

Identification of Parvalbumin as an Allergen in Horse Mackerel Muscle

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The crude extracts from the white muscle of five species of fish (horse mackerel *Trachurus japonicus*, Pacific mackerel *Scomber japonicus*, bigeye tuna *Thunnus obesus*, chum salmon *Oncorhynchus keta*, and walleye pollack *Theragra chalcogramma*) were evaluated for allergenicity by enzyme-linked immunosorbent assay (ELISA) using sera from two fish-allergic patients. All the extracts reacted with the IgE antibodies in one patient serum; the highest reaction was given by the horse mackerel extract and the lowest reaction by the bigeye tuna extract. In contrast, the bigeye tuna was the only positive sample as analyzed using another patient serum. Based on ELISAs using the former patient serum, a heat-stable allergen (named *Tra j 1*) in the horse mackerel was isolated by gel filtration on Sephadex G-75 and reverse-phase HPLC on TSKgel ODS-120T. In SDS-PAGE, *Tra j 1* gave a single band corresponding to a molecular weight of 10,500, regardless of the presence or absence of a reducing agent. The isoelectric point of *Tra j 1* was estimated to be 4.3 by IEF-PAGE. *Tra j 1* was rich in Ala, followed by Asx and Lys, but lacked Cys (half), His, and Trp. Furthermore, it showed an ability to bind to the monoclonal antibody against carp parvalbumin. From these physicochemical and immunological properties, *Tra j 1* was identified as parvalbumin of the horse mackerel.

Key words: *Trachurus japonicus*, allergen, parvalbumin, food allergy, horse mackerel, ELISA

Food allergy is generally considered as a type I allergy reaction mediated by antigen-specific IgE antibodies in patient sera. Some agricultural or livestock products, such as chicken egg, cow's milk, and soybean, are well known to be major allergenic foods and hence their allergenic proteins (allergens) have already been isolated and extensively studied for both chemical and immunological properties.^{1–3)} In some countries such as Scandinavian countries and Japan, where appreciable amounts of seafoods are consumed daily, allergy to seafoods is fairly common. Although previous studies on seafood allergens are limited in number, two classes of proteins, tropomyosins and parvalbumins, are now recognized as seafood allergens. Regardless of species, allergens isolated from three species of prawns *Penaeus indicus*,^{4,5)} *P. aztecus*,⁶⁾ and *Metapenaeus ensis*⁷⁾ have been all identified as tropomyosins. Very recently, allergens have been isolated from two species of mollusks, the oyster *Crassostrea gigas*⁸⁾ and the squid *Todarodes pacificus*,⁹⁾ and confirmed to be tropomyosins as in the case of prawn. On the other hand, fish allergens are not tropomyosins but parvalbumins as demonstrated in the Baltic cod *Gadus callarias*^{10,11)} and the Atlantic salmon *Salmo salar*.¹²⁾

In our recent screening for allergens in fish muscle by enzyme-linked immunosorbent assay using the crude extracts from five species of fish and the sera from two fish-allergic patients, the results differed markedly from patient to patient as well as from species to species. Furthermore, an allergen in the horse mackerel *Trachurus japonicus*, which was a strongly positive species to one patient serum, was isolated and identified to be parvalbumin. We report here the screening data and the isolation and identification of

parvalbumin as the allergen of horse mackerel.

Materials and Methods

Preparation of Crude Extracts

Five species of fish (horse mackerel *Trachurus japonicus*, Pacific mackerel *Scomber japonicus*, bigeye tuna *Thunnus obesus*, chum salmon *Oncorhynchus keta*, and walleye pollack *Theragra chalcogramma*) were purchased from a local fish market. The white muscle of each species (about 10 g) was homogenized with 4 volumes of 0.01 M phosphate buffer (pH 7.0) and centrifuged at 18,800 × g for 20 min. The supernatant was heated in a boiling water bath for 20 min. After removal of insoluble materials by centrifugation, the supernatant obtained was used as crude extract.

