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Purification of a 41 kDa cod-allergenic protein

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Abstract

Cod fish is one of the foods most frequently involved in allergy. Only the cod allergen Gad c I, a 12.3 kDa parvalbumin, has been purified and characterized. Recently, we have detected allergen bands which have not previously been described, in particular a 41 kDa protein, by Western-blot. In the present work, this protein has been purified from a crude cod extract by ammonium sulfate fractionation, hydroxyapatite chromatography and preparative electrophoresis; a single band with an M_r of 41×10^3 was found in silver-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The amino acid composition and the isoelectric point of the protein were determined. The purified protein (p41) was shown to bind specifically to reaginic IgE from sera of cod-allergic individuals and to a monoclonal anti-parvalbumin which recognizes specifically the first calcium binding site of parvalbumins. p41 may therefore contain a calcium binding site corresponding to an IgE-epitope similar to that of Gad c I. © 1998 Elsevier Science B.V.

Keywords: IgE reactive protein bands; Cod allergy; Hydroxyapatite chromatography; Proteins

1. Introduction

Fish is one of the most common food allergens of the pediatric population [1–10]. A broad panel of symptoms are reported such as urticaria, asthma or vomiting [1,4,6,8–11]. Fish ingestion may also cause fatal anaphylactic reactions [12,13]. Cod is often responsible for fish allergy [1,2,7,11].

The major cod allergen Gad c I has been extensively studied. This allergen has been purified by ion-exchange chromatography on DEAE-cellulose [14], gel permeation on Sephadex G-75 [15] and isoelectrofocusing [16]. Gad c I is a 12.3 kDa parvalbumin [17] with an isoelectric point of 4.75 [16]. Five allergenic sequences were obtained, in particular two located on the calcium binding sites of Gad c I [18–22].

Cod fish also contains other intermediate or minor allergens of total or semi-purified cod proteins as demonstrated by Western-blot with salmon or cod allergic sera [2,23]. Our group has shown that cod-surimi contains a single 63 kDa allergenic protein [24]. In a previous study, we have adapted a Western-blot procedure in order to improve the immunodetection of allergens from cod extracts. Allergen bands which had not previously been described were detected, in particular one band at 41

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kDa. So far, none of these allergens has been purified [25].

In this paper, we report the purification and characterization of the 41 kDa allergen from a crude cod extract. The protein was purified by ammonium sulfate fractionation, hydroxyapatite chromatography and preparative electrophoresis. Allergenicity and calcium-binding sites were tested by immunodetection with cod allergic sera and with an anti-parvalbumin monoclonal antibody, respectively. The isoelectric point and amino acid composition of the protein were determined.

2. Experimental

2.1. Materials

2.1.1. Cod fillets

Cod was sampled by IFREMER at the time of an oceanographic drive off Britain. The *pre rigor mortis* cod corresponded to cod fillets which were cut and placed immediately into liquid nitrogen before being stored at −80°C.

2.1.2. Cod allergic sera

Patients involved in this study had undergone an adverse reaction against cod. Blood samples were collected in dry tubes and centrifuged at 1000 *g* for 5 min. Sera were aliquoted and stored at −80°C. Patients had a positive IgE-RIA to cod with our crude cod protein extract [25].

2.2. Purification

All procedures were carried out at 4°C or below.

2.2.1. Preparation of crude cod extract [25]

Cod muscles (100 g fresh weight) were ground in a blender and homogenized with an UltraTurax in 200 ml of 0.07 *M* phosphate buffer (pH 7.0) for 3×20 s. The homogenate was clarified by centrifugation at 20 000 *g* for 20 min. The resulting supernatant was filtered through a 0.45-μm membrane filter (Sartorius, Göttingen, Germany) and subjected to ammonium sulfate fractionation.

2.2.2. Ammonium sulfate fractionation

The crude cod extract was brought to 80% $(NH_4)_2SO_4$ saturation and left under agitation for 30 min. The mixture was centrifuged at 20 000 *g* for 20 min. The supernatant was carefully removed and dialyzed overnight against a 375-fold volume of 0.01 *M* phosphate buffer (pH 7.0). The dialysate was concentrated in an Amicon ultrafiltration apparatus using a 10 kDa cut-off filter (Amicon, Beverly, MA, USA) and filtered.

