



Analytical Methods

A sensitive enzyme-linked immunosorbent assay for the determination of fish protein in processed foods

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ABSTRACT

Fish is one of the most common causes of food allergy and its major allergen is parvalbumin, a 12 kDa muscular protein. In this study, a sandwich enzyme-linked immunosorbent assay (ELISA) for the determination of fish protein in processed foods was developed using a polyclonal antibody raised against Pacific mackerel parvalbumin. The developed sandwich ELISA showed 22.6–99.0% reactivity (based on the reactivity to Pacific mackerel parvalbumin) to parvalbumins from various species of fish. The limits of detection and quantitation were estimated to be 0.23 and 0.70 µg protein per g of food, respectively. When the sandwich ELISA was subjected to inter-laboratory validation, spiked fish protein was recovered from five model processed foods in the range of 69.4–84.8% and the repeatability and reproducibility relative standard deviations were satisfactorily low ($\leq 10.5\%$). Thus, the sandwich ELISA was judged to be a useful tool to determine fish protein in processed foods.

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1. Introduction

Fish is a valuable source of highly assimilated proteins but is also among the most common causes of immunoglobulin E (IgE)-mediated food allergy (Akiyama, Imai, & Ebisawa, 2011). Immediately after ingestion of fish, adverse reactions, such as urticaria, asthma and diarrhea, are induced in fish-allergic subjects; fatal anaphylaxis may occur in severe cases (O'Neil, Helbling, & Lehrer, 1993; Pascual, Esteban, & Crespo, 1992). The major fish allergen was first identified in cod *Gadus callarias* as parvalbumin (known as Gad c 1), a 12 kDa muscle tissue protein (Elsayed & Aas, 1971a; Elsayed & Bennich, 1975). Subsequent molecular studies have established that parvalbumin is the major allergen in various fish species, such as Japanese eel (Shiomi, Hamada, Sekiguchi, Shimakura, & Nagashima, 1999), salmon (Lindström, van Dō, Hordvik, Endresen, & Elsayed, 1996), horse mackerel (Shiomi, Hayashi, Ishikawa, Shimakura, & Nagashima, 1998), mackerel (Hamada, Tanaka, Ishida, Nagashima, & Shiomi, 2003) and bigeye tuna (Shiomi et al., 1999). Parvalbumin is an EF-hand Ca^{2+} -binding protein and is found only in vertebrates, particularly at high levels in the muscle of fish and amphibians (Kretsinger & Nockolds, 1973). IgE cross-reactivity is recognised not only between fish parvalbumins (Van Dō, Elsayed, Florvaag, Hordvik, & Endresen, 2005) but also between fish and frog parvalbumins (Hamada, Nagashima, & Shiomi, 2004; Hilger et al., 2004).

Due to the lack of effective medical treatment for food allergy, the only measure to prevent possible life-threatening reactions is strict avoidance of consumption of allergenic foods. However, severe allergic reactions can be induced by the accidental intake of processed food products containing allergenic ingredients. Therefore, adequate information regarding potentially allergenic ingredients in processed food products is essential for sensitised subjects. Along this line, the Japanese Ministry of Health, Labour and Welfare (MHLW) enforced an allergen-labelling system for allergenic food ingredients in 2002. Although the Joint FAO/WHO Codex Alimentary Commission Session included fish among the eight kinds of foods to be labelled as “containing allergenic ingredients” in 1999, labelling of fish is not mandatory in the Japanese allergen-labelling system, in which only two species of fish, mackerel and salmon, is recommended to be labelled (Akiyama et al., 2011). This is largely because no reliable analytical method for fish protein in foods is available to ensure the labelling of fish.

Recently, a sandwich enzyme-linked immunosorbent assay (ELISA) with a polyclonal anti-cod parvalbumin antibody has been developed for the quantification of fish in foods (Fæsta & Plassen, 2008). However, the developed ELISA has some drawbacks with respect to sensitivity and specificity. Furthermore, it is unclear whether the developed ELISA is applicable to processed foods. In this study, therefore, a sandwich ELISA with high sensitivity and specificity was newly developed using a polyclonal antibody against Pacific mackerel (*Scomber japonicus*) parvalbumin and validated for practical use. We provide evidence that this sandwich ELISA satisfies the validation criteria described in the official

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guidelines published by the Japanese MHLW (Akiyama et al., 2011), being useful for the determination of fish protein in processed foods.

2. Materials and methods

2.1. Food samples

A total of 113 food ingredients (refer to Tables 1 and 2 and the Section 3.2 for the names of ingredients) and 37 commercial processed foods (refer to Table 4 for the names of processed foods) were all purchased at local stores in Japan.