Sera

Patient sera were obtained from two subjects (subjects 1 and 2), who had been diagnosed to be hypersensitive to fish by doctors. Total IgE levels of subject 1 and 2 sera were 360 and 90 IU/ml, respectively. Control serum was obtained from a subject (subject 3) showing no allergic reactions following ingestion of any foods. All sera were stored at –20°C until use.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was performed using a flat-bottomed polystyrene plate (ELISA Plate H type with 96 wells; Sumitomo Bakelite, Tokyo, Japan), as reported previously.⁸⁾ In brief, the plate was coated with 100 µl of sample and blocked with 350 µl of 1% bovine serum albumin. Then, the plate

was incubated with 100 μ l of serum solution (diluted 1:50), followed by 100 μ l of peroxidase-conjugated goat anti-human IgE antibody (Kirkegaard & Perry Laboratories, MD, USA; diluted 1:2,500). Finally, following the enzyme reaction using 100 μ l of substrate solution (0.1% *o*-phenylenediamine dihydrochloride and 0.03% H_2O_2 in 0.05 M phosphate-citrate buffer, pH 5.0), the developed color was read at 490 nm on a kinetic microplate reader Model 450 (Bio-Rad Laboratories, CA, USA). For the isolated allergen, the reactivity with the monoclonal antibody against carp muscle parvalbumin (Sigma Chemical, MO, USA; diluted 1:500,000) was also examined; in this case, peroxidase-conjugated goat anti-mouse IgG antibody (The Binding Site, Birmingham, UK; diluted 1:50,000) was used instead of the peroxidase-conjugated goat anti-human IgE antibody. All ELISAs were carried out in triplicate and the data were expressed as the mean values.

Isolation Procedure

Throughout the following isolation procedure, allergens were traced by ELISA using the serum from subject 1. The serum from subject 3 was used in control experiments.

The crude extract, which was prepared from 78 g of horse mackerel muscle as described above, was dialyzed against distilled water using a Spectra/Por 6 membrane (molecular weight cut-off 3500; Spectrum Medical Industries, TX, USA). The inner solution was concentrated to about 30 ml with a rotary evaporator. Two separate portions (about 15 ml each) were applied to a Sephadex G-75 column (2.5 $\times$ 96 cm; Pharmacia Biotech, Uppsala, Sweden) equilibrated with 0.01 M phosphate buffer (pH 7.0) containing 0.15 M NaCl. Fractions of 10 ml were collected at a flow rate of about 30 ml/h and measured for absorbance at 280 nm. Allergen-containing fractions were combined and dialyzed against distilled water. Finally, the inner solution was concentrated to about 2 ml and subjected to reverse-phase HPLC on a TSKgel ODS-120T column (0.46 $\times$ 25 cm; Tosoh, Tokyo, Japan). The column was eluted at a flow rate of 1 ml/min by a gradient of acetonitrile (see Fig. 3 for details) in 0.1% trifluoroacetic acid. Proteins were continuously monitored at 220 nm with a UV detector.

Analytical Methods

Protein was determined by the method of Lowry *et al.*,¹³⁾ using bovine serum albumin as a standard. Both sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and polyacrylamide gel electrophoresis (IEF-PAGE) were performed on a PhastSystem apparatus (Pharmacia Biotech, Uppsala, Sweden). Running, staining, and destaining were all conducted by following the recipes of the manufacturer. Ready-made gels (PhastGel High Density and PhastGel IEF 3-9), ready-made buffer strips (PhastGel SDS Buffer Strips), a molecular weight calibration kit (MW 2,512-16,949), and a pI calibration kit 3-10 were purchased from Pharmacia Biotech. Prior to SDS-PAGE, samples were treated with 1% SDS in the presence or absence of 2-mercaptoethanol at 100°C for 10 min. For the amino acid analysis, the isolated allergen (44 μ g) was hydrolyzed with 50 μ l of 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole in an evacuated tube at 115°C for 24 h. Following hydrolysis,

the solution was neutralized with 50 μ l of 3.5 M NaOH and then diluted with 600 μ l of 0.2 M citrate buffer (pH 2.2). A 400 μ l portion of the dilution was subjected to amino acid analysis using an Atto MLC-703 analyzer.

