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Note

Fish allergy in patients with parvalbumin-specific immunoglobulin E depends on parvalbumin content rather than molecular differences in the protein among fish species

Ayako Kobayashi¹, Yukihiro Kobayashi^{2,3,*} and Kazuo Shiomi¹

¹Department of Food Science and Technology, Tokyo University of Marine Science and Technology, Tokyo, Japan;

²Department of Marine Biosciences, Tokyo University of Marine Science and Technology, Tokyo, Japan; ³Course of Safety Management in Food Supply Chain, Tokyo University of Marine Science and Technology, Tokyo, Japan

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Allergenic characteristics of purified parvalbumins from different fish species have not been thoroughly investigated. We revealed that purified parvalbumins from nine different fish species have identical IgE-reactivities and high cross-reactivities. We also showed that fish allergenicity is associated with the parvalbumin content of the fish species, rather than species-specific differences in the molecular characteristics of the individual parvalbumin proteins.

Key words: allergen; fish allergy; immunoglobulin E (IgE); parvalbumin

Parvalbumin is a major fish allergen,^{1,2)} and parvalbumin-specific immunoglobulin E (IgE) shows cross-reactivity among a wide range of fish species.^{1,3–5)} Recently, we revealed that parvalbumin content widely varies among fish species, e.g. large-sized migratory fish contain less parvalbumin than small-sized sedentary fish.⁶⁾ Concentrations of parvalbumin in fish can vary among species by up to several dozens of times.⁶⁾ As a result, it is often difficult for clinicians to diagnose which fish elicits allergies because of the differing parvalbumin content. For example, a fish-allergic patient may eat tuna, which contains low levels of parvalbumin, without showing adverse reactions as found in oral challenges.⁷⁾ Therefore, an improved understanding of fish allergies is required to give appropriate dietary advice to those who are susceptible.

Currently, allergenicity of parvalbumin is considered to mainly depend on parvalbumin content.^{6,8,9)} However, a quantitative analysis examining the association between parvalbumin content and allergenicity in various fish species is yet to be conducted. Furthermore, allergenicity per fixed amount of immobilized parvalbumin in various fish species is unclear. Moreover, the analysis of cross-reactivities using purified parvalbumins has not been thoroughly performed for

many fish species, although parvalbumins are known to cross-react among various fish species.^{1,3–5)} In most cases, either crude extracts were used to investigate fish allergenicities or few purified parvalbumins were used for assessments. Therefore, this study was conducted to evaluate the following two aspects related to fish allergenicity. First, we aimed to determine the affinities of human IgE to purified parvalbumins instead of crude extracts (molecular characteristics). Second, we aimed to provide accurate information regarding the parvalbumin content in fish meat. Finally, we tried to reveal which factor determines the allergenicities of fish, characteristics of parvalbumin proteins or their contents in fish meat.

We assessed (i) IgE reactivities and cross-reactivities of pure parvalbumins and (ii) the parvalbumin content in the following nine fish species: Japanese eel (*Anguilla japonica*), Japanese sardine (*Sardinops melanostictus*), Japanese jack mackerel (*Trachurus japonicus*), crimson sea bream (*Erythrinus japonicus*), red sea bream (*Pagrus major*), Pacific mackerel (*Scomber japonicus*), skipjack (*Euthynnus pelamis*), bigeye tuna (*Thunnus obesus*), and Japanese flounder (*Paralichthys olivaceus*). All fish samples used in this study were adult fishes. Parvalbumin purification was performed as previously described.¹⁰⁾ Briefly, a heated extract was prepared from the pink muscle (type IIa; skipjack and bigeye tuna) or white muscle (type IIb; other fish species) of the dorsal part of the fish body. The extract was applied to gel filtration chromatography, followed by reverse-phase high-performance liquid chromatography, and the purified parvalbumins showed apparent homogeneity on SDS-PAGE (Supplementary Fig. S1). At each step, parvalbumin was monitored by mouse anti-frog parvalbumin monoclonal antibody PARV-19 (Cat. No. P3088, Sigma-Aldrich, St. Louis, MO, USA). Because four of these fish species (Japanese eel, Japanese sardine, crimson sea bream, and red sea bream) had several parvalbumin isoforms, the major isoform was used for the following experiments. The

