

Reactivity of serum immunoglobulin E to bullfrog *Rana catesbeiana* parvalbumins in fish-allergic patients

YUKI HAMADA,^a YUJI NAGASHIMA AND KAZUO SHIOMI*

Department of Food Science and Technology, Tokyo University of Marine Science and Technology, Minato, Tokyo 108-8477, Japan

ABSTRACT: Parvalbumin, a calcium-binding sarcoplasmic protein of approximately 12 kDa, represents the cross-reactive, major allergen in fish. In consideration of the fact that parvalbumin is contained at high levels not only in fish muscle but also in frog muscle, the present study was undertaken to clarify whether fish-allergic patients react to two parvalbumins (α - and β -parvalbumins) purified from the bullfrog *Rana catesbeiana*, which is sometimes consumed as a delicacy in Japan. In enzyme-linked immunosorbent assays (ELISA), sera from 12 of the 14 patients tested reacted equally to both parvalbumins purified from the Pacific mackerel *Scomber japonicus* and the bigeye tuna *Thunnus obesus*. Of the 12 sera positive to fish parvalbumins, eight sera also reacted to α - and β -parvalbumins of the bullfrog with different spectra: one serum reacted strongly to α -parvalbumin, six sera reacted strongly to β -parvalbumin and one serum reacted equally to both α - and β -parvalbumins. In addition, inhibition ELISA experiments revealed cross-reactivity between fish and bullfrog parvalbumins. Based on these results, it is proposed that fish-allergic patients should avoid the consumption of frog meat unless they are accurately diagnosed as lacking immunoglobulin E against frog.

KEY WORDS: allergen, cross-reactivity, fish, frog, parvalbumin, *Rana catesbeiana*.

INTRODUCTION

Fish is one of the most frequent causes of food allergy mediated by immunoglobulin (Ig)E antibodies, especially in coastal countries such as Japan and Scandinavia where large quantities of fish are consumed daily. Following ingestion of fish, acute symptoms such as urticaria, asthma and vomiting are induced in sensitized patients; even fatal cases with anaphylactic reactions have been recorded.^{1,2} Extensive studies performed with cod-fish *Gadus callarias* first demonstrated that the major allergen, known as Gad c 1, is parvalbumin.^{3,4} Subsequent molecular studies^{5–10} and immunoblotting studies^{1,11–13} have led to the conclusion that parvalbumin is the cross-reactive, major allergen that various species of fish have in common, although minor allergens such as collagen^{14–16} and aldehyde phosphate dehydrogenase¹⁷ have also been detected.

Parvalbumin is a calcium-binding sarcoplasmic protein with a molecular mass of approximately 12 kDa and is assumed to be involved in the process of muscle relaxation.¹⁸ This protein is found in various tissues of vertebrates¹⁹ and is especially found at high levels in the muscle of lower vertebrates such as fishes and amphibians.²⁰ Of amphibians, several species of bullfrogs belonging to the genus *Rana* are eaten as a delicacy by a limited number of people in the world. Although these are not very frequent, incidents of food allergy as a result of the ingestion of frog meat have been reported.^{21,22} Interestingly, a 47-year-old male patient with frog allergy was shown to react to α -parvalbumin of *Rana* sp. (unidentified species).²² Unfortunately, no information was obtained as to whether this patient was also sensitive to fish parvalbumin.

As frog parvalbumins are allergenic as mentioned above, it is very likely that fish-allergic patients recognize frog parvalbumins as well as fish parvalbumins and develop hypersensitivity reactions after eating frog meat. In the present study, therefore, sera from fish-allergic patients were evaluated for their reactivities to α - and β -parvalbumins purified from the bullfrog *Rana catesbeiana*, which is sometimes consumed in Japan, as well as parvalbumins from two species of

*Corresponding author: Tel: 81-3-5463-0601.

Fax: 81-3-5463-0669. Email: shiomi@s.kaiyodai.ac.jp

^aPresent address: Laboratory of Fishery Nutritional Science, Faculty of Fisheries, Nagasaki University, Nagasaki 852-8521, Japan.

