

Reduction in the IgE reactivity of Pacific mackerel parvalbumin by mutations at Ca²⁺-binding sites

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ABSTRACT: Parvalbumin is a sarcoplasmic Ca²⁺-binding protein of 12 kDa and represents the major fish allergen. Several peptide segments are identified as immunoglobulin E (IgE)-binding epitopes of cod parvalbumin. However, carp parvalbumin (Cyp c 1) shows a markedly reduced IgE-binding ability upon depletion of Ca²⁺, suggesting the importance of conformational epitopes associated with Ca²⁺-chelating. In this study, the IgE reactivity of Pacific mackerel *Scomber japonicus* parvalbumin (Sco j 1) was demonstrated to be markedly reduced (60–100% reduction) by Ca²⁺-depletion, similar to Cyp c 1. Three Sco j 1 mutants (D51A, D90A, D51/90A), with modifications in either one or both of the two Ca²⁺-binding sites, were then constructed by site-directed mutagenesis, followed by expression in *Escherichia coli*, and evaluated for their IgE reactivity. Interestingly, the double mutant (D51/90A), probably devoid of Ca²⁺-binding capacity, exhibited a significantly reduced IgE reactivity (equivalent to 0.0–7.5% of the IgE reactivity of natural Sco j 1). The results suggest that the IgE-binding ability of Sco j 1 largely depends on the solid conformation mediated by Ca²⁺-chelating, and that the hypoallergenic D51/90A will be a useful tool for the specific immunotherapy of fish allergy.

KEY WORDS: Ca²⁺-binding, IgE reactivity, mutant, Pacific mackerel, parvalbumin.

INTRODUCTION

Fish is among the most common causes of immunoglobulin E (IgE)-mediated food allergy, especially in coastal countries such as Scandinavia and Japan where fish is daily consumed as an important source of proteins and nutrients. Following the ingestion of fish, adverse reactions such as urticaria, angioedema, asthma, diarrhea and anaphylaxis are induced in sensitized subjects who have elevated IgE specific to fish; even fatal cases have been recorded.^{1,2} Extensive studies with cod *Gadus callarias* have demonstrated that the major allergen is parvalbumin (known as Gad c 1), a sarcoplasmic protein of approximately 12 kDa.^{3,4} Subsequently, parvalbumin has been identified at the molecular level as the major allergen in common with various species of fish such as eel *Anguilla japonica*,⁵ carp *Cyprinus carpio*,^{6,7} Atlantic salmon *Salmo salar*,⁸ horse mackerel *Trachurus japonicus*,⁹ mackerel *Scomber japonicus*¹⁰ and tuna *Thunnus obesus*.⁵ It is now accepted that parvalbumin is a

major cross-reactive fish allergen, although fish collagen has also been reported to be IgE-reactive.^{11,12}

Parvalbumin belongs to a family of Ca²⁺-binding proteins that are structurally characterized by the presence of EF-hand motifs (helix-loop-helix motifs), and has three EF-hand motifs called AB, CD and EF domains.^{13,14} The CD and EF domains individually contain a Ca²⁺-binding site (corresponding to the loop portion composed of 12 amino acid residues; refer to Fig. 1), while the AB domain is not involved in the binding of Ca²⁺.^{15,16} It is known that the folded structure of parvalbumin is maintained by the chelating of Ca²⁺ and perturbed upon depletion of Ca²⁺. As to the relationship between the IgE reactivity (= allergenicity) and the Ca²⁺-chelating of parvalbumin, different results have been obtained with Gad c 1 and carp parvalbumin (Cyp c 1). In the case of Gad c 1, its IgE reactivity is somewhat reduced (approx. 25% reduction) when bound-Ca²⁺ is released,¹⁷ but is very stable to heating, extreme pH and treatment with denaturing agents.¹⁸ It is therefore considered that the amino acid sequence is much more associated with the IgE-binding epitopes of Gad c 1 than the steric conformation. Indeed, some

