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CLINICAL EVALUATION OF ω-5 GLIADIN-SPECIFIC IgE TEST

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Objective: To reveal the clinical utility of an IgE test specific to ω-5 gliadin in the diagnosis of immediate-type wheat allergy.
Methods: We detected ω-5 gliadin-specific IgE in sera from all patients examined for wheat-specific IgE in our allergy clinic between January and October, 2008. The diagnostic value of the test was analyzed against the true diagnosis of wheat allergy based on oral wheat challenge or convincing clinical history. Subjects comprised 233 patients (median age, 3.6 years), 59 patients were diagnosed with wheat allergy, and 174 were judged to have no wheat allergy.
Results: The prevalence of being diagnosed as wheat allergy was 68% in the patients with CAP score 2 to ω-5 gliadin IgE (n = 31), and 87% in those with CAP score 3 (n = 15). All of the 3 patients with CAP score 4 or more were wheat allergic. However, 24% of patients with wheat allergy showed negative results (< 0.35UA/ml). According to the data, we proposed the probability curve of ω-5 gliadin-specific IgE.
Conclusions: IgE testing specific to ω-5 gliadin can offer a useful clinical marker for the diagnosis of immediate-type wheat allergy. But the data should always be evaluated with wheat-specific IgE titers, because of the low clinical sensitivity to detect the patients with wheat allergy.

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Original

A Major IgE Epitope of Rainbow Trout Collagen α2 Chain

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Bovine collagen is allergenic and its major IgE epitope has already been identified. Fish collagen is also allergenic but shows no IgE cross-reactivity with bovine collagen, implying that it has specific IgE epitopes. Therefore, this study was initiated to elucidate IgE epitopes of rainbow trout collagen α2 chain. Five overlapping proteins (R1–5; 221 or 225 amino acids long with an offset of 205 amino acids) covering the entire sequence of the rainbow trout collagen α2 chain were expressed in *Escherichia coli.* Immunoblotting experiments using 10 patients' sera reacting to fish collagen revealed that the major IgE epitope is included in the R5 protein (region 821–1,041). Then, 26 overlapping peptides (20 or 21 amino acids long with an offset of 8 amino acids) encompassing the sequence of the R5 protein were chemically synthesized and examined for IgE-binding ability by fluorescence ELISA. Region 941–960 was found to be most IgE-reactive. When evaluated by inhibition ELISA, this region accounted for more than 50% of the IgE reactivity to the R5 protein. Moreover, the same region was found to be IgE-reactive in bastard halibut and zebrafish collagen α2 chains, but not in bovine collagen α2 chain. Our results strongly suggest that region 941–960 is a major common IgE epitope of fish collagen α2 chains.

Key words: allergen; α2 chain; collagen; fish allergy; IgE epitope; rainbow trout

Introduction

Collagen is ubiquitously found in animals as a major protein in the skin, bone and intramuscular connective tissue of animals. It is composed of three α chains (in the form of $(\alpha1)_2\alpha2$ or $\alpha1\alpha2\alpha3$) of about 110–120 kDa, which are twisted together to form a triple helix[1]. The denatured collagen (gelatin) from skin or bone of bovine and porcine is widely utilized as a raw material of jelly and as a supplement in cosmetics and foods. Because gelatin has long been believed to be nonimmunogenic and nonallergenic to humans, it has also been used as a stabilizer in vaccines, such as those for measles, mumps and rubella. However, immunoglobulin E (IgE)-mediated adverse reactions, including anaphylaxis, to the vaccines have been reported in some children and demonstrated to be mostly ascribable to gelatin in the vaccines[2, 3]. Subsequently, of the α1 and α2 chains constituting bovine collagen, only the latter chain was found to be IgE-reactive[4]. Moreover, the major IgE epitope of the bovine collagen α2 chain has been elucidated to reside in region 485–494[5].

In fish, the major allergen is parvalbumin, a calcium-binding sarcoplasmic protein with a molecular mass of about 12 kDa, as demonstrated with various species of fish, including cod[6], carp[7], Atlantic salmon[8] and Pacific mackerel[9]. Besides parvalbumin, collagen has also been identified as a fish allergen, although not a major one[10, 11]. In contrast to bovine collagen, hetero α-chains of fish collagen all seem to be IgE-reactive[11, 12]. Importantly, collagens from various species of fish are cross-reactive with one another, but not with those from mammals[10, 12], implying that fish collagens have specific IgE epitopes distinct from those of mammalian collagens. To achieve a better understanding of the allergenicity of fish collagens, it is requisite to elucidate their specific IgE epitopes. Furthermore, information about IgE epitopes of fish collagens would be helpful to develop not only hypoallergenic processed fish products, but also hypoallergenic collagen molecules for immunotherapy of fish allergy.

All three α chains of collagen have been completely sequenced for only two species of fish, rainbow trout[13] and zebrafish *Danio rerio*[14]. Rainbow trout is an important edible fish that is widely consumed, while zebrafish is often used as a model fish in biology, but is not edible. Furthermore, the major IgE epitope has already been identified for the bovine collagen α2 chain[5]. Here, we selected the rainbow trout *Oncorhynchus mykiss* collagen α2 chain as a target for analysis of IgE epitopes. First, overlapping recombinant proteins covering the entire sequence of the rainbow trout collagen α2 chain were examined for IgE-binding ability. Next, epitope mapping experiments were performed using synthetic overlapping peptides encompassing the sequence of the most IgE-reactive protein. Our results indicate that the

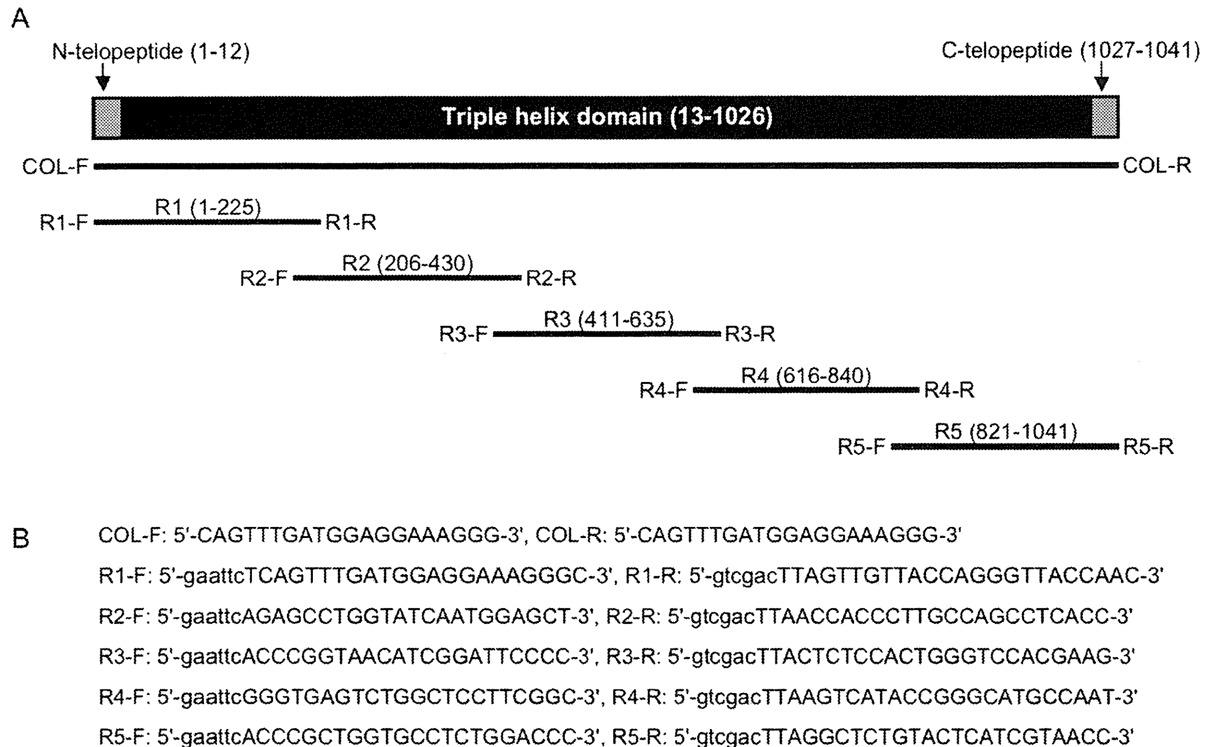

Fig. 1. Schematic presentation of the rainbow trout collagen $\alpha 2$ chain and five overlapping proteins (R1–5) covering the full sequence (A) and nucleotide sequences of the primers used to amplify the mature protein and R1–5 proteins (B)

(A) The regions corresponding to the N-telopeptide, triple helix domain, C-telopeptide and R1–5 proteins are indicated in parentheses. Forward and reverse primers are shown at the left and right sides, respectively, for each mature protein and R1–5 proteins. (B) The *Eco*RI and *Sal*I restriction sites are shown in small letters for forward and reverse primers, respectively.

major IgE epitope of the rainbow trout collagen $\alpha 2$ is included in the region 941–960. We also provide evidence that the same region is likely to be a common IgE epitope of fish collagen $\alpha 2$ chains.