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fish (Pacific mackerel and bigeye tuna). Antigenic cross-reactivity between frog and fish parvalbumins was also examined.

MATERIALS AND METHODS

Biological samples

Live specimens of the bullfrog *R. catesbeiana* were purchased at the Tokyo Central Wholesale Market and fresh specimens of the Pacific mackerel *Scomber japonicus* and fresh muscle lumps of the bigeye tuna *Thunnus obesus* were purchased from a local fish market in Tokyo. These samples were immediately subjected to experiments or stored at -20°C until use.

Human sera

Sera were obtained from 14 fish-allergic patients (patients 1–14) with a clinical history of immediate hypersensitivity reactions after ingestion of fish. These patients were all diagnosed at hospitals to have elevated serum IgE against several species of fish, such as cod and tuna, by radioallergosorbent tests. In the present study, sera from two healthy subjects 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

Enzyme-linked immunosorbent assay (ELISA) was performed to detect IgE against different parvalbumins as has been reported previously.²³ In brief, each sample (0.1 $\mu\text{g}/\text{well}$) coated on a flat-bottomed polystyrene plate with 96 wells (Type H Multi Well Plate for ELISA; Sumitomo Bakelite, Tokyo, Japan) was reacted with patient or control serum (diluted 1:50), followed by peroxidase-conjugated goat antihuman IgE antibody (diluted 1:2500; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Enzyme reaction was carried out using substrate solution (0.1% o-phenylenediamine and 0.03% hydrogen peroxide) and the developed color was measured by absorbance at 490 nm. ELISA was also performed to detect parvalbumins during chromatographic steps. In this case, monoclonal antifrog muscle parvalbumin antibody (diluted 1:5000; Sigma, St Louis, MO, USA) was used as a primary antibody and peroxidase-conjugated goat antimouse IgG

antibody (diluted 1:5000; Kirkegaard & Perry Laboratories) as a secondary antibody.

Cross-reactivity between Pacific mackerel and bigeye tuna parvalbumins or between fish and bullfrog parvalbumins was examined by inhibition ELISA experiments. Each patient's serum (diluted 1:25) was incubated at 37°C for 1 h with an equal volume of fish or bullfrog parvalbumin solution (10 $\mu\text{g}/\text{mL}$). A 100- μL portion of this solution was then added to a microtiter plate that had previously been coated with fish or bullfrog parvalbumin at a concentration of 0.1 $\mu\text{g}/\text{well}$. The subsequent procedure was the same as that for the ELISA described above.

All ELISA (including inhibition ELISA) were performed in triplicate and the data were expressed as mean values.

Purification of parvalbumin

Pacific mackerel and bigeye tuna parvalbumins were purified from the white muscle by a combination of gel filtration and reverse-phase high-performance liquid chromatography (HPLC), as has been reported previously.^{10,16} The Pacific mackerel parvalbumin (Scom j 1) has been confirmed to be a member of β -type parvalbumin from its determined primary structure,¹⁰ whereas it is unknown to which type of parvalbumins the bigeye tuna parvalbumin (Thun o 1) belongs, the α - or β -type. Essentially the same method was adopted for the purification of bullfrog parvalbumins as follows. The bullfrog skeletal muscle was homogenized with three volumes of 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.0) and centrifuged at $18000 \times g$ for 20 min at 4°C . The supernatant obtained was concentrated *in vacuo* and subjected to gel filtration on a Sephadex G-75 column (2.5 cm $\times$ 110.0 cm; Amersham Pharmacia Biotech, Buckinghamshire, UK), which was eluted with 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.0). Fractions of 10 mL were collected, measured for absorbance at 280 nm and assayed for parvalbumin by ELISA. Parvalbumin-containing fractions were pooled and then applied to reverse-phase HPLC on a TSKgel ODS-120T column (0.46 cm $\times$ 25.00 cm; Tosoh, Tokyo, Japan). The column was eluted at a flow rate of 1 mL/min with 0.1% trifluoroacetic acid initially for 5 min, followed by a linear gradient of acetonitrile (0.0–38.5% in 2 min, 38.5–45.5% in 60 min and 45.5–70.0% in 2 min) in 0.1% trifluoroacetic acid. Proteins were monitored at 220 nm with a UV detector. Thus, two parvalbumin components (PA1 and PA2) were obtained and used in the subsequent experiments.

