

Cloning of Two Distinct cDNAs Encoding Parvalbumin, the Major Allergen of Atlantic Salmon (*Salmo salar*)

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Allergy to fish is common in Northern Europe. Variable reactions to different fish species are usually experienced among fish allergic patients. The allergens of cod fish and particularly the major allergen parvalbumin β (*Gadus callarias*) have been extensively studied in Norway. In the present communication, the white muscle parvalbumin was similarly found to be a major allergen in Atlantic salmon (*Salmo salar*, *Sal s1*). A purified salmon parvalbumin was obtained by anion exchange chromatography, gel filtration chromatography (GFC) and high-performance liquid chromatography (HPLC) of the muscle extracts. The antigenicity and allergenicity of salmon parvalbumin were confirmed using various immunologic and electrophoretic techniques. The protein is representative for several isoallergens judged by the amino acid (AA) sequence variance at certain sites in the AA sequence of CNBr cleavage peptides. Using sera from patients with cod and salmon allergy *Sal s1* was demonstrated to be the major allergen of Atlantic salmon, as judged by RAST- and ELISA-inhibitions and crossed radioimmuno-electrophoresis (CRIE) techniques. The protein was also demonstrated to be antigenic by the use of polyclonal cod and salmon antibodies in IgG ELISA and immunoelectrophoretic methods. Cloning of parvalbumin cDNA from Atlantic salmon was performed based on an alignment of parvalbumin AA sequences from other species. A probe was generated by PCR and used for screening a salmon muscle cDNA-library. Subcloning and sequencing of two hybridizing clones revealed transcripts from two different parvalbumin genes. The translated sequences of both clones belong to the β -lineage of parvalbumins and include the entire coding region.

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INTRODUCTION

Atlantic salmon is the most important fish species in Norwegian aquaculture and consumption of this fish is increasing. The muscles of fatty fish contain polyunsaturated eicosanoids known to be of particular prophylactic health benefits [1–4]. Despite the nutritional merits, fish consumption may cause food hypersensitivity, fish allergy or enteropathy [5–8]. Fish allergies are common in Europe, particularly among male children and young adults. Children allergic to fish react variably against different fish species. Cod is among the most common offenders while salmon is the one best tolerated [9]. In a Norwegian

population, an estimated fish allergy was found to be ≈ 1 per 1000 individuals, and nearly 50% of cod hypersensitive patients also react to salmon [10]. Studies in Scandinavia reported 32 cases of fish allergy among 82 children with general food hypersensitivity [11]. In a general food allergy population in Italy ($n = 72$) 18% of the patients were reported to have cod specific IgE antibodies; among those only 20% were allergic to salmon [12].

The allergy-eliciting protein in cod has been found to be parvalbumin, designated Allergen M *Gad c1* according to the International Nomenclature of Allergens [13]. Parvalbumins are Ca^{2+} binding proteins of low molecular weight, initially detected in relatively high amounts in white muscle of lower vertebrates [14]. Likewise they were subsequently found, although in lesser amounts, in the fast twitch skeletal muscles of higher vertebrates

The nucleotide sequences reported in this article have been submitted to the GenBank Sequence Database and have been assigned the accession No.x97824 and x97825.

[15], as well as in a variety of non-muscle tissues including testis, endocrine glands, skin and specific neurones [16–20]. In the muscle, parvalbumin was suggested to be important in the relaxation of fibre [21]. Parvalbumins are divergent proteins in terms of their primary structure, and several isotypes can be present in the same muscle cell. Two distinct phylogenetic lineages of parvalbumins named α and β were inferred after more than a dozen amino acid (AA) sequences were compared [22]. Most muscles contain only parvalbumin of either α or of β -lineage, although in frog skeletal muscles both α and β parvalbumins have been found [23].

Among fish species, crystal structure has been revealed for both carp β -parvalbumin [24,25] and pike parvalbumin [26]. Parvalbumin from cod (*Gad c1*, pI 4.75) has been extensively studied in Norway [7]. It belongs to the β -lineage and a large homology was found between cod parvalbumin and other fish species [27].

Cloning and expression of allergens from different sources have been widely reported [28–32]. In the present study, muscle parvalbumin was found to be the major allergen in Atlantic salmon. The major aim of the present study was to establish cDNA encoding parvalbumin, and subsequently to generate a new recombinant (r) *Sal s1*. The availability of the r-*Sal s1* in homogenous form and sufficient amounts in addition to the r-*Gad c1* [33] and the synthetic peptides [7], could allow the study of molecular cross-reactivity between fish parvalbumins using allergen-specific T-cell clones [34].

MATERIALS AND METHODS

Extraction, isolation and purification of salmon parvalbumin. Atlantic salmon (*Salmo salar*) were obtained from a fish farm near Bergen and raised in tanks at the High Technology Centre, University of Bergen. The fish muscle extract was purified by anion exchange chromatography, DEAE cellulose (Cellulose *N,N*-diethyl aminoethyl ether, Cat. 1192723, Eastman Kodak Company, Rochester, NY, USA). DEAE was washed with 100 mM phosphate buffer pH 5.4 and packed into a column (d 50.35 cm). The same buffer was used for equilibrium and elution of the first two peaks (DI and DII). The buffer was upgraded to 150 mM, at which fraction DIII was eluted. After dialysis and lyophilization, the purified protein was applied on a Sephadex G-75 GFC column, d 40.15 cm, Pharmacia Biotechnology, Uppsala, Sweden, as described previously [35, 36]. The homogeneity of the protein was demonstrated using high-performance liquid chromatography (HPLC), sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and by analysing N-terminal AA sequence of cleavage peptides [37, 38]. HPLC utilizing the Micro Separation System (Applied Biosystems Inc., Foster City, CA 94404, USA), including a reversed phase (RP) cartridge d 30.21 mm (Aqua Pore RP-300, 7 Micron, Part no. 0711-0056) bonded silica phase and trifluoro acetic acid gradient [38].

