Full title:

Molecular and Immunological Characterization of β'-component (Onc k 5), a major IgE-binding protein in chum salmon roe

Running title: Molecular characterization of salmon roe allergen

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1 **Keywords:** Vitellogenin, β'-component, IgE-binding ability, recombinant allergen,

2 salmon roe allergy

3

Abstract (236 words)

Salmon roe has a high allergic potency and often causes anaphylaxis in Japan. The major allergic protein of salmon roe is β'-component, which is a 35 kDa-vitellogenin-fragment consisting of two subunits. To elucidate structural information and immunological characteristic, β'-component and the subunit components were purified from chum salmon (*Onchorhincus keta*) roe and vitellogenin-encoding mRNA was used to prepare β'-component subunit-encoding cDNA. This was PCR-amplified, cloned and sequenced, and the deduced amino acid sequence compared with partial sequences of β'-component obtained by peptide mapping. The recombinant β'-component subunit was produced by bacterial expression in *Escherichia coli* and its IgE-binding ability was measured by ELISA using the sera of a patient allergic to salmon roe. This was then compared with that of the native β'-component with and without carboxymethylation. Following successful cloning of the cDNA encoding the β'-component subunit, 170 amino acid residues were deduced and matched with the amino acid sequences of 121 and 88 residues in the 16 kDa- and 18 kDa-subunits, respectively. The sequences of both β'-component subunits were almost identical, and the predicted secondary structure of the β'-component showed a high content of β-pleated sheets and no α-helices. There was no

difference in IgE-binding ability between the native and recombinant β'-component subunits at the same protein concentration, regardless of carboxymethylation. In conclusion, β'-component is a homodimer protein composed of two isoform subunits having the same level of IgE-binding ability, and, therefore, allergenic identity.

Abbreviations used:

β'-c, β'-component

CAP-RAST, capsulated hydrophilic carrier polymer-radioallergosorbent test

CD, circular dichroism

Lv, lipovitellin

MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Pv, phosvitin

Rβsub, recombinant β'-c subunit

SDS-PAGE, Sodium dodecylsulfate-polyacrylamide gel electrophoresis

Vg, vitellogenin

Introduction

Several kinds of fish roes, including sturgeon, paddlefish, salmon, cod, lumpfish, capelin and herring are becoming popular seafood worldwide (1). However, as individuals have been widely reported to experience immediate allergic reactions following the consumption of king salmon caviar (2), Russian beluga caviar (3), and the roe of white fish and rainbow trout (4), the trend could cause a new risk of fish-roe allergies at a global level. In Japan, where more than 500 kinds of marine bioresources are consumed, the cases of allergy to salmon roe, particularly among children, have increased over the last decade and the anaphylaxis by ingestion of salmon roe also have been reported (5). Based on the number of cases of actual illnesses and the degree of seriousness, salmon roe is listed as a potential allergen (one of subspecific allergenic ingredients) under Japanese food sanitation laws (6).

Lipovitellin (Lv) and β'-component (β'-c) are major constituents of yolk proteins in teleost fish roe (7, 8), and have high IgE-binding ability (9). β'-c in particular is a major allergic protein of chum salmon (*Oncorhynchus keta*) roe as specific IgE reactions to β'-c occur in the sera of almost all patients with salmon roe allergies (9). Additionally, IgE cross-reactivities among fish roes, such as those from salmon (*Oncorhynchus keta*),

herring (*Clupea pallasii*) and walleye pollock (*Theragra chalcogramma*), have been reported in case studies (10). The authors also confirmed that yolk proteins from Atka mackerel (*Pleurogrammus azonus*), dusky sole (*Pleuronectes mochigarei*), slime flounder (*Microstomus achne*), shishamo smelt (*Spirinchus lanceolatus*), and Atlantic capelin (*Mallotus villosus*) were bound to specific IgE of salmon-roe-allergic patient sera (11). These findings clearly indicate the importance of research on β'-c as a food allergen, and β'-c was registered as a new allergen 'Onc k 5' in the official allergen list of the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Subcommittee in 2012 (http://www.allergen.org/viewallergen.php?aid=764).

Lv and β'-c are degradation fragments of vitellogenin (Vg), a protein synthesized in fish liver that is carried to the oocytes through the bloodstream. Accumulated Vg in fish oocytes is proteolytically degraded to the three major yolk proteins: Lv, β'-c and phosvitin (Pv) (7, 8, 12, 13) at proteolytic cleavage sites. Lv and β'-c amino acid sequences present in Vg have previously been confirmed in barfin flounder (*Verasper moseri*) (14), haddock (*Elanogrammus aeglefinus*) (15), Japanese conger (*Conger myriaster*) (16), red sea bream (*Pagrus major*) (17) and yellowfin goby (*Acanthogobius*

flavimanus) (18). Lv and Pv are sources of embryonic nutrients in oviparous vertebrates, while β'-c appears to be stable to proteolysis during development of the teleost fish embryo since it remains during oocyte growth and the early cleavage stage of the embryo (8, 12). The proteolytic tolerance of β'-c is probably related to its high allergenicity. Indeed, pepsin–trypsin digestion had little effect on the IgE-binding ability of β'-c prepared from chum salmon roe (19).

Thus far, there is little information regarding the biochemical characteristics of allergic proteins in fish roe compared with other food allergens. Most cases of fish roe allergy are classified as Type I allergies that are triggered by the binding of an allergen to a specific IgE, and the recognition of specific amino acid sequences in allergic proteins. Therefore, clarifying the structure of β'-c is the first step toward understanding fish roe allergy.

