

Expression and Analysis of Recombinant Salmon Parvalbumin, the Major Allergen in Atlantic Salmon (*Salmo salar*)

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The parvalbumin from white muscle of Atlantic salmon was previously found to be a major allergen, and designated *Sal s1*. Two distinct cDNAs, 14.1 and 24.1, which comprise the entire parvalbumin-encoding regions, were cloned, revealing transcripts from two different parvalbumin genes. In the present study, the protein-coding regions of these cDNAs were subcloned into an *Escherichia coli* expression vector (pET-19b). Both proteins were expressed and the generated target proteins were localized in both soluble and insoluble fractions of the expression host. The recombinant products in the soluble fraction were purified using the His tag-purification system and analysed on Western blots with anti-salmon parvalbumin polyclonal rabbit sera and sera from patients allergic to fish. Both recombinant products (His₁₀-14.1 and His₁₀-24.1) reacted positively with salmon parvalbumin-specific immunoglobulin G (IgG) from rabbits, and with specific immunoglobulin E (IgE) from the sera of six fish-allergic patients. The allergenicity of His₁₀-14.1 was confirmed using enzyme-linked immunosorbent assay (ELISA). The 14.1 cDNA of salmon parvalbumin was shown to be the dominant type represented in a muscle cDNA library.

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INTRODUCTION

Atlantic salmon (*Salmo salar*) belongs to the fatty fish group (>2% fat) whose muscles contain polyunsaturated eicosanoids that are known to be of particular prophylactic health benefits [1–6]. Despite the nutritional merits, the ingestion of salmon may cause allergy. In a Norwegian study, it was estimated that ≈ one per 1000 individuals are allergic to fish and that almost 50% of cod-hypersensitive patients also react to salmon [7]. The percentage of fish-allergic persons who also have specific immunoglobulin E (IgE) to salmon varies among different populations, e.g. 5% in Italy [8] and 15.2% in South Africa [9].

The major allergy-eliciting protein in fish has been found to be parvalbumin [10]. Cod and salmon major allergenic proteins were, respectively, designated Allergen M (*Gad c1*) and *Sal s1*. A purified salmon parvalbumin was obtained by anion-exchange chromatography, gel-filtration chromatography and high-performance liquid chromatography, and tested for its allergenicity and antigenicity using various immunoelectrophoretic techniques. Based on an alignment of parvalbumin sequences from other species, a salmon-specific DNA probe was generated by reverse

transcription–polymerase chain reaction (RT–PCR) and used to screen an oligo dT-primed muscle cDNA library. Two distinct full-length parvalbumin cDNAs were characterized and designated 14.1 and 24.1 [11].

Parvalbumins are high-affinity Ca²⁺-binding proteins of the ‘EF-hand type’ [12], of low molecular weight (MW) and were initially detected in relatively high amounts in white muscle of lower vertebrates [13]. They were subsequently found, although in smaller amounts, in the fast-twitch skeletal muscles of higher vertebrates [14]. Parvalbumins were suggested to be important in the relaxation of muscle fibre [15]. They are divergent proteins in terms of their primary structure, and several isotypes can be present in the same muscle. Two distinct phylogenetic lineages of parvalbumins, named α and β, are generally recognized in lower and higher vertebrates [16]. They can be distinguished by their different isoelectric points (α > pI 5, β < pI 5), sequence characteristics, affinities for Ca²⁺ and Mg²⁺, crystal structures, cell-type specific expression, chromosomal localization and physiological functions. Most muscles contain only parvalbumins of either α- or β-lineage, although in frog skeletal muscles both α and β parvalbumins have been found. Parvalbumins from

cod and salmon belong to the β -lineage and an amino acid identity of 51–58% was found between them. The two parvalbumin variants characterized in salmon have 70% identical amino acid residues [11].

Sufficient quantities of allergens are essential for the development of *in vitro* and *in vivo* diagnosis of allergic diseases. Standardization of allergenic protein extracts could not be performed without the availability of purified allergens. However, the purification of multiple allergenic components from a given extract is a difficult, sometimes impossible, task. Moreover, it is possible that during the purification process some allergens become rapidly denatured or are missing, and some are present in low concentrations or are poorly extracted. To overcome these problems, recombinant DNA technology has been applied. The cDNA of several allergen protein genes has been cloned, and the corresponding recombinant allergens have been synthesized, as reviewed elsewhere [17]. Molecular genetic techniques are also essential in mapping B- and T-cell epitopes of allergens and in analysing the cell interactions that are responsible for development of immediate, late and delayed allergic symptoms, as reviewed elsewhere [18].