2.2. Purification of parvalbumin

Purification of parvalbumins from five species of fish (Japanese eel *Anguilla japonica*, horse mackerel *Trachurus japonicus*, crimson sea bream *Evynnis japonica*, Pacific mackerel *S. japonicus* and bigeye tuna *Thunnus obesus*) and one species of amphibian (bullfrog *Rana catesbeiana*) was achieved by a combination of gel filtration on a Sephadex G-75 column (GE Healthcare, Buckinghamshire, UK) and reverse-phase HPLC on a TSKgel ODS-120T column (Tosoh, Tokyo, Japan), as described elsewhere (Guo, Kubota, & Shiomi, 2012; Hamada, Nagashima et al., 2004; Hamada et al., 2003; Shiomi et al., 1998, 1999). Although at least two isoforms of parvalbumin were detected in these fish and bullfrog, the following major isoforms were used in this study: Ang j 1 for Japanese eel (Shiomi et al., 1999), Tra j 1 for horse mackerel (Shiomi et al., 1998), PA II for crimson sea bream (Guo et al., 2012), Sco j 1 for Pacific mackerel (Hamada et al., 2003), Thu o 1 for bigeye tuna (Shiomi et al., 1999) and PA2 for bullfrog (Hamada, Nagashima et al., 2004). In addition to these parvalbumins, those of three species of fish (sardine *Sardinops melanostictus*, skipjack *Katsuwonus pelamis* and Japanese flounder *Paralichthys olivaceus*) were also purified by the same method as employed for the above fish and bullfrog. Of the parvalbumin isoforms detected in each fish, only the major isoform was used in experiments. In the case of Pacific mackerel, recombinant parvalbumin was also expressed in *Escherichia coli* and purified according to the previously reported procedure (Hamada et al., 2004). All the purified parvalbumin preparations were confirmed for their homogeneity by SDS-PAGE, which was performed on an XCell SureLock Mini-Cell system (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer.

2.3. Preparation of fish protein powder

The horse mackerel muscle was lyophilised and ground to powder (named fish protein powder), which was used for spiking into

model processed foods and making standard solutions in sandwich ELISA. Protein content in the fish protein powder was determined to be 470 mg/g using a 2-D Quant Kit (GE Healthcare).

2.4. Preparation of model processed foods

Five model processed foods (creamy croquette, chicken meatball, pork meatball dumpling, vegetable and chicken soup and rice gruel) individually spiked with the fish protein powder at a final concentration of 10 µg/g (fish protein weight/sample weight) were prepared and preserved in a deep-freezer at –20 °C until use. The creamy croquettes (composed of potato, onion, corn, milk, butter, flour, starch, bread crumbs and fish protein powder) and the chicken meatballs (composed of chicken breast, eggs, white part of green onion, ginger, starch, salt, soy sauce and fish protein powder) were prepared according to the method of Sakai et al. (2008) and the pork meatball dumplings (composed of pork, onion, sesame oil, ginger, starch, salt, soy sauce, flour and fish protein powder) essentially by the same procedure as employed for the chicken meatballs. In the case of the vegetable and chicken soup, precooked vegetables (carrot, potato, onion, pumpkin and cabbage), chicken soup and fish protein powder were thoroughly mixed, sealed in an aluminum pouch and boiled for 10 min. As for the rice gruel, rice was first cooked in water with a rice cooker. After being cooled to room temperature, the rice gruel was spiked with the fish protein powder and sealed in an aluminum pouch.

For each model processed food, a blank sample without the fish protein powder was prepared for confirming potential sample contamination, false positives, interference and matrix effects. All the values determined with the blank processed food samples using the sandwich ELISA were less than the limit of detection.

2.5. Preparation of sample solution

A 1.0 g portion of each test material (food ingredient, commercial processed food or model processed food) was added to 19 ml of 0.12 M Tris–HCl buffer (pH 7.4) containing 0.1% bovine serum albumin (BSA), 0.05% Tween 20, 0.5% SDS and 2% β-mercaptoethanol (Morishita et al., 2008; Watanabe et al., 2005) and the mixture was shaken horizontally for 16 h at room temperature. After centrifugation at 3000g for 20 min, the supernatant was filtered through a 5AB paper (Advantec Toyo, Tokyo, Japan). The filtrate thus obtained was diluted 20-fold with 0.02 M MOPS buffer (pH 8.2) containing 0.1% BSA, 0.01% Triton X-100, 1 mM CaCl₂ and 0.1% ProClin 300 (Sigma–Aldrich, St. Louis, MO, USA) and used as a sample solution for sandwich ELISA.

