

A new oligomeric parvalbumin allergen of Atlantic cod (*Gadus morhua*) encoded by a gene distinct from that of Gad cI

Background: The major allergen of Baltic cod (*Gadus callarias*) is a 12.3-kDa parvalbumin with two calcium-binding sites corresponding to EF-hand motifs. Our group found a 24-kDa IgE-reactive band that was also recognized by a monoclonal antiparvalbumin antibody in Atlantic cod (*Gadus morhua*). Our purpose was to purify and to determine the cDNA deduced sequence of this new cod allergen.

Methods: Proteins from *pre rigor mortis* Atlantic cod were separated by gel filtration and the eluted peaks were analysed by SDS-PAGE and Western blotting with sera of sensitized patients and with antiparvalbumin. Protein bands were microsequenced, RNA transcripts were amplified by reverse transcription and polymerase chain reaction (RT-PCR) using primer combinations overlapping the open reading frame.

Results: Four IgE and antiparvalbumin reactive proteins (12.5, 24, 38 and 51 kDa) were detected in gel filtration eluate. The cDNA deduced sequence of the 24 kDa protein had 109 amino acid residues with a molecular weight of 11.5 kDa and a theoretical pI of 4.34. The 24 kDa band corresponded therefore to a dimer of a β -parvalbumin. Its homology was higher with Sal sI than with Gad cI. This new allergen was named Gad mI.

Conclusion: We have characterized a new parvalbumin allergen in *Gadus morhua*. This protein formed oligomers in native and in reducing conditions. Gad mI and Gad cI may correspond to two distinct genes of *Gadus* species.

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Introduction

Fish is one of the foods most commonly involved in allergy in children (1, 2). Gad cI, the major allergen of Baltic cod (*Gadus callarias*), has been purified and characterized by Aas et al. (3, 4). This protein is a 12.3-kDa parvalbumin with two calcium-binding sites corresponding to EF-hand motifs (5–7).

The whole panel of cod allergens detected in Atlantic cod (*Gadus morhua*) remains to be characterized. Our group found, by Western blot of cod (*Gadus morhua*) protein extract, several IgE reactive bands over a wide (12–130 kDa) molecular range (8). In this study, a 24-kDa IgE-reactive band was also recognized by a monoclonal antiparvalbumin antibody (8). The purpose of the present work was to purify and to determine the cDNA deduced sequence of this new cod allergen.

Materials and methods

Sera

The patients of our study had an adverse reaction to Atlantic cod and were investigated to detect serum specific IgE against cod. Blood

samples were collected in dry tubes and centrifuged at 1000g for 5 min. Sera were aliquoted and stored at -80°C . A pool of control sera was collected from healthy subjects, with a total IgE serum level lower than 30 kU/l.

Preparation of crude cod extract

Cod used in this study was Atlantic cod (*Gadus morhua*) caught during a British oceanographic expedition. It was in the *pre rigor* state. Fillets were cut before the *post mortem* rigidity and placed immediately into liquid nitrogen before being stored at -80°C . Muscles were homogenized in phosphate buffer 0.07 M pH 7.0 in a 2-ml solution per gram of tissue. The mixture was centrifuged at 20 000g for 20 min. The samples were then dialysed against distilled water, lyophilized and stored at -20°C before use.

Protein concentration

The protein concentrations of the samples were determined by using the Coomassie[®] Plus Protein Assay Reagent (Pierce, Rockford, IL, USA), according to the manufacturer's instructions. Bovine serum albumin (BSA) was used as reference.

Gel filtration chromatography

Cod proteins were separated by gel filtration on Superdex HR200 (Pharmacia, Uppsala, Sweden). Twenty-five milligrams of samples were diluted with 1 ml of water containing 30% isopropanol (v/v) and 0.001% Tetrafluoroacetic acid (v/v) pH 4.6. The constant flow

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rate was 0.3 ml/min. The elution of the proteins was detected by UV-absorbance at 280 nm. The peaks were collected, dialysed against distilled water and analysed by SDS-PAGE and Western blotting.