The purpose of the present work was to produce parvalbumin, the major allergen of Atlantic salmon (*Sal s1*) by recombinant DNA technology, and to study its reactivities with specific IgE from fish-allergic persons. Moreover, the ratio of 14.1 : 24.1-type parvalbumin clones in a salmon muscle cDNA-library was estimated.

MATERIALS AND METHODS

Human sera. Sera were obtained from patients ($n=6$) with clinical histories of allergy to fish. Patient 1: total serum IgE = 460 kU/l, specific IgE (CAP-RAST, Pharmacia Diagnostics AB, Uppsala, Sweden) against cod class 4; patient 2: serum IgE > 2000 kU/l, specific IgE (CAP-RAST) against cod class 5, salmon class 5; patient 3: total serum IgE = 644 kU/l, specific IgE (CAP-RAST) against cod class 4; patient 4: total serum IgE = 5620 kU/l, serum-specific IgE (CAP-RAST) against cod class 5, salmon class 4; patient 5: total serum IgE = 269 kU/l, specific IgE (CAP-RAST) against cod class 4; and patient 6: total serum IgE = 2700 kU/l, CAP-RAST specific IgE against cod class 3. One negative control serum with CAP-RAST class 0 to fish and total serum IgE = 165 kU/l was included.

Rabbit sera. Rabbit polyclonal antibodies against the major allergen of salmon (*Sal s1*) were prepared as described previously [19, 20].

Subcloning into an expression vector. Expression plasmids containing cDNA inserts coding for two variants of salmon parvalbumin were constructed in the vector pET-19b (Novagen, Madison, WI, USA). The 5' primer 14NDEI (5'-CATATGGCCTGTGCCCATCTGT-3') and the 3' primer 14BAMHI (5'-GGATCCCTTGGTCAGTTGCTCTTA-3') were used in the PCR of cDNA 14.1 in order to produce a DNA fragment with appropriate restriction sequences at its 5' and 3' ends. Correspondingly, the primers 24NDEI (5'-CATATGTCCTTCGCCGGTCT-3') and 24BAMHI (5'-GGATCCGTGTTGGTCAGGCCCTTCA-3') were used in the PCR of cDNA 24.1. The primers 14NDEI and 24NDEI add an *NdeI* restriction site to the 5' end of the PCR products (underlined). The primers 14BAMHI and 24BAMHI add a *BamHI* site to the 3' end of the PCR products (underlined). PCR conditions were as recom-

mended by the supplier of *Taq* polymerase (Pharmacia Biotech AB, Uppsala, Sweden). PCR was conducted for 30 cycles using the following profiles: 1 min denaturation at 94 °C, 1 min annealing at 55 °C and 1 min extension at 72 °C, with a final extension for 10 min at 72 °C. The PCR products were ligated into vector pGEM-T (Promega, Madison, WI, USA). Fragments from positive plasmid clones were excised with *NdeI* and *BamHI* and purified by agarose-gel electrophoresis. The *NdeI*–*BamHI* fragments were inserted into *NdeI*/*BamHI*-digested vector pET-19b. These expression constructs were designated His₁₀-14.1 and His₁₀-24.1.

DNA sequencing and analyses. Double-stranded plasmid templates were sequenced manually using the Thermo Sequenase sequencing kit (Amersham Life Science, Cleveland, OH, USA) and/or with reagents for automatic sequencing (ABI Prism BigDye™ Terminator Cycle Sequencing Kit; Perkin Elmer Corporation, Foster City, CA, USA). Sequences were analysed using MACMOLLY® Tetra software (©Soft Gene GmbH, Berlin, Germany).