2.2.3. Hydroxyapatite chromatography [26]

The proteins of the dialysate were separated by hydroxyapatite chromatography (Ultragel HA, Sepracor, Marlborough, MA, USA) using a two-pump gradient system (Gilson, Middleton, WI, USA). The column (20×1.6 cm I.D.) was eluted with a linear gradient (0.01 *M*–0.5 *M*; 200 ml total phosphate buffer, pH 7.0) at a constant flow-rate of 1.0 ml/min. The amount of loaded proteins was 5 mg per run. Fractions were collected in 5-ml aliquots. Protein elution was detected by measuring the absorbance at 280 nm. Fractions corresponding to the first peak were pooled, dialyzed as described above and lyophilized.

2.2.4. Preparative electrophoresis

The first peak obtained after hydroxyapatite chromatography was subjected to preparative electrophoresis. Preparative electrophoresis was performed using a Prep Cell (Bio-Rad, Hercules, CA, USA), with a stacking gel of 4% and a separating gel of 12% acrylamide. The sample (1.5 mg, 500 μl) contained 10 m*M* Tris–HCl (pH 8.0), 1 m*M* EDTA, 1% SDS, 40 m*M* DTT, 10% glycerol, 0.015% bromophenol blue and was heated at 100°C for 3 min before loading. The column (7×2.8 cm I.D) was eluted with 25 m*M* Tris–HCl buffer (pH 8.3), 0.18 *M* glycine, 0.1% SDS at a constant flow-rate of 0.4 ml/min. The separation was done under 30 mA for 20 h. Fractions were collected in 2-ml aliquots. Protein elution was detected by measuring the absorbance at 280 nm. Fractions corresponding to the first peak (retention time of 560–620 min) were pooled and dialyzed as described above.

2.3. Protein determination

Protein concentrations were determined with the bicinchoninic acid (BCA) protein reagent assay (Pierce, Rockford, Il, USA) according to the manufacturer's instructions [27]. Bovine serum albumin (BSA) was used as protein standard.

2.4. Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Mini-protean II electrophoresis unit (Bio-Rad) with stacking gel of 4% and separating gel of 12% acrylamide in 25 m*M* Tris–HCl (pH 8.3), 0.18 *M* glycine, 0.1% SDS. Each sample contained 10 m*M* Tris–HCl (pH 8.0), 1 m*M* EDTA, 1% SDS, 40 m*M* DTT, 10% glycerol, 0.015% bromophenol blue and was heated at 100°C for 3 min before loading. The separation was carried out under 30 mA for 1 h. The following polypeptides were used as molecular mass markers: 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) (LMW Calibration Kit Proteins, Pharmacia, Uppsala, Sweden). Protein bands were detected by silver staining.

2.4.1. Silver staining

Proteins were fixed with 40% methanol, 10% acetic acid for 1 h. The gel was then washed with 30% ethanol for 3×20 min. Proteins were reduced with 0.02% sodium thiosulfate for 1 min, washed with ultrapure water for 3×20 s, stained with 0.2% silver nitrate, 0.02% formaldehyde for 20 min. The gel was washed and developed with 3% sodium carbonate, 0.05% formaldehyde, 0.0005% sodium thiosulfate for 3–5 min. Coloration was stopped with 0.5% glycine for 5 min and the gel was washed with ultrapure water for 2×30 min.

