



The major allergen (parvalbumin) of codfish is encoded by at least two isotypic genes: cDNA cloning, expression and antibody binding of the recombinant allergens[☆]

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Abstract

The major allergen (parvalbumin) from cod, designated Allergen M *Gad c 1*, has been intensively studied both from the structural and immunological sides. In the present study, transcripts of two isotypic parvalbumin genes in Atlantic cod were identified and characterized. Subsequently, subfragments were inserted into the expression vector pET-19b, generating plasmids with coding capacity for complete parvalbumin polypeptides fused to an N-terminal his₁₀ tag. Most of the recombinant products were found in the soluble fraction of the expression host *Escherichia coli*. The target proteins showed to react with polyclonal antibodies raised against Allergen M and demonstrated binding to specific IgE from 12 sera of patients allergic to cod in ELISA inhibition experiments. Sera with classes 4 and 5 CAP FEIA exhibited also strong binding to recombinant parvalbumins in immunoblots.

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1. Introduction

Despite the prophylactic health benefits related to fish diet, the ingestion and inhalation of fish allergens may cause serious symptoms for fish hypersensitive individuals. Fish proteins are among the most common causes of food allergy (Sampson and Metcalfe, 1992). For a large number of workers in the seafood industry, fish hypersensitivity is a serious problem (Jeebhay et al., 2000). In a Norwegian population, the estimated frequency of fish allergic individuals was found to be approximately one per 1000 (Aas, 1987).

Codfish is traditionally an important species in the fish industry of coast countries around the Atlantic ocean, the North sea and the Baltic sea (Kurlansky, 1997). The allergens of codfish have been extensively studied (Elsayed, 2002). The major allergy-eliciting protein in cod is parvalbumin, designated Allergen M *Gad c1*, 12 kDa, pI 4.75 (Elsayed, 2002; Elsayed and Bennich, 1975; Elsayed and

Apold, 1983). Parvalbumins are Ca²⁺ binding proteins of low molecular weight. They were detected in relatively high amounts in white muscle of lower vertebrates (up to 5 mg/g wet weight in white muscle of many fish species (Gerday, 1982)) and in lesser amounts in the fast twitch skeletal muscles of higher vertebrates (Lehky et al., 1974). Parvalbumin is assumed to be important in the relaxation of fibre in muscles by soaking up free calcium in the cell (Gillis et al., 1982; Pauls et al., 1996). Furthermore, parvalbumin is hypothesized to protect non-muscle cells, particularly neurons, from calcium induced damage (Heizmann and Braun, 1992). Two distinct phylogenetic lineages of parvalbumins, named α and β , are recognized in lower and higher vertebrates. They can be distinguished by sequence characteristics. Most parvalbumin sequences described from fish belong to the β type (Lindstrøm et al., 1996).

Many cDNAs encoding allergens have been cloned and the recombinant counterparts have become available. Future research to generate recombinant hypoallergenic mutants may provide new information on allergenicity and insight into the generic structure of allergenic epitopes (Lorenz et al., 2001).

The aim of the present work was to characterize parvalbumin mRNA sequences of Atlantic cod, produce recombinant parvalbumin in *E. coli*, study the reactivity with polyclonal

[☆] The parvalbumin cDNA sequences T1 and T2 of cod have been submitted to the GenBank Sequence Database and have been assigned the accession numbers AY035584 and AY035585, respectively.

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antibodies raised against Allergen M and specific IgE from fish-allergic persons.

2. Materials and methods

2.1. Patients' sera

Twelve sera from patients with positive clinical histories of type I fish allergy and high specific IgE binding (CAP FEIA system classes 3–5; Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden) were selected from patients referred to the Central Laboratory, Haukeland University Hospital, Bergen. Blood samples were donated after informed consent and pooled in small aliquots and stored at -80°C until used. Three negative control sera with CAP FEIA class 0 to fish were included.

