to human APDH also had a class 5–6 CAP-RAST IgE level to codfish, but two other patients with a class 5 had a negative APDH-Sepharose IgE-RIA. Relative content of APDH was higher in extracts of commercial nonfrozen fish, compared to *pre rigor mortis*, *post rigor mortis* and frozen commercial codfish. A high homology of codfish APDH was found with the corresponding human enzyme. A significant inhibition of APDH-Sepharose by human and, to a lesser extent, by rabbit APDH was observed. Western blot of APDH codfish extract showed two bands at 41 and 36 kDa, respectively.

Conclusions: We have characterized a new allergen from codfish, which had a high level of homology in different species. The p41 relative content of extracts from nonfrozen codfish was higher than in the other samples assessed.

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Introduction

Food allergy concerns 4–6% of children (1, 2) and 2% of the adult population (3). Fish has been reported to be one of the most commonly involved foods, after eggs, cow milk and peanuts (4–7). Moreover, fish allergy typically persists into adulthood (1). Symptoms are urticaria, atopic dermatitis, angioedema, asthma, diarrhoea or anaphylactic shock (5, 6, 8–12). Most children allergic to fish have specific IgE reacting against cod and patients allergic to cod also react to other species (13).

The major cod allergen, a 12.3-kDa parvalbumin, is well characterized. Nevertheless, many studies have detected other potential allergens (14–18). In our laboratory, a 41-kDa IgE-reactive protein (p41) was purified from raw cod extract by ammonium sulphate fractionation, hydroxyapatite chromatography and preparative electrophoresis (19). This protein is homologous to an aldehyde phosphate dehydrogenase (APDH) (unpublished data).

The present study aims to evaluate the IgE-binding and the cross-reactivity of this protein in patients with codfish allergy.

Materials and methods

Patients

Sera were collected from 13 patients allergic to codfish. One of them was from Italy, two were from France and the other 10 were from Spain. IgE antibodies specific to codfish were measured by RAST (CAP System Pharmacia, Uppsala, Sweden). All the patients had a positive RAST (class 3–6) (Table 1). Control sera were from individuals without allergy.

IgE-RIA

The assay was performed as described by Olszewski et al. (20). Briefly, human erythrocytes APDH (Sigma, St. Louis, USA) was covalently bound to epoxy-activated Sepharose 6B (Amersham-Pharmacia) (21–23) by mixing 0.65 g of gel expanded in water with 1 mg of human APDH. Some 50 µl of gel suspension was then incubated with 50 µl of sera from allergic patients for 3 h at room temperature. The solid phase was washed three times with 2 ml of 0.05 M phosphate buffer pH 7.4, 0.5 M NaCl, 0.02% BSA and was subsequently incubated with 50 µl of ¹²⁵I-labelled antihuman IgE (Immunotech, Marseille, France) for 18 h at room temperature. The reactive phase was washed again three times with the same buffer. Bound γ-radioactivity was counted to quantify specific IgE. The RIA was considered positive when the percentage of uptake of labelled anti-IgE with the patient sera (P) was two-fold higher than that of

control sera (C). The inhibition step was performed by incubating successively the sera with APDH solutions from different species for 1 h and with human APDH-Sepharose solid phase for 3 h. The inhibition percentage (IP) of the APDH used was estimated by γ -counting: $IP = [(cpm_i - cpm_0)/cpm_0] \times 100$, where cpm_0 was the radioactivity adsorbed without preincubation with APDH and cpm_i was the radioactivity adsorbed with preincubation with the APDH extract.

