1 TITLE

2 Development of an enzyme-linked immunosorbent assay system for detecting

3 β' -component (Onk k 5), a major IgE-binding protein in salmon roe

4

5 RUNNING TITLE

6 Development of ELISA for detecting salmon roe yolk protein

7

8 Authors

9 Yutaka Shimizu^a, Hiroshi Oda^b, Kohsuke Seiki^b, Hiroki Saeki^{a*}

10

11 Affiliations

12 ^a Faculty of Fisheries Sciences, Hokkaido University, Minato 3, Hakodate, Hokkaido

13 041-8611, Japan

14 ^b Central Research Institute, Maruha Nichiro Corporation, Wadai 16, Tsukuba, Ibaraki

15 300-4295, Japan

16 * Corresponding Author. Tel. and fax. : +81-138-40-5516.

17 E-mail address: saeki@fish.hokudai.ac.jp (Hiroki Saeki).

18 **Abstract**

19 A novel enzyme-linked immunosorbent assay (ELISA) system has been established for
20 selective detection of chum salmon (*Oncorhynchus keta*) yolk protein (SYP). Rabbit and
21 rat polyclonal Immunoglobulin G antibodies to β' -component (the major allergic protein
22 in fish roe; anti- β) were applied for designing the ELISA system. The sandwich ELISA
23 using rabbit anti- β for the capture antibody and horseradish peroxidase-labelled F(ab')₂
24 fragment of rat anti- β for the detection antibody obtained high sensitivity and narrow
25 specificity for SYP. Protein extraction using sodium dodecylsulfate and
26 2-mercaptoethanol ensured strict specificity of the ELISA, and components of three
27 popular processed foods had no effect on the ELISA response. The limits of
28 determination and quantification of SYP were estimated to be 0.78 $\mu\text{g/g}$ and 2.60 $\mu\text{g/g}$ of
29 food sample, respectively. In conclusion, the developed ELISA system has a probability
30 to be applied for the detection of contaminated chum salmon roe in processed food.

31

32 **Keywords**

33 Salmon roe; β' -component; food allergy; enzyme immunoassay; yolk protein; ELISA.

34 **1. Introduction**

The increase in the number of food allergy cases is a serious medical and social issue in developed countries (Urisu, Ebisawa, Mukoyama, Morikawa, & Kondo, 2011). Food allergen labelling regulations are enforced in many countries and regions as a countermeasure for this problem (Gendel, 2012). The first step in the treatment of patients with food allergy is avoidance of allergenic foods, which is important to prevent exacerbation of food hypersensitivities and to ensure daily food safety. Since food-derived hypersensitivity is triggered by small amounts of food allergens (Morisset et al., 2003; Crevel et al., 2008; Blom et al., 2013), establishment of procedures for detecting trace amounts of allergenic proteins in processed foods is important to ensure the health of allergic patients and maintain the reliability of food labelling systems. Highly sensitive procedures for detecting trace amounts of allergenic materials have been established using enzyme-linked immunosorbent assay (ELISA) and/or polymerase chain reaction (Akiyama, Imai, & Ebisawa, 2011), which detect specific proteins or genes originating from allergenic materials such as egg, milk, wheat, buckwheat, peanuts, crustacean, kiwifruit, soybean, and gelatins. At present, food allergen detection kits are available worldwide to detect various major food allergens, however there are still allergenic foods for which detection methods are not yet available.

52 In Japan, the major allergenic foods reported with high-frequency are egg, milk, and
53 wheat, which is similar to those reported in other countries. Seafood is also recognized as
54 one of major allergenic foods next to three the major allergens (Ebisawa, Nishima,
55 Ohnishi, & Kondo, 2013). Since seafood provides approximately 40% of animal protein
56 intake in Japan (Ministry of Health, Labour and Welfare, 2014) and children tend to
57 suffer from food allergy (Urisu, Ebisawa, Mukoyama, Morikawa, & Kondo, 2011), the
58 morbidity of seafood-allergic patients has reached 10% among elementary school
59 children and their families (Kanagawa Prefectural Institutes of Public Health, 2006).
60 Hypersensitivities to various kinds of fish, shrimp, crab, and processed seafood have been
61 reported in Japan, where more than 500 kinds of marine bio-resources are consumed.
62 Japan's food allergen labelling system has designated seven
63 kinds of seafood as allergenic foods: shrimp, crab, abalone, squid, salmon, mackerel, and
64 salmon roe (Akiyama et al., 2011), listed according to the frequency of adverse food
65 reactions in allergic patients (Ministry of Health, Labour and Welfare, 2008). Fish,
66 shellfish/mollusk, and crustaceans have been designated as allergenic foods worldwide,
67 but fish roe (salmon roe) has also been specified in Japan's food allergen labelling system
68 as one of the foods that should be labelled for potential allergic reactions, a decision that

69 reflects frequent intake of fish roe in Japan. Salted fish roes, such as those of salmon,
70 walleye pollock, and herring, are commonly consumed, and hypersensitivity to various
71 kinds of fish roes has been reported, particularly among children (Kanagawa Prefectural
72 Institutes of Public Health, 2006; Akiyama et al., 2011). In addition, several kinds of fish
73 roes, including sturgeon, paddlefish, salmon, cod, lumpfish, capelin and herring, are
74 becoming popular worldwide (Bledsoe, Bledsoe, & Rasco, 2003) and this trend suggests
75 a potential increase in fish roe allergies on a global level.