2.6. Preparation of standard solutions for calibration

A 0.1 g portion of the fish protein powder was added to 20 ml of 0.05 M Tris–HCl buffer (pH 7.3) containing 0.5 M NaCl, 0.5% SDS, 2% β-mercaptoethanol, 5 mM EDTA and 1% Protease Inhibitor Cocktail Kit (Thermo Fisher Scientific, Waltham, MA, USA). The mixture was shaken for 16 h at room temperature and centrifuged at 10000g for 20 min. The supernatant was filtered through a 0.8 µm micro-filter paper (DISMIC-25cs; Advantec Toyo) and the filtrate used as an extract. After heating at 100 °C for 10 min, the extract was determined for protein concentration using the 2-D Quant Kit. Based on the determined protein concentration, the extract was diluted to 50 ng/ml with 0.02 M Tris–HCl buffer (pH 7.3) containing 0.15 M NaCl, 0.1% BSA, 0.01% Triton X-100, 1 mM CaCl₂ and 0.1% ProClin 300. The diluted extract was stored at 4 °C until used as the calibration standard solution for ELISA.

Table 1
Reactivity of various parvalbumin samples in the sandwich ELISA method.

Sample	RC ₅₀ ^a (ng/ml)	Reactivity ^b (%)
<i>Fish</i>		
Pacific mackerel (<i>Scomber japonicus</i>)	6.6	100.0
Japanese eel (<i>Anguilla japonica</i>)	20.2	32.9
Sardine (<i>Sardinops melanostictus</i>)	15.6	42.6
Horse mackerel (<i>Trachurus japonicus</i>)	9.2	72.1
Crimson sea bream (<i>Evynnis japonica</i>)	11.5	57.9
Skipjack (<i>Katsuwonus pelamis</i>)	9.4	70.7
Bigeye tuna (<i>Thunnus obesus</i>)	6.7	99.0
Japanese flounder (<i>Paralichthys olivaceus</i>)	29.4	22.6
<i>Amphibian</i>		
Bullfrog (<i>Rana catesbeiana</i>)	550.9	1.2

^a RC₅₀ represents the concentration of parvalbumin giving a 50% response.

^b Reactivity = 100 × (RC₅₀ of Pacific mackerel parvalbumin/RC₅₀ of target parvalbumin).

Table 2
Reactivity of fish, cephalopod and fish roe samples in the sandwich ELISA method.

Sample	Fish protein concentration (μg/g)
<i>Fish</i>	
Spiny dogfish (<i>Squalus acanthias</i>)	4.0
Conger eel (<i>Conger myriaster</i>)	2980
Sardine (<i>Sardinops melanostictus</i>)	22,600
Pacific herring (<i>Clupea pallasii</i>)	24,300
Capelin (<i>Mallotus villosus</i>)	19,900
Silver salmon (<i>Oncorhynchus kisutch</i>)	43,800
Rainbow trout (<i>Oncorhynchus mykiss</i>)	35,900
Pacific cod (<i>Gadus macrocephalus</i>)	46,100
Pacific saury (<i>Cololabis saira</i>)	315
Red gurnard (<i>Chelidonichthys spinosus</i>)	4460
Yellowtail (<i>Seriola quinqueradiata</i>)	13,300
Horse mackerel (<i>Trachurus japonicus</i>)	34,000
Japanese whiting (<i>Sillago japonica</i>)	8860
Red sea bream (<i>Pagrus major</i>)	10,600
Sailfin sandfish (<i>Arctoscopus japonicus</i>)	14
Cutlass Fish (<i>Trichiurus lepturus</i>)	102
Pacific mackerel (<i>Scomber japonicus</i>)	1620
Skipjack (<i>Katsuwonus pelamis</i>)	2770
Bigeye tuna (<i>Thunnus obesus</i>)	183
Japanese flounder (<i>Paralichthys olivaceus</i>)	18,400
Rock sole (<i>Pleuronectes mochigarei</i>)	5820
Japanese file fish (<i>Stephanolepis cirrhifer</i>)	9590
<i>Cephalopod</i>	
Common octopus (<i>Octopus vulgaris</i>)	<1.0
Ocellated octopus (<i>Octopus ocellatus</i>)	<1.0
Giant octopus (<i>Octopus dofleini</i>)	1.3
Chestnut octopus (<i>Octopus conispadiceus</i>)	3.8
Japanese common squid (<i>Todarodes pacificus</i>)	<1.0
Spear squid (<i>Loligo bleekeri</i>)	1.2
Big fin reef squid (<i>Sepioteuthis lessoniana</i>)	1.3
Common cuttlefish (<i>Sepia officinalis</i>)	2.4
<i>Fish roe</i>	
Cod roe	1.9
Salmon roe	2.8

2.7. Preparation of polyclonal anti-parvalbumin antibody

Polyclonal anti-parvalbumin antibody was raised in a female Japanese white rabbit (3.0 kg; Takasugi Experimental Animals Supply, Kasukabe, Japan) using recombinant Pacific mackerel parvalbumin as an immunogen. The rabbit was immunized with 300 μg of recombinant parvalbumin in Freund's complete adjuvant (Sigma–Aldrich). Injections were repeated six times at intervals of 2 weeks. Whole blood was collected 2 weeks after the final immunization and the serum was separated. The polyclonal antibody was purified from the serum using a HiTrap Protein G HP column (1 ml; GE Healthcare) and labelled with horseradish peroxidase using a Peroxidase Labeling Kit-NH₂ (Dojindo Laboratories, Kumamoto, Japan).

