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Purification, immunological properties and molecular cloning of two allergenic parvalbumins from the crimson sea bream, *Evynnis japonica*

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ABSTRACT

Parvalbumin, a calcium-binding sarcoplasmic protein of 12 kDa, represents a major allergen of fish. Previous immunoblotting experiments suggested the presence of a 14 kDa allergen, together with parvalbumin, in sea breams. In this study, the 14 kDa allergen (PA I) and parvalbumin (PA II) were purified from the white muscle of crimson sea bream, *Evynnis japonica*, by gel filtration and reverse-phase HPLC. Amino acid sequencing of lysylendopeptidase peptide fragments demonstrated that both PA I and PA II were isoforms of parvalbumin. As analysed by ELISA, PA I showed weaker reactivities with IgG (monoclonal or polyclonal antibody) and serum IgE in fish-allergic patients than did other fish parvalbumins, including PA II. The amino acid sequences of PA I and PA II were elucidated by cDNA cloning. PA I was found to have more specific amino acid residues at several positions compared to the other fish parvalbumins.

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1. Introduction

Food allergy is a representative of type I allergies, mediated by immunoglobulin E (IgE) antibodies, and is a public concern in industrialised countries since even death may occur in severe cases. Among a number of allergenic foodstuffs, fish is recognised as an important cause of food allergy, especially in coastal countries, including Japan, where their consumption is high. The major fish allergen is parvalbumin, a calcium-binding sarcoplasmic protein with a molecular mass of 12 kDa, as demonstrated with various species of fish, such as carp (Bugajska-Schretter et al., 1999; Swoboda et al., 2002), salmon (Lindström, van Dô, Hordvik, Endresen, & Elsayed, 1996), cod (Elsayed & Aas, 1971a, 1971b; Elsayed & Bennich, 1975), horse mackerel (Shiomi, Hayashi, Ishikawa, Shimakura, & Nagashima, 1998), mackerel (Hamada et al., 2003) and tuna (Shiomi, Hamada, Sekiguchi, Shimakura, & Nagashima, 1999). Besides parvalbumin, three classes of proteins, namely collagen comprised of three 100 kDa α chains (Hamada, Nagashima, & Shiomi, 2001; Sakaguchi et al., 2000), aldehyde phosphate dehydrogenase of 41 kDa (Das Dores et al., 2002) and transferrin of 94 kDa (Kondo et al., 2006), have so far been identified as fish allergens, although not major ones.

It should be noted that unidentified allergens, such as a 25 kDa allergen in swordfish (Kelso, Jones, & Yunginger, 1996) and a 63 kDa allergen in codfish (Mata et al., 1994), still exist in some species of fish. Our previous immunoblotting experiments have also revealed that the red sea bream, *Pagrus major*, contains a

14 kDa allergen, together with parvalbumin (Kobayashi et al., 2006). The 14 kDa allergen was considered to be distinguishable from parvalbumin, since it did not react with the monoclonal anti-frog muscle parvalbumin antibody. Interestingly, the 14 kDa allergen (having no reactivity with the monoclonal anti-parvalbumin antibody) was also found in the crimson sea bream, *Evynnis japonica*, in our preliminary experiments, suggesting that the 14 kDa allergen seems to be commonly contained in sea breams. For a better understanding of the sea bream allergy, this study was initiated to identify the 14 kDa allergen in the crimson sea bream. As a result, the 14 kDa allergen (named PA I) was demonstrated to be a parvalbumin isoform showing less IgG and IgE reactivities than the other 12 kDa parvalbumin (named PA II) of crimson sea bream and parvalbumins of horse mackerel, *Trachurus japonicus*, and Pacific mackerel, *Scomber japonicus*. We here report the purification, immunological properties and molecular cloning of two allergenic parvalbumin isoforms, PA I and II, from the crimson sea bream.

2. Materials and methods

2.1. Fish samples

Live specimens of crimson sea bream were purchased at the Tokyo Central Wholesale Market and fresh specimens of horse mackerel and Pacific mackerel at a local retail shop. White muscle samples collected from these fish were stored at -20°C until used. For molecular cloning experiments, the white muscle obtained from a live specimen of crimson sea bream was immediately frozen in liquid nitrogen and stored at -80°C until used.