Protein determination

Protein was determined by the method of Lowry *et al.* using bovine serum albumin as a standard protein.²⁴

Sodium dodecylsulfate–polyacrylamide gel electrophoresis

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a PhastSystem (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Ready-made gels (PhastGel High Density), ready-made buffer strips (PhastGel SDS Buffer Strips) and a peptide marker kit (2 512–16 949 Da) were purchased from Amersham Pharmacia Biotech. Prior to electrophoresis, each sample was treated with 2.5% sodium dodecylsulfate containing 1% dithiothreitol at 100°C for 10 min. Proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Nacalai Tesque, Kyoto, Japan).

Enzyme digestion and isolation of peptide fragments

Bullfrog parvalbumin PA2 (100 µg) was digested with 2 µg of lysylendopeptidase (E.C. 3.4.21.50; Wako Pure Chemical, Osaka, Japan) in 1 mL of 25 mM Tris HCl buffer (pH 8.5) containing 1 mM EDTA at 37°C for 24 h. In order to separate peptide fragments, the digest was applied to reverse-phase HPLC on a TSKgel ODS-120T column (0.46 cm × 25.00 cm), which was eluted at a flow rate of 1 mL/min by a linear gradient of acetonitrile (0–50% in 120 min) in 0.1% trifluoroacetic acid. Peptides were monitored at 220 nm with a UV detector.

Amino acid sequencing

Amino acid sequencing was performed by the automated Edman degradation method using a gas-phase protein sequencer (LF-3400D TriCart with high sensitivity chemistry; Beckman Coulter, Fullerton, CA, USA).

RESULTS

Purification of bullfrog parvalbumins

In gel filtration on Sephadex G-75, bullfrog parvalbumins were eluted at fractions 35–45 (Fig. 1a).

