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Original article

Allergy to fish collagen: Thermostability of collagen and IgE reactivity of patients' sera with extracts of 11 species of bony and cartilaginous fish

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ELISA, enzyme-linked immunosorbent

assay; PBS, phosphate-buffered saline;

PBST, PBS containing 0.05% Tween 20;

SDS, sodium dodecyl sulfate;

SDS-PAGE, SDS-polyacrylamide gel

electrophoresis; SPT, skin prick test

ABSTRACT

Background: Parvalbumin was identified as a major fish allergen, and has been well investigated. Collagen was identified as a second allergen; however, its allergenic properties remain uncharacterized. Although fish is an important staple in coastal countries, its thermostability is unknown. Therefore, we aimed to determine the thermostability of fish collagen as an allergen.

Methods: Meat of seven bony and four cartilaginous fishes was heated at various temperatures and times, and extracts were analyzed using SDS-PAGE, IgE-ELISA, and SPTs.

Results: Collagen was dissolved from heated meat of Pacific mackerel into a crude extract. Collagen in the extracts was degraded at a high heating load—140 °C (10 min) or 100 °C (320 min). However, ELISA revealed the IgE reactivities of patients' sera with the extracts were unchanged even after heating the samples. Patients strongly reacted to extract proteins of other bony fish, which were detected by patients' IgE even after heating at 100 °C (320 min). In contrast, reactivities of the extracts of cartilaginous fish were lower than those of bony fish. SPTs in one patient revealed that all bony and cartilaginous fish extracts prepared from heated meat elicited allergic reactions.

Conclusions: The IgE reactivity of patients' sera to fish collagen in extracts was retained even when fish meat was treated by a high heating load. As for the fish collagen, the IgE reactivities to cartilaginous fish were lower than that to bony fish. Reducing IgE reactivity to fish meat using heat is difficult, and other modalities will be required to produce hypoallergenic fish meat.

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Introduction

Food allergy mediated by IgE is a serious public health problem not only in developed but also, recently, in developing countries.^{1,2} In parallel with the increase of fish consumption,³ immediate-type allergy induced by dietary fish is becoming a serious health problem worldwide, particularly in coastal countries, including Japan, Korea, Hong Kong, Philippines, Indonesia, Mexico, Brazil, and South Africa.^{2,4} Particularly in Asia, allergies to fish are more common than those to nuts, peanuts, and wheat because fish is a part of the

staple.² In sensitized subjects, adverse reactions such as asthma, urticaria, angioedema, and diarrhea are provoked immediately after the ingestion of fish, and life-threatening anaphylaxis may occur in severe cases.^{5–7}

Parvalbumin was first identified as a fish allergen derived from Baltic cod (*Gadus callarias*).⁸ Parvalbumin is a sarcoplasmic Ca²⁺-binding protein present only in vertebrates, and its concentrations are particularly high in the muscles of fish and amphibians.⁹ Parvalbumin is a major fish allergen,^{10–15} eliciting a reaction in the IgE of 67%–100% of patients with fish allergies.^{16–19} Further, parvalbumin is recognized as a highly cross-reactive fish pan-allergen.^{14,16,17,19,20} Parvalbumin has been reported to be heat-stable,⁸ and its content varies by fish species.^{20,21}

In the early 2000s, type I collagen was identified as a second fish allergen.^{22,23} Collagen contained in muscle has been identified as

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type I collagen.²⁴ Type I collagen forms a helical trimer (γ -chain). When collagen is denatured, trimers are easily dissociated to form monomers (α -chain) or dimers (β -chain). Type I collagens comprise extracellular fibers that are noncovalently cross-linked via inter-chain hydrogen bonds and covalently linked through pyridinoline or deoxypyridinoline cross-links.²⁵ Collagen is present in abundance compared with other proteins. Collagen accounts for 2%–12% of crude protein in the muscles, and approximately 50%, 5%–40%, and 40%–50% of lyophilized dry samples, the skin, fins, and bones, respectively.^{26,27} Denatured collagen (gelatin) from not only cattle and swine but also fish is widely used in various foods and nutritional supplements.

Although approximately 15 years have passed since collagen was identified as an allergen, there are few reports on fish collagen as an allergen. Recently, we revealed that 50% of Japanese patients with fish allergies have specific IgE against fish collagen.²⁸ Antibodies against fish collagen cross-react with collagens of various fish species but not with mammalian and avian collagens.^{23,28} The IgE reactivities of patients' sera against fish collagen are stable after collagen is heated at 100 °C for 120 min.²³ However, the thermostability of fish collagen at temperatures higher than 100 °C, or upon heating for more than 120 min, is unknown. When fish is cooked, it is usually heated at over 100 °C or for a prolonged time of over 120 min by various methods, such as boiling, steaming, grilling, baking, and frying, especially in Asian countries. Therefore, in the present study, we investigated the IgE reactivity of patients' sera to fish extracts prepared from heated fish meat. Further, to determine the thermostability of the allergenicity of fish collagen, we administered skin prick tests (SPTs) to a patient sensitized to fish collagen.