Results

ELISA of the Crude Extracts

In the case of the horse mackerel, no significant differences in ELISA were observed between the heated extract and the non-heated extract. Furthermore, the non-specific reaction with the control serum was markedly decreased when the heated extract was used in ELISA. Thus, the allergenicity of five species of fish was assessed using the heated extracts. As shown in Fig. 1A, all the crude extracts reacted with the IgE antibodies in the subject 1 serum. Highly positive reactions were recognized with the crude extracts from horse mackerel, walleye pollack, and Pacific mackerel, among which the horse mackerel extract was most reactive. The chum salmon extract was negative at 0.1 μ l/well but clearly positive at 1.0 μ l/well, while the bigeye tuna extract was weakly positive even at 1.0 μ l/well. In the case of the subject 2 serum, however, only the bigeye tuna extract was weakly positive at 1.0 μ l/well

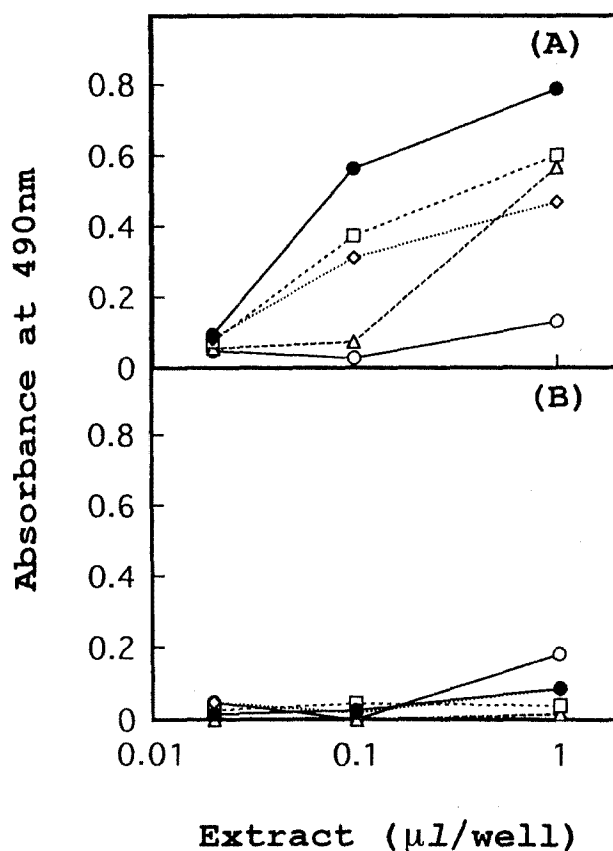


Fig. 1. Analysis by ELISA of the reactivity of the crude extracts from five species of fish with subject 1 (A) and 2 (B) sera.

Crude extract: —●—, horse mackerel; ...◇..., Pacific mackerel; —○—, bigeye tuna; ...△..., chum salmon; ...□..., walleye pollack. Each point is shown by subtracting the absorbance obtained with the control serum (subject 3) from that of the patient serum (subject 1 or 2).

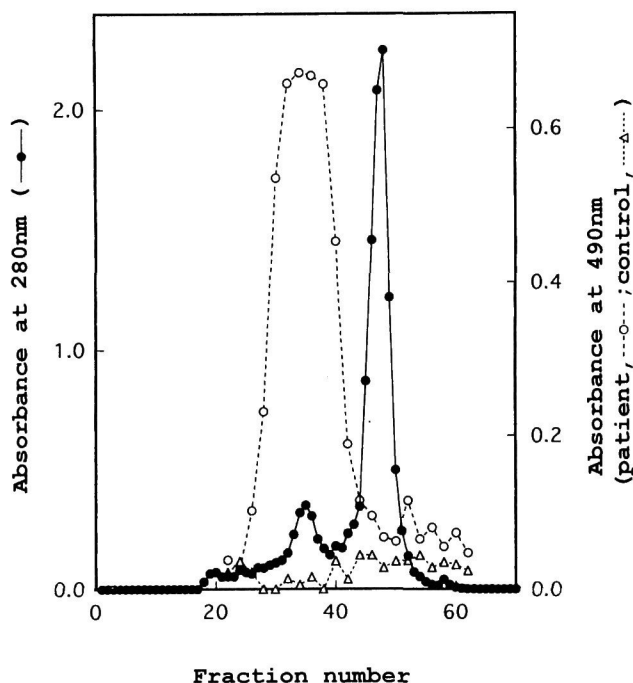


Fig. 2. Gel filtration of the crude extract.

