

Original Article

Reactivity of IgE in fish-allergic patients to fish muscle collagen

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ABSTRACT

Background: In addition to parvalbumin, the well-known major allergen in fish, collagen was recently identified as a new allergen in the muscle of bigeye tuna and in the skin of several species of fish. The aim of the present study was to evaluate fish muscle collagens for their reactivity with IgE in fish-allergic patients and antigenic cross-reactivity.

Methods: Collagen was purified from the white muscle of five species of fish (Japanese eel, alfonso, mackerel, skipjack and bigeye tuna) by acid extraction and salt precipitation, whereas parvalbumin was purified from bigeye tuna by gel filtration and reverse-phase HPLC. The IgE reactivities to collagen and parvalbumin were examined by ELISA, whereas antigenic cross-reactivity among fish muscle collagens was investigated by ELISA inhibition experiments.

Results: When 15 sera from fish-allergic patients were subjected to ELISA using bigeye tuna collagen and parvalbumin, 10 sera reacted only to parvalbumin, two reacted only to collagen, two reacted to both collagen and parvalbumin and one reacted to neither collagen nor parvalbumin. The sera containing specific IgE to bigeye tuna collagen also reacted to collagens from the other four species of fish. In the ELISA inhibition experiments, bigeye tuna collagen inhibited the binding of IgE not only to bigeye tuna collagen, but

also to that from the other four species of fish, suggesting cross-reactivity among the collagens from five species of fish.

Conclusions: These results demonstrate that some Japanese fish-allergic patients have specific IgE to fish muscle collagen and that fish muscle collagen is a cross-reactive allergen among various species of fish.

Key words: allergen, collagen, fish, muscle, parvalbumin.

INTRODUCTION

Fish allergy is prevalent in coastal countries, such as Japan and Scandinavia, where fish consumption is high. Ingestion of fish causes acute symptoms, such as urticaria, asthma and vomiting, in sensitized patients^{1–3} and, in severe cases, may be fatal with anaphylactic reactions.^{4,5} Extensive studies with codfish (*Gadus callarias*) established that the major allergen, known as Gad c 1, is parvalbumin, a calcium-binding sarcoplasmic protein with a molecular mass of 12 kDa.^{6,7} Subsequently, the major allergen in three species of fish, namely carp,^{8,9} Atlantic salmon¹⁰ and Japanese horse mackerel,¹¹ was similarly identified as parvalbumin at the molecular level. Several immunoblotting studies^{2,12–14} also suggested the major fish allergen to be parvalbumin.

As for fish allergens other than parvalbumin, Sakaguchi *et al.*¹⁵ reported that three of 10 fish-allergic patients have specific IgE to gelatin (denatured collagen) prepared from the skin of several species of fish. Independently of that study, we recently detected a new allergen in the muscle of Japanese eel and bigeye tuna

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that has a higher molecular weight than parvalbumin.¹⁶ In addition, we have purified the higher molecular weight allergen from bigeye tuna muscle and have demonstrated it to be collagen.¹⁷ It is worth mentioning that as many as five of eight sera from fish-allergic patients reacted to bigeye tuna muscle collagen. These results suggest that fish collagen, regardless of its origin, is an important fish allergen. From the viewpoints of the diagnosis and treatment of fish allergy, it is essential to confirm the suggested importance of collagen, especially muscle collagen, as a fish allergen. Therefore, in the present study, sera obtained from 15 fish-allergic patients were first examined for their IgE reactivities to collagen and parvalbumin purified from bigeye tuna muscle. Then, collagens purified from five species of fish were compared with one another as to IgE binding ability. Finally, antigenic cross-reactivity among fish muscle collagens was estimated.

METHODS

Fish

Live or fresh specimens of Japanese eel (*Anguilla japonica*), alfonsoin (*Beryx splendens*), mackerel (*Scomber japonicus*) and skipjack (*Euthynnus pelamis*), as well as fresh muscle lumps of bigeye tuna (*Thunnus obesus*), were purchased from a local fish market and either immediately subjected to experiments or stored at -20°C until use.

Human sera

Sera were obtained from 15 fish-allergic patients (Table 1), who had a documented clinical history of immediate hypersensitivity reactions, such as urticaria, asthma and diarrhea, after ingestion of fish. In the present study, sera from three healthy subjects without adverse reactions after ingestion of any foods were used as controls. All sera were stored at -20°C until use.

