

Original Article

Expression and evaluation of IgE-binding capacity of recombinant Pacific mackerel parvalbumin

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ABSTRACT

Background: Parvalbumin is the major and cross-reactive allergen in fish. Sufficient amounts of IgE-reactive recombinant fish parvalbumin are needed for diagnosis and immunotherapy of fish allergy.

Methods: A DNA fragment corresponding to parvalbumin of the Pacific mackerel *Scomber japonicus* was synthesized and cloned into the expression vector pGEX-6p-3 to produce glutathione S-transferase (GST)-fusion parvalbumin in *Escherichia coli*. The GST-free recombinant parvalbumin was purified using the RediPack GST Purification Module (Amersham Pharmacia Biotech, Buckinghamshire, UK). Parvalbumins of seven species of fish (Japanese eel, horse mackerel, red sea bream, Pacific mackerel, skipjack, bigeye tuna and Japanese flounder) were purified by gel filtration and reverse-phase HPLC. The IgE-binding capacity was examined by ELISA and antigenic cross-reactivity by inhibition ELISA.

Results: The GST-free recombinant Pacific mackerel parvalbumin was obtained in an electrophoretically pure state. Data from ELISA and inhibition ELISA revealed that the recombinant parvalbumin contains most of the IgE-binding epitopes of the natural counterpart. In addition, the recombinant parvalbumin inhibited the IgE reactivities of the pooled patient serum to parvalbumins purified from six

species of fish in almost the same magnitude as the natural Pacific mackerel parvalbumin.

Conclusions: Because the recombinant Pacific mackerel parvalbumin bearing the IgE-binding capacity of the natural counterpart is cross-reactive with various fish parvalbumins, it can be a useful tool for the diagnosis and immunotherapy of fish allergy.

Key words: cross-reactivity, expression, IgE-binding capacity, mackerel, parvalbumin.

INTRODUCTION

Fish is one of the most important causes of food allergy in some coastal countries, such as Japan and Scandinavia, where the fish consumption is high. Following ingestion of fish, acute hypersensitivity reactions, such as urticaria, asthma and vomiting, are induced in sensitized patients;^{1–3} even fatal cases with anaphylaxis have been reported.^{4,5} The first identified fish allergen is parvalbumin of cod *Gadus callarias*, a calcium-binding sarcoplasmic protein with a molecular mass of approximately 12 kDa.^{6,7} Subsequent immunoblotting studies^{2,8–10} and molecular studies^{11–18} have established that parvalbumin represents the major and cross-reactive allergen in common with various species of fish, although minor allergens, such as collagen^{19–21} and aldehyde phosphate dehydrogenase,²² have been detected simultaneously.

Sufficient amounts of pure allergens are essential for both the diagnosis and immunotherapy of allergy. Although it is generally laborious and time-consuming to purify allergens from biological samples, recent molecular genetic techniques enable us to easily obtain large amounts of recombinant allergens at need. Along this line, the cDNA coding for allergens (parvalbumins) of

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Atlantic salmon *Salmo salar*,¹⁵ carp *Cyprinus carpio*¹⁶ and Atlantic cod *Gadus morhua*¹⁷ have been cloned and expressed in *Escherichia coli* and the resulting recombinant allergens evaluated for IgE-binding capacity. However, these previous studies have been all performed in Europe and, hence, no recombinant fish parvalbumins are available in Japan.

In Japan, mackerels, together with salmon, are considered to be most frequently responsible for fish allergy and the Ministry of Health, Labour and Welfare of Japan has recommended that any processed food products containing these fish as raw materials have labels indicating their use on the packages or bottles. We have already identified parvalbumin as the major allergen in three species of mackerels (Pacific mackerel *Scomber japonicus*, spotted mackerel *S. australasicus* and Atlantic mackerel *S. scombrus*) and isolated a cDNA coding for Pacific mackerel parvalbumin.¹⁸ The production of recombinant Pacific mackerel parvalbumin mimicking the IgE-binding capacity of the corresponding natural allergen will facilitate the accurate diagnosis and future immunotherapy of fish allergy, especially in Japan. Therefore, in the present study we expressed the Pacific mackerel parvalbumin cDNA in *E. coli* and examined the immunological properties of the purified recombinant preparation.