76 Teleost fish roe contains three major yolk proteins: lipovitellin, phosvitin, and
77 β' -component. Vitellogenin, a precursor of these yolk proteins, is synthesized in fish liver
78 and transported in the bloodstream to accumulate in oocytes. Vitellogenin is fragmented
79 during the process of oocyte growth, and β' -component is the structural equivalent of
80 vitellogenin heavy chains (Hiramatsu, Cheek, Sullivan, Matsubara, & Hara, 2005).

81 Shimizu et al. (Shimizu et al., 2009) reported that β' -component was the major allergenic
82 protein of chum salmon (*Oncorhynchus keta*) roe, as specific Immunoglobulin E (IgE)
83 reactions to β' -component were observed in the sera of almost all patients with salmon
84 roe allergy. β' -component has been registered as a new allergen named 'Onc k 5' in the
85 official allergen list of the World Health Organization and International Union of

Immunological Societies Allergen Nomenclature Subcommittee in 2012 (<http://www.allergen.org/>). β' -component is a homodimer protein composed of two isoform subunits with similar allergenic identity (Shimizu et al., 2014), and the IgE-binding sites (IgE epitopes) in β' -component showed strong proteolytic resistance (Fujita et al., 2012), which may account for its strong allergenicity as a food allergen. IgE-cross reactivity among fish roes has been reported in case studies (Kondo et al., 2005), and IgE-cross reactivity of β' -component has been confirmed in fish roes from chum salmon, walleye pollack (*Theragra chalcogramma*), Atka mackerel (*Pleurogrammus azonus*), dusky sole (*Pleuronectes mochigarei*), slime flounder (*Microstomus achne*), shishamo smelt (*Spirinchus lanceolatus*) and Atlantic capelin (*Mallotus villosus*) (Shimizu & Saeki, 2010). This information suggests that β' -component is an appropriate target protein for detecting fish roe as an allergenic ingredient in processed food, and overcoming cross-reactivity caused by the structural similarity among fish β' -component is key to developing highly selective methods for detecting fish roe contamination in processed food.

At present, although IgE-cross reactivity has been established among fish roe, only salmon roe is listed as a potential allergenic food in the Japanese food allergen regulation

system, as noted above. Salmon roe is easily distinguishable by sight, but contamination with the yolk protein transferred from the roe raises a risk in processed foods containing salmon. Additionally, considering that precursor of yolk proteins, which reacted with salmon-roe-allergic patients' sera because β' -component is a part of vitellogenin heavy chain (Hiramatsu, Cheek, Sullivan, Matsubara, & Hara, 2005), reach the fish roe through the blood stream as described above, the establishment of the detection methods to evaluate contamination of yolk protein and its precursors in salmon meat is important for risk assessment of processed seafood.

The objective of this study is to develop a novel sandwich enzyme-linked immunosorbent assay (ELISA) system with a high sensitivity and strict species-specificity to salmon yolk protein, that is the key technology for determining trace amounts of chum salmon yolk protein in processed food. The developed ELISA system was established using rabbit and rat polyclonal immunoglobulin (IgG) antibodies raised against chum salmon β' -component. Additionally, the refinement of specificity to chum salmon roe using protein extraction in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (ME) is also discussed in this study.

120 **2. Materials and Methods**

121 **2.1. Fish roe**

122 Six kinds of fish roe, chum salmon, walleye pollock, Pacific herring (*Clupea pallasii*),
123 dusky sole, slime flounder and capelin, were collected from matured fresh fish, washed
124 with cold 0.16 M NaCl, and frozen at -60 °C until use. Each matured fresh fish was
125 purchased from the wholesale fish market at Hakodate city in Hokkaido, Japan.

126

127 **2.2. Experimental animals**

128 Three-month-old male New Zealand White rabbits and 13-week-old male Wistar rats
129 were purchased from Charles River Japan (Yokohama, Japan) and Japan SLC, Inc.,
130 (Shizuoka, Japan), respectively.

131

132 **2.3. Chemicals and food materials**

133 Bovine serum albumin (BSA; RIA grade), SDS (Biochemical grade) and ME
134 (Biochemical grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka,
135 Japan). All food materials were purchased at local retail stores (Tsukuba, Japan). The
136 manufacturers of all other chemicals are indicated in each section.

137

138 **2.4. Extraction of intact yolk protein from fish roes**

139 Each fish roe sample was homogenized in five volumes of 0.5 M NaCl containing 20
140 mM Tris-HCl (pH 8.0) using a Potter-Elvehjem homogenizer. The homogenates were
141 centrifuged at 2,000xg for 15 min to remove floating oil layer and then centrifuged at
142 20,000xg for 30 min. The sample temperature was kept below 5°C through all steps. The
143 resulting intact yolk protein extracts (YEx) were frozen at -30°C until use.

144

145 **2.5. Protein assay of fish roe extract**

146 The protein concentration of fish yolk extracts was determined by 2D Quant Protein
147 Assay Kit (GE Healthcare UK Ltd., Little Chalfont, England). SDS–polyacrylamide gel
148 electrophoresis (SDS-PAGE) analysis (Laemmli, 1970) was carried out to investigate the
149 protein components of fish yolk protein, and immunoblotting with anti-β'-component
150 IgG was performed as previously described (Shimizu et al., 2009).

151

152 **2.6. Removal of biotin from yolk protein extracts**

153 Magnetic streptavidin beads (MagnaBind Streptavidin Beads, PIERCE, Rockford, IL)

were used to remove biotin from fish yolk extracts according to the manufacturer's protocol. Briefly, the streptavidin-binding beads were suspended in TBS and mixed with chum salmon and walleye pollack YExs in a microcentrifuge tube. After gently stirring for 30 min at room temperature, the beads were removed by centrifugation and the supernatant was collected as biotin-free YEx.