ELISA

The IgE reactivities of the sera to fish collagen and parvalbumin were examined by ELISA using a flat-bottomed polystyrene plate with 96 wells (Type H Multi Well Plate for ELISA; Sumitomo Bakelite, Tokyo, Japan), as reported previously.¹⁸ In brief, the plate was coated with 100 μL sample and blocked with 350 μL of 1% bovine serum albumin (BSA) in Dulbecco's phosphate-buffered saline (DPBS; pH 7.4). The plate was then reacted with patient or control serum, diluted 1 : 50 with 0.1% BSA in DPBS, followed by peroxidase-conjugated goat antihuman IgE antibody (diluted 1 : 2500 with 0.1% BSA in DPBS; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The enzyme reaction was performed using a substrate solution containing 0.1% o-phenylenediamine and 0.03% hydrogen peroxide and arrested by addition of 1 mol/L sulfuric acid. The color developed was measured by absorbance at 490 nm. In the ELISA to detect parvalbumin in chromatographic steps, the serum was replaced by monoclonal antibody

Table 1 CAP-RAST class to fish

Patient	Age (years)	Sex	CAP-RAST class
1	1	Male	Salmon (2), cod (1), sardine (3), tuna (1), flatfish (3)
2	1	Female	Salmon (3), cod (3)
3	2	Male	Sardine (3), salmon (2), cod (2), horse mackerel (2)
4	2	Male	Salmon (3), cod (3), horse mackerel (4)
5	2	Female	Salmon (3), cod (3), horse mackerel (3), mackerel (3)
6	3	Female	Horse mackerel (4), mackerel (3), tuna (3)
7	3	Male	Cod (2), tuna (2)
8	4	Male	Salmon (3)
9	4	Male	Mackerel (2)
10	4	Male	Salmon (3), cod (2), horse mackerel (3), flatfish (2)
11	4	Female	Salmon (4), cod (3)
12	15	Male	Sardine (3), salmon (3), cod (3), horse mackerel (3), mackerel (3), tuna (3)
13	19	Male	Cod (4), horse mackerel (3), mackerel (3), tuna (4)
14	22	Female	Salmon (4)
15	27	Female	Sardine (3), salmon (4), cod (4), horse mackerel (4), mackerel (3), tuna (3), flatfish (4)

against frog muscle parvalbumin (diluted 1 : 5000 with 0.1% BSA in DPBS; Sigma, St Louis, MO, USA) and peroxidase-conjugated goat antihuman IgE antibody by peroxidase-conjugated goat antimouse IgG antibody (diluted 1 : 5000 with 0.1% BSA in DPBS; Kirkegaard & Perry Laboratories).

Cross-reactivity among collagens from five species of fish was examined by ELISA inhibition experiments. Each patient's serum was diluted 1 : 25 with 0.1% BSA in DPBS and incubated at 37°C for 1 h with an equal volume of bigeye tuna collagen solution (10 µg/mL) made in DPBS. A 100 µL aliquot of this solution was then added to a microtiter plate that had been coated previously with collagens from five species of fish at a concentration of 0.1 µg/well. The subsequent procedure was the same as that for the ELISA described above.

All ELISA were performed in triplicate and the data are expressed as mean values.

Protein determination

Proteins were determined by the method of Lowry *et al.*¹⁹ using BSA as a standard.

Purification of collagen and parvalbumin

Collagen (type I collagen) was purified from the white muscle of each species of fish by extraction with 0.5 mol/L acetic acid and precipitation with 0.8 mol/L NaCl, according to the method of Miller and Rhodes.²⁰ Because almost pure collagen is quantitatively extractable with 0.5 mol/L acetic acid, the collagen content in the muscle was calculated based on the amount of protein in the acetic acid extract and the weight of the starting material.