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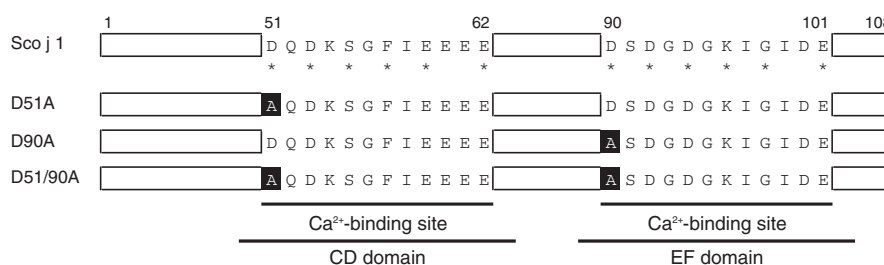


Fig. 1 Schematic amino acid sequences of Pacific mackerel parvalbumin (Sco j 1) and its three mutants (D51A, D90A, D51/90A) constructed in this study. Coordinating residues in Ca²⁺-binding are indicated by asterisks below the sequence of Sco j 1. Modified residues in the mutants are shown in white on a black background.

peptide segments of Gad c 1 have so far been identified as IgE-binding epitopes.¹⁹ However, the IgE reactivity of Cyp c 1 has recently been reported to be remarkably reduced (30–90% reduction) by depletion of Ca²⁺,⁷ indicating the significance of conformational IgE-binding epitopes. In accordance with this, some amino acid residues of Cyp c 1 have been proposed to comprise conformational epitopes by analysis of mimotopes.²⁰

Which type of epitopes, linear-type or conformational-type epitopes is more deeply related to the IgE-binding of fish parvalbumin is not fully understood due to the limited studies. To expand knowledge on this issue, Pacific mackerel *Scomber japonicus* parvalbumin (Sco j 1) was selected as a sample in this study because mackerels are considered to be most frequently responsible for fish allergy in Japan, and processed food products containing them as raw materials are recommended to be labeled, indicating their use on packages or bottles. First, the IgE reactivity of Sco j 1 was examined in the absence and presence of a chelating reagent (EGTA). In the next step, three kinds of Sco j 1 mutants, which have modifications in one or both of the two Ca²⁺-binding sites, were prepared and evaluated for their IgE-reactivity. It is reported here that the IgE-binding of Sco j 1 largely depends on the conformation, similar to Cyp c 1, but dissimilar to Gad c 1, and that the mutant with modifications in both of the Ca²⁺-binding sites exhibits a significantly reduced IgE-binding capacity, being a useful tool for the specific immunotherapy of fish allergy. During the preparation of this paper, a Cyp c 1 mutant with two modifications in each of the two Ca²⁺-binding sites was reported to have little IgE-binding capacity.²¹ The present study with Sco j 1 is of particular value in demonstrating that only one modification in each of the two Ca²⁺-binding sites is sufficient to produce the same degree of reduction in IgE-binding capacity as the Cyp c 1 mutant.

MATERIALS AND METHODS

Purification of Pacific mackerel parvalbumin

Pacific mackerel parvalbumin was purified from the white muscle of Pacific mackerel by gel filtration on Sephadex G-75 (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) and reverse-phase HPLC on TSKgel ODS-120T (Tosoh, Tokyo, Japan), as reported previously.¹⁰

Construction of mutant plasmids

It is generally known for Ca²⁺-binding proteins containing EF-hand motifs that the first amino acid residue of the 12 residues forming a Ca²⁺-binding site is particularly important for the chelating of Ca²⁺.²² Therefore, in this study, Asp-51 (corresponding to the first residue of the Ca²⁺-binding site in the CD domain) and Asp-90 (corresponding to the first residue of the Ca²⁺-binding site in the EF domain) were targeted for the preparation of the following three kinds of Sco j 1 mutants: (i) D51A with a replacement of Asp-51 by Ala; (ii) D90A with a replacement of Asp-90 by Ala; and (iii) D51/90A with replacements of both Asp-51 and Asp-90 by Ala (Fig. 1). Modification of the Sco j 1 cDNA was performed by site-directed mutagenesis using a Quick Changes II Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Inverse polymerase chain reaction (PCR) was conducted using the Sco j 1 cDNA cloned into the expression vector pGEX-6p-3²³ as a template. The following primers, each of which has a modified codon (underlined), were used; a forward primer (5'-CTTTCGCCATGATTGCCAGGACAAGAGC-3') and a reverse primer (5'-GCTCTTGTCCTGGGCAATGATGGCGAAAG-3') for the mutant D51A, and a forward primer (5'-CTCAAGGCCGGTGCCAGCGACGGTG-3') and a reverse primer (5'-CACCGTCGCTGGCACCGGC