2.7. Extraction of yolk protein in the presence of SDS and ME

Fish yolk proteins were extracted with a buffer containing protein denaturants (referred to as SDS-ME reagent). Tris-HCl (120 mM), pH 7.4, containing 0.5% (w/v) SDS, 2% ME, 0.1% BSA, and 0.05% Tween 20 was used as the extraction reagent, and was prepared according to the guidelines for food allergen detection in Japan (Consumer Affairs Agency, Japan, 2010; Shibahara, Uesaka, Wang, Yamada, & Shiomi, 2013). Fish roe was homogenized in 19 volumes of SDS-ME reagent using a Potter-Elvehjem homogenizer, shaken at a room temperature for 14 h, and centrifuged at 2,000g for 30 min. The yolk protein extracts (artificially denatured yolk-protein-extracts, referred to as AEx) were stored at -25 °C until use. AEx was diluted 20-fold in 1% BSA (ELISA blocking buffer) to reduce the inhibitory effect of SDS and ME on the protein response in the ELISA.

171 Furthermore, yolk protein extracted with the SDS-BSA extraction reagent (pH 7.4)

172 without ME was also subjected to ELISA analysis.

173

174 **2.8 Preparation of rabbit and rat IgG antibodies against chum salmon**

175 **β' -component**

176 Rabbits (n=3) and rats (n=10) were immunized with chum salmon β' -component with

177 high purity as previously reported (Shimizu et al. 2009). The purity of immunized antigen

178 was confirmed by silver staining of SDS-PAGE analysis using commercial silver staining

179 kit (EzSrtain Silver, Atto cooperation, Tokyo, Japan). All animal experiments in this

180 study were approved by the Hokkaido University Animal Care and Use Committee

181 (Permit number: 080198), and the experiments were performed according to the

182 Guidelines Concerning Animal Experiments at Hokkaido University.

183 The resulting antisera were applied to a Protein A HP column (GE Healthcare) to purify

184 the IgG fraction, according to the manufacturer's protocol. The purified rabbit and rat

185 anti- β' -component IgG antibodies (referred to as anti- β (Rabbit) and anti- β (Rat),

186 respectively) were dialyzed against PBS. ProClin (0.1%; Sigma-Aldrich, St. Louis,

187 Missouri, US) was added as preservative and the antibodies were stored at 5°C until use.

188

189 **2.9. Preparation of biotin conjugated anti- β' -c IgG**

190 The Sulfo-NHS-LC-Biotinylation Kit (Pierce Biotechnology, Illinois, US) was used to
191 prepare biotin-labelled anti- β (Rabbit) or anti- β (Rat) according to the manufacturer's
192 instructions.

193 **2.10. Preparation of horseradish peroxylase labelled F(ab')₂ fragments of anti β' -c**
194 **IgG**

195 F(ab')₂ fragments of anti- β (Rabbit) and anti- β (Rat) conjugated with horseradish
196 peroxidase (HRP) were prepared by Medical & Biological Laboratories Co., Ltd.
197 (Nagoya, Japan) using immobilized peptic digestion, Protein-A-column chromatography
198 (GE Healthcare), and the maleimide conjugation method (Hashida, Imagawa, Inoue,
199 Ruan, & Ishikawa, 1984), for use as detection antibodies in the sandwich ELISA systems
200 described in Table 1.

201

202 **2.11. Design of sandwich ELISA for detecting β' -component in yolk protein extracts**

203 Six kinds of sandwich ELISA systems were designed and are listed in Table 1. To
204 optimize the antibody pair for the sandwich ELISA, anti β' -component IgG and

205 HRP-conjugated F(ab')₂ fragments were combined as the capture and detection
206 antibodies, respectively, using either antibodies from the same species (rabbit or rat) as
207 both capture and detection antibodies (ELISA-A, ELISA-B, ELISA-C, ELISA-D), rabbit
208 IgG as capture antibody and rat F(ab')₂ fragment as detection antibody (ELISA-E), or rat
209 IgG as capture antibody and rabbit F(ab')₂ fragment as detection antibody (ELISA-F).
210 Protein concentration of each antibodies used in this study was also shown in Table 1.

211

212 **2.12. Evaluation of response to β'-component in each ELISA system**

213 Polystyrene 96-well micro-titer plates (Nalge Nunc International, New York, US) were
214 coated with 100 μL/well of the capture antibody which was diluted with 50 mM sodium
215 carbonate (pH 9.6) to the protein concentration listed in Table 1, and incubated at 4 °C for
216 15 h. The plate was washed 3 times with 150mM NaCl and 20 mM Tris-HCl (pH 7.5;
217 TBS), blocked with 1 % BSA in TBS at 25 °C for 1.5 h, and washed 4 times with TBS
218 containing 0.05% Tween 20 (TTBS). Next, 100 μL of the protein extracts (YEx, AEx) or
219 food extracts spiked with AEx were added to each well, followed by incubation at 25°C
220 for 1 h. After removal of unbound protein from each well by washing with TTBS, 100 μL
221 of the detection antibody, diluted in TBS containing 1% BSA (listed in Table 1), was

added to each well. The plate was incubated at 25 °C for 1 h and washed with TTBS. In the case of ELISA-A and ELISA-B, which incorporated biotinylated detection antibodies, 100 µL/well of diluted (1:20,000) streptavidin-HRP conjugate (Immunopure streptavidin, Pierce Biotechnology, Rockford, Illinois, US) was first added and incubated at 25 °C for 1 h before substrate addition. All ELISAs were developed using 100 µL/well of 3, 3', 5, 5'-tetramethylbenzidine solution (BM Blue POD Substrate, Roche Diagnostics, Indianapolis, Indiana, US) at 25°C for 20 min. The reactions were terminated by adding 100 µL/well of 1 N sulfuric acid, and absorbance of each well at 450 nm was measured using a micro plate reader. All the experiments were conducted twice, and when any the one data was higher than the cut-off value (the mean of the blank value + three standard deviations (n=5)), ELISA-response was judged as “positive”.