Transformation, bacterial growth and preparation of cell lysates. Competent cells of *Escherichia coli*, strain BL21(DE3)pLysS, which houses the gene T7 RNA polymerase under the control of the isopropylthio- β -D-galactoside (IPTG)-inducible lac UV5 promoter, were transformed by electroporation with the resulting recombinant plasmids. The cells were grown at 30 °C or 37 °C in Luria Bertani medium (supplemented with 100 μ g/ml of ampicillin and 34 μ g/ml of chloramphenicol) to an optical density (OD) of 0.6 at 600 nm. IPTG was then added to the culture to a final concentration of 1 mM, and incubation was continued for another 4 h. After expression of the recombinant proteins, the cells were harvested by centrifugation at 5000 *g* for 5 min. The pellet was resuspended in ice-cold binding buffer (20 mM Tris-HCl, pH 7.9, 5 mM imidazole, 0.5 M NaCl) and disrupted by sonication. Insoluble material was removed by centrifugation at 20 000 *g* for 30 min. The supernatant containing the expressed protein was filtered through a 0.45-micron membrane and stored at –20 °C until used.

Purification of target protein. Purification of the fusion protein was performed according to the pET manual (Novagen). Briefly, 2.5 ml of ProBond™ resin (Invitrogen BV, Groningen, the Netherlands) was washed with 3 vol of sterile deionized water and equilibrated with 3 vol of binding buffer (20 mM Tris-HCl, pH 7.9, 5 mM imidazole, 1 M NaCl). The column containing equilibrated resin was loaded with the protein extract in binding buffer and then washed with 10 vol of binding buffer and 6 vol of wash buffer (20 mM Tris-HCl, pH 7.9, 60 mM imidazole, 1 M NaCl). The bound proteins were eluted with 5 ml of elution buffer (20 mM Tris-HCl, pH 7.9, 1 M imidazole, 0.5 M NaCl).

Protein concentrations. The concentrations of expressed proteins were measured using the DC (detergent-compatible) Protein Assay kit (Bio-Rad, Hercules, CA, USA). Briefly, 5 μ l of sample was pipetted into the wells of a microtitre plate. The sample wells then received 25 μ l of reagent A and 200 μ l of reagent B. After 15 min, the absorbance was read at 690 nm. Bovine serum albumin (BSA) was used as a standard.

Digestion with enterokinase. Digestion of recombinant products were performed using enterokinase, according to the protocol of EkMax™ (Invitrogen). The recombinant protein was concentrated using the Centricon centrifugal filters (Amicon; Millipore Corporation, Bedford, MA, USA) and subsequently dialysed against EKMax™ buffer (500 mM Tris-HCl, pH 8.0, 10 mM CaCl₂, 1% Tween-20 [vol/vol]) for 24 h at 4 °C. One unit (1 μ l) of enterokinase was used to digest 25 μ g (25 μ l) of protein for 1.5 h. The digested protein was then mixed with ProBond metal-affinity resin and incubated at room temperature. After 15 min of

incubation, the resin was centrifuged at 1000*g* for 1 min and the supernatant containing the purified protein without the His tag was collected and stored at 4°C until use.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotting. Proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), according to the procedure of Laemmli *et al.* [21]. The samples, together with molecular-weight standards (Bio-Rad low range SDS–PAGE standards and prestained standards) were resolved in a 15% separating gel at 200 V. The individual protein bands were identified with Brilliant Blue R-250 (Sigma Chemical Co, St Louis, MO, USA) or the Silver stain kit (Sigma). Separated proteins were transferred to a nitrocellulose (NC) membrane in a 25 mM Tris, 192 mM glycine, 40% methanol transfer buffer [22] for 1 h at 100 V. After blocking with 10% BSA or 0.5% Tween-20 in Tris-buffered saline (TBS), the NC membrane was washed three times with TBS containing 0.05% Tween-20 (TBS-T) and then probed with individual patient serum samples (1:5 dilution) for 3 h at room temperature. After three separate washes with TBS-T, horseradish peroxidase-labelled anti-human IgE conjugate was added (1:1000 TBS-T) and incubated at room temperature, with rocking, for 2 h. Following a further wash with TBS, the blot was incubated for 1 min with enhanced chemiluminescence (ECL) Western blot detection reagents (Amersham International plc, Little Chalfont, Bucks, UK), exposed for ≈15 s to Hyperfilm-ECLTM and then developed.