METHODS

Purification of parvalbumin

Parvalbumins of Japanese eel *Anguilla japonica*,¹³ horse mackerel *Trachurus japonicus*,¹² Pacific mackerel¹⁸ and bigeye tuna *Thunnus obesus*²¹ were purified by a combination of gel filtration and reverse-phase HPLC, as reported previously. Essentially the same method was adopted for the purification of parvalbumins from red sea bream *Pagrus major*, skipjack *Katsuwonus pelamis* and Japanese flounder *Paralichthys olivaceus*. The white muscle of each fish species was homogenized with three volumes of 150 mmol/L NaCl in 10 mmol/L phosphate buffer (pH 7.0) and heated in a boiling water bath for 10 min. After centrifugation (at 18 000 g for 10 min at 4°C), the supernatant (extract) was concentrated in vacuo and subjected to gel filtration on a Sephadex G-75 column (2.5 × 110 cm; Amersham Pharmacia Biotech, Buckinghamshire, UK), which was eluted with 150 mmol/L NaCl in 10 mmol/L phosphate buffer (pH 7.0). Parvalbumin-containing fractions detected by ELISA were pooled and then 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 with 0.1% trifluoroacetic acid (TFA) for an initial 5 min, followed by a linear gradient of acetonitrile in 0.1% TFA. The eluate containing parvalbumin was collected manually and used in subsequent experiments.

Expression and purification of recombinant parvalbumin

A DNA fragment corresponding to the mature Pacific mackerel parvalbumin, with *Bam*H1 and *Eco*R1 sites at the 5' and 3' ends, respectively, was amplified by polymerase chain reaction (PCR), using the previously purified plasmids¹⁸ containing the full-length parvalbumin cDNA as a template. The following primers were used: a primer specific for the 5' end with a *Bam*H1 site (underlined) 5'-GGATCCGCCTTTGCAAGTGTACTGAAAGATG-3' and a primer specific for the 3' end with an *Eco*R1 site (underlined) 5'-GAATTCTTATCCCTTAATCATGGCTGCAAAC-3'. The PCR was performed under the following conditions: 95°C for 10 min; 35 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 2 min; and 72°C for 2 min. The amplified product was ligated to the *Bam*H1/*Eco*R1 site of the expression vector pGEX-6p-3 (Amersham Pharmacia Biotech). Following introduction of the ligation product into *E. coli* JM 109, transformed cells were grown overnight at 37°C in 5 mL LB medium containing 0.005% ampicillin. Then, 1 mL of the culture was inoculated into 100 mL of the same medium and incubated at 37°C until the absorbance of the medium at 600 nm was increased to 0.5. To induce protein expression, 1 mmol/L isopropyl β-D-thiogalactopyranoside (IPTG) was added to the culture and the culture was further incubated at 37°C for 3 h. Bacteria harvested by centrifugation were suspended in 5 mL of 50 mmol/L Tris-HCl buffer (pH 7.0) containing 150 mmol/L NaCl and 1 mmol/L EDTA and sonicated on ice. After centrifugation (at 2000 g for 15 min at room temperature), a target protein fused with glutathione S-transferase (GST) was found in the supernatant (cell lysate), but not in the precipitate. The GST-free recombinant parvalbumin was purified using the RediPack GST Purification Module (Amersham Pharmacia Biotech) according to the manufacturer's instructions. In brief, the cell lysate was subjected to affinity chromatography on a Glutathione Sepharose 4B column. The column was washed with a cleavage buffer (50 mmol/L Tris-HCl buffer, pH 7.0, containing 150 mM NaCl, 1 mM EDTA and 50 mmol/L

dithiothreitol) to remove unadsorbed proteins and then treated with PreScission Protease (RediPack GST Purification Module; 40 units/mL Sepharose 4B) at 5°C for 4 h. Finally, the recombinant mackerel parvalbumin released from the GST-fusion protein was eluted from the column with the cleavage buffer. The purified GST-free recombinant preparation was desalted by dialysis against distilled water and used in subsequent experiments.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a PhastSystem (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Ready made gels (PhastGel 8-25), ready made buffer strips (PhastGel SDS Buffer Strips) and a low molecular weight calibration kit were purchased from Amersham Pharmacia Biotech. Prior to running, each sample was dissolved in 10 mmol/L Tris-HCl (pH 6.8) containing 5% SDS and 5% dithiothreitol and heated at 100°C for 5 min. Proteins were visualized by staining with Coomassie Brilliant Blue R-250.

