

Grey mullet roe contains yolk protein having IgE cross-reactivity to chum salmon roe major allergen (Onc k 5)

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Abstract The major allergenic protein in grey mullet roe was immunologically characterized using rabbit IgG against the β' -component, which is a vitellogenin fragment and the major allergen in chum salmon roe, and the IgE cross-reactivity between salmon and mullet roe was examined using chum salmon roe-allergic patients' sera. Grey mullet β' -component was identified in the yolk protein and consisted of 16 and 30 kDa proteins that showed marked reaction with the anti- β' -component IgG and shared N-terminal amino acid sequence similarity with vitellogenin. The grey mullet β' -component consisted of six different isoforms generated from vitellogenin A and vitellogenin B, and they inhibited the IgE-binding reaction between chum salmon β' -component and a chum salmon roe-allergic patient's serum. Thus, grey mullet roe may be an allergenic seafood capable of introducing IgE cross-reactivity to chum salmon roe through the β' -component.

Keywords Grey mullet roe · Chum salmon roe · Allergen · β' -component · IgE cross-reactivity · Seafood allergy

Abbreviations

a- β	Anti-salmon β' -c IgG
a-Lv	Anti-salmon lipovitellin IgG
β' -c	β' -component
CAP-RAST	Capsulated hydrophilic carrier polymer-radioallergosorbent test
ELISA	Enzyme-linked immunosorbent assay
Ig	Immunoglobulin

KP	Potassium phosphate
Lv	Lipovitellin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
SDS	Sodium dodecylsulfate
Vg	Vitellogenin
YP	Yolk protein

Introduction

Salted fish roe is a popular seafood in the Asia-Pacific region, where marine bioresources occupy an important position in dietary habits. Due to taste and abundant nutritive value [1, 2], the whole ovary or grated roe has high economic value, and various kinds of fish roe such as salmon, walleye pollack, herring, and flying fish are commonly consumed throughout the year. Mullet is a popular fish species in the aquaculture industry worldwide [3], and grey mullet *Mugil cephalus* roe is a popular material for salted and semidried products in the Asia-Pacific region, particularly in Japan (*Karasumi*) and Taiwan (*Wuyuzi*), and also in Mediterranean countries such as Italy, Spain, Egypt, and Turkey (*Botarrga*).

On the other hand, immediate hypersensitivity caused by fish roe ingestion is frequently observed in cases of food allergy. Japan's food allergen labeling system has specified that salmon roe was labeled as a potential cause of allergic reactions [4], as allergy to salmon roe is the most frequently reported of fish roe-derived foods. Indeed, fish roe as a cause of anaphylaxis accounts for 3% of all food allergic patients in Japan, and in particular, the incidence rate from fish roe ingestion is around 7% of food allergy cases in infants [5].

The yolk proteins of teleost fish are generated from vitellogenin (Vg) synthesized in the liver [6]. Vg is carried

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in the bloodstream and accumulates in oocytes, where it is enzymatically degraded into the major constituents of fish yolk proteins, lipovitellin (Lv), phosvitin (Pv), and β' -component (β' -c) during oocyte maturation (vitellogenesis) [7, 8]. The authors have previously reported that β' -c, one of the degradation fragments of Vg, was the major IgE-binding protein in chum salmon roe [9] and it has been registered as the novel allergen 'Onc k 5' in the official allergen list of the World Health Organization and International Union of Immunological Societies Allergen Nomenclature Subcommittee (<http://www.allergen.org/>). Indeed, almost all of salmon roe-allergic patients' sera contain specific IgE against β' -c [9], and the high digestive tolerance of β' -c to gastrointestinal proteases [9] may contribute to its potential as a sensitizing antigen with strong allergenicity. Additionally, yolk proteins from various kinds of fish also reacted with IgE specific to chum salmon β' -c, indicating establishment of IgE cross-reactivity among fish roes mediated by β' -c [10–12], and Liu et al. [13] also reported the cross-immunoreactivity of β' -c between the roe of large yellow croaker and various kinds of fish roe. These findings suggest the importance of research focusing on β' -c for characterization of fish roe allergy.

In the case of grey mullet roe, Amano et al. [14, 15] characterized a series of yolk proteins with respect to their comparative physiology and embryology, and reported the existence of β' -c isoforms derived from three subtypes of Vg having high amino acid sequential homology to other teleost Vg proteins. These findings suggest that seafood products from grey mullet roe may also be a risk in food allergies in which IgE cross-reactivity through β' -c is involved, the same as chum salmon roe. However, despite its importance in the seafood industry, there is no information regarding potential allergenic proteins contained in mullet roe.