Materials and Methods

Fish

A live specimen of rainbow trout was kindly supplied by Professor S. Sato of our university. The white muscle obtained from the specimen was immediately immersed in liquid nitrogen and kept at −80℃ until use.

Construction of a cDNA library

Total RNA was extracted from 2 g of the rainbow trout white muscle with TRIzol reagent (Life Technologies, Rockville, MD, USA) and poly(A)$^+$ mRNA was purified using an mRNA Purification Kit (GE Healthcare, Piscataway, NJ, USA). A Marathon cDNA library was constructed by conversion of a part of the purified mRNA to cDNA, followed by ligation of AP1 adapters, using a Marathon cDNA Amplification Kit (Clontech Laboratories, Palo Alto, CA, USA).

Expression and purification of recombinant proteins

Five overlapping proteins, R1–5 (221 or 225 amino acids in length with an offset of 205 amino acids), which cover the entire sequence of the rainbow trout collagen$\alpha 2$ chain (Fig. 1A), were individually expressed in *Escherichia coli* as a glutathione-*S*-transferase (GST)-fusion form using the pGEX-6P-3 expression vector (GE Healthcare). First, a cDNA encoding the $\alpha 2$ chain[13] was amplified by PCR using the Marathon cDNA library as a template. Then, cDNAs of the R1–5 proteins, with addition of *Eco*RI and *Sal*I restriction sites at the 5′ and 3′ ends, respectively, were individually amplified by PCR using the $\alpha 2$ chain cDNA as a template. Nucleotide sequences of the primers used in PCR are summarized in Fig. 1B. Each PCR product and the expression vector were digested with *Eco*RI and *Sal*I and ligated using a DNA Ligation Kit (Takara, Otsu, Japan). *E. coli* JM109 was transformed with the ligated product and cultured overnight on LB agar containing 0.005% ampicillin at 37℃. A single colony was selected and grown in 500 mL of LB medium containing 0.005% ampicillin at 37℃ until the absorbance at 600 nm reached 0.6. Then, isopropyl-β-D-thiogalactoside (IPTG) was added to the culture to give a concentration of 1 mmol/L and incubation was continued for 3 hr. Bacteria were harvested by centrifugation and resuspended in 25 mL of 50 mmol/L Tris–HCl buffer (pH 7.5) containing 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride and 0.01% lysozyme. The bacterial suspension was sonicated and centrifuged,

and the GST-fusion protein recovered in the supernatant was purified by affinity chromatography on a GSTrap HP column (GE Healthcare) according to the manufacturer's instructions.

Human sera

Sera from 10 fish-allergic patients (patients 1–10) were used. This study was approved by the ethics committees of Chiba University, Fujita Health University and Yokohama City University. All patients had been diagnosed to be allergic to fish at hospitals, based on documented clinical histories of immediate hypersensitivity reactions after ingestion of fish and capsulated hydrophilic carrier polymer-radioallergosorbent test (CAP-RAST) classes of 2–4 against fish such as mackerel and tuna. In addition, their sera were all confirmed to contain specific IgE to fish collagen by ELISA, which was performed using either parvalbumin or collagen purified from bigeye tuna *Thunnus obesus* muscle as an antigen, as described in our previous paper[12]. Sera from two healthy volunteers without adverse reactions after ingestion of any foods were used as controls.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

SDS-PAGE was performed on a ready-made gel (PhastGel Gradient 8–25; GE Healthcare) using a PhastSystem apparatus (GE Healthcare), as recommended by the manufacturer. Each sample was dissolved in 62.5 mmol/L phosphate buffer (pH 7.5) containing 2% SDS, 3 mol/L urea and 100 mmol/L dithiothreitol, heated at 100℃ for 10 min and subjected to electrophoresis. Precision Plus Protein Standards (Bio-Rad Laboratories, Hercules, CA, USA) were run as a reference, along with samples. After running, proteins were stained with Coomassie Brilliant Blue R-250.

Immunoblotting was carried out as reported previously[15]. In brief, the proteins separated by SDS-PAGE were electrotransferred to a polyvinylidene difluoride membrane, which was reacted successively with patients' serum (diluted 1 : 250) and horseradish peroxidase-conjugated goat anti-human IgE antibody (diluted 1 : 5,000; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Antigen-antibody binding was visualized using an ECL Plus Western Blotting Detection System (GE Healthcare) and an ECL Mini Camera (GE Healthcare) according to the manufacturer's manual.

Peptide synthesis

The R5 protein (region 821–1041) was found to be most IgE-reactive, as described below. Therefore, 26 overlapping peptides (20 or 21 amino acids in length with an offset of 8 amino acids) covering the entire sequence of the R5 protein were chemically synthesized. Peptide 16 (region 941–960) was judged to be the most IgE-reactive as described below, and hence peptides corresponding to region 941–960 of bastard halibut *Paralichthys olivaceus*[16], zebrafish[14] and bovine[17] collagen α2 chains were additionally synthesized (named peptides Bh, Zf and Bv, respectively).

Peptide synthesis was performed on a PMMS-8 peptide synthesizer (Shimadzu, Kyoto, Japan) using 9-fluorenylmethyloxycarbonyl as an amino-protecting group and (benzotriazol-1-yloxy)tripyrrolidino-phosphonium hexafluorophosphate as a coupling reagent, according to the manufacturer's instructions. After synthesis, each peptide was purified by reverse-phase HPLC on a TSKgel ODS-120T column (0.46×25 cm; Tosoh, Tokyo, Japan), which was eluted with a linear gradient of acetonitrile (49–63% in 40 min) at a flow rate of 1 mL/min. The molecular weight of each purified peptide was confirmed by MALDI-TOFMS using a KOMPACT MALDI I instrument (Shimadzu).

Enzyme-linked immunosorbent assay (ELISA)

IgE reactivity of synthetic peptides was assessed by fluorescence ELISA as reported previously[18]. In brief, a Nunc Immobilizer Amino plate for peptide (Nalge Nunc International, Rochester, NY, USA) was coated with 50 μL of peptide solution (10 μg/mL) and reacted successively with patients' serum (diluted 1 : 250) and β-galactosidase-conjugated goat anti-human IgE antibody solution (0.25 μg/mL; American Qualex, San Clement, CA, USA). Enzyme reaction was carried out using substrate solution (0.1 mg/mL 4-methylumbelliferyl-β-D-galactoside) and stopped by addition of 100 mmol/L glycine–NaOH buffer (pH 10.3). Fluorescence intensity was measured on a SPECTRAmax GEMINI XS (Molecular Devices, Sunnyvale, CA, USA) with excitation at 367 nm and emission at 453 nm. Inhibition ELISA was also carried out to estimate what percentage of IgE reactivity to the R5 protein is accounted for by peptide 16. Pooled patients' serum (diluted 1 : 125) was incubated with an equal volume of inhibitor (peptide 16 or R5 protein) solution (0.002–20 μg/mL) at 37℃ for 1 hr and 50 μL of the mixture was then added to a microplate that had previously been coated with the R5 protein (1 μg/mL). The subsequent procedure was the same as in ELISA. All ELISAs (including inhibition ELISA) were performed in triplicate and the data obtained were expressed as mean±SD.

Results

IgE-binding ability of recombinant R1–5 proteins

Five proteins (R1–5) covering the entire sequence of the rainbow trout collagen α2 chain were individually expressed in *E. coli* as GST-fusions. In each expression experiment, a 45–48 kDa band was prominently observed in both soluble and insoluble fractions from IPTG-induced bacteria as analyzed by SDS-PAGE (data not shown). Based on the molecular masses of GST (about 26 kDa) and R1–5 proteins (about 20 kDa), these bands were attributed to the GST-fusion proteins. Following affinity chromatography on a GSTrap HP column, each GST-fusion protein was obtained in electrophoretically pure state from the soluble fraction (Fig. 2A).