Detection of the blotted proteins with a pool of cod allergic sera

Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 12% polyacrylamide gel in 20 mM Tris HCl, pH 8.3, 0.18 M glycine, and 0.1% SDS using a vertical Mini Protean® II apparatus (Biorad, Richmond, CA, USA) under 35 mA, 250 V, for 1 h. Proteins were transferred overnight at 20 V onto a nitrocellulose membrane (0.2 µm Trans-Blot® Transfer Medium, Bio-Rad) using a Trans-Blot® cell (Bio-Rad). 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 three washings with 0.05% (v/v) Tween 20, 20 mM Tris-HCl pH 7.4, and 0.15 M NaCl, the nitrocellulose membrane was incubated for 3 h with 1 ml of serum diluted 1:10 in 0.1% (v/v) Tween 20, 20 mM Tris-HCl pH 7.4 and 0.15 M NaCl. The membrane was washed three times with the 0.05% Tween buffer and incubated for 3 h, at room temperature, with 2 ml anti-IgE diluted 1/10 in the 0.1% Tween buffer. After an additional three-step washing, the membrane was incubated overnight with antimouse IgG (whole molecule) horse-radish peroxidase conjugate (Amersham International) diluted 1/500 in the 0.1% Tween buffer. The membrane was rinsed 3 times during 5 min with 0.05% Tween at room temperature and incubated in the detection reagent ECL+® (Amersham International, UK). The membrane was washed briefly, placed subsequently in contact with an autoradiography film (Hyperfilm®, Amersham International) during 2 min and developed immediately.

Detection of the blotted proteins with antiparvalbumin

The membrane was blocked as described above. It was incubated with monoclonal antiparvalbumin (clone PA-35, Sigma-Aldrich, Chesham, France) 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, washed three times, incubated during an additional 3 h with an antimouse IgG alkaline phosphatase conjugate (Sigma-Aldrich) diluted 1:10 000 (v/v) in 0.1% Tween 20, 20 mM Tris HCl, pH 7.4, and NaCl 0.15 M. The membrane was washed again 3 times and incubated with 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium (BCIP/NBT Buffered Substrate Tablet, Sigma-Aldrich).

Peptides microsequencing

Semi-purified gel filtration fraction was run in SDS-PAGE and transferred on a PVDF membrane (Amersham International) using 50 mM Tris-borate buffer. The protein band of interest was cut and microsequenced for determination of peptides by the Institut Pasteur (Paris, France) on an Applied Biosystems Procise Sequencer (Applied Biosystems, Courtaboeuf, France).

Amplification of RNA transcripts by Reverse Transcription (RT), Polymerase Chain Reaction (PCR) and sequencing of cDNA

Primers used for cDNA synthesis and PCR amplification were, respectively:

- S6:
5'-GA(AG)AT(ACT)AT(ACT)GA(CT)CA(AG)GA(CT)AA-3';
- As2:
5'-ATCAT(ATGC)GC(ATGC)CC(AG)AA(TC)TC(GA)TC-3';
- Ints2:
5'-AGGACAAGAGTGACTTTGTT-3';
- As7:
5'-GCCTTGATCATGGCGCCAAA-3';
- ParvII:
5'-CCTTCGCCGGTCTTAACGAT-3';

- PolydT-Anchor:
5'-GACCACGCGTATCGATGTCTGA(T₁₆)(AGC)-3';
- Anchor:
5'-GACCACGCGTATCGATGTCTGA-3'.

Primers S6, AS2, and AS7 were selected on the basis of the N-terminal and internal amino acid sequences obtained by micro-sequencing and INT S2 from the first generated PCR fragment. ParvII was chosen by alignment with already known sequences of the 5'-Salmo salar parvalbumin gene.