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2.2. Preparation of heated extract

Each muscle sample was homogenised with three volumes of 0.15 M NaCl–0.01 M phosphate buffer (pH 7.0). The homogenate was heated in a boiling water bath for 10 min, cooled to room temperature and centrifuged at 18,800g for 20 min. The supernatant obtained was regarded as a heated extract.

2.3. Purification procedure

Parvalbumins of horse mackerel and Pacific mackerel were individually purified from the heated extract by a combination of gel filtration and reverse-phase HPLC, as reported previously (Hamada et al., 2003; Shiomi et al., 1998). PA I and PA II of crimson sea bream were similarly purified as now described. The heated extract was subjected to gel filtration on a Sephadex G-75 column (2.5 × 100 cm; GE-Healthcare, Buckinghamshire, UK), which was eluted with 0.15 M NaCl–0.01 M phosphate buffer (pH 7.0) at a flow rate of about 35 ml h⁻¹. Fractions of 10 ml were collected, measured for absorbance at 280 nm and subjected to SDS–PAGE to identify the presence of PA I and/or PA II. The fractions containing both PA I and PA II were combined and applied to reverse-phase HPLC on a TSKgel ODS-120T column (0.46 × 25 cm; Tosoh, Tokyo, Japan). The column was eluted at a flow rate of 1 ml min⁻¹ with a linear gradient of acetonitrile, 0–70% (v/v) in 60 min, in 0.1% (v/v) trifluoroacetic acid. Proteins were monitored at 220 nm with a UV detector and the eluate corresponding to each peak was manually collected and analysed by SDS–PAGE.

2.4. SDS–PAGE

SDS–PAGE was performed on a PhastSystem apparatus (GE-Healthcare), using ready-made gels (PhastGel Gradient 8–25; GE-Healthcare) and ready-made buffer strips (PhastGel SDS Buffer Strips; GE-Healthcare), according to the manufacturer's instructions. Each sample was dissolved in 0.0625 M Tris–HCl buffer (pH 7.4) containing 2% (w/v) SDS, 4 M urea and 0.1 M dithiothreitol, heated at 100 °C for 10 min and subjected to electrophoresis. Precision Plus Protein Standards (10–250 kDa; Bio-Rad Laboratories, Hercules, CA, USA) were run as references, along with samples. After running, the gel was stained with Coomassie Brilliant Blue R-250.

2.5. Protein determination

Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as a standard protein.

2.6. Human sera

Sera were obtained from 12 fish-allergic patients. Written informed consent was obtained from each patient. Also, this study (using the sera) was approved by the ethics committees of Chiba University, Fujita Health University and Yokohama City University. All patients had been diagnosed to be allergic to fish at hospitals, based on the documented clinical histories of immediate hypersensitive reactions after ingestion of fish and capsulated hydrophilic carrier polymer-radioallergosorbent test (CAP-RAST) classes of 2–4 against some fish, such as salmon, mackerel and tuna. Furthermore, the patient sera were all verified to have specific IgE to fish parvalbumin by ELISA, in which both parvalbumin and collagen (purified from bigeye tuna, *Thunnus obesus* muscle) were used as antigens, as described in our previous paper (Hamada et al., 2001). In this study, a pooled serum from 10 healthy volunteers,

without adverse reactions after ingestion of any foods, was used as a control.

2.7. ELISA

IgG reactivity of parvalbumins was examined by visible-light ELISA, according to the method of Ishikawa, Shimakura, Nagashima, and Shiomi (1997). In brief, a flat-bottomed polystyrene microtitre plate, with 96 wells (Type H; Sumitomo Bakelite, Tokyo, Japan), was coated with 50 µl of parvalbumin solution (0.001–10 µg ml⁻¹) and reacted successively with the monoclonal anti-frog muscle parvalbumin antibody (diluted 1:5000; Sigma–Aldrich, St. Louis, MO, USA) and peroxidase-conjugated rabbit anti-mouse IgG antibody (diluted 1:10000; Zymed Laboratories, San Francisco, CA, USA) or with the antiserum (diluted 1:5000; a gift from Mr. H. Nakajima, Maruha Co., Tokyo, Japan) raised in rabbits against Pacific mackerel parvalbumin and peroxidase-conjugated goat anti-rabbit IgG antibody (diluted 1:10000; American Qualex, San Clemente, CA, USA). Enzyme reaction was carried out at room temperature for 30 min, using substrate solution, 0.1% (w/v) o-phenylenediamine and 0.03% (v/v) hydrogen peroxide in 0.05 M phosphate–citrate buffer (pH 5.0), and stopped by addition of 1 M sulphuric acid. The developed colour was measured at 490 nm.