This study therefore aimed to elucidate structural information and immunological characteristics of chum salmon β'-c. Vg mRNA was sampled from fresh livers of female chum salmon and 170 amino acid sequences corresponding to the β'-c subunit were analyzed by cDNA cloning and sequencing. The primary structure and IgE-binding ability of recombinant β'-c were then examined by comparison with native β'-c.

Materials and Methods

Salmon roe and total RNA from liver

Chum salmon roe from fresh mature individuals (*Oncorhynchus keta*) was washed with cold 0.16 M NaCl and frozen at -60 °C until required. Liver for total RNA sampling was collected from a mature female fish caught within 8 h, and was immediately treated by RNAlater (Qiagen, Hilden, Germany).

Sera from patients allergic to salmon roe

Sera from patients diagnosed with salmon roe allergies were used for this study. Patient clinical data are listed in Table 1. Total and specific IgE levels were determined by the capsulated hydrophilic carrier polymer-radioallergosorbent test (CAP-RAST system; Phadia AB, Uppsala, Sweden). After being stored at < -60 °C for 2–12 months, the patients' sera were mixed with the same volume of phosphate-buffered saline (PBS: pH 7.5) containing 0.2% NaN_3 and then stored at 4 °C until required. We confirmed that the specific IgE was contained in all sample sera by ELISA using purified β'-c. The study was approved by the local ethical committee and all subjects provided written informed consent before enrollment in the study.

Preparation of β'-c and its subunit components

β'-c was prepared from chum salmon roe according to the method of Hiramatsu et al. (20). Briefly, roes were homogenized in 0.5 M NaCl and 20 mM Tris-HCl (pH 8.0) and the yolk protein extract was dropped into 10 × volumes of cold distilled water. The precipitate generated in this step was collected by centrifugation at 20,000 × *g* for 30 min, dissolved in 0.5 M NaCl (pH 8.0), and the 67%-saturated ammonium sulfate precipitate was collected by centrifugation at 20,000 × *g* for 30 min. The precipitate was redissolved in 0.5 M NaCl (pH 8.0) and loaded onto a Sephacryl S-200HR column (GE Healthcare, Piscataway, NJ) to purify β'-c. The protein fractions were detected at 280 nm, and the concentration was determined by the Biuret method (21). All steps were performed at temperatures below 5 °C, and the purified proteins were frozen at -30 °C until required. The subunit components of purified β'-c were separated with a preparative SDS-PAGE system (AE-6750S, Atto Corp., Tokyo, Japan) in the presence of 2-mercaptoethernol. The purified subunits were dialyzed against 1 mM sodium bicarbonate, lyophilized, and stored at -60 °C until required.

SDS-PAGE analysis and immunoblotting

SDS-PAGE was performed according to the method of Laemmli (22), using 4.5% and 12.5% polyacrylamide slab gels for stacking and resolving gels, respectively. The protein bands were stained with 0.25% Coomassie Brilliant Blue R (Sigma Aldrich, St. Louis, USA) dissolved in 9% acetic acid and 45% methanol. Proteins subjected to SDS-PAGE were transferred onto a polyvinylidene difluoride membrane and reacted with the patients' serum (P1 in Table 1). The blotting picture was gray-scaled and reversed using a computer.

CD spectroscopy

The CD-spectrum of β'-c (0.8 mg/mL) was measured at 25 °C using a spectropolarimeter (J-725, Jasco Inc., Tokyo, Japan).

MALDI-TOF-MS

Mass spectrometry was performed using MALDI-TOF system (AB4700, Applied Biosystems Inc., California, USA) equipped with a 335 nm YAG laser in the reflection mode. α-Cyano-4 hydroxycinnamic acid (α-CHCA) was used

as matrix. Samples, desalted with a C18-micro column (ZipTip, Millipore Corp., Massachusetts, USA) and dissolved in 50% acetonitrile containing 0.1% trifluoroacetic acid, were spotted onto a sample-target plate using the dry droplet method. The sample-coted plate was subsequently subjected to the MALDI-TOF system under the positive-ion-mode, after applying a drop of the matrix-only solution (10 mg/mL of α-CHCA dissolved in acetonitrile-trifluoroacetic acid) to the sample droplet.

Molecular cloning of β'-c subunit cDNA

Vitellogenin is a precursor of β'-c as described in the introduction, and the DNA sequences encoding major yolk proteins in salmonid are located in the Vg gene in the following order: NH2-(Lv heavy chain)-(Pv)-(Lv light chain)-(β'-c)-(C-terminal peptide)-COOH (8). Although we have no structural information about chum salmon Vg, the N-terminal 20 amino acid sequence of chum salmon β'-c was almost identical to that of rainbow trout (*Oncorhynchus mykiss*) β'-c (9), suggesting high similarity of the primary structures. Thus, the partial base sequence of rainbow trout Vg (EMBL: X92804) was used as a cloning refer (23). The cDNA cloning strategy is shown in Fig. 1. Briefly,

the forward primer (5'-CCCTGTTCTCTGCCATTTGA-3') was designed upstream of the coding to the N-terminal amino acid sequence of β'-c, and the reverse primer (5'-CTGGGTGCTTCCTTCTGATA-3') was designed downstream from the sequence encoding the 170th amino acid residue in order to cover the whole amino acid sequence of the 18kDa-subunit. cDNA cloning was carried out as described previously (24), with the exception of primer differences. The nucleotide sequence was determined using the DNA Sequencer 3130 (Life Technologies, Carlsbad, CA) after labeling the DNA with the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies).

Carboxymethylation of β'-c

Native and recombinant β'-cs were carboxymethylated according to the modified method of Crestfield et al. (25). β'-c was dissolved in 20 mM Tris-HCl (pH 8.5) containing 8 M urea, 10 mM 2-mercaptoethanol, and 5 mM EDTA, then incubated for 3 h at room temperature. The β'-c solution was then treated by 10 mM monoiodoacetic acid for 30 min with shaking. After dialysis against 1 mM $NaHCO_3$, the β'-c solution was lyophilized and stored at -60 ºC until required.

