TITLE

In vitro digestion of major allergen in salmon roe and its peptide portion with proteolytic resistance

RUNNING TITLE

Effect of digestion on IgE-binding ability of salmon roe allergen

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Abstract (141 words)

A fish yolk protein, β'-component (β'-c), is the major allergen in chum salmon roe. The effect of proteolysis on the allergenicity of β'-c was estimated. Changes in the IgE-binding ability of β'-c upon pepsin and trypsin digestion were investigated by monitoring the proteolytic cleavage. In the pepsin-trypsin digestion of chum salmon yolk protein, the β'-c contained therein was degraded in a manner similar to that of other yolk proteins, but digestion fragments with a molecular mass of > 10 kDa remained throughout the digestion process. Specifically, the peptide sequence between 31-Y and 119-Q (10 kDa) was stable to pepsin-trypsin digestion and the portion showed high IgE-binding ability. As a result, pepsin-trypsin digestion had little effect on the IgE-binding ability of β'-c. These results suggest that β'-c reaches the small intestine in the form of high-molecular-mass components with IgE-binding ability *in vivo*.

Keywords: Food allergen; salmon roe; allergenicity; digestibility; β'-component; yolk protein; proteolytic tolerance; IgE-binding ability

1. Introduction

Food allergy is a serious medical problem in Japan. The number of people who have hypersensitivity to a specific food is increasing, and the morbidity of seafood-allergic patients has reached 10% among elementary school children and their families (Kanagawa Prefectural Institutes of Public Health, 2006). Food allergy is closely related to dietary habits, and seafood is recognized as a major allergen in Japan, where more than 500 kinds of seafood are consumed. Hypersensitivity to various kinds of fish, shrimp, crab, and processed seafood has been reported, and the number of cases of salmon roe allergy, particularly among children, has increased in the last decade. Therefore, salmon roe has been listed as one of the potential allergen food materials in the Japanese food sanitation law (Ministry of Health, Labour and Welfare, Japan, 2008). Outside of Japan, there have been reports of individuals experiencing immediate allergic reactions to the consumption of king salmon caviar (Flais, Kim, Harris, & Greenberger, 2004), Russian beluga caviar (Untersmayr et al., 2002), and the roe of whitefish and rainbow trout (Kilijunen, Kiistala, & Varjonen, 2003). Immunoglobulin E (IgE) cross-reactivities among fish roes, such as those from salmon, herring, and walleye pollock (Kondo et al., 2005), have been reported in case studies. Therefore, we need to recognize fish roe as a potential allergenic seafood.

55 Teleost roe contains three major yolk proteins, lipovitellin (Lv), phosvitin (Pv), and β'-
56 component (β'-c) (Matsubara & Sawano, 1995; Hiramatsu & Hara, 1996), which are
57 utilized as sources of embryonic nutrients in oviparous vertebrates (Hiramatsu,
58 Matsubara, Fujita, Sullivan, & Hara, 2006). In some marine teleosts that spawn pelagic
59 eggs, these yolk proteins are further cleaved in oocytes and supply a pool of free amino
60 acids (i.e., diffusible nutrients and osmotic effectors) during ovarian follicle maturation,
61 whereas this thorough proteolysis does not occur in freshwater species such as salmonids
62 (Hiramatsu, Cheek, Sullivan, Matsubara, & Hara, 2005; Hiramatsu, Matsubara, Fujita,
63 Sullivan, & Hara, 2006). Additionally, in both cases, some or all yolk proteins are not
64 fully digested during follicle maturation; researchers have found that some β'-c exists in
65 the ovulated egg (Hiramatsu et al., 2002; Amano et al., 2007), suggesting that β'-c
66 remains in the yolk during the early cleavage stage of the embryo. It is known that the
67 enzymatic cleavage of the yolk proteins described above is caused by cathepsin families
68 (Cavalli, Kashiwagi, & Iwai, 1997; Hiramatsu et al., 2002; Imamura, Yabu, & Yamashita,
69 2008; Raldua, Fabra, Bozzo, Weber, & Cerda, 2006). Therefore, the structure of β'-c
70 seems to be stable to proteolysis during development of fish embryo. However, there is
71 no information about gastrointestinal digestion of β'-c. Investigation of the relationship
72 between the digestion behaviour of β'-c and its IgE-reactivity is important for

73 understanding overview of fish roe allergy.

74 High proteolytic resistance is one of the important characteristics of food-allergen
75 proteins (Bannon, Fu, Kimber, & Hinton, 2003; Besler, Steinhut, & Paschke, 2001;
76 Untersmayr & Jensen-Jarolim, 2006). For example, food allergens such as ovomucoid in
77 egg white (Kovacs-Nolan, Zhang, Hayakawa, & Mine, 2000), β-lactoalbumin in cow
78 milk (Astwood, Leach, & Fuchs, 1996), trypsin inhibitor in peanut (Ara h 2) (Lehmann et
79 al., 2006), lipid transfer protein in grape (Vassilopoulou et al., 2006) and β-conglycininin
80 soybean (Astwood, Leach, & Fuchs, 1996), actinidin in kiwifruit (Bublin et al., 2008),
81 and tropomyosin in crab (Liu et al., 2010) have high stability to digestion by
82 gastrointestinal enzymes. The authors found that all the sera of salmon-roe-allergic
83 patients (n=20) contained a specific IgE that showed a strong reaction to β'-c, suggesting
84 β'-c as a common major allergen in salmon roe allergy (Shimizu et al., 2009). Thus, the
85 proteolytic resistance of β'-c may contribute to its high allergenicity in the case of fish
86 roe allergy. However, little information about the structure of salmonid β'-c has been
87 published.

88 The objective of this work was to estimate the effect of proteolysis on the allergenicity
89 of chum salmon β'-c. Changes in the IgE-binding ability of β'-c during pepsin and
90 trypsin digestion were investigated by monitoring its proteolytic cleavage. β'-c that

degraded the subunit structure by carboxymethylation was also examined in order to understand the involvement of a tertiary structure in the proteolytic resistance of IgE-binding ability. Additionally, the structure of β'-c in terms of its contribution to proteolytic resistance was studied by analyzing amino acid sequences of the digestion fragments.

2. Materials and methods

2.1. Fish roe

Fresh chum salmon (*Oncorhyncus keta*) roe was purchased at a local fish market. It was washed with 0.16 M NaCl and stored at -60 °C until use.