2.8. Sandwich ELISA

A 96-well microtiter plate (Thermo Fisher Scientific) was coated with 100 μl/well of polyclonal antibody solution (20 μg/ml) at 4 °C for 16 h and washed twice with phosphate-buffered saline (PBS). The plate was then blocked with 200 μl/well of PBS containing 1% BSA, 0.5% polyvinyl alcohol and 0.1% ProClin 300 at 4 °C for 16 h. After double washings with PBS containing 0.05% Tween 20 (PBST), the polyclonal antibody-coated plate was added with 100 μl/well of sample solution or standard solution (0.78, 1.56, 3.13, 6.25, 12.5 or 50 ng/ml). The plate was incubated at 25 °C for 1 h, washed five times with PBST and reacted with 100 μl/well of the horseradish peroxidase-labelled polyclonal antibody solution (0.7 μg/ml) at 25 °C for 1 h. After five washings with PBST, the plate was added with 100 μl/well of 3,3',5,5'-tetramethylbenzidine solution (TMB

One Component HRP Microwell Substrate; SurModics, Eden Prairie, MN, USA) and allowed to stand at 25 °C for 20 min. Enzyme reaction was stopped by addition of 100 μl/well of 0.5 M sulphuric acid and the absorbance was measured at 450 nm with a reference wavelength of 650 nm. Data were analysed using a SoftMax Pro 2.2.1 software (Molecular Devices, Sunnyvale, CA, USA) based on the four-parameter logistic method. The concentration of fish protein in the sample solution was calculated from the calibration curve prepared by a four-parameter logistic analysis and converted to that in the given food using the dilution factor ($\times 400$).

2.9. Assay validation

Determination of the limit of detection (LOD) and limit of quantitation (LOQ) was performed according to the guidelines of International Standards Organization (ISO) and International Union of Pure and Applied Chemistry (IUPAC) (ICH Steering Committee., 1996). The LOD was defined as the mean measured content of 20 blank samples plus 3.3 times the standard deviation (SD) of the mean value and the LOQ as the mean measured content of 20 blank samples plus 10 times the SD of the mean value.

The purified parvalbumin preparations from eight species of fish and one species of amphibian (bullfrog) were subjected to the sandwich ELISA in the concentration range from 0.078 to 250 ng/ml. The concentration–response curve obtained for each parvalbumin was fitted to the four-parameter logistic equation, $y = \{[A - D]/[1 + (x/C)^B]\} + D$, where A is the maximum absorbance at infinite concentration, B is the curve slope at the inflection point, C is the concentration of analyte giving 50% response (RC_{50}) and D is the minimum absorbance for no analyte. The reactivity of a target parvalbumin relative to the Pacific mackerel parvalbumin was calculated by the following equation: reactivity (%) = $100 \times (RC_{50} \text{ of Pacific mackerel parvalbumin} / RC_{50} \text{ of target parvalbumin})$.

The precision of the sandwich ELISA was validated by intra- and inter-laboratory experiments using five model processed foods. In intra-laboratory experiments, repeatability was evaluated by the relative standard deviation (RSD) calculated from 10 replicates for each model processed food and daily reproducibility by the RSD from double analyses on 6 different days. Inter-laboratory experiments, in which three laboratories (Nissui Pharmaceutical Co., Ltd., Nippon Suisan Kaisha, Ltd., and Tokyo University of Marine Science and Technology) participated, were performed according to the method of Sakai et al. (2008). As an organizer, Nissui Pharmaceutical Co., Ltd. calculated a four-parameter logistic calibration curve based on the absorbance data of the standard solution and calculated the fish protein concentration in the test food sample. Repeatability and reproducibility RSDs were also calculated by the organizer.

3. Results

3.1. LOD and LOQ

Fig. 1 shows the calibration graph of the sandwich ELISA. A satisfactory correlation was recognised between 0.78 and 50 ng/ml. The sandwich ELISA LOD and LOQ were estimated to be 0.58 ng/ml (equivalent to 0.23 μg fish protein/g in food) and 1.76 ng/ml (equivalent to 0.70 μg/g), respectively. It is thus considered that fish protein can be practically determined in the range of 0.78–50 ng/ml (equivalent to 0.31–20 μg/g) by the sandwich ELISA.