For the purification of bigeye tuna parvalbumin, the white muscle was extracted with three volumes of 0.15 mol/L NaCl in 0.01 mol/L phosphate buffer (pH 7.0) in a boiling water bath for 10 min. The heated extract was applied to gel filtration on a Sephadex G-75 column (2.5 × 98 cm; Amersham Pharmacia Biotech, Buckinghamshire, UK), which was eluted with 0.15 mol/L NaCl in 0.01 mol/L phosphate buffer (pH 7.0). Fractions (10 mL) were collected, measured for absorbance at 280 nm and assayed for parvalbumin by ELISA. Parvalbumin-containing fractions were pooled and then subjected to reverse-phase HPLC on a TSKgel ODS-120T column (0.46 × 25 cm; Tosoh, Tokyo, Japan). The column was eluted first with 0.1% trifluoroacetic acid

(TFA), followed by two steps of linear gradients of acetonitrile (0–35% in 2 min and 35–49% in 60 min) in 0.1% TFA, at a flow rate of 1 mL/min. The eluate judged to contain parvalbumin by ELISA was collected manually, lyophilized and dissolved in desired solvents for subsequent experiments.

Electrophoresis

Purified collagens were analyzed by sodium dodecyl-sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on ready made 7.5% gels (PAGEL NPU-7.5 L; Atto, Tokyo, Japan), according to the method of Laemmli.²¹ Prior to SDS-PAGE, collagen samples were dissolved in 0.05 mol/L Tris-HCl buffer (pH 6.8) containing 1% SDS and 20% glycerol and kept at 37°C for 2 min. Electrophoresis was performed at 100 V and 20 mA for 2 h. Together with collagen samples, a HMW-SDS calibration kit containing five kinds of proteins of 53–212 kDa (Amersham Pharmacia Biotech) was run as a reference. After electrophoresis, the gel was stained with Coomassie brilliant blue R-250 (Nacalai Tesque, Kyoto, Japan).

In the case of parvalbumin, 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 molecular weight calibration kit (containing five kinds of proteins or peptides of 2512–16 949 Da) were purchased from Amersham Pharmacia Biotech. Samples were treated with 2.5% SDS containing 5% dithiothreitol at 100°C for 10 min and then subjected to electrophoresis. Proteins were visualized by staining with Coomassie brilliant blue R-250.

Enzyme digestion and isolation of peptide fragments

The purified bigeye tuna parvalbumin (100 µg) was digested with 2 µg lysylendopeptidase (EC 3.4.21.50; Wako Pure Chemicals, Osaka, Japan) in 0.3 mL of 0.025 mol/L Tris-HCl buffer (pH 8.5) containing 1 mmol/L 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 × 25 cm; Tosoh), 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% TFA. Peptides were monitored at 220 nm with an ultraviolet detector.

Amino acid sequencing

Sequence analyses were 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 collagen and parvalbumin

Collagen (type I collagen) was purified from the muscle of five species of fish by a well-established method. When analyzed by SDS-PAGE, each collagen purified