Electrophoretic detection and IgE-immunoblot of the p41 allergen extracted from *pre rigor* and *post rigor mortis* codfish and from commercially available codfish

p41 protein was purified from raw cod extract as described by Galland et al. by ammonium sulphate fractionation, hydroxyapatite chromatography and preparative electrophoresis (19).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed using a Mini-protein II electrophoresis unit (Bio-Rad, Hercules, USA). The stacking and the separating gel contained 4% and 12% of acrylamide, respectively. Proteins were separated for 2 h at 125 V in 25 mM Tris-HCl (pH 8.3), 0.192 M glycine, 0.1% SDS (w/v). The molecular mass markers used were LMW (Amersham-Pharmacia): 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) or Kaleidoscope (pre-stained marker from Bio-Rad): myosin (209 kDa), β -galactosidase (125 kDa), BSA (70 kDa), carbonic anhydrase (42.1 kDa), soybean trypsin inhibitor (32.6 kDa), lysozyme (17.6 kDa), and aprotinin (7.5 kDa). Protein bands were stained with Coomassie blue. Proteins were electrotransferred onto nitrocellulose membrane (0.2 μ m, Bio-Rad) in 25 mM Tris buffer containing 192 mM glycine and 20% (v/v) methanol (pH 8.3). The transfer was done using a Trans-blot semidry transfer cell (Bio-Rad) during 30 min at 15 V. The membrane was blocked by incubation in 20 mM Tris-HCl (pH 7.4), 0.15 mM NaCl (TBS), 3% (w/v) BSA for 2 h at room temperature and was then washed 2 times with gentle rocking in 20 mM Tris-HCl buffer containing 0.15 M NaCl (TBS) with 0.05% (v/v) Tween 20. Nitrocellulose was then probed overnight with the serum from cod-allergic patient (diluted 1:5 in TBS with 0.1% Tween 20). It was washed again 5 times and incubated for one more night with ^{125} I-anti-IgE (Immunotech) (diluted 1:10 in TBS with 1% (w/v) BSA). All the incubations were performed at 4°C. IgE binding was detected by autoradiography. The film (Hyperfilm-MP from Amersham-Pharmacia) was kept at -80°C and exposed for 7 days.

Results and discussion

Among the 13 patients allergic to codfish, only 4 had specific IgE to human APDH. (Table 1). The two patients who had the highest level of specific IgE to human APDH also had a class 5–6 CAP-RAST IgE level to codfish (Table 1). Nevertheless, two other patients with a class 5 CAP-RAST had a negative APDH-Sepharose IgE-RIA. Taken together, these results suggested that no cross-reactivity of APDH occurs with the parvalbumin Gad cI. APDH was present as a soluble protein in the supernatant of ammonium sulphate fractionation of fish from 6 distinct species (Fig. 1). Its relative content was slightly increased in extracts of commercial nonfrozen codfish, compared to *pre rigor* and *post rigor mortis* codfish and to frozen commercial codfish (Fig. 1). It was detected in non-frozen commercial codfish extract but not in *pre rigor* and *post rigor mortis* frozen extracts, using Western blot

Table 1 Serum IgE specific to APDH determined by APDH-Sepharose IgE-RIA

Patient	CAP-RAST for codfish (kU/l)	Class	IgE-RIA with APDH-Sepharose		
			% uptake*	P/C†	Interpretation
1	>100	6	22.9	7.6	+
2	61	5	2.9	1	–
3	54.8	5	20.8	6.9	+
4	53	5	4.5	1.5	–
5	48.7	4	2.8	0.9	–
6	43.5	4	7.2	2.4	+
7	40.8	4	2.6	0.9	–
8	31	4	1.8	0.6	–
9	23.6	4	2.3	0.8	–
10	23.4	4	1.8	2	±
11	22.4	4	2.5	0.8	–
12	19.5	4	3.4	1.1	–
13	16.9	3	6.1	5	+
Controls	<2		≈1.7		

*% uptake corresponds to the ^{125} I-anti IgE bound to the solid reactive phase incubated with patient sera, as described in the section on Materials and methods. †The IgE-RIA was considered positive when the uptake of the tracer was at least 2-fold higher with patient sera (P) than with control sera (C).

performed with patient sera (15). This suggested that APDH could be an allergenic marker of storage conditions. In fact, APDH is an enzyme located within the cell. It may be therefore hypothesized that it is released from the cytosol after cell death and that this release increases in nonfrozen fish.