IgE reactivity of parvalbumins was examined by fluorescence ELISA, as reported previously (Hamada et al., 2004). Briefly, a microtitre plate (Type H (black); Sumitomo Bakelite) was coated with 50 µl of parvalbumin solution (1 µg ml⁻¹) and reacted successively with patient or control serum (diluted 1:250) and β-galactosidase-conjugated goat anti-human IgE antibody (0.25 µg ml⁻¹; American Qualex, San Clement, CA, USA). Enzyme reaction was carried out at 37 °C for 18 h using substrate solution, 0.1 mg ml⁻¹ 4-methylumbelliferyl-β-D-galactoside-1 mM MgCl₂–0.01 M phosphate buffer (pH 7.0), and stopped by addition of 0.1 M glycine–NaOH buffer (pH 10.3). Fluorescence units were measured with excitation and emission wavelengths at 367 and 453 nm, respectively.

2.8. Enzymatic digestion and isolation and amino acid sequencing of peptide fragments

The purified preparations (50 µg each) of PA I and PA II were individually digested with 1 µg of lysylendopeptidase (E.C. 3.4.21.50; Wako Pure Chemicals, Osaka, Japan) in 1 ml of 0.025 M Tris–HCl buffer (pH 8.5) containing 1 mM EDTA and 2 M urea at 37 °C for 24 h. To isolate peptide fragments produced, the digest was subjected to reverse-phase HPLC on the same TSKgel ODS-120T column as described above. The column was eluted at a flow rate of 1 ml min⁻¹ by a linear gradient of acetonitrile, 0–70% (v/v) in 120 min, in 0.1% (v/v) trifluoroacetic acid. Peptides were monitored at 220 nm with a UV detector. Amino acid sequencing of the isolated peptides was performed on a 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems, Foster City, CA, USA) by the MS/MS positive method. α-Cyano-4-hydroxycinnamic acid was used as a matrix.

2.9. Cloning experiments

Total RNA was extracted from 2 g of the white muscle of crimson sea bream with TRIzol reagent (Life Technologies, Rockville, MD, USA) and poly(A)⁺ mRNA was purified by affinity chromatography on oligo(dT)-cellulose, using a mRNA Purification Kit (GE-Healthcare). A part of the purified mRNA was converted to cDNA, followed by ligation of AP1 adapters, using a Marathon cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA, USA). The Marathon cDNA library thus constructed was used as a

template in 3' and 5' rapid amplification cDNA ends (RACE). In combination with the AP1 adapter primer, a forward primer (PA-F with the sequence of 5'-TTGAGGAGGAGGAGCTGAAGCT-3') and a reverse primer (PA-R with the sequence of 5'-AGCTTCAGCTCCTCCTCTCAA-3'), which encode the amino acid sequence 58-IEEELKL-65 conserved in some fish parvalbumins (refer to Fig. 5, see below), were used in 3'RACE and 5'RACE, respectively. Amplification was performed using Ex Taq DNA polymerase (Takara, Otsu, Japan) under the following conditions: pre-incubation at 95 °C for 2 min; 35 cycles consisting of denaturation at 95 °C for 10 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min; final extension at 72 °C for 7 min. The PCR products were subcloned into the pT7Blue-2 T-vector (Novagen, Darmstadt, Germany) and their nucleotide sequences were determined using a BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems) and a PRISM 310 genetic analyzer (Applied Biosystems). Based on the determined partial nucleotide sequences, 3'RACE, using a forward primer (PA I-F with the sequence of 5'-CCTTTCAAAGGACTGCAGGAT-3' corresponding to 1-PFKGLQD-7) and AP1, was performed for PA I and 5'RACE using AP1 and a reverse primer (PA II-R with the sequence of 5'-TGCTTAACAAGGGCAGCAAA-3' corresponding to 102-FAALVKA-108) for PA II. Amplified products were similarly subcloned and sequenced.