2.2 Sera of fish-roe-allergic patients

Sera from 13 patients diagnosed with salmon roe allergy were selected for this study (age range, 5 months-12 years). Sera from two nonallergic individuals (age, 31 and 50 years) were also used as the control. **Table 1** contains the clinical information of the patients. Each serum was subjected to capsulated hydrophilic carrier polymer-radio allergosorbent test (CAP-RAST) (Sampson & Ho, 1997) to determine the total IgE and

specific IgE levels for chum salmon roe allergy. In the diagnostic system (ImmunoCAP, Phadia AB, Uppsala, Sweden), the whole extract of chum salmon roe was used as a solid-phase antigen. CAP-RAST score was determined from 0 (negative) to 6 (strong positive), according to the level of the specific IgE concentration. All patients' sera were evaluated as "positive". The patients' sera were frozen at < -60 °C for 2-12 months and were then thawed and mixed with the same volume of Dulbecco's phosphate buffered saline (pH 7.5; PBS) containing 0.2% NaN_3. They were stored at 4 °C until use.

2.3. IgG anibody against β'-c

The purified chum salmon β'-c was emulsified with Freund's incomplete adjuvant (Pierce, Rockford, IL). The emulsions were injected into rabbits (New Zealand White, male, 3 months old) once a week for 4 weeks. One week after the fourth injection, rabbit blood was gathered and centrifuged at 3000 g for 15 min to collect the supernatant. Forty per cent of saturated ammonium sulphate at the final concentration was added to the supernatant, and the mixture was centrifuged at 30,000 g for 30 min. The supernatant was dialysed against PBS and diluted with the same volume of PBS containing 0.2% NaN_3. The antibody against β'-c (anti-β') thus obtained was stored at 5 °C until use. The animal experiment was performed according to the Guidelines Concerning Animal Experiments

at Hokkaido University.

2.4. Preparation of yolk protein

Yolk protein extract (YPE) and β'-c were prepared from chum salmon roe by the method of Hiramatsu and Hara (1996), with a slight modification. Briefly, the roe was homogenised in 2-fold weight of 0.5 M NaCl containing 20 mM Tris-HCl (pH 8.0) using a potter homogenizer. The salt-soluble extract was centrifuged at 2000 g for 15 min to remove the floating oil layer and was further centrifuged at 20,000 g for 30 min. The supernatant was dropped into a 10-fold volume of cold distilled water, and the precipitate generated in this step was collected by centrifugation at 20,000 g for 30 min and dissolved in 0.5 M NaCl (pH 8.0). The fraction used in this study was YPE. β'-c was prepared from the YPE thus obtained. Sixty-seven per cent of saturated ammonium sulphate at the final concentration was added to the YPE. After centrifugation at 15,000 g for 30 min, the precipitate was redissolved in 0.5 M NaCl (pH 8.0) and loaded onto a Sephacryl S-200HR column (ø1.6 x 60 cm, GE Healthcare, Piscataway, NJ) to purify the β'-c. The protein fractions were detected at 280 nm, and the concentration was determined by using Colour-producing Solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan). All steps were performed at temperatures < 5 °C, and the purified proteins

were frozen at < -60 °C until use.

2.5. Assay for proteolytic activity

The proteolytic activity of pepsin (from porcine mucosa, Sigma, St. Louis, MO) was analyzed by using haemoglobin as a substrate (Kageyama & Takahashi, 1980). In this work, we defined 1 unit as the ability to increase absorbance at 280 nm by 0.001 unit per minute. The proteolytic activity of trypsin (from bovine pancreas, Sigma, St. Louis, MO) was analyzed by the method of Hummel (1959). In this work, we defined 1 unit as the ability to increase absorbance at 247 nm by 0.001 unit per minute.

2.6. Protein digestion

YPE and β'-c were digested using pepsin and subsequently trypsin for 3 h each. The proteins were dissolved in 0.5 M NaCl (pH 8.0) at 3.0 mg/ml, and the pH was shifted to 2.0 by 1.2 M HCl. Pepsin was also adjusted to 100 μg/ml at pH 2.0. The proteins and the pepsin solutions were preincubated at 37 °C for 5 min using a water bath, and the protein digestion was started by adding pepsin to the β'-c solution at 39.4 units/mg of protein (enzyme:substrate = 1: 100 (w/w)). After the 3-h pepsin digestion, the digested protein solution was shifted to pH 8.0 by 1 M NaOH,

and trypsin (100 μg/ml dissolved in 0.5 M NaCl (pH 8.0)) was subsequently added to the solution at 201 units/mg of protein (enzyme:substrate = 1:100 (w/w)). After the reaction at 37 °C for 3 h, the digested solution was boiled for 15 min and added to 1 mM phenylmethylsulphonyl fluoride to terminate the digestion.

2.7. SDS-PAGE analysis

Digested proteins were mixed with an equal volume of 2% sodium dodecyl sulphate (SDS) containing 8 M urea, 2% 2-mercaptoethanol, and 20 mM Tris-HCl (pH 8.0) and then heated in boiling water for 2 min. Observation of the digestion fragments was performed by Tricine-SDS-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) (Schagger & Vonjagow, 1987), with 4, 10, and 16% acrylamide slab gels used as the stacking, spacer, and resolving gels, respectively. The protein bands were soaked in 10% acetic acid and 40% methanol solution for 30 min and then stained by Coomassie Brilliant Blue G (Sigma, St. Louis, MO). The molecular mass of each stained band was calculated from the mobility of Tricine-SDS-PAGE analysis.

2.8. Carboxymethylation of β'-c

β'-c was dissolved in 0.25 M NaCl and 50mM Tris-HCl (pH 8.0) containing 8 M urea, 5

mM ethylenediaminetetraacetic acid, and 10 mM 2-mercaptoethanol, and the solution pH was precisely adjusted to 8.0 using 1 M NaOH. After agitation for 3 h at room temperature, 0.25 M monoiodoacetic acid was gently and dropwise added to the β'-c solution, and the pH of the protein solution was kept at 8.0 by adding 1 M NaOH. When the final concentration of monoiodoacetic acid reached 10 mM, the reaction mixture was agitated for 30 min at room temperature in the dark, dialysed against 1 mM $NaHCO_3$ for 18 h, and subsequently dialysed against 0.1 mM $NaHCO_3$ for 6 h. After dialysis, the carboxymethylated β'-c thus obtained (two subunits were separated) was lyophilised, stored at < -60 °C until use, and dissolved in 0.5 M NaCl-20 mM Tris-HCl (pH 8.0) just before the experiment.