Methods

Fish samples

The 11 selected species of fish, which are widely consumed in Japan, were as follows: Pacific mackerel (*Scomber japonicus*), chicken grunt (*Parapristipoma trilineatum*), red seabream (*Pagrus major*), yellowfin tuna (*Thunnus albacares*), silver salmon (*Oncorhynchus kisutch*), Japanese sardine (*Sardinops melanostictus*), goldeye rockfish (*Sebastes thompsoni*), bigeye thresher (*Alopias superciliosus*), shortfin mako shark (*Isurus oxyrinchus*), blue shark (*Prionace glauca*), and mottled skate (*Raja pulchra*). Four species (bigeye thresher, shortfin mako shark, blue shark, and mottled skate) are cartilaginous fish and the seven others are bony fish. The bigeye thresher, shortfin mako shark, and blue shark were caught in the Pacific Ocean and stored at –60 °C. Fresh fillets (for three species of fish including yellowfin tuna, silver salmon, and mottled skate) or whole fish samples (for the remaining five species) were purchased at local retail shops and immediately used in the experiments.

Preparation of fish extracts

White muscle obtained from each specimen was well minced and not heated. To prepare heated muscle samples, a 0.5 g sample of a mince was placed in a closed vessel and heated at 20 °C, 40 °C, 60 °C, or 80 °C using a Dry Bath Incubator MD-01N (Major Science, Saratoga, CA, USA), at 100 °C in boiling water and at 120 °C or 140 °C using a Joule heating apparatus (Hano Manufacturing, Fukuoka, Japan) as follows: (i) 100 °C heating, 5, 10, 20, 40, 80, 160, or 320 min for Pacific mackerel and 10 or 320 min for the remaining fish; and (ii) 100–140 °C heating, 10 min for Pacific mackerel. In addition, Pacific mackerel meat was heated at 120 °C for 60 min as above for SPTs. The unheated and heated muscle samples were

individually homogenized with four volumes of phosphate-buffered saline (PBS, pH 7.0; 150 mM NaCl in 10 mM phosphate buffer) at 30,000 rpm for 10 min on ice using a handy homogenizer MH-1005 (As One, Osaka, Japan). The homogenate was centrifuged at 12,000 $\times g$ for 10 min at 4 °C, and the supernatant was used to prepare unheated or heated extracts.

Purification of parvalbumin and collagen

Parvalbumin was purified from the white muscle of the Pacific mackerel, according to the method described by Hamada *et al.*¹⁴ Briefly, a heated extract was prepared from the muscle. The extract was applied to gel filtration chromatography, followed by reverse phase-high-performance liquid chromatography, and parvalbumin was obtained in pure state. At each step, parvalbumin was monitored by mouse anti-frog parvalbumin monoclonal antibody PARV-19 (Cat. No. P3088, Sigma–Aldrich, St. Louis, MO, USA). Collagen was purified from the skin of Pacific mackerel, according to the method described by Hamada *et al.*²³ Briefly, fish skin was homogenized with PBS. After centrifugation, the pellet was dissolved with 10 mM phosphate buffer containing 600 mM KCl. After centrifugation, the pellet was dissolved with 100 mM NaOH. After it was neutralized, the pellet was obtained by centrifugation. It was dissolved with 10 mM phosphate buffer containing 600 mM KCl. After centrifugation, the pellet was dissolved with 500 mM acetic acid. It was dialyzed with water, and purified collagen was obtained. The concentrations of these two proteins were determined, according to the method described by Lowry *et al.*,²⁹ using bovine serum albumin as a standard.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed using a WSE-1100 PageRun-R apparatus (Atto, Tokyo, Japan). The unheated extract and extracts prepared from heated meat were separately dissolved in an equal volume of 80 mM Tris–HCl buffer (pH 6.8), containing 140 mM SDS, 200 mM dithiothreitol, 8 M urea, and 20% glycerol (SDS sample buffer), incubated at 37 °C for 120 min, and a 20- μ l aliquot was subjected to electrophoresis through a 10%–20% gradient polyacrylamide gel (e-Pagel E-T/R1020L, Atto). A 10- μ l aliquot of 125 μ g/ml of purified Pacific mackerel collagen was prepared in the same way and applied separately to the gel. The BLUE Star Prestained Protein-Ladder (Nippon Genetics, Tokyo, Japan) was used as a reference. Proteins were detected by staining the gels with Coomassie brilliant blue solution (CBB Stain One; Nacalai Tesque, Kyoto, Japan), which can detect several dozen ng of proteins.

Human sera

Patients' sera were obtained at Yokohama City University School of Medicine, National Defense Medical College, and Shinshu University Hospital in Japan (Table 1). All patients ($n = 8$) had clinical histories of immediate hypersensitivity reactions after the ingestion of raw and cooked fish. Written informed consent was obtained from each patient, and patient anonymity was assured. These patients had elevated specific IgE levels that reacted with the purified Pacific mackerel collagen but not with purified Pacific mackerel parvalbumin as determined by the enzyme-linked immunosorbent assay (ELISA) described below. Each serum sample from patient was sensitized to collagen but not parvalbumin and a pooled serum, which was a mixture of equal volumes of patients' sera (diluted 1:50), was used. In the present study, a pool of sera from 10 healthy non-atopic volunteers (Cosmo Bio, Tokyo, Japan) was used as a control. All experiments using human sera

Table 1

Profile of patients in the study.