3. Results and discussion

3.1. Purification of PA I and PA II

In order to purify PA I and PA II, the heated extract from the white muscle of crimson sea bream was first chromatographed on a Sephadex G-75 column. As monitored by absorbance at 280 nm, three peaks were observed at fractions 23, 47 and 56 (Fig. 1A). Analysis by SDS-PAGE (data not shown) revealed that PA I and PA II were co-eluted in fractions 37–42. It is not strange that the fractions 37–42 have no substantial absorbance at 280 nm, since PA I is devoid of both Trp and Tyr residues, responsible for the absorbance at 280 nm, and PA II has only one Tyr residue (refer to Fig. 5, see below). When the fractions 37–42 were combined and subjected to reverse-phase HPLC on a TSKgel ODS-120T column, two prominent peaks (I and II) were recognised at retention times of 37.5 and 41.0 min (Fig. 1B). On SDS-PAGE, homogeneous PA I and PA II were found in the peaks I and II, respectively (Fig. 1C).

3.2. Identification of PA I and II as parvalbumin isoforms

Following the digestion of the purified PA I with lysylendopeptidase, peptide fragments were isolated from the digest by reverse-phase HPLC on TSKgel ODS-120T (Fig. 2A). Among them, three peptides (named peptides I-1, I-2 and I-3, in the order of elution) were randomly selected and sequenced by MALDI TOF/MS/MS, as shown in Fig. 2A. A homology search by the BLAST programme (Altschul, Gish, Miller, Myers, & Lipman, 1990) proved that the peptides I-1, I-2 and I-3 correspond to the regions 20–27, 97–107 and 45–54 of fish parvalbumins, respectively (refer to Fig. 5, see below). Thus, PA I was definitely identified as an isoform of parvalbumin. In the case of PA II, three (named peptides II-1, II-2 and II-3, in the order of elution) of the lysylendopeptidase peptide fragments isolated by reverse-phase HPLC, were similarly sequenced, as shown in Fig. 2B. Obviously, the peptides II-1, II-2 and II-3, respectively, correspond to the regions 73–80, 84–96 and 55–64 of fish parvalbumins (refer to Fig. 5), supporting the idea that PA II is an isoform of parvalbumin. It should be noted that the nucleotide sequence and deduced amino acid sequence of crimson sea bream parvalbumin can be seen in the DDBJ/EMBL/GenBank databases under the accession number AB375264. From the determined amino acid sequences of the lysylendopeptidase peptide fragments, however, either PA I or PA II was judged to differ from the crimson sea bream parvalbumin so far deposited in the databases. Therefore, the parvalbumin in the databases is hereinafter called PA III.

3.3. Immunological properties of PA I and PA II

Analysis by visible-light ELISA showed that PA II had almost the same IgG reactivity with the monoclonal anti-frog muscle parvalbumin antibody as had horse mackerel and Pacific mackerel parvalbumins, while PA I was remarkably low in reactivity compared to the other parvalbumins (Fig. 3A). As for the reactivity with the antiserum raised against Pacific mackerel parvalbumin, PA I reactivity was also considerably lower than the other parvalbumins (Fig. 3B). IgE reactivity of PA I and II was assessed by fluorescence ELISA, using 12 patient sera. Although one patient serum (patient 9 serum) reacted equally to both PA I and PA II, the remaining 11 patient sera apparently showed less reactivity to PA I than to PA II; even no substantial reactivity to PA I was observed in patient 6 serum (Fig. 4A). To compare the IgE reactivity of crimson sea bream parvalbumins (PA I and PA II) with that of horse mackerel and Pacific mackerel parvalbumins, a pooled serum from 12 patients was used in ELISA, due to the scarcity of several sera. As