The amino acid sequence deduced from the cDNA sequence of the protein was compared to the corresponding enzyme from other animal species. In fact, the protein is well conserved in evolution, because a high level of homology was found with the codfish and the corresponding human enzyme. We investigated therefore the cross-reactivity between both enzymes by IgE-

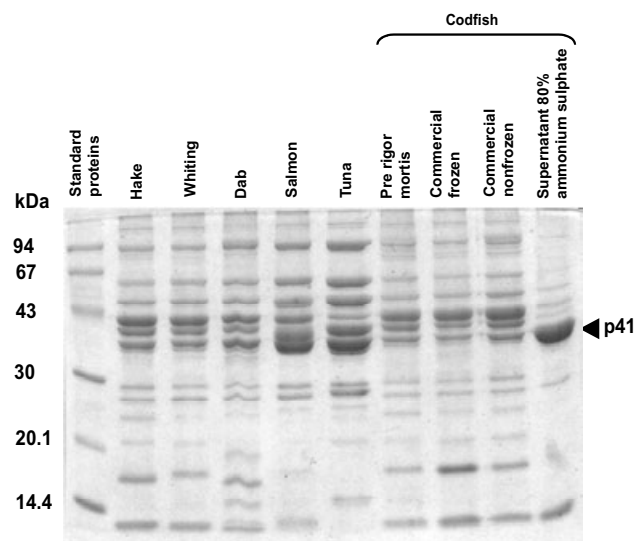


Figure 1. Sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) of proteins from fish extracts of 5 different fish species, as well as of codfish in various storage conditions: *pre rigor mortis* frozen, commercial frozen and nonfrozen. The last lane (right side) corresponds to an enriched fraction obtained by 80% ammonium sulphate precipitation. The p41 protein was observed in all extracts.

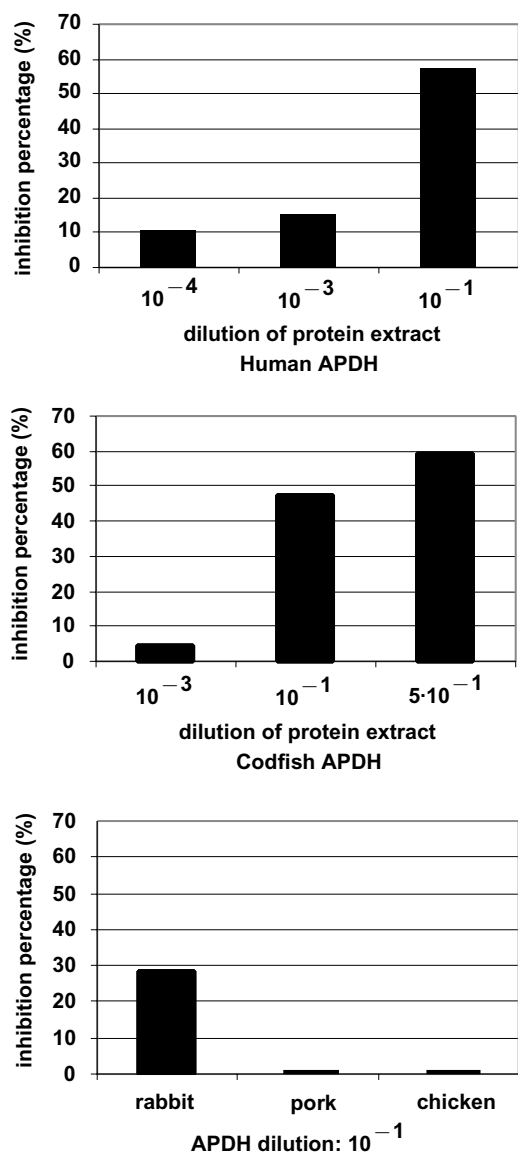


Figure 2. Inhibition tests of APDH-Sepharose IgE-RIA with different APDH extracts. The tests were performed by incubating the serum of patient 1 with APDH from human erythrocytes (top), codfish muscle (middle), rabbit, pork and chicken muscle (bottom). The serum was then subjected to APDH-Sepharose IgE-RIA as described in the methods section. A cross-inhibition was found between human and codfish ADPH. No inhibition was observed with pork and chicken APDH.