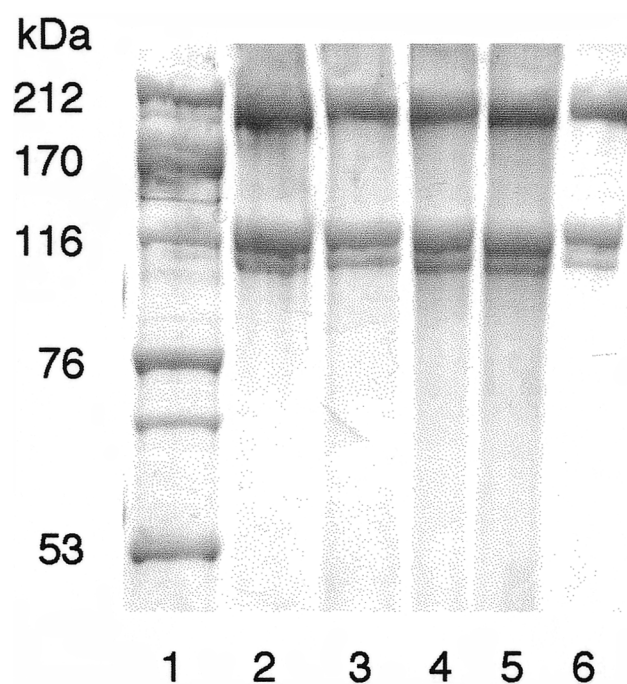
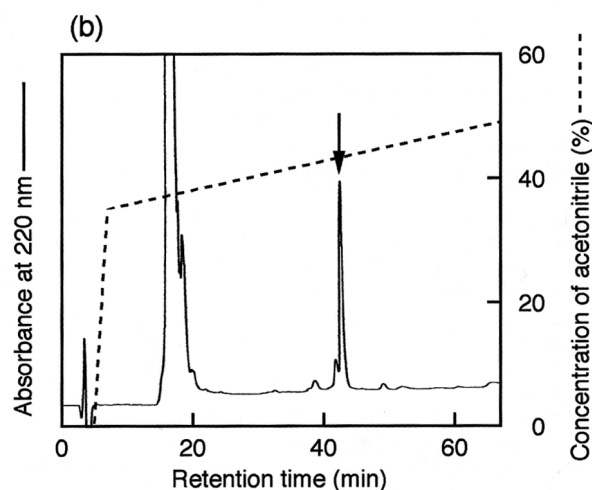
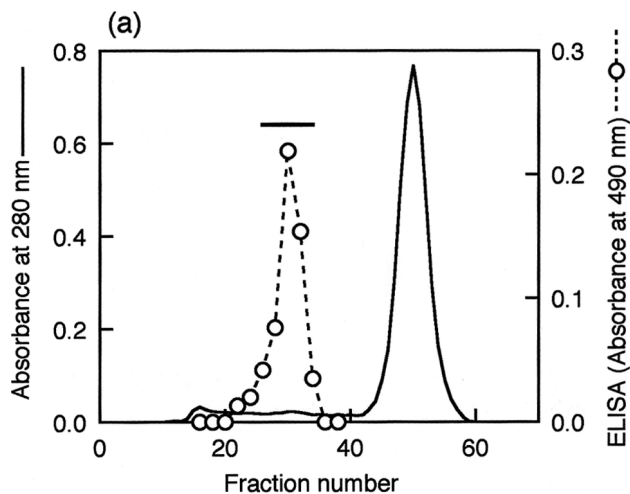


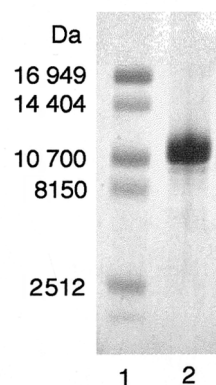
Fig. 1 Sodium dodecylsulfate–polyacrylamide gel electrophoresis of collagens purified from five species of fish. Lane 1, standard proteins; lane 2, Japanese eel; lane 3, alfonsin; lane 4, mackerel; lane 5, skipjack; lane 6, bigeye tuna.

Fig. 2 Purification of parvalbumin from bigeye tuna. (a) Gel filtration. Sample, heated extract; column, Sephadex G-75 (2.5 × 98 cm); solvent, 0.15 mol/L NaCl in 0.01 mol/L phosphate buffer (pH 7.0); volume/fraction, 10 mL. A bar represents the pooled fractions. (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 trifluoroacetic acid; flow rate, 1 mL/min. An arrow represents the peak containing parvalbumin. (c) Sodium dodecylsulfate–polyacrylamide gel electrophoresis of purified parvalbumin. Lane 1, standard proteins; lane 2, purified parvalbumin.

afforded three bands, all of which showed the meta-chromasy characteristic of collagen (Fig. 1). The two bands in the range of 110–120 kDa are attributable to hetero α -chains of collagen and the one band of approximately 210 kDa is attributable to the β -chain (dimer of the α -chain).



(c)



Parvalbumin in the muscle of bigeye tuna was purified by gel filtration on Sephadex G-75 (Fig. 2a) and reverse-phase HPLC on TSKgel ODS-120T (Fig. 2b). In SDS-PAGE, the purified preparation migrated as a single band at a position corresponding to approximately 11 kDa, supporting its homogeneity (Fig. 2c). Analysis by a protein sequencer proved that the N-terminus of the purified preparation is blocked, as known for parvalbumins from cod²² and Atlantic salmon.¹⁰ Therefore, the purified preparation was digested with lysylendopeptidase to obtain information about its partial amino acid sequence. Four of the peptide fragments isolated from the digest by reverse-phase HPLC were selected and sequenced (Fig. 3). Compared with the amino acid sequences reported for fish parvalbumins, such as those from cod,⁷ Atlantic salmon¹⁰ and carp,⁹ AFAIIDQDK, SGYLDEDELK, LFLQNFKLDAK and IGMDEFAAK were easily assignable to the segments of 46–54, 55–64, 65–75 and 97–105, respectively, demonstrating that the purified preparation is certainly parvalbumin.