First strand cDNA was synthesized from 3.4 µg of total RNA in a reaction volume of 20 µl with Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase (Life Technologies, Cergy-Pontoise, France). All PCR were performed with a pair of primers under the following conditions: 94°C for 15 s; hybridization temperature depending on the couple of primers used for 50 s; 72°C for 50 s; 35 cycles. For S6/AS2, the annealing temperature was 47°C, 51°C for INTS2/Anchor(3'-Rapid Amplification of cDNA Ends) and 57°C for parvII/AS7 couple. Sequencing of the purified PCR and 3'RACE products was achieved by the dideoxynucleotide chain termination method and ABI PRISM Dye Terminator kit as indicated by the manufacturer (PE France, Courtaboeuf, France). Analyses were performed on a ABI 377 DNA sequencer (Applied Biosystems) by Genome Express (Montreuil, France).

Sequences alignment

Alignment was performed with ClustalW (version 1.81) (9), using the protein complete sequence. Homology research relied on the Blosum matrix series.

Results

The 7 proteins observed in silver stained SDS-PAGE of 30% isopropanol cod protein extract were cleared in 3 peaks in gel filtration chromatography. The second eluted peak contained the 24 kDa protein and 3 other proteins with molecular mass estimated, respectively, at 20.4 kDa, 18.5 kDa and 12.5 kDa (Fig. 1). Western-blotting of samples eluted from gel filtration was performed with chromatography with specific IgE sera against cod (Fig. 2). Four proteins with respective molecular weights at 12.5 kDa, 24, 38 and 51 kDa were detected in the first peak, while only the 12.5 and the 24 kDa were found in the second and the third peak (Fig. 2). The antiparvalbumin antibody also detected the 12.5 kDa, 24 and 38 kDa bands (Fig. 2). The N-terminal residue of the 24 kDa protein was not accessible to the Edman reaction. The tryptic digestion of the protein produced 40 peptides separated by HPLC. Three peptides had the following sequences: val-phe-glu-ile-ile-asp-gln-asp; ile-gly val-asp-glu-phe-gly ala-met-ile; ala-phe-phe-val-ile-asp-gln-asp.

The similarity search computed on the European Bioinformatic Institute server (Program FASTA 3) confirmed that the 24 kDa protein belonged to the parvalbumin family. The primers combination generated cDNA overlapping fragments of the open reading frame. The sequence of the 20 first nucleotides of the cDNA was uncertain when compared to those of Gad cI and Sal sI. It was verified by performing additional RT-PCR experiments with a primer encom-

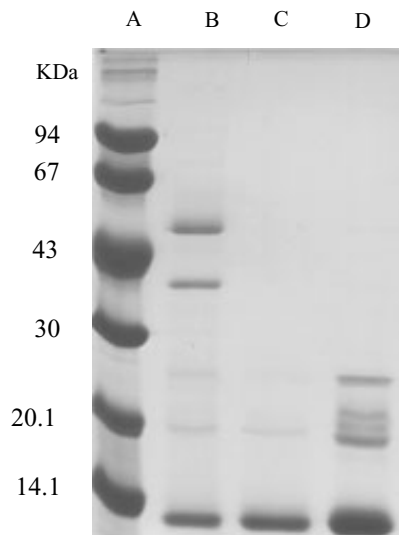


Figure 1. SDS-PAGE analysis of gel filtration protein. Electrophoresis was run in a 12% acrylamide gel. Ten micrograms of protein were loaded per line. Lane A: molecular weight markers (phosphorylase b: 94 kDa, albumin: 67 kDa, ovalbumin 43 kDa, carbonic anhydrase 30 kDa, soybean trypsin inhibitor 20.1 kDa, lactalbumin 14.4 kDa). Lane B: peak 1 of the gel filtration chromatography; Lane C: peak 3 of the gel filtration chromatography; Lane D: peak 2 of the gel filtration chromatography.

passing the 5'-untranslated region and the ATG codon, using the sequence of the corresponding region of Sal sI. The 500 bp cDNA sequence (Fig. 3) corresponded to a protein of 109 amino acids with a molecular weight of 11.5 kDa and a theoretical pI of 4.34. The 24 kDa band observed in SDS-PAGE corresponded therefore to a dimeric form. Sequence alignment of amino acids confirmed that the 11.5 kDa protein was a β -parvalbumin with typical characteristics: calcium-binding sites, a cysteine in position 18, an arginine in position 75 and a quadruplet of acidic residues in the region 59–62 (10). The sequence was compared with that of parvalbumins from other species (data not shown). The homology of the 11.5 kDa protein was higher with Sal sI clone 24.1 (11) than with Gad cI, the Baltic cod major allergen (12). According to the international nomenclature, this new allergen was called Gad mI.