Enzyme-linked immunosorbent assay. IgE reactivities to native and recombinant allergens were determined using a direct enzyme-linked immunosorbent assay (ELISA) technique [23]. Plates (96-well microtitre) (Falcon 3911, Micro Test III, Flexible Assay Plate; Becton-Dickinson Labware, Oxnard, CA, USA) were coated with 1 µg of antigen (native or recombinant salmon parvalbumin) dissolved in 0.1 ml of 0.1 M sodium carbonate buffer, pH 9.6, and incubated overnight at 4°C. Native salmon protein was used as a positive control. The plates were washed five times with TBS, pH 7.4, containing 0.05% Tween-20, 0.1 ml of undiluted human serum was added and the plates were incubated overnight at 4°C. After repeating the washing procedure, a 1:1000 dilution of anti-human IgE alkaline phosphatase conjugate (Sigma; 0.1 ml/well) was added, and the plates were incubated for 2 h at room temperature. Finally, after another wash procedure, the colour reaction was developed by incubating with 0.1 ml/well of Tris buffer, pH 9.5, containing 1 mg/ml *p*-nitrophenylphosphate (Sigma). After 1 h of development, the absorbance was read at 405 nm in an ELISA reader (Labsystems Multiskan Bichromatic, Helsinki, Finland). The enzyme reaction was terminated by the addition of 50 µl of 5N NaOH.

RESULTS

Construction of expression plasmids

Expression plasmids of two parvalbumin-coding genes in salmon were constructed from previously cloned cDNA fragments (Fig. 1). Restriction sites were introduced at both ends of the cDNA subfragments by PCR, generating plasmid constructs with coding capacity for the entire parvalbumin polypeptide, plus a short N-terminal peptide of 22 amino acids (MGHHHHHHHHSSGHI DDDDKH). The His tag facilitates purification of the target protein, and an adjacent cleavage site for enterokinase (underlined) allows removal of the tag if conditions are appropriate.

		M		A	C	A	H	L	C	K	E	A	D	I		12
14.1	<u>CAT</u>	<u>ATG</u>	GCC	TGT	GCC	CAT	CTG	TGC	AAG	GAG	GCT	GAC	ATC			39
		M		S	F	A	G	L	-	N	D	A	D	V		11
24.1	<u>CAT</u>	<u>ATG</u>	TTC	TTC	GCC	GGT	CTT		AAC	GAT	GCT	GAT	GTT			36
		K	T	A	L	E	A	C	K	A	A	D	T	F		25
14.1	AAA	ACC	GCA	TTG	GAA	GCA	TGC	AAA	GCC	GCC	GAC	ACC	TTC			78
	A	A	A	L	A	A	C	T	A	A	D	S	F			24
24.1	GCT	GCA	GCC	CTC	GCT	GCT	TGC	ACA	GCT	GCT	GAC	TCC	TTC			75
		S	F	K	T	F	F	H	T	I	G	F	A	S		38
14.1	AGC	TTC	AAG	ACC	TTC	TTC	CAC	ACG	ATT	GGC	TTT	GCC	TCG			117
	N	H	K	T	F	F	A	K	V	G	L	A	S			37
24.1	AAC	CAC	AAG	ACT	TTC	TTT	GCC	AAG	GTT	GGC	CTG	GCC	AGC			114
		K	S	A	D	D	V	K	K	A	F	K	V	I		51
14.1	AAG	TCC	GCC	GAC	GAC	GTC	AAG	AAG	GCT	TTC	AAG	GTC	ATT			156
	K	S	S	D	D	V	K	K	A	F	Y	V	I			50
24.1	AAG	TCC	AGC	GAT	GAT	GTG	AAG	AAG	GCC	TTC	TAC	GTC	ATT			153
		D	Q	D	A	S	G	F	I	E	V	E	E	L		64
14.1	GAC	CAG	GAT	GCA	AGT	GGC	TTT	ATT	GAG	GTG	GAG	GAG	CTG			195
	D	Q	D	K	S	A	F	I	E	E	D	E	L			63
24.1	GAC	CAG	GAC	AAG	AGT	GCC	TTC	ATT	GAG	GAG	GAT	GAC	CTC			192
		K	L	F	L	Q	N	F	C	P	K	A	K	E		77
14.1	AAG	CTG	TTC	CTT	CAG	AAC	TTT	TGC	CCC	AAG	GCC	AGG	GAA			234
	K	L	F	L	Q	N	F	S	A	S	A	R	A			76
24.1	AAG	CTG	TTC	CTG	CAG	AAC	TTC	TCT	GCC	TCT	GCC	AGA	GCC			231
		L	T	D	A	E	T	K	A	F	L	K	A	G		90
14.1	CTT	ACC	GAT	GCA	GAG	ACA	AAG	GCT	TTC	CTC	AAG	GCT	GGT			273
	L	T	D	A	E	T	K	A	F	L	A	D	G			89
24.1	CTG	ACT	GAC	GCT	GAG	ACC	AAG	GCT	TTC	CTG	GCT	GAC	GGG			270
		D	A	D	G	D	G	M	I	G	I	D	E	F		103
14.1	GAT	GCC	GAT	GGT	GAT	GGC	ATG	ATT	GGA	ATT	GAT	GAG	TTC			312
	D	K	D	G	D	G	M	I	G	V	D	E	F			102
24.1	GAC	AAA	GAT	GGT	GAT	GGC	ATG	ATT	GGA	GTT	GAT	GAG	TTC			309
		A	V	L	V	K	Q	---								109
14.1	GCT	GTA	TTG	GTT	AAG	CAA	TAA	AGAGCAACTGACCAACGGATCC								355
	A	A	M	I	K	G	---									108
24.1	GCT	GCC	ATG	ATC	AAG	GGA	TAA	ATGAAGGCCTGACCAACACGGATCC								355