The objective of this work was to characterize the allergenic protein in grey mullet roe. A series of β' -c (isoforms) was identified as the major allergenic yolk proteins and the IgE cross-reactivity between salmon roe and mullet roe were examined using chum salmon allergic patients' sera.

Materials and methods

Grey mullet roe

Grey mullet roe, as a material for the high-quality *Karasumi*, was provided by the Fujii Karasumi Company (Kitaura, Nagasaki, Japan), a producer of processed roe in Nagasaki prefecture, Japan. The roe was collected from fresh female grey mullet caught off the Nagasaki shore and stored at -25°C until use.

Preparation of antibodies against chum salmon yolk proteins

Chum salmon β' -c and Lv were prepared from fresh frozen roe as described by Hiramatsu and Hara [16], and the purified proteins were used to immunize rabbits (NZW, male, 3 months old) as previously described [9]. The rabbit anti sera were applied to a Protein A HP column (GE Healthcare, Piscataway, NJ, USA) and the purified IgG antibodies were dialyzed against PBS. The resulting anti-salmon β' -c IgG (α - β) and anti-salmon lipovitellin IgG (α -Lv) antibodies were mixed with 0.2% sodium azide and stored at 5°C until use. Animal experiments were performed according to the Guidelines Concerning Animal Experiments at Hokkaido University.

Allergic patients' sera

Sera from 13 childhood patients diagnosed with salmon roe allergy were used from the provided written informed consents by parents to the patients' attending doctors before enrollment in the study. Sample sera were used according to the approval of the ethics committee of the Japan Society of Nutrition and Food Science. Total IgE and specific IgE to chum salmon roe levels of the sera were determined by the capsulated hydrophilic carrier polymer-radioallergen sorbent test for chum salmon roe allergy (CAP-RAST system; Phadia AB, Uppsala, Sweden). The frozen-stored patients' sera were thawed and mixed with the same volume of phosphate-buffered saline (PBS; pH 7.5) containing 0.2% sodium azide and then stored at 4°C until use.

The patients' clinical data are listed in Table 1. Sera from five non-allergic individuals were used as a negative control in the enzyme-linked immunosorbent assay (ELISA).

Preparation of yolk protein extract and β' -c isoforms from grey mullet roe

The thawed grey mullet roe was homogenized with four volumes of 20 mM Tris-HCl (pH 8.0) containing 0.5 M NaCl and centrifuged for 15 min at 20,000g. The extracted yolk protein (YP)-containing supernatant was filtered through filter paper, and grey mullet β' -c was prepared from the YP according to the method of Amano et al. [14] with a slight modification. Briefly, YP was salted-out using ammonium sulfate at 50 and 67% saturation. The precipitate fraction was dissolved and dialyzed against 50 mM potassium phosphate (KP) buffer (pH 6.8) prior to being subjected to hydroxylapatite (HA) chromatography. The HA media (Bio-Rad, Hercules, CA, USA) was packed into a $\phi 3 \times 21$ -cm glass column and equilibrated with 50 mM KP buffer, and the dialyzed samples were eluted

Table 1 Characteristics of allergic patients with hypersensitivity to chum salmon roe

Serum	Age (Years)	Gender	Total IgE (IU/mL)	Specific IgE (IU/mL) [CAP-RAST score]	Symptoms
P1	3	F	204	ND	Ur
P2	4	F	–	–	OAS
P3	2	F	–	–	OAS
P4	1	F	–	–	OAS
P5	1	F	85	0.74 [2]	AD
P6	2	F	212	10.3 [3]	AD
P7	5	F	135	ND	Ur
P8	3	M	337	84.2 [5]	Ur
P9	2	M	–	4.2 [3]	Ur
P10	4	M	–	1.9 [2]	Ana
P11	3	M	–	2.95 [2]	Ana
P12	4	M	–	>100 [6]	No information
P13	3	M	6130	>100 [6]	Ad, BA