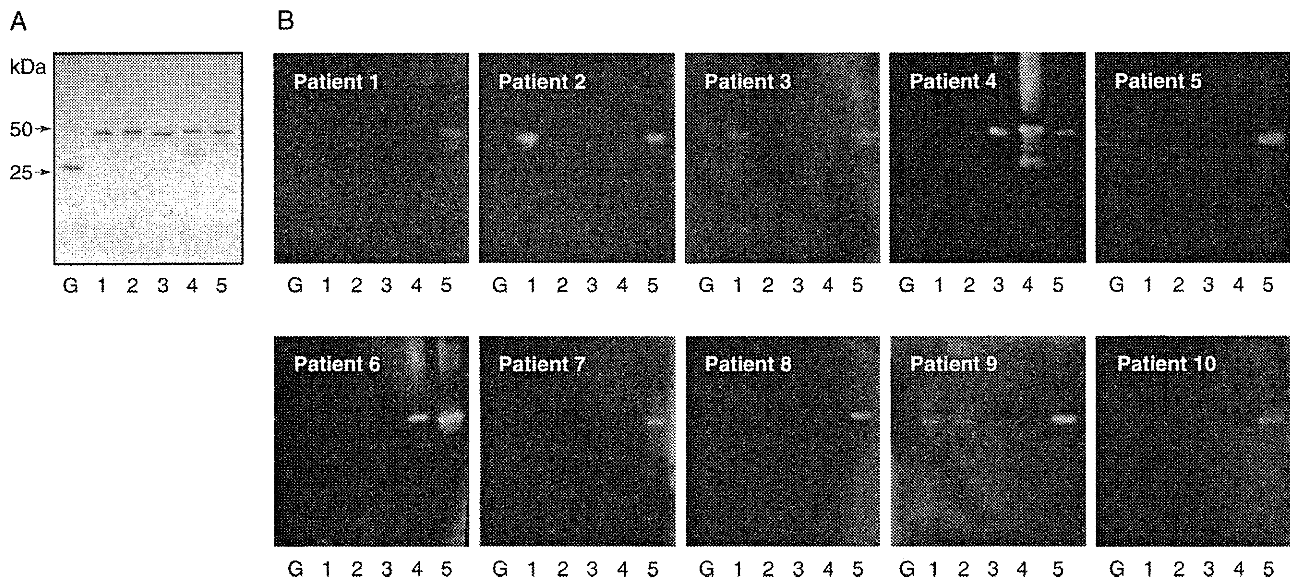

Fig. 2. Analysis of GST and recombinant R1–5 proteins by SDS-PAGE (A) and immunoblotting (B)
Lanes: G, GST; 1, GST-fusion R1; 2, GST-fusion R2; 3, GST-fusion R3; 4, GST-fusion R4; 5, GST-fusion R5.

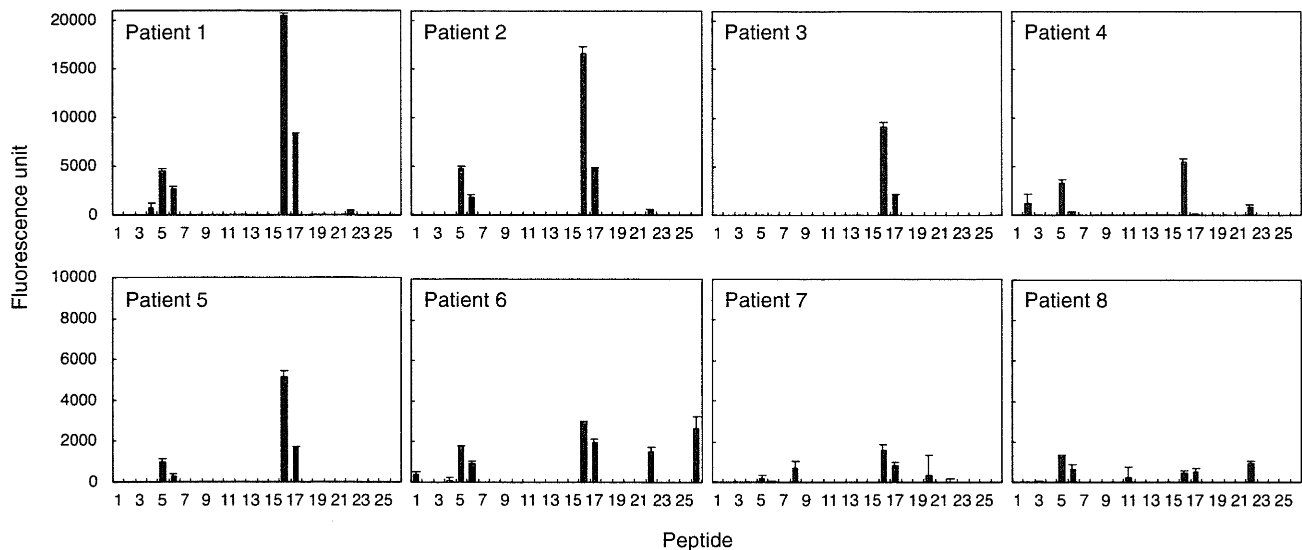

Fig. 3. IgE reactivity of peptides 1–26 analyzed by fluorescence ELISA
Data are expressed as mean±SD (n=3).

When analyzed by immunoblotting, sera of 10 patients reacted to at least one of the five GST-fusion proteins (Fig. 2B), while two control sera showed no reactivity to any of them (data not shown). IgE reactivity to GST was not recognized in any of the patients' sera, supporting the view that the reactivity between the patients' sera and the GST-fusion proteins is attributable to the R1–5 portions of the fusion proteins. Sera from patients 2, 3, 4, 6 and 9 reacted not only to the R5 protein, but also to one or two of the R1–4 proteins; for example, serum of patient 2 also reacted to the R1 protein and serum of patient 4 to the R3 and 4 proteins. The remaining five patients' sera showed positive reactivity only to the R5 protein. These results indicate that the major IgE epitopes of the rainbow trout collagen $\alpha 2$ chain are included in the C-terminal R5 protein (region 821–1,041).

IgE-binding ability of synthetic peptides 1–26

To evaluate further the major IgE epitopes, 26 overlapping peptides covering the entire sequence of the R5 protein were synthesized and examined for IgE-binding ability by fluorescence ELISA. Since insufficient serum was available from patients 9 and 10, only the remaining eight sera were subjected to fluorescence ELISA. Each serum reacted to several peptides (Fig. 3), although control sera reacted to none of the peptides (data not shown). Among the 26 peptides, peptides 5, 16 and 17 were recognized at high frequencies by the patients' sera. Seven sera showed the highest reactivity to peptide 16; the only exception was serum from patient 8 serum, in which the reactivity to peptide 16 was

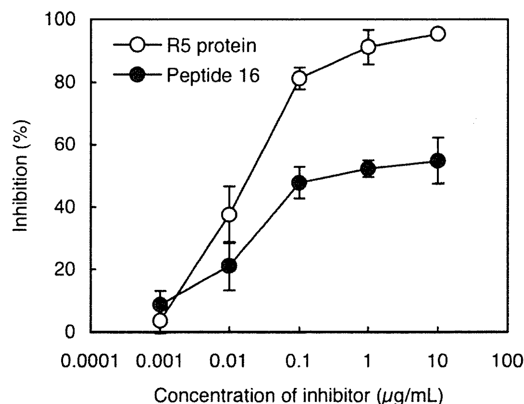

Fig. 4. Inhibition of the IgE reactivity to R5 protein by peptide 16 and R5 protein

Pooled serum (1 : 125 dilution) from patients 1–5 was preincubated with an equal volume of various concentrations of inhibitor (peptide 16 or R5 protein) and added to a plate coated with R5 protein (1 μg/mL). Data are ex- pressed as mean±SD ($n=3$).

minimal. It was thus concluded that the major IgE epitope of the rainbow trout collagen $\alpha2$ chain is located in peptide 16 (region 941–960). As evaluated by inhibition ELISA using pooled serum from patients 1–5, peptide 16 accounted for more than 50% of the IgE reactivity to the R5 protein (Fig. 4).

