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A Major IgE Epitope of Rainbow Trout Collagen $\alpha 2$ 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 $\alpha 2$ 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 $\alpha 2$ 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 $\alpha 2$ chains, but not in bovine collagen $\alpha 2$ chain. Our results strongly suggest that region 941-960 is a major common IgE epitope of fish collagen $\alpha 2$ chains.

Key words: allergen; $\alpha 2$ 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 $(\alpha 1)_2\alpha 2$ or $\alpha 1\alpha 2\alpha 3$) of about 110-120 kDa, which are twisted together to form a triple helix¹. 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 $\alpha 1$ and $\alpha 2$ chains constituting bovine collagen, only the latter chain was found to be IgE-reactive⁴. Moreover, the major IgE epitope of the bovine collagen $\alpha 2$ chain has been elucidated to reside in region 485-494⁵.

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 cod⁶, carp⁷, Atlantic salmon⁸ and Pacific mackerel⁹. 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 trout¹³ 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 $\alpha 2$ chain⁵. Here, we selected the rainbow trout *Oncorhynchus mykiss* collagen $\alpha 2$ chain as a target for analysis of IgE epitopes. First, overlapping recombinant proteins covering the entire sequence of the rainbow trout collagen $\alpha 2$ 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

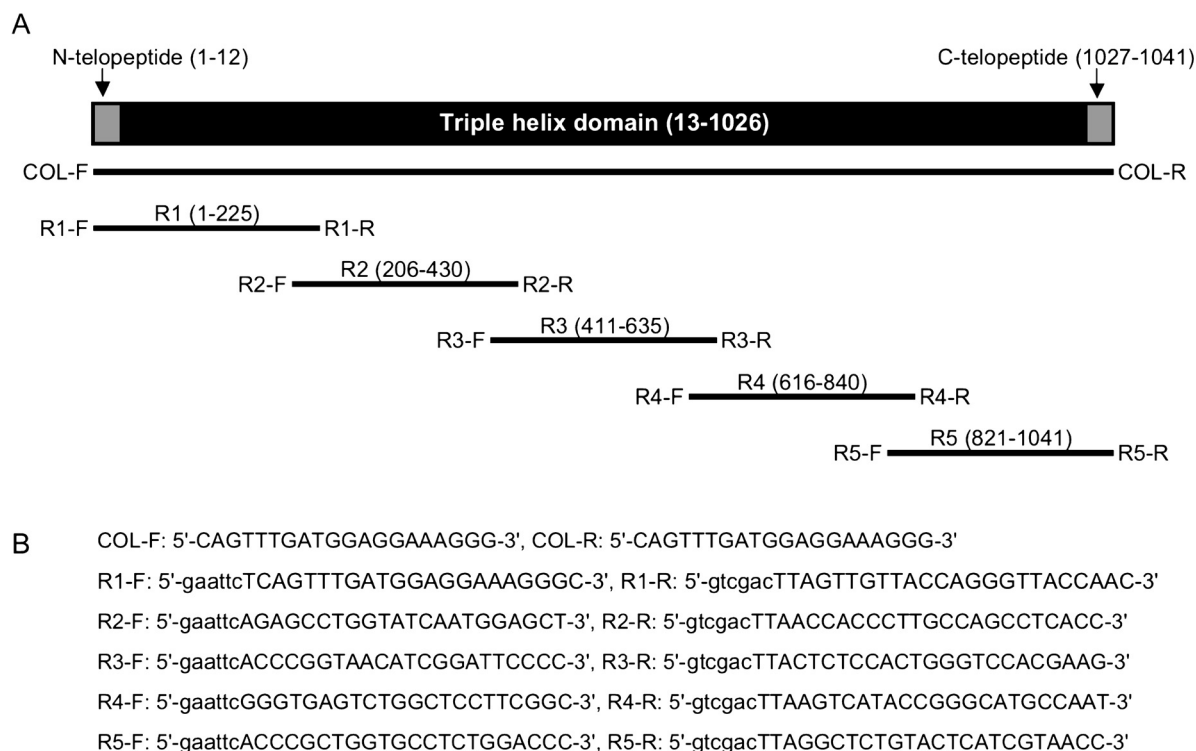


Fig. 1. Schematic presentation of the rainbow trout collagen $\alpha 2$ chain and five overlapping proteins (R1–5) covering the full sequence (A) and nucleotide sequences of the primers used to amplify the mature protein and R1–5 proteins (B)

(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°C 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 $\alpha 2$ chain¹³⁾ 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°C . A single colony was selected and grown in 500 mL of LB medium containing 0.005% ampicillin at 37°C 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 paper¹²⁾. 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°C 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 previously¹⁵⁾. 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⁾, zebrafish¹⁴⁾ and

bovine¹⁷⁾ collagen $\alpha 2$ 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 previously¹⁸⁾. In brief, a Nunc Immobilizer Amino plate for peptide (Nalge Nunc International, Rochester, NY, USA) was coated with 50 μ L of peptide solution (10 μ g/mL) and reacted successively with patients' serum (diluted 1:250) and β -galactosidase-conjugated goat anti-human IgE antibody solution (0.25 μ g/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 μ g/mL) at 37°C for 1 hr and 50 μ L of the mixture was then added to a microplate that had previously been coated with the R5 protein (1 μ g/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 $\pm$ SD.