Protein determination

Proteins were determined according to the method of Lowry *et al.*²³ using bovine serum albumin (BSA) as a standard.

Human sera

Sera were obtained from nine fish-allergic patients with documented clinical histories of immediate hypersensitivity reactions after the ingestion of fish. In our previous paper²¹ using both parvalbumin and collagen purified from bigeye tuna, these sera were all shown to react to parvalbumin, but not to collagen, by ELISA. In the present study, the patient sera were used as a pooled sample unless stated otherwise. Written informed consent was obtained from each patient.

ELISA

The IgE reactivities of the patient sera to fish parvalbumins were examined by fluorescence ELISA using a flat-bottomed polystyrene plate with 96 wells (Type H Multi Well Plate for ELISA; Sumitomo Bakelite, Tokyo, Japan), essentially by the same procedure as described elsewhere.²⁴ Parvalbumin is a Ca²⁺-binding protein and its

IgE-binding capacity seems to be sensitive to Ca²⁺ depletion, as observed previously with carp parvalbumin.¹⁶ Therefore, prior to ELISA all purified parvalbumins, including both natural and recombinant preparations, were dissolved in 5 mmol/L CaCl₂ solution at a concentration of 1 mg/mL and incubated at 37°C for at least 30 min. In brief, ELISA was performed as follows. Each parvalbumin sample coated on the plate at 0.1 µg/well was reacted with the patient sera diluted 1 : 100 with 0.1% BSA in Dulbecco's phosphate-buffered saline (pH 7.4; DPBS), followed by β-galactosidase-conjugated goat antihuman IgE antibody (American Qualex, San Clemente, CA, USA) diluted 1 : 1000 with 0.1% BSA in DPBS. The enzyme reaction was performed using substrate solution (0.01% 4-methylumbelliferyl β-D-galactoside in 0.1 mol/L phosphate buffer (pH 7.8) containing 1 mmol/L MgCl₂) and arrested by the addition of 0.1 mmol/L glycine-NaOH buffer (pH 10.3). The fluorescence intensity was measured at 500 nm with an extinction of 340 nm. To detect parvalbumin in chromatographic steps, the mouse anti-frog muscle parvalbumin monoclonal antibody (Sigma, St Louis, MO, USA) diluted 1 : 5000 with 0.1% BSA in DPBS was used as a primary antibody and peroxidase-conjugated goat antimouse IgG antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) diluted 1 : 5000 with 0.1% BSA in DPBS as a secondary antibody. The enzyme reaction was performed using a substrate solution containing 0.1% o-phenylenediamine and 0.03% H₂O₂. After the addition of 1 mol/L sulfuric acid, the color developed was measured by absorbance at 490 nm.

For inhibition ELISA experiments, the pooled patient serum was diluted 1 : 50 with 0.1% BSA in DPBS and incubated at 37°C for 1 h with an equal volume of inhibitor solution (0.2–10 µg parvalbumin/mL) made in DPBS. A 100 µL portion of this solution was then added to a microtiter plate that had been coated previously with fish parvalbumins at a concentration of 0.1 µg/well. The subsequent procedure was the same as that for the fluorescence ELISA described above.

RESULTS

Expression and purification of recombinant Pacific mackerel parvalbumin

Recombinant Pacific mackerel parvalbumin was expressed successfully in *E. coli* as a GST-fusion protein using the pGEX-6p-3 system and recovered only in the

cell lysate. As analyzed by SDS-PAGE, a prominent protein band with a molecular mass of 38 kDa was observed in the lysate of *E. coli* induced by IPTG (lane 3 in Fig. 1). Based on the molecular masses of GST (approximately 26 kDa) and Pacific mackerel parvalbumin