Fig. 1. Expression fragments of salmon parvalbumin (from cDNA clones 14.1 and 24.1). The parvalbumin-coding regions were inserted into the *NdeI/BamHI* sites of vector pET-19b by a polymerase chain reaction (PCR) approach, implying that the resultant protein was N-terminally fused to a His₁₀ tag. The nucleotide sequences of the inserted fragments are shown with the sense and antisense primers underlined. Coding regions are in bold. Deduced amino acids are denoted above the nucleotide sequences and those identical in both clones are presented in italic. The stop codon is indicated with a dashed line (---).

Expression of recombinant salmon parvalbumin

Recombinant parvalbumin was expressed in transformed *E. coli* under two different temperatures. After induction of protein synthesis by addition of IPTG for 4 h, bacterial cells were harvested. Samples collected after 1, 2, 3 and 4 h of induction showed a rapid increase of target protein production. There was no significant difference between the concentration of target protein from the cells harvested after 4 h of induction and those of cells sampled after 3 h of induction (data not shown). Recombinant parvalbumin was found in both soluble and insoluble fractions of the cultures. We used only the soluble proteins for further purification of the target products. The level of recombinant proteins in the soluble fractions was higher when the bacteria grew at 30°C than at 37°C (7.7 mg/l and 5.2 mg/l for 14NDEI and 0.9 mg/l and 0.6 mg/l for 24NDEI). As deduced