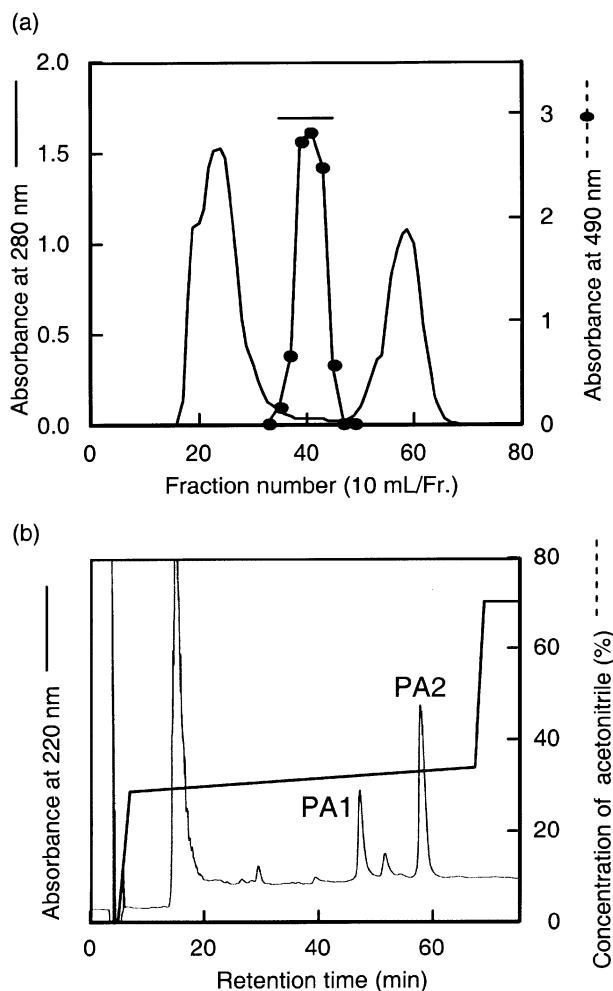


Fig. 1 Purification of parvalbumins from the muscle of bullfrog. (a) Gel filtration: sample, muscle extract; column, Sephadex G-75 (2.5 cm × 110.0 cm; Amersham Pharmacia Biotech, Buckinghamshire, UK); solvent, 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.0); pooled fractions are indicated by a bar. (b) Reverse-phase high-performance liquid chromatography: sample, parvalbumin-containing fraction obtained by gel filtration; column, TSKgel ODS-120T (0.46 cm × 25.00 cm; Tosoh, Tokyo, Japan); elution, linear gradient of acetonitrile in 0.1% trifluoroacetic acid; flow rate, 1 mL/min; two parvalbumins (PA1 and PA2) were eluted in the labeled peaks.

When the parvalbumin-containing fraction obtained by gel filtration was subjected to reverse-phase HPLC on TSKgel ODS-120T, parvalbumins detected by ELISA appeared in three peaks with retention times of 47.0, 51.5 and 58.0 min (Fig. 1b). The preparation in the smallest peak, with a retention time of 51.5 min, was found to be a mixture of at least two parvalbumin components as analyzed by SDS-PAGE (data not shown). In contrast, both

preparations in the other two peaks, similar to parvalbumins purified from Pacific mackerel and bigeye tuna, migrated as a single band at a position corresponding to approximately 11 kDa in SDS-PAGE, supporting their homogeneity (Fig. 2). In the present study, therefore, only these two preparations (named PA1 and PA2 in the order of elution) were used in the subsequent experiments.

Direct application of PA1 to a protein sequencer identified the first 30 amino acid residues as follows: MHMTDVLPAAGDISKAVEAFAAPDSFNHKKF. This sequence was completely consistent with the N-terminal sequence of α -type parvalbumin previously purified from *R. catesbeiana* (Fig. 3).²⁵ In contrast, analysis of PA2 by a protein sequencer was unsuccessful, suggesting the blocking of its N-

terminal residue. Therefore, PA2 was digested with lysylendopeptidase to obtain information as to its partial amino acid sequence. Peptide fragments were separated from the digest by reverse-phase HPLC on TSKgel ODS-120T and three of them were selected and sequenced (Fig. 4). The sequences determined, DIEAALESVK, AEGSFNYK and VFEILDRDRSGFIEEEELCLFLQNFK, were identical to segments 10–19, 20–27 and 46–71 of the β -type parvalbumin previously purified from *R. catesbeiana*, respectively (Fig. 3).²⁶ Thus, PA1 and PA2 were unequivocally identified as the α - and β -parvalbumins of *R. catesbeiana*, respectively.

Reactivity of immunoglobulin E to fish and bullfrog parvalbumins in fish-allergic patients

When analyzed by ELISA, 12 of 14 sera from fish-allergic patients reacted to both Pacific mackerel parvalbumin (Sco j 1) and bigeye tuna parvalbumin (Thu o 1); each serum reacted almost equally to both parvalbumins but the potencies varied largely among the sera (Fig. 5). In contrast, the remaining two sera (from patients 13 and 14) reacted to neither Sco j 1 nor Thu o 1. In the preliminary experiments, these sera were found to react to collagen purified from the bigeye tuna muscle, further supporting the previous finding that some fish-allergic patients react to not parvalbumin but collagen.^{15,16}

The reactivities of patient sera to the two bullfrog parvalbumins (PA1 and PA2) were much more complicated than expected compared with reactivities to fish parvalbumins. One serum (from patient 1) reacted much more strongly to PA1 than to PA2 and six sera (from patients 2–7) showed a reverse pattern. However, one serum (from patient 8) reacted almost equally to both PA1 and PA2. In addition, although four sera (from patients 9–12) significantly reacted to both Sco j 1 and Thu o 1, they were substantially negative to both PA1 and PA2. No reactivities to bullfrog