2.9. Detection of digested allergenic fragments using immunoblotting

Digested products of YPE and β'-c were subjected to Tricine-SDS-PAGE and then transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA) using a semidry blotting system (ATTO, Tokyo, Japan). The membrane was soaked in blocking buffer (3% casein dissolved in 150 mM NaCl and 20 mM Tris-HCl (pH 7.5; TBS)) at room temperature for 1 h and incubated with the patients' sera (pooled P1-P9, diluted 1:10 with the blocking buffer) or rabbit antibody against β'-c (diluted 1:20,000

199 with the blocking buffer) at 5 °C overnight. After being washed six times with TBS
200 containing 0.05% Tween 20 (TTBS), the antibodies bound to the protein or digestion
201 fragments were reacted with the peroxidase-conjugated rabbit anti-human IgE antibody
202 (Biosource, Camarillo, CA) or peroxidase-conjugated goat anti-rabbit IgG antibody
203 (BioRad, Hercules, CA) at 37 °C for 3 h. After three washings with TTBS and TBS had
204 been completed, the specific reaction with the antigen and antibodies was detected with
205 Immobilon (Millipore, Billerica, MA) for patients' sera or with the ECL photosystem (GE
206 Healthcare, Piscataway, NJ) for rabbit antibody as a detection reagent.
207
208 *2.10. Evaluation of IgE-binding ability using competitive ELISA*
209 The IgE-binding ability of digested products of β'-c was investigated using competitive
210 enzyme-linked immunosorbent assay (ELISA) by employing pooled sera of patients
211 (P10-P13) and pooled sera of nonallergic individuals (C1 and C2). A 96-well ELISA plate
212 (IWAKI, Tokyo, Japan) was coated with 2.5 μg/ml β'-c (100 μl/well) that had been
213 dissolved in PBS and then incubated overnight at 5 °C. After being washed
214 with PBS containing 0.05% Tween 20 (TPBS), the residual blocking sites in
215 each well were coated with a blocking buffer (1% casein in PBS) at 37 °C for 1.5
216 h. Simultaneously, 125 μl of sera of patients or nonallergic individuals

217 (diluted 1:50-100 times with the blocking buffer) were mixed with an equal
218 volume of β'-c or its digested products as an inhibitor (0.002-200 μg/ml diluted
219 with the blocking buffer). After incubation at 37 °C for 2 h, 70 μl of each solution
220 were placed into the β'-c coated ELISA plate and incubated again at 37 °C for
221 2 h. After the plate was washed with TPBS, 100 μl/well of peroxidase-
222 conjugated rabbit anti-human IgE antibody (Biosource, Camarillo, CA) diluted with
223 the blocking buffer (1:2000) were added to each well and incubated at 37 °C for 1.5 h.
224 The enzyme-substrate reaction was performed using BM Blue POD Substrate (Roche
225 Diagnostics, Indianapolis, IN) at 25 °C for 20 min. The reaction was terminated by
226 adding 100 μl/well of 4 N sulphuric acid. The detection of the enzyme reaction
227 was carried out by measuring the absorbance at 450 nm using a microplate reader
228 (MTP-300, Corona Electric, Ibaraki, Japan). The loss of the specific IgE-binding ability
229 of the patients' sera resulting from the treatment with the inhibitors was represented by
230 calculating the inhibition rate using the following formula: inhibition rate (%) = ((X-Y)
231 /(X-Z)) ×100, where X is the absorbance of each patient's serum without the inhibitors
232 and Y and Z, respectively, are the absorbance of the patients' sera and that of nonallergic
233 individuals' sera treated with various concentrations of inhibitors.
234 In this study, the inhibitory concentration obtaining a 50% inhibition rate (defined as

235 IC_{50}) was calculated from the inhibition curve of the competitive ELISA as an indicator
236 of the allergenicity of digested β'-cs.
237
238 *2.11. Identification of digestion fragments of β'-c*
239 The portions of intact β'-c corresponding to the digestion fragments were identified by
240 comparing their N-terminal first to fifth amino acids. The IgE-binding fragments of the
241 β'-cs generated by the pepsin and trypsin digestion were subjected to *N*-terminal amino
242 acid sequence analysis, and the *N*-terminal 5-amino acid sequences were attributed to the
243 amino acid sequence of chum salmon β'-c. The amino acid sequence of the intact β'-c
244 was deduced from the c-DNA sequence (DNA Data Bank of Japan, Accession number:
245 AB560769). The protein-blotting membranes were stained with 0.1% Coomassie Brilliant
246 Blue R in 30% methanol and 7.5% acetic acid, and the stained protein bands coinciding
247 with the IgE-binding fragments in immunoblotting were subjected to the automatic
248 Edman sequence analyzer (Procise 492, Perkin-Elmer, Waltham, MA). The validity of the
249 N-terminus of the digestion fragments in relation to the cleavage site specificity of pepsin
250 and trypsin was confirmed by PeptideCutter (Gasteiger et al., 2005), presented by the
251 Swiss Institute of Bioinformatics (http://www.expasy.org/tools/peptidecutter/). The
252 digestion fragment of β'-c was estimated by the information of *N*-terminal amino acid

sequence analysis, mobility of SDS-PAGE, and substrate specificity of the proteases.

2.12. Statistical Analysis

The results of each measurement in Fig. 5 were the average of three determinations, and error bars corresponded to the standard deviations. Statistical differences were tested using Dunn's procedure as a multiple comparison procedure (Bonferroni/ Dunn method) at the 1 or 5% significance level with the Statcel software ver. 1.0 (OMS-Publishing, Saitama, Japan).

3. RESULTS

3.1. Pepsin-trypsin digestion of YPE

Figure 1A shows the enzymatic degradation of YPE by continuous pepsin-trypsin digestion, monitored by Tricine-SDS-PAGE. The intact YPE consisted of four major components (6, 8, 16.8, and 19 kDa; indicated by allows) and high-molecular components stacked onto the stacking gel. These components were fragmented with the progress of peptic degradation, and the components with a molecular mass of >16 kDa disappeared after 3 h of digestion. The digestion fragments were further degraded by the subsequent trypsin digestion, but several components with 4-16 kDa remained even after

271 3 h of digestion.

272 The β’-c contained in YPE was subjected to continuous pepsin-trypsin digestion for 3 h,
273 and the proteolytic degradation was monitored using immunoblotting. As shown in Fig.
274 1B, β’-c in the intact YPE was detected as a 17 kDa single band and disappeared after
275 pepsin-trypsin digestion, whereas its immunoblotting signal by anti-β’ clearly remained.
276 That is, the digestion fragments of β’-c (14-16 kDa) were observed after the enzymatic
277 degradation. These results suggest that β’-c contains a large portion having proteolytic
278 resistance, unlike other yolk proteins in YPE.