Cyanogen bromide cleavage and N-terminal amino acid sequence analyses. Carboxy methylation and cyanogen bromide (CNBr) cleavage at the methionyl peptide bonds was done using 1 μ M of Atlantic salmon parvalbumin by the methods described previously [39–41]. The AA sequences of two cyanogen bromide cleavage fractions were determined using an automatic Edman degradation method, Applied Biosystems Model 477 A Protein/Peptide Sequencer, and the phenylthiohydantoin

(PTH) AA were analysed on an on-line PTH-Analyser model 120 A, Applied Biosystem Inc., Foster City, CA, USA. Otherwise the procedure was performed as described previously [38].

Human sera. Eleven sera from patients with clinical histories of fish allergy, positive skin prick test, and with CAP/RAST class 3–6 radio-allergosorbent test (Pharmacia) were selected from patients referred to the Central Laboratory, University Hospital. Blood samples were taken after informed consent had been given, and were pooled in small aliquots then stored at -80°C until used.

Immuno-electrophoretic techniques. Antibodies to purified salmon parvalbumin (*Sal s1*) and to cod Allergen M (*Gad c1*) were raised in rabbits, Animal House Vivarium, University Hospital. Crossed rocket immuno-electrophoresis (CIE), Fused rocket immuno-electrophoresis (FRIE) and Fused rocket radioimmuno-electrophoresis (FRRIE), were performed as described in detail in previous publications and used for locating the allergenic fractions [42].

ELISA inhibition. This was done using the procedure adapted from [43]. Briefly, 96-well microtitre plates (Microtitre plates, Dynatech Laboratories Inc., Chantilly, VA, USA) were coated with 1.25 μ g *Gad c1* (100 μ l/100 mM sodium carbonate pH 9.6). The inhibition was performed by overnight (O-N) pre-incubation of a series of ascending concentrations of the antigens and 1000-fold dilution of the respective antisera, at room temperature (RT). The plates were washed five times with 250 μ l Tris-Tween pH 7.4; thereafter 100 μ l of the inhibited sera were added, incubated for 2 h at RT and the washing procedure was repeated. A solution of 100 μ l/well of 1000-fold diluted freshly prepared alkaline phosphatase conjugate (A-8025 Sigma, Sigma Chemical Co., St. Louis, MO, USA) was then added, and the plates were further incubated for 2 h at RT. After washing a further five times, freshly prepared *p*-dinitrophenyl phosphate (100 μ g) (Sigma 104 phosphatase substrate) and 100 μ l of Tris buffer pH 9.5 were added to each well. The absorbance was read at 405 nm in an ELISA reader (Labsystems Multiscan Biochromatic, type 348, Pultitie 8, 00880 Helsinki, Finland). The enzyme reaction was terminated by addition of 50 μ l of 5 N NaOH.

Specific IgE inhibition. Radioallergosorbent test (RAST) inhibition was done employing a modified procedure routinely used in our laboratory [44]. Briefly, the allergen solution (100 μ g in 100 μ l) was mixed with 50 μ l undiluted patient serum. After 3 h at RT under gentle shaking the allergen discs (f3 Phadebas RAST, Pharmacia) were added to the tubes and incubated O-N at RT with gentle shaking. The discs were washed three times with 2.5 ml RAST wash and incubated O-N with 50 μ l anti-IgE (^{125}I anti-Fc ϵ , Pharmacia). After a further washing procedure, the activity was determined using the γ -counter LKB Wallac 1282 compugamma (Wallac Oy, PO Box 10, 20101 Turku 10, Finland). Percentage inhibitions were calculated:

$$\frac{\text{cpm of non-inhibited sample} - \text{cpm of inhibited sample}}{\text{cpm of non-inhibited sample}} \times 100$$

Electrophoresis and immunoblotting. The electrophoresis was carried out essentially as described in the operating manual (BIO-RAD Protean II Slab Cell, BIO-RAD Laboratories, Richmond, CA, USA). It was run at 25 mA for the first hour and then 35 mA for the next 2 h. Briefly, 30 μ l of samples (containing 60 μ g protein) were applied to each well. The separated proteins were transferred from the gel to a nitrocellulose (NC) membrane (0.1 μ m) by electrophoretic transfer. Blotting was performed in a BIO-RAD Transblot Cell for 1 h at 50 V. After transfer, non-occupied protein-binding sites were blocked by incubation with a phosphate buffer, pH 7.4 containing 0.5% Tween 20 and 10% bovine serum albumin for 1 h. The NC membrane was cut into strips, twice washed for 10 min and incubated O-N (at 4°C) with fish allergic patient