IgE-binding ability of region 941–960 of collagen α2 chains

Fish collagen $\alpha2$ chains have been completely sequenced for chum salmon *Oncorhynchus keta* (DDBJ/EMBL/GenBank accession number: AB075699), bastard halibut[16] and zebrafish,[14] as well as for rainbow trout[13]. As compared to the amino acid sequence of the rainbow trout collagen $\alpha2$ chain, that of the chum salmon collagen $\alpha2$ chain is completely conserved in region 941–960 (the most IgE-reactive region of the rainbow trout collagen $\alpha2$ chain) and those of the bastard, halibut and zebrafish collagen $\alpha2$ chains are highly conserved, with only one or two alterations (Fig. 5). On the other hand, the amino acid sequences of mammalian collagen $\alpha2$ chains are homologous with one another in region 941–960 but are significantly different from those of fish collagen $\alpha2$ chains. To examine whether region 941–960 is a common IgE epitope in fish collagen $\alpha2$ chains, synthetic peptides Bh, Zf and Bv corresponding to region 941–960 of the bastard halibut, zebrafish and bovine collagen $\alpha2$ chains, respectively, were analyzed for IgE-binding ability by fluorescence ELISA using sera from patients 1–7, in which significant reactivity to peptide 16 was observed. As shown in Fig. 6, no significant difference in reactivity with serum from patient 1, 2 or 3 was observed among peptides 16, Bh and Zf. However, the reactivity to peptides Bh and Zf was low in sera from patients 4–6 compared to that to peptide 16. No reactivity with serum from patient 7 was displayed by peptide Bh or Zf. In the case of the

```
                941      950           960
Rainbow trout   MKGLRGHGGLQGMPGPNGPS
Chum salmon     --------------------
Bastard halibut -------P--------S---
Zebrafish       -------P------------
Human           LP--K--N----L--IA-HH
Bovine          LP--K--N----L--LA-HH
Rabbit          LP-IK--N----L--LA-QH
Mouse           LP--K-YS----L--LA-LH
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Fig. 5. Amino acid sequence alignment of the 941–960 region of collagen $\alpha2$ chains from fish and mammals

DDBJ / EMBL / GenBank accession numbers: rainbow trout, AB052837; chum salmon, AB 075699; bastard halibut, AB196514; zebrafish, BC071278; human, BC042586; bovine, AB 008683; rabbit, D49399; mouse, AK132496.

peptide Bv, little reactivity with patients' sera was seen, except for patient 6. Patient 6 may be allergic to bovine collagen as well as fish collagen, although this was not confirmed. Nevertheless, in view of the fact that there are marked differences in amino acid sequence between peptides 16 and Bv, it seems unlikely that the patient 6 IgE reacting to peptide 16 can cross-react to peptide Bv.

Discussion

Collagen α chains, including $\alpha2$ chain, are too large for direct analysis of their IgE epitopes by usual epitope mapping experiments with synthetic peptides. In this study, therefore, five overlapping proteins (R1–5) covering the entire sequence of the rainbow trout collagen $\alpha2$ chain were expressed in *E. coli* and their IgE reactivity was examined by means of immunoblotting to identify major IgE-binding regions. This strategy was successful; the R5 protein was found to be the most IgE-reactive. Subsequent epitope mapping experiments, using synthetic overlapping peptides encompassing the sequence of the R5 protein, clearly demonstrated that the major IgE epitope of the rainbow trout collagen $\alpha2$ chain is located in region 941–960 (MKGLRGHGGLQGMPGPNGPS). The major IgE epitope of the bovine collagen $\alpha2$ chain has previously been reported to be included in region 485–494 (IPGEFGKPGP)[5]. Clearly, there is no overall sequence homology between the major IgE epitopes of the rainbow trout and bovine collagen $\alpha2$ chains.

The chum salmon collagen $\alpha2$ chain has the same amino acid sequence in region 941–960 as the rainbow trout collagen $\alpha2$ chain (Fig. 5). In the case of the bastard halibut and zebrafish collagen $\alpha2$ chains, one or two replacements (replacements of Gly by Pro at position 948 and Asn by Ser at position 957 for the bastard halibut collagen $\alpha2$ chain and replacement of Gly by Pro at position 948 for the zebrafish collagen $\alpha2$ chain) are recognized in region 941–960 compared to the amino acid sequence of the rainbow trout collagen $\alpha2$ chain. However, the peptides Bh and Zf, corresponding to region 941–960 of the bastard halibut and zebrafish collagen $\alpha2$ chains, respectively, were found to be reac-

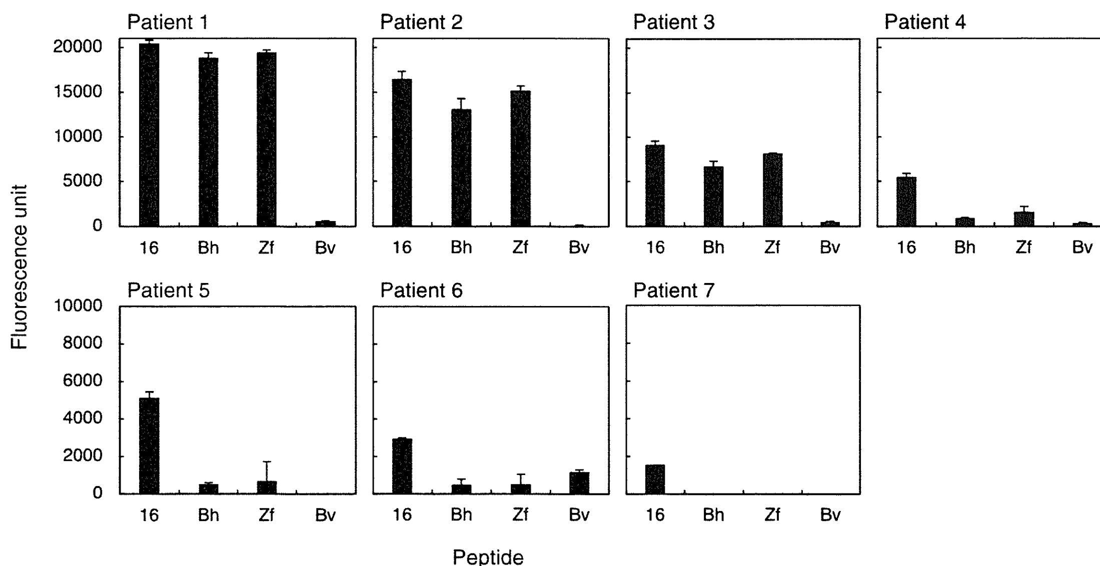

Fig. 6. IgE reactivity of the 941–960 region of fish and bovine collagen $\alpha2$ chains analyzed by fluorescence ELISA
Data are expressed as mean±SD ($n=3$). Peptides: 16, rainbow trout; Bh, bastard halibut; Zf, zebrafish; Bv, bovine.

tive with patients' sera, although their IgE-binding ability was reduced in three sera and lost in one (Fig. 6). It is thus likely that region 941–960 is a major common IgE-binding epitope in fish collagen $\alpha2$ chains. To confirm this, further amino acid sequence data on fish collagen $\alpha2$ chains should be accumulated.

Bovine type I collagen is composed of two $\alpha1$ chains and one $\alpha2$ chain, like other mammalian type I collagens, and only the $\alpha2$ chain has been demonstrated to be IgE-reactive[4]. The major IgE-binding epitope elucidated for the bovine collagen $\alpha2$ chain is not found in collagen $\alpha1$ chains from mammals, including bovine[5]. Differing from mammalian collagens, fish collagens take the form of either $(\alpha1)_2\alpha2$ or $\alpha1\alpha2\alpha3$, depending on the fish species and tissues. Importantly, IgE cross-reactivity has been found among fish collagen α chains[11),12)]. Nevertheless, the same sequence as that of the major IgE-binding epitope (region 941–960) determined for the rainbow trout $\alpha2$ chain is not found in fish collagen $\alpha1$ and $\alpha3$ chains, including the rainbow trout collagen $\alpha1$ and $\alpha3$ chains. Further study on which residues in the major IgE epitope of the rainbow trout collagen $\alpha2$ chain are crucial for IgE-binding would be helpful to understand the cross-reactivity among different α chains of fish collagens.