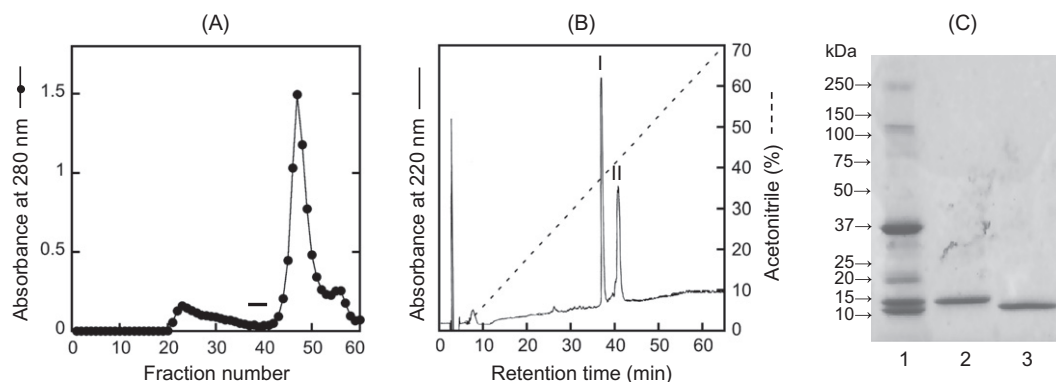


Fig. 1. Purification of PA I (14 kDa allergen) and PA II (12 kDa parvalbumin) from the crimson sea bream. (A) Gel filtration. Sample, heated extract from the white muscle of crimson sea bream; column, Sephadex G-75 column (2.5 × 100 cm); solvent, 0.15 M NaCl–0.01 M phosphate buffer (pH 7.0); volume/fraction, 10 ml. A horizontal bar indicates the fractions (fractions 37–42) containing both PA I and PA II. (B) Reverse-phase HPLC. Sample, fractions 37–42 obtained by gel filtration; column, TSKgel ODS-120T column (0.46 × 25 cm); elution, linear gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid; flow rate, 1 ml min⁻¹. (C) SDS-PAGE. Samples: lane 1, heated extract; lane 2, purified PA I (peak I in B); lane 3, purified PA II (peak II in B).

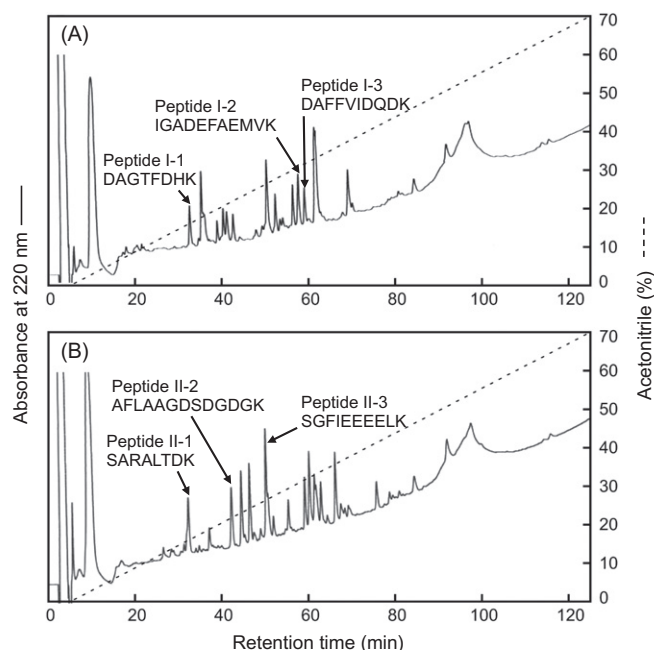


Fig. 2. Isolation of lysylendopeptidase peptide fragments from PA I (A) and PA II (B) by reverse-phase HPLC on TSKgel ODS-120T. The amino acid sequences of three peptides (I-1, I-2 and I-3) from PA I and three peptides (II-1, II-2 and II-3) from PA II, which were elucidated by MALDI TOF/MS/MS, are included in (A) and (B), respectively.