279

280 *3.2. Digestion of β’-c and IgE-binding ability of its digestion fragments*

281 Intact and carboxymethylated β’-cs were subjected to pepsin-trypsin digestion, and their
282 digested products were detected by Tricine-SDS-PAGE. As shown in Fig. 2, the intact β’-
283 c was degraded to eight major fragments by pepsin digestion for 3 h, and marked
284 proteolysis was followed by trypsin digestion. Finally, three major components (5.4, 10.6,
285 and 14.6 kDa; arrows in the figure) and a small amount of β’-c (arrow in the figure)
286 remained after 3 h trypsin digestion. On the other hand, the digested pattern of
287 carboxymethylated β’-c was different from that of intact β’-c; that is, the 17 kDa-
288 component disappeared, and five major fragments (5.0 -11.3 kDa; arrows in the figure)

289 were observed after 3 h of trypsin digestion.

290 The digested β'-cs obtained by pepsin-trypsin digestion for 3 h each were subjected to
291 SDS-PAGE and subsequently reacted with the serum of a salmon-roe-allergic patient
292 (Pooled P1-P9) in immunoblotting to clarify the fragments with IgE- binding ability in
293 digested β'-c and to estimate their approximate molecular weight. The amounts of
294 applied proteins used in the digested-sample-experiments were larger than that used in
295 the intact β'-c (See Figure 3). A large number of digestion fragments were developed in 3
296 h by pepsin digestion and subsequent 3 h trypsin digestion, and the digestion fragments
297 with the molecular mass of < 10k Da had no IgE-binding activity in all digested samples,
298 whereas some digestion fragments with a molecular mass of >10 kDa reacted markedly
299 with IgE in the patient serum. In the intact β'-c (the upper photos in Fig. 3), three IgE-
300 binding fragments were detected after 3 h of pepsin digestion (F-*a, -b, and -c*: 16.9, 15.9,
301 and 12.1 kDa, respectively), and the subsequent 3 h of trypsin digestion (F-*d, -e, and -f*:
302 16.9, 14.9, and 11.7 kDa, respectively). These results indicate that β'-c can retain its IgE-
303 binding ability upon proteolysis by high concentrations of pepsin and trypsin.
304 Three IgE-binding fragments were also observed in the pepsin digestion of
305 carboxymethylated β'-c (lower photos in Fig. 3. F-*g, -h,* and -*i*: 16.9, 14.9, and 12.1 kDa,
306 respectively), whereas the largest fragment (F-*g*) disappeared and two IgE-reactive

fragments (F-*j* and -*k*: 11.3 and 10.0 kDa, respectively) were developed after subsequent trypsin digestion. The result of carboxymethylated β'-c suggests that the tertiary structure participates in the acquisition of tryptic resistance.

3.3 Identification of digestion fragments of β'-c

The eleven kinds of IgE-binding fragments generated from the intact and carboxymethylated β'-cs (Fig. 3) were subjected to amino acid sequence analysis, and the *N*-terminal 5-amino acid sequences were attributed to the amino acid sequence of intact β'-c. Figure 4 shows the positional relation among the IgE-binding fragments in β'-c. The *N*-terminal 5-amino acid sequences of all the digestion fragments were found in the sequence between the *N*-terminal first and 36th amino acid residues. The *N*-terminus of β'-c lacked all digestion fragments except F-*a*, -*d*, and -*g*. The *N*-termini of the peptic digestion fragments (F-*b*, -*c*, -*h*, and -*i*) and the tryptic digestion fragments (F-*d*, -*f*, -*j*, and -*k*) were matched to the cleavage-specific site of pepsin, and only the *N*-terminus of F-*e* was matched to the cleavage-specific site of trypsin. As shown in Figs. 3 and 4, all of the digestion fragments overlapped with F-*k* (10.0 kDa). The portion corresponding to F-k in β'-c was estimated to be the sequence from 31-Y to 119-Q.

325 *3.4. Quantitative evaluation of change in IgE-binding activity of β'-c*

326 The effect of enzymatic digestion on the allergenicity of β'-c was evaluated by
327 competitive ELISA using the intact β'-c and the digested β'-cs as inhibitor antigens. As
328 shown in Fig. 5A, the reaction between the specific IgE and the solid-phase β'-c on the
329 ELISA plate was effectively suppressed with an increase in the concentration of the intact
330 β'-c added to the patient's serum (P10). The inhibition rate was 70% at 0.1 μg/ml and
331 reached 100% at 100 μg/ml. The inhibition rate was significantly lower in the
332 pepsin- and the pepsin-trypsin-digested β'-cs than in the intact β'-c at the inhibitor
333 concentration of >0.1 μg/ml (p< 0.01). The IC_{50} of the intact β'-c was 0.07 μg/ml and
334 increased to 3.86 and 5.21 μg/ml after 3 h pepsin digestion and pepsin-trypsin
335 digestion, respectively. That is 55- and 74-fold increase in the IC_{50} change upon the
336 respective enzymatic digestions. However, the inhibitory effect of both digested β'-cs
337 was diminished with increasing concentration. The inhibition rates finally reached 100%
338 at 100 μg/ml. A similar result by competitive ELISA was also found with the sera of
339 other patients (P11-P13; data not shown). These results imply that major IgE-binding
340 sites (epitopes) remained through pepsin-trypsin digestion.

341 As shown in Fig. 5B, the inhibitory effect of carboxymethylated β'-c was significantly
342 lower than that of intact β'-c at the inhibitor concentration of > 0.1 μg/ml ($p < 0.01$).