2.5. Western-blot [25]

Samples were run on 12% SDS-PAGE as described above, except that the following polypeptides were used as molecular mass markers: myosin (208 kDa), β-galactosidase (144 kDa), albumin (87 kDa), carbonic anhydrase (44.1), soybean trypsin inhibitor (32.7 kDa), lysozyme (17.7 kDa), aprotinin (7.1 kDa) (Kaleidoscope Prestained Standards, Bio-Rad). The proteins were transferred onto nitrocellulose (0.2-μm Trans-blot transfer medium, Bio-Rad) by blotting using a Trans-blot cell (Bio-Rad). The transfer was done for 15 h with 20 V in 25 m*M* Tris HCl (pH 8.3), 192 m*M* glycine, 20% methanol. Unoccupied protein-binding sites of the nitrocellulose sheet were blocked by incubating it for 1 h with 0.3% Tween 20 in 20 m*M* Tris–HCl buffer (pH 7.4), 0.15 *M* NaCl (TBS). The nitrocellulose was washed three times with 0.05% Tween 20 in TBS and incubating with sera from cod-allergic patients (diluted 1:10 with 0.1% Tween 20 in TBS) for 3 h, washed and incubated for an additional 3 h with ^{125}I-anti-IgE (Immunotech, Marseille, France) (diluted 1:10 with 0.3% Tween 20 in TBS). Incubations were performed under gentle rocking. IgE binding was detected by exposure for 72 h on a phosphor screen (Phosphor Imager, Molecular dynamics). The screen was scanned with the PhosphorImager 445 SI apparatus. The nitrocellulose was also incubated with a monoclonal anti-parvalbumin (clone PA-235; Sigma, St. Louis, MO, USA) (diluted 1:1000 with 0.1% Tween 20 in TBS) for 3 h, washed, incubated for an additional 3 h with anti-mouse IgG alkaline phosphatase conjugate (Sigma) (diluted 1:10 000 with 0.3% Tween 20), washed and revealed for 5 min with the alkaline phosphatase substrate, 5-bromo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium (BCIP–NBT buffered substrate tablet; Sigma).

2.6. Slot-blot

Proteins (0, 1, 2 and 5 μg) were applied onto nitrocellulose (0.2-μm Trans-blot transfer medium, Bio-Rad) using the Bio-Slot microfiltration apparatus (Bio-Rad). The nitrocellulose was blocked and incubated as described in the Western-blot protocol, except that for incubation with a cod allergic serum (dilution 1:10, 1:5 and 1:2) IgE binding was detected by autoradiography on Kodak X-Omat film (Sigma) (exposure at −80°C for 5 days).

2.7. Isoelectrofocusing

Isoelectrofocusing was performed using the automated Phast-System (Pharmacia) with a PhastGel Dry IEF pH 3–10 according to the manufacturer's instructions (Pharmacia). The following polypeptides were used as isoelectric point markers: amyloglucosidase 3.5, methyl red 3.75, trypsin inhibitor 4.55, β-lactoglobulin A 5.20, carbonic anhydrase B 5.85 and 6.55, myoglobin 6.85 and 7.35, lentil lectin 8.15, 8.45 and 8.65, trypsinogen 9.30 (isoelectric focusing calibration kit, Pharmacia). Protein bands were detected by silver staining.

2.8. Amino acid analysis

Samples containing approx. 20 μg of purified cod protein were hydrolyzed in gas phase under 1% phenol–6 *M* HCl as 150°C vapors for 1 h. Amino acid analysis was performed in an Applied Biosystem analyzer (Foster City, CA, USA) equipped with a C_{18} reversed-phase column (220 mm×2.1 mm I.D.; Brownlee PTC type).

3. Results

3.1. Purification

The protein content of the cod muscle was estimated at 19 mg/g. Eighty percent ammonium sulfate saturation was needed to obtain a fractionation of the crude cod extract proteins (Fig. 2). SDS-PAGE analysis showed eighteen proteins in the crude cod extract with M_r values ranging from 13×10^3 to 130×10^3. Only the protein with M_r 41×10^3 and four minor bands with M_r 13, 28, 39 and 49×10^3 were found in the ammonium sulfate fractionation supernatant. The supernatant aliquots gave three peaks in hydroxyapatite chromatography (Fig. 1). The protein with M_r 41×10^3 was identified mainly in the first peak, with a contaminant protein with M_r 66×10^3, but it was also present in the second peak and slightly detectable in the third one (Fig. 2). A protein with M_r 13×10^3 and three proteins with M_r 28×10^3, 45×10^3 and 49×10^3 were also detected in the second and third peak, respectively. Purification of