IgE reactivity to bigeye tuna collagen and parvalbumin

Fifteen patient sera and three control sera were evaluated for IgE reactivities to collagen and parvalbumin

purified from the bigeye tuna muscle by ELISA. As shown in Fig. 4, as many as 10 sera (from patients 2–6, 8–10, 12 and 15) reacted to parvalbumin only with varying potencies; especially high reactivity was displayed by

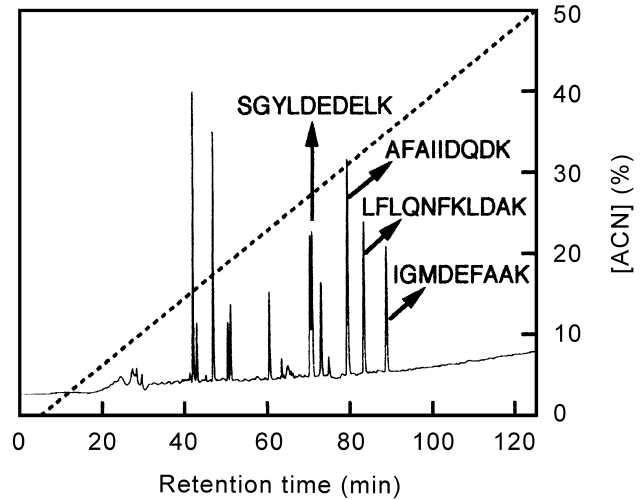


Fig. 3 Separation of peptide fragments produced by digestion of purified bigeye tuna parvalbumin with lysylendoprotease. Column, TSKgel ODS-120T (0.46 × 25 cm); elution, linear gradient of acetonitrile (ACN) in trifluoroacetic acid; flow rate, 1 mL/min. Four peptides were sequenced and the results are included. (-----), concentration of ACN.