2.13. Limits of determination and quantification in ELISA system

AEx from chum salmon roe was used to construct the standard calibration curve using the antibody pair described in ELISA-E. A dilution series of AEx ranging from 0.20 to 100 ng/mL was prepared to generate the calibration curve. Protein concentration of AEx was measured using 2D Quant Protein Assay Kit. The limit of determination (LOD) and

the limit of quantification (LOQ) were calculated according to the Japanese Pharmacopoeia (Minister of Health, Labour and Welfare, Japan, 2011). LOQ and LOD were calculated as the 3 times and 10 times the standard deviation of the mean value of the buffer blank, respectively, in 30 experiments.

2.14. Preparation of food extracts for investigating effect of processed food components on ELISA-response

The popular processed foods freeze-dried egg soup, meatball, and rice porridge were prepared as follows. Freeze-dried egg soup: 1.6 kg of egg soup containing lactose (72 g), sucrose (25 g), bonito bouillon powder (42 g), salt (112 g), corn-starch (67 g), and whole egg (224 g) were frozen at -80 °C and freeze-dried. Chicken meat ball: chicken breast tender (325 g) mixed with lard (50 g), corn-starch (50 g), sugar (25 g) and water (50 g) was homogenized with a food processor, and each 50 g of the meat paste was boiled in water. Rice porridge: 45 mL of 1% sucrose solution was added to 5 g of polished rice and put into a retort pouch. After sealing, the pouch was heated at 121 °C for 10 min with an autoclave. All materials were purchased from local food markets. These processed foods were homogenized in the SDS-ME reagent in the same manner as AEx, and the resulting

extract was mixed with AEx. The recovery rate (%) was calculated to investigate the effect of food components on ELISA response of ELISA-E.

3. Results and Discussion

3.1. Detection of β' -component in fish yolk extract

The protein content/composition of YEx extracted from six different fish roes was examined by SDS-PAGE, and the reactivity/binding activity of the extracted proteins with rabbit anti- β' -component IgG was determined by immunoblotting (Fig. 1). As presented in Lane No. 2, chum salmon β' -component as an immunized antigen showed a high purity. Lipovitellin heavy chain (around 90 kDa) was found to be the predominant component in all fish yolk extracts, and the 16 kDa and 18 kDa subunits of β' -component (Shimizu et al., 2014) were observed in chum salmon YEx. In the immunoblot analysis (Lane3), protein components detected by anti- β (Rabbit) were also found in the all other fish YEx, except for Pacific herring. On the other hand, the reaction between YExs and anti- β (Rat) was extremely weak and no response was observed in walleye pollack and Pacific herring YExs (Lane 4). These results observed in the electrophoresised YExs

suggests that anti- β (Rat) has high species-specificity than anti- β (Rabbit).

The difference in the subunit composition of β' -component among YExs may reflect the characteristics of the oocyte maturation process, including transformation of precursor protein (vitellogenin) to yolk proteins. Indeed, the protein components detected by anti- β (Rabbit) with molecular weights > 30 kDa were found in chum salmon, walleye pollack and Atlantic capelin. These components may be a fragment of vitellogenin in the process of being degraded to the smaller molecule component. The results of the immunoblot analysis indicate the existence of IgG cross-reactivity among fish yolk protein fractions through the β' -component.

3.2. Applicability of a biotin-streptavidin ELISA for detecting fish roe protein

Figure 2 shows the response of various kinds of YEx in the sandwich ELISA using anti-salmon β' -component antibodies and the biotin-streptavidin detection system (ELISA-A and ELISA-B in Table 1). In ELISA-A using anti- β (Rabbit) for the capture and detection antibodies, no response was observed with YEx of dusky sole, slime flounder, and Atlantic capelin, whereas YEx of walleye pollack responded as the same as that of chum salmon as a positive control. The difference in the ELISA response depending on

fish species was also observed in ELISA-B using anti- β (Rat). However, when YEx of chum salmon and walleye pollack were subjected to the biotin-removal treatment and then applied to ELISA-A, the ELISA-responses diminished markedly. It is known that most of the biotin in food is covalently attached to food protein and walleye pollack roe contains 17.6 $\mu\text{g}/100\text{ g}$ of biotin (The Standard Tables of Food Composition in Japan, Sixth edition, 2010). YEx was likely contaminated with biotin-protein complex having specific IgG-binding activity, as about 15% of protein loss occurred in the chum salmon YEx through the biotin-removal treatment (data not shown). The results of Fig. 2 indicate that the biotin-streptavidin detection system is inadequate for quantitative analysis of fish roe proteins.