Patient no.	Age	Gender	Symptom	ImmunoCAP (class)	SPT (positive)	Oral challenge test (positive)	Absorbance in ELISA (Pacific mackerel)			Notes
							Purified collagen (1 µg/ml)	Purified parvalbumin (1 µg/ml)	Extract prepared from heated meat (100 °C, 10 min)	
1	24	F	Urticaria, itching of skin, abdominal pain, oral allergy syndrome, anaphylaxis	Salmon (5), tuna (5), cod (4), flatfish (3) horse mackerel (3), sardine (3), mackerel (2)		Horse mackerel, mackerel	1.734	0.000	0.940	Anaphylaxis after ingestion of a jelly containing 1000 mg of fish collagen
2	33	F	Urticaria	Tuna (6), salmon (6), mackerel (4), sardine (4)	Cod, horse mackerel, salmon, skipjack, tuna, yellowtail		1.587	0.065	0.928	
3	41	M	Urticaria	Salmon (4), cod (3), flatfish (2) tuna (2), sardine (1)	Eel, Japanese seaperch, tuna, yellowtail		0.567	0.010	0.648	
4	17	F	Urticaria, abdominal pain, vomiting	Salmon (4), tuna (4), cod (3), flatfish (3), horse mackerel (3), sardine (2), mackerel (1)	Cod, horse mackerel, rose fish, shishamo smelt, yellowtail		0.535	0.017	0.434	
5	31	F	Urticaria, itching of skin, oral allergy syndrome, dyspnea	Gelatin (3), mackerel (2)	Fish gelatin		0.371	0.007	0.303	Eczema after using cosmetics containing hydrolyzed fish collagen. Oral allergy syndrome and dyspnea after drinking beverage containing fish collagen
6	28	F	Urticaria	Tuna (3), mackerel (2), sardine (1)		Horse mackerel, mackerel, salmon	0.200	0.000	0.208	
7	7	M	Urticaria	Cod (4), flatfish (4), horse mackerel (4), salmon (4), sardine (4), mackerel (3), tuna (3)			0.288	0.000	0.139	
8	1.5	M	Urticaria	Salmon (4), cod (3), eel (2), mackerel (1), sardine (1)			0.216	0.006	0.128	

were performed, according to the Ethical Guidelines of Tokyo University of Marine Science and Technology (Permit Number: 26-002). The study was performed in accordance with the principles embodied in the Declaration of Helsinki.

Western blotting

At first, two aliquots of 100 µg/ml of purified Pacific mackerel collagen were incubated at 4 °C or 100 °C for 320 min using MJ Mini thermal cycler (BioRad, Hercules, CA, USA). The unheated and heated purified collagen was then separately dissolved in an equal volume of SDS sample buffer, incubated at 37 °C for 120 min, and 20-µl aliquots were subjected to electrophoresis as described above. In western blotting, the proteins separated by SDS-PAGE were first electrotransferred from the gel to a polyvinylidene difluoride membrane using a blotting apparatus NA-1512S (Nihon Eido, Tokyo, Japan). The membrane was then washed with PBS containing 0.05% Tween 20 (PBST) and blocked with Blocking One solution (Nacalai Tesque) at room temperature for 1 h. After washing with PBST, the membrane was reacted with patient's serum obtained from patient 1 (diluted 1:300) in Immuno Shot Reagent 1 (Cosmo Bio) at room temperature for 2 h. The membrane was washed and reacted at room temperature for 1 h with the peroxidase-labeled goat anti-human IgE antibody (20 ng/ml; Cat. No. 074-1004, Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) in Immuno Shot Reagent 2 (Cosmo Bio) at 37 °C for 1 h. After re-washing with PBST, antigen–antibody binding was visualized using an ECL Prime Kit (GE Healthcare, Little Chalfont, UK) and the emitted luminescence was photographed using LumiCube apparatus (Liponics, Tokyo, Japan).

ELISA

A microtiter plate (MaxiSorp; Thermo Fisher Scientific, Hudson, NH, USA) was coated with 50 µl of 1 µg/ml of purified parvalbumin or collagen, or fish extracts (diluted 1:1000), mentioned in the section on “preparation of fish extract” except for SPTs, with 25 mM carbonate buffer (pH 9.5) at 37 °C for 2 h. After washing 4 times with 400 µl of PBST, the wells were blocked with 300 µl of 20% Blocking One solution (Nacalai Tesque) at 4 °C overnight. After

washing with PBST, the plate was individually reacted with 50 µl of all human sera describe above (diluted 1:50 with PBST containing 5% Blocking One) at 37 °C for 2 h. The plate was then washed with PBST and reacted with 50 µl of horseradish peroxidase-conjugated goat anti-human IgE antibody (400 ng/ml in PBST containing 5% Blocking One; Kirkegaard & Perry Laboratories) at 37 °C for 1 h. After washing with PBST, the plate was incubated with 50 µl of substrate solution (ELISA POD Substrate TMB Kit, Popular; Nacalai Tesque) at 37 °C for 10 min. The enzyme reaction was terminated using 50 µl of 1 M sulfuric acid, and absorbance was measured at 450 nm using a ChroMate Microplate Reader (Awareness Technology, Palm City, FL, USA). All ELISAs were performed in triplicate, and the data are expressed as mean values + standard deviation.