2.2. Native parvalbumin of Atlantic cod

Codfish (*Gadus morhua*) was purchased live at the fish market in Bergen. Parvalbumin was purified from muscle tissue extract as described previously (Elsayed and Aas, 1971).

2.3. Polyclonal antibodies against Allergen M

Rabbit antisera against parvalbumin of Atlantic cod were prepared using Allergen M as antigen as described elsewhere (Elsayed and Apold, 1983).

2.4. Characterization of parvalbumin mRNA sequences in Atlantic cod

Total RNA was isolated from white muscle, utilizing Trizol (Gibco BRL, MD, USA). First strand cDNA was synthesized from an oligo-dT₁₈ primer with an *EcoRI* anchor (dT₁₈-*EcoRI*) on 2 μg muscle RNA, using Superscript reverse transcriptase (Gibco BRL). A 143 bp parvalbumin cDNA fragment was amplified by PCR and sequenced by use of the degenerated primers "Sense-PV" (5'-AGYGGCTT-YATHGARGARGAYGARYT-3') and "Antisense-PV2" (5'-GAATTCRTCRACHCCDATYTTTHCC-3'), designed for homology cloning in a previous study (Lindström et al., 1996). "Sense-PV" was also used for PCR in combination with the dT₁₈-*EcoRI* primer, revealing the 3' part of a parvalbumin mRNA. Following PCR profile was used: 94°C , 1 min; 55°C , 1 min; 72°C , 1 min; 35 cycles, linked to 72°C , 10 min. Enzyme and buffer for PCR were purchased from Pharmacia Biotech AB (Uppsala, Sweden). Some sequence discrepancies between the two parvalbumin cDNAs that had been amplified indicated that two parvalbumin transcripts were present. The remaining part of the type 1 sequence was obtained in 5' RACE by use of the gene specific antisense primer T1.*BamHI*; 5'-GGATCCAGCACATCCTCAGTACTCAG-3'. The type 2 mRNA sequence was assembled in 5' and 3' RACE,

respectively, with the two gene specific primers T2.anti; 5'-CCTTGGTCTCAGCGTCACTC-3' and *NdeI*.T2; 5'-CATATGGCATTGCTGGAATTCTCG-3'. Rapid Amplification of CdnA Ends (RACE) were performed by use of reaction kits from Gibco BRL, and their standard recommendations regarding RNA concentration, PCR profiles, etc. were followed. The ABI Prism BigDye™ Terminator Cycle Sequencing Kit was used for DNA-sequencing reactions (Perkin-Elmer, 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 (www.expasy.ch).

2.5. Cloning of parvalbumin cDNA subfragments into pET-19b

PCR-amplified subfragments of cod parvalbumin cDNAs, digested with *NdeI* and *BamHI*, were subcloned into the expression vector pET-19b (Novagen, Madison, WI, USA). Following primers were used for PCR-amplification: *NdeI*.T2 (sequence shown above), T1.*BamHI* (sequence shown above), *NdeI*.T1; 5'-CATATGGCATTGCTGGAATTCTCG-3', and T2.*BamHI*; 5'-GGATCCGCATCTGGTCTAGTTGGTGG-3'. The PCR was conducted for 30 cycles using the following profile: 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 plasmid insert and regions flanking the insert were controlled by DNA sequencing before the plasmid was used for expression.

2.6. Expression and analysis of recombinant cod parvalbumin

Competent *E. coli* strain BL21 Star™ (DE3) pLysS cells were transformed chemically with the resulting recombinant plasmids. Bacterial growth, preparation of cell lysates and purification of recombinant parvalbumin were performed as described by the manufacturer (Invitrogen BV, Groningen, The Netherlands). The homogeneity of the protein was demonstrated using high performance liquid chromatography (HPLC). HPLC was performed using 130 A Separation System and Brownlee™ C18, including Aquapore RP-300, 7 micron, 30 mm $\times$ 2.1 mm cartridge (Applied Biosystems Inc., Foster City, CA, USA). The used solvents were, A: 0.1% trifluoroacetic acid (TFA) and B: 70% acetonitrile CH₃CN in 0.085% TFA. A gradient of 10–95% B was run for 5–35 min with 200 $\mu\text{l}/\text{min}$ flow.