3.3. Characterization of rabbit and rat anti β' -component IgG in the sandwich

ELISA system

Combinations of anti- β (Rabbit) and anti- β (Rat) as the capture and detection antibodies in the sandwich ELISA were investigated by measuring the ELISA responses to the series of YEx from various roes (Fig. 3(Left side)). The F(ab')_2 fragment of anti- β (Rabbit) and anti- β (Rat), instead of whole IgG, was labelled with HRP and used as the detection

307 antibody. Figure 3(Left side) shows the response to the series of YEx tested in ELISA-C,
308 ELISA-D, ELISA-E, and ELISA-F. In general, an advantage of using the F(ab')₂
309 fragment in ELISA systems is the reduction of non-specific reactions, an important
310 characteristic for establishing an ELISA. Indeed, using F(ab')₂ fragment here showed low
311 blank values (Absorbance: 0.10-0.30), even when YEx samples were added at a
312 concentration of 1,000 ng/mL.

313 As shown in Fig. 3(Left side), each antibody pair showed different species-specific
314 responses. Little to no response was observed when Pacific herring YEx was tested using
315 all antibody pairs, which corresponded to the results of the immunoblot analysis shown in
316 Fig. 1. The strongest response to chum salmon YEx was observed in ELISA-C
317 (anti-β(Rabbit) was used as the capture and detection antibodies), however, other YEx
318 samples, with the exception of Pacific herring, were also detected. In particular, walleye
319 pollack showed a marked response when this antibody pair was used. On the other hand,
320 in ELISA-D, only chum salmon and walleye pollack showed clear responses, but those
321 responses were weak when compared to ELISA-C in which smaller amount of the
322 antibodies were used, as listed in Table 1. These results indicate that anti-β(Rabbit) had a
323 higher antibody titer than anti-β(Rat), and the results of ELISA-D suggest that

anti- β (Rat) has a narrower antigen specificity when compared to anti- β (Rabbit).

To utilize the strong antibody titer in anti- β (Rabbit) and the superior antigen specificity in anti- β (Rat), ELISA-E and ELISA-F were designed by combining both antibodies. As a result, the low species-specificity of anti- β (Rabbit) observed in ELISA-C and the low reactivity of anti- β (Rat) observed in ELISA-D were simultaneously obtained in ELISA-E in which anti- β (Rabbit) was used as the capture antibody and anti- β (Rat) was used as the detection antibody. However, the response to chum salmon was reduced in ELISA-F, even when anti- β (Rabbit) was used as the detection antibody. The results of the ELISA system shown in Fig. 3(Left side) suggest the importance of the capture antibody when designing a highly sensitive sandwich ELISA. That is, the intensity of the ELISA response seems to reflect the quantity of antigen captured by the capture antibody. However, neither ELISA-E nor ELISA-F was able to distinguish YEx of chum salmon from walleye pollack.

3.4. Elimination of IgG cross-reactivity between fish yolk protein extracted with SDS and ME

Yolk protein from the six fish roes was extracted with 19 volumes of SDS-ME reagent,

and the resulting extract (AEx) was diluted 20 times with blocking buffer and then tested in the different sandwich ELISA systems. The responses of each AEx samples in ELISA-C, ELISA-D, ELISA-E, and ELISA-F are shown in Fig. 3(Right side), along with the protein concentration of each sample. Chum salmon AEx at 460 ng/mL displayed a marked response in ELISA-C and ELISA-D, indicating that the use of SDS and ME is available for the extraction reagent. On the other hand, AEx samples from walleye pollack, slime flounder, and Antarctic capelin were also detected in ELISA-C, whereas ELISA-D detected only chum salmon AEx. These results indicate that SDS-ME reagent improved antigen specificity of anti- β (Rat).

Chum salmon β' -component is a homodimer protein composed of two subunits linked by disulfide bonds (Shimizu et al., 2014), and the IgE-binding ability of β' -component, which was examined using salmon roe-allergic patients' sera, was found to be diminished with collapse of the subunit structure caused by carboxymethylation (Shimizu et al., 2014), suggesting that the β' -component-specific IgE may recognize structural epitopes on the surface of β' -component. In the same way, structural IgG-epitopes responsible for the cross-reactivity between chum salmon and walleye pollack YEx may have been abolished by SDS-ME reagent, and this change may have contributed to the increase in

358 selectivity to chum salmon yolk protein in ELISA-D.

359 As described above, ELISA-D, which used anti- β (Rat) as the capture and the detection
360 antibodies, could detect chum salmon AEx with high specificity, but its detection
361 sensitivity would be limited for practical use because the high amount of the capture
362 antibody were required to establish the ELISA system as shown in Table 1. To
363 compensate for this drawback, anti- β (Rabbit) was used instead of anti- β (Rat) as the
364 capture antibody. As a result, ELISA-E had high sensitivity for the detection of chum
365 salmon AEx and the specificity to chum salmon roe remained even when high protein
366 concentrations (295-470 ng/mL) were used. As indicated in Table 1, ELISA-E was
367 designed using the combination of anti- β (Rabbit), with a high antibody titer, as the
368 capture antibody, and anti- β (Rat), having superior antigen specificity, as the detection
369 antibody. In contrast, the high selectivity for chum salmon β' -component was abrogated
370 in ELISA-F using anti- β (Rat) for the capture antibody and anti- β (Rabbit) for the
371 detection antibody. These differences in the detection characteristics of the sandwich
372 ELISA observed here suggest that the immunological properties of the capture antibody
373 and the detection antibody played a role in the sensitivity and the species selectivity of the
374 ELISA, respectively. Additionally, the high specificity shown in ELISA-E was not

observed when fish yolk proteins were extracted using SDS reagent without the addition of ME (ELISA-E(-ME) in Fig. 3). Therefore, it is apparent that the cooperative effect of SDS and ME are indispensable for improving the species specificity of the ELISA system for detecting salmon yolk protein.