SPTs

Patient 1 was subjected to SPTs using unheated extract and extracts prepared from heated meat. All SPTs were performed using steel lancets. Histamine dihydrochloride (10 mg/ml) and PBS were used as positive and negative controls, respectively. Responses were read 15 min after pricking and graded as scores 1+, 2+, 3+, and 4+ that were defined as 25%, 50%, 100%, and 200% of the wheal area [(long diameter + short diameter)/2], respectively, compared with the wheal induced by histamine. Scores ≥2+ were considered positive. The response was defined as negative (–) if the wheal was equal or less than the wheal area of the negative-control.

Results

SDS-PAGE analysis of the unheated extract and extracts prepared from heated meat of Pacific mackerel

The proteins detected in extracts of unheated and heated muscle samples using the conditions described in Methods are shown in Figure 1A, B. Purified collagen from the skin of the Pacific mackerel migrated as to dominant bands at (125 kDa and approximately 250 kDa) and two minor bands (115 kDa and 300–400 kDa). The 115 kDa and 125 kDa bands correspond to the molecular masses of the collagen α-chain (monomer). The other two bands (approximately 250 kDa and 300–400 kDa) corresponded to the molecular

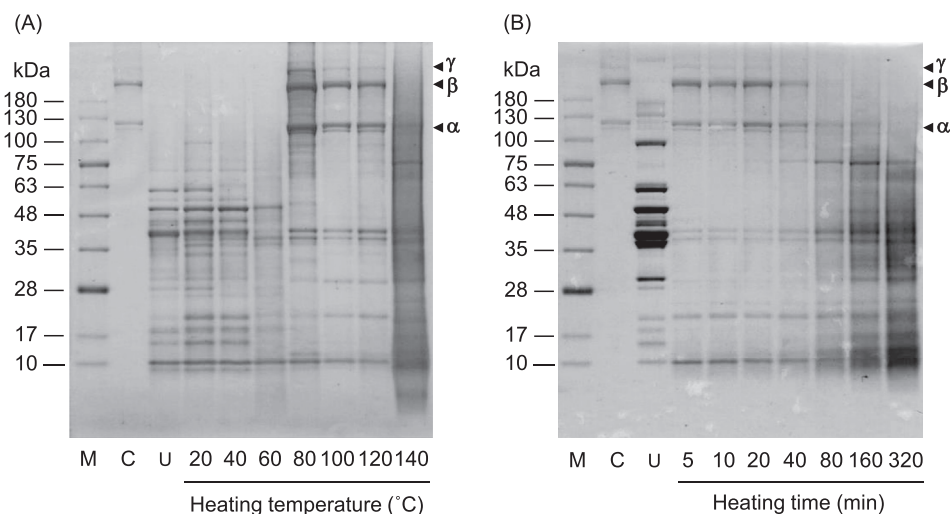


Fig. 1. SDS-PAGE analysis of unheated extract and extracts prepared from heated meat of Pacific mackerel. Fish meat was heated at various temperatures for 10 min (A) or at 100 °C for various times (B). Lanes M, C, and U denote molecular mass markers, purified Pacific mackerel collagen, and an unheated extract, respectively. The positions of the collagen α-, β-, and γ-chains are indicated on the right.

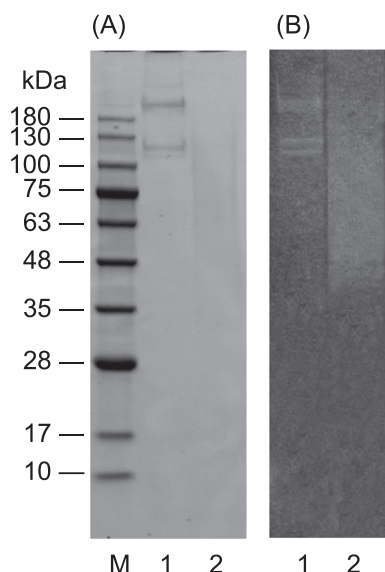


Fig. 2. SDS-PAGE (A) and western blotting (B) analyses of unheated and heated purified Pacific mackerel collagen. Western blotting was conducted using serum obtained from patient 1. Lanes: M, molecular mass markers; 1, unheated purified Pacific mackerel collagen; 2, purified Pacific mackerel collagen heated at 100 °C for 320 min.

masses of the β -chain (of the dimer) and γ -chain (of the trimer) of collagen, respectively. Bands that migrated similarly to collagen subunits were not detected in the unheated extracts and the extract prepared from meat heated at 20 °C–60 °C. In samples heated at 80 °C–120 °C, there were dominant bands at 125 kDa and approximately 250 kDa that corresponded to collagen α - and β -chains, respectively, and minor bands at 115 kDa and 300–400 kDa, corresponding to the α - and γ -chains, respectively. However, the electrophoretic pattern of samples heated at 140 °C was poorly resolved, and α -chains were difficult to detect.

Samples incubated at 100 °C for 5–20 min exhibited bands that migrated similarly to collagen α -, β - and γ -chains that were not detected in unheated sample. After heating for 40 min, bands corresponding to the collagen α - and β -chains were present at low levels, and bands corresponding to collagen γ -chains were undetectable. When samples were heated for 80–160 min, only bands corresponding to collagen α -chains were detected but at low intensity. The extract prepared from meat heated for 320 min did not generate bands corresponding to collagen.