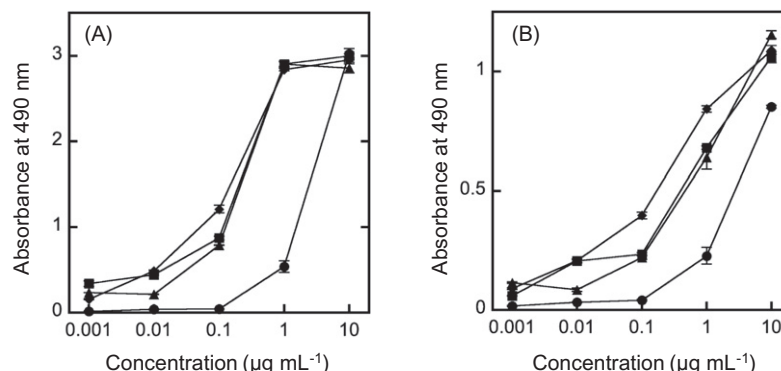


Fig. 3. Analysis of IgG reactivity of PA I (●), PA II (■), horse mackerel parvalbumin (◆) and Pacific mackerel parvalbumin (▲) by visible-light ELISA. Each datum is expressed as mean $\pm$ SD ($n = 3$). (A) Reactivity with the monoclonal anti-frog muscle parvalbumin antibody. (B) Reactivity with the antiserum raised against Pacific mackerel parvalbumin.

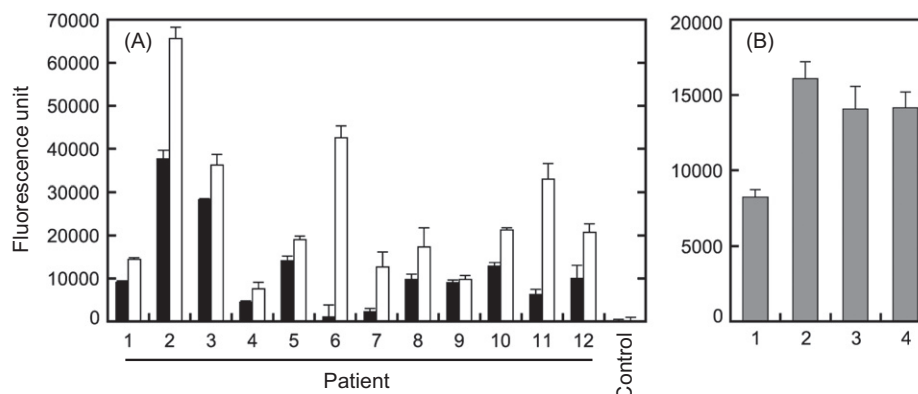


Fig. 4. Analysis of IgE reactivity of PA I, PA II, horse mackerel parvalbumin and Pacific mackerel parvalbumin by fluorescence ELISA. Each datum is expressed as mean $\pm$ SD ($n = 3$). (A) Reactivity of PA I (■) and II (□) with 12 patient sera. (B) Reactivity of PA I (1), PA II (2), horse mackerel parvalbumin (3) and Pacific mackerel parvalbumin (4) with a pooled serum from 12 patients.

a result, PA I was apparently lower in IgE reactivity than were the other parvalbumins (Fig. 4B).

In our previous immunoblotting experiments, PA II reacted with the monoclonal anti-parvalbumin antibody but PA I did not, leading us to assume that PA I differs from parvalbumin (unpublished data). This erroneous assumption was unavoidable, in view of the fact that PA I is low in IgG reactivity, as quantitatively demonstrated by ELISA in this study. Moreover, it should be noted that the 14 kDa allergen in the red sea bream showed no IgG reactivity and was similarly assumed to differ from parvalbumin (Kobayashi et al., 2006). The 14 kDa allergen in the red sea bream is also likely to be a parvalbumin isoform with low IgG and IgE reactivities as in the case of PA I (14 kDa allergen in the crimson sea bream).