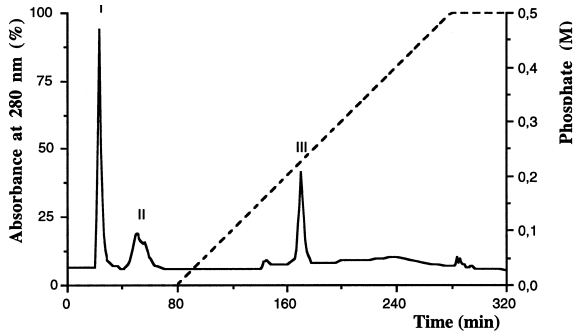

Fig. 1. Elution profile of cod proteins of the ammonium sulfate fractionation supernatant on hydroxyapatite chromatography (Ultragel HA). The column (20×1.6 cm I.D.) was eluted with a linear gradient (0.01–0.5 *M*; 200 ml total phosphate buffer, pH 7.0) at a flow-rate of 1 ml/min. The amount of proteins was 5 mg. Fractions were collected in 5-ml aliquots. Protein elution was detected by measuring the absorbance at 280 nm. Three peaks were observed; the 41 kDa protein was found in peak I (see Fig. 2).

the protein with M_r 41×10^3 was completed by preparative electrophoresis. The purified 41 kDa protein (p41) was eluted at a retention time of 560–620 min. A single protein with a M_r of 41×10^3 was found in silver stained SDS-PAGE (Fig. 2). This purification procedure yielded a total of 200 μg of pure p41 from 1100 μg of crude cod extract proteins.

3.2. Biochemical characterization

The protein fractions of the different purification steps were analyzed by Western-blot (Fig. 3). Western-blot was performed with a pool of sera from cod-allergic patients (Fig. 3a). It showed six allergenic bands in the crude cod extract, with M_r values of 13×10^3, 22×10^3, 28×10^3, 41×10^3, 49×10^3 and 60×10^3, respectively. Western-blot was also performed with a monoclonal anti-parvalbumin (Fig. 3b). Four proteins were detected in the supernatant with M_r values of 13×10^3, 28×10^3, 41×10^3 and 49×10^3, respectively. In immuno-blot, the purified p41 protein was shown to bind specifically to reaginic IgE from sera of cod allergic individuals (Fig. 3a and Fig. 4) and to a monoclonal anti-parvalbumin (Fig. 3b and Fig. 5). Isoelectric point (p*I*) of the purified allergen p41 was estimated to pH 5.8. The amino acid composition of the purified p41 is listed in Table 1. It was characterized by a high

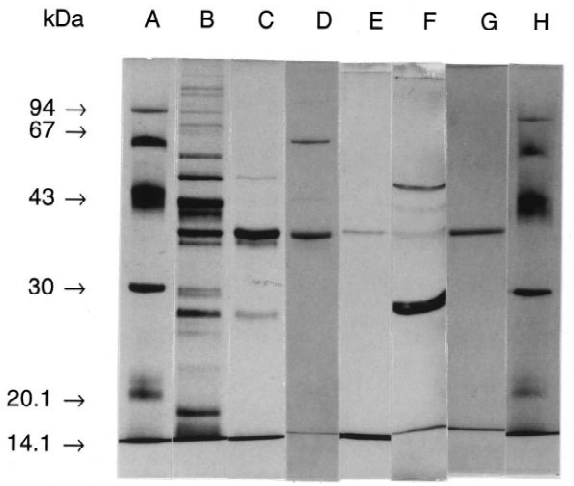

Fig. 2. SDS-PAGE analysis of samples from the different purification steps. Electrophoresis was run in a 12% acrylamide gel (30 mA per gel) and protein bands were detected by silver staining. Lane A: standard proteins (phosphorylase b 94; albumin 67; ovalbumin 43; carbonic anhydrase 30, soybean trypsin inhibitor 20.1, lactalbumin 14.4), lane B: crude cod extract (2.5 μg), lane C: supernatant from 80% ammonium sulfate fractionation (2 μg), lane D: peak I of hydroxyapatite chromatography (see Fig. 1) (0.5 μg). Two protein bands were observed, with respective M_r at 41 and 66 kDa, lane E: peak II of hydroxyapatite chromatography (0.5 μg). A main protein band was obtained, with M_r estimated at 13 kDa, lane F: peak III of hydroxyapatite chromatography (0.5 μg). A main protein band was observed, with M_r estimated at 28 kDa, lane G: purified p41 obtained after preparative electrophoresis (0.3 μg), lane H: standard proteins.

relative content of valine and alanine, and was also rich in isoleucine, leucine, glutamate and glutamine and poor in tyrosine and lysine. The N-terminal amino acid of the p41 protein, determined by Edman degradation, was aspartate.