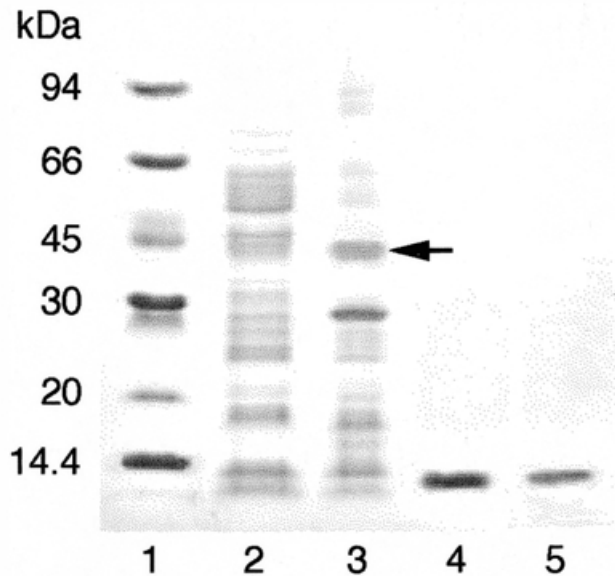


Fig. 1 Sodium dodecylsulfate–polyacrylamide gel electrophoresis of recombinant Pacific mackerel parvalbumin. Lane 1, standard proteins; lane 2, cell lysate (– isopropyl β -D-thiogalactopyranoside (IPTG)); lane 3, cell lysate (+IPTG); lane 4, purified recombinant parvalbumin; lane 5, purified natural parvalbumin. The arrow indicates a putative glutathione S-transferase-fusion parvalbumin.

(approximately 12 kDa), this band was attributable to the GST-fusion parvalbumin. When the cell lysate was applied to affinity chromatography on a Glutathione Sepharose 4B column, the GST-fusion parvalbumin was specifically retained on the column. Upon treatment with PreScission Protease on the column, the GST-free parvalbumin was released and eluted from the column. In SDS-PAGE, the GST-free recombinant parvalbumin thus obtained migrated as a single band of approximately 12 kDa (lane 4 in Fig. 1) at the same position as the natural counterpart (lane 5 in Fig. 1) purified as reported previously.¹⁸ In a typical run, the yield of the GST-free recombinant parvalbumin was 24 mg/L of the bacterial culture.

Comparison of IgE-binding capacity between recombinant and natural Pacific mackerel parvalbumins

When analyzed by ELISA using pooled patient serum and two individual sera, the recombinant Pacific mackerel parvalbumin was found to have almost the same IgE-binding capacity as the natural mackerel parvalbumin (Fig. 2). In inhibition ELISA, the IgE reactivity of the pooled patient serum to either the recombinant mackerel parvalbumin or the natural counterpart was inhibited to almost the same extent by both recombinant and natural mackerel parvalbumins (Fig. 3), demonstrating that the recombinant mackerel parvalbumin contains most of the IgE-binding epitopes of the natural mackerel parvalbumin.

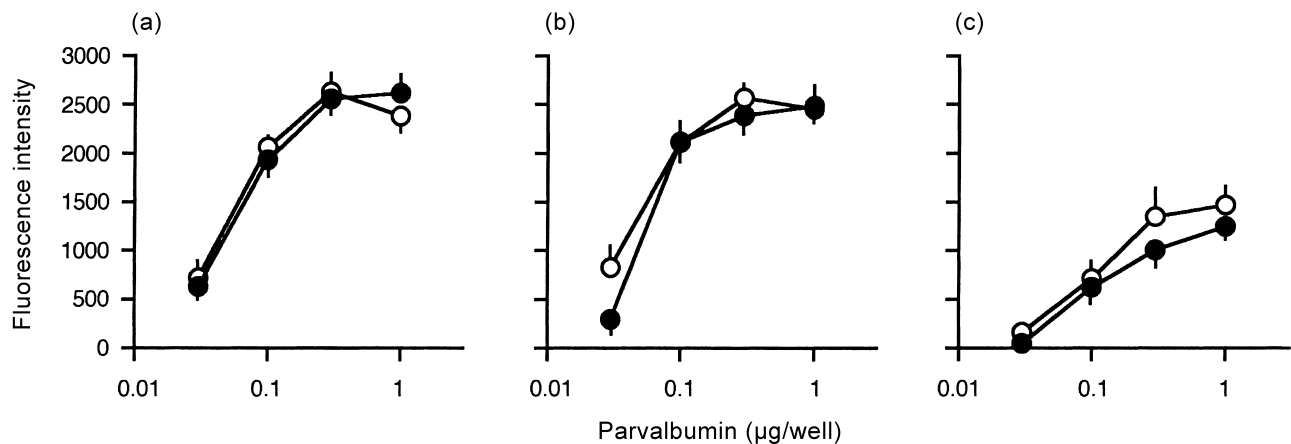


Fig. 2 Analysis by ELISA of IgE reactivities of patient sera to natural (●) and recombinant (○) Pacific mackerel parvalbumins. (a) Pooled patient serum; (b) serum from patient 1; (c) serum from patient 2. Data are the mean \pm SD ($n = 3$).