AD atopic dermatitis, Ana anaphylaxis, BA bronchial asthma, ND not detected, OAS oral allergy syndrome, Ur urticaria

by step-wise additions of 0.05, 0.1, 0.15, 0.25, and 0.5 M KP buffer at a flow rate of 0.5 ml/min. The eluted fractions were collected in a volume of 10 ml/tube. All steps were performed at temperatures below 5 °C, and the protein elution was monitored at 280 nm. To prepare β' -c isoforms from the eluents, the fractions collected using 0.1, 0.15, and 0.25 M KP buffer, respectively, were mixed with an equal volume of 2% sodium dodecylsulphate (SDS) containing 8 M urea and 20 mM Tris–HCl (pH 8.0) and subjected to polyacrylamide gel preparative electrophoresis (ATTO Corp., Tokyo, Japan) according to the manufacturer's protocol. The proteins were separated using a 12.5% acrylamide disk gel equipped with the preparative electrophoresis system and continuously captured in 800 μ l of 20% sucrose with Tris–HCl (pH 8.8) at the bottom of the disk gel. The elution of the separated proteins was monitored at 280 nm, and the isolated β' -c isoforms were dialyzed against 1 mM NaHCO₃ at 4 °C for 48 h, and stored at –60 °C until use.

SDS-PAGE analysis

SDS–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [17], using 4.5 and 12.5% polyacrylamide slab gels for stacking and resolving gels, respectively. The protein bands were stained with 0.2% Coomassie Brilliant Blue (Sigma Aldrich, St. Louis, MO, USA) dissolved in 9% acetic acid and 45% methanol. Molecular masses of protein subunits were evaluated using a LMW marker kit (GE Healthcare).

Immunoblot assay

Protein bands separated by SDS-PAGE were transferred onto a polyvinylidene difluoride membrane (Immobilon-P;

Millipore, Billerica, MA, USA) using a semidry blotting system (ATTO, Tokyo, Japan) for 45 min at 120 mA. Transferred bands were visualized by soaking the membrane for 3–5 min in 0.1% Ponceau S (in 1% acetic acid) and were marked with a pen after destaining with 20 mM Tris–HCl buffer containing 0.15 M NaCl and 0.05% Tween (TTBS; pH 7.5). The membranes were blocked with TTBS containing 3% casein for 1 h at room temperature, and incubated with anti-chum salmon yolk protein antibodies (a- β and a-Lv, diluted 1:20,000 with blocking buffer) or with allergic patient's serum containing IgE (P12, diluted 1:50 with blocking buffer) at 4 °C overnight. After washing six times with TTBS, the antibodies binding to the blotted proteins were probed with peroxidase-conjugated goat anti-rabbit IgG antibody (BioRad; diluted 1:20,000 in blocking buffer) or peroxidase-conjugated rabbit anti-human IgE anti-body (Dako, Amsterdam, The Netherlands; diluted 1:20,000 with blocking buffer.) at 37 °C for 3 h. The membranes were then washed three times with TTBS and three times with 0.15 M NaCl (pH 7.5), and bound antibodies were visualized using ECL Western Blotting Detection Reagent (GE Healthcare) for a- β and a-Lv antibodies or Immobilon Western Chemiluminescent HRP Substrate (Millipore) for patient's serum. The chemiluminescent images were obtained by an image analyzer, and the reversed images are presented.

Direct and competitive ELISAs

For the direct ELISA, a 96-well black ELISA plate (Greiner Bio-One GmbH, Frickenhausen, Germany) was coated with 2.5 μ g/ml of YP (100 μ L/well) diluted in coating buffer (50 mM H₂CO₃, pH 9.6) and incubated overnight at 4 °C. The plate was washed three times with

TTBS, and then blocked with blocking buffer (1% casein in TTBS) at 37 °C for 1.5 h, followed by washing four times with TTBS. Continuously, sera from allergic patients or non-allergic individuals (125 µl, diluted 1:400 in TTBS containing 0.1% casein) was added to the wells and incubated at 37 °C for 2 h. After washing three times with TTBS containing 1 M NaCl, peroxidase-conjugated rabbit anti-human IgE antibody (100 µl/well, diluted 1:1000 (v/v) in TTBS containing 0.1% casein) was added to each well and incubated at 37 °C for 1.5 h. After washing again with TTBS containing 1 M NaCl, the plate was incubated with the fluorescence substrate (0.1 M phosphate buffer containing 0.1 mg/ml 4-methylumbelliferyl- β -D-galactoside and 1 mM MgCl₂, pH 7.8) at 37 °C for 1.5 h, and the reaction was stopped by adding 100 µl/well of 0.1 M glycine-NaOH (pH 10.3). The fluorescence intensity was read using excitation at 365 nm and emission at 450 nm. The ELISA response of the five negative control sera were measured, and the value obtained by adding ten times the standard deviation to the average was used as the cut-off value [18, 19] for evaluating positive ELISA results in Fig. 1a.