Finally, it should be noted that the major IgE epitope of the rainbow trout collagen $\alpha2$ chain contains three Pro residues and one Lys residue. This is also the case with the major IgE epitope of the bovine collagen $\alpha2$ chain. Pro residues are abundant in collagen, and many of them are hydroxylated by post-translational modification. Some Lys resides are also known to be hydroxylated. In the case of the major IgE epitope of the bovine collagen $\alpha2$ chain, hydroxylation of Pro and Lys residues was suggested to be independent of the interaction with IgE. However, this has not been experimentally verified. Further study using analogous synthetic peptides with replacements of Pro and Lys residues by hydroxy-Pro and hydroxy-Lys residues, respectively, is needed to assess in more detail the IgE-binding ability of the major IgE epitopes determined for the rainbow trout and bovine collagen $\alpha2$ chains.

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New extraction method suitable for immunoblotting analysis of fish allergens

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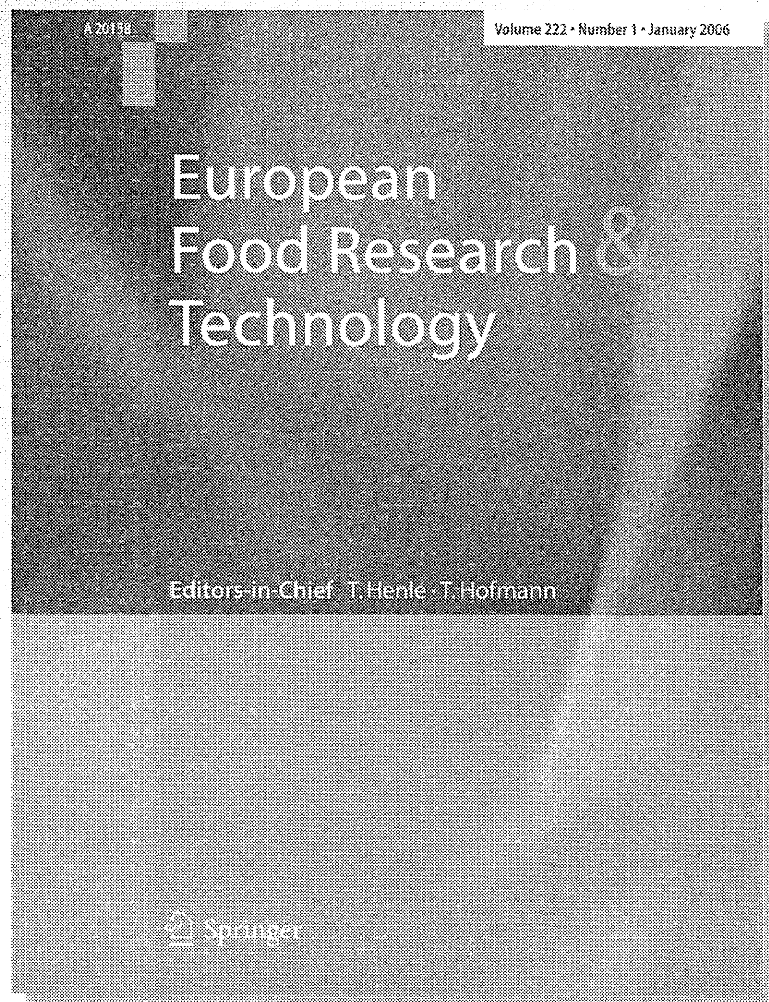

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ORIGINAL PAPER

New extraction method suitable for immunoblotting analysis of fish allergens

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Abstract Immunoblotting is a simple method to analyze allergens in biological samples. In previous immunoblotting studies on fish allergens, however, collagen, an important allergen next to parvalbumin (major fish allergen), has not been detected in fish muscle extracts probably due to its unique chemical properties. This study was aimed to develop an extraction method suitable for immunoblotting analysis of fish allergens including collagen as well as parvalbumin. When various extracts from the Japanese eel white muscle were analyzed by SDS–PAGE, heating of the muscle homogenate at 80 °C for 20 min was found to be the most effective method to extract collagen as well as parvalbumin. The same extraction method was also effective for the other five species of fish analyzed (rainbow trout, Japanese horse mackerel, crimson sea bream, Pacific mackerel, and Japanese flounder). Furthermore, parvalbumin and/or collagen were successfully identified as allergens in the six species of fish by immunoblotting using the heated extracts prepared by the method described above. It can be concluded that the extraction method (heating of the muscle homogenate at 80 °C for 20 min) developed in this study is useful not only for analyzing fish allergens by immunoblotting but also for preparing antigens for diagnosis of fish allergy by RAST (radioallergosorbent test).

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Introduction

Food allergy mediated by immunoglobulin E (IgE) is frequently fatal due to anaphylactic shock, being a serious problem in industrialized countries. Fish is obviously among the most common causes of food allergy, especially in coastal countries including Japan. The major fish allergen is parvalbumin, a calcium-binding sarcoplasmic protein with a molecular mass of 12 kDa, as demonstrated first with cod (*Gadus callarias*) [1, 2] and subsequently with a number of fish such as carp (*Cyprinus carpio*) [3, 4], salmon (*Salmo salar*) [5], mackerel (*Scomber japonicus*) [6], and tuna (*Thunnus obesus*) [7]. Besides parvalbumin, three classes of proteins, collagen [8, 9], aldehyde phosphate dehydrogenase [10], and transferrin [11] have also been identified as fish allergens, although not major ones. Aldehyde phosphate dehydrogenase was found as an allergen in codfish and transferrin as a cross-reactive allergen between tuna and marlin. However, these two classes of proteins have not yet been recognized as allergens in other species of fish, suggesting that they are allergens in limited species of fish. In contrast, our inhibition ELISA data revealed that collagen is a cross-reactive allergen among various species of fish [12]. Therefore, collagen is considered to be an important fish allergen next to parvalbumin.

An immunoblotting technique, which comprises separation of proteins by SDS–PAGE, transferring of proteins from a polyacrylamide gel to a membrane and immunological detection of targeting proteins on the membrane, has been widely used to identify allergens in crude extracts from biological samples. So far, fish allergens including

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parvalbumin and/or other allergens (aldehyde phosphate dehydrogenase, transferrin, and unknown allergens) have also been effectively analyzed by a number of previous immunoblotting experiments [10, 11, 13–21]. However, it should be noted that immunoblotting is naturally helpless if targeting allergens are absent in the crude extract. As for parvalbumin, it is water-soluble and very stable to heating and hence can be easily extracted from the fish muscle under both non-heated and heated conditions. On the other hand, it may be not so easy to extract collagen from the fish muscle, due to its unique chemical properties. Firstly, native collagen, which is composed of three α-chains [in the form of $(\alpha1)_2\alpha2$ or $\alpha1\alpha2\alpha3$] twisted together to form a triple helix [22], is insoluble in water at low temperatures, being usually absent in the non-heated extract from the fresh fish muscle. Secondly, native collagen is readily converted to a mixture of peptides (called gelatin) during heating of the fish muscle, as a result of both heat denaturation and digestion with muscle proteases, being hardly present in the heated extract. Thus, collagen as a fish allergen has so far been overlooked in previous immunoblotting experiments [10, 11, 13–21]. Recently, Weber et al. [23] prepared fish muscle extracts using urea solution (containing 8 M urea and 2% cyclohexylaminopropane) and detected collagen in the extracts by immunoblotting with polyclonal antibodies against fish collagen. It is, however, unknown whether their extraction method is suitable for analysis of fish allergens (both parvalbumin and collagen) by immunoblotting with patient sera.

As described above, collagen is not an ignorable fish allergen. To analyze fish allergens by immunoblotting, therefore, it is necessary to devise a sophisticated method to extract collagen without fragmentation, together with parvalbumin, from the fish muscle. We report here an extraction method suitable for immunoblotting analysis of fish allergens including collagen as well as parvalbumin.