*Corresponding author. Email: yukihiro@kaiyodai.ac.jp

Abbreviations: IgE, immunoglobulin E; ELISA, enzyme-linked immunosorbent assay.

results of SDS-PAGE (Supplementary Fig. S1) showed that parvalbumins of most fish species migrated at position 10 kDa, except for 8.5 kDa for Japanese jack mackerel, 12.9 kDa for red sea bream, and 12.3 kDa for Japanese flounder. Amino acid sequence identities of parvalbumins among eight fish species (no data for bigeye tuna) were $73 \pm 4\%$ (67–82%) based on the database. As for isoforms of parvalbumin from crimson sea bream, differences in IgE reactivity of each isoform have been investigated.¹¹⁾ Because there is no drastic difference in IgE reactivity among these isoforms,¹¹⁾ it is unlikely that minor isoforms of the Japanese ell, Japanese sardine, and red sea bream would have a great influence on total IgE reactivity in a mixture of all isoforms.

To assess IgE reactivity per fixed amount of immobilized parvalbumin, 1 $\mu\text{g}/\text{ml}$ of each purified parvalbumin was immobilized on a microtiter plate, and an enzyme-linked immunosorbent assay (ELISA) was conducted using sera obtained from eight parvalbumin-allergic patients (Fig. 1(A)), as previously described.¹⁰⁾ Most types of purified parvalbumins bound to the patient's IgE to similar degrees, although patient 3

showed a more intense reaction to skipjack parvalbumin than to other fish parvalbumins. Therefore, there seems to be no differences in IgE reactivity of parvalbumin, which may be reflected by the structural differences of IgE-binding epitopes of species tested. Because inhibition ELISA (Fig. 1(B); details are shown in the next paragraph) using the serum from patient 3 showed a high inhibition rate, IgE-binding epitopes of skipjack for patient 3 may almost be same as that for other patients. Presumably, the affinity of IgE in patient 3 to skipjack parvalbumin is higher than that of IgE in other patients.

To verify the similarity of the IgE-binding epitopes on these parvalbumins, we performed inhibition ELISA, as previously described.¹²⁾ Purified parvalbumins from the nine fish species were used as inhibitors against IgE reactivity to purified Pacific mackerel parvalbumin (Fig. 1(B)). Purified parvalbumins from most fish species inhibited IgE reactivity to purified Pacific mackerel parvalbumin at high levels, except for Japanese eel parvalbumin in patients 4, 5, and 8; skipjack parvalbumin in patient 1; and Japanese flounder parvalbumin in patient 5. These data indicate that each