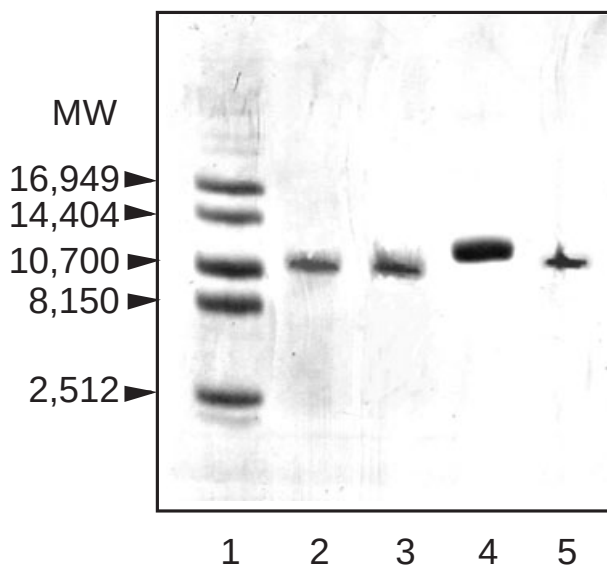


Fig. 2 Sodium dodecylsulfate-polyacrylamide gel electrophoresis of parvalbumins purified from Pacific mackerel *Scomber japonicus* (Sco j 1), bigeye tuna *Thunnus obesus* (Thu o 1) and bullfrog *Rana catesbeiana* (PA1 and PA2). Lane 1, standard proteins; lane 2, Sco j 1; lane 3, Thu o 1; lane 4, PA1; lane 5, PA2.

	1	10	20	30	40	50	60
<i>Rana catesbeiana</i> α	M	H	M	T	D	V	L
<i>Rana catesbeiana</i> β	S	I	-	I	V	S	Q
<i>Rana esculenta</i> α	P	-	-	L	-	-	-
<i>Rana esculenta</i> β	S	I	-	I	V	S	Q
<i>Rana sp.</i> α	-	P	-	-	-	-	-
<i>Rana sp.</i> β	-	S	I	-	I	V	S
	61	70	80	90	100	110	
<i>Rana catesbeiana</i> α	E	D	E	L	C	L	M
<i>Rana catesbeiana</i> β	-	E	-	-	-	-	-
<i>Rana esculenta</i> α	K	E	-	-	-	-	-
<i>Rana esculenta</i> β	Q	-	-	-	-	-	-
<i>Rana sp.</i> α	K	-	-	-	-	-	-
<i>Rana sp.</i> β	K	-	-	-	-	-	-

Fig. 3 Amino acid sequences of α - and β -parvalbumins from three species of frogs (*Rana catesbeiana*, *Rana esculenta* and *Rana sp.*). Residues identical to *R. catesbeiana* α -parvalbumin are indicated by dashes. Thirteen unique residues, which are suggested to be responsible for the selective reactivity of a frog-allergic patient to the α -parvalbumin of *Rana sp.*, are boxed.²²

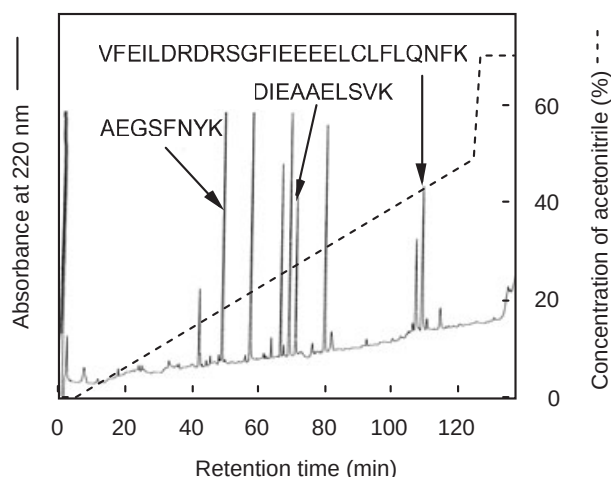


Fig. 4 Isolation of peptide fragments from the lysylendopeptidase digest of bullfrog parvalbumin PA2 by reverse-phase high-performance liquid chromatography on TSKgel ODS-120T (Tosoh, Tokyo, Japan). The amino acid sequences determined from three peptide fragments are included.

parvalbumins were observed in two sera (from patients 13 and 14) that reacted to fish collagens but not fish parvalbumins.