343 This result suggests that the disruption of the subunit structure by
344 carboxymethylation led to the loss of the IgE-binding ability of β'-c. Furthermore, the
345 IgE-binding ability of carboxymethylated β'-c was diminished with the progress of the
346 pepsin-trypsin digestion ($p < 0.05$ at an inhibitor concentration of 0.01 μg/ml
347 and $p < 0.01$ at > 0.1 μg/ml). However, it is apparent that IgE-binding ability
348 remained high in the digested carboxymethylated β'-c, as it did in intact β'-c.
349 Therefore, the results shown in Fig. 5 indicate that the proteolytic stability of the IgE-
350 binding ability stems mainly from the polypeptide structure of β'-c.
351
352 **4. Discussion**
353 In this work, the IgE-binding ability of digested β'-c was investigated in relation to
354 proteolytic characteristics. The results show that β'-c has the same digestion tolerance to
355 pepsin-trypsin digestion as these allergens have. β'-c in YPE was digested in a manner
356 similar to the digestion of other yolk proteins such as lipovitellin by pepsin and trypsin
357 (Fig. 1), but the digestion fragments with molecular masses of >10 kDa remained through
358 the digestion process and had IgE-binding ability, as shown in Fig. 1B. Such a digestion
359 behaviour is an intrinsic characteristic of β'-c because the residual fragments with IgE-
360 binding ability were developed from purified intact β'-c (Fig. 3). Additionally, the

361 stability of the IgE-binding ability against pepsin-trypsin digestion was found in
362 carboxymethylated β'-c that had lost its subunit structure. These results indicate that β'-c
363 contains peptide regions that are stable to gastrointestinal digestion and that contain the
364 IgE-binding site.
365 The detection of the *N*-thermal amino acid sequence of the digestion fragments in this
366 work contributes to an understanding of the digestion behaviour of β'-c. Since there was
367 a difference in the digested peptide pattern between intact and carboxymethylated β'-cs
368 (Fig. 2), 11 kinds of digestion fragments having IgE-binding ability (F-*a* to F-*k*) were
369 obtained. As described in Fig. 3, the *N*-termini of F-*a* and F-*d* coincided with that of
370 intact β'-c, and a slight decline in their mobility during pepsin-trypsin digestion occurred
371 in Tricine-SDS-PAGE. These results suggest that a small amount of β'-c that has partially
372 digested its C-terminus site remains after pepsin-trypsin digestion *in vivo*. On the other
373 hand, a large fragment corresponding to F-*d* was lost by trypsin digestion in
374 carboxymethylated β'-c, suggesting that the subunit structure is partially involved in the
375 proteolytic resistance of β'-c.
376 The identification of the region of each fragment in the amino acid sequence of intact
377 β'-c was useful to estimate the portion involved in proteolytic resistance. In a series of
378 IgE-binding fragments, the mobility of the peptic digestion fragments in Trisine-SDS-

PAGE was slightly diminished by continuous trypsin digestion. Chum salmon consists of two subunits (16 and 18 kDa), and the 16 kDa component seems to be a subfragment of the 18 kDa component lacking a C-terminal region because the *N*-terminal 20 sequences coincided (Shimizu et al., 2009). Therefore, relationships between peptic digestion fragments and the subsequent tryptic digestion fragments can be determined by comparing their N-terminal amino acid sequences. The relationships among digestion fragments were as follows: F-*f* (11.7 kDa in Tricine-SDS-PAGE) was derived from F-*c* (12.1 kDa), and F-*j* (11.3 kDa) and F-*k* (10.0 kDa) were derived from F-*i* (12.1 kDa). Additionally, the tryptic digestion fragment, F-*e* (14.9 kDa), was derived from F-*a* (16.9 kDa) or F-*b* (15.9 kDa). The mobility of the digestion fragments was slightly diminished by subsequent trypsin digestion, whereas the loss of molecular mass between F-*a* or F-*b* and F-*e* estimated from the mobility change was apparently larger than that of the lacking *N*-termini (EVNAVKCSMVGDTLTTFNNR or TTFFNNR). Therefore, it is apparent that the C-terminus sides of the peptic digestion fragments were also cleaved by the subsequent trypsin digestion. In Fig. 3, the IgE-binding intensity of each β'-c fragments seemed to be different and the higher molecular weight bands showed a strong intensity. This is probably caused by the loss of IgE-binding epitope that located in the N- and C-terminus regions with the digestion process.

F-*k* was the smallest digestion fragment having IgE-binding ability, and the portion that matched F-*k* in β'-c could be estimated as the sequence of 31-Y to 119-Q. Since β'-c consists of two subunits with the same amino acid sequence, the range of F-*k* holds around 60% of β'-c. There are many cleavage sites of pepsin or trypsin in the amino acid sequence of F-*k.* For example, one arginine and nine lysine residues exist as trypsin cleavage sites, and 16 cleavage sites by pepsin were also predicted by PeptideCutter analysis. Therefore, it is apparent that the existence of F-*k* gives high proteolytic resistance to β'-c, and proteolytic resistance of allergenicity of β'-c is probably due to IgE-binding epitopes located in the portion of F-k. Clarifying the peptide structure of F-*k* and estimating degree of IgE-binding ability of each peptides would contribute to an understanding of the mechanism by which β'-c has high proteolytic resistance as a food allergen.

A quantitative evaluation using competitive ELISA showed the effect of proteolytic digestion on the IgE-binding ability of β'-c (Fig. 5), and it was apparent that pepsin digestion decreased the IgE-binding ability of the digested β '-c. On the other hand, the subsequent trypsin digestion showed a weaker effect on the IgE-biding ability although the digestion of β'-c was further progressed as shown in Figure 2. The results in Figs. 2, 3, and 5 indicate that trypsin digestion has no effect on the structure of sequential epitopes

415 remaining in β'-c fragments after pepsin digestion. Therefore, stomach digestion seems to
416 play an important role in the loss of allergenicity of β'-c in human gastrointestinal tract.
417 However, as described above, allergenicity could remain in part through stomach
418 digestion and reach the small intestine.
419 Although most food allergens are stable to proteolysis, some reports have shown that
420 proteolysis in food processing could reduce food allergy risks. Fermentation by microbial
421 proteolytic enzymes degradated some allergens: IgE-binding abilities were reduced in
422 soybean allergen in soy sauce (Song, Frias, Martinez-Villaluenga, Vidal-Valdeverde, & de
423 Mejia, 2008), shrimp tropomyosin in kimchi (Park et al., 2007), and β-lactoglobulin in
424 yogurt (Ehn, Ekstrand, Bengtsson, & Ahlstedt, 2004). Figure 5A also shows the loss of
425 IgE-binding ability in β'-c, and a 74-fold increase in IC_{50} was observed after pepsin-
426 trypsin digestion. However, compared with those of other allergenic proteins, the degree
427 of the increase in IC_{50} was slightly changed; for example, the IC_{50} values of Cor a 1
428 (hazelnut) (Vieths, Reindl, Muller, Hoffmann, & Haustein, 1999) and parvalbumin
429 (codfish) (Untersmayr et al., 2005) increased 1000-10,000 times with pepsin or trypsin
430 digestion. Therefore, enzymatic degradation seems to have little effect on the direction of
431 the allergenic risk of β'-c.
432 The reduction of IgE-binding ability with the progress of tertiary structural change has