CTTGAG-3') for the mutant D90A. After PCR, the template plasmid was digested with *Dpn* I and the single mutant plasmids were therefore obtained. To construct the double mutant (D51/90A) plasmid, the D51A plasmid was subjected as a template to PCR with the same primers as employed for the preparation of the D90A plasmid. *Escherichia coli* (*E. coli*) JM109 was transformed with each mutant plasmid and cultured on LB agar containing 0.005% ampicillin at 37°C overnight. A single colony was selected and cultured in LB medium containing 0.005% ampicillin at 37°C overnight. The plasmid was purified from the bacterial suspension using a Quantum Prep Plasmid Mini Prep Kit (Bio-Rad Laboratories, Hercules, CA, USA), and sequenced using a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

Expression and purification of mutants

Escherichia coli JM109 was transformed with each mutant plasmid, for which correct mutations were confirmed by nucleotide sequencing, and grown in 500 mL of LB medium containing 0.005% ampicillin at 37°C until the absorbance at 600 nm reached 0.7. The culture was then added with isopropyl- β -D-thiogalactoside (IPTG) at a concentration of 1 mM and further incubated for 3 h. Bacteria were harvested by centrifugation, resuspended to be lysed in 25 mL of 5 mM Tris-HCl buffer (pH 8.0) containing lysozyme (0.2 mg/mL), 0.15 M NaCl, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride and sonicated. After centrifugation, the GST-fusion protein recovered in the supernatant was purified by affinity chromatography on a GStrap

HP column (GE Healthcare Bio-Sciences) according to the manufacturer's manual, and digested with PreScission Protease (GE Healthcare Bio-Sciences) at 4°C for 4 h. The digest was again applied to the GStrap HP column and the GST-free recombinant mutant was obtained in the flow-through fraction.

Sodium dodecylsulfate–polyacrylamide gel electrophoresis

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a PhastSystem (GE Healthcare Bio-Sciences) as recommended by the manufacturer. Ready-made gels (PhastGel Gradient 8–25) and ready-made buffer strips (PhastGel SDS Buffer Strips) were purchased from GE Healthcare Bio-Sciences, and a molecular weight calibration kit (Precision Plus Protein Standards) from Bio-Rad Laboratories. Prior to running, each sample was heated at 100°C for 10 min in 0.0625 M Tris-HCl buffer (pH 7.5) containing 2% SDS, 0.1 M dithiothreitol and 3 M urea. Proteins were visualized by staining with Coomassie Brilliant Blue R-250.

Human sera

Sera were obtained from 12 fish-allergic patients who had a documented clinical history of immediate hypersensitive reactions after ingestion of fish. These patients were all diagnosed to be allergic to fish, based on the determined capsulated hydrophilic carrier polymer-radioallergosorbent test classes of 2–4 against various species of fish such as mackerel and tuna (Table 1). Written informed

Table 1 Clinical data of 12 fish-allergic patients

Patient	Age (years)	Gender	Total IgE (IU/mL)	CAP-RAST class
1	3	Female	408–918	Sardine 3, salmon 3, tuna 3
2	8	Male	1 579–5 174	Sardine 3, salmon 3, cod 3, horse mackerel 3, mackerel 3, tuna 3
3	20	Female	1 262	Horse mackerel 3, tuna 3
4	3	Female	5 322	Tuna 3
5	2	Male	221	Tuna 3, mackerel 3
6	1	Male	15 481	Tuna 4
7	4	Female	Unknown	Salmon 4, cod 3
8	1	Female	Unknown	Salmon 3, cod 3
9	Unknown	Unknown	2 150	Sardine 3, salmon 3, horse mackerel 3, mackerel 2, tuna 3, flounder 2
10	Unknown	Unknown	13 170	Tuna 4
11	Unknown	Unknown	3 420	Horse mackerel 4, tuna 4
12	15	Male	6 300	Sardine 3, salmon 3, cod 3, horse mackerel 3, mackerel 3, tuna 3

CAP-RAST, carrier polymer-radioallergosorbent test; IgE, immunoglobulin E.

consent was obtained from each patient. In this study, sera from two healthy volunteers without adverse reactions after ingestion of any foods were used as controls. All sera were stored at -20°C until use.

