

Characterization of parvalbumin, the major allergen in Alaska pollack, and comparison with codfish Allergen M

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Abstract

Increased fish consumption has led to frequent reporting of fish allergy and adverse reactions. Alaska pollack (*Theragra chalcogramma*) is a globally important commercial fish species, belonging to the Gadidae family. This family of fish also includes cod whose parvalbumin, Allergen M (*Gad c 1*), has been thoroughly studied and considered as a reference to sensitization in fish allergy. In the present study, parvalbumin from Alaska pollack, designated *The c 1*, was purified by use of anion exchange chromatography. To demonstrate the homogeneity of the purified protein, reverse phase high performance liquid chromatography was performed and showed two distinct fractions which had similar IgG and IgE binding capacities. Accordingly, cDNA cloning revealed two isotypic parvalbumin transcripts in pollack muscle. Recombinant parvalbumins of pollack exhibited low IgG and IgE binding capacities, in contrast to the native counterparts, which were almost as potent as cod *Gad c 1*. The allergenicity of *The c 1* was assayed by ELISA inhibition, and compared to cod, the concentration required for obtaining 50% ELISA inhibition (C 50%) was only 18% higher for *The c 1*.

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1. Introduction

Fish becomes an increasingly important food source due to its high consumption and dietary value. Simultaneously, fish is one of the most frequent causes of food allergy, especially in coastal areas and fish processing communities (Elsayed, 2002; Sampson, 2003). Fish allergy in children and young adults is an IgE-mediated disease with clinical manifestations ranging from urticaria, angioedema to anaphylaxis (Aas, 1966; Hansen et al., 1997). It is reported that 1/1000 of the general population in Norway is hypersensitive to fish (Aas, 1987). The prevalence of fish and shellfish allergy in the United States is 0.5% of food-hypersensitive pa-

tients, which are about 2% of the adult population (Sampson, 1999).

The major allergy-eliciting protein in fish has been found to be a parvalbumin (Elsayed and Bennich, 1975). Parvalbumins are Ca²⁺-binding proteins with low molecular weight and acidic isoelectric point. They are present in relatively high amounts in white muscle of lower vertebrates and in lesser amounts in the fast twitch skeletal muscles of higher vertebrates (Lehky et al., 1974; Pechère, 1977). Parvalbumin has been suggested to be important in the relaxation of fibre in muscles (Gillis et al., 1982; Heizmann et al., 1982). The structure of parvalbumin is homologous in different fish species (Elsayed and Bennich, 1975), and antibody cross-reactivities to different fish species have been demonstrated (De Martino et al., 1990; Bernhisel-Broadbent et al., 1992; Pascual et al., 1992; Bugajska-Schretter et al., 1998). Some fish species have been shown to display from two to five parvalbumin isotypes. In various fishes, different parvalbumin isotypes

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have been detected during muscle ontogeny. This differential expression is most probably related to the physiological requirements of the developmental stages of the growing fish (Huriaux et al., 2002).

Alaska pollack (*Theragra chalcogramma*, also called walleye pollack or snow cod) is one of the highly commercial fish species. It was the third most frequently consumed seafood in the United States in 1990–1997, while canned tuna occupied the first place, shrimp the second, salmon the fourth and cod the fifth (Lehrer et al., 2003). Pollack belongs to the Gadidae family, which also includes codfish. The cod major allergen (parvalbumin), designated Allergen M (*Gad c 1*), is well studied and considered as a representative for fish allergens (Elsayed and Apold, 1983). Despite of the commercial value of Alaska pollack and its close relation to codfish, limited studies were reported on this allergen. These studies demonstrated that cross-reactivity among various species of fish might exist. RAST and skin test were performed with fish extracts (Helbling et al., 1992, 1996, 1999).

The aim of the present study was: (a) to isolate parvalbumin from Alaska pollack in a homogeneous form; (b) to reveal its primary structure by cDNA cloning; (c) to produce recombinant allergen and (d) to examine the cross-reactivities between this and cod.

2. Materials and methods

2.1. Patients' sera

Six sera from patients with clinical histories of fish allergy, positive skin prick test, and CAP FEIA (Pharmacia AB, Uppsala, Sweden) classes 4–5 were selected from patients referred to the Central Laboratory, Haukeland University Hospital, Bergen, Norway. Blood samples were taken after informed consent had been given, pooled in small aliquots and stored at -80°C until used. Many control sera from non-allergic individuals with CAP FEIA class 0 to fish were included, three of which were reported in the present study.