Cross-reactivity between recombinant Pacific mackerel parvalbumin and parvalbumins from six species of fish

Six species of fish (Japanese eel, horse mackerel, red sea bream, skipjack, bigeye tuna and Japanese flounder), which are widely consumed in Japan, were selected for analysis. It should also be noted that two of these fish (skipjack and bigeye tuna) are phylogenetically close to Pacific mackerel, whereas the remaining species are remote from the Pacific mackerel.

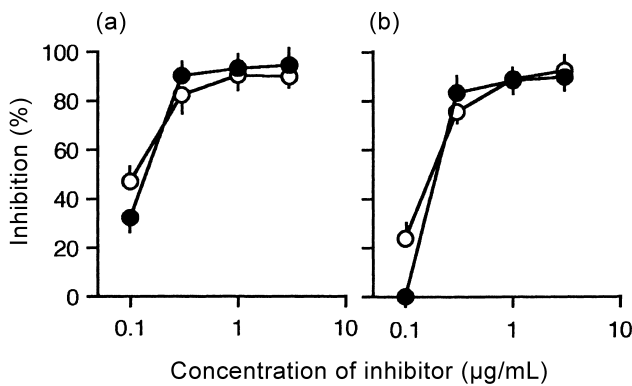


Fig. 3 Inhibition of IgE reactivities of the pooled patient serum to natural (●) and recombinant (○) Pacific mackerel parvalbumins by both parvalbumins. The pooled patient serum (1 : 50 dilution) was preincubated with an equal volume of natural (●) or recombinant (○) Pacific mackerel parvalbumin solution (0.2, 0.6, 2 or 6 µg/mL) and added to the plate coated with natural (a) or recombinant (b) Pacific mackerel parvalbumin (0.1 µg/well). Data are the mean ± SD (n = 3).

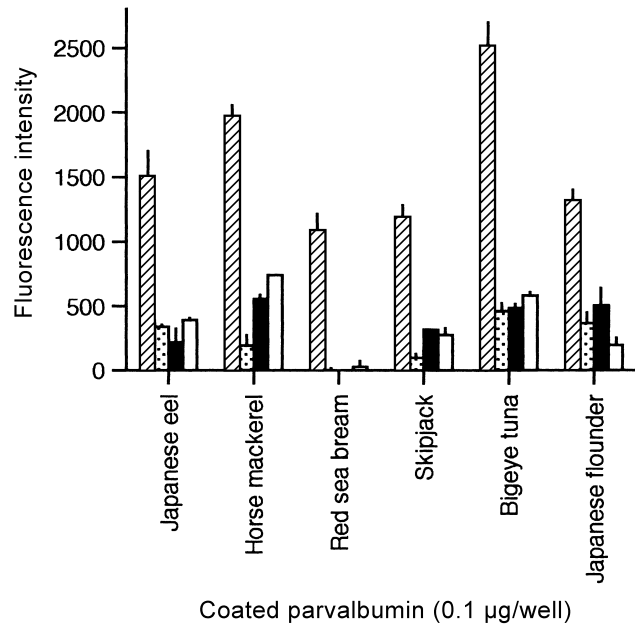


Fig. 5 Inhibition of IgE reactivities of the pooled patient serum to parvalbumins from six species of fish by each fish parvalbumin, natural Pacific mackerel parvalbumin and recombinant Pacific mackerel parvalbumin. The pooled patient serum (1 : 50 dilution) was pre-incubated with an equal volume of inhibitor solution (10 µg/mL) and added to the plate coated with parvalbumins from six species of fish (0.1 µg/well). Inhibitors: (▨), none; (▧), each fish parvalbumin; (■), natural Pacific mackerel parvalbumin; (□), recombinant Pacific mackerel parvalbumin. Data are the mean ± SD (n = 3).

