

Identification of Collagen as a New Fish Allergen

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This study was intended to identify a high molecular weight allergen that had been detected in fish. Analyses by ELISA of five protein fractions prepared from bigeye tuna muscle showed that the high molecular weight allergen was contained in the myostromal protein fraction. Based on the results of SDS-PAGE, immunoblotting and amino acid analysis of the myostromal protein fraction, the high molecular weight allergen was judged to be collagen. Five of the eight patient sera used were found to react to the bigeye tuna collagen. In competitive ELISA inhibition experiments, the bigeye tuna collagen almost completely inhibited the IgE reactivity to the heated extracts from five species of fish, suggesting that collagen is commonly allergic regardless of fish species. However, no antigenic cross-reactivity was observed between collagens from fish and other animals.

Key words: allergen; bigeye tuna; collagen; fish; gelatin

Fish is one of the most common causes of food allergy, especially in coastal countries, such as Japan and Scandinavia, where large amounts of fish are daily consumed. For example, according to the investigation conducted in 1997 by the Ministry of Health and Welfare, Japan, fish is ranked second to crustaceans among various causes of food allergy for adults in Japan; approximately 20% of food allergic adults are sensitive to fish. As for fish allergens, extensive studies with the codfish *Gadus callaris* showed that the major allergen (known as Gad c 1) is parvalbumin, a low molecular mass (12 kDa), thermostable, calcium-binding sarcoplasmic protein in the ordinary muscle.^{1,2)} Recently, allergens in three species of fish, Atlantic salmon,³⁾ carp,⁴⁾ and Japanese horse mackerel,⁵⁾ have also been identified as parvalbumins at the molecular level. These results, together with some immunoblotting analyses,^{6–10)} led to the conclusion that the major fish allergen is parvalbumin independent of fish species.

It is interesting to note that several studies are inconsistent with the above conclusion on fish aller-

gens. In two species of tuna fish (albacore and yellowfin), parvalbumin was recognized by only one of the eight tuna fish-positive patient sera.¹¹⁾ Though in a rare case, not parvalbumin but a 25 kDa protein was detected as the allergen in swordfish, to which only a peculiar patient monospecific to swordfish was sensitive.¹²⁾ Quite strange results were also obtained in our study on allergens in Japanese eel and bigeye tuna.¹³⁾ When analyzed by gel filtration, allergens in both fish were separated into two fractions (named Frs. I and II in the order of elution). The allergen in Fr. II, recognized specifically by two of the five sera from Japanese fish-sensitive subjects, was isolated by reverse-phase HPLC and certainly identified as parvalbumin. On the other hand, two sera exhibited almost specific reactivity to the high molecular weight protein in Fr. I and the remaining one was equally reactive to both parvalbumin and the high molecular weight allergen. Our results strongly suggested that fish-sensitive subjects recognizing the high molecular weight allergen are present in no small numbers, at least in Japan. Not only for the diagnosis and medical treatment of fish allergy but also for the future development of hypoallergenic fish processed products, it is essential to identify the high molecular weight allergen. Very recently, we found an IgE-reactive protein in *surimi* from walleye pollack by ELISA using one patient serum positive to the high molecular weight allergen and suggested it to be collagen.¹⁴⁾ In accordance with this suggestion, we provide here evidence that the high molecular weight allergen recognized by plural patient sera is collagen, regardless of fish species.

Materials and Methods

Preparation of heated extracts. Three kinds of meats (beef, pork, and chicken), five species of fish (Japanese eel *Anguilla japonica*, alfonsoin *Beryx splendens*, mackerel *Scomber japonicus*, skipjack *Katsuwonus pelamis*, and bigeye tuna *Thunnus obesus*), two species of crustaceans (tanner crab *Chionoecetes*

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

opilio and giant tiger shrimp *Penaeus monodon*) and three species of mollusks (oyster *Crassostrea gigas*, turban shell *Turbo cornutus*, and Japanese common squid *Todarodes pacificus*) were purchased from a local supermarket or a seafood market in Tokyo. All samples were stored at -20°C until used.