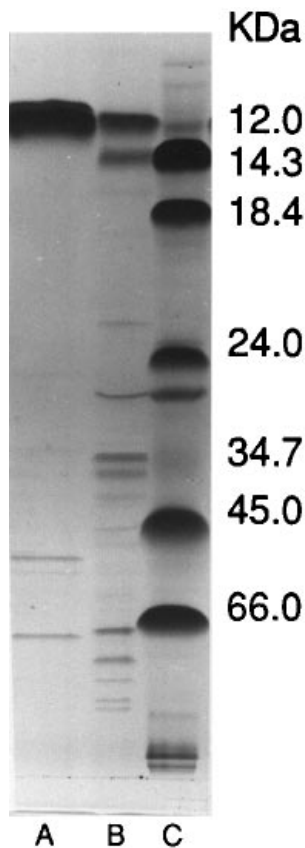


Fig. 1. SDS PAGE analysis of cod and salmon parvalbumin. Parvalbumin from cod is shown (A) next to parvalbumin from salmon (B) and a molecular weight marker (C). Parvalbumin from salmon showed two bands (12 kDa and 14 kDa) while those of cod were located at ≈ 12 kDa.

serum. The NC strips were washed five times for 6 min, and then incubated O-N at RT with anti-human IgE alkaline phosphatase conjugate (A-3525 Sigma Chemical Co., St. Louis, MO, USA). After further washing, the NC strips incubated with anti-IgE were incubated with Sigma Fast, Fast Red TR/Naphthol AS-MX alkaline phosphatase substrate (F-4523) for detection of specific IgE. After 1 h at RT the reactions were terminated by washing the strips in water.

Isolation of RNA and construction of a salmon muscle cDNA library. Total RNA from fast contracting/relaxing muscle was isolated as described in [45]. Chemicals and enzymes for cDNA synthesis and cloning were obtained from Promega (Madison, WI, USA). First strand cDNA was synthesized in a reaction mixture of 50 μ l containing 15 μ g of total RNA and 4 μ g oligo dT15. Otherwise, procedures from the suppliers were followed. The ds-cDNA was ligated to *Eco*RI adaptors. Removal of excess adaptors was done employing spin columns containing Sephacryl S-400 slurry before ligation to λ -gt10 arms. Packaging was carried out using the Packagene system.

Polymerase chain reaction (PCR) analysis and synthesis of a salmon specific parvalbumin probe. A species specific parvalbumin DNA-probe was synthesized by reverse transcriptase polymerase chain reaction (RT-PCR). Mixed primers designed for cDNA-synthesis and PCR were Sense-PV: 5'AGYGGCTTYATHGARGARGAYGARYT, Antisense-PV1: 5'YTGYTTNACNAANACNGCRAAYTC and Antisense-PV2: 5'GAATTCRTCRACHC-CDATYTTTCC (IUB symbols). First strand cDNA was synthesized from 20 μ g of total RNA in a reaction volume of 28 μ l using AMV-reverse transcriptase (Promega) and 2 μ g of either Antisense-PV1 or Antisense-PV2. Reaction volumes for PCR were 100 μ l, containing 1 $\times$ amplification buffer, 1 μ M of each primer, 0.2 mM of each dNTP and 2.5 u *Taq* polymerase (Perking Elmer Cetus). In accordance with information from the computer program *Primer*, the following profile was used with both primer combinations: 94°C, 0.5 min; 55°C, 1 min; 72°C, 1 min; 40 cycles. The PCR-product based on Sense-PV and Antisense-PV2 was 32-P-labelled by random priming [46].

Screening and subcloning. Plaque lifts were hybridized using a high

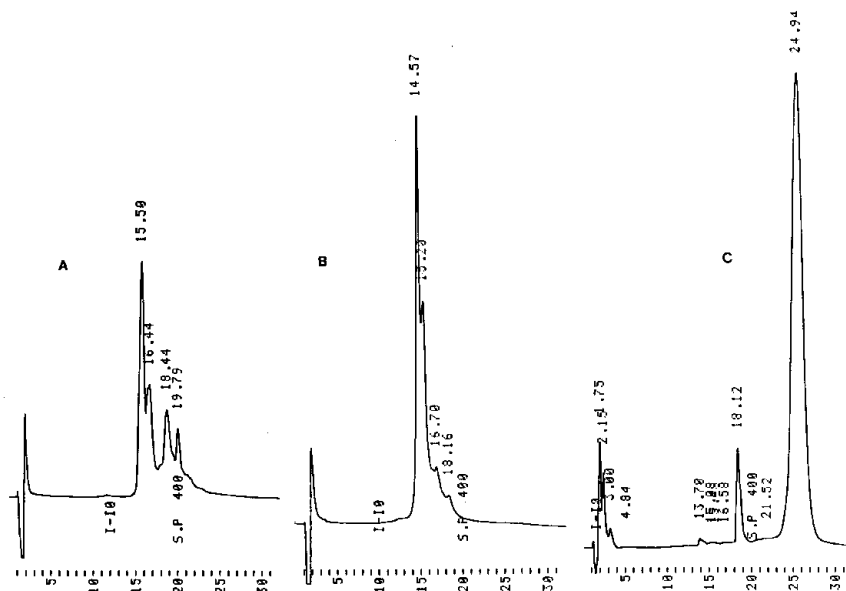


Fig. 2. Analysis of the homogeneity of salmon parvalbumin and CNBr-fragments. RP/HPLC (A) partially purified salmon parvalbumin after anion exchange chromatography; (B) Salmon parvalbumin after GFC; (C) Cyanogen bromide fractions of parvalbumin R_i 18.12 was used to ascertain the homogeneity of the isolated fraction used in testing the antigenicity and allergenicity.

stringency hybridization solution [47]. The most stringent wash of the filters was performed at 60°C in 0.2% standard sodium citrate buffer (SSC) and 0.1% SDS. Affirmed positive λ gt10 clones were isolated and PCR amplified with λ -specific primers. The PCR-amplified inserts of λ -clones 24.1 and 14.1 were inserted into pGEM7zf(+) as described [48].