Antigenic cross-reactivity between fish and bullfrog parvalbumins

Inhibition ELISA experiments were performed using five sera from patients 2, 5, 6, 8 and 9. Regardless of the sera, reactivities to Sco j 1 and Thu o 1 were all reduced by preabsorption with Sco j 1, suggesting cross-reactivity between both fish parvalbumins (Fig. 6). In the case of three sera (from patients 2, 5 and 6) that reacted strongly to PA2 but weakly to PA1, preabsorption with PA2 inhibited reactivities to Sco j 1, Thu o 1 and PA2, and preabsorption with Sco j 1 inhibited reactivity to PA2. Moreover, preabsorption with PA1 inhibited reactivities to Sco j 1, Thu o 1 and PA2, although to a lesser extent than in the case of preabsorption with PA2. These results reveal that PA1

Fig. 5 Analysis by enzyme-linked immunosorbent assay of immunoglobulin E reactivities of sera from 14 patients and two controls to Pacific mackerel *Scomber japonicus* (Sco j 1), bigeye tuna *Thunnus obesus* (Thu o 1) and bullfrog *Rana catesbeiana* (PA1 and PA2) parvalbumins. Each parvalbumin was coated on the plate at 0.1 µg/well and reacted with patient or control serum (1:50 dilution).

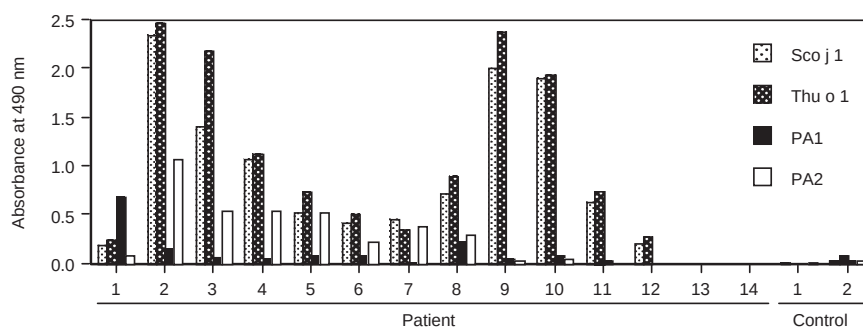
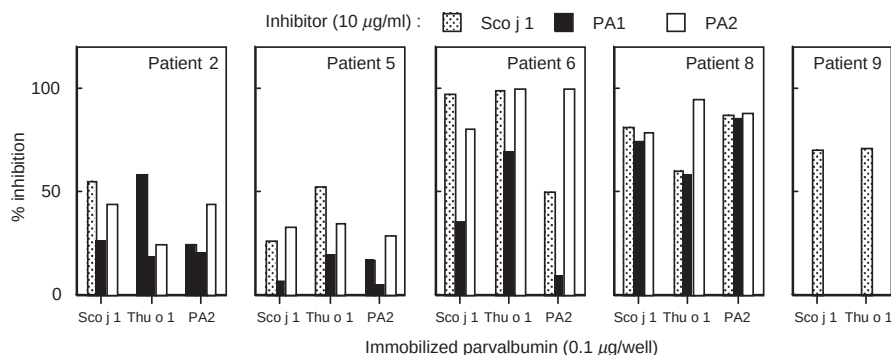


Fig. 6 Analysis by inhibition enzyme-linked immunosorbent assay of antigenic cross-reactivity between fish and bullfrog parvalbumins. Each patient serum (1:25 dilution) was incubated with an equal volume of inhibitor (fish or bullfrog parvalbumin) solution (10 µg/mL) and added to a microtiter plate previously coated with fish or bullfrog parvalbumin (0.1 µg/well). For control experiments, the serum (1:25 dilution) incubated with an equal volume of buffer was used. By comparing the absorbance in inhibition experiments with that in control experiments, the percentage inhibition was calculated.



and PA2 are cross-reactive with each other and also with fish parvalbumins. This was more obviously evidenced by the inhibition ELISA experiments using the patient 8 serum, which reacted equally to PA1 and PA2; the reactivities to Sco j 1, Thu o 1 and PA2 were all significantly inhibited by Sco j 1, PA1 and PA2.