2.7. Enzyme-linked immunosorbent assay (ELISA) inhibition

IgE reactivity to native and recombinant cod allergens was determined by ELISA inhibition assay (Lindström et al., 1996) using sera from 12 codfish allergic patients. Inhibition was performed by incubating 0.5 mg allergen and 100 μl of

two-fold diluted patient serum overnight at 4 °C. Native cod parvalbumin (1 µg per well) was used as coating allergen. Concentration-related ELISA inhibition studies were performed using a serum pool of three patients (specific IgE against cod CAP FEIA classes 4–5) preincubated with serial 10-fold dilutions (0.00005, 0.0005, 0.005, 0.05, 0.5 and 5.0 µg/0.1 ml serum per well) of Allergen M or recombinant cod His₁₀-T1 parvalbumin.

2.8. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Proteins were separated by SDS-PAGE according to the procedure of Laemmli (1970). The samples along with molecular weight standards (Bio-Rad low range SDS-PAGE standards and prestained standards) were resolved in a 15% separating gel at 200 V. Immunoblotting was carried out as described previously (Van Do et al., 1999).

3. Results

3.1. Identification of two distinct parvalbumin transcripts in Atlantic cod

Muscle RNA of cod was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) with primers designed to anneal to fish parvalbumin mRNA in general (Lindström et al., 1996). Two distinct parvalbumin transcripts were identified and designated T1 and T2. The complete coding region of these, plus some flanking sequences, were obtained by RACE. The nucleotide and translated amino acid sequences of the cDNAs are shown in Fig. 1.

3.2. Analysis of the deduced parvalbumin sequences

Both parvalbumin mRNA sequences which have been characterized in the present study encode 108 amino acid long polypeptides (plus the Met start codon), and show unambiguously strongest relationship to the β lineage of parvalbumin sequences, as illustrated in Fig. 2. The amino acid sequence of T1 had identity indices with β-type parvalbumin sequences from teleost fish spanning from 73.1% (Northern pike) to 92.6% (whiting). For T2, the identity indices were 71.3% (whiting) to 86.0% (European hake). The identity indices with α-type parvalbumins ranged from 53.9% (human) to 59.8% (Northern pike). The identity with mammalian oncomodulin was from 46.1% (human) to 48.0% (mouse).

3.3. Construction of parvalbumin expression plasmids

Expression plasmids were constructed on the basis of the vector pET-19b. Restriction sites were introduced at both ends of the cDNA subfragments by PCR, generating plasmid constructs with coding capacity for complete parvalbumin polypeptides, plus a short N-terminal peptide of 22 amino

acids (MGHHHHHHHHHSSGHIDDDDKH). The His₁₀ tag facilitates the purification of the target protein and an adjacent cleavage site for enterokinase (underlined) allows removal of the tag if conditions are appropriate. The translated parvalbumin sequences of the three expression clones are shown in Fig. 3, one recombinant identical to the translated T1 sequence (His₁₀-T1), one almost identical to the translated T2 sequence (His₁₀-c2), and a third recombinant comprising one part of each isotype (His₁₀-c13). The hybrid clones were constructed by PCR, utilizing one sense primer designed to anneal to the T1 isotype in combination with an antisense primer designed to anneal to the T2 isotype.