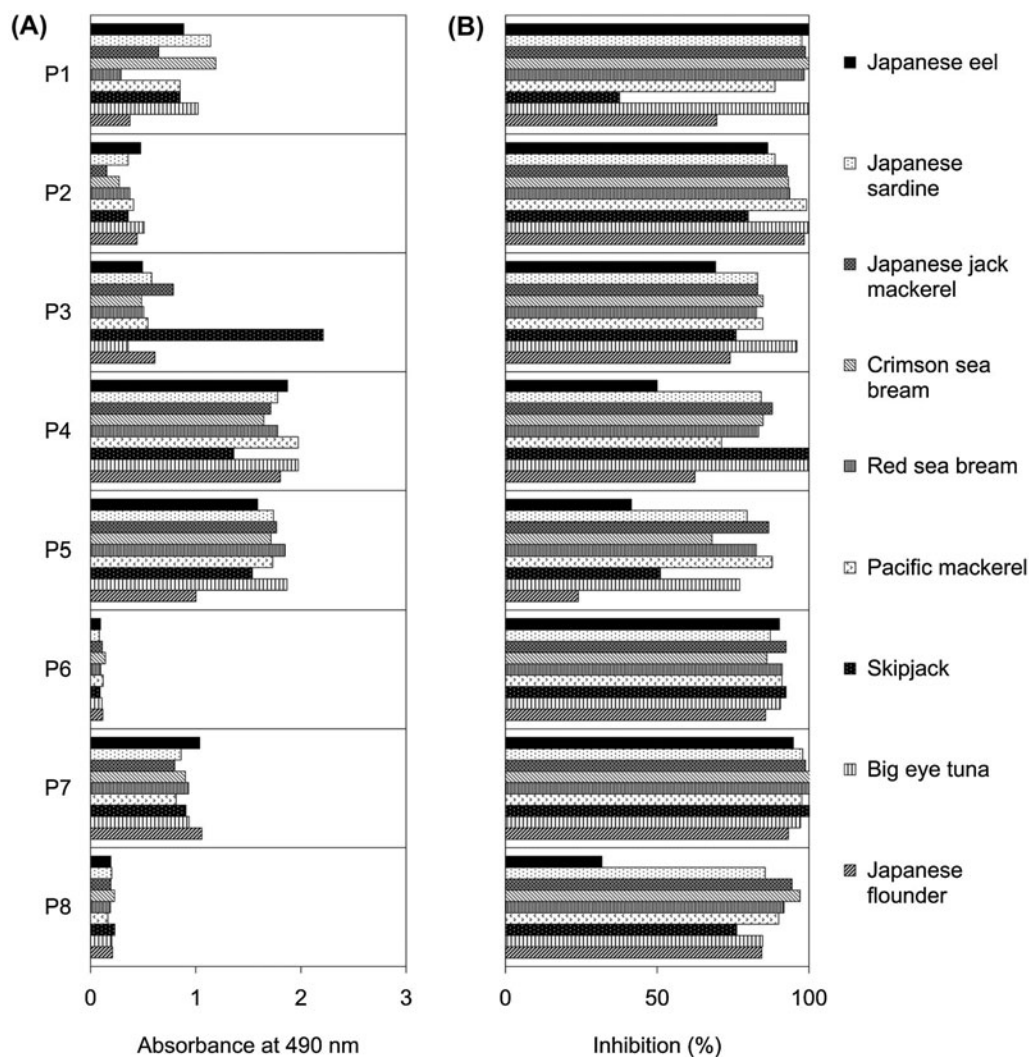


Fig. 1. IgE reactivity and cross-reactivity to purified parvalbumins from the nine fish species.

Notes: (A) Analysis of IgE reactivities (absorbance at 490 nm) of patient sera to parvalbumins purified from nine fish species using ELISA. (B) Analysis of antigenic cross-reactivity between fish parvalbumins using inhibition ELISA. IgE reactivities of immobilized Pacific mackerel parvalbumin were inhibited by parvalbumins purified from nine types of fish as inhibitors. P1–8 indicates patients 1–8.

parvalbumin has the same IgE-binding epitopes, and their structures are highly conserved. In some patients, Japanese eel, skipjack, and Japanese flounder might have species-specific IgE-binding epitope(s).