Peptide mapping of native β'-c subunits

The 16 kDa- and 18 kDa-subunits of β'-c were digested with lysyl endopeptidase (Wako, Osaka, Japan) dissolved in 20 mM Tris-HCl (pH 8.0), endoproteinase Glu-C (Sigma Aldrich) dissolved in 50 mM PBS (pH 7.8) or trypsin (Sigma Aldrich) dissolved in 20 mM Tris-HCl (pH 8.0), at 1% of enzyme to substrate weight ratio and at 37 °C for 2 h. The digested peptides were lyophilized, dissolved in 1% acetonitrile containing 0.1% trifluoroacetic acid, and then applied to reverse-phase high performance liquid chromatography (HPLC) on Mightysil RP-18 GP columns (4.6 × 250 mm: Kanto Chemical Co., Inc., Tokyo, Japan). Columns were eluted at a flow rate of 1.0 mL/min by a liner gradient of acetonitrile (1–60% in 120 min) containing 0.1% trifluoroacetic acid. Peptides were monitored at 228 nm with a UV detector. Digested peptides fractionated with HPLC thus obtained were subjected to the automatic Edman sequence analyzer (Procise 492, Perkin Elmer, Waltham, USA) to identify the amino acid sequences. Up to 20 residues from the N-terminus were detected in this experiment.

Preparation of recombinant β'-c subunit (Rβsub)

The coding region of β'-c was amplified by reverse transcription polymerase chain

reaction (RT-PCR) using the forward primer (5'–TTAGGATCCGAAGTCAACGCAGT–3'), reverse primer (5'–TTAGGCAAAGCTGACTGAGCTCT–3'), and Prime STAR HS DNA polymerase (Takara Bio Inc., Shiga, Japan). The amplified fragment was then inserted into the *BamH* I and *Sma* I sites of the pGEX-6P-1 vector (GE Healthcare). The vector was transformed into competent *Escherichia coli* (BL21 (DE3), Nippon Gene Co., Ltd., Tokyo, Japan), which were cultured in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) containing 50 μg/mL ampicillin. The expression of the GST-fused protein was induced by 1.0 mM isopropyl-β-thiogalactosidase at 37 ˚C for 3 h and the cultured *E. coli* were sonicated with a detergent buffer (0.05% sodium deoxycholic acid-1% Triton X-100). After adding Benzonase (Novagen Inc. Madison, USA) on ice and removing the residues by centrifugation at 10,000 × *g* for 10 min, Rβsub was purified by running the supernatant through a GSTrap HP column (GE Healthcare). Cleavage between GST and Rβsub was performed using PreScission protease (GE Healthcare) within the column, and elution was carried out by PBS (pH 7.4). Expression and purification of Rβsub were confirmed by SDS-PAGE under reducing condition, immunoblotting using rabbit anti-β'-component serum, and measuring N-terminal amino acid sequence. Rβsub thus obtained

was concentrated by ultrafiltration and stored at -80 ˚C until required.

Quantitative evaluation of β'-c IgE-binding ability

The inhibiting effect of recombinant β'-c on the reaction between native β'-c (solid phase antigen) and the specific IgE in patients' sera was evaluated using competitive ELISA to evaluate the IgE-binding ability (9). Carboxymethylation-induced dissociation of the two native β'-c subunits was also examined to investigate the IgE-binding ability of the subunits. The IgE-binding ability of the proteins was evaluated by measuring fluorescence intensity using a β-galactosidase-conjugated rabbit anti-human IgE antibody (American Qualex Manufactures, San Clemente, CA) and 4-methylliferyl-β-D-galactoside with excitation at 365 nm and emission at 450 nm.

Results

Structural information of β'-c and its reactivity to specific IgE in serum of allergic patient

The molecular weight of β'-c purified from yolk protein extract (Fig. 2-1, Lane A) revealed a major 35 kDa- and a minor 16 kDa-band in SDS-PAGE analysis under non-reducing conditions (Fig. 2-1, Lane B), and the two subunit components (16 kDa and 18 kDa) were confirmed under reducing conditions (lane C). Both subunits were completely separate and collected with the polyacrylamide gel preparative electrophoresis system (Lanes D and E) and were capable of binding the specific IgE in the serum from the patient allergic to chum salmon (lane F). These results indicate that IgE-binding sites exist in both subunits of β'-c that are covalently cross-linked with disulfide bonds. Both N-terminal amino acid sequences of the β'-c subunits (Lanes D and E) were identical to EVNAVKCSMVGDTLTTFNNR, indicating the similarity of their primary structures. As shown in Fig. 2-2, CD spectrum of native β'-c showed only a clear negative peak at 216 nm, suggesting β'-c contains only β-pleated sheet as a regular structure.

As presented in Figure 3, MALDI-TOF mass spectrum of native β'-c showed a single peak (M/Z, 36,395) with a small shoulder (M/Z, around 35,200), and two signals

were observed in carboxymethylated β'-c (The increment of the molecular mass of each β'-c subunit by carboxymethylation was 290, because they contained five cysteine residues).

cDNA cloning and deduced amino acid sequence of β'-c

cDNA cloning of the partial base sequence of Vg was performed to determine the primary structure of β'-c according to the method shown in Fig. 1. Three 579-bp cDNA fragments were obtained and their deduced amino acid sequences (Cβ1, Cβ2 and Cβ3) consisted of 170 amino acid residues as shown in Fig. 4. The deduced N-terminal 20 amino acid sequences (EVNAVKCSMVGDTLTTFNNR) were consistent with those of the purified 16 kDa- and 18 kDa-subunits shown in Fig. 5. On the other hand, Cβ1 and Cβ3 differed by a single amino acid at position 151. Seven differences in amino acid residues at positions 88, 90, 94, 101, 103, 104, and 151 were also found between Cβ1 and Cβ2. The sequences have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers AB474573, AB474574, and AB560769, respectively.