Results

IgE-binding ability of recombinant R1–5 proteins

Five proteins (R1–5) covering the entire sequence of the rainbow trout collagen $\alpha 2$ 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).

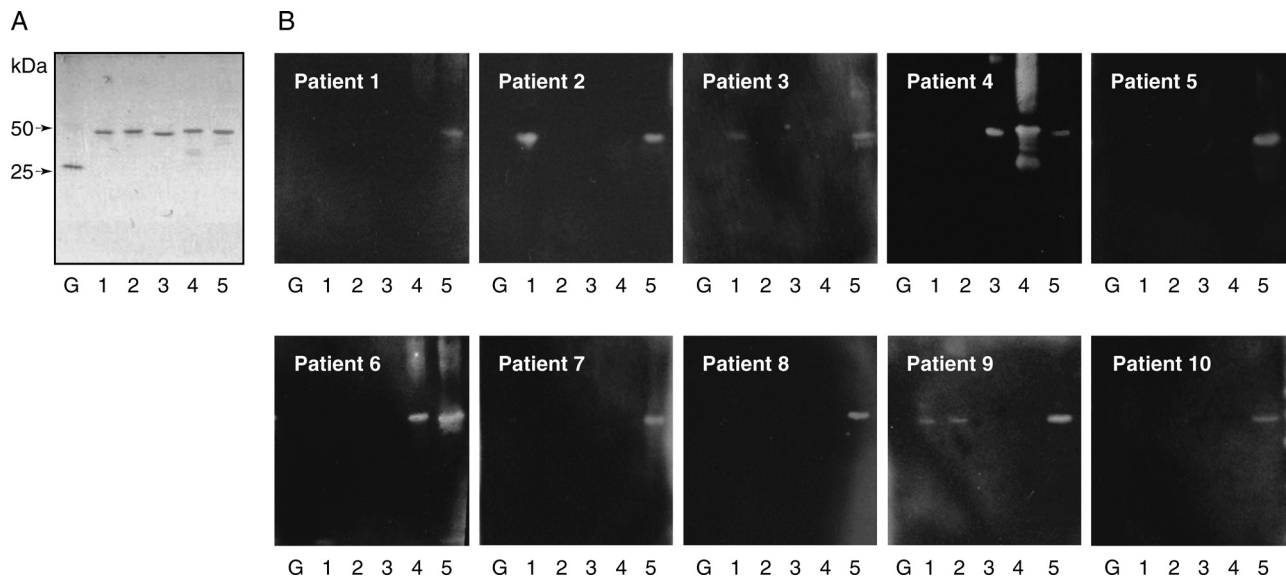


Fig. 2. Analysis of GST and recombinant R1-5 proteins by SDS-PAGE (A) and immunoblotting (B)
Lanes: G, GST; 1, GST-fusion R1; 2, GST-fusion R2; 3, GST-fusion R3; 4, GST-fusion R4; 5, GST-fusion R5.