3.4. Molecular cloning of PA I and PA II

About 450 bp products were amplified by 3'RACE, using forward (PA-F) and reverse (AP1) primers. After subcloning into the pT7Blue-2 T-vector, eight clones were obtained and analysed for nucleotide sequencing. As a result, five clones were found to code for PA I, two clones for PA II and one clone for PA III. On the other hand, 5'RACE, using forward (AP1) and reverse (PA-R) primers, afforded about 250 bp products. Nucleotide sequencing of five clones obtained by subcloning showed that two clones code for PA I and three clones for PA III. On the basis of the partial nucleotide sequences, the remaining sequences of the cDNAs encoding PA I and II were analysed by 3'RACE, using forward (PA I-F) and reverse (AP1) primers, and 5'RACE, using forward (AP1) and reverse

(PA II-R) primers, respectively. Sequencing data, after subcloning, revealed that all 13 clones coded for PA I in 3'RACE and all six clones for PA II in 5'RACE. Thus, the nucleotide sequences of the full-length cDNAs (705 bp for PA I and 654 bp for PA II) encoding PA I and PA II were determined and deposited into the DDBJ/EMBL/GenBank databases under the accession numbers AB622511 and AB622512, respectively.

3.5. Amino acid sequences of PA I and PA II

An open reading frame composed of 324 bp is contained in the PA I cDNA and that of 327 bp in the PA II cDNA. The amino acid sequences of PA I (107 residues, excepting the initiating Met), and PA II (108 residues, excepting the initiating Met) are shown in Fig. 5, together with those of some fish parvalbumins, including PA III. The peptides I-1, I-2 and I-3 are all recognised in the sequence of PA I and the peptides II-1, II-2 and II-3 in the sequence of PA II, supporting the accuracy of our nucleotide sequencing.

PA I and PA II are highly homologous (72% identity) with each other and also show high identities (52–80%) with parvalbumins from various species of fish; especially high identities (75–100%) are recognised in the two calcium-binding sites (positions 51–62 and 90–101). Based on the chain length and sequence features, parvalbumins are divided into two types (α - and β -types). Both PA I and PA II are obviously β -type parvalbumins, similar to many fish parvalbumins. First, they are shorter in chain length compared to α -type parvalbumins having 109 residues or more (Moncrief, Goodman, & Kretsinger, 1990). Second, they have six residues (Ala-13, Leu-15, Cys-18, Phe-66, Gln-68 and Thr-78) characteristic of β -type parvalbumins (Goodman & Pecheré, 1977), except for the replacement of Ala-13 by Lys in PA I. PA I is a β -type parvalbumin and its molecular mass (11582.0, calculated from the amino acid sequence) is comparable to those of the other fish β -type parvalbu-

mins. It is still unclear why PA I behaves as a larger protein (14 kDa protein) than its molecular mass in SDS–PAGE.

The IgE cross-reactivity among fish parvalbumins has been established (Hamada et al., 2004; Van Dô, Elsayed, Florvaag, Hordvik, & Endresen, 2005) but their common IgE-binding epitopes, responsible for the cross-reactivity, have been little understood. The linear-type IgE epitopes so far proposed for cod parvalbumin (Elsayed & Apold, 1983) and Pacific mackerel parvalbumin (Yoshida, Ichimura, & Shiomi, 2008) are considered to be not always applicable to various fish parvalbumins. Recently, the IgE reactivities of carp parvalbumin (Swoboda et al., 2007) and Pacific mackerel parvalbumin (Tomura, Ishizaki, Nagashima, & Shiomi, 2008) have been reported to be significantly reduced by depletion of Ca^{2+} , indicating the importance of conformational-type IgE epitopes for the cross-reactivity among fish parvalbumins. Although conformational IgE epitopes of the carp parvalbumin have been suggested, based on the elucidation of mimotopes (Untersmayr et al., 2006), they have not been experimentally confirmed. In the light of current knowledge, therefore, it is difficult to explain why the IgE reactivity of PA I is considerably lower than that of other fish parvalbumins. Nevertheless, it is worth mentioning that several amino acid residues, such as Lys-13, Ala-41 and Asp-45, are specifically included only in PA I, being responsible for the low IgE reactivity.