Each of the amino acid sequences had a deduced molecular mass of about 19 kDa (Cβ1: 19,108 Da; Cβ2: 19,099 Da; Cβ3: 19,131 Da). The C-terminus of the β'-c subunits could not be clarified by cDNA cloning as β'-c is a cleavage fragment of Vg in fish egg

yolk. However, it was apparent that the obtained amino acid sequence contains the 16 kDa-and 18 kDa-subunits.

Determination of the amino acid sequence of β'-c subunits

The 16 kDa-subunit was digested by three kinds of proteases and the digested peptides were loaded onto reverse-phase HPLC. The 17 peptide peaks (*a* to *q* in Fig. 5) were collected and subjected to amino acid sequential analysis, and their determined N-terminal sequences were found in the deduced amino acid sequences shown in Fig. 4. Consequently, we identified the locations of the 17 peptides in the primary structure of the 16 kDa-subunit, as shown in Fig. 5, and the 121 amino acid residue sequence was almost consistent with that of Cβs, except for three residues in peptide *a* (57D), peptide *p* (64M), and peptide *m* (140G).

Following tryptic digestion, the peptide chromatogram of the 18 kDa-subunit resembled that of the 16 kDa-subunit as shown in Fig. 5 (chromatograms). Additionally, as shown in the peptide mapping, the amino acid sequences of the 18 kDa-subunit-peptides (*r*-*z*) were almost consistent with those of the peptides from the 16 kDa-subunit, except for three residues (88N, 94E, and 128G), whereas 88N and 94E were consistent

with the deduced amino acids from Cβ2. These results indicate the high structural similarity between the 16 kDa- and 18 kDa-subunits.

Comparison of the IgE-binding ability between native and recombinant β'-c subunits

The recombinant β'-c subunit (Rβsub) based on Cβ3 was subjected to competitive ELISA using serum from a patient allergic to salmon roe (P2-P5 in Table 1), and the IgE-binding ability of Rβsub was compared with that of native β'-c (Fig. 6(A)). Apparently, Rβsub inhibited the reaction between native β'-c and the specific serum IgEs, and Rβsub completely inhibited the IgE-binding between native β'-c and the specific serum IgEs at the high protein concentrations (>0.01 μg/mL). Additionally, the inhibition effect of Rβsub was not significantly different from that of β'-c at the same protein concentration. Carboxymethylated β'-c (16 kDa- and 18 kDa-subunits mixture) also showed the inhibitory effect as the same as carboxymethylated Rβsub, and the inhibition rate of the both carboxymethylated proteins was larger than 80% at 1 μg/mL (Fig. 6(B)). These results indicate that the 16 kDa- and 18 kDa-subunits have a similar level of IgE-binding ability.

On the other hand, the competitive ELISA-curves of native β'-c and Rβsub (Fig. 6(A))

were shifted to the right side of the figures by carboxymethylation (Fig. 6(B)), and the ELISA-curves of native β'-c were markedly shifted to the right side rather than that of Rbsub except for the case of P2. These changes in the competitive ELISA-curves indicate that the carboxymethylation diminished the IgE-binding ability of the proteins, particularly native β'-c.

Discussion

Structural determination of an allergen is the first step toward understanding its sensitization as most IgEs that induce food allergies bind to allergens by recognizing their specific amino acid sequences. In this work, the primary structure of two subunits of β'-c were investigated by cDNA cloning and peptide mapping. The 16 kDa and 18 kDa-subunits were purified and subjected to the peptide mapping, separately. Consequently, the sequences of 170 amino acid residues were obtained (Fig. 4) and matched with the sequences of 121 residues in the 16 kDa-subunit and 88 residues in the 18 kDa-subunit (Fig. 5).

As described above, the DNA sequences encoding major yolk proteins are located in the Vg gene. Since the gene encoding β'-c does not exist independently and the structure of the C-terminal peptide located downstream of β'-c was not identified, the C-terminus of β'-c was not disclosed in this work. However, the entire amino acid sequences of both subunits of native β'-c shown here appear to be covered by the deduced amino acid sequences (Cβ1, Cβ2, Cβ3) as their molecular mass was calculated to be 19 kDa.

The authors have successfully detected five kinds of sequential IgE epitopes in β'-c in continuous research works (data not shown), and the identified IgE epitopes did not

contain the substituted amino acid sequences among the isoforms shown in Fig. 4. Therefore, the allergenicity of 3-isoform recombinants seems to be identical each other.

Vg consists of two disulfide-bonded heavy chains, and enzymatic degradation during oocyte growth generates β'-c (8). Although there is no information about cysteine pairs involving in dimerization of β'-c, we suggest that, following cleavage, the fragments of Vg heavy chains, that is the 16 kDa and 18 kDa subunits of β'-c connect with disulfide bonds as shown in Fig. 2-1(Lane B, C). HPLC elution patterns of the tryptic-digested peptides derived from the 16 kDa- and 18 kDa-subunits were quite similar, as shown in Fig. 5. Moreover, the peptides contained in the peaks with identical elution times (o and w, q and z) were consistent, excluding only two amino acid residues between the 16 kDa-subunit (121 residues) and the 18 kDa-subunit (88 residues). These results clearly indicate that native β'-c is a homodimer protein composed of two isoform subunits. Thus, the 16 kDa-subunit appears to be a subfragment of the 18 kDa-subunit lacking the C-terminal region.