2.2. Rabbit sera

Rabbit polyclonal antibodies against Alaska pollack parvalbumin were prepared as described elsewhere (Harboe and Ingild, 1983; Elsayed et al., 1986). Rabbit antisera against cod Allergen M were prepared previously (Lindström et al., 1996; Van Do et al., 2003).

2.3. Extraction, isolation and purification of parvalbumin

Alaska pollack was purchased as frozen fillet produced by FRoSTA AG, Bremerhaven, Germany. The fish muscle extract was purified by anion exchange chromatography, using DEAE cellulose (Cellulose N, *N*-diethyl aminoethyl ether,

Eastman Kodak Company, Rochester, NY, USA). DEAE was washed with 100 mM phosphate buffer pH 5.4 and packed into a column. The same buffer was used for equilibrium and elution of the first two fractions designated DI and DII. The buffer was upgraded to 150 mM, at which fraction DIII was eluted. After dialysis and lyophilization, the purified protein DIII was applied on a Sephadex G-75 GFC column (Pharmacia Biotechnology, Uppsala, Sweden), as described previously (Aas and Jebsen, 1967; Elsayed and Aas, 1971). The partially purified Alaska pollack parvalbumin was designated *The c 1*, according to the Allergy Nomenclature Subcommittee of the International Union of the Immunological Societies (King et al., 1995). Reverse phase high performance liquid chromatography (RP/HPLC) was performed to demonstrate the homogeneity of the parvalbumin using 130 A separation system and BrownleeTM C18, including Aquapore RP-300, 7 μm , 30 mm $\times$ 2.1 mm cartridge (Applied Biosystems Inc., Foster City, CA, USA). The solvents used were as following: A: 0.1% trifluoroacetic acid (TFA) and B: 70% acetonitrile (CH_3CN) in 0.085% TFA. The run was conducted with a gradient of 10–95% B for 5–35 min with a flow of 200 $\mu\text{l}/\text{min}$.

2.4. Isolation of RNA, RT-PCR and cloning

Total RNA was isolated from white muscle using TRIZOL[®] Reagent (GIBCO BRL, Life TechnologiesTM, Rockville, MD, USA). First strand cDNA was synthesized from an oligo-dT₁₈ primer (with an EcoRI anchor sequence) on 2 μg muscle RNA of Alaska pollack. In order to amplify parvalbumin cDNA, the two primers Sense.PV and TP.s were used in 3'RACE. Sense.PV (5'-AGYGGCTTYATHGARGARGAYGARYT-3') was designed for homology cloning of fish parvalbumin genes in a previous study (Lindström et al., 1996), while TP.s (5'-GACCGATGCTGAGACCAAGG-3') was designed during the identification of parvalbumin transcripts in cod (Van Do et al., 2003). Two distinct parvalbumin cDNAs from Alaska pollack were identified by use of these primers. Following PCR profile was used: 94 $^{\circ}\text{C}$, 1 min; 55 $^{\circ}\text{C}$, 1 min; 72 $^{\circ}\text{C}$, 1 min; 35 cycles, linked to 72 $^{\circ}\text{C}$, 10 min. Full-length cDNA sequences of the two isotypic parvalbumin genes, named P1 and P2, were obtained by use of specific primers in 5'RACE. A restriction subfragment of P1 encoding the complete parvalbumin polypeptide was subcloned into the expression vector pET19b (Novagen, Madison, WI, USA). The primers P.NdeI-3 (5'-CATATGTCCTTCGCTGGAGTACTCG-3') and P.BamHI-NY (5'-GGATCCGTTACTGAGTCTTGCAGGG-3') were used to amplify the subfragment. A restriction subfragment of P2 was subcloned by a corresponding approach, utilizing the primers NdeI-2 (5'-CATATGGCATTTCGAGGAATTCTGA-3') and P.BamHI-ISO (5'-GGATCCTTGTTGTTTCATCAGAGC-3'). The plasmid constructs were controlled by DNA sequencing before they were used for expression. The ABI Prism BigDyeTM Terminator Cycle Sequencing Kit was used for DNA-sequencing reactions (Perkin-Elmer Corporation,

Foster City, CA, USA). Sequences were analyzed by use of BLAST (Altschul et al., 1990; Gish and States, 1993), Clustal W (Thompson et al., 1994), Clustal X (Thompson et al., 1997) and tools at the ExPasy home page on the internet (<http://www.expasy.ch/>).

2.5. Expression and purification of recombinant protein

Competent *Escherichia coli* strain BL21 StarTM (DE3) pLysS cells were transformed chemically with the resulting recombinant plasmid. Bacterial growth, preparation of cell lysates and purification of recombinant parvalbumin were performed as described by the manufacturer (Invitrogen BV, Groningen, The Netherlands).