3.4. Production of recombinant parvalbumin

Recombinant parvalbumins were expressed in transformed *E. coli*, and the target proteins were found in both soluble and insoluble fractions of the cultures. The soluble fractions were used for purification of recombinant protein. The homogeneity of His₁₀-T1 was demonstrated by HPLC (Fig. 4). One symmetrical peak was obtained indicating satisfactory degree of homogeneity. The HPLC peak with retention time (*R*_t) 25.05 min was used in further experiments. The yields of the recombinant proteins His₁₀-T1, His₁₀-c2 and His₁₀-c13 were 1.8, 1.6 and 1.7 mg/200 ml of bacterial cultures, respectively.

3.5. Specific antibody binding

The purified recombinant products were tested in immunoblots with rabbit antisera raised against native cod parvalbumin, revealing strong reaction, comparable with native protein (Fig. 5).

Analysis of IgE binding to native and recombinant cod parvalbumins showed that the concentrations of Allergen M and His₁₀-T1 required for 50% inhibition were 0.0037 µg/0.1 ml serum and 0.0388 µg/0.1 ml, respectively (Fig. 6). The IgE binding of all 12 sera to native cod parvalbumin was inhibited by at least one of the recombinant proteins (Fig. 7). The ranges of inhibition were as follows: from 20 to 92% for His₁₀-T1, from 0 to 92% for His₁₀-c2, from 0 to 79% for His₁₀-c13 and from 35 to 99% for Allergen M. The mean values of inhibition by His₁₀-T1, His₁₀-c2, His₁₀-c13 and native cod parvalbumin were 56, 41, 46 and 79%, respectively.

IgE-binding capacity of the recombinant cod allergens was further assessed by immunoblots with cod-allergic patients' sera. Initially, a pool of five sera was tested for IgE-binding to recombinant cod parvalbumin. The serum pool displayed strong IgE-binding to all three recombinant cod parvalbumins, as well as to Allergen M. Subsequently, sera from each of the five patients were tested (Fig. 8). The recombinant proteins had the ability to bind IgE of all tested sera. The results from the immunoblotting were in concordance with the CAP FEIA values. Patients' sera LI and PY with CAP FEIA class 5 against cod exhibited highest IgE

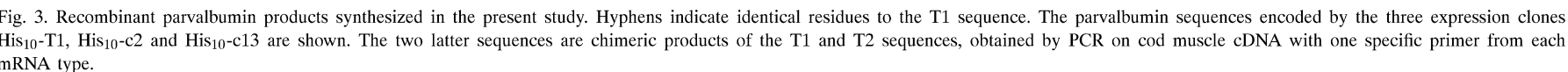
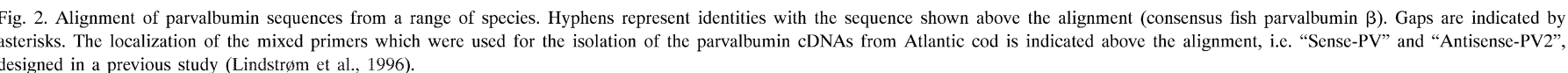
T1