When the secondary structure of the β'-c subunit was predicted from the deduced amino acid sequence using the computer programs Jpred 3 (26) and PROF-sec (27), approximately half of the entire structure was shown to be composed of β–pleated sheets

with no α-helices (Supplementary data, Figure S1). Additionally, the CD spectrum of native β'-c (Fig. 2-2) agreed with these predicted characteristics. Such a high content of β–pleated sheets and a homodimer structure may contribute to the observed high thermal stability of β'-c in which the water solubility remained unchanged after heating at 98 °C for 20 min (Data not shown).

Figure 6 showed that the 16 kDa- and 18 kDa-subunits of native β'-c have an equal IgE-binding ability. Thus, a slight difference in the primary structure between the isoforms and the absence of a C-terminal region in the 16 kDa-subunit seems to have no effect on the allergenicity of β'-c. Furthermore, the reaction between native β'-c and the specific IgE was effectively suppressed by carboxymethylated β'-c and Rβsub. This result, although structural IgE epitopes may be reconstructed with refolding of Rβsub, suggests that sequential IgE-epitopes are more likely to be involved in sensitization to β'-c in salmon roe allergies than structural IgE-epitopes.

On the other hand, the IgE-binding abilities of β'-c and Rβsub were impaired by the carboxymethylation. These changes were presumably caused by loss of structure epitopes with collapse of the subunit structure of native β'-c or by the modification of cysteine residues in sequential epitopes.

It is unclear that which of higher order structure or internal structure of β'-c participates in the structural IgE-epitopes. Further structural information of native and recombinant proteins before and after carboxymethylation are required to clarify this issue, and these discussion could contribute to identifying IgE epitopes in β'-c.

In oviparous animals including fish and bird, Vg is mainly fragmented to Lv, Pv, and specific proteins such as β'-c in teleost fish (7, 8) and 40 kDa-glycoprotein in chiken (*Gallus gallus*) (28). However, no amino acid sequence of β'-c clarified in this study was found out in chicken Vg (Accession number: D89547, M18060 (29)), indicating that no protein with a similar structure to β'-c exists in hen egg proteins. Additionally, the patients' sera containing specific IgE with strong reactivity to native β'-c and Rβsub (Fig. 6) showed little IgE-reactivity against chicken egg yolk and white proteins as presented in CAP-RAST (Table 1). These results lend support to the previous study (10) that denied the existence of IgE cross-reactivity between fish roe and hen egg.

Since some substitutions of amino acid residues were observed between the deduced amino acid sequences and the digested peptides, as shown in Fig. 5, the two subunits prepared by preparative electrophoresis could contain their own isoforms. Buisine et al. (30) reported that the Vg gene of salmonid fish forms a complex Vg gene cluster and that

the *Oncorhynchus* group including chum salmon contains 28–30 Vg genes. The reason for the difference in molecular mass between β'-c subunits might be caused by variations in enzyme cleavage sites as a result of the slight difference in the C-terminal amino acid sequences. In MALDI-TOF MS, one major signal (M/Z, 36,395) with a shoulder part on the low molecular weight side was observed in native β'-c. Additionally, in carboxymethylated β'-c, 18 kDa-subunit was shown as a broad and tailing signal (M/Z, 18,068) as compared with 16 kDa-subunit (M/Z, 17,269). Since only one type of amino acid sequence was observed in N-terminus of β'-c subunits as shown in Fig. 5, the results of MALDI-TOF MS suggest that native β'-c contains 18 kDa-subunit having different cleavage sites at the C-terminal side.

In conclusion, β'-component, a major allergen in chum salmon roe, is a homodimer protein composed of two isoform subunits having the same level of IgE-binding ability. These findings are critical data for identifying conformational IgE epitopes and for understanding allergenic cross-reactivity among fish roes. An attempt to identify conformational IgE epitopes is in progress using the structural information obtained in this study.

Acknowledgments

We appreciate Mr. Tomohiro Hirose, Instrumental Analysis Division, Equipment Management Center, Creative Research Institution, Hokkaido University, for excellent technical assistance in the amino acid sequence analysis.

Funding

This study was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (18580200), a grant from the Japanese Health Sciences Foundation (KHB1212), and a grant from the Japanese Ministry of Health, Labor, and Welfare (H21-meneki-ippann-005).

Supplementary data

Supplementary data are available at *International Immunology* Online.

Conflict of interest

The authors have no financial conflicts of interest.

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1

Table 1. Allergic patient characteristics with hypersensitivity to chum salmon roe

Serum	Age [years]	Gender	Total IgE [IU/mL]	Specific IgE [IU/mL, (CAP-RAST score)]			Symptoms
				Salmon roe	Egg yolk	Egg white	
P1	1	Male	66	20.0 (4)	4.6 (2)	0.5 (1)	AD
P2	6	Male	1,017	18.9 (4)	2.5 (2)	1.1 (1)	BA, OAS
P3	1	Male	221	35.9 (4)	4.0 (2)	0.7 (1)	AD
P4	3	Male	337	84.2 (5)	2.6 (2)	6.0 (3)	Ur
P5	4	Male	-	>100(6)	-	-	AD, BA

AD, Atopic dermatitis; BA, Bronchial asthma; OAS, Oral allergy syndrome;
Ur, Urticaria.

Legends of Figures

Fig. 1. DNA sequences encoding major yolk proteins located in the vitellogenin gene (**A**) and cDNA cloning strategy for the expression of β'-component subunit gene (**B**). Lipovitellin heavy chain and light chain (Lv-Hc, Lv-Lc), phosvitin (Pv), β'-component (β'-c), and C-terminal component (Ct).