4. Discussion

We report here the method used to purify the 41 kDa allergen from a crude cod extract. The ammonium sulfate fractionation was found to be an efficient step for increasing the relative content of p41 in the extract. The supernatant of the 80% ammonium sulfate fractionation contained only five proteins, including the 41 kDa allergen, compared to the eighteen proteins detected in the crude extract (Fig. 2). The mixture of partially purified proteins was used for the hydroxyapatite chromatography. Hydroxyapatite is a weak ion-exchanger recommended for difficult separation [28]. This technique was chosen as the protocol was rapid and required a single chromatographic step. In addition, hydroxyapatite gels can be used in batch which could be interesting for the purification of our protein on a large scale [29]. As hydroxyapatite chromatography is based on several interaction mechanisms, the 41 kDa protein may be recovered in different peaks. The number of contaminant proteins was efficiently reduced to only one 66 kDa protein in peak I (Fig. 2). This 66 kDa contaminating band was not identified in the starting material. This protein may be more concentrated in the eluted peak I than in the crude extract, explaining why it was only detected after hydroxyapatite chromatography. Preparative electrophoresis with separating gel of 12% acrylamide was chosen to remove the 66 kDa contaminating protein as the 41 kDa and 66 kDa proteins were well separated on 12% acrylamide mini-gel. Preparative electrophoresis gave a good resolution for the 41 kDa protein purification. The homogeneity of the purified product was first assessed by the presence of a single band in silver-stained SDS-PAGE and a single isoprotein in IEF (Fig. 2). The homogeneity of the purified protein was verified by the determination of a single N-terminal amino acid. As the 41 kDa protein has been purified and characterized under reducing conditions and in presence of SDS, we cannot exclude the possibility that it corresponded to a subunit of a higher M_r protein complex.

A 13 kDa protein was the major cod allergen (crude cod extract and supernatant) in Western-blot with cod-allergic sera (Fig. 3a) and may correspond to the major cod allergen Gad c I described in the literature [16]. This allergen was separated from the 41 kDa allergen by hydroxyapatite chromatography and it was eluted in the second peak. The effect of denaturating agents (reduction and alkylation) on Gad c I showed that allergenic activity was shared by a sequential rather than by a conformational epitope [30]. The 41 kDa protein obtained through purification was still allergenic as shown by Western-blot with cod-allergic sera (Fig. 3a, Fig. 4).

Following Western-blot analysis Gad c I, the 41 kDa allergen and two proteins of M_r 28 000 and 49 000 were detected with a pool of sera from cod