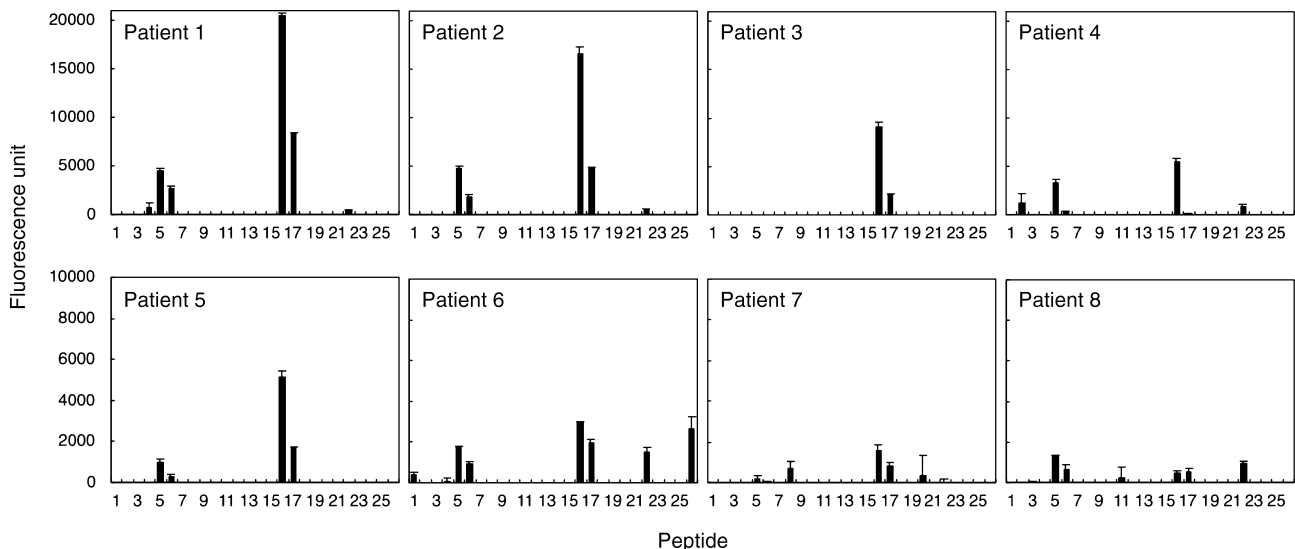


Fig. 3. IgE reactivity of peptides 1-26 analyzed by fluorescence ELISA
Data are expressed as mean $\pm$ SD ($n=3$).

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 R

5 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

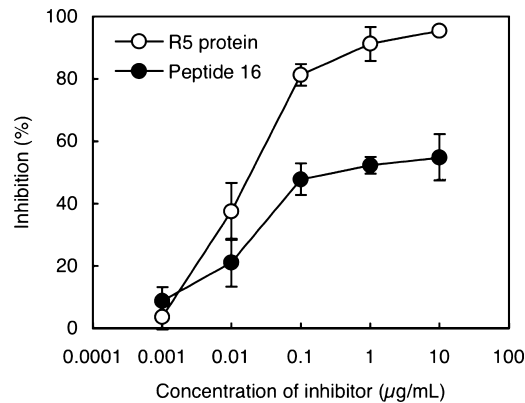


Fig. 4. Inhibition of the IgE reactivity to R5 protein by peptide 16 and R5 protein

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 expressed as mean $\pm$ SD ($n=3$).

minimal. It was thus concluded that the major IgE epitope of the rainbow trout collagen $\alpha 2$ 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 $\alpha 2$ chains

Fish collagen $\alpha 2$ chains have been completely sequenced for chum salmon *Oncorhynchus keta* (DDBJ/EMBL/GenBank accession number: AB075699), bastard halibut¹⁶⁾ and zebrafish,¹⁴⁾ as well as for rainbow trout¹³⁾. As compared to the amino acid sequence of the rainbow trout collagen $\alpha 2$ chain, that of the chum salmon collagen $\alpha 2$ chain is completely conserved in region 941–960 (the most IgE-reactive region of the rainbow trout collagen $\alpha 2$ chain) and those of the bastard, halibut and zebrafish collagen $\alpha 2$ chains are highly conserved, with only one or two alterations (Fig. 5). On the other hand, the amino acid sequences of mammalian collagen $\alpha 2$ chains are homologous with one another in region 941–960 but are significantly different from those of fish collagen $\alpha 2$ chains. To examine whether region 941–960 is a common IgE epitope in fish collagen $\alpha 2$ chains, synthetic peptides Bh, Zf and Bv corresponding to region 941–960 of the bastard halibut, zebrafish and bovine collagen $\alpha 2$ 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	M K G L R G H G G L Q G M P G P N G P S		
Chum salmon	- - - - -	- - - - -	- - - - -
Bastard halibut	- - - - - P - - - - -	- - - - -	S - - - -
Zebrafish	- - - - - P - - - - -	- - - - -	- - - - -
Human	L P - - K - - N - - - L - - I A - H H		
Bovine	L P - - K - - N - - - L - - L A - H H		
Rabbit	L P - I K - - N - - - L - - L A - Q H		
Mouse	L P - - K - Y S - - - L - - L A - L H		

Fig. 5. Amino acid sequence alignment of the 941–960 region of collagen $\alpha 2$ chains from fish and mammals

DDBJ / EMBL / GenBank accession numbers: rainbow trout, AB052837; chum salmon, AB075699; bastard halibut, AB196514; zebrafish, BC071278; human, BC042586; bovine, AB008683; 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 $\alpha 2$ 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 $\alpha 2$ 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 $\alpha 2$ chain is located in region 941–960 (MKGLRGHGGLQGMPGPNGPS). The major IgE epitope of the bovine collagen $\alpha 2$ 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 $\alpha 2$ chains.