2.6. Protein concentration

The concentrations of the native and recombinant parvalbumins were determined using DC protein assay (BIO-RAD, Richmond, CA, USA).

2.7. ELISA inhibition

Examination of the reactivities of native and recombinant allergens from Alaska pollack and codfish allergen M were analyzed by ELISA inhibition using rabbit serum specific IgG and human IgE (Van Do et al., 2003).

2.8. SDS-PAGE and immunoblotting

Parvalbumins were analyzed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were visualized by Coomassie brilliant blue R-250 staining (Sigma Chemical Co., St. Louis, MO, USA) and silver staining (Gelcode Color Silver Stain; Pierce, Rockford, Illinois, USA). For immunoblot analyses, proteins were transferred onto nitrocellulose membranes (0.45 µm, Schleicher and Schuell, Dassel, Germany) in a mini trans-blot cell (BIO-RAD) for 1 h at 100 V. Immunodetection was performed with polyclonal rabbit antisera (1:3000 dilution, overnight incubation) and patients' sera (1:4 dilution, overnight incubation). After antibody binding, the colour reaction was developed using SIGMA FASTTM BCIP/NBT tablets (Sigma).

3. Results

3.1. Purification and allergenic reactivities of parvalbumin from pollack

The native parvalbumin *The c 1* from Alaska pollack was isolated and partially purified by anion exchange chromatography. Fractions were collected and tested for allergenic activity. The active fractions displayed a similar pattern as parvalbumin of cod in SDS-PAGE (Fig. 1). The partially puri-

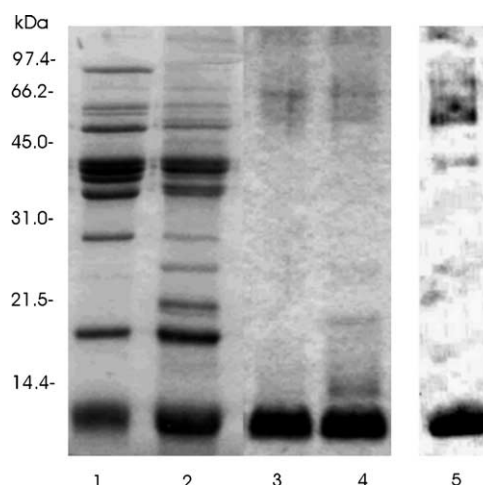


Fig. 1. Isolation of Alaska pollack parvalbumin. The purified parvalbumin of Alaska pollack was compared to cod parvalbumin by SDS-PAGE and Coomassie brilliant blue R-250 staining. Lane 1: cod crude extract, lane 2: Alaska pollack crude extract, lane 3: cod parvalbumin, lane 4: pollack parvalbumin, lane 5: IgE blot using pooled sera from fish-allergic patients and SDS-PAGE of Alaska pollack crude extract.

fied pollack *The c 1* was then applied to RP/HPLC revealing two distinct fractions of parvalbumin (Fig. 2). The fractions with retention time (R_t) 33.12 and 34.18 were collected separately and demonstrated to have similarly high IgE- and IgG-binding capacities. This indicated strongly the presence of two parvalbumin isotypes in Alaska pollack.

Pollack *The c 1* was tested in immunoblots with polyclonal rabbit anti-cod and anti-pollack. Both antibody samples reacted with pollack *The c 1* (Fig. 3) with almost identical binding intensities.

The IgE-binding capacity of Alaska pollack parvalbumin was demonstrated by immunoblotting with a panel of six sera from fish-allergic patients. All sera displayed IgE-binding to pollack *The c 1* as well as to cod *Gad c 1* (Fig. 4). No antibody binding was detected with the control sera.

The allergenicity of pollack *The c 1* was substantiated by ELISA inhibition assays. The concentration required for obtaining 50% inhibition of IgE-binding to cod parvalbumin was 18% higher for *The c 1* than *Gad c 1* (Fig. 5).