DNA sequencing and sequence analyses. Plasmid subclones were sequenced by the chain termination elongation reaction with dideoxynucleotides (Sequenase, USB, Cleveland, OH, USA). Plasmid sequencing primers were used as well as specific oligonucleotides. The following software was used: (1) *Primer* (Scientific & Educational Software, PA) in selection of primers; (2) *Geneworks* (Intelligenetics) for the multiple AA sequence alignment upon which an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree was based. Description of alignment algorithm was described in [49] and [50]; and (3) *Clustal V* (higgins@embl-heidelberg.de) for the other DNA and protein sequence alignments. Alignment algorithm is described in [51]. Alignment matrices and parameters used in alignments can be requested from C. d.V. Lindstrøm.

RESULTS

Extraction, isolation and purification of salmon parvalbumin

Fractionation of salmon muscle extract on DEAE cellulose column generated three peaks (DI, DII and DIII) of which DIII was shown to be analogous to DIII from cod (Mr 12.3 kDa) [7]. This fraction was further purified by GFC allowing two peaks DS1 and DS2. By CIE, CRIE, FRIE and FRRIE DS 2 was found to include the antigenic and allergenic activities. Both DIII of salmon and the corresponding one of cod could readily be resolved by SDS/PAGE. Salmon parvalbumin showed two bands, 12 and 14 kDa, while cod showed apparently one single band at $\approx$ 12 kDa (Fig. 1). DIII of salmon was further resolved by RP/HPLC allowing four peaks of which the first two were proximal. The partially purified fraction DS2 of salmon parvalbumin generated two extremely close proteins (Fig. 2A & B). Cyanogen cleavage of salmon parvalbumin revealed two polypeptide fragments, Rt 18.12 m and Rt 24.94 m. N-terminal AA sequence analysis was performed on both peptides. Peptide Rt 18.12 m was used for the construction of one primer and to mark out the grade of homogeneity of the intact protein. The integrity of the fractions after each step of purification was resolved by RP/HPLC (Fig. 2A & B).

N-terminal amino acid sequence analyses

No free terminal amino group was available for salmon parvalbumin to produce N-terminal PTH-AA in the Edman degradation. This was also the case for Allergen M *Gad c1* [52]. Therefore, the analysis of one of the cyanogen bromide fragments with the retention time (Rt) 18.12 (Fig. 2C) was performed, and a tentative AA sequence was deduced: (M), I, G (I/V), D, E, F (A/T/F) (V/A/T), L, V (R/V) (Q/E/R). The percentage of the average repetitive yield for all the PTH-AA was more than 90% and variances with most values close to 1.00.

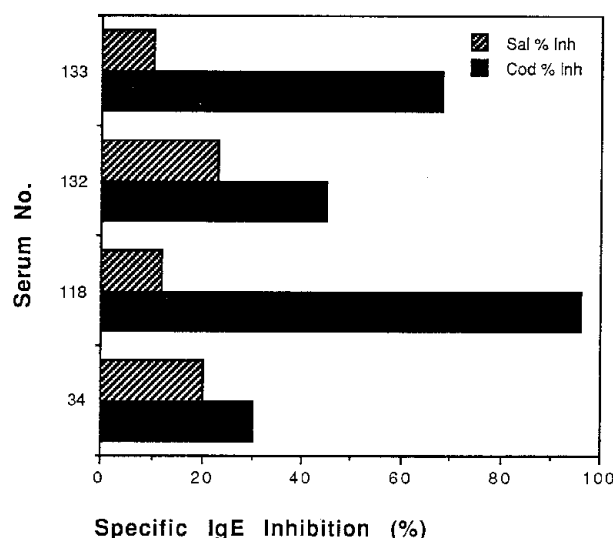


Fig. 3. Specific IgE inhibition using cod and salmon allergens. Determination of the differences between the levels (%) of cod inhibition and those of salmon inhibition using four fish allergic patients' sera.

The average and combined AA repetitive yield was between 92.8 and 100% (data not shown). The sequence analysis was highly reproducible in triplicate runs.

The allergenic and antigenic activity

The allergenicity was determined using RAST-inhibition, shown in Fig. 3. Four sera from cod fish allergic patients were used (Nos. 34, 118, 132, 133). Salmon parvalbumin inhibited the binding of specific IgE to cod at a lower level than that obtained for Allergen M from cod. The allergenicity was substantiated by the results from immunoblotting. Seven patients (Nos. 1, 2, 3, 5, 6, 7 and 11) out of 11 fish allergic sera have specific IgE to salmon (Fig. 4). All the sera giving positive reaction to salmon were also positive to cod (RAST class 3 4, except for serum No. 11 RAST class = 3).