433 been reported in peanut allergen (Ara h 2), a proteolytically stable allergen. The IgE-
434 binding ability of β'-c was also reduced by carboxymethylation, as shown in Fig. 5B,
435 suggesting that a part of the specific IgE in a patient's serum recognises the structure of
436 β'-c. Therefore, it is apparent that the reduction of IgE-binding ability in intact β'-c was
437 caused by not only the degradation of peptide bonds but also the loss of the intrinsic
438 structure of β'-c. However, the IgE-binding ability was highly retained after pepsin-
439 trypsin digestion, and the digestion fragment with proteolytic resistance (F-*k*) remained in
440 carboxymethylated β'-c after pepsin-trypsin digestion. The conformational change in β'-c
441 could have little effect on its proteolytic resistance.
442 In conclusion, β'-c had a wide peptide portion with proteolytic resistance (31-Y to 119-
443 Q), and it conferred stable IgE-binding ability against pepsin-trypsin digestion. The
444 results of this work suggest that β'-c can reach the small intestine in the form of high-
445 molecular-weight components with high allergenicity. An animal study with the
446 proteolytic fragments or skin prick testing under the supervision of medical professions
447 will further enhance this work.
448
449
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Figure and Table captions

Table 1. List of allergic patients hypersensitive to chum salmon roe.

Fig. 1. Pepsin-trypsin digestion of β'-c in YPE. YPE was digested at 37 °C with pepsin at pH 2.0 for 3 h and subsequently with trypsin at pH 8.0 (A), and the fragmentation of β'-c in YPE during the proteolysis was detected by immunoblotting using anti-β (B). P: protein staining. IB: immunoblotting.

Fig. 2. Fragmentation of chum salmon β'-c and its carboxymethylated product during enzymatic digestion. Intact and carboxymethylated β'-cs (digestion time: 0 h) were subjected to pepsin-trypsin digestion, as noted in Fig. 1.

Fig. 3. IgE-binding ability of digestion fragments from chum salmon β'-c. Intact and carboxymethylated β'-cs were subjected to pepsin-trypsin digestion (each for 3 h), as noted in Fig. 1. P: protein staining of the digestion fragments in Tricine-SDS-PAGE. IB: Immunoblotting using the serum of a salmon-roe-allergic patient (P-9 listed in Table 1).

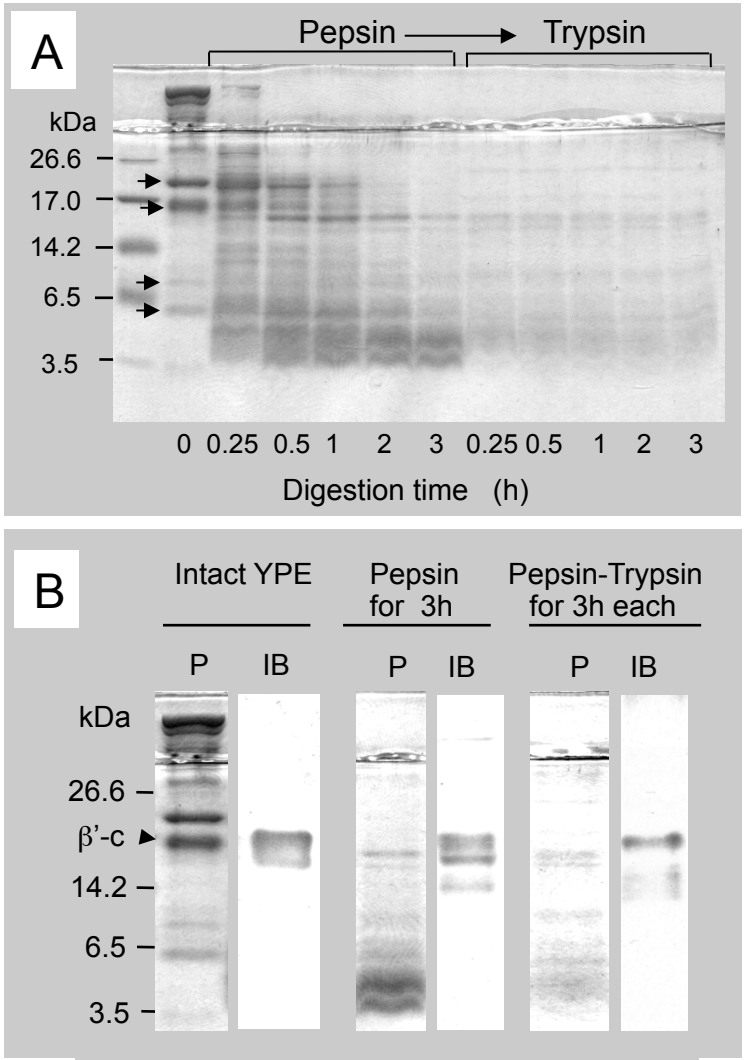
Fig. 4. Positional relation among digestion fragments by pepsin and trypsin in the amino acid sequence in intact β'-c. N-terminal 5-amino acid sequences of the digestion fragments shown in Fig. 3 (*a*-*k*) were attached to the sequence of intact β'-c.

Fig. 5. Effect of enzymatic digestion on the IgE-binding ability of chum salmon β'-c. Patient's serum (P10) was mixed with intact β'-c, carboxymethylated β'-c, or the digestion fragments, and the protein-serum mixtures were reacted with intact β'-c in competitive ELISA. Inhibitors used in this experiment were as follows: (A) pepsin-digested (open triangle) and pepsin-trypsin-digested (open square) β'-cs. (B) carboxymethylated β'-c (closed circle), its digested samples by pepsin (closed triangle), and the pepsin-trypsin (closed square). Intact β'-c (open circle) was also examined as a control.

Table 1 List of allergic patients hypersensitive to chumsalmon roe

Serum	Sex	Age (year)	CAP-RAST		Hypersensitivity reaction
			Total IgE (IU/mL)	Specific IgE (UA/mL, class)	
P1	F	2	349	86.8 (5)	AD
P2	M	11 months	538	68.1 (5)	AD
P3	M	1	524	90.0 (5)	AD
P4	F	5 months	24	5.1 (3)	AD,Ur
P5	M	2	9120	42.0 (4)	AD
P6	F	1	668	76.6 (5)	AD
P7	M	2	2398	66.9 (5)	AD,Ur
P8	F	11	3452	>100 (6)	AD
P9	M	5	5210	29.8 (4)	AD、BA
P10	M	2	2398	66.9 (5)	AD、Ur
P11	M	3	756	>100 (6)	AD、BA
P12	M	6	2936	>100 (6)	Ur
P13	M	3	402	63.3 (5)	AD
C1	M	31	-	-	-
C2	M	50	-	-	-

AD, atopic dermatitis; BA, bronchial asthma; Ur, urticaria; -, no data.