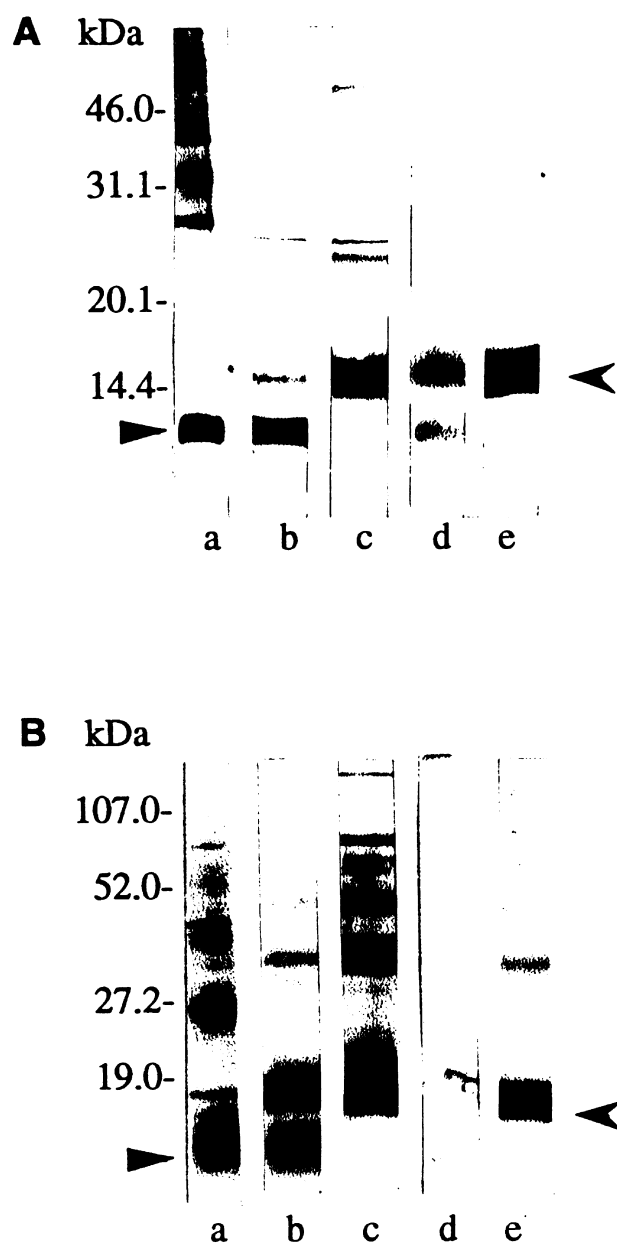


Fig. 2. (A) Silver-stained sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of native and recombinant salmon parvalbumin. (B) Immunoblot analysis using rabbit anti-salmon-specific immunoglobulin G (IgG) from a pool of two sera (Ø486 and Ø505). Lane a, purified native salmon parvalbumin. Lane b, enterokinase-digested His₁₀-14.1. Lane c, undigested His₁₀-14.1. Lane d, enterokinase-digested His₁₀-24.1. Lane e, undigested His₁₀-24.1. Arrowheads indicate the native parvalbumin (arrowhead pointing to the right) and the undigested recombinant product (arrowhead pointing to the left). Some nonspecific binding bands probably represent cross-reactivity to muscle proteins of Atlantic salmon (lane a), and a few contaminant bacterial proteins in the recombinant protein samples (lanes b–e).

from SDS–PAGE, the relative molecular mass (M_r) of the recombinant proteins was ≈ 14 kDa, which is slightly higher than, or in accordance with, the theoretical M_r . After purification by Probond resin, the expressed proteins still contained some *E. coli* proteins (Fig. 2).

Removal of the His₁₀ tag from the target proteins

In an attempt to remove the His tag, the recombinant proteins His₁₀-14.1 and His₁₀-24.1 were digested with enterokinase for different time intervals: 1 h, 1.5 h, 2 h and overnight. The fusion proteins were partly digested after 1.5 h and the target proteins without the His tag showed a MW similar to that of the native protein (Fig. 2). Longer digestion or a larger enterokinase concentration led to further degradation of the proteins, as experienced by several digestion attempts.

Antibody binding

Polyclonal immunoglobulin G (IgG) from rabbit sera bound to both His₁₀-14.1 and His₁₀-24.1 as well as to native *Sal s1* (Fig. 3).

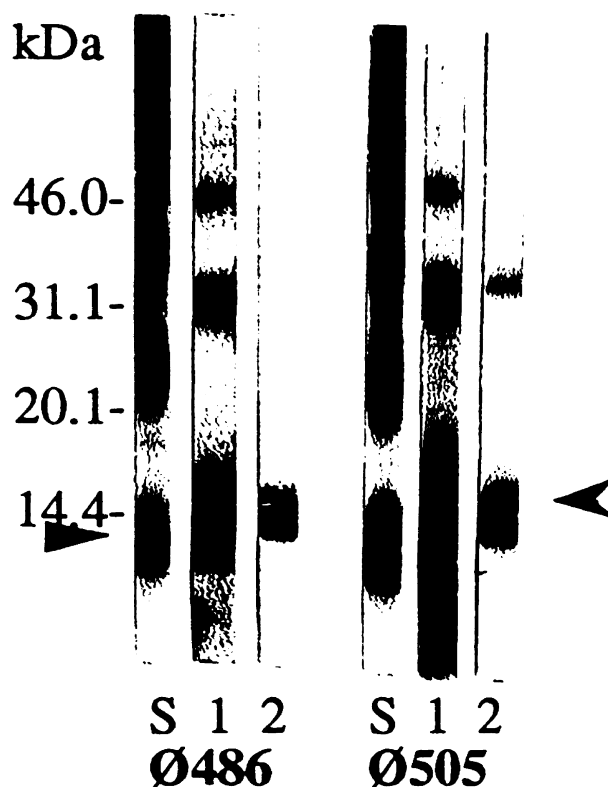


Fig. 3. Immunoblot analysis of native and recombinant salmon parvalbumin using two separate rabbit sera. Anti-salmon parvalbumin sera from two rabbits (Ø486 and Ø505) were probed with the following proteins: native salmon allergen (S), His₁₀-14.1 (1) and His₁₀-24.1 (2). Blots were exposed to Hyperfilm-ECLTM. Arrowheads indicate the native protein (arrowhead pointing to the right) and the recombinant protein (arrowhead pointing to the left). The reactivity pattern was similar for both sera.