3.2. Characterization of two distinct parvalbumin transcripts in pollack

Two isotypic parvalbumin cDNA sequences of Alaska pollack were detected and characterized by an RT-PCR approach. Firstly, partial sequences were obtained by degenerated primers previously used for the isolation of parvalbumin cDNAs from Atlantic salmon and cod (Lindström et al., 1996; Van Do et al., 2003). Subsequently, complete cDNA sequences were assembled by use of specific primers in 3' and 5'RACE, respectively. Both isoforms, designated P1 and P2, showed to belong to the β -lineage of parvalbumins (Fig. 6). The cDNAs encode 108 amino acid long proteins with a

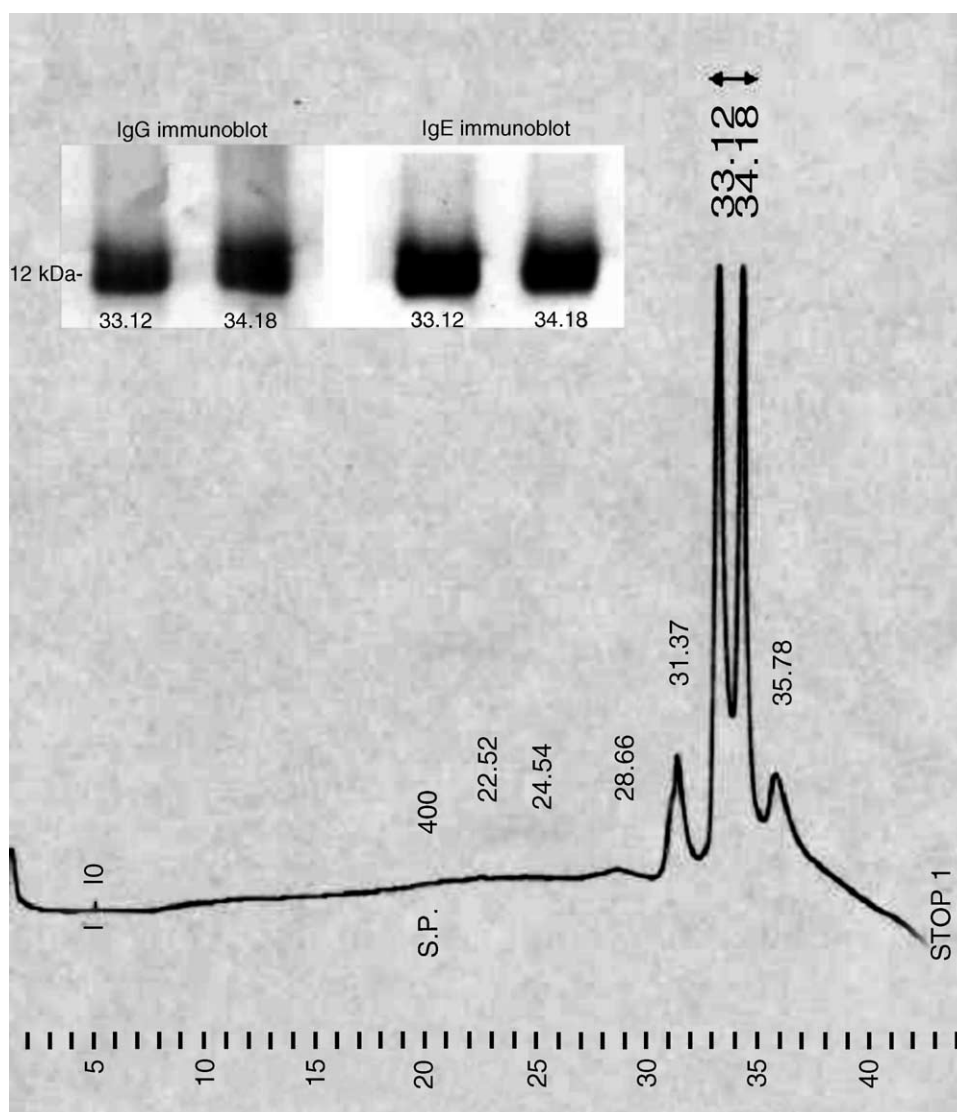


Fig. 2. Purification of pollack parvalbumin. Elution of RP/HPLC parvalbumin after anion exchange chromatography and Sephadex G-75 chromatography. Fractions R_t 33.12 and 34.18, indicated by a solid bar, were immunoblotted with an anti-pollack rabbit serum and a pool of fish-allergic sera, respectively. This demonstrated high IgE- and IgG-binding capacities.

theoretical molecular weight of 11.5 kDa and isoelectric points 4.39 for P1 and 4.60 for P2. The translated sequences of P1 and P2 were aligned with parvalbumin sequences of Atlantic cod, Baltic cod and Atlantic salmon (Fig. 7). Their amino acid sequence identities are shown in Table 1.