Discussion

In our experimental conditions, four of the seven proteins were IgE-reactive with respective molecular sizes at 12.5, 24, 38 and 51 kDa. Their solubility in 30% isopropanol indicated that they had a relative hydrophobic surface. The 12.5 kDa IgE reactive band could correspond to the major cod allergen Gad cI and the 24 and 38 kDa bands to parvalbumins, as they also were also recognized by antiparvalbumins (8, 13–16).

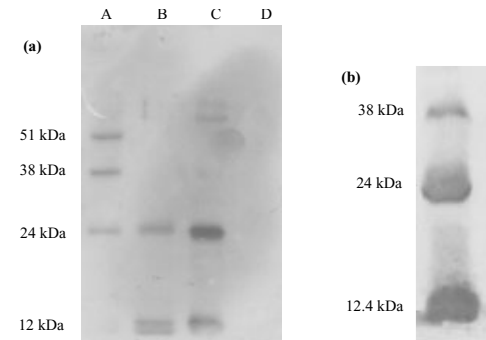


Figure 2. Western blot of eluted fractions from gel filtration. SDS-PAGE was run on a 12% polyacrylamide gel as described above. Some 10 μ g of proteins were transferred onto a 0.2- μ m nitrocellulose membrane. a) Eluted fractions of the gel filtration blotted with a pool of sera of sensitized patients. Lane A: Fraction 1 of the gel filtration chromatography; Lane B: Fraction 3 of the gel filtration chromatography; Lane C: Fraction 2 of the gel filtration chromatography; Lane D: molecular weight markers (no staining). b) Second fraction of gel filtration chromatography blotted with antiparvalbumin. Some 10 μ g of protein were transferred onto nitrocellulose membrane.

Fish parvalbumins represent the major allergen family involved in IgE-mediated hypersensitivity to fish and fish products. They belong to a family of calcium-binding proteins which may play a role in the relaxation of muscle tissue (6, 7, 17). Parvalbumin allergens have been already described in *Gadus callarias* (codfish from the Baltic Sea), *Salmo salar* (salmon) and *Cyprinus carpio* (carp) (4, 11, 18). This work is the first one providing the cDNA deduced sequence of a parvalbumin allergen in *Gadus morhua*. The parvalbumins can be divided into two evolutionary distinct lineages, α -type and β -type (5, 17). The present protein had a molecular size and an isoelectric point of a β -parvalbumin. The present parvalbumin had a 24-kDa molecular size in native conditions (e.g. gel filtration) and a 11.5-kDa molecular size deduced from the cDNA sequence. This showed therefore that it was present in dimeric form. In addition, western-blot after SDS-PAGE in reducing conditions of the semipurified protein also detected the 24 kDa and two other 38 and 51 kDa parvalbumins, which may therefore correspond to dimeric, trimeric and tetrameric forms, respectively. This indicated that reducing conditions did not completely dissociate the oligomers (Fig. 2). No indications of aggregation of fish parvalbumins have been previously described. They are regarded as soluble monomeric species (11, 18). Until now, only a few proteins of the parvalbumin family have been described in dimeric forms. Such an oligomerization has been previously observed in an avian thymic parvalbumin in the absence of reducing agents. Thymus parvalbumin oligomerization involved the presence of divalent ions and a disulphide bond in position Cys 72 (19). The consequences and the functions of the protein were not studied. By comparison, the dimerization of a related