The chum salmon collagen $\alpha 2$ chain has the same amino acid sequence in region 941–960 as the rainbow trout collagen $\alpha 2$ chain (Fig. 5). In the case of the bastard halibut and zebrafish collagen $\alpha 2$ 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 $\alpha 2$ chain and replacement of Gly by Pro at position 948 for the zebrafish collagen $\alpha 2$ chain) are recognized in region 941–960 compared to the amino acid sequence of the rainbow trout collagen $\alpha 2$ chain. However, the peptides Bh and Zf, corresponding to region 941–960 of the bastard halibut and zebrafish collagen $\alpha 2$ chains, respectively, were found to be reac-

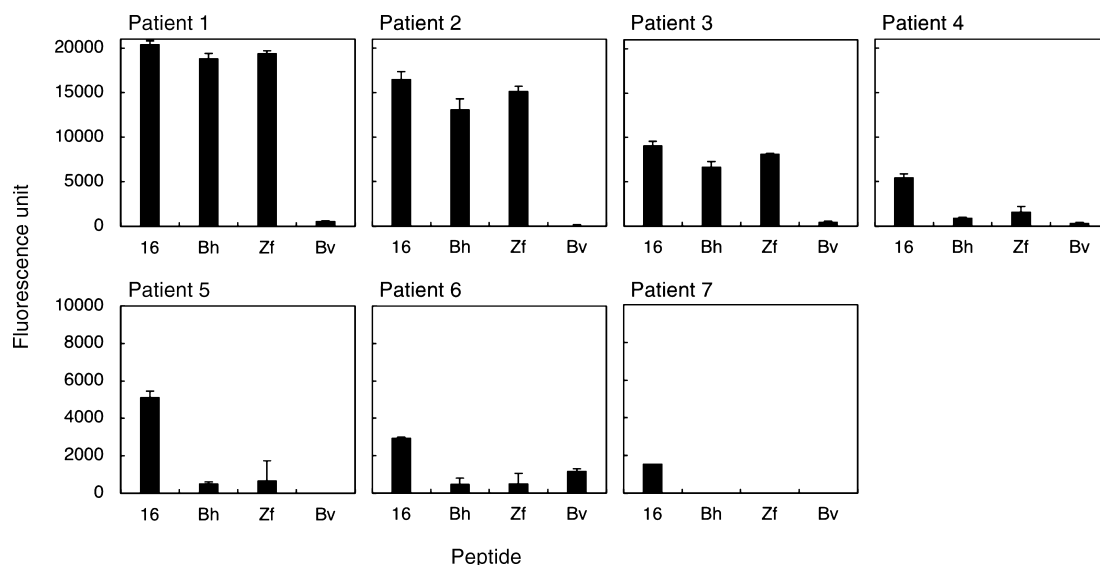


Fig. 6. IgE reactivity of the 941–960 region of fish and bovine collagen $\alpha 2$ chains analyzed by fluorescence ELISA. Data are expressed as mean $\pm$ SD ($n=3$). Peptides: 16, rainbow trout; Bh, bastard halibut; Zf, zebrafish; Bv, bovine.

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 $\alpha 2$ chains. To confirm this, further amino acid sequence data on fish collagen $\alpha 2$ chains should be accumulated.

Bovine type I collagen is composed of two $\alpha 1$ chains and one $\alpha 2$ chain, like other mammalian type I collagens, and only the $\alpha 2$ chain has been demonstrated to be IgE-reactive⁴). The major IgE-binding epitope elucidated for the bovine collagen $\alpha 2$ chain is not found in collagen $\alpha 1$ chains from mammals, including bovine⁵). Differing from mammalian collagens, fish collagens take the form of either $(\alpha 1)_2\alpha 2$ or $\alpha 1\alpha 2\alpha 3$, 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 $\alpha 2$ chain is not found in fish collagen $\alpha 1$ and $\alpha 3$ chains, including the rainbow trout collagen $\alpha 1$ and $\alpha 3$ chains. Further study on which residues in the major IgE epitope of the rainbow trout collagen $\alpha 2$ 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 $\alpha 2$ chain contains three Pro residues and one Lys residue. This is also the case with the major IgE epitope of the bovine collagen $\alpha 2$ chain. Pro residues are abundant in collagen, and many of them are hydroxylated by post-translational modification. Some Lys residues are also known to be hydroxylated. In the case of the major IgE epitope of the bovine collagen $\alpha 2$ 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 syn-

thetic 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 $\alpha 2$ chains.

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