Alaska pollack P1 and P2 were identical to each other in 67% of the residues. P1 revealed 72%, 65%, 59%, 75% and 67% similarity to Atlantic cod T1, Atlantic cod T2, Baltic cod, Atlantic salmon 24.1 and Atlantic salmon 14.1, respectively. For P2, the similarities to the above-mentioned

Table 1

Amino acid identities between Alaska pollack, Atlantic cod, Baltic cod and Atlantic salmon parvalbumins

	Pollack P2 (%)	Cod T1 (%)	Cod T2 (%)	Salmon 24.1 (%)	Salmon 14.1 (%)	Baltic cod (%)
Pollack P1	67	72	65	75	67	59
Pollack P2		72	77	75	65	62
Cod T1			71	74	66	65
Cod T2				75	59	68
Salmon 24.1					69	61
Salmon 14.1						68

Accession numbers are presented in legends of Figs. 6 and 7.

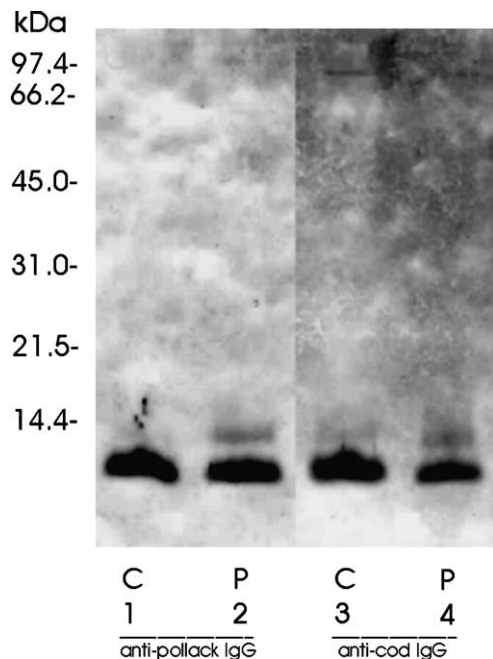


Fig. 3. Immunoblot analysis of cod (C) and pollack (P) parvalbumins (12 kDa) with rabbit anti-pollack sera, lanes 1 and 2, and anti-cod sera, lanes 3 and 4. Both antisera showed strong binding to cod and pollack parvalbumins.

sequences were 72%, 77%, 62%, 75% and 65%, respectively.

3.3. Expression and analysis of recombinant parvalbumin

Recombinant pollack parvalbumin P1 was expressed in transformed *E. coli*. The target protein was found in both soluble and insoluble fractions of the culture. The soluble fractions were used for purification of recombinant protein. The yield of the recombinant parvalbumin P1 was 1.4 mg per

200 ml bacterial culture. P1 showed low allergenicity in comparison to native pollack parvalbumin. In ELISA, inhibition recombinant P1 required 1000 times higher concentration than its native counterpart to reach 10% inhibition of IgE binding to cod parvalbumin and 50% inhibition of rabbit IgG binding to native pollack parvalbumin (Fig. 5).

A recombinant pollack P2 parvalbumin was also produced in *E. coli*. The expression clone of P2 had a deletion of a nucleotide in the coding region (the C in position 215, Fig. 6). This led to a frame shift and a stop-codon, 11 codons downstream of this position. Thus, the length of the recombinant P2 protein was 52 amino acids, plus the N-terminal His-tag and an 11 amino acid long C-terminal tail. The P2 protein was present in very low concentrations in both soluble and insoluble fractions of the culture, of which the former was used for purification. Both recombinant proteins of P1 and P2 showed low antibody binding in immunoblots (not shown) and ELISA inhibition (Fig. 5).

4. Discussion

This work reports the characterization of the parvalbumin in Alaska pollack. As in other fish species analyzed by us (Lindstrøm et al., 1996; Van Do et al., 2003), it was found that the muscle calcium-binding parvalbumin is the potent major allergen of Alaska pollack. All tested sera from fish-allergic patients were rich in specific IgE against parvalbumin from pollack. Therefore, this allergen was designated *The c 1*, according to the Allergy Nomenclature Subcommittee of the International Union of the Immunological Societies (King et al., 1995). Other bands were also observed in immunoblots of the crude extract of pollack (Fig. 1). These may represent minor allergenic proteins, similar to the situation in codfish (Galland et al., 1998).