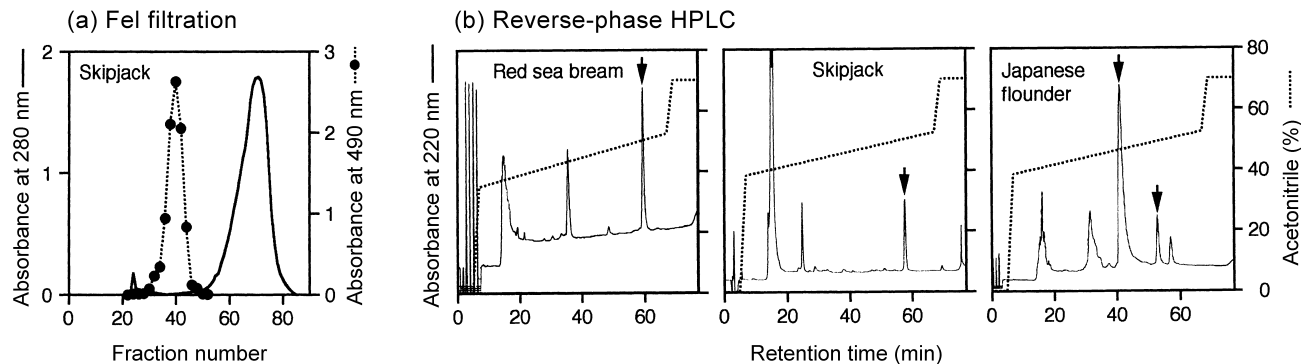


Fig. 4 Purification of parvalbumins from red sea bream, skipjack and Japanese flounder. (a) Gel filtration of the extract from skipjack. Column, Sephadex G-75 (2.5 × 110 cm); solvent, 150 mmol/L NaCl in 10 mmol/L phosphate buffer (pH 7.0); volume/fraction, 10 mL. Similar chromatograms were also obtained with extracts from red sea bream and Japanese flounder. (b) Reverse-phase HPLC. Sample, parvalbumin-containing fraction obtained by gel filtration; column, TSKgel ODS-120T (0.46 × 25 cm); elution, linear gradient of acetonitrile in 0.1% trifluoroacetic acid; flow rate, 1 mL/min. Arrows indicate the peaks containing parvalbumin.

Parvalbumins of the Japanese eel,¹³ horse mackerel¹² and bigeye tuna²¹ were purified as reported previously. In the present study, parvalbumins of red sea bream, skipjack and Japanese flounder were newly purified by a combination of gel filtration on Sephadex G-75 (Fig. 4a) and reverse-phase HPLC on TSKgel ODS-120T (Fig. 4b). In SDS-PAGE, all the newly purified parvalbumins afforded a single band of 11–12 kDa, as in the case of Pacific mackerel parvalbumin (lane 5 in Fig. 1), supporting their homogeneity. No isoforms of parvalbumin were detected in red sea bream and skipjack during the purification procedure, similar to the case with horse mackerel¹² and bigeye tuna.²¹ However, Japanese flounder was shown to contain two parvalbumin isoforms by reverse-phase HPLC (Fig. 4b), similar to the case with Japanese eel.¹³ For Japanese eel and Japanese flounder, only one major isoform was used in the following experiments.

Antigenic cross-reactivity of the recombinant Pacific mackerel parvalbumin with the parvalbumins purified from six species of fish was examined by inhibition ELISA. As shown in Fig. 5, the pooled patient serum reacted to the parvalbumins from six species of fish with varied potencies and the reactivity to each parvalbumin was markedly reduced by the same parvalbumin. In addition, similar inhibitory effects against the IgE reactivities of the pooled patient serum to the parvalbumins from six species of fish were displayed by both recombinant and natural Pacific mackerel parvalbumins, although rather less inhibition was observed in horse mackerel and skipjack parvalbumins compared with that by the same allergen as the immobilized one. These results strongly suggest that the recombinant Pacific mackerel parvalbumin, as well as the natural counterpart, has a wide cross-reactivity with various fish parvalbumins.