ACCCGGCTTTCAATTGGAGATAGCAGCAGCACCCAAGAACTCTCTAAAA														49	
M	A	F	A	G	I	L	A	D	A	D	C	A	A	A	14
ATG	GCA	TTC	GCT	GGA	ATT	CTC	GCC	GAC	GCT	GAC	TGC	GCC	GCA	GCC	94
V	K	A	C	E	A	A	E	S	F	S	Y	K	A	F	29
GTC	AAG	GCT	TGC	GAA	GCT	GCT	GAG	TCC	TTC	AGC	TAC	AAG	GCC	TTC	139
F	A	K	C	G	L	S	G	K	S	A	D	D	I	K	44
TTC	GCC	AAG	TGC	GGC	CTG	TCC	GGC	AAG	TCC	GCT	GAC	GAC	ATC	AAG	184
K	A	F	F	V	I	D	Q	D	K	S	G	F	I	E	59
AAG	GCT	TTC	TTC	GTG	ATT	GAC	CAG	GAC	AAG	AGT	GGA	TTC	ATT	GAG	229
E	D	E	L	K	L	F	L	Q	V	F	K	A	G	A	74
GAG	GAT	GAG	CTC	AAG	CTC	TTC	CTC	CAG	GTC	TTC	AAG	GCT	GGA	GCC	274
R	A	L	T	D	A	E	T	K	A	F	L	K	A	G	89
AGA	GCT	CTT	ACA	GAT	GCT	GAG	ACC	AAG	GCC	TTC	CTG	AAA	GCC	GGT	319
D	S	D	G	D	G	A	I	G	V	D	E	W	A	V	104
GAC	AGC	GAC	GGT	GAT	GGT	GCT	ATT	GGA	GTT	GAT	GAG	TGG	GCC	GTT	364
L	V	K	A	*											108
CTT	GTG	AAG	GCA	TAA	TCTGATTCTGTGGAACCACAGTTTCTGAGTACTGAGGAT										418
GTGCTGTCTGAGTCAAATAGTATATCTTTTATATGCATTTTATTTATAAAGTAACAGG															477
CAATATTGTAACCTCTATCCGAGAGCATTTCCTGTACACAATTTTTTTGTCTTTAGTG															536
CAATCATCGCATACCGAAGAGGAGAAAAATACACCACTGCTCATTTGTACTTTTGTAAAA															595
TGAAATAAACTCTGATTTGGTTTTAGAAAAAAAAAAAAAAAAAAAAA															639

T2

AAGAATCATCTCTTAGCGTTTTCTACCTCTATCCTTTAAGAGACAAACTCAGAGATAAA														59	
M	A	F	A	G	I	L	N	D	A	D	I	T	A	A	14
ATG	GCT	TTC	GCC	GGA	ATT	CTG	AAC	GAC	GCT	GAC	ATC	ACT	GCA	GCC	104
L	A	A	C	K	A	E	G	S	F	D	H	K	A	F	29
CTG	GCG	GCC	TGC	AAA	GCT	GAG	GGC	TCC	TTC	GAC	CAC	AAG	GCG	TTC	149
F	T	K	V	G	L	A	A	K	S	P	A	D	I	K	45
TTC	ACT	AAA	GTC	GGC	CTG	GCC	GCC	AAG	TCC	CCC	GCT	GAC	ATC	AAG	194
K	V	F	E	I	I	D	Q	D	K	S	D	F	V	E	59
AAG	GTG	TTT	GAG	ATC	ATT	GAC	CAG	GAC	AAG	AGT	GAC	TTT	GTT	GAG	239
E	D	E	L	K	L	F	L	Q	N	F	S	A	G	A	74
GAG	GAT	GAG	CTG	AAG	CTG	TTC	CTC	CAG	AAC	TTC	AGC	GCC	GGT	GCC	284
R	A	L	S	D	A	E	T	K	V	F	L	K	A	G	89
AGA	GCT	CTG	AGT	GAC	GCT	GAG	ACC	AAG	GTT	TTC	CTG	AAG	GCC	GGA	329
D	S	D	G	D	G	K	I	G	V	D	E	F	G	A	104
GAC	TCT	GAC	GGT	GAC	GGC	AAG	ATC	GGA	GTT	GAT	GAG	TTC	GGC	GCC	374
M	I	K	A	*											108
ATG	ATC	AAG	GCA	TAG	ACTGTTCTCCACCAACTAGACCAGATGCTTACCCACCT										428
ACTCAGAAGGAACACTGGAGCTGAGACCACCTCCTCACCCCTCTCTCGCTCATCTCTCAA															487
CAACAATTTTATATAAATAACCTTTAACCTAACCCCCCCC															527

Fig. 1. Parvalbumin cDNAs of Atlantic cod. The cDNAs T1 and T2 are shown with the deduced amino acid sequence above the nucleotide sequence. Both cDNAs comprises the entire parvalbumin-coding region. The stop codon is indicated by an asterisk and the polyadenylation signal in T1 is underlined. Reliable sequence of the 3' part of the untranslated tail in T2 was not obtained due to a long stretch of C-residues (sequence was not obtained from the other side because of the poly-A-tail).