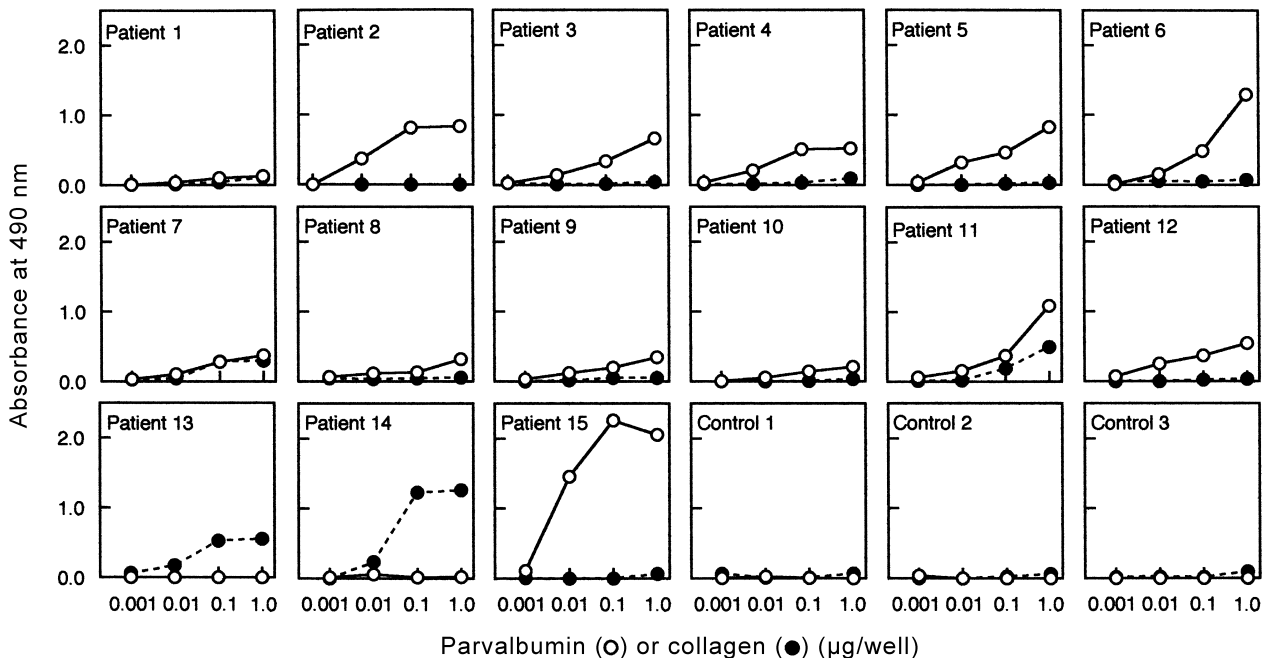


Fig. 4 Analysis by ELISA of IgE reactivities of 15 patient and three control sera to collagen and parvalbumin purified from bigeye tuna.

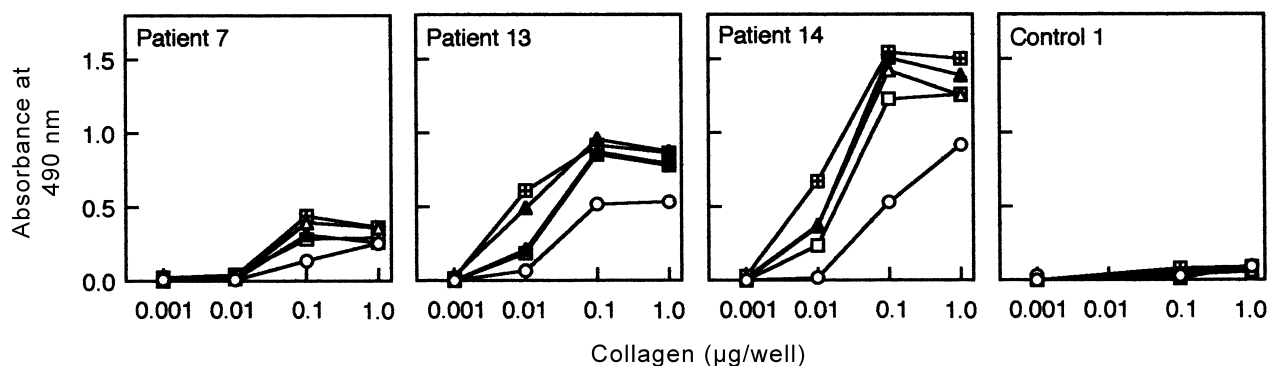


Fig. 5 Analysis by ELISA of IgE reactivities of three patient and one control sera to collagens purified from Japanese eel (○), alfonsin; (▲), mackerel; (△), skipjack; (■) and bigeye tuna (□).