3.2. Reactivity and specificity tests

The sandwich ELISA was first assessed for the reactivity to the parvalbumins purified from eight species of fish and one species

of amphibian (bullfrog). As shown in Table 1, the RC_{50} values ranged from 6.6 ng/ml (Pacific mackerel parvalbumin) to 551 ng/ml (bullfrog parvalbumin). Based on the RC_{50} values, the reactivity relative to the Pacific mackerel parvalbumin was estimated to be more than 22.6% for the parvalbumins of the seven species of fish. In contrast, the reactivity (1.2%) of the bullfrog parvalbumin was almost negligible.

In the next step, the sample solutions prepared from various kinds of food ingredients were tested for reactivity and specificity by sandwich ELISA. Cut-off for the reactivity of the sandwich ELISA was set to 1 µg/g, which is a little lower concentration than the threshold (a few micrograms of protein per millilitre of food or a few micrograms of protein per gram of food) of the Japanese labelling system (Akiyama et al., 2011). The results obtained with 22 kinds of fish, eight kinds of cephalopods and two kinds of fish roes are shown in Table 2. As expected, all the fish samples used afforded positive results, although the estimated values (4.0–46100 µg/g) remarkably differed from fish to fish in reflection of the fact that parvalbumin contents greatly vary among fish species (Kuehn, Scheuermann, Hilger, & Hentges, 2010; van Dô et al., 2005). Weakly positive results with a value range of 1.2–3.8 µg/g were obtained with five of the eight kinds of cephalopods and two kinds of fish roes. However, no false-positive results were observed in the remaining three kinds of cephalopods and also in the following 79 kinds of food ingredients: one kind of echinoderm (Japanese common sea cucumber *Stichopus japonica*), nine kinds of crustaceans (acorn barnacle *Balanus rostratus*, Antarctic krill *Euphausia superba*, kuruma prawn *Penaeus japonicus*, pink shrimp *Pandalus eous*, Japanese spiny lobster *Panulirus japonicus*, snow crab *Chionoecetes opilio*, Chinese mitten crab *Eriocheir sinensis*, king crab *Paralithodes camtschaticus* and mantis shrimp *Oratosquilla oratoria*), four kinds of gastropods (disk abalone *Haliotis discus discus*, blacklip abalone *Haliotis ruber*, turban shell *Turbo cornutus* and ivory shell *Babylonia japonica*) and nine kinds of bivalves (blue mussel *Mytilus galloprovincialis*, Yesso scallop *Patinopecten yessoensis*, Japanese oyster *Crassostrea gigas*, brackishwater clam *Corbicula japonica*, surf clam *Pseudocardium sachalinensis*, hen clam *Macra chinensis*, short-neck clam *Ruditapes philippinarum*, hard clam *Meretrix petechialis* and geoduck *Panopea japonica*), five kinds of seaweeds (sea mustard *Undaria pinnatifida*, kelp *Laminaria* sp., hiziki *Hizikia fusiforme*, mozuku *Nemacystus decipiens* and dry laver), hen's egg, cow's milk, gelatin, sesame, three kinds of meats (beef, chicken and pork), six kinds of vegetables (onion, tomato, cabbage, eggplant, spinach and yam), five kinds of fruits (orange, peach, apple, banana and kiwifruit), eight kinds of cereals (wheat, barley, rye, oat, rice, foxtail millet, barnyard millet and buckwheat), two kinds of mushrooms (common mushroom *Agaricus bisporus* and

hen of the woods *Grifola frondosa*), four kinds of beans (green bean, soybean, azuki bean and kidney bean), four kinds of nuts (peanut, walnut, cashew nut and almond), five kinds of teas (green tea, black tea, rose hip tea, oolong tea and coffee) and 10 kinds of spices (thyme, rosemary, paprika, cinnamon, nutmeg, cumin, curry powder, chile pepper, wasabi and oriental mustard).

3.3. Validation of sandwich ELISA

Performance of the sandwich ELISA was first evaluated by intra-laboratory experiments using the five model processed foods spiked with fish protein at a concentration of 10 µg/g. Regardless of the model food samples, fish protein was recovered at high levels (63.6–81.8% recovery) and both repeatability and daily reproducibility RSDs were low (below 9.5%) (Table 3). The sandwich ELISA was then subjected to inter-laboratory evaluation using the same model processed foods as used in the intra-laboratory experiments. As in the intra-laboratory experiments, the recovery of fish protein from the food samples was significantly high (69.4–84.8%) and the repeatability RSDs (1.2–3.3%) and reproducibility RSDs (3.1–10.5%) were also satisfactorily low (Table 3).