For the competitive ELISA, a 96-well black ELISA plate was coated with chum salmon β' -c and blocked with blocking buffer (1% casein in PBS) in the same way as the direct ELISA. Patient serum (P13) and non-allergic control serum (diluted 1:200 in TTBS containing 0.2% casein) were pre-incubated with equal volumes of grey mullet β' -c isoforms as inhibitors at concentrations ranging from 10^{-5} to 10^1 µg/ml (diluted in TTBS) at 37 °C for 2 h. Seventy µL of each solution was then placed into the blocked ELISA plate to react with the coated chum salmon β' -c as a solid phase antigen. After incubation at 37 °C for 2 h, the detection and measurement of fluorescence intensity was carried out in the same way as the direct ELISA. The loss of the specific IgE-binding ability of the patient's serum resulting from the treatment with the inhibitors was determined by calculating the inhibition rate (Y) using the following formula: $Y = (FI_0 - FI_{inhibitor}) / (FI_0 - FI_{control}) \times 100$, where FI_0 is the fluorescence intensity of a patient's serum without the inhibitors, and $FI_{inhibitor}$ and $FI_{control}$ is the fluorescence intensity of the patient's serum and non-allergic control serum incubated with the various concentrations of inhibitors, respectively.

Amino acid sequence analysis

The proteins were separated by SDS-PAGE and blotted in the same way as the immunoblotting, and the protein-blotting membrane was then stained with 0.1% Coomassie Brilliant Blue R in 30% methanol and 7.5% acetic acid. The stained protein bands were cut out from the membrane and subjected to amino acid sequence analysis using an automated Edman sequence analyzer (Procise 492,

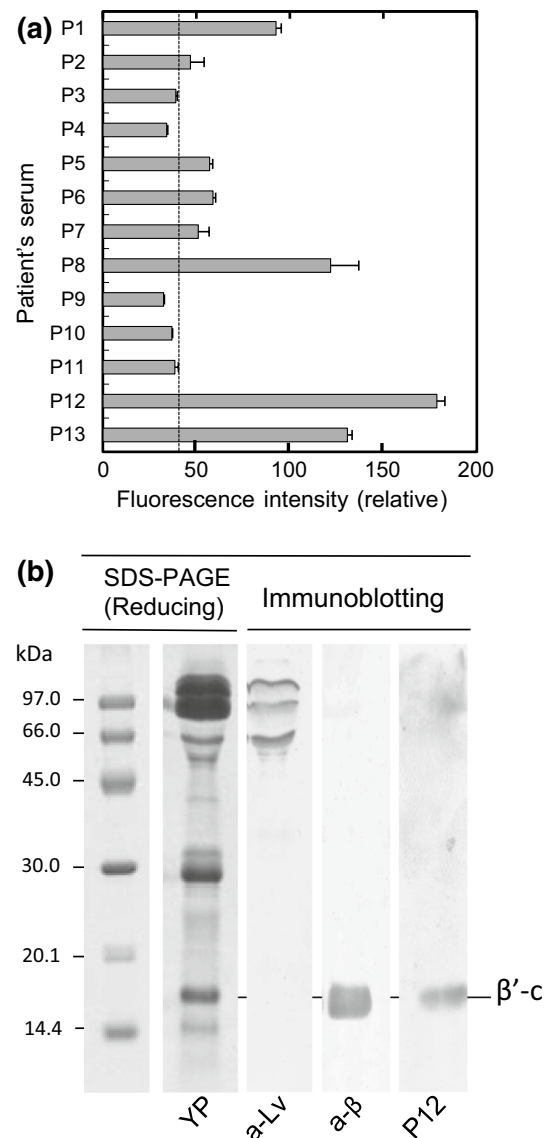


Fig. 1 IgE binding between salmon roe-allergic patients' sera and grey mullet YP and SDS-PAGE and immunoblotting analysis of YP. **a** Salmon roe-allergic patients' sera (Table 1) were analyzed for specific IgE binding to grey mullet YP by direct ELISA, and fluorescence intensity was measured as the ELISA response. The dotted line indicates the cut-off value calculated as the mean of five negative control sera plus ten standard deviations. Values above cut-off (dotted line) were considered a positive response. Data are expressed as mean $\pm$ standard deviation ($n = 5$). **b** Grey mullet YP was analyzed by SDS-PAGE under reducing conditions. Immunoblotting was carried out using a-Lv, a- β , and salmon roe-allergic patient's serum (P12) shown in Fig. 1a

Perkin-Elmer, Waltham, MA, USA), and 20 N-terminal amino acid residues of every protein band were determined. The resulting N-terminal amino acid sequences were searched and identified by the BLAST search of the DNA Data Bank of Japan database.