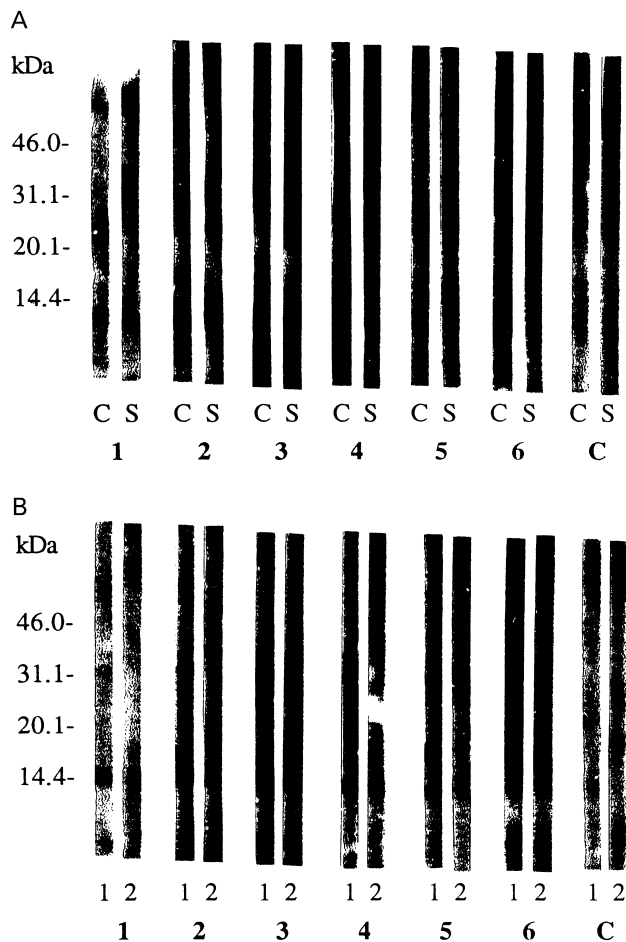


Fig. 4. (A) Immunoblot analyses of cod Allergen M (C) and native salmon major allergen (S). (B) Immunoblot analyses of His₁₀-14.1 (1) and His₁₀-24.1 (2). The strips were probed with sera from six fish-allergic patients (1–6) and with one nonallergic serum sample as a control (C). The blots were analysed using enhanced chemiluminescence (ECL) Western blot detection reagents and exposed to film.

However, there were marked differences in the ability of each recombinant product to bind IgG from the two different rabbit sera. Both rabbits displayed the strongest IgG binding with His₁₀-14.1.

The IgE-binding ability of recombinant and native parvalbumin was tested on immunoblots using six sera from patients with fish allergies (CAP-RAST class 3–5), and serum from a non-allergic person as a control (Fig. 4). In all sera, IgE binding to native as well as to recombinant proteins was observed.

The degree of IgE binding to His₁₀-14.1 was also tested by ELISA. Specific IgE from all fish-allergic patients' sera used in this assay (patients 3, 4, 5 and 6) showed a positive reaction, but the binding capacity of this recombinant allergen was less than that of the natural allergen (Fig. 5).