Pollack *The c 1* was separated into two fractions by RP/HPLC (Fig. 2), indicating that there are two parvalbu-

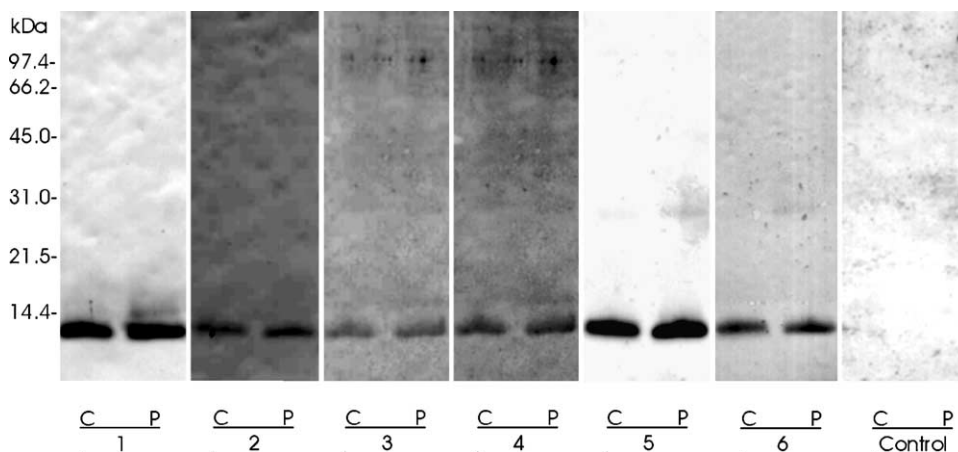


Fig. 4. Immunoblotting of cod (C) and pollack (P) parvalbumins (12 kDa). Sera from six fish-allergic patients (Nos. 1–6) displayed positive IgE reactivity to nitrocellulose blotted cod and pollack parvalbumins. One representative normal serum was included as control.