The meats were used as they were for the preparation of heated extracts. For the other animal samples, the ordinary muscles were obtained from fish; the leg muscle from tanner crab; the tail muscle from giant tiger shrimp; the soft tissue from oyster; the foot muscle from turban shell; the mantle muscle from Japanese common squid. Each tissue sample was homogenized with three volumes of 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.0). After heating in a boiling water bath for 10 min, the homogenate was cooled to room temperature and centrifuged at 18,000 g for 20 min. The supernatant obtained was used as the heated extract.

Fractionation of bigeye tuna muscle proteins. Bigeye tuna muscle proteins were fractionated into several classes essentially by the method of Sato *et al.*¹⁵⁾ All operations were done at 4°C in a cold room or using a centrifuge with a refrigerator. The ordinary muscle (30 g) was extracted three times with 90 ml of 0.15 M NaCl in 0.01 M phosphate buffer (pH 7.0) and then three times with 90 ml of 0.6 M KCl in 0.01 M phosphate buffer (pH 7.0). The former extract was regarded as the sarcoplasmic protein fraction and the latter as the myofibrillar protein fraction. In the next step, the residue was extracted three times with 90 ml of 0.1 M NaOH. After neutralization with 1 M HCl, the NaOH extract was separated into supernatant and precipitate by centrifugation at 18,000 g for 20 min. The supernatant was thoroughly dialyzed against distilled water and lyophilized. The dried material was dissolved in 270 ml of 0.05 M sodium carbonate buffer (pH 9.5) and used as the alkali soluble protein fraction (supernatant). On the other hand, the precipitate was extracted three times with 90 ml of 0.6 M KCl in 0.01 M phosphate buffer (pH 7.0) and the extract was used as the alkali soluble protein fraction (precipitate). Finally, the residue obtained after extraction with 0.1 M NaOH was washed three times with 90 ml of distilled water and extracted with 90 ml of 0.5 M acetic acid with constant stirring for 3 d. The same extraction procedure was repeated twice more and the combined acetic acid extract was regarded as the myostromal protein fraction.

Isolation of bigeye tuna parvalbumin. Parvalbumin (Thu o 1.01, one of parvalbumin isomers) was isolated from the bigeye tuna muscle by gel filtration and reverse-phase HPLC, as reported previously.¹³⁾

Sera. Sensitized sera were obtained from eight subjects (subjects 1–8, Table 1), who had a clinical histo-

Table 1. Characteristics of Patients

Subject	Age	Sex	RAST score
1	3	F	Sardine (3), cod (2), horse mackerel (4), mackerel (3)
2	1	F	Tuna (3), carb (2), shrimp (2), egg white (4), milk (3)
3	19	M	Cod (4), horse mackerel (3), mackerel (3), tina (4), crab (2)
4	8	M	Horse mackerel (3)
5	3	M	Salmon (3), egg white (4), milk (4), peanut (4)
6	1	M	Tuna (4), beef (3), pork (3), soybean (4), rice (4)
7	0	F	ND*
8	16	F	ND*

* ND: not determined

ry of immediate hypersensitivity reactions, such as urticaria, asthma, and diarrhea, after ingestion of fish. Subjects 1–6 had been demonstrated to have elevated serum IgE to fish by RAST done at hospitals. It should be noted that the sera from subjects 1 and 2 had IgE reactivity only to parvalbumin, while those from subjects 3 and 4 were almost specific to a higher molecular weight protein than parvalbumin and that from subject 5 equally to parvalbumin and the high molecular weight allergen.¹³⁾ In this study, serum from one subject (subject 9) without adverse reactions after ingestion of any foods was used as a control. All sera were divided into portions and stored at -20°C until use.