In conclusion, this study showed that the 14 kDa allergen (PA I), detected in the crimson sea bream, behaves as a larger protein in SDS–PAGE than usual parvalbumins of 12 kDa but is none other than parvalbumin. PA I is low in IgE reactivity and hence will be a useful model molecule in future study on the conformational IgE epitopes of fish parvalbumins. Such a 14 kDa allergen as PA I has already been found in the red sea bream as well (Kobayashi et al., 2006), suggesting its wide distribution in sea breams. For a better understanding of fish allergy, especially sea bream allergy,

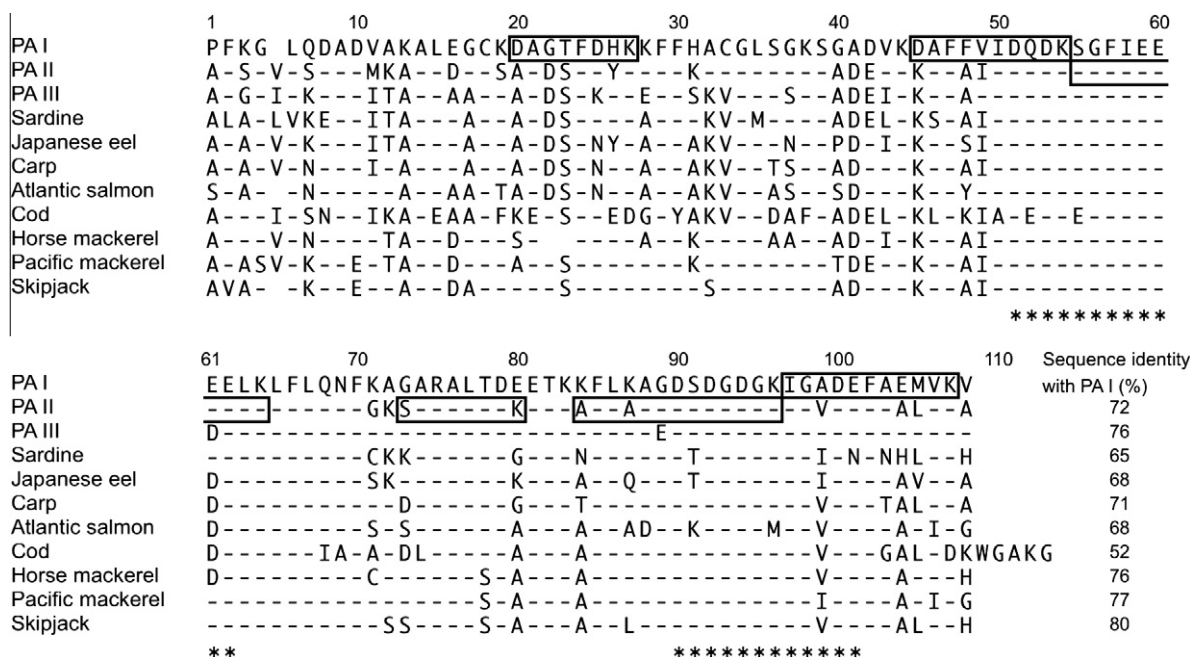


Fig. 5. Alignment of the amino acid sequences of crimson sea bream parvalbumins (PA I–III) with those of some fish parvalbumins. The residues identical with those of PA I are shown by dashes. Calcium-binding sites are indicated by asterisks under the sequence of skipjack parvalbumin. Lyslendopeptidase peptide fragments (peptides I-1, I-2 and I-3 from PA I and peptides II-1, II-2 and II-3 from PA II) are boxed. Accession numbers for parvalbumins (UniprotKB/Swiss-Prot databases for carp and cod parvalbumins and DDBJ/EMBL/GenBank databases for the other parvalbumins); PA I, AB622511; PA II, AB622512; PA III, AB375264; sardine (*Sardinops melanostictus*), AB375262; Japanese eel (*Anguilla japonica*), AB375263; carp (*Cyprinus carpio*), P02618; Atlantic salmon (*Salmo salar*), X97825; cod (*Gadus callarias*), P02622; horse mackerel (*Trachurus japonicus*), AB211364; Pacific mackerel (*Scomber japonicus*), AB091470; skipjack (*Katsuwonus pelamis*), AB375265. Note that gaps are inserted at position 5 of PA I, Atlantic salmon parvalbumin and skipjack parvalbumin and at positions 22 and 23 of horse mackerel parvalbumin.

future study is needed to identify the 14 kDa allergen in sea breams and assess its IgE reactivity.

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