To avoid false positive reactions caused by IgG cross-reactivity, monoclonal antibodies with high antigen specificity are often used in the development of ELISAs for detecting trace amounts of food allergens (Seiki et al., 2007; Gavrović-Jankulović et al., 2008; Ma et al., 2010; Zhang, Lu, Ushio, & Shiomi, 2014). However, ELISA-E, using polyclonal antibodies from two different animals, showed high allergen specificity for the detection of salmon roe among various fish species, without using expensive monoclonal antibodies. The combination of IgG antibodies from different animal species could be useful to avoid false positive caused by IgG cross-reactivity when developing food allergen detection systems.

3.5. Sensitivity of developed ELISA system

The standard curve generated using ELISA-E with chum salmon AEx is shown in Fig. 4. The calculated LOD and LOQ were 1.95 ng/mL and 6.51 ng/mL, respectively. Since

AEx was extracted from chum salmon roe with 19 volumes of the extraction reagent and further diluted 20 times with the blocking buffer as described in Materials and Methods, LOD and LOQ of chum salmon roe were estimated as 0.78 µg/g and 2.60 µg/g in food sample, respectively. Concerning the Labelling System for foods containing allergens in the Food Sanitation Act, the Consumer Affairs Agency, Government of Japan has stated that: “Foods containing more than a few µg/mL concentration level or a few µg/g content level of specified ingredients in terms of the amount of total protein need labelling. (Ministry of Health, Labour and Welfare, Japan. (2008) ”. Indeed, a microgram level of allergenic food has been reported to induce hypersensitivity symptoms (Bindslev-Jensen, Briggs, & Osterballe, 2002; Morisset et al., 2003). Therefore, we determined that the detection sensitivity of the antibody pair described here in ELISA-E using the SDS-ME reagent reached a practical level for the detection of contaminated chum salmon allergen in processed food.

Finally, the effect of food components on the response of ELISA-E was investigated by measuring the recovery rate of AEx mixed with an extract of processed foods. As listed in Table 2, no false negative response was observed in all processed foods tested and the recovery rates reached >90%. These results indicate no suppressive influence of the

processed food components on the performance of the ELISA.

4. Concluding remarks

A novel ELISA system for detecting trace contamination of chum salmon roe in processed food has been established in this study. Rabbit and rat polyclonal IgG antibodies against chum salmon β' -component were used for designing the ELISA system. The optimized ELISA using rabbit anti- β -component for the capture antibody and HRP-labelled F(ab')₂ fragment of rat anti- β -component for the detection antibody, showed high sensitivity and good species-specificity for detecting chum salmon yolk protein. Additionally, sample extraction in the presence of SDS and ME enhanced the high selectivity of the ELISA system. The authors have estimated that the developed ELISA system has a probability to be applied for the detection of contaminated chum salmon allergen in processed food. Therefore, the next development step will be a validation study (including allergen recovery test using processed foods) in line with Japanese analysis guidelines for food allergen detection systems (Akiyama et al., 2011).

Acknowledgments

426 We thank Dr. Kazuo Shiomi (Professor emeritus, Tokyo University of Marine Science
427 and Technology) for his incisive comments at the start of this study. We also thank Dr.
428 Hiroshi Akiyama (National Institute of Health Sciences, Japan) who offered the detailed
429 information about Japan's food allergen labelling system. This work was supported by
430 Japan Science and Technology Agency, JST-project to develop "innovative seeds 2008"
431 and the project on the development of determination methods for allergic materials in
432 foods by the Ministry of Health, Labour, and Welfare of Japan.
433

434 **References**

- 435 Akiyama, H., Imai, T., & Ebisawa, M. (2011). Japan food allergen labeling
436 regulation--history and evaluation. *Advances in Food and Nutrition Research*,
437 62,139–171.
- 438 Bindslev-Jensen, C., Briggs, D., & Osterballe, M. (2002). Can we determine a threshold
439 level for allergenic foods by statistical analysis of published data in the literature?
440 *Allergy*, 57, 741–746.
- 441 Bledsoe, G. E., Bledsoe, C. D., & Rasco, B. (2003). Caviars and fish roe products.
442 *Critical Reviews in Food Science and Nutrition*, 43, 317–356.
- 443 Blom, W. M., Vlieg-Boerstra, B. J., Kruizinga, A. G., van der Heide, S., Houben, G. F., &
444 Dubois, A. E. J. (2013). Threshold dose distributions for 5 major allergenic foods in
445 children. *The Journal of Allergy and Clinical Immunology*, 131, 172–179.
- 446 Crevel, R. W. R., Ballmer-Weber, B. K., Holzhauser, T., Hourihane, J. O., Knulst, A. C.,
447 Mackie, A. R., et al. (2008). Thresholds for food allergens and their value to
448 different stakeholders. *Allergy*, 63, 597–609.

449 Consumer Affairs Agency, Japan. (2010). Notification No. 286 of September 10, 2010
 450 (in Japanese). Retrieved from <http://www.caa.go.jp/foods/pdf/syokuhin1178.pdf> .
 451 Accessed 14.08.14.

452 Ebisawa, M., Nishima, S., Ohnishi, H., & Kondo, N. (2013). Pediatric allergy and
 453 immunology in Japan. *Pediatric Allergy and Immunology*, 24, 704–714.

454 Fujita, S., Shimizu, Y., Kishimura, H., Watanabe, K., Hara, A., & Saeki, H. (2012). In
 455 vitro digestion of major allergen in salmon roe and its peptide portion with
 456 proteolytic resistance. *Food Chemistry*, 130, 644–650.