Enzyme-linked immunosorbent assay (ELISA). ELISA was done as described elsewhere,¹⁶⁾ using a flat-bottomed polystyrene plate with 96 wells (Type H Multi Plate for ELISA; Sumitomo Bakelite, Tokyo, Japan). In brief, each sample was reacted with sensitized or control serum, diluted 1:100 with 0.1% bovine serum albumin in Dulbecco's phosphate-buffered saline (PBS, pH 7.4), followed by peroxidase-conjugated goat anti-human IgE antibody (Kirkegaard & Perry Laboratories, Gaithersburg, USA), diluted 1:2,500 with 0.1% bovine serum albumin in Dulbecco's PBS. The enzyme reaction was done using a substrate solution (0.05 M phosphate-citrate buffer, pH 5.0, containing 0.1% *o*-phenylenediamine and 0.03% H_2O_2) and the developed color was measured by absorbance at 490 nm.

Inhibitory effects of the purified allergen (myostromal protein fraction) from bigeye tuna on the reactivity between the subject 3 serum and the heated extracts from five species of fish were examined by competitive ELISA. The purified allergen was dissolved in Dulbecco's PBS containing 0.1% bovine serum albumin at a concentration of $10\text{ }\mu\text{g/ml}$ and incubated with an equal volume of the subject 3 serum (diluted 1:50 with Dulbecco's PBS containing 0.1% bovine serum albumin) at 37°C for 1 h. A $100\text{-}\mu\text{l}$ portion of this solution was then added to the plate

previously coated with heated extract of each fish (diluted 1:500 with 0.05 M sodium carbonate buffer, pH 9.5). The subsequent procedure was the same as in the ELISA described above.

All ELISAs (including competitive ELISA) were done in triplicate and the data were expressed as the mean values.

Protein measurement. Protein was measured by the method of Lowry *et al.*,¹⁷⁾ using bovine serum albumin as a standard.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE were done on a PhastSystem apparatus (Pharmacia Biotech, Uppsala, Sweden) using ready-made slab gels (PhastGel Gradient 10–15) and ready-made buffer strips (PhastGel SDS Buffer Strips). Running and staining of the gel with Coomassie Brilliant Blue R-250 were followed by the manufacturer's instruction. The purified allergen (myostromal protein fraction) from bigeye tuna was dissolved in 0.02 M phosphate buffer (pH 7.2) containing 2% SDS and 3.5 M urea and kept at room temperature for 10 min before it was put on the gel. An HMW-SDS calibration kit (Pharmacia Biotech), composed of myosin (212 kDa), α_2 -macroglobulin (170 kDa), b-galactosidase (116 kDa), transferrin (76 kDa), and glutamate dehydrogenase (53 kDa), was used as a reference.

Immunoblotting. The proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane with the PhastSystem apparatus according to the manufacture's instruction. After confirming the transfer of the proteins by staining with 0.2% Ponceau S solution containing 3% trichloroacetic acid and 3% sulfosalicylic acid, the membrane was blocked with 2% skimmed milk in Dulbecco's PBS at 37°C for 12 h. The membrane was then immunoreacted successively with the subject 3 serum or control serum (diluted 1:100 with 0.1% skimmed milk in Dulbecco's PBS) at 37°C for 3 h and peroxidase-conjugated goat anti-human IgE antibody (diluted 1:2,500 with 0.1% skimmed milk in Dulbecco's PBS) at 37°C for 3 h. Finally, the antigen-IgE complex formed was made visible by enzyme reaction using a substrate solution (0.05 M phosphate-citrate buffer, pH 5.0, containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride and 0.1% H₂O₂).

Amino acid analysis. The purified bigeye tuna allergen (myostromal protein fraction, 40 μ g) was hydrolyzed with 50 μ l of 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl) indole in an evacuated tube at 115°C for 24 h. After hydrolysis, the solution was neutralized with an equal volume of 3.5 M NaOH and diluted with 500 μ l of 0.2 M citrate buffer (pH 2.2). A 400- μ l portion of the dilution was

put into an Atto MLC-703 amino acid analyzer.