SDS-PAGE and western blotting analyses of the unheated and heated purified Pacific mackerel collagen

The unheated and heated (at 100 °C for 320 min) of purified Pacific mackerel collagen were subjected to SDS-PAGE (Fig. 2A). The heated collagen generated smearing pattern from 40 kDa to 120 kDa and α -, β -, and γ -chains of collagen were disappeared. Western blotting analysis using serum obtained from patient 1 (Fig. 2B) exhibited positive IgE reaction to this smear. This indicates that collagen was degraded randomly at a range (40 kDa–120 kDa) that retains IgE reactivity. The IgE reactivity was not distinctly observed presumably because of the smearing where collagen molecules were not consolidated into one band.

ELISA analysis of unheated extract and extracts prepared from heated meat of Pacific mackerel subjected to different heating conditions

Minced meat of the Pacific mackerel was heated at 100 °C, 120 °C, or 140 °C for 10 min, and unheated extract and extracts prepared from heated meat (samples used in Fig. 1) were subjected to the ELISA using sera obtained from patients sensitized to fish collagen (Fig. 3). Only low levels of reactivity against the unheated extracts were detected, which is consistent with the findings described above indicating that such extracts lack detectable levels of collagen. Except for the serum of patient 3 with markedly reduced IgE reactivities to extracts prepared from meat heated at 120 °C or 140 °C, all patients' sera had equivalent IgE reactivities to extracts prepared from meat heated at 120 °C or 140 °C compared with those heated at 100 °C. The pool of control sera did not react with any of the extracts.

We next used an ELISA to test sera obtained from patients sensitized to fish collagen using extracts prepared from the meat of Pacific mackerel heated at 100 °C for 10 min or 320 min (Fig. 4; the same samples in Fig. 1). IgE reactivities to the extract prepared from meat heated for 320 min were slightly reduced compared with that for 10 min. The pooled control serum did not react with either extract.

SDS-PAGE analysis of the extracts prepared heated meat of six bony and four cartilaginous fish

SDS-PAGE of six bony and four cartilaginous fishes other than Pacific mackerel are shown in Figure 5A. All samples heated at

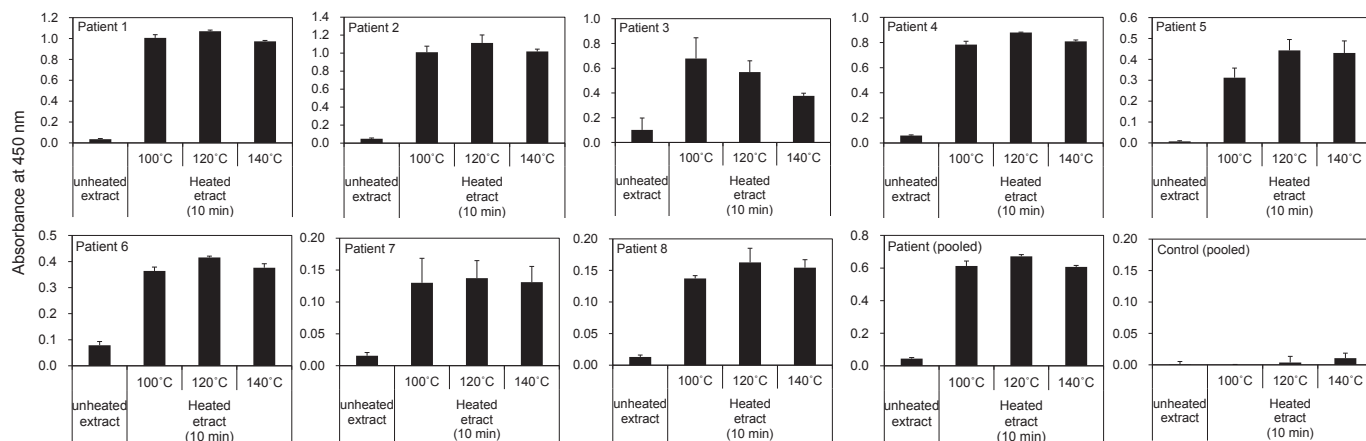


Fig. 3. ELISA analysis of the IgE reactivities of sera obtained from patients sensitized fish collagen against extracts from Pacific mackerel. Extracts were prepared from meat heated at 100 °C, 120 °C, and 140 °C for 10 min. Data are expressed as the mean + standard deviation ($n = 3$).