Materials and methods

Fish samples

The following six species of fish were used in extraction experiments: Japanese eel (*Anguilla japonica*), rainbow trout (*Oncorhynchus mykiss*), Japanese horse mackerel (*Trachurus japonicus*), crimson sea bream (*Evynnis japonica*), Pacific mackerel (*Scomber japonicus*), and Japanese flounder (*Paralichthys olivaceus*). In the case of Japanese eel, live specimens were purchased at the Tokyo Central Wholesale Market, transported to our laboratory, and used for experiments immediately after killing. For the remaining five species, fresh specimens purchased at a local supermarket were immediately subjected to experiments.

Preparation of extracts

In this study, the Japanese eel white muscle was used as a model sample to prepare various extracts. The white muscle was collected from a specimen and well macerated with a mortar and a pestle. The macerate (4 g) was homogenized in three volumes of PBS (150 mM NaCl-10 mM phosphate buffer, pH 7.0) using an Ace Homogenizer AM10 (Nippon Seiki, Tokyo, Japan) under low temperature with ice. After centrifugation of the homogenate at 18,000×*g* and 4 °C for 20 min, the supernatant obtained was regarded as a non-heated extract. On the other hand, heated extracts were prepared as follows. The homogenate was transferred into a 50-mL polypropylene conical tube (30 × 115 mm; Nippon Becton–Dickinson, Tokyo, Japan), which was capped tightly and heated at 20, 40, 60, 80, or 100 °C in a water bath (about 2 L) for 20 min with shaking for initial 5 min. Then, the homogenate was cooled in an ice bath and centrifuged at 18,000×*g* and 4 °C for 20 min. The supernatant was used as a heated extract. In the case of the five species of fish other than Japanese eel, the heated extract was similarly prepared from the white muscle by heating of the homogenate at 80 °C for 20 min.

Purification of parvalbumin and collagen

Purification of parvalbumin from the white muscle of Pacific mackerel and bigeye tuna was achieved by gel filtration and reverse-phase HPLC, as reported previously [6, 7]. Collagen was purified from the white muscle of both species of fish by extraction with 500 mM acetic acid, followed by precipitation with 800 mM NaCl, according to the method of Miller and Rhodes [24].

SDS–PAGE

SDS–PAGE was carried out on a PhastSystem apparatus (GE Healthcare, Piscataway, NJ, USA) using ready-made polyacrylamide gels (PhastGel Gradient 8–25; GE Healthcare), as recommended by the manufacturer. Each sample was dissolved in 62.5 mM Tris–HCl buffer (pH 7.4) containing 2% SDS, 4 M urea, and 100 mM dithiothreitol, denatured by heating at 100 °C for 10 min and subjected to electrophoresis. Precision Plus Protein Standards (Bio-Rad Laboratories, Hercules, CA, USA) were used as references. After running, proteins were visualized by staining with Coomassie Brilliant Blue (CBB) R-250.

Human sera

Sera were obtained from eight fish-allergic patients (Table 1). These patients had been diagnosed to be allergic

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Table 1 Characteristics of patients

Patient	Age	Gender	CAP-RAST class
1	27	Female	Sardine (3), salmon (4), cod (4), mackerel (3), horse mackerel (4), tuna (3)
2	4	Male	Mackerel (2)
3	3	Female	Mackerel (3), horse mackerel (4), tuna (3)
4	22	Female	Salmon (4)
5	19	Male	Sardine (2), cod (4), mackerel (3), horse mackerel (2), tuna (4)
6	18	Female	Horse mackerel (3), tuna (3), flounder (3)
7	4	Female	Salmon (4), cod (3)
8	3	Male	Tuna (3)

to fish at medical institutions (hospitals of Chiba University, Fujita Health University and Yokohama City University), based on the clinical histories of immediate allergic reactions after ingestion of fish and the CAP-RAST (capsulated hydrophilic carrier polymer-radioallergosorbent test) data. Written informed consent for the research use of serum was obtained from each patient.

ELISA

ELISA was performed to examine whether each patient serum reacts to bigeye tuna parvalbumin, bigeye tuna collagen, or both, as reported previously [12]. In brief, a flat-bottomed polystyrene plate with 96 wells (Type H Multi Well Plate for ELISA; Sumitomo Bakelite, Tokyo, Japan) was coated with bigeye tuna parvalbumin or collagen solution (0.001–1.0 μg/mL) and incubated with patient serum (diluted 1:50), followed by horseradish peroxidase-conjugated goat antihuman IgE antibody (diluted 1:2,500; Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Enzyme reaction was carried out using a substrate solution (0.1% *o*-phenylenediamine and 0.03% hydrogen peroxide), and the developed color was measured by absorbance at 490 nm. All ELISAs were performed in triplicate, and the data were expressed as the mean values.

Immunoblotting and inhibition immunoblotting

Immunoblotting was performed as described elsewhere [20]. Briefly, the proteins separated by SDS–PAGE were electrotransferred from the polyacrylamide gel to a polyvinylidene difluoride membrane. Then, the membrane was incubated successively with patient serum (diluted 1:500) and horseradish peroxidase-conjugated goat antihuman IgE antibody (diluted 1:5,000). Antigen–antibody binding was detected using an ECL Plus Western Blotting Detection System (GE-Healthcare) and an ECL Mini Camera (GE-Healthcare), according to the manufacturer's instructions. For inhibition immunoblotting, patient serum (diluted 1:500) was preincubated with either Pacific mackerel parvalbumin (final concentration: 10 μg/mL) or Pacific mackerel collagen (final concentration: 10 μg/mL) at 37 °C for 2 h and used as a primary antibody. The subsequent procedure was the same as that for the immunoblotting described above.

Results

Analysis of fish muscle extracts by SDS–PAGE