Results

IgE reactivity of the subject 3 serum to the protein fractions from bigeye tuna

The IgE of subject 3 serum reacted more or less to all the five protein fractions prepared from the bigeye tuna muscle (Fig. 1). In the four fractions other than the myostromal protein fraction, however, almost the same or rather higher reactivities were displayed by the control serum, implying that the positive reactions observed with the subject 3 serum were non-specific. On the other hand, the subject 3 serum reacted most strongly to the myostromal protein fraction, to which the reactivity of the control serum was negligible. The high molecular weight allergen against subject 3 was thus judged to be recovered in the myostromal protein fraction.

Properties of the bigeye tuna allergen

On SDS-PAGE, the myostromal protein fraction from the bigeye tuna muscle afforded single and double bands at positions equivalent to about 240 and 120 kDa, respectively (Fig. 2A). As compared to the dark blue color of the reference protein bands, the three bands were all stained reddish blue, indicating the occurrence of metachromasy. In immunoblotting, the subject 3 serum reacted to all the three bands (Fig. 2B) but the reactivity of the control serum was negative (data not shown), demonstrating that the proteins corresponding to the three bands are allergenic.

According to Sato *et al.*,¹⁵⁾ pure collagen is usually obtainable in the myostromal protein fraction. In-

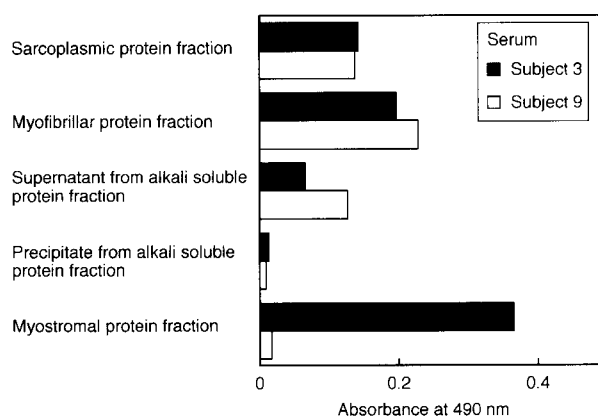


Fig. 1. Analysis by ELISA of the IgE Reactivity of Patient (Subject 3) and Control (Subject 9) Sera against the Five Protein Fractions from the Bigeye Tuna Muscle. Each serum (1:100 dilution) was added to the plate coated with the five fractions (1:100 dilution) with the following protein concentrations (μ g/ml): sarcoplasmic protein fraction, 95; myofibrillar protein fraction, 278; supernatant from alkali soluble protein fraction, 437; precipitate from alkali soluble protein fraction, 35; myostromal protein fraction, 0.45.

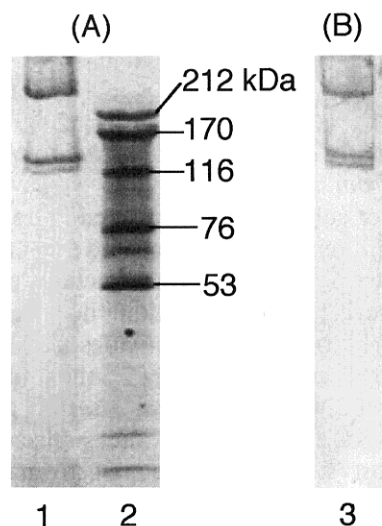


Fig. 2. SDS-PAGE (A) and Immunoblotting (B) of the Myostromal Protein Fraction from Bigeye Tuna. Lanes: 1 and 3, myostromal protein fraction; 2, standard proteins. Proteins were stained with Coomassie Brilliant Blue R-250 in SDS-PAGE and detected by the IgE reactivity of patient (subject 3) serum in immunoblotting.