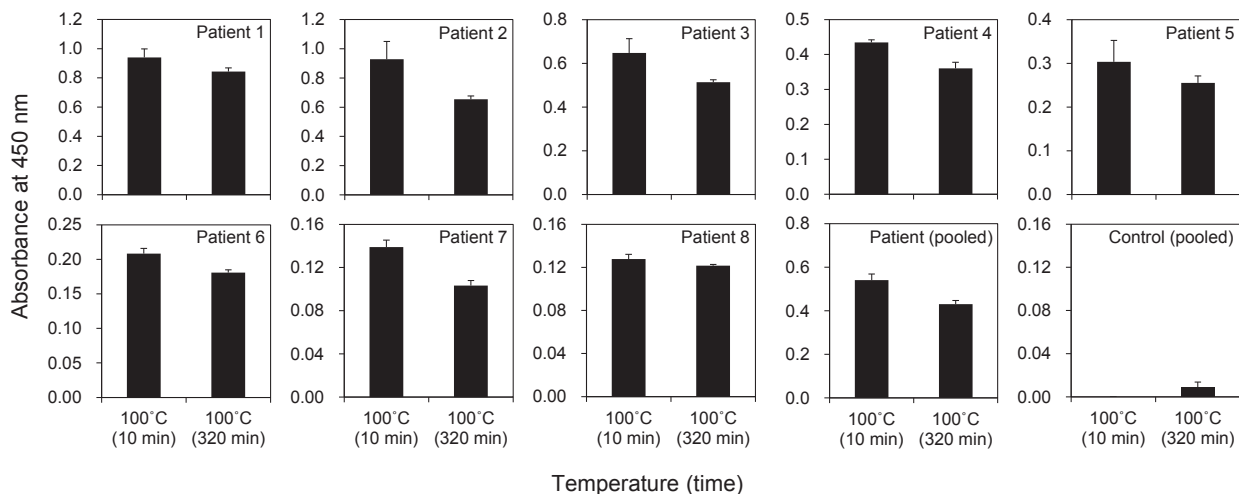


Fig. 4. ELISA analysis of the IgE reactivities of sera obtained from patients sensitized to fish collagen against fish extracts from Pacific mackerel. Extracts were prepared from meats heated at 100 °C for different times. Data are expressed as the mean + standard deviation ($n = 3$).

100 °C for 10 min gave α - and β -chains of collagen. In some of the fish, γ -chain was not detected because of low concentrations of collagens. In case of samples heated at 100 °C for 320 min for all fish species, electrophoretic patterns were smeared and all α -, β -, and γ -chains of collagen were not detected; smearing patterns were observed like in the case of Pacific mackerel.

IgE reactivities of patients' sera with extracts prepared from heated meat of 10 species of fish

Reactivities to extracts prepared from heated meat of IgEs in sera from patients sensitized to fish collagen from six bony fish and four cartilaginous fish were determined using an ELISA (Fig. 5B). Samples were heated for 10 min or 320 min (the same samples in Fig. 5A). The IgE reactivities to extracts of bony fish were high and IgE reactivities to extracts of cartilaginous fish were low. The reactivities of IgEs to extracts of bony fish (yellowfin tuna, silver salmon, and the Japanese sardine) were not prominently changed, even if the samples were heated for 320 min. In contrast, IgE reactivities to extracts of the chicken grunt, red seabream, and goldeye rockfish were reduced by heating for 320 min compared with heating for 10 min. The IgE reactivities to extracts of the bigeye thresher, shortfin mako shark, and blue shark (sharks) were lower compared with that of the mottled skate (ray).

SPTs of extracts of 11 fish species

Table 2 shows the results of SPTs for patient 1. The scores for all extracts prepared from heated meat of the Pacific mackerel (4+) were higher than those of the unheated extract (3+). The diameters of wheals and erythemas of extract prepared from meat heated at 100 °C for 320 min or at 120 °C for 60 min were larger than those induced by extracts prepared from meat heated at 100 °C for 10 min. Other fish extracts elicited wheals and erythemas. For wheals and erythemas induced by the extracts prepared from heated meat, the scores for bony fish were 4+ and those for cartilaginous fish were 3+.

Discussion

In the present study, we investigated the heat stabilities of the IgE reactivities to fish extracts of sera from fish allergic patients

sensitized to fish collagen. Although SDS-PAGE analysis detected bands corresponding to the collagen α - (monomer), β - (dimer), and γ - (trimer) chains that disappeared depending on heat load (Fig. 1), the IgE-ELISA revealed that IgE reactivities of sera from patients sensitized to fish collagen remained after samples were heated, for example, at 100 °C for 320 min and at 140 °C for 10 min (Figs 3, 4). The reasons why the various conditions were selected are because of the following: first, there was a possibility that the allergenicity of fish collagen could be reduced by applying a heavy heating load because denatured collagen (gelatin) has been known to be degraded by heating; second, fish is commonly boiled for several hours or up to over 10 h for a preparing soup stock of *ramen* noodles in Japanese *ramen* restaurants; third, retort pouch foods can be heated at 140 °C; and fourth, 140 °C treatment can heat fish without singeing.

SPTs using extracts prepared from meat heated at 100 °C for 320 min and at 120 °C for 60 min elicited wheals and erythemas in a patient sensitized by fish collagen (Table 2). Further, IgE in patients' sera reacted to extracts of six types of bony fish meat as well as the extract of Pacific mackerel meat heated at 100 °C for 320 min. Interestingly, the IgE reactivities in the ELISA (Fig. 5B) and the responses to the SPT (Table 2) to extracts of cartilaginous fish markedly lower compared with those of bony fish.

A continuous increase of the heat load imposed on the meat of Pacific mackerel gradually caused the disappearance of the bands corresponding to the collagen β - and γ -chains (Fig. 1). For example, after the meat was heated at 140 °C for 10 min, only a low level of the putative α -chain remained, which was undetectable in the extract prepared from meat heated at 100 °C for 320 min. These results may be explained by the disassociation of multimers to monomers because of the increased heat load that degraded the putative collagen α -chain. Along with the disappearance of three putative collagen bands from the SDS-PAGE gels, smear was detected at a range from 40 kDa to 120 kDa, which showed IgE reactivity when purified Pacific mackerel collagen was heated at 100 °C for 320 min (Fig. 2), demonstrating that although collagen is degraded by heating, IgE-binding epitopes remained after degradation of collagen.