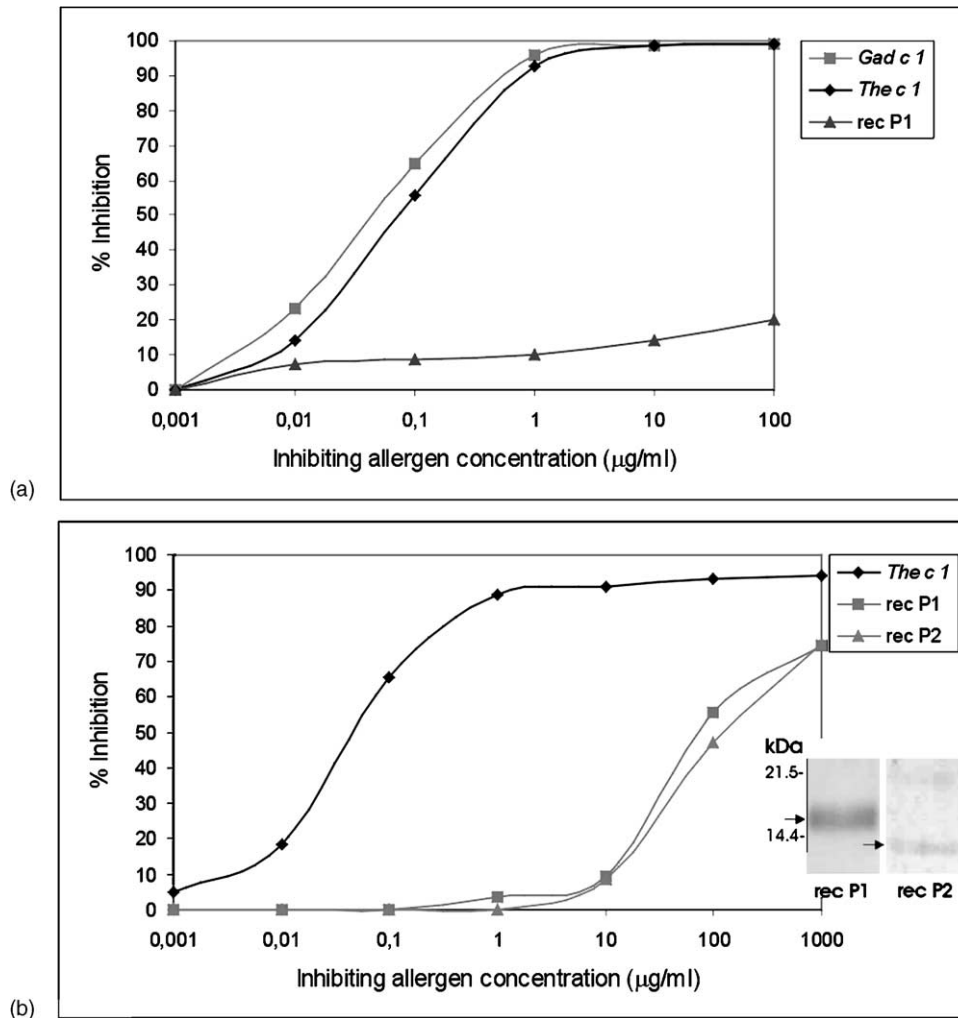


Fig. 5. ELISA inhibition of specific IgE and IgG antibodies. (a) IgE-binding to cod Allergen M was inhibited by relevant concentrations of native cod and native and recombinant pollack parvalbumins. The fish-allergic patient's serum pool was pre-incubated with increasing amounts of cod *Gad c 1*, pollack *The c 1* and recombinant pollack P1 (rec P1). The IgE-binding capacity of the recombinant protein was weaker than that of the native cod and native pollack parvalbumins. (b) Inhibition of rabbit anti-pollack IgG by pollack *The c 1* and recombinant pollack P1 (rec P1) and P2 (rec P2). SDS-PAGE and silver staining of rec P1 and rec P2 (indicated by arrows) are shown in the right lower part of the figure. Rec P1 is constituted of a complete parvalbumin polypeptide and an N-terminal His-tag. Rec P2 has a shorter length that is composed of the first 52 amino acids of the P2 protein fused to an N-terminal His-tag.

min isotypes present in similar concentration in the muscle of this fish species. Accordingly, RT-PCR revealed parvalbumin transcripts from two isotypic genes. It is plausible to assume that these transcripts correspond to the two fractions of parvalbumin isolated. The amino acid sequences of the two parvalbumin isotypes in pollack showed 67% identity to each other, and both proteins belong to the β -lineage of parvalbumins. Previous work in our laboratory has revealed parvalbumin isotypes from both Atlantic cod and Atlantic salmon (Lindström et al., 1996; Van Do et al., 2003), indicating that more than one parvalbumin isotype is generally present in the muscle of fish. However, we could not find any clear-cut pattern of inter-species correspondence regarding the isotypes (Table 1). Both P1 and P2 have 72% identical residues with T1,

while P2 was somewhat more similar to T2 (77%) than P1 (65%).

In order to produce recombinant pollack parvalbumin, the pET-19b expression system was used to express the recombinant parvalbumin. This system allows N-terminal fusion to a cleavable His-tag for rapid affinity purification of the target protein. The pET-system typically generates large amounts of protein. However, we have experienced that expression of different fish parvalbumins in the pET-19b system can result in highly variable amounts of recombinant protein. The yield of recombinant protein in the present study was low compared to the recombinant proteins that we derived in a previous study of cod parvalbumin (Van Do et al., 2003). Since the yield of recombinant pollack parvalbumin was low, and since it displayed low IgE-binding capacity, the recombinant cod

P1

ACACTTGAGCTCCACTTGGTTTTCTACTCGCCCTCAACGCAGCGCAAAAAGATAGAAAA 59

M S F A G V L A D A D V K A A 14
ATG TCC TTC GCT GGA GTA CTC GCT GAC GCT GAC GTC AAG GCC GCC 104

L A G C A A A D S F N Y K T F 29
CTC GCC GGC TGC GCA GCT GCT GAC TCC TTC AAC TAC AAG ACC TTC 149

F K A C G L A A K S H E E V K 44
TTC AAG GCA TGC GGT CTG GCC GCC AAG TCC CAC GAG GAA GTC AAG 194

K A F F V I D Q D Q S G F I E 59
AAG GCT TTC TTC GTC ATT GAC CAG GAC CAG AGC GGC TTC ATT GAG 239

E D E L K L F L Q T F G A G A 74
GAG GAT GAG CTT AAG CTC TTC CTG CAG ACC TTC GGC GCC GGA GCA 284

R E L T A A E T K A F L A A G 89
AAG GAA CTC ACC GCT GCT GAG ACC AAG GCC TTC CTC GCC GCT GGT 329

D E D G D G M I G V D E F V T 104
GAC GAG GAT GGT GAT GGC ATG ATC GGA GTT GAT GAG TTT GTC ACC 374

L V K A * 108
CTT GTG AAG GCA TAA ATCTCCATTGACCAACATCACAACAACCTCTACACGGAAC 428

CGCGCACTCCCTGCAAGACTCAGTAACCATCAGCTGAAATAATATTTTATATCTTAT 487
TTATGAATTGCCTGCAGACTTTTCTCAACATCTGACACATCGTGTAATGTTAATGTT 546
ACTCGATGTGTTAAATGTTCTGGAATTGACCTGTGCAAAATGTACAACTTATCTGCAC 605
TTTGTAGGAAGAGTGAAAAACCAGGAATAAATGCTCTTTTGTCTG 650