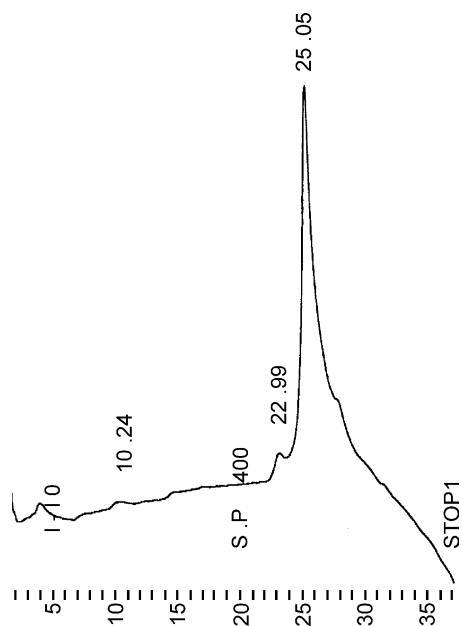


Fig. 4. Analysis of the homogeneity of His₁₀-T1. The homogeneity was demonstrated by use of RP/HPLC. A symmetrical peak with R_t 25.05 min was revealed using a gradient of 10–95% B running for 5–35 min with 200 μ l/min flow.

reactivities for the recombinant cod allergens. Immunoblot using non-allergic serum revealed no IgE binding to any of the recombinant proteins.

4. Discussion

In the present study, recombinant cod parvalbumins are documented to bind specific antibodies against Allergen M and specific IgE from fish allergic persons. The IgE binding capacities of the recombinant proteins were lower than that of native cod parvalbumin, but our results revealed no significant differences between Allergen M and His₁₀-T1.

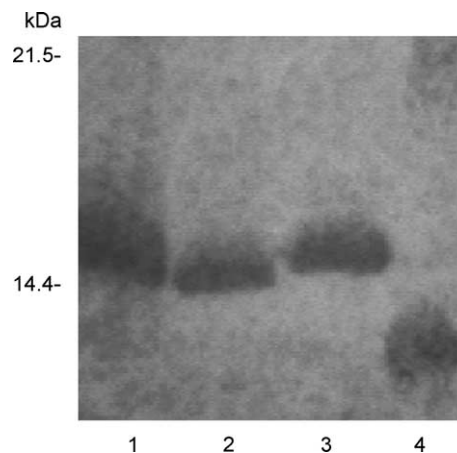


Fig. 5. IgG immunoblots. Native and recombinant cod parvalbumins were recognized by rabbit anti native cod parvalbumin sera. Lane 1: His₁₀-T1. Lane 2: His₁₀-c2. Lane 3: His₁₀-c13. Lane 4: native cod parvalbumin.

Similar findings have been published for other recombinant allergens (Ferreira et al., 1993; Slunt et al., 1995). The band intensity on the immunoblots correlated with the respective CAP FEIA values measured previously. A pool of two rabbit anti-Allergen M sera displayed a similar IgG binding capacity to the recombinant allergens as well as to the native cod parvalbumin. The retention of the allergenicity of the recombinant codfish allergens further indicates that the biological activity is sequentially determined (Elsayed and Bennich, 1975).