Then, we assessed the effect of parvalbumin content on fish allergenicity. We quantified parvalbumin content in the nine fish species by ELISA (Fig. 2(A)) using each purified parvalbumin as a standard to construct calibration curves (Supplementary Fig. S2), as previously described.¹⁰ Japanese eel, red sea bream, and Japanese flounder contained high levels of parvalbumin, ranging 4.76–7.33 mg/g (Fig. 2(A)). Conversely, parvalbumin concentrations in skipjack (0.42 mg/g) and bigeye tuna (0.33 mg/g) were markedly low (Fig. 2(A)). These data are consistent with our previous findings that small-sized sedentary fish contain more parvalbumins than large-sized migratory fish.⁶ In addition, skipjack and bigeye tuna have a developed pink

muscle. It has been known that pink muscle contains lower parvalbumin than white muscle.¹³

As a next step, we evaluated allergenicity of each parvalbumin using a unique way, “an allergenicity index,” which is calculated based on affinity of parvalbumin molecule against IgE and content of parvalbumin in muscle as follows. First, the relative IgE reactivity per fixed amount of immobilized parvalbumin was calculated from IgE reactivity of each parvalbumin when IgE reactivity of Japanese eel was assumed to be 1 by an exponential function (Supplementary Fig. S3). Second, we fixed the parvalbumin content of Japanese eel as 1, and calculated the relative concentrations of parvalbumin in the remaining eight fish using linear functions. Third, relative IgE reactivities to purified parvalbumins were multiplied by relative concentrations of parvalbumins, and the obtained values were the allergenicity indices (Fig. 2B). The formula is described below:

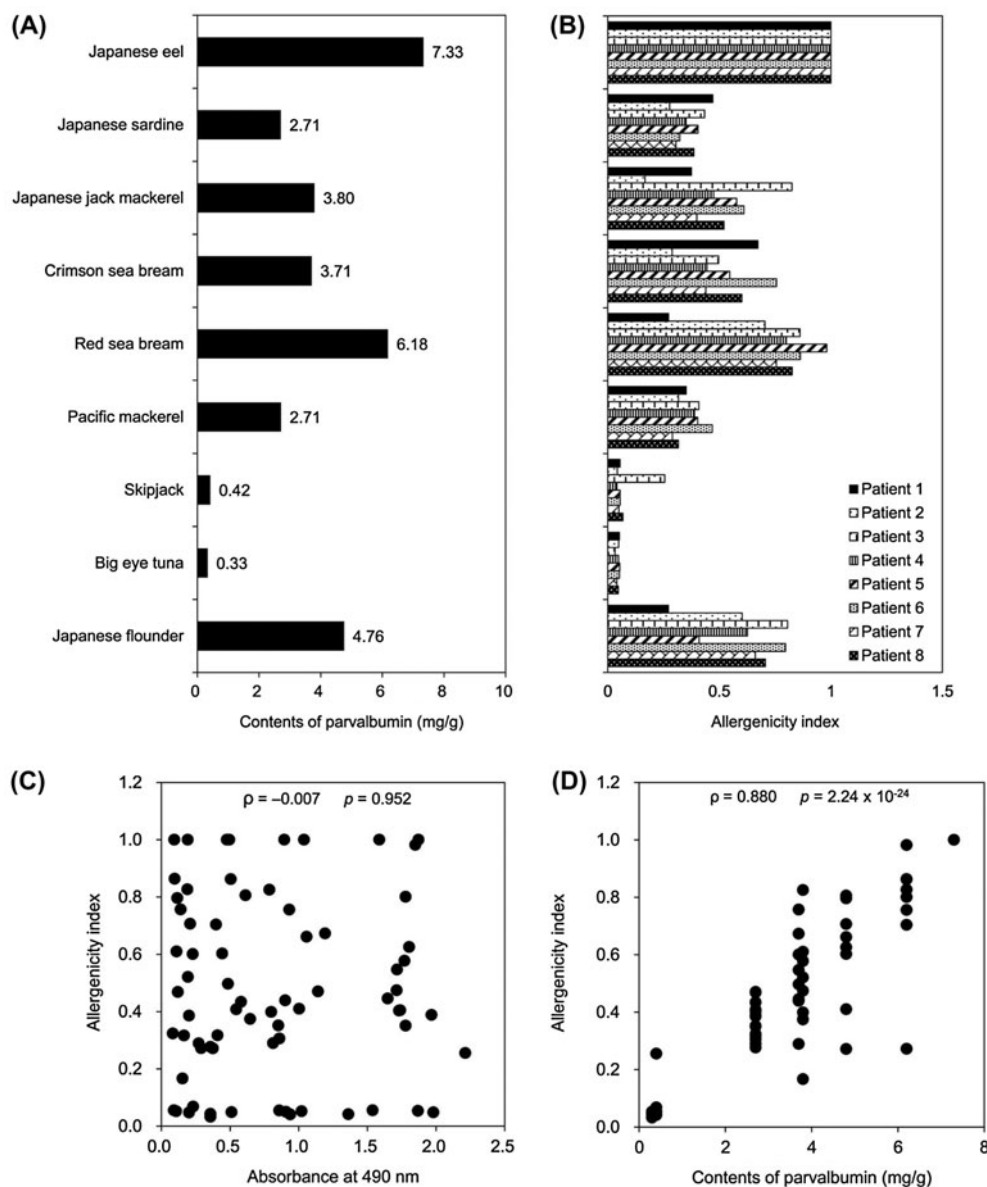


Fig. 2. Parvalbumin content, allergenicity indices, and relationships between allergenicity indices versus IgE reactivities or parvalbumin contents. Notes: (A) Parvalbumin content in fish meat for the nine fish species. (B) Allergenicities (allergenicity index) based on IgE reactivities and parvalbumin content. (C) Correlation between IgE reactivities per fixed amount of immobilized parvalbumin (absorbance at 490 nm in ELISA) and allergenicity indices. (D) Correlation between parvalbumin content and allergenicity indices.

$$\text{Allergenicity index} = \left[\frac{\text{relative IgE reactivity per fixed amount of immobilized parvalbumin (affinity)}}{\times [\text{relative concentration of parvalbumin in muscle (quantity)}]} \right]$$

As a result, allergenicity indices of Japanese eel, red sea bream, and Japanese flounder were high and that of skipjack, and big eye tuna were low.

To appraise whether fish allergenicity is affected by IgE reactivity per fixed amount of immobilized purified parvalbumin or parvalbumin content, we performed Spearman's rank order correlation analysis for added clarity. IgE reactivities per fixed amount of immobilized purified parvalbumin (absorbance at 490 nm in ELISA) did not correlate with allergenicity indices ($\rho = -0.007$, $p = 0.925$; Fig. 2(C)). On the other hand, parvalbumin content significantly correlated with allergenicity indices ($\rho = 0.880$, $p = 2.24 \times 10^{-24}$; Fig. 2(D)). These results indicate that allergenicities of fish are not determined by qualitative differences of parvalbumin molecules but by the overall parvalbumin content in fish meat.

In summary, our results indicate that IgE-binding epitopes of parvalbumin are highly conserved among various fish species. Furthermore, we clarified that fish allergenicities are determined by the overall parvalbumin content in fish muscles. Therefore, our data further support that the evaluation of fish allergenicities should focus on parvalbumin content as previously reported.⁶⁾ Moreover, Asian countries, such as Japan, consume a particularly large variety of fish species in their diet. Although the number of fish whose parvalbumin content has been clarified is currently limited, we have recently developed an easy method to quantify parvalbumin content in fish meat.⁶⁾ Therefore, this method will make it possible to create a database of the parvalbumin content of many fish species. Such information would be useful for clinicians to accurately diagnose fish allergies and provide patients with appropriate dietary advice.

Ethics statement

Written informed consent was obtained from each patient. All experiments using human sera were performed in compliance with the Ethical Guidelines of Tokyo University of Marine Science and Technology (Permit Number: 26-002). The study was conducted in accordance with the principles embodied in the Declaration of Helsinki.

Author contributions

Ayako Kobayashi conducted the experiments. Yukihiro Kobayashi conducted experiments and wrote the article. Kazuo Shiomi supervised all experiments.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplementary material

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