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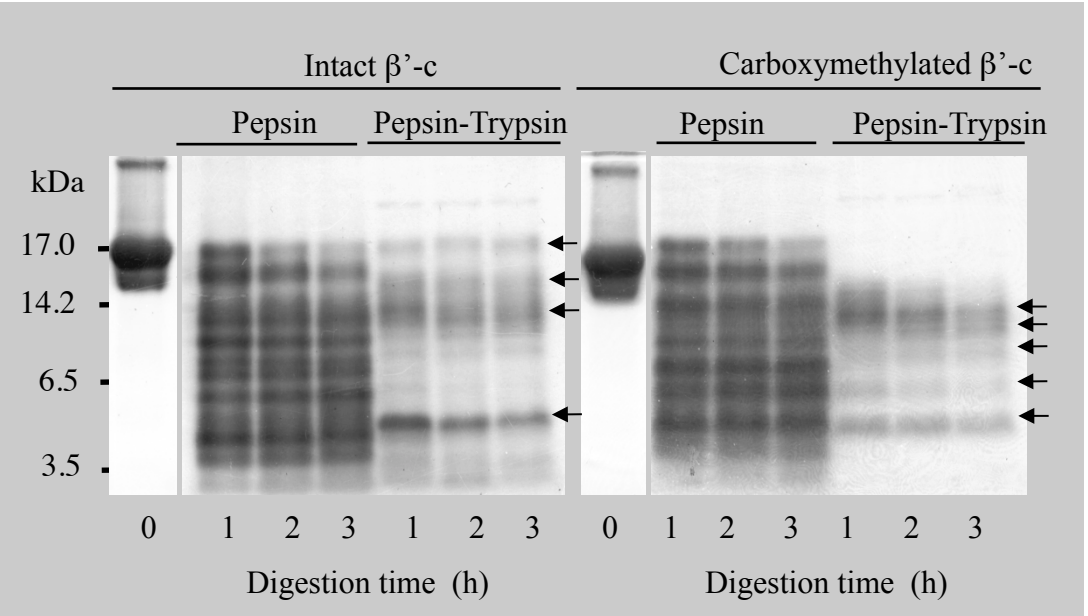

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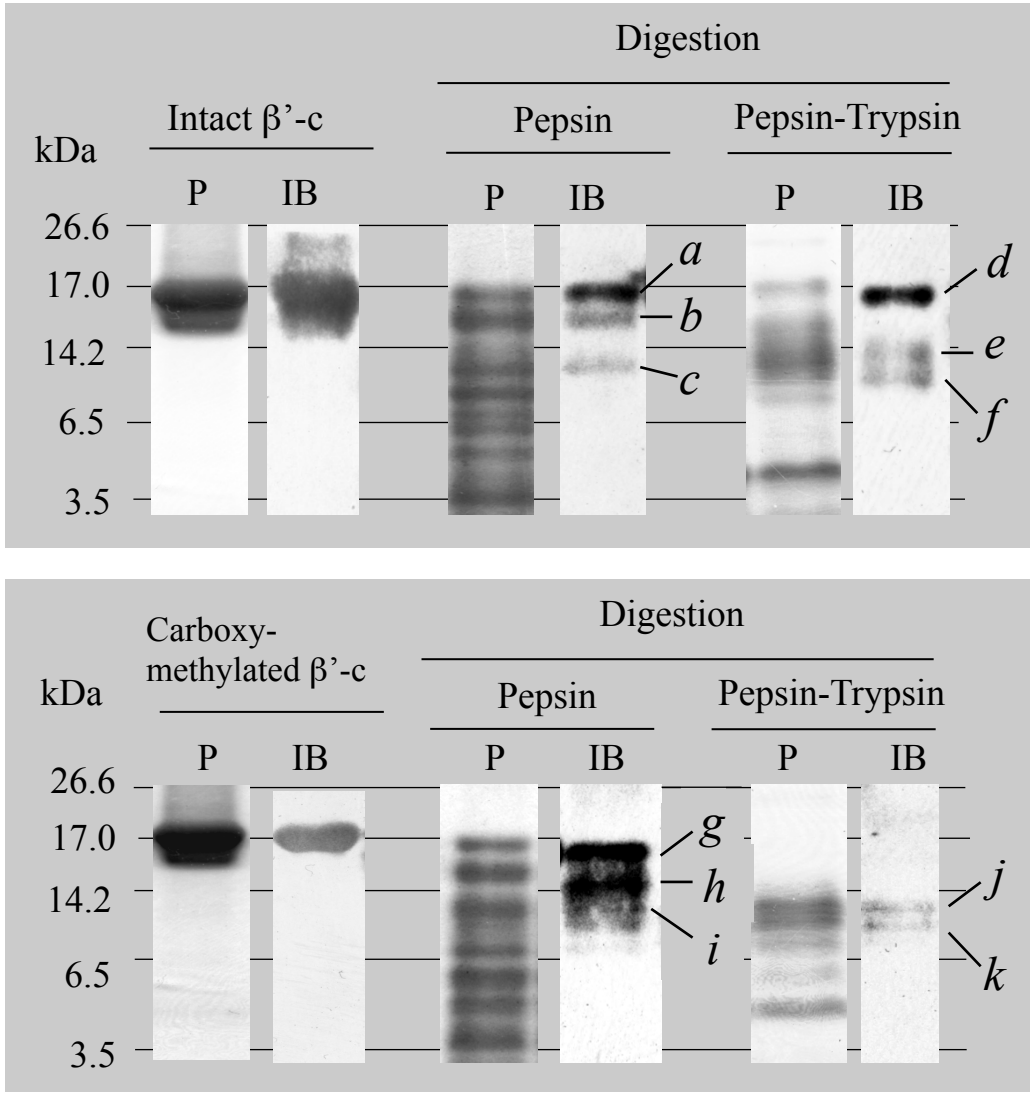