A major IgE-binding epitope of rainbow trout (*Oncorhynchus mykiss*) collagen resides near its C-terminus (amino acid residues 941–960).³⁰ This epitope is recognized by 10 of 10 patients sensitized to fish collagen.³⁰ However, four of 10 patients with allergies

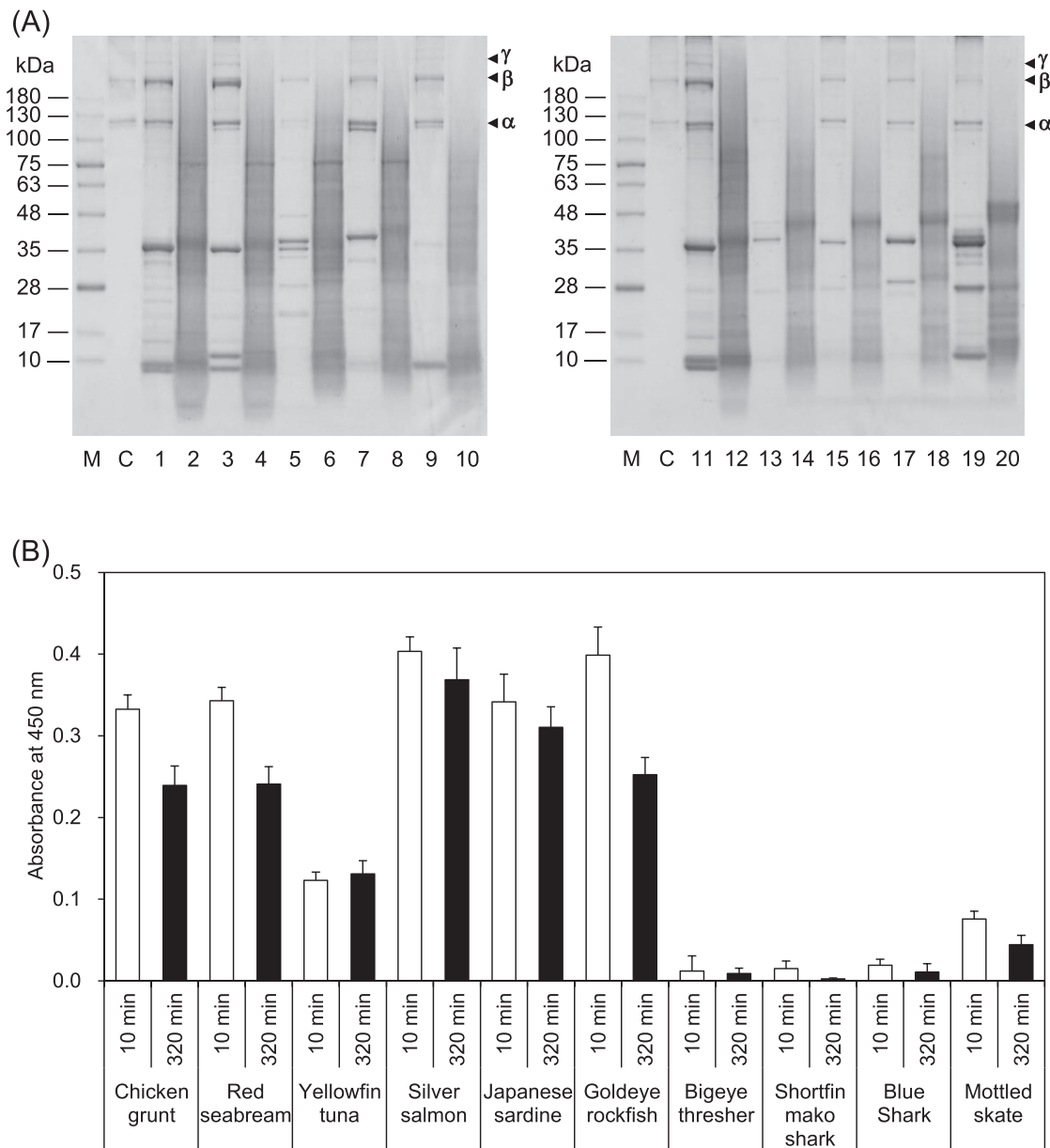


Fig. 5. SDS-PAGE analysis of extracts prepared from heated meat of six bony and four cartilaginous fish (A) and ELISA analysis of the IgE reactivities of pooled serum obtained from eight patients sensitized to fish collagen against those extracts (B). (A) Lanes: M, molecular mass markers; C, purified Pacific mackerel collagen. Extracts were prepared from meats heated at 100 °C for 10 min (n) and 320 min (n + 1): chicken grunt (1, 2); red seabream (3, 4); yellowfin tuna (5, 6); silver salmon (7, 8); Japanese sardine (9, 10); goldeye rockfish (11, 12); bigeye thresher (13, 14); shortfin mako shark (15, 16); blue shark (17, 18); and mottled skate (19, 20). α , β , and γ denote collagen chains. (B) Extracts of six bony and four cartilaginous fish were prepared from meats heated at 100 °C for 10 or 320 min. Data are expressed as the mean + standard deviation (n = 3).