Results

IgE reactivity of salmon roe-allergic patients' sera to grey mullet YP

Sera from 13 chum salmon roe-allergic patients, listed in Table 1, were analyzed by direct ELISA to screen for specific IgE binding to grey mullet YP. As shown in Fig. 1a, the ELISA response of eight of the 13 sera was determined to be “positive” compared with the negative control sera, and in particular, markedly higher ELISA responses were observed in three of the sera (P8, P12, and P13) corresponding to the high specific IgE levels detected by CAP-RAST (Table 1). The result of Fig. 1a suggests that grey mullet roe may be capable of inducing IgE-mediated hypersensitivity in chum salmon roe-allergic patients.

Immunochemical analysis of grey mullet YP

Grey mullet YP was subjected to SDS-PAGE and immunoblotting assays to identify the protein binding specific IgE in chum salmon roe-allergic patient's serum. The chum salmon patient's serum, P12 shown in Fig. 1a, was used in this experiment. The SDS-PAGE pattern of grey mullet YP under reducing conditions (Fig. 1b; Lane YP) revealed six major proteins with molecular masses of 16, 30, 60, 66, 90 and >97 kDa. In the immunoblot analysis using a-Lv antibodies, the three bands with higher molecular masses were determined to be the Lv heavy chain and its fragments (Fig. 1b; Lane a-Lv) because grey mullet Lv was enzymatically degraded with oocyte maturation in the same way as other teleost fish [15]. The 30 kDa Lv light chain, which was reported previously [9], was not detected by a-Lv antibodies. The 16 kDa component was identified as β' -c due to the marked reaction with a- β antibodies (Fig. 1b; Lane a- β), and it showed significant IgE reactivity when probed with salmon roe-allergic patient's serum with a high IgE level in CAP-RAST (P12). Taken together, the results of Fig. 1a, b; Lane P12) suggest the establishment of IgE cross reactivity between mullet and chum salmon roe through β' -c.

Isoform identification of grey mullet β' -c

Grey mullet β' -c (16 kDa component shown in Fig. 1b) was purified from YP in order to investigate its structural form. The β' -c fraction purified by ammonium sulfate precipitation was applied to stepwise gradient HA column chromatography. Figure 2a, b show the elution profile of the HA column separation and the SDS-PAGE analysis of the eluted proteins under reducing conditions, respectively. As shown in Fig. 2a, different protein fractions were eluted by each concentration of KP buffer. Apparently, β' -c (16 kDa) was present in the eluted fractions at 0.1, 0.15 and 0.25 M

KP, and Lv was separately eluted at 0.5 M KP in SDS-PAGE under reducing conditions (Fig. 2b).

To investigate the reason that β' -c was eluted in a wide range of KP, the β' -c-containing fractions were then subjected to polyacrylamide gel preparative electrophoresis under non-reducing conditions. Two major components with molecular masses of 16 and 30 kDa were clearly identified in all eluted fractions as shown in Fig. 3a, and both exhibited marked reactivity with a- β antibodies (Fig. 3b). Teleost vitellogenin (Vg) has three major subtypes; VgA and VgB are referred to as complete Vgs containing three major yolk proteins (Lv, Pv and β' -c), and VgC is referred to as an incomplete Vg consisting of only the Lv domain [7]. Therefore, the N-terminal 20 amino acids of the proteins reacting with a- β antibodies [Fig. 3b; 16 kDa (β 1, β 3, β 5) and 30 kDa (β 2, β 4, β 6)] were compared with the amino acid sequence of grey mullet VgA and VgB [14, 15]. As shown in Table 2, the amino acid sequences of five of the six proteins (β 1, β 2, β 3, β 4 and β 6) matched well with