The crude extract of horse mackerel was put onto a Sephadex G-75 column (2.5 × 96 cm), which was equilibrated and eluted with phosphate buffer (pH 7.0) containing 0.15 M NaCl. Fractions of 10 ml were collected at a flow rate of about 30 ml/h.

(Fig. 1B). In this study, therefore, the horse mackerel was selected as a fish sample for the isolation of allergens and the subject 1 serum was used in ELISAs to trace allergens.

Isolation of an Allergen

When chromatographed on Sephadex G-75, the crude extract from the horse mackerel muscle afforded two peaks with absorbance at 280 nm (Fig. 2). Analyses by ELISA demonstrated that allergens were contained in the small peak at around fraction 34. In reverse-phase HPLC on TSKgel ODS-120T of the allergen-containing fraction obtained by gel filtration on Sephadex G-75, six small peaks (peaks 1-6) and one large peak (peak 7) were observed as monitored by absorbance at 220 nm (Fig. 3). The major allergen (named *Tra j 1* according to the suggestions of the International Union of Immunological Nomenclature) was eluted in the large peak at a retention time of 39 min. Thus, 31 mg of *Tra j 1* was isolated from 78 g of the muscle sample. Although positive reactions to the patient serum were recognized in peaks 2 and 5 by ELISA, they were not further examined in this study.

Properties of the Isolated Allergen

Tra j 1 gave a single band in SDS-PAGE, regardless of the presence or absence of 2-mercaptoethanol, supporting its homogeneity (Fig. 4). As compared to the mobilities of the reference proteins, the molecular weight of *Tra j 1* was estimated to be 10,500. In IEF-PAGE, *Tra j 1* also afforded a single band at the position corresponding to pI 4.3, demonstrating again its homogeneity (Fig. 5). When analyzed by ELISA, *Tra j 1* was found to bind to the

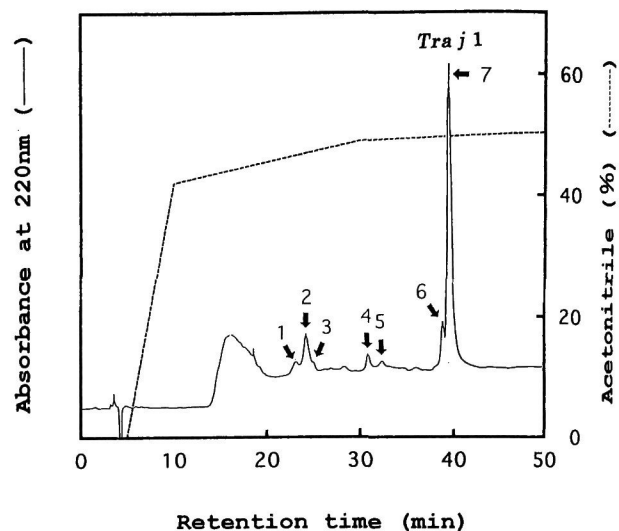


Fig. 3. Reverse-phase HPLC of the allergenic fraction obtained by gel filtration.

The allergenic fraction obtained by gel filtration (see Fig. 2) was applied to a TSKgel ODS-120T column (0.46 × 25 cm) equilibrated with 0.1% trifluoroacetic acid. Elution was achieved at a flow rate of 1.0 ml/min by a gradient of acetonitrile in 0.1% trifluoroacetic acid. Positive reactions to the patient (subject 1) serum were recognized in peaks 2, 5, and 7 (*Tra j 1*).