When the non-heated and heated extracts from the white muscle of Japanese eel were analyzed by SDS–PAGE, a prominent 12 kDa protein band corresponding to parvalbumin was observed in all extracts (Fig. 1a). This is reasonably understood by the fact that parvalbumin is a thermostable sarcoplasmic (water-soluble) protein, being extractable with aqueous solvents regardless of heating temperatures. On the other hand, collagen α chain of about 120 kDa and β chain (dimer of α chain) of about 240 kDa were recognized in the extracts prepared by heating at 60, 80, and 100 °C but not in the other extracts. Based on the CBB-staining intensity, collagen concentration was assumed to be slightly higher in the 80 °C-heated extract than in the 60 °C-heated extract. In the case of the 100 °C-heated extract, both bands corresponding to collagen α and β chains were rather smear, suggesting that collagen was partly degraded. In the next step, heated extracts were prepared by heating at 80 °C for different times (10, 20, and 60 min) and similarly analyzed by SDS–PAGE. As shown in Fig. 1b, all the three kinds of extracts afforded bands of collagen α and β chains as well as that of parvalbumin. However, the collagen bands were considerably smeared in the 60 min-heated extract probably due to the gradual degradation of collagen during long heating.

The results described above allowed us to conclude that collagen as well as parvalbumin can be most effectively extracted from the Japanese eel white muscle with PBS by heating at 80 °C for 20 min. To evaluate whether this extraction method is commonly applicable to fish, the extracts from five species of fish (rainbow trout, Japanese horse mackerel, crimson sea bream, Pacific mackerel, and

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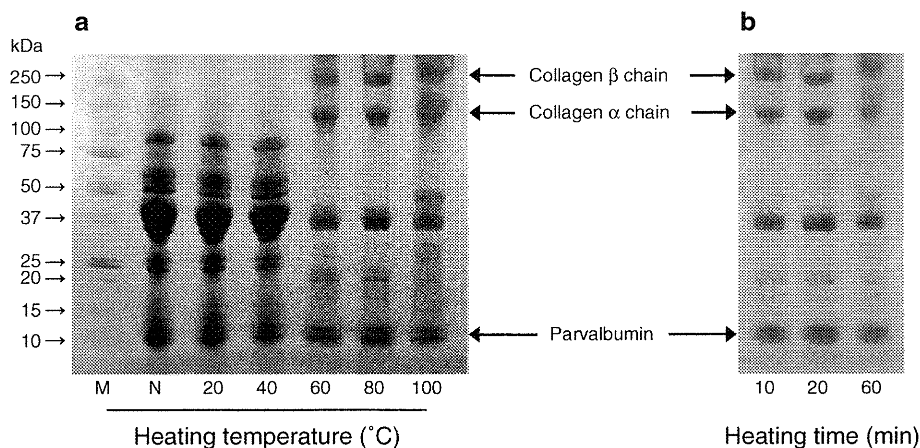

Fig. 1 SDS–PAGE of non-heated and heated extracts from the Japanese eel white muscle. **a** Extracts were prepared from the muscle by non-heating (labeled by N) or heating at different temperatures (20–100 °C) for 20 min. *Lane M*: molecular weight markers (Precision Plus Protein Standards). **b** Extracts were prepared from the muscle by heating at 80 °C for different times (10–60 min)

Japanese flounder) were similarly prepared by heating of the muscle homogenate at 80 °C for 20 min and analyzed by SDS–PAGE in comparison with the heated extract from the Japanese eel muscle. As shown in Fig. 2, both parvalbumin and collagen were certainly detected in the heated extracts from the five species of fish, as in that from Japanese eel. The observed CBB-staining intensity suggested no significant difference in collagen concentration at least among the six species of fish. In contrast, parvalbumin concentration markedly varied from fish to fish; the parvalbumin concentrations in rainbow trout and Pacific mackerel were much lower than those in the other four species of fish. It should be noted that a 14 kDa protein, together with parvalbumin (12 kDa), was clearly found only in crimson sea bream. We have already purified both 14 and 12 kDa proteins and identified them as isoforms of parvalbumin based on the determined partial amino acid sequences (unpublished data).

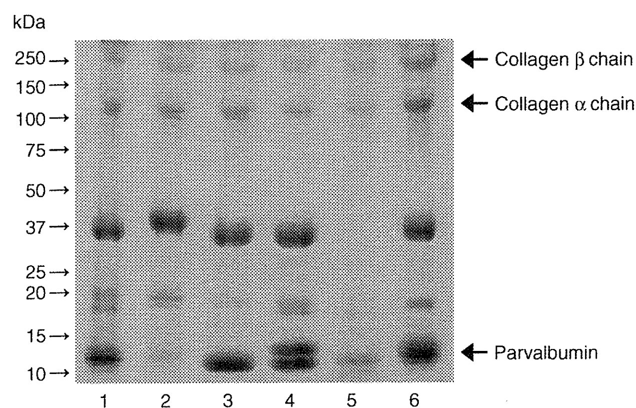

Fig. 2 SDS–PAGE of 80 °C-heated extracts from the white muscles of six species of fish. Fish samples: *lane 1*, Japanese eel; *lane 2*, rainbow trout; *lane 3*, Japanese horse mackerel; *lane 4*, crimson sea bream; *lane 5*, Pacific mackerel; and *lane 6*, Japanese flounder

Reactivity of patient sera to bigeye tuna parvalbumin and collagen

Prior to immunoblotting, eight patient sera were assessed for reactivity to bigeye tuna parvalbumin and collagen by ELISA. As a result, the sera were divided into the following three types: parvalbumin type (sera from patients 1–3) reacting only to parvalbumin, collagen type (sera from patients 4–6) reacting only to collagen, and parvalbumin/collagen type (sera from patients 7 and 8) reacting to both parvalbumin and collagen. In Fig. 3, the reactivities of the sera from patients 1, 4, and 7 to parvalbumin and collagen are shown as the typical examples of the parvalbumin, collagen, and parvalbumin/collagen types, respectively.

Analysis of fish muscle extracts by immunoblotting and inhibition immunoblotting

Allergens in the heated extracts from the six species of fish (Japanese eel, rainbow trout, Japanese horse mackerel, crimson sea bream, Pacific mackerel, and Japanese flounder) were analyzed by immunoblotting using the eight patient sera. In the case of the parvalbumin-type sera (from patients 1–3), a 12 kDa blot corresponding to parvalbumin was observed in all the six species of fish although its intensity considerably varied among fish (Fig. 4). Similarly, the collagen-type sera (from patients 4–6) reacted to both collagen α and β chains of all species of fish and the parvalbumin/collagen-type sera (from patients 7 and 8) to parvalbumin, collagen α chain, and collagen β chain of all species of fish. Interestingly, the parvalbumin-type and parvalbumin/collagen-type sera reacted to the 12 kDa parvalbumin isoform in crimson sea bream but did not to another parvalbumin isoform of 14 kDa, conforming well to the fact that the latter isoform presents considerably weaker IgE reactivity than the former (unpublished data). Besides parvalbumin and collagen, 50–60 kDa proteins in rainbow trout, Japanese horse mackerel, and Japanese

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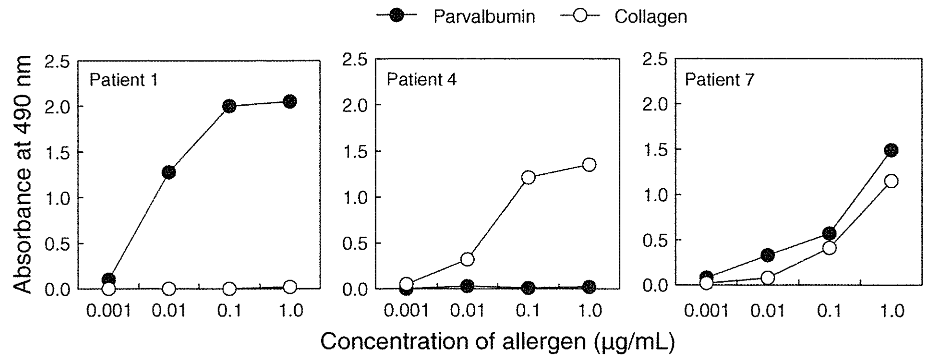

Fig. 3 ELISA analysis of reactivity of three patient sera to collagen and parvalbumin purified from bigeye tuna

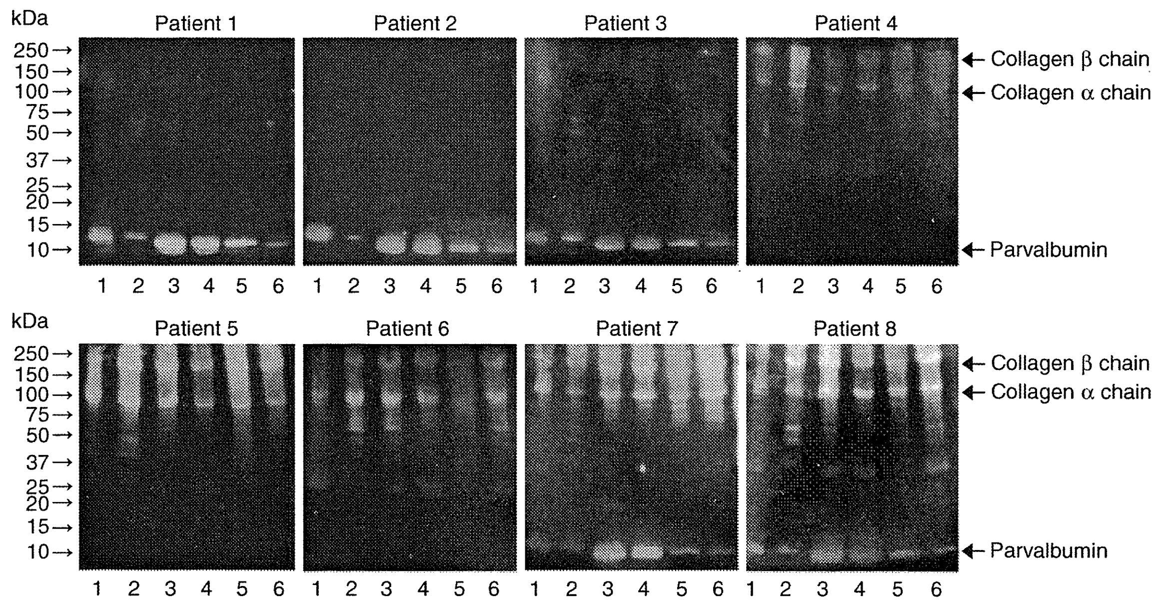

Fig. 4 Immunoblotting analysis of allergens reacting with eight patient sera in heated extracts from six species of fish. Fish samples: *lane 1*, Japanese eel; *lane 2*, rainbow trout; *lane 3*, Japanese horse mackerel; *lane 4*, crimson sea bream; *lane 5*, Pacific mackerel; and *lane 6*, Japanese flounder

flounder weakly reacted with certain sera (e.g., patient 6 serum) and 37 kDa proteins in Japanese eel and Japanese flounder with the patient 8 serum, indicating the presence of minor fish allergens to be identified in future.

When preincubated with Pacific mackerel parvalbumin, the patient 1 serum (parvalbumin type) showed no reactivity to any of the parvalbumins from the six species of fish (Fig. 5). Similarly, preincubation with Pacific mackerel collagen almost completely abolished the reactivity of the patient 4 serum (collagen type) to the collagen α and β chains of the six species of fish. In the case of the patient 7 serum (parvalbumin/collagen type), the reactivity to parvalbumins was completely inhibited by preincubation with Pacific mackerel parvalbumin. Although not completely, the reactivity of the patient 7 serum to collagens was greatly inhibited by preincubation with Pacific mackerel collagen.