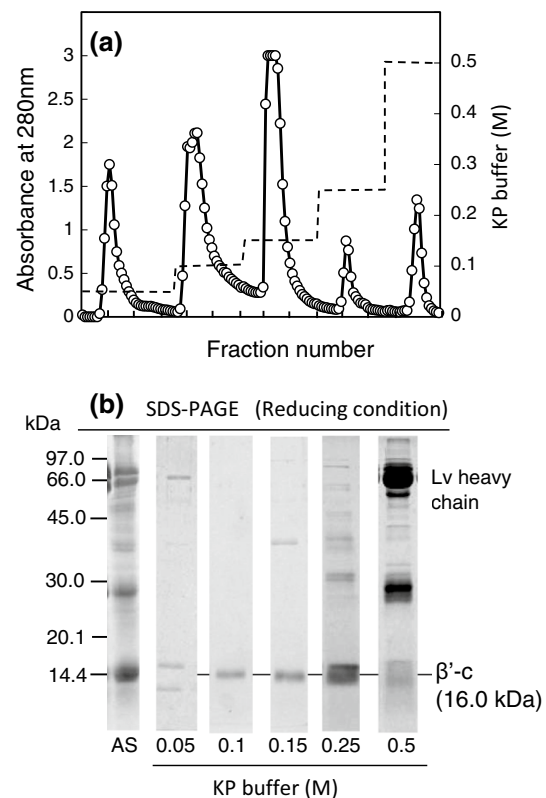


Fig. 2 Separation of grey mullet YP by HA column chromatography. Grey mullet YP was salted out using 50–67% saturated ammonium sulfate, and the resulting precipitate (AS) was subjected to stepwise gradient HA column chromatography using 0.05, 0.1, 0.15, 0.25 and 0.5 M KP buffer, and elution was monitored at 280 nm (a). The resulting eluted fractions were analyzed by SDS-PAGE under reducing conditions; an aliquot of total volume of each fraction was sampled, subjected to SDS-treatment, and loaded for SDS-PAGE analysis (b)

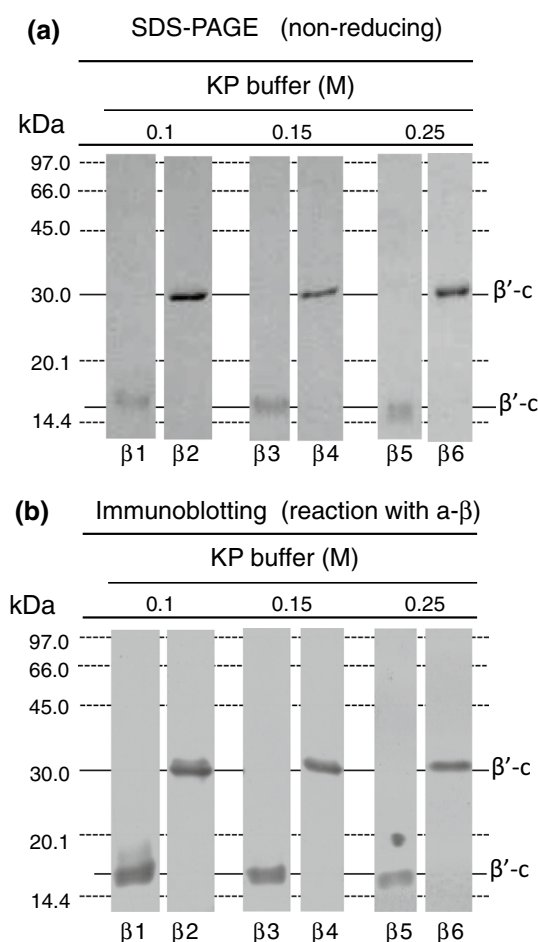


Fig. 3 Detection of grey mullet β' -c isoforms separated by HA column chromatography. Protein fractions eluted from the HA column using 0.1, 0.15 and 0.25 M KP buffer (Fig. 2) were subjected to preparative polyacrylamide gel electrophoresis under non-reducing conditions to fractionate β' -c isoforms. The resulting β' -c isoforms were analyzed by non-reducing SDS-PAGE (a) and immunoblotting using α - β (b). β 1– β 6 were grey mullet β' -c isoforms

that of grey mullet VgB (Accession Number: AB288933) from I-1423 to N-1442 [15], while β 5 matched that of VgA (AB288932), from A-1425 to N-1444, with 95% identity. These results indicate that grey mullet roe contains multiple isoforms of β' -c generated from VgA and VgB. The 30 kDa components appeared to be dimers composed of two identical subunits, as they were not observed in SDS-PAGE under reducing conditions (Figs. 1, 2) and different amino acid residues were not detected by amino acid sequence analysis.