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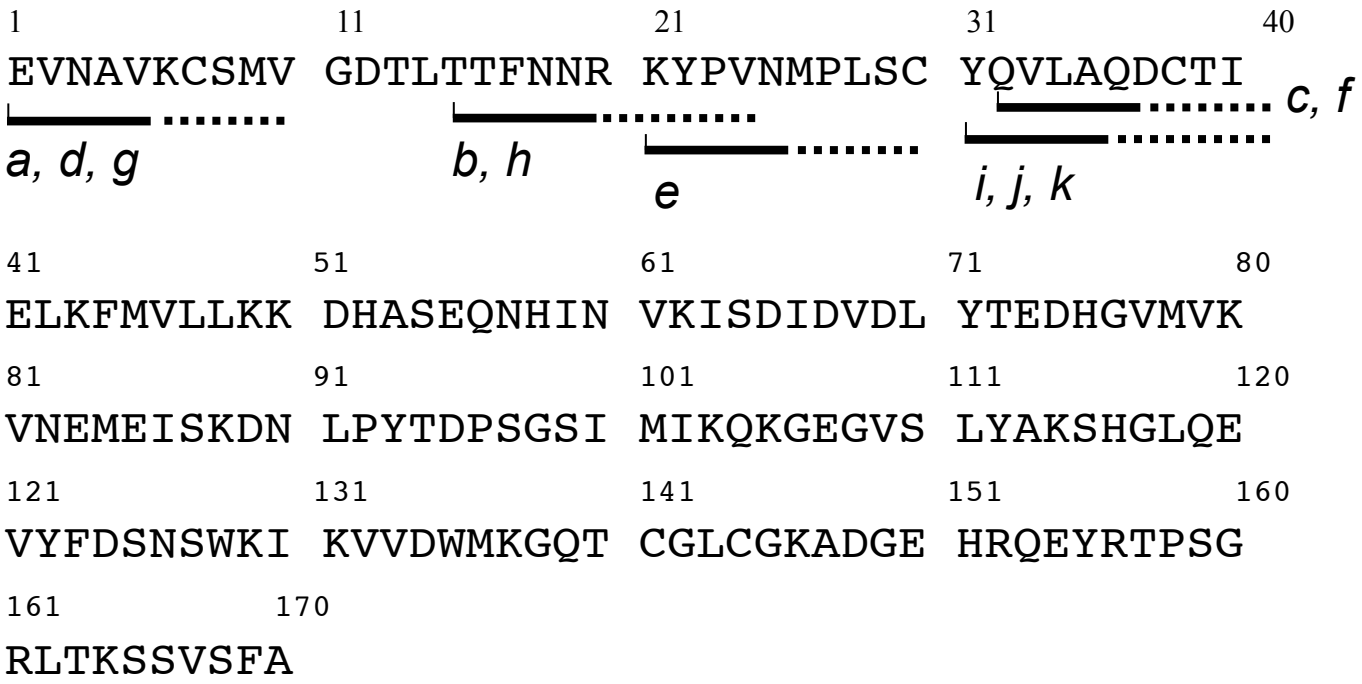

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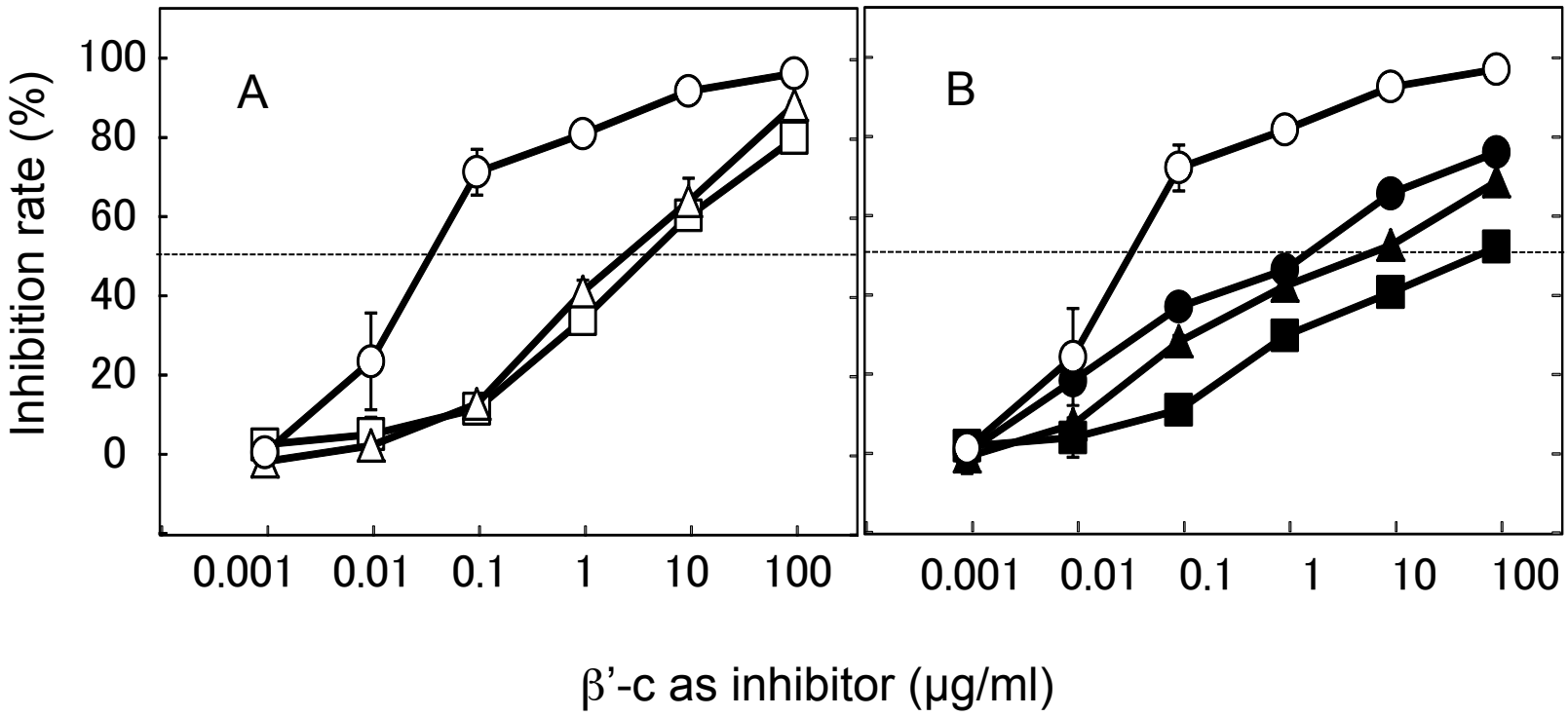

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