deed, the three bands observed in the myostromal protein fraction from the bigeye tuna muscle were reasonably attributable to collagen; the two bands of 120 kDa corresponded to α -chain of collagen and the 240 kDa band to β -chain (dimer of α -chain). The metachromasy observed for the three bands was also characteristic of collagen. Further evidence for collagen was obtained by amino acid analysis. The amino acid composition of the myostromal protein fraction from bigeye tuna is shown in Table 2, in which that of skipjack ordinary muscle collagen¹⁸⁾ is included for comparison. Apparently, the myostromal protein fraction was closely similar in amino acid composition to the skipjack collagen. Two amino acids (hydroxyproline and hydroxylysine) specific for collagen were detected in the myostromal protein fraction. In addition, the myostromal protein fraction contained a large amount of glycine and lacked half-cystine and tryptophan. Based on the results in SDS-PAGE, immunoblotting and amino acid analysis, the high molecular weight allergen in the bigeye tuna muscle, recognized by the subject 3 serum, was identified as collagen (probably type I collagen, known to be the representative collagen in the fish muscle). Therefore, the myostromal protein fraction was used as a pure bigeye tuna collagen preparation in the following experiments.

When tested by ELISA, the subject 4 serum strongly reacted in a dose-dependent manner to the bigeye tuna collagen as in the case of subject 3 serum (Fig. 3A). Though to a lesser extent, sera from subjects 5, 6 and 8 also reacted to the collagen. In contrast, the reactivity of subjects 1, 2 and 7 sera to the collagen was negligible, being comparable to that of

Table 2. Amino Acid Compositions of the Myostromal Protein Fraction from Bigeye Tuna and Skipjack Ordinary Muscle Collagen

Amino acid	Residues/1000 residues	
	Bigeye tuna	Skipjack*
Hyp	59	80
Asp	41	43
Thr	26	31
Ser	37	33
Glu	77	75
Pro	93	102
Gly	404	337
Ala	112	126
Val	13	18
Cys (half)	0	0
Met	8	15
Ile	6	11
Leu	21	23
Tyr	7	3
Phe	12	14
Hyl	10	7
Lys	23	26
His	6	6
Trp	0	0
Arg	46	52

* Cited from Zhu and Kimura.¹⁸⁾

the control (subject 9) serum. We have previously reported that the IgE reactivities to Japanese eel and bigeye tuna parvalbumins are strongly positive in subjects 1 and 2 sera, almost negative in subjects 3 and 4 sera and moderately positive in subject 5 serum.¹³⁾ As for the other patient sera, the subject 6 and 7 sera moderately and strongly reacted to the bigeye tuna parvalbumin, respectively, while the reactivity was negative in the subject 8 serum (Fig. 3B).

The bigeye tuna collagen was found to be very thermostable as to its allergenicity. Even when denatured to gelatin by heating in a boiling water bath for 120 min, the bigeye tuna collagen retained 90% of its original binding ability to the IgE in the sera from subjects 3–5.

Inhibition of IgE reactivity of the subject 3 serum to fish muscle extracts by the bigeye tuna collagen

In competitive ELISA inhibition experiments, the bigeye tuna collagen almost completely reduced the IgE reactivities of the subject 3 serum against all the heated extracts from Japanese eel, alfonsoin, mackerel, skipjack, and bigeye tuna (Fig. 4). This indicated that collagen is allergic to subject 3 in common with the five species of fish used.

IgE reactivity of patient sera to collagens from various animals

In ELISA experiments, none of the sera from subjects 3–5 reacted to any of the heated extracts (containing gelatin) from three kinds of meats (beef, pork and chicken), two species of crustaceans (tanner crab

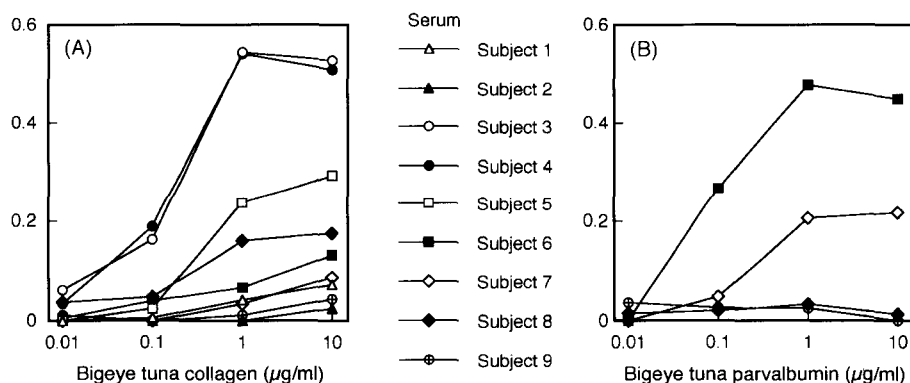


Fig. 3. Analysis by ELISA of the IgE Reactivity of Sera from Eight Patients (subjects 1-8) and One Control (subject 9) against the Bigeye Tuna Collagen (A) and Parvalbumin (B).