IgE cross-reactivity between grey mullet and chum salmon β' -c

A competitive ELISA was carried out to investigate the possible IgE cross-reactivity between grey mullet and chum

salmon β' -c. Pre-incubation with the different isoforms of grey mullet β' -c inhibited the specific IgE reactivity of the salmon roe-allergic patient's serum (P13) to chum salmon β' -c (Fig. 4), indicating that the specific IgE contained in the salmon roe-allergic patient's serum was reacted with the series of grey mullet β' -c isoform. A minimum 40% inhibition rate was observed upon incubation with 10^{-5} μ g/ml of grey mullet β' -c as the inhibitor, and the inhibition rate increased with increasing inhibitor concentration. These results clearly indicate the presence of IgE cross-reactivity between β' -c from grey mullet and chum salmon roe. On the other hand, there was no difference in the inhibition effect among β' -c isoforms, suggesting that the grey mullet β' -c isoforms have the same level of IgE-binding ability.

Discussion

Grey mullet and chum salmon, important resources in the seafood industry, belong to a different form in fish systematics. However, the present study revealed the possibility that grey mullet β' -c acts as an allergenic food the same as chum salmon roe.

Grey mullet yolk proteins with reactivity to anti-chum salmon IgG were found in grey mullet roe, and the 16 kDa component in SDS-PAGE under reducing condition was identified as the β' -c by immunochemical experiments using anti-chum salmon β' -c IgG and chum salmon roe-allergic patients' sera (Fig. 1b). Salmonid β' -c exists as a homodimeric protein [7, 16] and chum salmon β' -c was shown to be comprised of 16 and 18 kDa subunits with different cleavage sites of the C-terminal region upon proteolysis from Vg [9, 20]. Here, grey mullet roe appeared to contain 16 and 30 kDa β' -c proteins (Fig. 3), however, only the 16 kDa β' -c was detected by SDS-PAGE under reducing conditions (Fig. 1b). These results indicate that unlike salmonid fish roe, β' -c exists as both monomeric and dimeric forms in grey mullet roe. Additionally, there was no difference in the inhibitory effect on the reaction between salmon β' -c and the specific IgE among grey mullet β' -cs having monomeric and dimeric forms, as described in Fig. 4. This result suggests that there is no structural epitope due to the dimeric structure of the β' -c.

As listed in Table 2, VgB was a precursor for five of the six different isoforms detected, which seems to reflect the genetic variation of fish Vg; 28–30 Vg genes have been identified in salmonid fish [21] and three different β' -c isoforms were found in chum salmon roe [20]. The appearance of β' -c isoforms in grey mullet could also be related to a complex Vg gene cluster often found in oviparous vertebrates [22], but the reason that the 16 and 30 kDa β' -c exist in the different state is unclear at present. Since egg yolk proteins derived from Vg were degraded with oocyte

Table 2 N-terminal 20 amino acid sequence of grey mullet β' -c isoforms

VgB 1423	I	T	K	A	H	A	V	E	C	N	L	V	R	D	T	L	I	T	F	N	1442	
$\beta 1$	¹	I	T	K	A	H	A	V	E	E	N	L	V	R	D	T	L	I	T	F	N	20
$\beta 2$	¹	I	T	K	A	H	A	V	E	E	N	L	V	V	D	T	X	I	T	F	N	20
$\beta 3$	¹	I	T	K	A	H	A	V	E	V	E	L	L	R	D	T	L	T	I	F	N	20
$\beta 4$	¹	I	T	K	A	H	A	V	E	S	D	L	V	R	N	T	T	I	T	F	D	20
$\beta 6$	¹	I	T	K	A	H	A	V	E	S	N	L	V	R	D	T	L	I	T	F	N	
VgA	1425 A	K	A	G	A	A	E	C	S	L	I	G	D	T	L	T	T	F	N	N	1444	
$\beta 5$	¹ S	K	A	G	A	A	E	C	N	L	I	G	D	T	T	T	T	F	N	N	1	
CS β	¹	E	V	N	A	V	K	C	S	M	V	G	D	T	L	T	T	F	N	N	R 20	

VgA: Deduced amino acid sequence of gray mullet vitellogenin A (AB288932), residues 1425-A to 1444-N.

VgB: Deduced amino acid sequence of gray mullet vitellogenin B (AB288933), residues 1423-I to 1442-N.

CS β : N terminal 20 amino acid sequence of chum salmon β' -c (AB474573).

$\beta 1$ to $\beta 6$: grey mullet β' -c isoforms shown in Fig. 3.

The lightly shaded sequences represent amino acid identity between VgA/VgB and β' -c isoforms in grey mullet.