Fig. 2. Molecular characteristics of β'-c.

[1]: SDS-PAGE patterns of yolk protein and β'-c and their IgE-reactivity in immunoblotting. Whole yolk proteins extracted with 0.5M NaCl (pH 8.0) (A), native β'-c (B, C), 16 kDa- and 18 kDa-subunits of β'-c (D, E) were electrophoresed. Samples were loaded under reducing (A, C, D, E, F) or non-reducing (B) conditions. β'-c was immunoblotted using patient's (F) and control (G) sera. The 16 kDa- and 18 kDa-subunits of β'-c were prepared using the preparative SDS-PAGE system in the presence of 2-mercaptoethernol.

[2]: CD spectrum of native β'-c.

Fig. 3. Molecular mass distribution of β'-c. Native (A) and carboxymethylated β'-c (B) were applied to MALDI-TOF system.

Fig. 4. cDNA sequences of chum salmon β'-c and deduced amino acid sequences. Accession numbers (DDBJ nucleotide sequence database) are shown and the deduced amino acid sequences from the cDNAs are represented as Cβ1, Cβ2 and Cβ3. Shaded bases and amino acids in Cβ2 and Cβ3 differed from those of Cβ1.

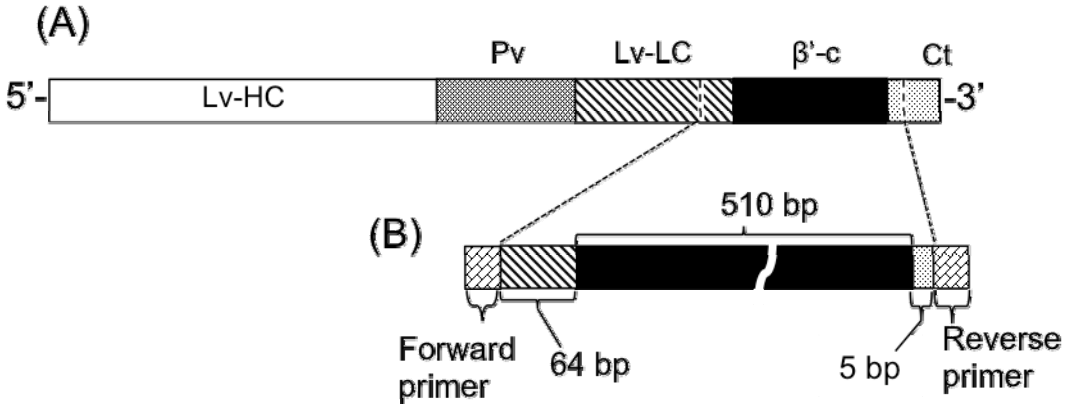
Fig. 5. Peptide mapping of β'-c subunits.

Upper figures: HPLC chromatogram of digested β'-c subunits (16 kDa and 18 kDa) by lysyl endopeptidase, endoproteinase Glu-C and trypsin. The solid line represents the elution curve of the digested peptides, and the dotted line is the gradient curve of the acetonitrile concentration. The inserted symbol letters match the peptide sequence shown in the lower figure.

Lower figure: Positions of digested peptides in the entire amino acid sequence of β'-c subunits. The digested peptides (shadowed) were matched to the predicted amino acid

sequences of β'-c. Abbreviations are as follows: 18-Nt, N-terminal of 18 kDa-subunit; 16-Nt, N-terminal of 16 kDa-subunit; 16-Le, 16 kDa-subunit digestion by lysyl endopeptidase; 16-EG, 16 kDa-subunit digestion by endoprotease Glu-C; 16-Tr, 16 kDa-subunit digestion by trypsin; 18-Tr, 18 kDa-subunit digestion by trypsin.

Fig. 6. Inhibitory effect of recombinant β'-c on the reaction between specific IgE and native β'-c. (A): Serum from 4 patients allergic to salmon roe (P2-P5) were mixed with recombinant (Rβsub) and native β'-cs as inhibitors in a competitive ELISA. (B): The inhibitors were carboxymethylated before examining the competitive ELISA. The ELISA plates were coated with native β'-c.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Pv
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β'-c
Ct
5'-
Lv-HC
-3'
(B)
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Forward primer
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5 bp
Reverse primer