1	---	-CC	TTC	GCC	GGT	CTT	N	D	A	D	I	T
13	A	A	L	A	A	C	K	A	E	G	S	F
37	GCA	GCC	CTG	GCG	GCC	TGC	AAA	GCT	GAG	GGC	TCC	TTC
AB loop												
25	D	H	K	A	F	F	T	K	V	G	L	A
73	GAC	CAC	AAG	GCG	TTC	TTC	ACT	AAA	GTC	GGC	CTG	GCC
37	A	K	S	P	A	D	I	K	K	V	F	E
109	GCC	AAG	TCC	CCC	GCT	GAC	ATC	AAG	AAG	GTG	TTT	GAG
49	I	I	D	Q	D	K	S	D	F	V	E	E
145	ATC	ATT	GAC	CAG	GAC	AAG	AGT	GAC	TTT	GTT	GAG	GAG
CD loop										Acidic residues		
61	D	E	L	K	L	F	L	Q	N	F	S	A
181	GAT	GAG	CTG	AAG	CTG	TTC	CTC	CAG	AAC	TTC	AGC	GCC

73	G	A	R	A	L	S	D	A	E	T	K	V
217	GGT	GCC	AGA	GCT	CTG	AGT	GAC	GCT	GAG	ACC	AAG	GTT
85	F	L	K	A	G	D	S	D	G	D	G	K
253	TTC	CTG	AAG	GCC	GGA	GAC	TCT	GAC	GGT	GAC	GGC	AAG
EF loop												
97	I	G	V	D	E	F	G	A	M	I	K	A
289	ATC	GGA	GTT	GAT	GAG	TTT	GGC	GCC	ATG	ATC	AAG	GCA
Stop												
325	TAG	ACTGTTCTCCACCAACTAGACCAGATGCTTACCCACCTACTCAGAAGGAACACTG										
383	GAGCTGAGACCACCTCCTCACCCCTCTCTCGCTCATCTCTCAACAACAATTTTATATAAATAA											
446	CCTTTAACCTAACCCCCCCCCCCCCCCCCCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT											

Figure 3. cDNA and deduced amino acid sequence of cod parvalbumin. The quadruplet of acidic residues and AB, CD, EF loops are underlined. Cysteine in position 18 and arginine in position 72 are encircled.

protein, oncomodulin (20), was formed by a disulphide bond in Cys 18 in presence of Ca^{2+} , and it conferred a calmodulin-like activity. In addition, in a recent study using circular dichroism EF-hand peptides have been shown to bind calcium and to dimerize (21). Only 2 of the 3 “helix-loop-helix” EF-hand domains of parvalbumin can chelate divalent cations. The other domain may cover the hydrophobic surface of this pair of domains (22). Hydrophobic interactions related to apo- and holo-forms may be therefore implicated into oligomerization. Indeed, a disulphide bridge should be possible in Cys 18, but reductive conditions of SDS-PAGE could not separate the subunits. In regard to this hypothesis, it has been shown recently that the holo-form of carp parvalbumin is more favourable than the apo-form for IgE-binding (18). In the present work, we found an IgE-binding for both mono and oligomeric

forms of cod parvalbumin. Additional work should be performed to determine whether the oligomerization has an influence on the allergenicity of this protein.

IgE antibodies bind directly to the Ca^{2+} -binding loops of parvalbumin. This motif is the most conserved structural motif in different Ca^{2+} -binding proteins. It is often present in plant and animal protein allergens and may be responsible of cross-reacting sensitization (23). For example, distinct cross-reacting EF-hand type epitopes have been identified in pollen and vegetable allergens such as the major birch pollen allergen Bet v1 and profilins (24).

The Gad mI is a new allergen which had only 62.3% identity with Gad cI from *Gadus callarias*. This was surprising considering that its identity was higher (75%) with *Salmo salar* allergen (Sal sI). Since *Gadus morhua* is phylogenetically much closer to *Gadus callarias* than

Salmo salar, it may be hypothesized that Gad mI and Sal sI are encoded by the same gene in the two different species and that Gad mI and Gad cI correspond to two distinct genes which may be present in both *Gadus*

species. In conclusion, we have characterized a new parvalbumin allergen in Atlantic cod, which was named Gad mI. This protein formed oligomers in native and in reducing conditions.

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