3.4. Determination of fish protein in commercial food products

Thirty-seven commercial processed food samples were analysed for fish protein by the sandwich ELISA. As shown in Table 4, fish protein was detected in 18 of the 21 processed foods with a label including fish in the ingredient list, although the concentration varied widely from 1.5 (tuna pasta sauce) to 1850 µg/g (chikuwa 2); false-negative results were displayed by fish sauce 1, shuto and collagen powder. On the other hand, 13 of the 16 food samples without a label including fish in the ingredient list showed negative results (<1.0 µg/g). However, three samples, Japanese rice cookie (squid), octopus fritter and pasta sauce (cod roe), gave false-positive results although at low concentrations (1.4–4.2 µg/g).

3.5. Reactivity to heated parvalbumin

To examine whether the reactivity of the sandwich ELISA is influenced by heating of parvalbumin, the fish protein powder was dissolved in water at a concentration of 0.6 mg/ml and heated (100 °C) or retorted (121 °C) for 5, 15, 30 or 60 min. After being cooled to room temperature, the solution was diluted to 5 ng/ml and subjected to the sandwich ELISA. As shown in Fig. 2, heating at 100 °C caused no marked reduction in reactivity; even after heating for 60 min, 85% of the original reactivity was recognized. In the case of heating at 121 °C, however, the reactivity was considerably reduced with the heating time and only 18% of the original reactivity remained after heating for 60 min.

4. Discussion

In the Japanese allergen-labelling system, two specific species of fish, mackerel and salmon, are recommended to be labelled. Considering this, we first attempted to establish a sandwich ELISA for the determination of mackerel using monoclonal antibodies against Pacific mackerel parvalbumin. However, this attempt was unsuccessful because no monoclonal antibodies with specific reactivity only to mackerel parvalbumin were obtained. In addition, no monoclonal antibodies universally recognising fish parvalbumins were also found, suggesting that it is impossible to determine fish protein by a sandwich ELISA with monoclonal anti-mackerel parvalbumin antibodies. Therefore, this study was aimed to develop a sandwich ELISA for the determination of fish protein using polyclonal antibodies against Pacific mackerel parvalbumin.

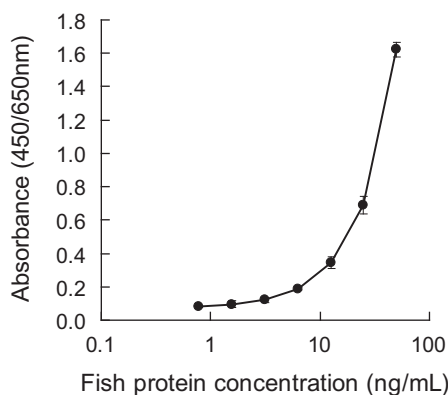


Fig. 1. Calibration graph for the sandwich ELISA method. Each datum is expressed as mean $\pm$ 3SD ($n=8$). The calibration curve was prepared by a four-parameter logistic analysis.

Table 3

Intra- and inter-laboratory evaluations of the sandwich ELISA method using five model processed foods spiked with 10 µg fish protein/g.

Food sample	Intra-laboratory evaluation			Inter-laboratory evaluation		
	Recovery of fish protein (%)	RSD (%)		Recovery of fish protein (%)	RSD (%)	
		Repeatability (n = 10)	Daily reproducibility (n = 6)		Repeatability (n = 3)	Reproducibility (n = 3)
Chicken meatball	73.5	6.0	9.5	71.7	2.4	4.7
Creamy croquette	63.6	8.1	9.0	69.4	2.3	4.9
Pork meatball dumpling	81.8	1.2	8.1	84.8	3.3	10.5
Vegetable and chicken soup	78.7	1.6	8.1	77.2	1.5	3.1
Rice gruel	78.8	2.4	2.4	79.4	1.2	4.5

Table 4

Determination of fish protein concentration in various commercial processed foods using the sandwich ELISA method.