The dark shaded sequences with white letters represent amino acid identity between CS β and grey mullet VgA or VgB.

The lightly shaded sequences represent amino acid identity between VgA/VgB and β' -c isoforms in grey mullet. The dark shaded sequences with white letters represent amino acid identity between CS β and grey mullet VgA or VgB

VgA Deduced amino acid sequence of gray mullet vitellogenin A (AB288932), residues 1425-A to 1444-N, VgB Deduced amino acid sequence of gray mullet vitellogenin B (AB288933), residues 1423-I to 1442-N. CS β N-terminal 20 amino acid sequence of chum salmon β' -c (AB474573), $\beta 1$ – $\beta 6$ grey mullet β' -c isoforms shown in Fig. 3

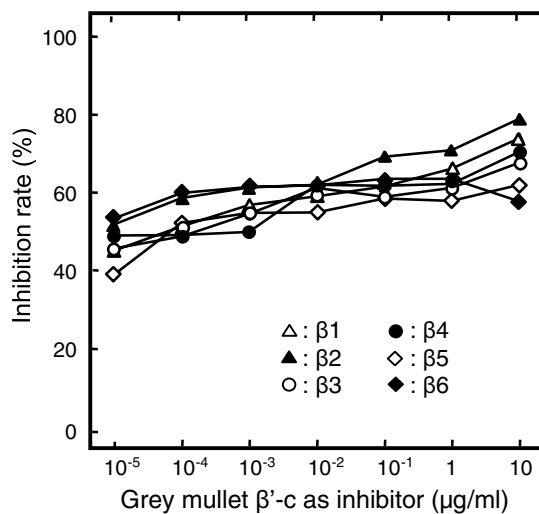


Fig. 4 IgE cross-reactivity between grey mullet β' -c isoforms and chum salmon β' -c. The inhibition effect of the grey mullet β' -c isoforms $\beta 1$, $\beta 2$, $\beta 3$, $\beta 4$, $\beta 5$, and $\beta 6$ on the IgE reaction between salmon roe-allergic patient's serum (P13) and chum salmon β' -c was examined using competitive ELISA. The patient's serum was pre-incubated with equal volumes of grey mullet β' -c isoforms as inhibitors at concentrations ranging from 10^{-5} to 10^1 $\mu\text{g/ml}$ at 37°C for 2 h, and the mixtures were reacted with chum salmon β' -c immobilized in ELSA plates. The loss of the specific IgE-binding ability of the patient's serum caused by the inhibitors was estimated by the method described in "Materials and methods"

maturation [7, 8], β' -c with different digestive stages could be mixed in the yolk, or progress of digestion could differ depending on each isoform.

The important finding in this study was that all six different isoforms of grey mullet β' -c showed IgE-cross reactivity to chum salmon β' -c. In the competitive ELISA shown in Fig. 4, no marked difference in the inhibition effect was observed among β' -c isoforms; and we also confirmed that purified grey mullet β' -c containing all isoforms and $\beta 4$ showed the same level of IgE-reactivity in the direct ELISA (data not shown). These results indicate that grey mullet β' -c consists of a series of isoforms having the same IgE-binding ability and suggest that grey mullet β' -c does not contain structural IgE-binding epitopes dependent on dimer structure. In other words, the 16 kDa subunit is the substance of the allergen.

In the competitive ELISA (Fig. 4), the grey mullet β' -c isoforms effectively inhibited the reaction between chum salmon β' -c and the patient's serum even at low concentrations, indicating the presence of common IgE epitopes between grey mullet and chum salmon β' -c. On the other hand, as shown in Table 2, there was little similarity in the N-terminal amino acid sequences between grey mullet and chum salmon β' -c. Although the IgE-binding ability of chum salmon β' -c has strong proteolytic tolerance, its N-terminal region is easily digested by gastrointestinal proteases [10]. Therefore, the N-terminal region of β' -c is likely not essential for establishing sensitization and may not participate in establishment of IgE-cross reactivity among fish β' -c proteins.

Generally, the maturational state of fresh roe affects the quality of the resulting products, and excessively matured fish roe is not suitable for raw materials of processed foods

because of its undesirable texture. The results of this study were obtained using grey mullet roe supplied for the production of high-quality salted and semidried products. Therefore, the IgE-cross reactivity to salmon roe mediated by β' -c should be considered an actual food safety risk in grey mullet roe products. In conclusion, attention should be given to the potential risks of grey mullet roe as one of the allergenic seafoods.

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