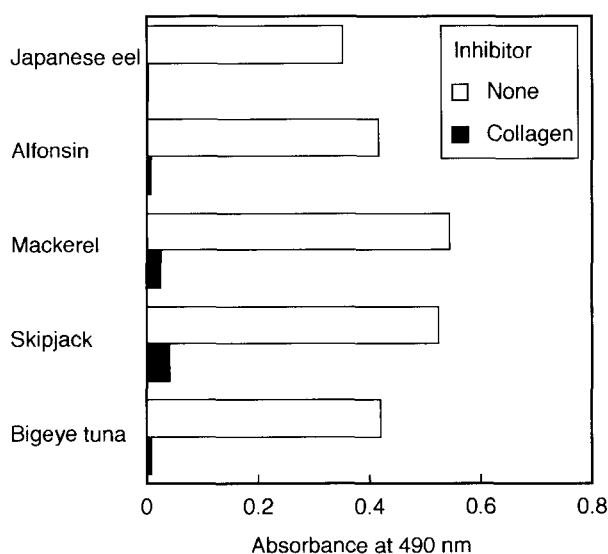


Fig. 4. Inhibition of the IgE Reactivity of Subject 3 Serum against the Heated Extracts from Five Species of Fish by the Bigeye Tuna Collagen. The plate was coated with the heated extracts (1:500 dilution) with the following protein concentrations ($\mu\text{g/ml}$): Japanese eel, 57; alfonsin, 65; mackerel, 26; skipjack, 28; bigeye tuna, 24. Before addition to the plate, the serum (1:50 dilution) was incubated with an equal volume of PBS without or with the inhibitor (bigeye tuna collagen) at a concentration of 10 $\mu\text{g/ml}$.

and giant tiger shrimp) and three species of mollusks (oyster, turban shell, and Japanese common squid). Also, they did not react to type I collagens (up to a concentration of 10 $\mu\text{g/ml}$) from bovine Achilles' tendon (Sigma, St. Louis, USA) and porcine Achilles' tendon (Wako Pure Chemicals, Osaka, Japan).

Discussion

In this study, the high molecular weight allergen in the bigeye tuna muscle against subject 3 was clearly demonstrated to be collagen, based on the results in SDS-PAGE (Fig. 2A), immunoblotting (Fig. 2B),

and amino acid analysis (Table 2). The IgE reactivity of the patient sera revealed that the bigeye tuna collagen is allergic to subjects 4, 5, 6, and 8 as well as subject 3 but not to subjects 1, 2, and 7 (Fig. 3A). Furthermore, ELISA inhibition experiments strongly suggested that collagen is allergic to subject 3 regardless of fish species (Fig. 4). This seems to be the case with subjects 4, 5, 6, and 8, although their sera were not tested by competitive ELISA due to scarcity.

As far as we know, this is the first time collagen was identified as a fish allergen. It should be pointed out that our new finding is supported by recent studies of two research groups. Sakaguchi *et al.*¹⁹⁾ examined the IgE reactivity of ten bovine gelatin-sensitive children to gelatins (denatured collagens) from various animals and found one child having strong IgE reactivity to gelatins from codfish and salmon. In their report, however, no information is given as to whether the child is fish-allergic or not. On the other hand, Yamada *et al.*¹¹⁾ analyzed allergens in two species of tuna fish (albacore and yellowfin) by immunoblotting, and presented an interesting figure (corresponding to Fig. 2 in their paper) indicating the presence of collagen as an allergen; of the eight patient sera, one serum showed IgE reactivity to two proteins of 120 and 240 kDa in albacore and two sera to the same proteins in two species of tuna fish. To our eyes, the 120 and 240 kDa proteins appear to be α -chain and β -chain of collagen, respectively, although Yamada *et al.* did not give any comments on these IgE reactive proteins. Considering our results and those of Sakaguchi *et al.* and Yamada *et al.*, we believe that collagen is not an unusual allergen in fish. Also, fish allergic subjects recognizing only collagen or both parvalbumin and collagen seem not to be rare at least in Japan, although the sensitized sera examined are limited in number.