DISCUSSION

In the present study, we successfully expressed Pacific mackerel parvalbumin in *E. coli* as a GST-fusion protein by use of the pGEX-6p-3 system. It should be noted that purification of the GST-free recombinant parvalbumin is easily achieved by specific adsorption of the GST-fusion protein on a Glutathione Sepharose 4B column, followed by cleavage with PreScission Protease on the column. Furthermore, the yield (24 mg/L of the bacterial culture) of the GST-free recombinant preparation was sufficient to its practical use.

The ELISA data revealed that the recombinant Pacific mackerel parvalbumin has almost the same IgE-binding capacity as the natural counterpart. This result was explained by inhibition ELISA experiments, which suggested that the recombinant parvalbumin contains most of the IgE-binding epitopes of the natural parvalbumin molecule. With regard to these results, it is worth mentioning that the recombinant mackerel parvalbumin is structurally distinct from the natural counterpart. First, the recombinant parvalbumin has a free N-terminal amino acid, whereas the N-terminus of the natural parvalbumin is blocked,¹⁸ as recognized in other fish parvalbumins, such as those of Atlantic salmon¹¹ and carp.¹⁶ Second, compared with the natural parvalbumin, the recombinant preparation produced by the pGEX-6p-3 system adopted in the present study has an additional sequence (Gly-Pro-Leu-Gly-Ser) composed of five amino acid residues at the N-terminus. No significant difference in IgE-binding capacity between the recombinant and natural parvalbumins implies that neither the N-terminal blocking of the natural parvalbumin nor the additional N-terminal sequence of the recombinant parvalbumin is involved in the binding to IgE.

Recombinant fish parvalbumins have so far been produced for Atlantic salmon,¹⁵ carp¹⁶ and Atlantic cod¹⁷ and confirmed to be IgE reactive. However, of the two recombinant parvalbumins of Atlantic salmon expressed as His-tag proteins, one was quantitatively analyzed for IgE reactivity by ELISA and shown to be much less reactive than the natural counterpart.¹⁵ This reduced IgE reactivity was explained by assuming that the recombinant Atlantic salmon parvalbumin is different in folding and glycosylation from the natural parvalbumin. Similarly, recombinant parvalbumins of Atlantic cod were expressed as His-tag proteins and proved to be lower in IgE-binding capacity than the natural parvalbumin.¹⁷ In contrast, the recombinant carp parvalbumin expressed as a non-fusion protein was judged to have almost the same IgE-binding capacity as the natural carp parvalbumin.¹⁶ This result suggests no implication of glycosylation in the IgE-binding capacity of the natural carp parvalbumin, although glycosylation was assumed to be associated with the IgE-binding capacity of the natural Atlantic salmon parvalbumin as described above. As for folding, circular dichroism analysis showed that the recombinant carp parvalbumin represents a folded structure, characterized by the abundance of α -helices, comparable to that of the natural carp parvalbumin. Our recombinant Pacific mackerel parvalbumin bearing the IgE-binding

capacity of the natural counterpart is also considered to have the same folded structure as the natural counterpart. Although not confirmed, the recombinant preparation was assumed to be correctly folded by dissolving in CaCl₂ solution.

In inhibition ELISA experiments, the recombinant Pacific mackerel parvalbumin was found to inhibit the IgE reactivities of the pooled patient serum to the parvalbumins purified from six species of fish, in almost the same magnitude as the natural counterpart. The observed wide cross-reactivity with the parvalbumins of various species of fish suggests that our recombinant Pacific mackerel parvalbumin can be used as a standard molecule in the diagnosis of fish allergy as well as in detailed studies of the properties of fish allergens. Moreover, the recombinant Pacific mackerel parvalbumin may be used in specific immunotherapy of fish allergy. In immunotherapy, however, administration of allergen, even at very low doses, may induce various side-effects, such as fatal anaphylaxis. To avoid side-effects in immunotherapy, it is desirable to produce hypoallergenic molecules by a site-directed mutagenesis technique. Elucidation of the B and T cell epitopes of Pacific mackerel parvalbumin, which is essential to develop hypoallergenic derivatives, is under progress.

In conclusion, our expression system is promising for the production of large amounts of recombinant Pacific mackerel parvalbumin bearing the IgE-binding capacity of the corresponding natural allergen. The recombinant Pacific mackerel parvalbumin is expected to be a useful tool in the diagnosis and immunotherapy of fish allergy, especially in Japan, where mackerels are frequently responsible for fish allergy incidents.

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