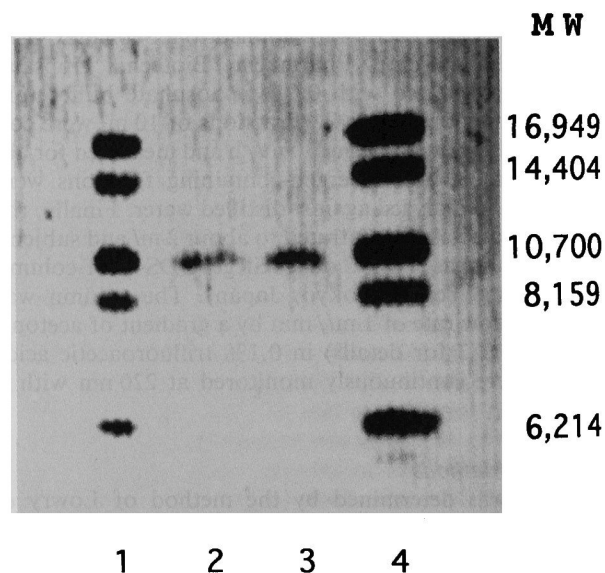


Fig. 4. SDS-PAGE of *Tra j 1*.

Lanes 1 and 4, standard proteins; lanes 2 and 3, *Tra j 1*. Electrophoresis was performed in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of 1% 2-mercaptoethanol.

monoclonal antibody against carp parvalbumin (Fig. 6), suggesting that both *Tra j 1* and carp parvalbumin have the same or similar antigenic determinant group(s). Amino acid analysis showed that *Tra j 1* was especially rich in Ala, followed by Asx and Lys, while it lacked Cys (half), His, and Trp (Table 1). These features in amino acid composition were recognized for the three kinds of fish parvalbumin which are listed in Table 1 for comparison.

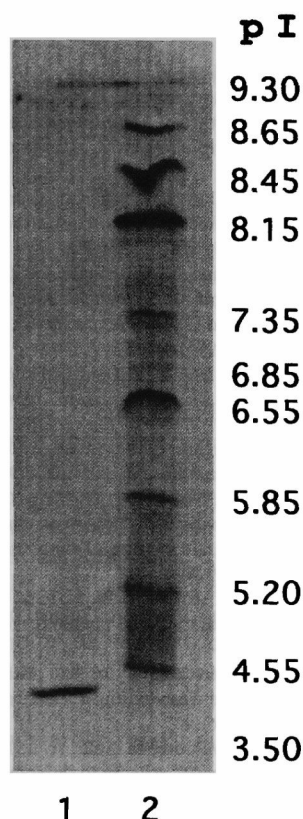


Fig. 5. IEF-PAGE of *Tra j 1*.
Lane 1, *Tra j 1*; lane 2, pI calibration kit.

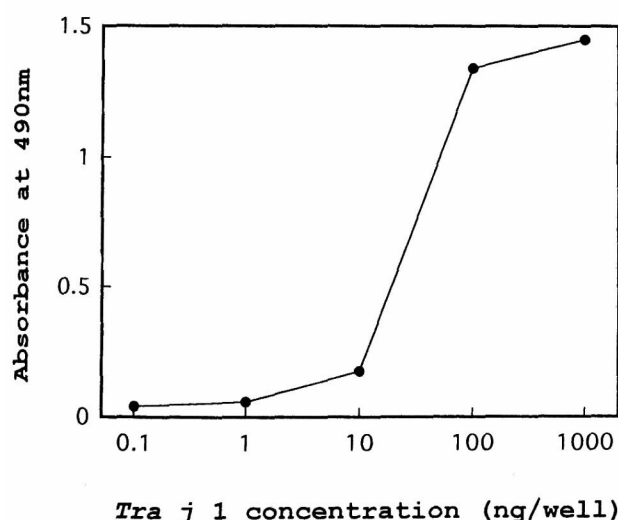


Fig. 6. Analysis by ELISA of the reactivity of *Tra j 1* with anti-carp parvalbumin monoclonal antibody.