Collagen has long been overlooked in previous studies on fish allergens. In relation to this, it is worth mentioning that an immunoblotting technique, which has been frequently used to easily identify allergens in crude extracts from biological samples, is

not very helpful in detecting collagen as an allergen in crude extracts due to its unique chemical properties. Native collagen, which is composed of three homo or hetero α -chains twisted together to form a triple helix, is insoluble in water at low temperatures, being absent in the non-heated extract. On the other hand, each α -chain is liberated from the triple helix if collagen is denatured; the denatured form of collagen, i.e. gelatin, is water-soluble. A small amount of denatured collagen may be produced during storage of the fish muscle even at low temperatures. In some cases, therefore, the denatured collagen contained in the non-heated extract may be detectable by immunoblotting, as in the study of Yamada *et al.*¹¹⁾ However, it seems to be very difficult to detect collagen in the heated extract by immunoblotting. Though not fully studied, collagen is generally considered to be converted to a mixture of protein fragments (also called gelatin) during heating of the fish muscle, as a result of denaturation by both heating and digestion with muscle proteases. Indeed, although the heated extracts from five species of fish were positive in ELISA, they afforded no protein bands indicative of collagen in SDS-PAGE. In order to analyze fish allergens by immunoblotting, it is important to devise a sophisticated method to extract collagen without fragmentation, together with parvalbumin, from the fish muscle.

In our previous study, allergens in Japanese eel and bigeye tuna were analyzed using the heated extracts, not by immunoblotting but by a combination of gel filtration and ELISA, which resulted in the discovery of the high molecular weight allergen (identified as collagen in this study), together with parvalbumin.¹³⁾ At the beginning of this study, purification of the high molecular weight allergen in bigeye tuna was further tried by cation-exchange FPLC on Mono S (Pharmacia Biotech, Uppsala, Sweden) and reverse-phase HPLC on TSKgel Phenyl-5PW RP (Tosoh, Tokyo, Japan). However, the trial was unsuccessful because the high molecular weight allergen displayed very complicated behaviors in each column chromatography. This is now realized by the fact that the high molecular weight allergen in the heated extract is not a single protein but is composed of multiple IgE-binding proteins derived from collagen. We thus set up another strategy, namely fractionation of muscle proteins, and eventually could identify the high molecular weight allergen as collagen.

Collagen is ubiquitously found as an extracellular matrix protein in animals. However, the sera from subjects 3–5 reacted to neither collagens from bovine and porcine Achilles' tendons nor the heated extracts (containing gelatins) from some meats, crustaceans and mollusks, suggesting no antigenic cross-reactivity between collagens from fish and other animals. Similarly, Sakaguchi *et al.*¹⁹⁾ reported that the IgE reactivity of one bovine gelatin-sensitive child to

gelatins from codfish and salmon was not inhibited by bovine gelatin. It is thus apparent that fish collagen contains IgE-binding epitopes with peculiar amino acid sequences which are not found in collagen molecules from other animals. Future structural study on IgE-binding epitopes of fish collagen will disclose the reason why fish collagen does not share antigenic properties with those from other animals.

In summary, collagen was newly identified as a fish allergen. Although collagen has been overlooked in previous studies, probably due to its unique chemical properties, it seems to be not an unusual allergen but one of the primary allergens in fish. In view of this, our finding will accelerate future study on fish allergens to focus on collagen.

Acknowledgments

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