The antigenicity of parvalbumin fractions were shown by FRIE/FRRIE. The antigenic fractions were located in the wells

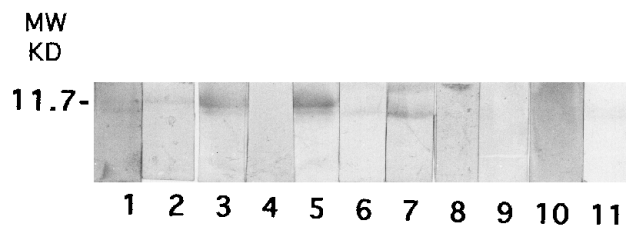


Fig. 4. The allergenic activity of *Sal s1*. Immunoblotting of SDS/PAGE gels to NC membranes which were further incubated with 11 fish allergic patients sera. Seven sera could detect parvalbumin from salmon as illustrated by anti human IgE alkaline phosphatase substrate and Fast Red/Naphthol coloured bands.

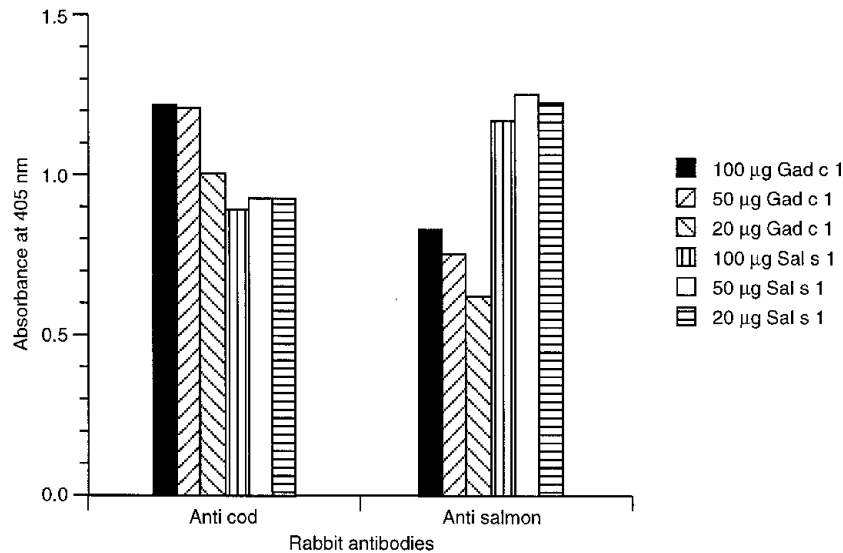


Fig. 5. The cross-reactivity of cod and salmon. Rabbit anti-cod and anti-salmon were used to demonstrate the cross-reactivity between cod and salmon fractions, respectively. Both antigens showed higher affinity to the autologous antibodies.

corresponding to the peak absorbency (280 nm) for DIII. The antigenic lines could bind fish specific IgE in patients' sera. Similar findings were obtained by CIE using fractions DIII and DS2. The results of autoradiography showed one dominant

antigen signal which could bind specific IgE and subsequently ^{125} I rabbit anti-Fc $_{\epsilon}$ (not shown). Further support was provided by IgG-ELISA inhibition assay using both parvalbumins from cod and salmon (not shown). Cod parvalbumin showed inhibition at

