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 (α1)α2 or α1α2α3) of about 110–120 kDa, which are twisted together to form a triple helix. 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 adults suffering from vaccine-induced anaphylaxis. These adverse reactions, including anaphylaxis, are found to reside in region 485–505 and nonallergenic to humans, it has also been used as a stabilizer 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 adults suffering from vaccine-induced anaphylaxis. These adverse reactions, including anaphylaxis, are often used as a model fish in biology, but is not edible.

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, carp, Atlantic salmon and Pacific mackerel. Besides parvalbumin, collagen has also been identified as a fish allergen, although not a major one. In contrast to bovine collagen, hetero-α-chains of fish collagen all seem to be IgE-reactive. Importantly, collagens from various species of fish are cross-reactive with one another, but not with those from mammals, 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 and zebrafish Danio rerio. 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. 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
major IgE epitope of the rainbow trout collagen a2 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 a2 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 a2 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 a2 chain13) was amplified by PCR using the Marathon cDNA library as a template. Then, cDNAs of the R1–5 proteins, with addition of EcoRI and SalI restriction sites at the 5′ and 3′ ends, respectively, were individually amplified by PCR using the a2 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 EcoRI and SalI 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 µg/ml ampicillin at 37°C. A single colony was selected and grown in 500 mL of LB medium containing 0.005 µg/ml 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 mM and incubation was continued for 3 hr. Bacteria were harvested by centrifugation and resuspended in 25 mL of 50 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl, 1 mM EDTA, 1 mM 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 capselfish and tuna. In addition, their sera were all confirmed to contain specific IgE to fish collagen by ELISA, which el 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 Cooamassie 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 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°C 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 a2 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).
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\(^{c8140}\)/R5 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 α2 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
minimal. It was thus concluded that the major IgE epitope of the rainbow trout collagen α2 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 α2 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 α2 chain, that of the chum salmon collagen α2 chain is completely conserved in region 941–960 (the most IgE-reactive region of the rainbow trout collagen α2 chain) and those of the bastard halibut and zebrafish collagen α2 chains are highly conserved, with only one or two alterations (Fig. 5). On the other hand, the amino acid sequences of mammalian collagen α2 chains are homologous with one another in region 941–960 but are significantly different from those of fish collagen α2 chains. To examine whether region 941–960 is a common IgE epitope in fish collagen α2 chains, synthetic peptides Bh, Zf and Bv corresponding to region 941–960 of the bastard halibut, zebrafish and bovine collagen α2 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 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 α2 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 α2 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 α2 chain is located in region 941–960 (MKGLRGHGGLQGMPGPNGPS). The major IgE epitope of the bovine collagen α2 chain has previously been reported to be included in region 485–494 (IPGEFKPGP). Clearly, there is no overall sequence homology between the major IgE epitopes of the rainbow trout and bovine collagen α2 chains.

The chum salmon collagen α2 chain has the same amino acid sequence in region 941–960 as the rainbow trout collagen α2 chain (Fig. 5). In the case of the bastard halibut and zebrafish collagen α2 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 α2 chain and replacement of Gly by Pro at position 948 for the zebrafish collagen α2 chain) are recognized in region 941–960 compared to the amino acid sequence of the rainbow trout collagen α2 chain. However, the peptides Bh and Zf, corresponding to region 941–960 of the bastard halibut and zebrafish collagen α2 chains, respectively, were found to be reac-
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 α2 chains. To confirm this, further amino acid sequence data on fish collagen α2 chains should be accumulated.

Bovine type I collagen is composed of two α1 chains and one α2 chain, like other mammalian type I collagens, and only the α2 chain has been demonstrated to be IgE-reactive⁴. The major IgE-binding epitope elucidated for the bovine collagen α2 chain is not found in collagen α1 chains from mammals, including bovine⁵. Differing from mammalian collagens, fish collagens take the form of either (α1)₂α2 or α1α2α3, depending on the fish species and tissues. Importantly, IgE cross-reactivity has been found among fish collagen α chains¹¹,¹². Nevertheless, the same sequence as that of the major IgE-binding epitope (region 941–960) determined for the rainbow trout α2 chain is not found in fish collagen α1 and α3 chains, including the rainbow trout collagen α1 and α3 chains. Further study on which residues in the major IgE epitope of the rainbow trout collagen α2 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 α2 chain contains three Pro residues and one Lys residue. This is also the case with the major IgE epitope of the bovine collagen α2 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 α2 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 α2 chains.

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References