457 Gavrović-Jankulović, M., Spasić, M., Cirković Velicković, T., Stojanović, M.,
 458 Inić-Kanada, A., Dimitrijević, L., et al. (2008). Quantification of the thaumatin-like
 459 kiwi allergen by a monoclonal antibody-based ELISA. *Molecular Nutrition & Food*
 460 *Research*, 52, 701–707.

461 Gendel, S. M. (2012). Comparison of international food allergen labeling regulations.
 462 *Regulatory Toxicology and Pharmacology*, 63, 279–285.

463 Hashida, S., Imagawa, M., Inoue, S., Ruan, K. H., & Ishikawa, E. (1984). More useful
 464 maleimide compounds for the conjugation of Fab' to horseradish peroxidase
 465 through thiol groups in the hinge. *Journal of Applied Biochemistry*, 6, 56–63.

466 Hiramatsu, N., Cheek, A. N., Sullivan, C. V., Matsubara, T., & Hara, A. (2005).
 467 Vitellogenesis and endocrine distribution. In T. P. Mommsen & T. W. Moon (Eds.),
 468 *Biochemistry and molecular biology of fishes* (Vol. 6, pp. 431–471).
 469 Amsterdam, The Netherlands: Elsevier Science Ltd.

470 Kanagawa Prefectural Institutes of Public Health. (2006). *Statistical analysis on the field*
 471 *survey of food allergy in Kanagawa, Japan (in Japanese)*. Retrieved from
 472 http://www.eiken.pref.kanagawa.jp/004_chousa/images/061206_allergyreport.pdf .
 473 Accessed 14.08.2014.

474 Kondo, Y., Kakami, M., Koyama, H., Yasuda, T., Nakajima, Y., & Kawamura, M., et al.
 475 (2005). IgE cross-reactivity between fish roe (salmon, herring and pollock) and
 476 chicken egg in patients anaphylactic to salmon roe. *Allergology International*, 54,
 477 317–323.

478 Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of
 479 bacteriophage T4. *Nature*, 227, 680–685.

480 Ma, X., Sun, P., He, P., Han, P., Wang, J., & Qiao, S., et al. (2010). Development of
 481 monoclonal antibodies and a competitive ELISA detection method for glycinin, an
 482 allergen in soybean. *Food Chemistry*, 121, 546–551.

483 Ministry of Health, Labour and Welfare, Japan. (2008). *FAQs on Labeling System for*
 484 *Foods Containing Allergens*. Retrieved from
 485 <http://www.caa.go.jp/foods/pdf/syokuhin13.pdf> . Accessed 14.08.2014.

486 Minister of Health, Labour and Welfare, Japan. (2011). *Japanese Pharmacopoeia*
 487 *Sixteenth Edition* (6 ed., pp. 2184–2185). Retrieved from
 488 <http://www.pmda.go.jp/kyokuhou/pdf/jpdata/JP16eng.pdf> . Accessed 18.08.2014.

489 Ministry of Health, Labour and Welfare, Japan (2014). The National Health and Nutrition
 490 Survey in Japan, 2012. Retrieved from
 491 <http://www.mhlw.go.jp/bunya/kenkou/eiyou/dl/h24-houkoku.pdf>. Accessed
 492 24.12.2014.

493 Morisset, M., Moneret-Vautrin, D. A., Kanny, G., Guénard, L., Beaudouin, E., &
 494 Flabbée, J., et al. (2003). Thresholds of clinical reactivity to milk, egg, peanut and
 495 sesame in immunoglobulin E-dependent allergies: evaluation by double-blind or
 496 single-blind placebo-controlled oral challenges. *Clinical and Experimental Allergy*,
 497 33, 1046–51.

498 Seiki, K., Oda, H., Yoshioka, H., Sakai, S., Urisu, A., & Akiyama, H., et al. (2007). A
 499 reliable and sensitive immunoassay for the determination of crustacean protein in
 500 processed foods. *Journal of Agricultural and Food Chemistry*, 55, 9345–9350.

501 Shibahara, Y., Uesaka, Y., Wang, J., Yamada, S., & Shiomi, K. (2013). A sensitive
 502 enzyme-linked immunosorbent assay for the determination of fish protein in
 503 processed foods. *Food Chemistry*, 136, 675–681.

504 Shimizu, Y., Nakamura, A., Kishimura, H., Hara, A., Watanabe, K., & Saeki, H. (2009).
 505 Major allergen and its IgE cross-reactivity among salmonid fish roe allergy. *Journal*
 506 *of Agricultural and Food Chemistry*, 57, 2314–2319.

507 Shimizu, Y., & Saeki, H. (2010). Characterization of fish roe allergens. In K. Shiomi & H.
 508 Saeki (Eds.), *Science of Seafood Allergens* (pp. 47-59). Tokyo:
 509 Koseisha-Koseikaku.

510 Shimizu, Y., Kishimura, H., Kanno, G., Nakamura, A., Adachi, R., Akiyama, H., et al.
 511 (2014). Molecular and immunological characterization of β' -component (Onc k 5), a
 512 major IgE-binding protein in chum salmon roe. *International Immunology*, 26,
 513 139–147.

514 Urisu, A., Ebisawa, M., Mukoyama, T., Morikawa, A., & Kondo, N. (2011). Japanese
 515 guideline for food allergy. *Allergology International : Official Journal of the*
 516 *Japanese Society of Allergology*, 60, 221–236.

517 Yoshitake, S., Imagawa, M., Ishikawa, E., Niitsu, Y., Urushizaki, I., Nishiura, M., et al.
 518 (1982). Mild and efficient conjugation of rabbit Fab' and horseradish peroxidase
 519 using a maleimide compound and its use for enzyme immunoassay. *Journal of*
 520 *Biochemistry*, 92, 1413–1424.