Enzyme-linked immunosorbent assay

Immunoglobulin E reactivity was examined by fluorescence enzyme-linked immunosorbent assay (ELISA), which was performed using a polystyrene microtiter plate with 96 wells (Type H [black]; Sumitomo Bakelite, Tokyo, Japan), as described in a previous paper.²³ In brief, the microtiter plate was coated with 50 μL of the antigen solution (1 $\mu\text{g}/\text{mL}$) and reacted successively with patient serum (diluted 1:200), which had been pre-incubated at 37°C for 2 h in 0.1% bovine serum albumin-0.05% Tween 20-Dulbecco's phosphate buffer (pH 7.0), with or without 5 mM EGTA, and β -galactosidase-conjugated goat antihuman IgE antibody (0.25 $\mu\text{g}/\text{mL}$; American Qualex, San Clement, CA, USA). Enzyme reaction was conducted using 0.1 mg/mL 4-methylumbelliferyl- β -D-galactoside and arrested by addition of 0.1 M glycine-NaOH buffer (pH 10.3). Fluorescence intensity was measured at 367 nm (excitation) and 453 nm (emission). All ELISA were performed in triplicate and the data were expressed in mean values.

RESULTS

Effect of Ca^{2+} -depletion on the immunoglobulin E reactivity of Pacific mackerel parvalbumin

Irrespective of the patient sera, the IgE reactivity of Sco j 1 was remarkably reduced (60–100% reduction) in the presence of EGTA compared to that in the absence of EGTA (Fig. 2), demonstrating the importance of Ca^{2+} for the binding between Sco j 1 and serum IgE. In the present separate experiments, the IgE-binding capacity of black tiger shrimp tropomyosin was not affected by the presence of EGTA. It is therefore apparent that EGTA itself exhibits no inhibitory activity against the binding of allergenic proteins, including Sco j 1 with serum IgE.

To examine the effect of Ca^{2+} -depletion on the IgE reactivity, Sco j 1 was coated on the microtiter plate without preincubation with EGTA and reacted with patient serum preincubated with EGTA. It should be noted that the IgE reactivity of Sco j 1 was preliminarily found to be independent of whether or not it was incubated with EGTA prior

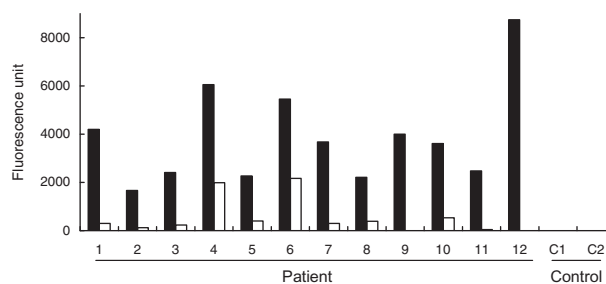


Fig. 2 Analysis of immunoglobulin E reactivity of Pacific mackerel parvalbumin in the absence (■) and presence of EGTA (□) by fluorescence enzyme-linked immunosorbent assay.

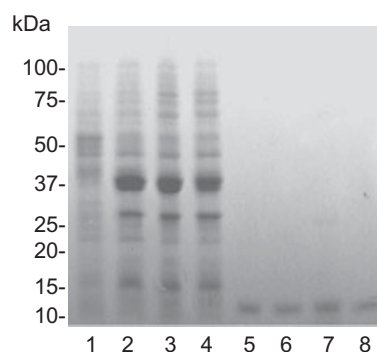


Fig. 3 Sodium dodecylsulfate-polyacrylamide gel electrophoresis of recombinant Pacific mackerel parvalbumin (Sco j 1) mutants. Lanes: 1, cell lysate (–IPTG); 2, lysate of *Escherichia coli* (*E. coli*) expressing GST-fusion D51A (+IPTG); 3, lysate of *E. coli* expressing GST-fusion D90A (+IPTG); 4, lysate of *E. coli* expressing GST-fusion D51/90A (+IPTG); 5, purified recombinant GST-free D51A; 6, purified recombinant GST-free D90A; 7, purified recombinant GST-free D51/90A; 8, natural Sco j 1.

to coating. This implies that Ca^{2+} -depleted Sco j 1 can readily rebinding to Ca^{2+} contained in patient serum.