Food sample	Fish protein concentration (µg/g)	Label of fish or fish product
Fish sauce 1	<1.0	Fish extract
Fish sauce 2	9.9	Fish
Shuto (salted tuna guts)	<1.0	Tuna
Collagen powder	<1.0	Fish collagen peptide
Seafood noodle	26.0	Fish meat, seafood extract
Kamaboko (steamed fish paste)	1640	Fish meat (cod, threadfin bream, red bigeye)
Chikuwa 1 (tubular kamaboko)	791	Fish meat (cod)
Chikuwa 2 (tubular kamaboko)	1850	Fish meat
Chikuwa 3 (tubular kamaboko)	524	Fish meat
Fish sausage 1	19.3	Fish meat (cod, Atka mackerel, threadfin bream)
Fish sausage 2	106	Fish meat (cod, Atka mackerel, sailfin sandfish)
Fish sausage 3	18.8	Fish meat (goatfish, Atka mackerel, cod)
Fish sausage 4	798	Fish meat (lizardfish, Atka mackerel)
Base of soup	9.9	Dried bonito flake (powder)
Dried bonito flake	127	Bonito flake
Furikake (rice seasoning)	399	Salmon extract, salmon, dried bonito flake
Canned fish 1	17.6	Atlantic salmon
Canned fish 2	257	Mackerel
Canned fish 3	11.3	Yellowfin tuna
Pasta sauce (tuna)	1.5	Boiled tuna
Rice gruel	163	Red salmon, salmon extract
Cookie	<1.0	Non-labelled
Snack (shrimp)	<1.0	Non-labelled
Japanese rice cookie (shrimp)	<1.0	Non-labelled
Japanese rice cookie (squid)	1.4	Non-labelled
Octopus fritter	2.3	Non-labelled
Canned crab	<1.0	Non-labelled
Hamburg steak	<1.0	Non-labelled
Dressing	<1.0	Non-labelled
Beef curry	<1.0	Non-labelled
Worcester sauce	<1.0	Non-labelled
Dumpling	<1.0	Non-labelled
Croquette	<1.0	Non-labelled
Pasta sauce (carbonara)	<1.0	Non-labelled
Pasta sauce (cod roe)	4.2	Non-labelled
Fried food (noodle)	<1.0	Non-labelled
Fried food (oyster)	<1.0	Non-labelled

To determine fish protein in foods, a sandwich ELISA method with a polyclonal anti-cod parvalbumin has recently been developed by Fæsta and Plassen (2008). However, in view of the thresh-

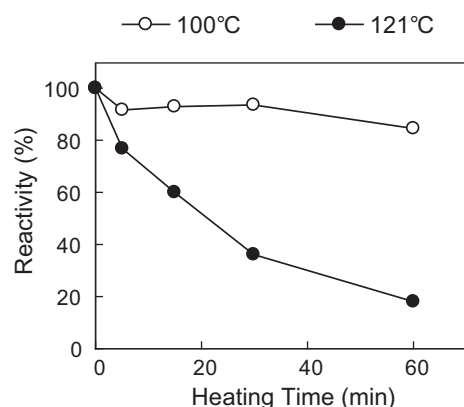


Fig. 2. Reactivity of the sandwich ELISA to heated or retorted fish parvalbumin. The fish protein powder was dissolved in water at a concentration of 0.6 mg/ml, heated (100 °C) or retorted (121 °C) and subjected to the sandwich ELISA.

old (a few micrograms of protein per millilitre of food or a few micrograms of protein per gram of food) of the Japanese labelling system (Akiyama et al., 2011), the LOQ (5 µg/g) reported for the developed ELISA is rather insufficient. It should be also noted that the ELISA method appears to be unsuitable for a considerable number of fish, since it gave extremely low recoveries (less than 1%) in as many as 12 of the 32 kinds of fish tested. As compared to the method of Fæsta and Plassen, our sandwich ELISA method is apparently more sensitive and specific. The LOQ of our method is 0.70 µg/g, satisfying the sensitivity required for the current Japanese food labelling system. Furthermore, our method exhibited high reactivity (more than 22.6% reactivity relative to Pacific mackerel parvalbumin) to parvalbumins from seven kinds of fish other than Pacific mackerel and also gave positive results in all 22 kinds of fish examined, suggesting the specificity of our method for a number of fish.

In addition to the sufficient sensitivity and specificity described above, intra- and inter-laboratory evaluations using the five model processed foods demonstrate our sandwich ELISA method to be highly precise and reliable. The Japanese government MHLW established the inter-laboratory validation protocol in the official guidelines published in 2006 (Akiyama et al., 2011). The outline of the inter-laboratory validation protocol is as follows: number of laboratories, ≥ 8 ; number of incurred samples, ≥ 5 ; number of dose level, ≥ 1 , including 10 µg/g (allergen protein weight/food weight); recovery, 50–150%; reproducibility RSD, $\leq 25\%$. Importantly, our sandwich ELISA satisfies all the validation criteria in the official guidelines, with the only exception that the number (three) of laboratories participated in our inter-laboratory experiments is small. It is thus considered that our sandwich ELISA is a precise and reliable method to determine fish protein in processed foods. Indeed, this study provided experimental evidence that our

ELISA is applicable to various commercial processed foods as discussed below in more detail, although the sandwich ELISA developed by Fæsta and Plassen (2008) remains to be clarified for applicability to commercial processed foods.