The present characterization of two distinct parvalbumin cDNAs from Atlantic cod suggests that isotypic variants are generally present in fish. The T1 isotype of cod was highly similar to the parvalbumin β sequence from whiting (92.6%), while the cod T2 isotype was considerably less similar to the same sequence (71.3%). Thus, it is plausible to assume that further characterization of isotypes from fish will reveal hitherto unknown patterns of parvalbumin phylogeny. The presence of an α -type parvalbumin in Northern

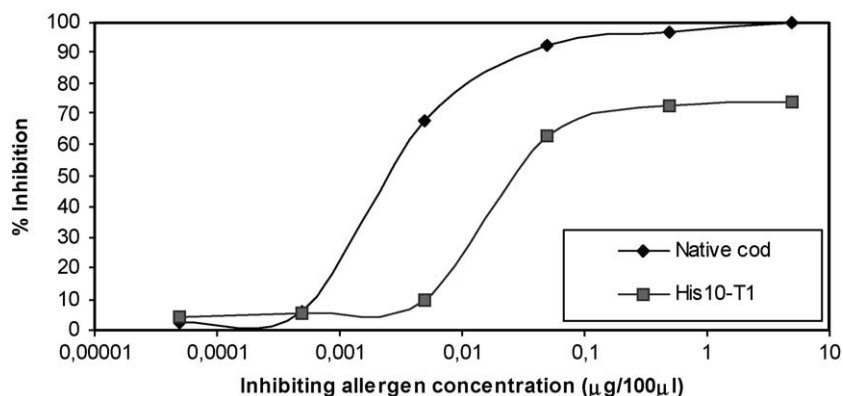


Fig. 6. Titration of IgE inhibition. IgE-binding to Allergen M was inhibited by native cod and recombinant cod His₁₀-T1 parvalbumins. Allergen M (1 μ g/0.1 ml) was used as coating allergen. Inhibitions were determined with *Gad c 1*-specific serum pool preincubated with different concentrations of native or recombinant cod parvalbumins.

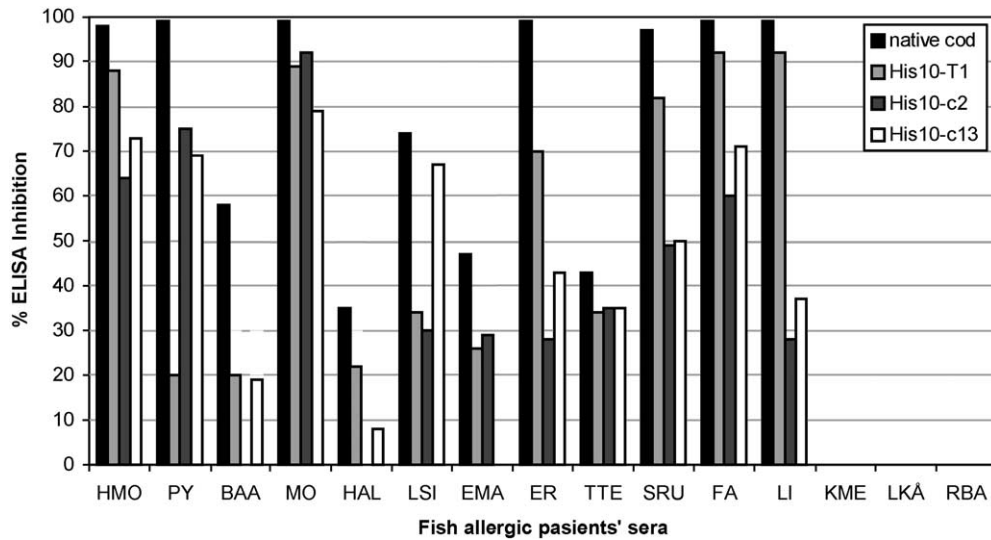


Fig. 7. IgE inhibition. Inhibitions of IgE reactivities of 12 codfish allergic sera against Allergen M by native and recombinant cod parvalbumins (His₁₀-T1, His₁₀-c2 and His₁₀-c13) were determined by ELISA. Three normal sera (KME, LKÅ and RBA) were used as negative controls.