521 Zhang, H., Lu, Y., Ushio, H., & Shiomi, K. (2014). Development of sandwich ELISA for
522 detection and quantification of invertebrate major allergen tropomyosin by a
523 monoclonal antibody. *Food Chemistry*, 150, 151–157.

524

525

Figure Captions

Fig. 1. SDS-PAGE and immunoblot analyses of proteins extracted from fish yolk.

Lane 1: CBB stained YEx, Lane 2: Silver stained chum salmon β' -component, Lane 3: Immunoblotting pattern of YEx using anti- β (Rabbit), Lane 4: Immunoblotting pattern of YEx using anti- β (Rabbit). Rabbit anti- β' -component serum was used only at the immunoblotting of Pacific herring YEx (Lane 3).

*: Subunits of chum salmon β' -component.

Fig. 2. Detection of β' -component in fish roe extracts with ELISA systems using biotin-streptavidin reaction.

YExs from chum salmon, walleye pollack, dusky sole, slime flounder, and Atlantic capelin were subjected to ELISA-A and ELISA-B using biotin-streptavidin reaction as a detection system (listed in Table 1). Biotin-free YExs from chum salmon and walleye pollack were also examined in ELISA-A. Blank indicates the absorbance of diluent without addition of protein sample. The dotted line indicates the cut-off value which is the mean of the blank value + three standard deviations (n=5). Asterisk in the figure

indicates that ELISA-response was judged as “positive”.

Fig 3. Sensitivity and species-specificity of anti- β' -component sandwich ELISA systems to detect chum salmon β' -component in fish roe extracts.

YExs (Left side figures) and AExs (Right side figures) from chum salmon, walleye pollack, Pacific herring, dusky sole, slime flounder, and Atlantic capelin were tested using ELISA systems listed in Table 1. The protein concentration of each YEx was adjusted to 1,000 ng/mL, and the protein concentrations of each AExs (ng/mL) were indicated in ELISA-C(AEx). Fish yolk proteins extracted using SDS reagent without the addition of ME were also subjected to ELISA-E (referred as to ELISA-E(-ME) with protein concentration of the extracts (ng/mL)). Blank indicates the absorbance of diluent without addition of protein sample. The dotted line indicates the cut-off value which is the mean of the blank value + three standard deviations (n=5). Asterisk in the figure indicates that ELISA-response was judged as “positive”.

Fig. 4. Standard curve of optimized sandwich ELISA for detection of salmon yolk

560 β' -component protein.

561 A dilution series of AEx was subjected to ELISA-E, and the mean absorbance values
562 (n=3) were plotted against the protein concentration. Limit of determination (LOD) and
563 Limit of quantification (LOQ) were calculated as three-fold and 10-fold standard
564 deviations of the average of the buffer blank value (n=30), respectively.

Table 1. Design of anti- β' -component sandwich ELISA for detection of trace amounts of chum salmon β' -component fish yolk protein.

Presentation	ELISA design	Extract	Capture antibody	Detection antibody
			Concentration ($\mu\text{g/mL}$)	Concentration ($\mu\text{g/mL}$)
Fig. 2	A	YEx	anti- β (Rabbit) 5	anti- β (Rabbit), Biotin labeled 0.2
Fig. 2	B	YEx	anti- β (Rat) 5	anti- β (Rat), Biotin labeled 0.2
Fig. 3L	C	YEx	anti- β (Rabbit) 5	anti- β (Rabbit)* 0.25
Fig. 3L	D	YEx	anti- β (Rat) 15	anti- β (Rat)* 1
Fig. 3L	E	YEx	anti- β (Rabbit) 5	anti- β (Rat)* 0.25
Fig. 3L	F	YEx	anti- β (Rat) 5	anti- β (Rabbit) * 0.25
Fig. 3R	C	AEx	anti- β (Rabbit) 5	anti- β (Rabbit)* 0.25
Fig. 3R	D	AEx	anti- β (Rat) 15	anti- β (Rat)* 1
Fig. 3R	E	AEx	anti- β (Rabbit) 5	anti- β (Rat)* 1
Fig. 3R	F	AEx	anti- β (Rat) 10	anti- β (Rabbit)* 2
Fig. 3R	E	-ME**	anti- β (Rabbit) 5	anti- β (Rat)* 0.25

Fig. 3L and Fig. 3R indicate left and right sides in Fig. 3, respectively.

Abbreviations were shown in the part of Materials and Methods.

*: HRP labeled F(ab')₂ fragment.

** : Fish yolk proteins extracted using SDS reagent without the addition of ME.

Table 2. Effect of food components on detection of chum salmon β' -component using ELISA-E.

Model processed food	β' -component added as AEx to model processed food extract						
	No addition	5 ng/mL added		10 ng/mL added		50 ng/mL added	
		Mean (ng/mL)	Recovery (%)	Mean (ng/mL)	Recovery (%)	Mean (ng/mL)	Recovery (%)
Chicken meat ball	N. D.	6.3	125	11.0	110	51.2	102
Egg soup	N. D.	5.4	108	9.4	94	51.5	103
Rice porridge	N. D.	4.5	90	9.0	90	52.7	105

Mean indicates average concentration of chum salmon β' -component (n=3).

Recovery = Mean/Added protein. N.D.: not detected.

Fig. 1 (Shimizu et al.).
Revised version

Fig. 2 (Shimizu et al.).

Fig. 3 (Shimizu et al.).

Fig. 4 (Shimizu et al.).