Commercial processed foods are generally exposed to severe conditions (e.g., heating, retorting and fermentation) during processing. Nevertheless, the developed sandwich ELISA could measure fish protein in 18 of the 21 processed foods that are labelled to contain fish. Parvalbumin is a thermostable protein, its IgE-binding ability being not significantly reduced by heating (Elsayed & Aas, 1971b). Similarly, our model experiments using the fish protein powder showed that fish parvalbumin is sufficiently detected by the sandwich ELISA even after heating at 100 °C. This is an explanation for the positive results with all the 12 kinds of heated foods (seafood noodle, kamaboko, three chikuwas, four fish sausages, base of soup, dried bonito flakes and furikake) that are labelled to include fish. Since it has previously been suggested that parvalbumin can be mostly removed by washing during processing of surimi (material of fish meat paste products) (Hamada, Genka, Ohira, Nagashima, & Shiomi, 2000), it should be discussed why the determined concentrations of fish protein (18.8–1850 µg/g) in the eight kinds of fish meat paste products (kamaboko, three chikuwas and four fish sausages) significantly differ from product to product. One possibility is that the amount of parvalbumin removed from the fish meat paste greatly varies depending on the washing condition employed by each supplier. Another possibility is that unwashed fish meat paste, together with washed fish meat paste, may have been used as a raw material in several fish meat paste products with high levels of parvalbumin.

Besides the above heated foods, all the five kinds of retorted foods (three canned fishes, pasta sauce and rice gruel) with a label including fish were also shown to contain fish protein by our sandwich ELISA. In our model experiments using the fish protein powder, the reactivity of the sandwich ELISA to fish parvalbumin was significantly reduced with the time of retorting (heating at 121 °C). Consequently, fish protein concentration is assumed to have been underestimated in the five retorted foods. This assumption seems to be realised by the results with the canned fish samples. Although the canned fish samples are mostly composed of fish meat, their fish protein concentrations are considerably low (11.3–257 µg/g). Similarly, reduced allergenicity of the canned fishes has previously been demonstrated (Bernhisel-Broadbent, Strause, & Sampson, 1992).

It is particularly worth mentioning that the reactivity of the sandwich ELISA was preliminarily found to be reduced to a large extent (74% reduction) in the presence of EDTA compared to that in the absence of EDTA. This indicates that the polyclonal anti-Pacific mackerel parvalbumin antibody used in our ELISA recognises Ca²⁺-associated conformational-type epitopes of parvalbumin much more strongly than linear-type epitopes, as previously observed with parvalbumin-specific IgE (Tomura, Ishizaki, Nagashima, & Shiomi, 2008). During retorting, therefore, fish parvalbumin might undergo conformational changes rather than degradation. It is also likely that heating at 100 °C causes no remarkable change in Ca²⁺-associated conformation of fish parvalbumin.

Our sandwich ELISA could not measure fish protein in two kinds of fermented foods (fish sauce 1 and shuto) and collagen powder, although a low concentration of fish protein (9.9 µg/g) was detected in fish sauce 2. These false-negative results are not strange. In the case of fermented foods, parvalbumin, together with other fish proteins, can be severely degraded into peptides and amino acids by protease digestion during fermentation, resulting in the loss of reactivity with anti-parvalbumin antibodies. Protease digestion is generally known as an effective way to reduce the allergenicity of foods (Kobayashi, Hashimoto, Taniuchi, & Tanabe, 2004;

Shimakura, Tonomura, Hamada, Nagashima, & Shiomi, 2005). On the other hand, the fish collagen powder is assumed to be not contaminated with parvalbumin. Collagen is distinguishable from parvalbumin in chemical properties; for example, collagen is soluble in acetic acid but sparingly soluble in water, while parvalbumin is readily soluble in water. Accordingly, parvalbumin appears to have been almost completely removed from collagen fractions during refining of the fish collagen powder.

Of the food ingredients tested by our sandwich ELISA, five kinds of cephalopods and two kinds of fish roes displayed false-positive results. In agreement with this, false-positive results were recognised in three kinds of commercial processed foods, Japanese rice cookie (squid), octopus fritter and pasta sauce (cod roe), which include either cephalopod or fish roe but not fish. Parvalbumin is contained only in vertebrates. It is thus relevant to consider that the anti-parvalbumin antibody used in our sandwich ELISA non-specifically react to an unknown protein in cephalopods and fish roes that partly shares a similar amino acid sequence with fish parvalbumins.

In conclusion, the sandwich ELISA developed in this study is a reliable, specific, and sensitive method for the determination of fish protein in processed foods. In EU and USA, fish is listed as one of allergenic ingredients in the labelling system. Therefore, our sandwich ELISA might be utilised to verify the labelling of fish in EU and USA. On the other hand, two species of fish, mackerel and salmon, are the subjects of labelling in Japan. In relation to this, we have recently established a qualitative salmon-specific polymerase chain reaction (PCR) method (Ishizaki et al., 2012). A combination of this salmon-specific PCR method with the developed sandwich ELISA will make it possible to determine salmon protein in processed foods. To ensure the Japanese labelling system for fish, a mackerel-specific PCR method is also under development.

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