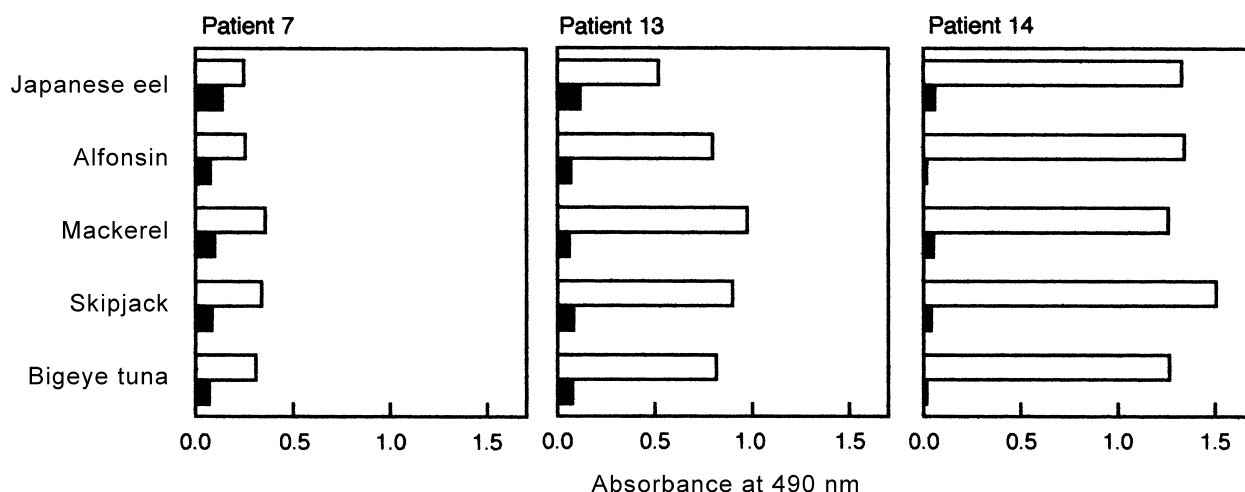


Fig. 6 Inhibition of IgE reactivities of three patient sera to collagens from five species of fish by bigeye tuna collagen. The serum (1 : 25 dilution) was preincubated with an equal volume of bigeye tuna collagen solution (10 μ g/mL; ■) or Dulbecco's phosphate-buffered saline (□) and added to the plate coated with collagens from five species of fish (0.1 μ g/well).

serum from patient 15. In contrast, two sera (from patients 13 and 14) reacted strongly to collagen but not to parvalbumin. Positive reactions to both collagen and parvalbumin were observed in two sera (from patients 7 and 11). Interestingly, serum from patient 1 reacted to neither collagen nor parvalbumin, as was observed for the three control sera, suggesting that patient 1 recognizes allergens other than collagen and parvalbumin.

IgE reactivity to collagen purified from the muscle of five species of fish

The three sera (from patients 7, 13 and 14) found to be positive to bigeye tuna collagen, together with

one control serum, were examined further for their IgE reactivities to collagens purified from five species of fish. As shown in Fig. 5, the IgE reactivities to fish collagens varied considerably. However, for each patient serum, there was no marked difference in IgE reactivity among the collagens from five species of fish, with the exception that rather low reactivity to Japanese eel collagen was observed in two sera (from patients 13 and 14). Moreover, the collagen contents in five species of fish were determined to be 2.3, 3.5, 1.5, 1.0 and 1.4 mg/g muscle for Japanese eel, alfonsin, mackerel, skipjack and bigeye tuna, respectively, being comparable with one another. It is thus assumed that the five species of fish tested are almost equally allergenic with respect to collagen.

The collagens purified from five species of fish were very thermostable as to IgE binding ability. When heated in a boiling water bath for 120 min, they afforded no bands in SDS-PAGE, indicating that they were greatly degraded by heating. Nevertheless, they retained more than 90% of their original binding ability to the IgE in the sera from patients 7, 13 and 14.

Antigenic cross-reactivity among fish muscle collagens

Antigenic cross-reactivity among fish muscle collagens was examined by ELISA inhibition experiments using sera from patients 7, 13 and 14. As a matter of course, IgE reactivity of the three sera to bigeye tuna collagen was remarkably reduced by preincubation with bigeye tuna collagen (Fig. 6). Similarly, for each serum, IgE binding to collagens from Japanese eel, alfonso, mackerel and skipjack was significantly inhibited by bigeye tuna collagen. The observed cross-reactivity suggests that fish muscle collagens share the same or closely similar IgE binding epitopes. In contrast, none of the sera from patients 7, 13 and 14 reacted to type I collagen from bovine Achilles tendon (Sigma), indicating no cross-reactivity between fish and bovine collagens.

DISCUSSION

When analyzed by ELISA using parvalbumin and collagen purified from bigeye tuna muscle, 10 of 15 sera from fish-allergic patients reacted to parvalbumin only, two reacted to collagen only, two reacted to both parvalbumin and collagen and one reacted to neither parvalbumin nor collagen. These results are of value on two points. First, the results clearly demonstrate that the major allergen in the bigeye tuna muscle is parvalbumin. Based on the results from immunoblotting studies, tuna fish have so far been assumed to be out of the general concept that the major fish allergen is parvalbumin. For example, Yamada *et al.*²³ analyzed allergens in two species of tuna fish (albacore *Thunnus alalunga* and yellowfin *Thunnus albacares*) by immunoblotting using eight sera probably from European and/or American patients and found that only one serum reacted to a 12 kDa protein (attributable to parvalbumin). The discrepancy between the results of the present study and those of previous studies may be explained by differences in either tuna species or racial characteristics. Second, the results of our ELISA experiments are more