Discussion

Collagen is insoluble in aqueous solvents at low temperatures and is readily converted to gelatin during heating. Due to these properties, collagen has been overlooked in previous immunoblotting studies [10, 11, 13–21], although it is an important fish allergen next to parvalbumin (major fish allergen). In this study, we found that collagen as well as parvalbumin can be most effectively extracted from Japanese eel by heating of the muscle homogenate at 80 °C for 20 min. The same extraction method was judged to be also effective for the other five species of fish (rainbow trout, Japanese horse mackerel, crimson sea bream, Pacific mackerel, and Japanese flounder). Furthermore, when the heated extracts prepared from the six species of fish by the method described above were applied to immunoblotting analysis, collagen and/or parvalbumin were successfully

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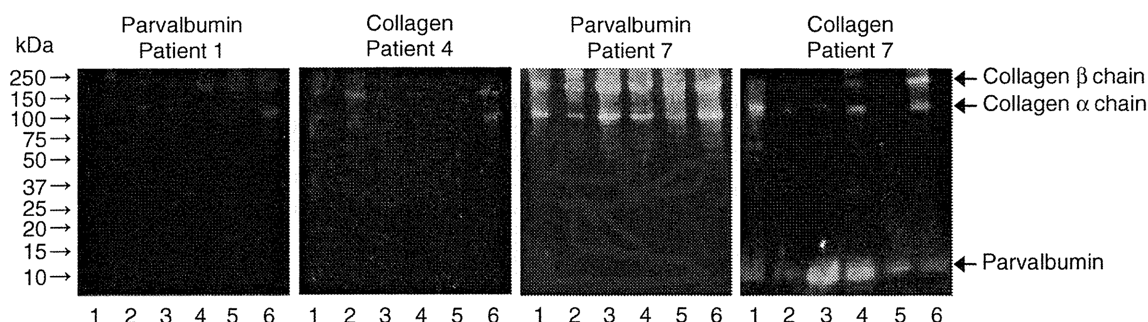

Fig. 5 Inhibition immunoblotting analysis of cross-reactivity among allergens in heated extracts from six species of fish. Fish samples: *lane 1*, Japanese eel; *lane 2*, rainbow trout; *lane 3*, Japanese horse mackerel; *lane 4*, crimson sea bream; *lane 5*, Pacific mackerel; and *lane 6*, Japanese flounder. Each patient serum was preincubated with the inhibitor (Pacific mackerel parvalbumin or collagen) indicated above the patient number and used as a primary antibody

identified as allergens in all species. Thus, it can be concluded that the extraction method (heating at 80 °C for 20 min) developed in this study is suitable for analyzing two important allergens (parvalbumin and collagen) in various species of fish by immunoblotting. However, it should be kept in mind that the extraction method from the fish muscle is useful to analyze thermostable allergens but is helpless for thermolabile ones.

With regard to the developed extraction method, two important points should be taken into account. Firstly, shaking of the muscle homogenate for initial 5 min during the heating process is essential for the effective extraction of collagen, because otherwise collagen bands (corresponding to both α and β chains) became smear on SDS–PAGE (data not shown) probably as a result of protease degradation. It is relevant to infer that the temperature of the muscle homogenate rapidly reaches the setting value (80 °C) through an efficient heat transfer by shaking, thereby preventing the degradation of collagen by muscle proteases (generally most active at 35–40 °C). Secondly, the fish muscle used for extraction is desirable to be as fresh as possible, since the native collagen (triple helix form) may be denatured into α-chains, which are susceptible to protease digestion, during storage of the fish muscle especially under unfavorable conditions. Indeed, our preliminary experiments showed that collagen bands were obscure on SDS–PAGE when the heated extract was prepared from the Japanese eel muscle previously kept overnight at room temperature.

It is particularly interesting to note that parvalbumin content differs among fish species as revealed by SDS–PAGE (Fig. 2); based on the CBB-staining intensity, parvalbumin content is significantly lower in rainbow trout and Pacific mackerel than in the other four species of fish. In consistence with this, immunoblotting experiments proved that the parvalbumin-recognizing sera (from patients 1, 2, 3, 7, and 8) weakly react to the parvalbumins from both rainbow trout and Pacific mackerel. Mackerels have previously been reported to be low in parvalbumin content and classified as low-allergenic fish species [19, 21]. This study newly showed that rainbow trout is also a low-allergenic fish species with low content of parvalbumin. In view of our findings, together with previous reports [19, 21], it can be assumed that there is a positive relationship between parvalbumin content and allergenicity of different fish species. Japanese flounder is an exception to this general assumption; although it has a large amount of parvalbumin as shown by SDS–PAGE, the IgE reactivity of its parvalbumin is weak (almost equivalent to the Pacific mackerel parvalbumin) as revealed by immunoblotting. Future molecular studies are needed to understand the weak IgE reactivity of the Japanese flounder parvalbumin.

For clinical diagnosis of allergic diseases, a RAST method (including a CAP-RAST method) is widely employed to determine antigens recognized by allergic subjects. Although commercial antigens (extracts from allergenic biological materials) are usually used for RAST, no careful attention is paid to whether or not they contain all allergens to be analyzed. If a commercial antigen does not contain an allergen recognized by a certain patient, its use in RAST leads to a wrong diagnosis that the patient is negative to the antigen. Since it is difficult to effectively extract collagen from the fish muscle, commercial fish antigens are likely to be devoid of collagen. In relation to this, it is worth mentioning that there are some fish-allergic patients who are judged to be negative to fish by RAST but manifest hypersensitive reactions after ingestion of fish. Such fish-allergic patients might recognize only collagen. To reduce false-negative results in the diagnosis of fish allergy by RAST, it is important to use fish antigens containing collagen. The extraction method developed for immunoblotting in this study is considered to be also applicable to the preparation of fish antigens for RAST.

In conclusion, an extraction method (heating at 80 °C for 20 min) suitable for immunoblotting analysis of fish allergens was developed in this study. Future

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immunoblotting experiments using the heated extracts prepared by the established extraction method will identify both parvalbumin and collagen as allergens and also detect unknown allergens in a variety of fish species. Moreover, RAST using the heated extracts as fish antigens will improve the accuracy in diagnosis of fish allergy.

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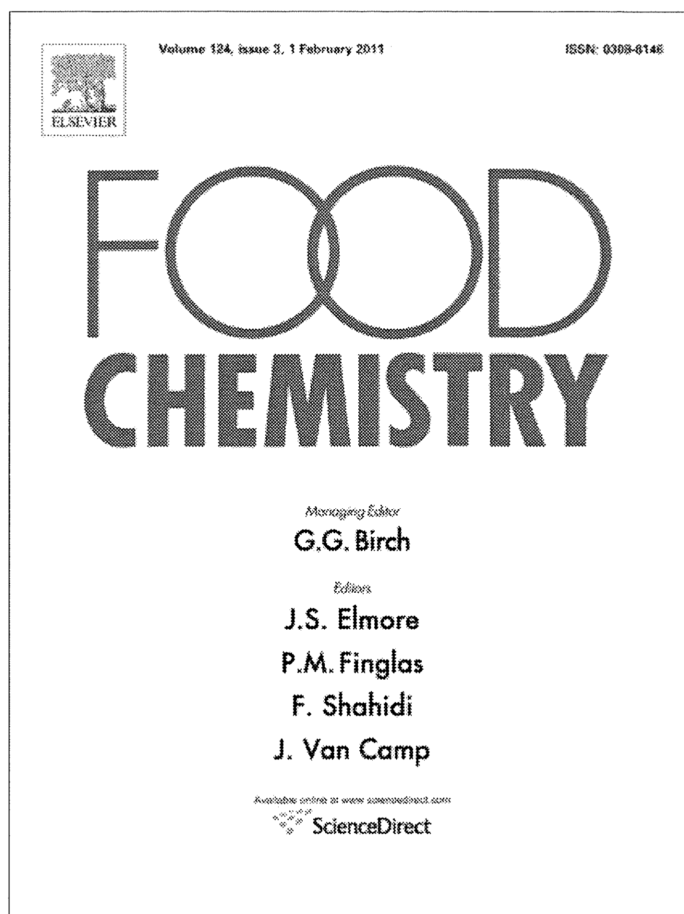

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