Expression and purification of recombinant Pacific mackerel parvalbumin mutants

Three kinds of Sco j 1 mutants were successfully expressed in *E. coli* as GST-fusion proteins, 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 37 kDa was observed in each lysate of IPTG-induced *E. coli* (lanes 2–4 in Fig. 3), but not in that of non-induced bacteria (lane 1, Fig. 3). Based on the molecular masses of GST (approx. 26 kDa) and Sco j 1 (approx. 12 kDa), this band was attributable to the recombinant GST-fusion Sco j 1 mutant. Each

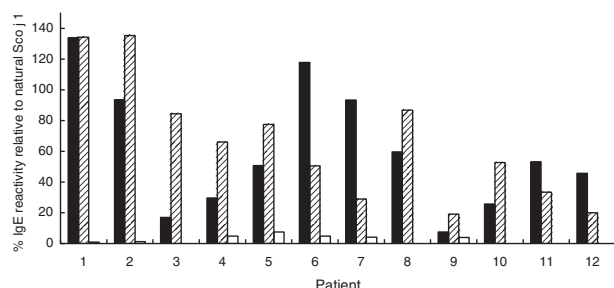


Fig. 4 Analysis of immunoglobulin E (IgE) reactivity of three Pacific mackerel parvalbumin (Sco j 1) mutants (D51A, D90A, D51/90A) by fluorescence enzyme-linked immunosorbent assay. Data are shown in percentage IgE reactivity by taking the IgE reactivity of natural Sco j 1 as 100%. D51A (■), D90A (▨), D51/90A (□).

GST-fusion mutant was then purified from the cell lysate by affinity chromatography on a GSTrap HP column and digested with PreScission Protease (GE Healthcare Bio-Sciences). The digest was again subjected to affinity chromatography to obtain the GST-free mutant. In SDS-PAGE, all the three GST-free mutants migrated as a single band of approximately 12 kDa (lanes 5–7, Fig. 3) at the same position as the natural counterpart (lane 8, Fig. 3).

Immunoglobulin E reactivity of Pacific mackerel parvalbumin mutants

The IgE-binding ability of the recombinant Sco j 1 mutants was evaluated by fluorescence ELISA using sera of 12 patient. Since there is no difference in the IgE reactivity between natural Sco j 1 and its recombinant counterpart,²³ the natural preparation was used as a reference in this study. The patient sera showed varied patterns of IgE reactivities to the mutants D51A and D90A, which have a single modification in the CD and EF domains, respectively (Fig. 4). As compared to the results with natural Sco j 1, two sera (from patients 1 and 6) displayed increased IgE reactivities to the mutant D51A and two sera (from patient 1 and 2) to the mutant D90A. However, the majority of the patient sera showed reduced IgE reactivity to both D51A and D90A; 6.5–92.5% reduction was recognized for D51A and 15.6–80.8% for D90A. However, the double mutant D51/90A, with one modification in each of the CD and EF domains, completely lost IgE reactivity with five sera (from patients 3, 8, 10, 11, 12). The IgE reactivity with the remaining seven sera was also very weak, being only 0.8–7.5% of that of natural Sco j 1.

DISCUSSION

The ELISA data obtained, in the absence and presence of EGTA, showed that the depletion of Ca^{2+} causes a significant reduction in the IgE reactivity of Sco j 1. This strongly suggests that the IgE-binding capacity (= allergenicity) of Sco j 1 mostly depends on the solid conformation (helix-loop-helix) of the CD and EF domains mediated by the chelating of Ca^{2+} . The importance of conformational IgE-binding epitopes associated with the binding of Ca^{2+} is also known for Cyp c 1.⁷ In contrast, the IgE-binding epitopes of Gad c 1 seem to be linear rather than conformational; several peptide segments have been proposed for the IgE-binding epitopes of Gad c 1.¹⁹ In relation to the discrepancy observed as to the IgE-binding epitopes among fish parvalbumins, it is worth mentioning that the amino acid sequences of parvalbumins from various species of fish are highly conserved in the CD and EF domains, but have considerable alterations in the other regions, especially in the first half (N-terminal portion) of the sequence. It is therefore assumed that fish-allergic patients commonly recognize the solid conformation of fish parvalbumins formed by the chelating of Ca^{2+} , and also recognize several specific peptide segments contained in some fish parvalbumins such as Gad c 1. To confirm this assumption, the effect of Ca^{2+} -depletion on the IgE reactivity should be examined with parvalbumins from many species of fish.