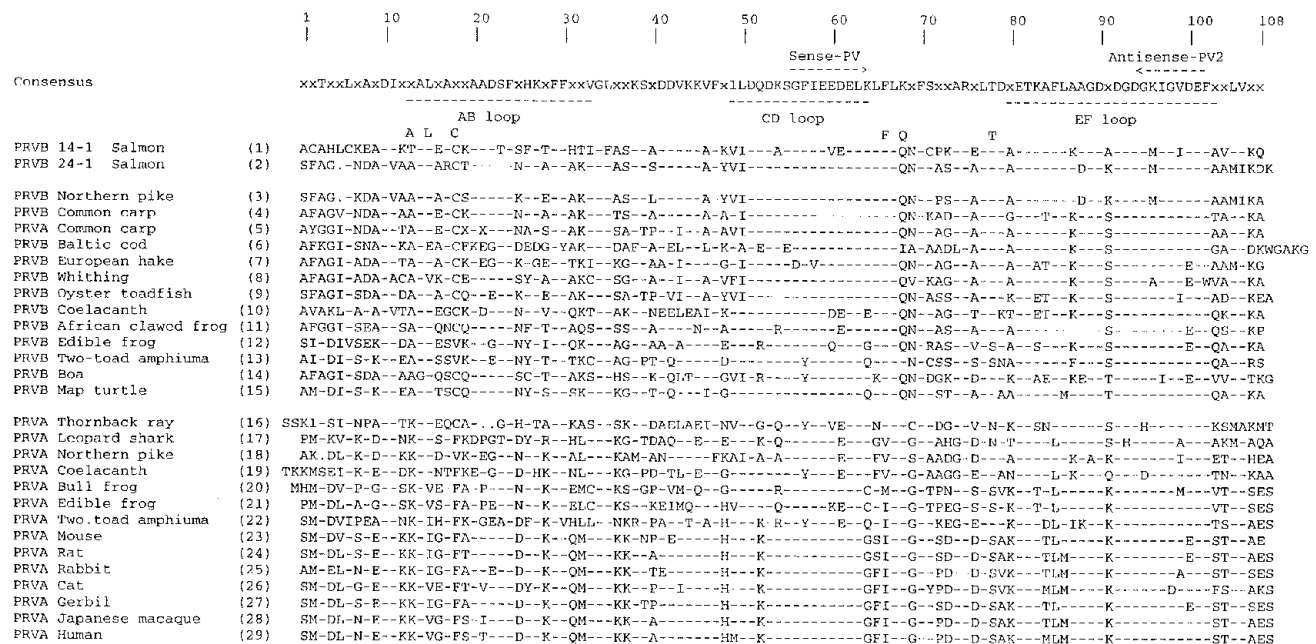


Fig. 6. Alignment of α and β parvalbumin amino acid sequences from different species with the purpose of finding conserved parvalbumin regions for the selection of parvalbumin specific primers. Parvalbumin α and β sequences are shown separately. The α sequence No. 5 has more β type characteristics and therefore is grouped among β sequences. Consensus sequence is shown with AA positioning above and marking of AB, CD and EF loops below. Hyphens represent identities with the consensus sequence. Gaps are indicated by dots. AA sequences were obtained from Swissprot. Numbers in parentheses refer to Table 1. Mixed primers, here represented by their predicted AA-sequence products, are shown above the alignment. The two mixed primers, *Sense-PV* and *Antisense-PV2* were chosen from the relatively conserved regions in the centre and carboxyl terminal region, respectively.

Table 1. References for the sequences used in alignments. % indicates AA sequence inferred from cDNA sequence indicates AA sequence inferred from cDNA sequence

Seq. no.	ID	AC	Molecule	Species	Common name
1			PRVB	<i>Salmo salar</i>	Atlantic salmon
2			PRVB	<i>Salmo salar</i>	Atlantic salmon
3	PRVB-ESOLU	P02619	PRVB	<i>Esox lucius</i>	Northern pike
4	PRVB-CYPCA	P02618	PRVB	<i>Cyprinus carpio</i>	Common carp
5	PRVA-CYPCA	P09227	PRVA	<i>Cyprinus carpio</i>	Common carp
6	PRVB-GADCA	P02622	PRVB	<i>Gadus callarias</i>	Baltic cod
7	PRVB-MERME	P02620	PRVB	<i>Merluccius merluccius</i>	European hake
8	PRVB-MERMR	P02621	PRVB	<i>Merlangius merlangus</i>	Whiting
9	PRVB-OPSTA	P05941	PRVB	<i>Opsanus tau</i>	Oyster toadfish
10	PRVB-LATCH	P02623	PRVB	<i>Latimeria chalumnae</i>	Coelacanth
11	PRVB-XENLA	P05940	PRVB	<i>Xenopus laevis</i>	African clawed frog
12	PRVB-RANES	P02617	PRVB	<i>Rana esculenta</i>	Edible frog
13	PRVB-AMPME	P02616	PRVB	<i>Amphiuma means</i>	Two-toed amphiuma
14	PRVB-BOACO	P02615	PRVB	<i>Boa constrictor</i>	Boa
15	PRVA-GRAGE	P02614	PRVB	<i>Graptemys geographica</i>	Map turtle
16	PRVA-RAJCL	P02630	PRVA	<i>Raja clavata</i>	Thornback ray
17	PRVA-TRISE	P30563	PRVA	<i>Triakis semifasciata</i>	Leopard shark
18	PRVA-ESOLU	P02628	PRVA	<i>Esox lucius</i>	Northern pike
19	PRVA-LATCH	P02629	PRVA	<i>Latimeria chalumnae</i>	Coelacanth
20	PRVA-RANCA	P18087	PRVA	<i>Rana catesbeiana</i>	Bullfrog
21	PRVA-RANES	P02627	PRVA	<i>Rana esculenta</i>	Edible frog
22	PRVA-AMPEME	P02626	PRVA	<i>Amphiuma means</i>	Two-toed amphiuma
23	PRVA-MOUSE%	P32848	PRVA	<i>Mus musculus</i>	Mouse
24	PRVA-RAT%#	P02625	PRVA	<i>Rattus norvegicus</i>	Rat
25	PRVA-RABIT	P02624	PRVA	<i>Oryctolagus cuniculus</i>	Rabbit
26	PRVA-FELCA	P80079	PRVA	<i>Felis catus</i>	Cat
27	PRVA-GERSP	P80080	PRVA	<i>Gerbillus sp.</i>	Gerbil
28	PRVA-MACFU	P80050	PRVA	<i>Macaca fuscata fuscata</i>	Japanese macaque
29	PRVA-HUMAN#	P20472	PRVA	<i>Homo sapiens</i>	Human

least two times higher as compared to salmon parvalbumin in an experiment using rabbit anti-cod antisera. The highest inhibition of salmon parvalbumin was $\approx 16\%$ as compared to cod, which had a maximum inhibition of $\approx 32\%$ using the same antigen concentration. The results were reproducible in triplicate runs.