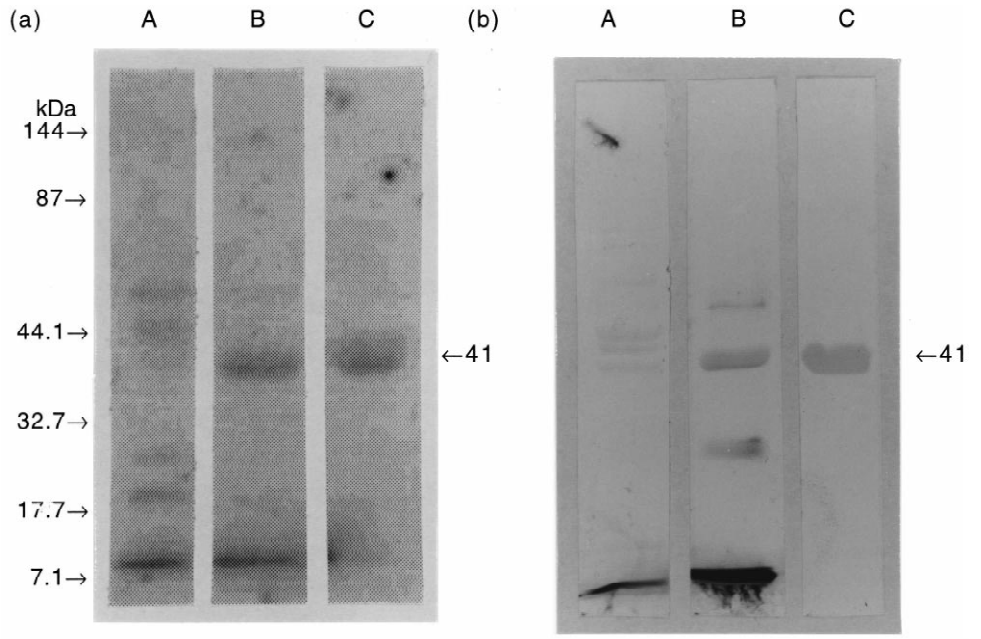

Fig. 3. Western-blot analysis of samples from different purification steps. Samples were run on 12% SDS-PAGE as described above. Proteins were transferred onto nitrocellulose. The membrane was blocked with 0.3% Tween 20 in 20 m*M* Tris–HCl buffer (pH 7.4), 0.15 *M* NaCl (TBS). (a) Incubation with cod allergic-sera. The membrane was incubated successively with sera from cod-allergenic individuals (diluted 1:10 with 0.1% Tween 20 in TBS) and with ^{125}I anti-IgE (diluted 1:10 with 0.3% Tween 20 in TBS). IgE binding was detected by exposure for 72 h on a phosphor screen. Lane A: crude cod extract (10 μg), lane B: supernatant (10 μg), lane C: p41 (10 μg). (b) Incubation with a monoclonal anti-parvalbumin. The membrane was incubated successively with a monoclonal anti-parvalbumin (diluted 1:1000 with 0.1% Tween 20 in TBS) and with anti-mouse IgG alkaline phosphatase conjugate (diluted 1:10 000 with 0.3% Tween 20 in TBS) and revealed for 5 min with the alkaline phosphatase substrate (BCIP/NBT). p41 was detected by both cod allergic-sera and monoclonal anti-parvalbumin.

allergic patients and with an anti-parvalbumin monoclonal antibody (Fig. 3b, Fig. 5). The monoclonal antibody stained specifically the calcium bound form of parvalbumin and was directed against an epitope located in the first calcium binding site [31,32]. This epitope corresponded to the same region that an IgE-epitope of Gad c I located in the first calcium binding loop (sequence 49–64) [33,21,22]. We previously showed that most of the IgE-reactive cod protein bands were recognized by anti-parvalbumins [25]. Three hypothesis could explain this immunoreactivity. First, some IgE-reactive protein bands may correspond to polymers or aggregates of the 12 kDa IgE-reactive band (Gad c I). Second, Gad c I is

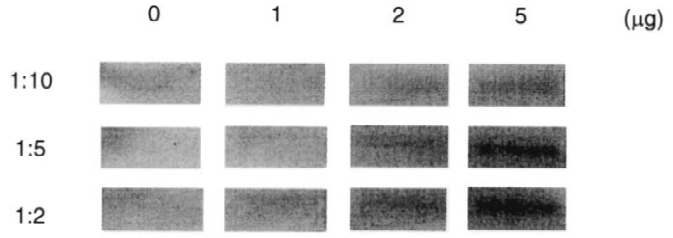

Fig. 4. Slot-blot analysis of the purified p41 protein with a cod allergic serum. p41 (0, 1, 2 and 5 μg) was applied to nitrocellulose. Incubation with serum of a patient allergic to cod fish (diluted 1:10, 1:5 and 1:2) was done as described above. IgE binding was detected by autoradiography on Kodak X-Omat film (exposure at −80°C for 5 days). A dose dependent binding of reaginic IgE to p41 was observed.

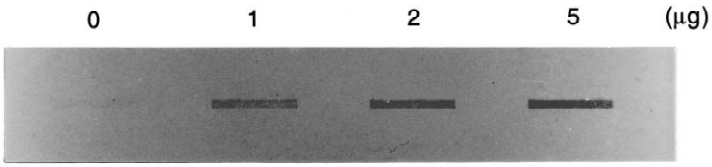

Fig. 5. Slot-blot analysis of the purified p41 allergen with a monoclonal anti-parvalbumin 0, 1, 2 and 5 μg of p41 were applied to nitrocellulose. Incubation with the monoclonal anti-parvalbumin was done as described above. p41 recognized specifically an anti-parvalbumin monoclonal antibody.

adsorbed to other proteins. Third, there is a common antigenic site corresponding to the first calcium binding site. The 41 kDa allergen may therefore contain a similar IgE epitope which corresponds to a calcium binding site homologous to that of Gad c I.