DISCUSSION

Enzyme-linked immunosorbent assay data demonstrated that sera from as many as 12 of the 14 patients used in the present study were reactive to both Sco j 1 and Thu o 1, in accordance with the current opinion that parvalbumin is the major allergen in fish. The two sera (from patients 13 and 14) with no reactivities to both Sco j 1 and Thu o 1 were found to have IgE directed to fish collagen. Importantly, eight of the 12 sera that reacted to fish parvalbumins reacted to the α - and β -parvalbumins of *R. catesbeiana*. Based on this finding, it is proposed that fish-allergic patients should avoid consumption of frog meat unless they are accurately diagnosed as lacking serum IgE against frog.

The 12 sera reacting to fish parvalbumins were interestingly classified into the following four groups in terms of reactivity to the α - and β -parvalbumins of *R. catesbeiana*: group 1 (patient 1 serum) reacted much more strongly to α -parvalbumin than to β -parvalbumin; group 2 (sera from patients 2–7) reacted much more strongly to β -parvalbumin than to α -parvalbumin; group 3 (patient 8 serum) reacted almost equally to both parvalbumins; and group 4 (sera from patients 9–12) reacted to neither α -parvalbumin nor β -parvalbumin. In the present study, three sera (from patients 2, 5 and 6) belonging to group 2, one serum (from patient 8) belonging to group 3 and one serum (from patient 9) belonging to group 4 were used in inhibition ELISA experiments. The results obtained prove the antigenic cross-reactivity between Sco j 1 and Thu o 1, regardless of the sera. Moreover, the cross-reactivities between fish and bullfrog parvalbumins and between bullfrog α - and β -parvalbumins were recognized in both group 2 and group 3. This is probably the case with the patient 1 serum (group 1), although this was not confirmed because of the scarcity of the available serum sample.

Despite the observed antigenic cross-reactivity between fish and bullfrog parvalbumins, the patient sera used in the present study showed markedly different reactivities to fish and bullfrog parvalbumins; almost equal reactivities to two fish parvalbumins (Sco j 1 and Thu o 1) were observed in all sera, whereas the reactivities to two bullfrog

parvalbumins (α - and β -parvalbumins) varied from serum to serum. These results are of particular importance in showing that at least some of the IgE binding epitopes of fish parvalbumins vary among fish-allergic patients. In future studies on the epitopes of fish parvalbumins, useful data will be obtained if, prior to use, sera from fish-allergic patients are classified into four groups based on reactivity to the α - and β -parvalbumins of *R. catesbeiana*.

Serum from only one frog-allergic patient (a 47-year-old man) has so far been examined for reactivity to frog parvalbumins.²² Interestingly, the reactivity was selective to the α -parvalbumin of *Rana* sp., with no cross-reactivities with the β -parvalbumin of *Rana* sp. and the α - and β -parvalbumins of *Rana esculenta*. Compared with the amino acid sequences of the latter three parvalbumins, the α -parvalbumin of *Rana* sp. has unique residues at 13 positions (Fig. 3), which are suggested to be responsible for the selective reactivity of the patient serum to the α -parvalbumin of *Rana* sp.²² However, none of the 13 unique residues contained in the α -parvalbumin from *Rana* sp. are recognized in the α - and β -parvalbumins of *R. catesbeiana*, except for one residue (Val at position 6) conserved in α -parvalbumin. Further detailed study is needed to confirm which residues are associated with the binding of frog parvalbumins to IgE.

The present study is the first to provide evidence that not all but many fish-allergic patients who react to fish parvalbumins are also sensitive to parvalbumins from the bullfrog *R. catesbeiana*. However, reactivities to the α - and β -parvalbumins of *R. catesbeiana* differ remarkably among fish-allergic patients. Frog parvalbumins might be useful references in future study on the IgE-binding epitopes of fish parvalbumins and also in the diagnosis of fish allergy.

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