P2

TGACTACAGTCAAAGTGTGTTGTCATCTGTACAAAGCAAAAGGCCTACCAGAGCTCAAA 58

M A F A G I L K D A E V A A A 14
ATG GCA TTC GCA GGA ATT CTG AAG GAT GCG GAA GTT GCT GCA GCC 103

L E A C K S A G S F D H T K F 29
CTG GAA GCC TGC AAA TCC GCC GGC TCA TTT GAC CAT ACA AAG TTT 148

F K S C G L A G K S S D D V K 44
TTC AAA TCC TGC GGT CTG GCC GGA AAG TCC TCC GAT GAC GTC AAG 193

K A F G I I D Q D Q S D F I E 59
AAG GCC TTC GGC ATC ATT GAC CAG GAC CAG AGC GAC TTC ATT GAG 238

E E E L K L F L Q N F S A S A 74
GAG GAG GAG CTG AAG CTG TTC CTG CAG AAC TTC AGC GCA AGT GCA 283

R A L S D A E T K A F L K A G 89
CGC GCG CTG AGC GAT GCT GAG ACA AAG GCT TTC CTG AAG GCC GGA 328

D S D G D G K I G V D E F A A 104
GAC TCC GAC GGG GAC GGC AAG ATA GGA GTT GAT GAG TTT GCT GCC 373

M V K A * 108
ATG GTG AAG GCA TAA ACAGACTGACCAACACCTGCTCTGATGAAACAACAAGGC 427

CACGAGACCTCCCCCTCTTCATCTGAATATACATAATTTTATACACTCGCTTGTGA 486
GACATTTCTCTATGCAACAACTGTCCAGAAGAAGAAGGAAAAATTATGATTGT 545
GAATGTAGCTTGGTTGTGTTCTGTTCTTAAATATGAATTTTGATGTATTGTAATCTG 604
TTATGCACCTTTTCAGAAACGGAACAGATTATTATTATTAACAGAAATAATAAATAAT 663
TTTTCAATGGTCACTTA 680

Fig. 6. Parvalbumin cDNAs of Alaska pollack. The cDNAs P1 and P2 are shown with the deduced amino acid sequences above the nucleotide sequence. Both cDNAs comprise the entire parvalbumin-coding region. The stop-codon is indicated by an asterisk and the polyadenylation signal is underlined. The sequences have been submitted to the GenBank Sequence database and have been assigned the accession nos. AY035586 for P1 and AY035587 for P2.

proteins might be better candidates for further studies. The low allergenicity of the recombinant pollack parvalbumins could be due to a conformational modification, since IgE antibodies bind directly to the calcium-binding loops of the parvalbumins, as demonstrated in cod Allergen M (Elsayed, 2002). However, isoallergens can also display significant differences in reactivity towards antibodies

and T-cells (Lowenstein et al., 1995). Thus, the recombinant pollack proteins, with their reduced allergenic activity, might be useful in experimental allergen specific immunotherapy in allergic animal model. To further evaluate the allergenicity of the pollack parvalbumin, studies with a large number of patients as well as skin prick test are in progress.

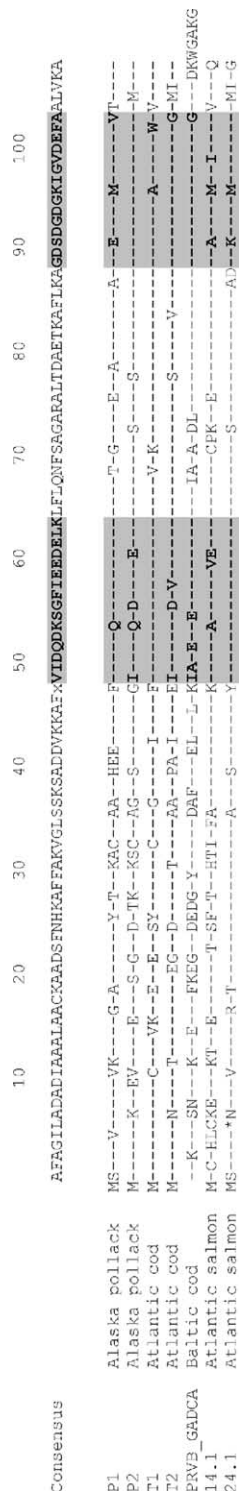


Fig. 7. Comparison of the deduced amino acid sequences of Alaska pollack P1 and P2 with parvalbumins of Atlantic cod (*Gadus morhua*) T1, GenBank accession no. AY035584; T2, GenBank accession no. AY035585; Baltic cod (*Gadus callarias*), GenBank accession no. P02622; and Atlantic salmon (*Salmo salar*) 14.1, GenBank accession no. X97824; 24.1, GenBank accession no. X97825. Hyphens represent identities with the fish parvalbumin β consensus sequence shown above. Gaps are indicated by asterisks. Shaded boxes indicate the IgE-binding motifs.

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