The antigenic cross-reactivities of *Gad c1* and *Sal s1* were demonstrated using rabbit anti-cod and rabbit anti-salmon parvalbumins at different antigen concentrations (Fig. 5). Salmon parvalbumin showed $\approx 80\%$ binding to rabbit anti-cod parvalbumin, while cod parvalbumin had relatively less binding affinity to rabbit anti-salmon parvalbumin ($\approx 60\%$). The parvalbumins from salmon showed high affinity to rabbit antibodies against cod allergen. Minimal differences were found at the salmon concentrations used. Lower affinities were obtained with *Gad c1* when reacted with rabbit anti-salmon antibodies.

Cloning of salmon parvalbumin cDNA

Cloning of parvalbumin cDNA from salmon was performed utilizing a RT-PCR strategy. One mixed primer, Antisense-PV1,

was selected based on the AA sequence of an N-terminal cyanogen bromide cleavage peptide from a homogeneous salmon parvalbumin fraction. Two other mixed primers, Sense-PV and Antisense-PV2, were based on a multiple alignment of characterized α and β -type parvalbumin sequences from other species (Fig. 6). The primer combinations Sense-PV/Antisense-PV1 and Sense-PV/Antisense-PV2 revealed fragments of the expected size, with no visible background detected by agarose gel electrophoresis. The fragment generated by Sense-PV and Antisense-PV2 was used for screening a muscle cDNA library constructed as described in the Methods section. Screening of $\approx 30,000$ plaques from the primary plating revealed 48 hybridizing signals, of which six were plaque-purified for further analysis.

Sequence analyses of salmon parvalbumin cDNA

The two λ -clones 24.1 and 14.1 that were chosen for subcloning and sequencing had both been shown to possess the entire parvalbumin coding regions as estimated by PCR analysis.

A

24.1

GAATC

ATCTTTGCGAATCCACTCAAGCCTTAAAGCCAAACCTGAGCTAAAC	M S F A G	4
	ATG TCC TTC GCC GGT	66
	* * *	
L N D A D V A A A L A R C T A A D		21
CTT AAC GAT GCT GAT GTT GCT GCA GCC CTC GCT CGT TGC ACA GCT GCT GAC		117
S F N H K A F F A K V G L A S K S		38
TCC TTC AAC CAC AAG GCT TTC TTT GCC AAG GTT GGC CTG GCC AGC AAG TCC		168
S D D V K K A F Y V I D Q D K S G		55
AGC GAT GAT GTG AAG AAG GCC TTC TAC GTC ATT GAC CAG GAC AAG AGT GGC		219
	* *	
F I E E D E L K L F L Q N F S A S		72
TTC ATT GAG GAG GAT GAG CTC AAG CTG TTC CTG CAG AAC TTC TCT GCC TCT		270
	*	
A R A L T D A E T K A F L A D G D		89
GCC AGA GCC CTG ACT GAC GCT GAG ACC AAG GCT TTC CTG GCT GAC GGG GAC		321
	99	
K D G D G M I G V D E F A A M I K	104	106
AAA GAT GGT GAT GGC ATG ATC GGA GTT GAT GAG TTC GCT GCC ATG ATC AAG		372
D K ---		108
GAT AAA TGA AGGCCTGACCAACACCTCTTGATCTGAGGGAACAACCTTGACCCAAGACATCCCCC		436
TTTTCATCTTAACACTTTTATACACGTGACTGTTACTGTGTCTGAGATGACACCCCTTTCCAGGCAT		503
AATGCAATACCTTTTCCAGAGCTGATGGGCAAAAGTAGTGCACTATATTGGGAACCTGGGGGTCATA		570
TCAGACACAGCCTGTGACTGTCTACCCATAGTGCATCTTTCTGTCCACTATGAATGTAAATGTAGCG		637
TGGCTGTGTCTCCCCCTGTCTCATTTGAGTTTGTCTTTAGTGTCCAATCAATGATGCACTT		704
TTTGGGAAATGGACGTCAGACGATATATAGAAAAGGAGGTGGCTTATGAGTATTTCTCCAGGGTAA		771
AAAGCAAAA		780

B

14.1

GTCTTCTCTATCAACCGTTCCACCAAGACATCAGAGCTCAAA	M A C A H L	5
	ATG GCC TGT GCC CAT CTG	60
	* *	
C K E A D I K T A L E A C K A A D		22
TGC AAG GAG GCT GAC ATC AAA ACC GCA TTG GAA GCA TGC AAA GCC GCC GAC		111
T F S F K T F F H T I G F A S K S		39
ACC TTC AGC TTC AAG ACC TTC TTC CAC ACG ATT GGC TTT GCC TCG AAG TCC		162
A D D V K K A F K V I D Q D A S G		56
GCC GAC GAC GTC AAG AAG GCT TTC AAG GTC ATT GAC CAG GAT GCA AGT GGC		213
	* *	
F I E V E E L K L F L Q N F C P K		73
TTT ATT GAG GTG GAG GAG CTG AAG CTG TTC CTT CAG AAC TTT TGC CCC AAG		264
	*	
A R E L T D A E T K A F L K A G D		90
GCC AGG GAA CTT ACC GAT GCA GAG ACA AAG GCT TTC CTC AAG GCT GGT GAT		315
	99	
A D G D G M I G I D E F A V L V K	104	107
GCC GAT GGT GAT GGC ATG ATT GGA ATT GAT GAG TTC GCT GTA TTG GTT AAG		366
Q ---		108
CAA TAA AGAGCAACTGACCAAGAAGTACCTGAAGGAACAATATTGCCCAAGACCTCCCCATTTC		431
ATCTGAATATAAATACTTTTATACATTTGCTTTGGCAAGAAGTTTCCTCCTGGGCGCACACTGTCC		498
AAAATGATGATTGTGAATGTAGCGGGATGTGTTTCATGTCTGATATGAATTTTGCTTACTGTAAAT		565
CTATGATGCACTTTTCAGGAAAGGACGATAGATAAAAAATAATCAATATTCTTTTGG		622