In this study, three Sco j 1 mutants (D51A, D90A, D51/90A) were constructed by site-directed mutagenesis, followed by expression in *E. coli*, to shed more light on the relationship between the depletion of Ca^{2+} and the IgE-binding ability of Sco j 1. Interestingly, the double mutant D51/90A with replacements of Asp-51 in the CD domain and Asp-90 in the EF domain by Ala displayed a markedly reduced IgE reactivity (only 0.0–7.5% of IgE reactivity compared to natural Sco j 1). Although not confirmed, the mutant D51/90A has possibly no Ca^{2+} -binding capacity and is in the unfolded structure. Therefore, the results with D51/90A seem to be a molecular basis for the fact that the IgE-binding epitopes of Sco j 1 are largely conformational.

Immediately before the submission of this paper, Swoboda *et al.*²¹ reported almost the same results with Cyp c 1 mutants as the present study's results with Sco j 1 mutants. Swoboda *et al.*²¹ prepared three kinds of Cyp c 1 mutants, two single mutants (Mut-CD and Mut-EF) and one double mutant (Mut-CD/EF), and demonstrated that the double mutant exhibits a particularly reduced IgE reactivity, which on average is equivalent to <5% of

the IgE reactivity of wild-type recombinant Cyp c 1. Although Mut-CD/EF has two modifications (replacements of Asp-51 and Asp-53 by Ala) in the CD domain and two modifications (replacements of Asp-90 and Asp-92 by Ala) in the EF domain, this study's double mutant D51/90A had only one modification in each of the CD and EF domains. Nevertheless, D51/90A showed a markedly reduced IgE-binding ability comparable to that of Mut-CD/EF, suggesting that the replacement of the first residue (Asp-51 or Asp-90) in the Ca^{2+} -binding sites contained in either the CD domain or the EF domain is sufficient to release the bound Ca^{2+} from fish parvalbumin.

Besides parvalbumin, some Ca^{2+} -binding proteins containing EF-hand motifs have so far been identified as allergens of pollens and displayed a Ca^{2+} -dependency of the IgE-binding ability, similar to parvalbumin. For example, the IgE reactivity of birch pollen allergen (Bet v 4),²⁴ Bermuda grass pollen allergen (Cyn d 7)²⁵ and olive pollen allergens (Ole e 3, Ole e 8)²⁶ is known to be reduced by Ca^{2+} -depletion. In addition to the similarity in the Ca^{2+} -dependency of IgE-binding ability, Ca^{2+} -binding proteins including parvalbumin also have a high sequence similarity in the EF-hand Ca^{2+} -binding sites. Although these facts may allow us to assume the involvement of the Ca^{2+} -binding sites for a wide cross-reactivity between allergenic Ca^{2+} -binding proteins, no cross-reactivity is not recognized at least between olive pollen allergens and frog parvalbumin.²⁶

Allergen-specific immunotherapy is currently performed for the effective treatment of inhalant allergies such as those by pollens and mites. In specific immunotherapy, increasing doses of allergen are continuously administered to patients until the responsiveness disappears.²⁷ However, in the case of food allergy, immunotherapy using allergens is known to be frequently accompanied by severe side-effects including anaphylactic shock, and hence is not commonly used for the treatment. In this context, the generation of hypoallergenic parvalbumin mutants (D51/90A, Mut-CD/EF) is of particular value in designing a future treatment of fish allergy. In view of the fact that the hypoallergenic parvalbumin mutants have only two (D51/90A) or four (Mut-CD/EF) modifications, they seem to preserve T cell epitopes. Therefore, it is expected that they can be administered to fish-allergic patients at high doses with a reduced risk of side-effects, leading to the rapid induction of tolerance. To evaluate whether the mutant D51/90A is a useful antigen for the specific immunotherapy of fish allergy, its tolerance-inducing effect is under study using a mouse model.

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