important in reconfirming our previous finding¹⁷ that some fish-allergic patients recognize bigeye tuna muscle collagen. In the present study, the sera positive to bigeye tuna muscle collagen were further shown to react to muscle collagens from the other four species of fish. In addition, ELISA inhibition experiments established the cross-reactivity among muscle collagens from five species of fish. It is therefore relevant to conclude that fish-allergic patients recognizing fish muscle collagen are not small in number, at least in Japan, and that fish muscle collagen is a cross-reactive allergen among various species of fish. In relation to this, it should be noted that Sakaguchi *et al.*¹⁵ recently came to the same conclusion for fish skin collagen, although the cross-reactivity between fish muscle and skin collagens remains to be elucidated.

Collagen is composed of three α -chains ($\alpha 1$)₂ $\alpha 2$ or $\alpha 1\alpha 2\alpha 3$, twisted together to form a triple helix. If collagen is denatured, each α -chain and β -chain, comprising two α -chains connected by an intramolecular or intermolecular bridge, is liberated from the triple helix and, therefore, denatured collagen gives two bands of 110–120 kDa (corresponding to hetero α -chains) and one band of 210 kDa (corresponding to the β -chain) in SDS-PAGE, as shown in Fig. 1. Hetero α -chains of bigeye tuna muscle collagen¹⁷ and tuna skin collagen¹⁵ have already been shown to be IgE reactive by immunoblotting using a purified preparation, suggesting that fish collagen is allergenic even though denatured and that the allergenicity of fish collagen is not ascribable to a specific α -chain. More importantly, the present study demonstrates that fish muscle collagen is very thermostable as to allergenicity. The purified collagens from five species of fish were greatly degraded by heating, but their IgE binding ability was not lost. When fish muscle is heated, much more severe degradation of collagen seems to occur by muscle proteases as well as heating. However, our previous ELISA and ELISA inhibition experiments revealed that peptide fragments derived from collagen in the heated extract from fish muscle are significantly IgE reactive.¹⁷ These results indicate that the allergenic activity of fish muscle collagen is dependent on amino acid sequence, but not on steric conformation. In the present study, no cross-reactivity was recognized between fish muscle collagen and bovine collagen. Similarly, no cross-reactivity between fish skin collagen and bovine collagen has been reported previously.¹⁵ Indeed, there are high sequence homologies (98% for $\alpha 1$ chain and 93% for $\alpha 2$ chain) between bovine and human

collagens.^{24,25} In contrast, rainbow trout collagen, the only fish collagen with an elucidated complete amino acid sequence, has lower homologies (77% for $\alpha 1$ chain and 68% for $\alpha 2$ chain) with human collagen,²⁶ accounting for no cross-reactivity between fish and bovine collagens.

It is very likely that fish-allergic patients respond to unknown allergens as well as parvalbumin and/or collagen or only to unknown allergens, as observed with patient 1. Immunoblotting is the most frequently used technique to analyze allergens in crude extracts from biological samples. Indeed, some minor allergens other than parvalbumin have been detected in various species of fish by immunoblotting.^{2,12–14} However, it should be pointed out that, due to peculiar chemical properties, collagen is not easily identified as a fish allergen by immunoblotting. Collagen is insoluble in water or buffers at low temperatures and, hence, is absent in the non-heated extract from fish muscle. When fish muscle is extracted at high temperatures (e.g. 100°C), collagen is degraded to a mixture of peptide fragments during heating and, hence, affords no bands typical of collagen in SDS-PAGE. The present study focused mainly on collagen; therefore, patient sera were examined for their IgE reactivities to purified parvalbumin and collagen by ELISA, although purification was time consuming and also unknown allergens were overlooked. It should also be noted that the non-heated extract from fish muscle containing little collagen is unsuitable as a fish antigen in RAST for diagnosis. Not only from the viewpoint of easy analysis of fish allergens by immunoblotting, but also from that of accurate diagnosis of fish allergy, it is essential to develop a sophisticated method to extract collagen without fragmentation, together with parvalbumin, from the fish muscle.

In summary, the present study provides evidence that fish muscle collagen is an important allergen for some fish-allergic patients. For a better understanding of fish allergy, further studies are needed to clarify the chemical and immunological properties of fish muscle collagen and its hetero α -chains.

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