RIA inhibition test (Fig. 2). This was performed with the serum having the highest level of specific IgE to APDH. We found 1) a significant uptake of IgE to human APDH-Sepharose, and 2) a dose-related significant inhibition of human APDH-Sepharose RIA by codfish APDH extract. The cross-reactivity was much lower with rabbit APDH, while no inhibition was found with ADPH from pork and chicken (Fig. 2). The cross-reactivity of antifish APDH IgE with the human enzyme should not occur *in vivo*, as the native protein is sequestered in the cell.

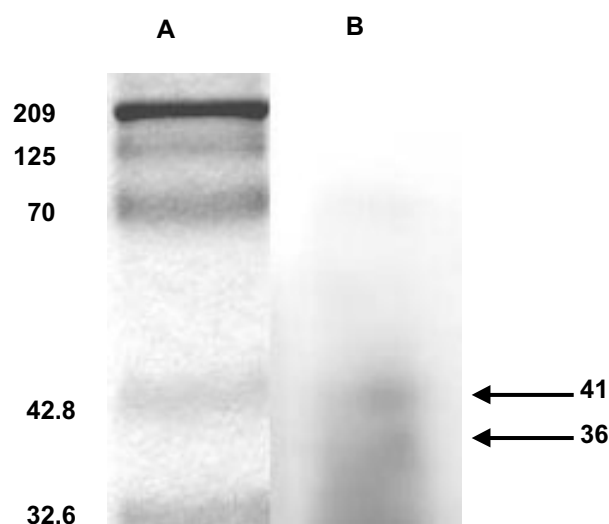


Figure 3. Western-blot of semipurified cod protein extract with IgE from an allergic patient. The proteins were blotted with the serum of patient 1 and radiolabelled with ^{125} I-iodinated anti-IgE (lane B). One main band with a molecular size of 41 kDa and a minor one of 36 kDa were identified by autoradiography. Standard proteins were run in lane A.

However, further studies should be performed to investigate whether a reactivity of IgE with the native enzyme occurs in chronic manifestations of fish allergy such as atopic dermatitis. The protein cross-reacts with an antiparvalbumin monoclonal antibody, in Western blot (15, 19). APDH shows a low homology with the calcium-binding domains of parvalbumins. The cross-reactivity with a monoclonal antiparvalbumin may therefore correspond to “nonspecific” binding. Another explanation could be that the acidic residues D, E and E of the calcium-binding domain **DEDKEG-FIEEDE** of parvalbumin are specifically recognized by the monoclonal antibody because these residues are separated by 3 distinct residues in both proteins. APDH may be one of the allergens, in addition to parvalbumin, which explains the cross-reactivity of allergen extracts from different fish species in patients allergic to codfish. A Western blot of codfish extract was performed with the serum of the patient who had the highest level of anti-p41 IgE (Fig. 3). We observed two bands, a main one at 41 kDa and a weaker band at about 36 kDa. This weaker band had been not previously observed by Galland et al. (19). The sequence of the cDNA of the open reading frame corresponded to a 333 amino acid protein with a molecular mass estimated at 36 kDa. The 36 kDa and the 41 kDa bands could correspond therefore to unglycosylated and glycosylated isoforms, respectively.

In conclusion, we have characterized a new allergen from codfish, which is an enzyme having a high level of homology in different animal species. The release of this intracellular enzyme depends on the storage conditions. This may explain our previous finding that IgE binding of p41 in nonfrozen codfish was higher than in the other samples assessed.

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