Fig. 1

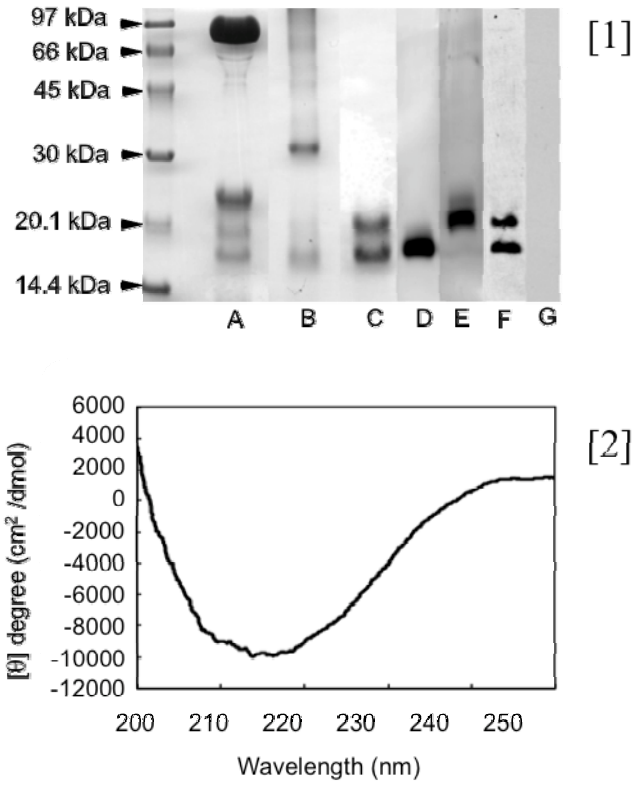
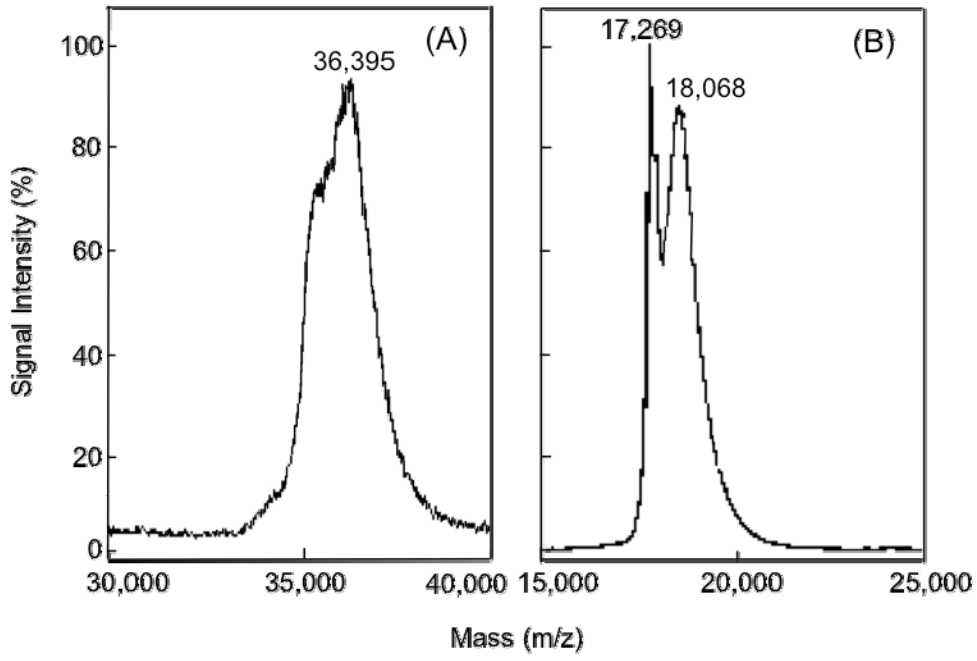


Fig. 2 (revised)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18,068
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80
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0
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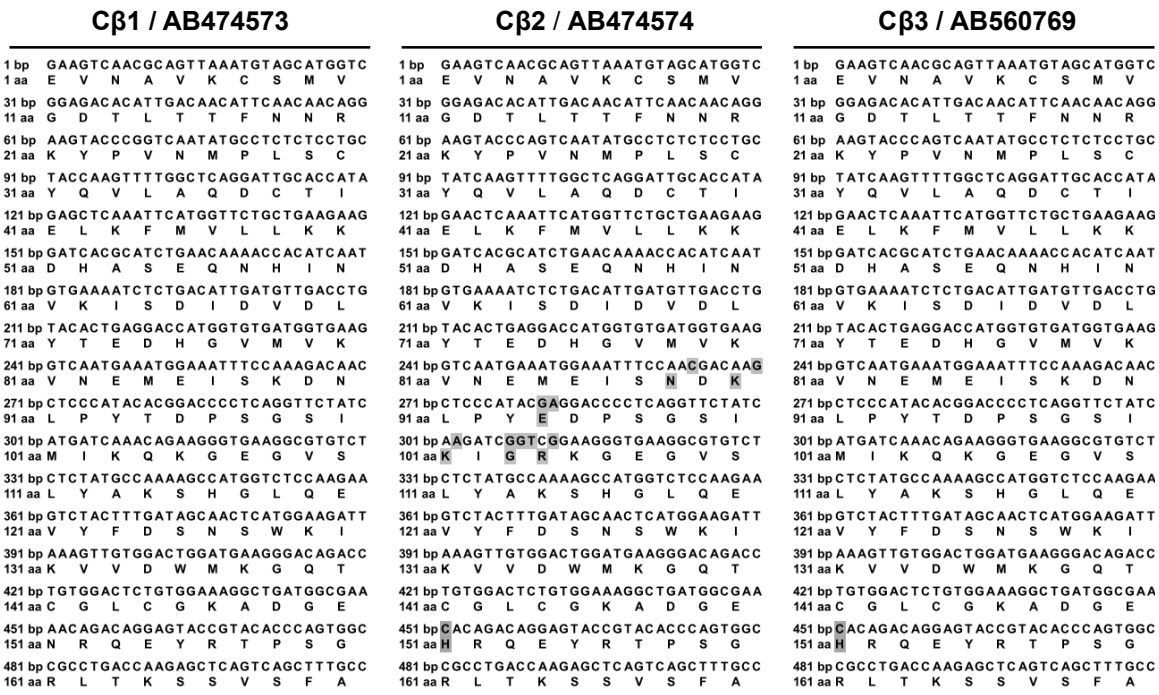
35,000
40,000
15,000
20,000
25,000
Mass (m/z)