Fig. 7. The cDNAs of clones 24.1 (A) and 14.1 (B) are shown with their deduced amino acid sequences. Both clones 24.1 and 14.1 encode parvalbumin β from salmon. Coding regions are in bold. Deduced AAs are denoted above the nucleotide sequence. Characteristic parvalbumin β -specific AA residues are indicated with stars. The stop codon is indicated with a dashed line (---). Numbering of the DNA and AA sequences are given at the right. The sequenced cyanogen cleavage peptide (residues 95-104) from the parvalbumin C-terminal region can be recognized in both figures (underlined).

This was performed using vector specific primers as well as primers used in the initial RT-PCR analysis of salmon muscle mRNA. Sequencing of clones 24.1 and 14.1 revealed transcripts from two different parvalbumin genes (Fig. 7A & B). PCR with clone specific primers indicated that all four of the remaining plaque purified λ -clones were of 14.1 type. Both clone 24.1 and clone 14.1 include the entire coding region and some nucleotides upstream. From the λ -clone analysis, the 3'-untranslated region of 24.1 was 430 bp long. Due to an internal *EcoRI* site in the 24.1 clone, only the 5' part of the untranslated tail was sequenced since only this part was subcloned. Nucleotide sequences of clones 24.1 and 14.1 were compared. The similarity indices between clone 24.1 and 14.1 were for the coding region: 69%, the upstream first 45 bp: 57%, and the 3'-untranslated first 200 bp: 51%. The translated AA sequences of clones 24.1 and 14.1 were identical in 65% of the residues. Translated sequences were aligned with the same α and β -type parvalbumin sequences that were utilized in the diagnosis of conserved parvalbumin regions for the selection of parvalbumin specific primers. A UPGMA tree was made based on this multiple alignment. The tree showed that the products of clone 24.1 and 14.1 groups with the β -type parvalbumins (not shown). The AA sequence of 24.1 had the following variable similarity indices with β -type parvalbumin sequences from other groups of animals; fish spanning from 91% (Northern pike) to 58% (cod), amphibia 70–59% and reptiles 66–62%. For 14.1 the similarities were: fish 69% (Northern pike) to 51% (cod), amphibia 66–56% and reptiles 59–57%.

DISCUSSION

The cloning of two distinct parvalbumin cDNAs from salmon muscle demonstrates the presence of at least two isotypic variants. This was anticipated by the AA sequence heterogeneity only at some variable sites in the CNBr cleavage peptide from *Sal s1*. A UPGMA tree, based on an alignment of the parvalbumins from salmon (clones 14.1 and 24.1), and parvalbumins from other species, indicated that both clones from salmon were of β -lineage. Upon closer examination, AA sequences of 14.1 and 24.1 were shown to possess the following characteristics of β -lineage parvalbumins: lack of the C-terminal AA 109 found in α -parvalbumins [53], and presence of characteristic residues for β -parvalbumins [54]. These are indicated with stars in the presentation of the sequences (Fig. 7A & B).

In spite of the fact that the products of both clones 14.1 and 24.1 belong to the parvalbumins of β -lineage, nucleotide sequence similarity was relatively low: 69% in the translated part and 51% in the 3'-untranslated part. A high incidence of duplicated genes in salmonids are assumed to be the result of a tetraploidization event in a salmonid ancestor [55]. However, the duplicated genes so far characterized from Atlantic salmon are much less diverged [56] than what 14.1 and 24.1 seem to be based on cDNA data. Still, it cannot be ruled out that the 14.1 and 24.1 genes are a result of a tetraploidization event, as loci located on separate chromosomes probably have established disomic inheritance at different times in evolution [55,56]. Several

parvalbumin cDNA-clones should be analysed to see whether there are additional transcripts that are more likely descendants of a tetraploid event.

Previous studies on Allergen M (*Gad c1*) have shown that the allergenic activity is located to particular epitopes encompassed in the three loops AB, BC and CD (Fig. 6) [7]. Similarity between salmon 24.1 and Allergen M was higher (58%) than the similarity between salmon 14.1 and Allergen M (51%). Similarities were higher within the CD and EF loops, the latter being the highest with percentage identity ranging from 79% (between 24.1 and Allergen M) to 83% (between the 14.1 and Allergen M). The activities of parvalbumin β of Baltic cod and Atlantic salmon allergens could be correlated to their primary structure homologies and to the length of the genetic distances between them and those of pike, carp, whiting and hake in the UPGMA tree [fig. not shown; ref. 26]. Mapping B-cell epitopes using synthetic peptides from parvalbumin of salmon are necessary to clarify the cross-reacting epitopes triggering the binding to specific IgE.

This study should allow the development of a recombinant major allergen of salmon parvalbumin. Further mapping of the B- and T-cell epitopes of parvalbumin from those species could reveal the molecular basis contributing to the relatively low allergenicity of salmon fish.

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