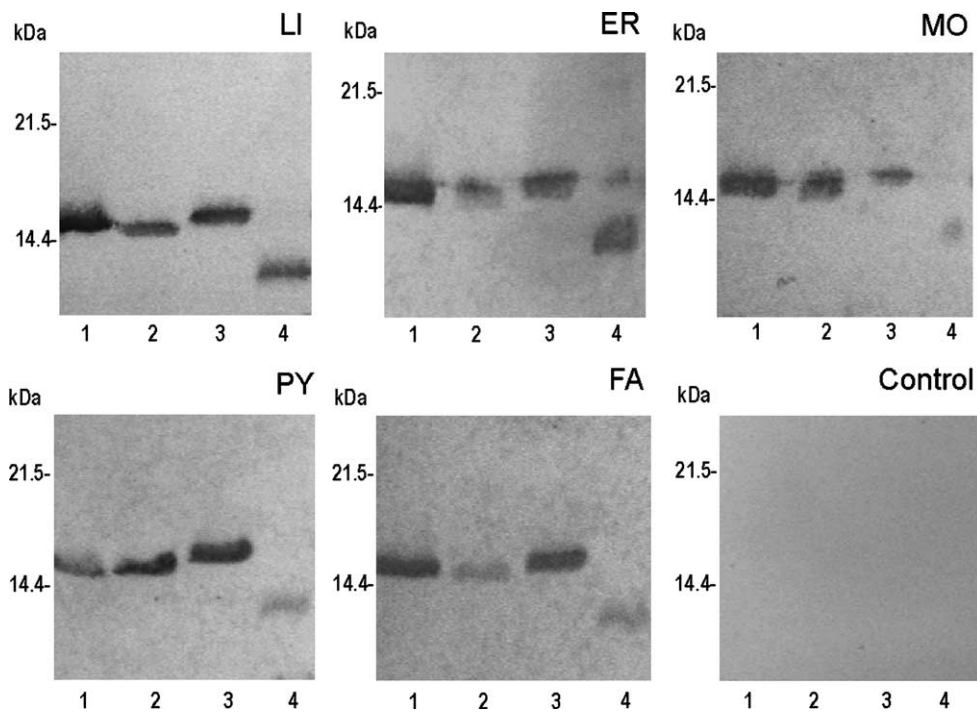


Fig. 8. IgE immunoblots. IgE-reactivities of sera from 5 cod allergic patients (initials: LI, ER, MO, PY and FA) against native and recombinant cod parvalbumins were detected by immunoblotting. A normal serum was included as control. Lane 1: His₁₀-T1. Lane 2: His₁₀-c2. Lane 3: His₁₀-c13. Lane 4: native cod parvalbumin.

pike indicate that there can be counterparts of this type in other teleost fish species as well. The relatively low similarity between the present parvalbumin sequences from Atlantic cod and the sequence of Baltic cod (peptide-based sequence; [Elsayed and Bennich, 1975](#)) indicate that there are additional parvalbumin genes in cod that have not been identified in the present study.

In order to produce recombinant cod parvalbumin, the pET-19b expression system was chosen, primarily since this vector most often generates large amounts of target protein, and since the recombinant product can easily be purified by affinity chromatography on a Ni²⁺ column. The three parvalbumin expression clones in the present study gave good yield and generated almost similar amounts of protein. From

earlier experience (Van Do et al., 1999), it was possible to remove the His₁₀ tag from the recombinant parvalbumins with enterokinase, but the digestion conditions have to be optimized to obtain complete removal of the tag. Interestingly, His₁₀-c2 and His₁₀-c13 without tag corresponded to the lower and upper parts of the native parvalbumin separated by SDS–PAGE (figure not shown). Further experimental analyses of parvalbumin isotype diversity in cod (e.g. Southern blot analysis) and comparative analyses of emerging sequence information on model fish species (e.g. zebrafish) will clarify the relationship between Allergen M and the parvalbumin transcripts present in codfish.

The availability of recombinant cod allergens will make relatively easy to obtain larger amounts of homogeneous allergen for use in diagnosis, research and therapy.

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