Fig. 3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11 aa G D T L T T F N N R
61 bp AAGTACCCGGTCAATATGCCTCTCTCCTGC
21 aa K Y P V N M P L S C
91 bp TACCAAGTTTTGGCTCAGGATTGCACCATA
31 aa Y Q V L A Q D C T I
121 bp GAGCTCAAATTCATGGTTCTGCTGAAGAAG
41 aa E L K F M V L L K K
151 bp GATCACGCATCTGAACAAAACCACATCAAT
51 aa D H A S E Q N H I N
181 bp GTGAAAATCTCTGACATTGATGTTGACCTG
61 aa V K I S D I D V D L
211 bp TACACTGAGGACCATGGTGTGATGGTGAAG
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271 bp CTCCCATACACGGACCCCTCAGGTTCTATC
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301 bp ATGATCAAACAGAAGGGTGAAGGCGTGTCT
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Cβ2 / AB474574
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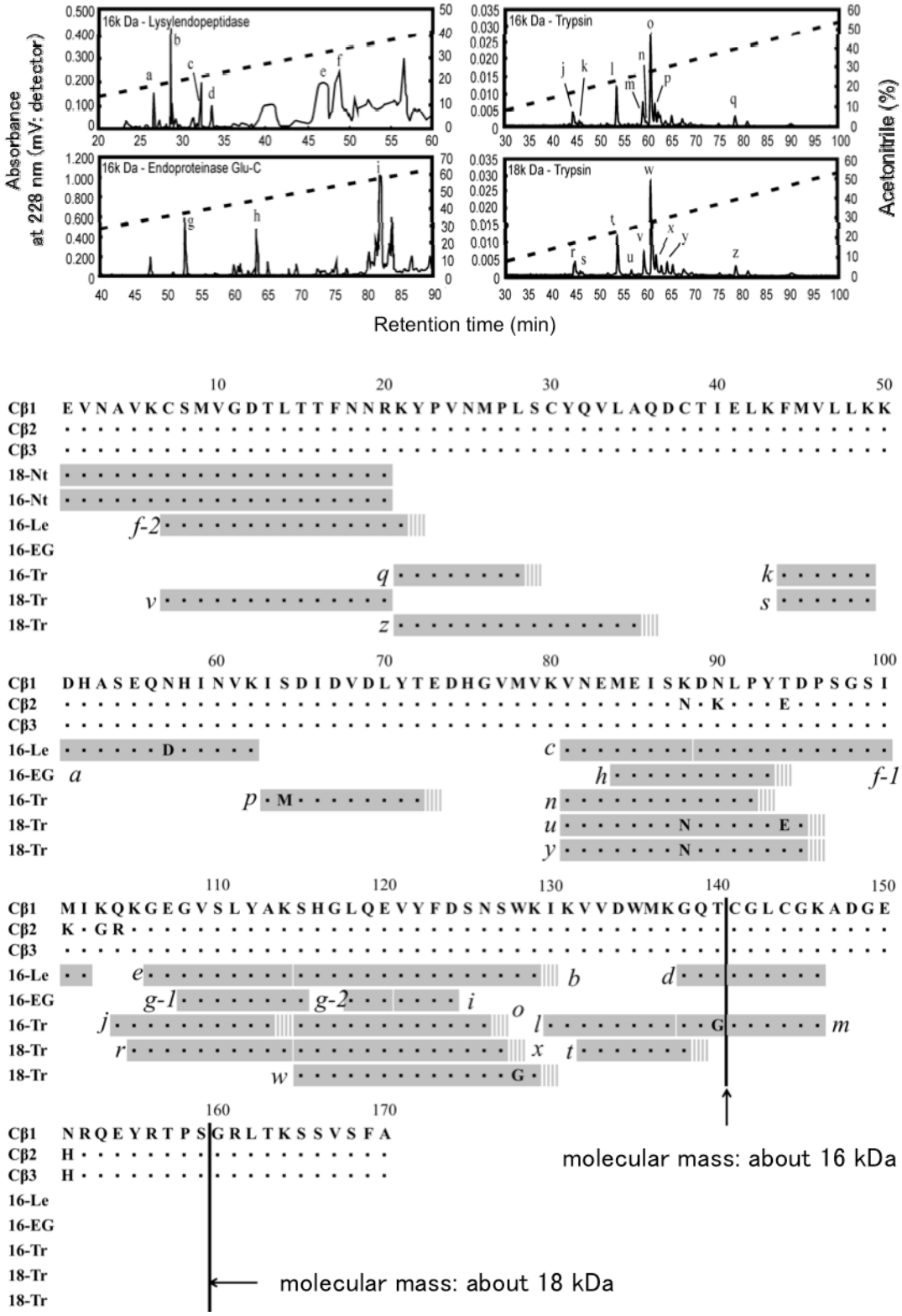
Fig. 4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16k Da - Trypsin
16k Da - Endoproteinase Glu-C
18k Da - Trypsin
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Retention time (min)
molecular mass: about 16 kDa
molecular mass: about 18 kDa

Fig. 5

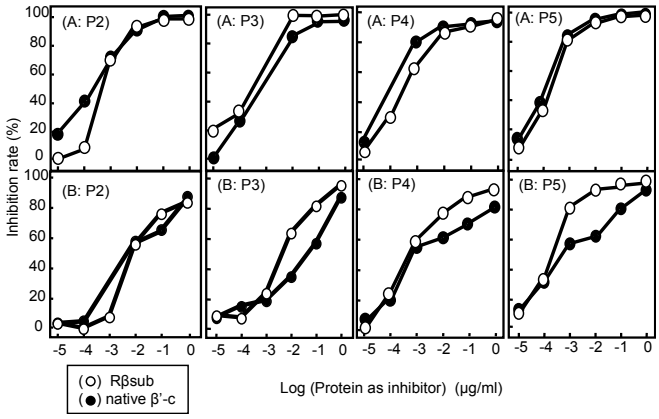
(A: P2)
(A: P3)
(A: P4)
(A: P5)
(B: P2)
(B: P3)
(B: P4)
(B: P5)
Inhibition rate (%)
100
80
60
40
20
0
-5 -4 -3 -2 -1 0
(○) Rβsub
(●) native β'-c
Log (Protein as inhibitor) (μg/ml)

Fig. 6