to fish collagen recognize only one region of the IgE-binding epitope of the collagen α -chain of rainbow trout.³⁰ X-ray analyses revealed that partial fragments of collagen can form a triple-helical structure (trimer).³¹ Further, γ -chains interact with each other to form the collagen fiber.²⁵ When symptoms of allergy occur, binding of the allergen to IgE already bound by the IgE receptor (Fc ϵ RI) on mast cells cross-links the bound IgEs with the allergen, and the aggregation of Fc ϵ RI leads to degranulation and the release of chemical mediators from the cell. Therefore, the cross-linked structure requires at least two IgE-binding epitopes. Presumably, thermolytic fragments of collagen form dimers, trimers, or fibers (assembly of multiple trimers), and if only one IgE-binding epitope is present on an α -chain, IgE molecules can form cross-links with collagens on the mast cell.

SDS-PAGE analysis of extracts prepared from meat heated at 100 °C for 10 min that were prepared from six types of bony fish other than the Pacific mackerel, revealed that the intensity of the putative collagen band of yellowfin tuna was weak, and those of extracts from the remaining bony fish were strong (Fig. 5A). Consistent with these findings, the IgE-reactivity with the extract of yellowfin tuna was lower compared with those of the five types of bony fish (Fig. 5B). These findings are consistent with those of the SPTs (Table 2) and indicate that the IgE reactivity of collagen is proportional to the collagen content of at least bony fish. In contrast, SDS-PAGE analysis of extracts prepared from the shortfin mako and blue sharks detected unambiguous bands consistent with those of the collagens of cartilaginous fish (Fig. 5A), and the IgE-ELISA reactivities were very low (Fig. 5B). Further, the

Table 2
Results of skin prick testing.

Sample	Extract	Wheal (mm × mm)/ erythema (mm × mm)	Score
Histamine	–	3 × 3/6 × 4	–
Control	–	–/–	–
Pacific mackerel	Unheated	5 × 5/13 × 13	3+
	Heated (100 °C, 10 min)	5 × 8/15 × 13	4+
	Heated (100 °C, 320 min)	13 × 9/19 × 16	4+
	Heated (120 °C, 60 min)	9 × 8/17 × 16	4+
Chicken grunt	Heated (100 °C, 10 min)	12 × 7/17 × 18	4+
Red seabream	Heated (100 °C, 10 min)	12 × 8/19 × 20	4+
Yellowfin tuna	Heated (100 °C, 10 min)	7 × 6/13 × 18	4+
Silver salmon	Heated (100 °C, 10 min)	21 × 9/18 × 32	4+
Japanese sardine	Heated (100 °C, 10 min)	12 × 9/25 × 16	4+
Goldeye rockfish	Heated (100 °C, 10 min)	11 × 9/25 × 26	4+
Bigeye thresher	Heated (100 °C, 10 min)	3 × 3/11 × 11	3+
Shortfin mako shark	Heated (100 °C, 10 min)	5 × 6/12 × 13	3+
Blue shark	Heated (100 °C, 10 min)	4 × 4/12 × 13	3+
Mottled skate	Heated (100 °C, 10 min)	5 × 5/19 × 19	3+

responses to cartilaginous fish extracts detected using the STP were lower than those prepared from bony fish (Table 2). The IgE reactivities of extracts from rays are weak when analyzed using western blotting with sera obtained from patients with allergies to fish collagen.³² Therefore, the amino acid sequences of type I collagens from cartilaginous fish may vary widely from those of from bony fish.

Because reducing the IgE reactivities of fish collagen by heating is difficult, we expect that investigations of the degradation of IgE-binding epitopes by proteases or removal of collagens from fish meat will be required to develop hypoallergenic fish products. Interestingly, we show here that the IgE reactivities of cartilaginous fish was lower than that of bony fish. The meat of cartilaginous fish is used for meat-paste products such as Japanese *surimi*. Therefore, it might be possible to produce hypoallergenic products using the meat of cartilaginous fish. However, sharks' fins contain large amount of collagens²⁷ and are widely used in Chinese cuisine. Although the IgE reactivity to collagens from cartilaginous fish is low, attention should be paid to their massive consumption.

A previous study revealed that the sera of 50% of Japanese patients with fish allergies showed IgE reactivities to fish collagens.²⁸ In the study, IgE in pooled serum collagen-mono-sensitized patients who have experienced allergic symptoms when eating raw and cooked fish recognized only three collagen bands on western blotting analysis using heated crude extract.²⁸ All patients used in current study were among the patients used in the previous study.²⁸ Therefore, there is no possibility that patients used in the current study developed allergic reactions to proteins other than fish collagen when they ate cooked fish.

Nevertheless, few studies focus on collagen as an allergen. Therefore, future studies must be designed to determine the cross-reactivities of fish collagens, the determination of the amino acid sequences of type I collagens of numerous fish species, and the development of hypoallergenic fish products for patients with allergies to fish collagen.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

YK supervised the entire research, conducted *in vitro* experiments, and wrote the manuscript. TK conducted *in vitro* experiments. RN conducted *in vivo* experiments. MA and NH-S contributed toward manuscript revision. All authors read and approved the final manuscript.

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