Ratio of 14.1 : 24.1-type clones in a muscle cDNA library

The cDNA library from which cDNA clones 14.1 and 24.1 were

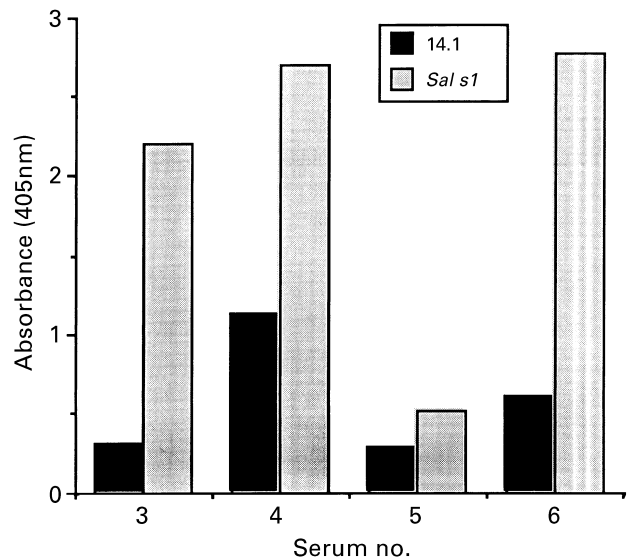


Fig. 5. Measurement of His₁₀-14.1 and *Sal s1*-specific immunoglobulin E (IgE) by enzyme-linked immunosorbent assay (ELISA) using sera from four patients with allergies to fish. The reactivities of the His₁₀-14.1 product were weaker than those of the native *Sal s1*. The absorbances (at 405 nm) of the specific IgE of these sera against the recombinant and native allergen were as follows: patient 3: 0.305 (recombinant) and 2.208 (native); patient 4: 1.116 (recombinant) and 2.695 (native); patient 5: 0.281 (recombinant) and 0.516 (native); and patient 6: 0.604 (recombinant) and 2.770 (native).

isolated contained $\approx 30\,000$ clones, and 48 of these gave a positive signal in the primary screening [11]. In the present study, 40 of these clones were defined by a PCR approach as being of the 14.1 type, while only two were defined as the 24.1 type. The remaining clones might have been false positive, other parvalbumin subvariants, cDNAs to pretranscripts, or partial cDNA fragments ligated to other cDNAs in the library. The latter clones will be analysed further in a follow-up study. In the course of the present study, three discrepancies in cDNA 24.1 (Acc. no. x97825) were revealed and reported to the GenBank sequence database.

DISCUSSION

In the present study, we have produced recombinant products of two distinct parvalbumin-encoding genes in salmon (type 14.1 and type 24.1, respectively). The native parvalbumin was previously shown to be the major allergen in Atlantic salmon [11]. Comparison of either specific human IgE or rabbit IgG binding reactivities of native and recombinant parvalbumin by immunoblots showed no significant differences in the band intensities, whereas the IgE-binding capacity of the recombinant products, demonstrated by ELISA, was less than that of their natural counterparts. This difference in binding affinity is probably attributed to differences in the folding and glycosylation of the recombinant versus the native allergen [10].

Parvalbumins have previously been identified as the major and

cross-reactive allergens in various fish species [7, 24–26]. Accordingly, the present IgE-immunoblotting experiment revealed cross-reaction between salmon and cod. All the patients in this study who had IgE against native and recombinant salmon parvalbumin, also had IgE against cod. This supports the finding that cod and salmon parvalbumin have some homologous allergenic epitopes. Reactivity in Western blots between patients' sera IgE and the present recombinant products is probably attributed to linear epitopes, as previously found with cod Allergen M [26, 27].

We used the pET-19b expression system to express the recombinant proteins. This system was chosen primarily because it allows N-terminal fusion to a cleavable His tag for rapid affinity purification of the target protein and because the pET system usually generates large amounts of target protein. We found that the His₁₀-14.1 product showed a higher level of expression ($\approx$ eightfold) than the His₁₀-24.1 product. Following purification, the target proteins were contaminated by bacterial proteins. It was possible to obtain a purer preparation of recombinant protein by optimizing the relationship between the bacterial culture volume and the volume of resin in the purification process, but some bacterial proteins were still evident. During expression of the recombinant proteins, the temperature used was 30°C instead of 37°C. This choice was made owing to the frequent formation of inclusion bodies at 37°C. Previous studies have linked inclusion body formation to temperature-sensitive denaturation and indicates that lowering the expression temperature from 37°C to 30°C reduces the number of inclusion bodies [28].

The purified target proteins were digested with enterokinase, revealing that both recombinant proteins are similar to the native allergen in terms of M_r . The results showed that it is possible to remove the His tag from both proteins by enterokinase digestion, but the digested proteins appear to be susceptible to further degradation.

In conclusion, the expressed proteins were recognized by rabbit antisera, as well as by specific IgE from persons allergic to fish. These results show that this expression system is promising for the production of large amounts of pure allergen. Clinical studies using skin prick test and histamine release are currently in progress on larger numbers of individuals with fish allergies, and the results of these will be reported separately.

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