Discussion

The allergen (*Tra j 1*) in the horse mackerel muscle was isolated by the following two steps: gel filtration on Sephadex G-75 and reverse-phase HPLC on TSKgel ODS-120T. Judging from its clarified properties, *Tra j 1* can be identified as a parvalbumin of horse mackerel. It is a heat-sta-

Table 1. Amino acid compositions of *Tra j 1* and parvalbumins the Baltic cod *Gadus callarias*, the Atlantic salmon *Salmo salar*, and the common carp *Cyprinus carpio*

Amino acid	Mol%			
	<i>Tra j 1</i>	Baltic cod*	Atlantic salmon*	Carp*
Asx	12.2	14.1	12.0	15.7
Thr	2.4	1.8	5.6	4.6
Ser	5.3	3.5	3.7	4.6
Glx	8.8	9.7	9.3	7.4
Pro	0.8	0	0.9	0
Gly	8.1	10.6	5.6	7.4
Ala	22.2	16.8	15.7	18.5
Val	3.0	2.7	4.6	4.6
Cys(1/2)	0	0.9	3.7	0.9
Met	1.8	0	0.9	0
Ile	5.1	5.3	5.6	4.6
Leu	7.5	9.7	9.3	8.3
Tyr	1.0	1.8	0	0
Phe	9.5	9.7	10.2	9.3
His	0	0	1.9	0.9
Trp	0	0.9	0	0
Lys	11.3	12.4	12.0	8.3
Arg	1.2	0.9	0.9	0.9

* Calculated from the amino acid sequences deposited with the database.

ble, acidic (pI 4.3), and relatively small (MW 10,500) protein, in accordance with the known parvalbumins from other sources. Its amino acid composition, characterized by the abundance of Ala, Asx, and Lys and the absence of Cys (half), His, and Trp, is comparable to those of other parvalbumins. In addition to these chemical similarities, the positive reaction of *Tra j 1* with the monoclonal antibody against carp parvalbumin provided immunological evidence for its identity with parvalbumin. Following the isolation of *Gad c 1* from Baltic cod,^{10,11} and *Sal s 1* from Atlantic salmon,¹² *Tra j 1* is the third example of fish allergens. Importantly, *Gad c 1*, *Sal s 1*, and *Tra j 1* are parvalbumins without exception. Furthermore, the cross-reactivity of allergens in various species of fish has previously been reported by many researchers.¹⁴⁻¹⁹ These circumstances strongly suggest that fish allergens are commonly parvalbumins.

It should be noted that the crude extracts from five species of fish gave fairly different results from species to species in ELISAs using the patient serum from subject 1 (Fig. 1A). For example, a marked difference is seen between the extracts from horse mackerel and bigeye tuna. This leads us to assume that there is a large difference in the content of parvalbumin or in the ability of parvalbumin to bind IgE antibodies in the patient serum between horse mackerel and bigeye tuna. In order to confirm this assumption, it is essential to determine parvalbumins from various species of fish and evaluate their allergenicity.

It is also worth mentioning that the results in ELISAs using the subject 2 serum were quite different from those using the subject 1 serum, although the total IgE level of the subject 2 serum was one-fourth that of the subject 1 serum. The horse mackerel extract exhibited the highest reactivity with IgE antibodies in the subject 1 serum, but did not react with IgE antibodies in the subject 2 serum. Moreover, the bigeye tuna extract, the only positive sample in ELISAs using the subject 2 serum, was least reactive

with IgE antibodies in the subject 1 serum. These results suggest that both IgE antibodies in the sera from subjects 1 and 2 recognize different portions in the antigens (parvalbumin) or that the IgE antibodies in the subject 2 serum react with not parvalbumin but unknown substances.

In conclusion, fish allergy due to parvalbumins seems to be much more complicated than expected since the allergenicity of parvalbumins differ not only from species to species but also from patient to patient. Further study is needed to compare the structures of IgE-binding epitopes of fish parvalbumins, using many fish species and patient sera.

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