Correlation between the amino acid composition of the 41 kDa allergen and that of different cod proteins is reported in Table 2. No correlation was found between the 41 kDa allergen and the cod proteins already described in the literature. The amino acid composition of the 41 kDa allergen (Table 1) differed from that of Gad c I [34,35,17], principally by a higher percentages of the sulfur-containing amino acids, threonine and valine, and a lower percentage of lysine. The high relative content of dicarboxylic amino acids may explain the acidic nature of the 41 kDa allergen (p*I* 5.8). However, these amino acids were in a lower proportion than those observed in Gad c I. Gad c I has also a lower p*I* (p*I* 4.75) than p41. The hydrophobic amino acids were in a higher proportion in the 41 kDa protein (59%) than in Gad c I (45%). Hydrophobic domains may be present on the 41 kDa allergen and interact with reaginic IgE of cod allergic sera.

Table 1
Amino acid composition of the purified p41 allergen

Amino acid	Composition (%)
Asx	6.2
Glx	8.4
Ser	4.2
Gly	ND
His	4.3
Arg	3.9
Thr	6.2
Ala	10.6
Pro	6.6
Tyr	1.4
Val	11.4
Cys[a]	4.8
Ile	8.8
Leu	8.1
Phe	6.0
Lys	2.9
Met[b]	6.2
Trp	ND
Total	100

[a] Measured as cysteic acid.
[b] Measured as methionic sulfone.
ND=Not determined.

Considering the biochemical characteristics of the p41 and Gad c I allergens one may wonder about the elution behavior of these proteins on hydroxyapatite. p41 and Gad c I were eluted in the first and second peaks, respectively. Three hypotheses may explain this elution order:

(a) Calcium binding protein affinity for calcium ions of the solid-phase [39]. Potential interactions may occur between Ca^{2+} ions on hydroxyapatite ($(Ca_3(PO_4)_2)_3Ca(OH)_2$) and calcium binding sites of Gad c I and p41. Gad c I may have a higher affinity Ca^{2+} binding than p41, due to particular configurational requirement of binding groups.

(b) Ionic interactions [26,40]: both Gad c I (p*I* 4.75) and p41 (p*I* 5.8) were anionic at the elution pH of 7.0. Ion-exchange interactions may occur between negative groups of the proteins and Ca^{2+} ions of the solid-phase. Gad c I, which has a lower p*I*, may therefore be bound more strongly to the solid phase.

(c) Protein hydrophobicity: p41 is potentially more hydrophobic than Gad c I and has potentially a lower adsorption capacity to hydrated hydroxyapatite.

In conclusion, the purification of a 41 kDa protein from cod fish has been achieved after hydroxyapatite chromatography, with an additional step of preparative electrophoresis. This purified protein is an allergen as it was detected in Western-blot using a pool of sera from patients allergic to cod fish. It

Table 2
Correlation between amino acid composition of the 41 kDa allergen and that of different cod proteins

		a	b	c	d	e	f	g	h
p41	a	1	0.34	0.67	0.55	0.44	0.65	0.65	0.61
Allergen Gad c I	b	0.34	1	0.60	0.40	0.35	0.38	0.41	0.38
Alcohol dehydrogenase	c	0.67	0.60	1	0.44	0.25	0.34	0.78	0.75
Cytochrome *b*	d	0.55	0.40	0.44	1	0.78	0.92	0.52	0.38
NADH ubiquinone Oxidoreductase chain 2	e	0.44	0.35	0.25	0.78	1	0.84	0.43	0.33
Cytochrome *c* oxidase polypeptide I	f	0.65	0.38	0.34	0.92	0.84	1	0.44	0.33
Chymotrypsin A precursor	g	0.65	0.41	0.78	0.52	0.43	0.44	1	0.94
Chymotrypsin B	h	0.61	0.38	0.75	0.38	0.33	0.33	0.94	1

[a] This study.
[b] Elsayed et al. [17,34,35].
[c] Danielsson et al. [36].
[def] Johansen et al. [37].
[gh] Leth-Larsen et al. [38].

remains to determine the frequency of anti-p41 IgE in allergic patients.

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