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A sandwich ELISA for the detection of fish and fish products

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ABSTRACT

Fish is classified as one of the major allergenic foods. However, a reliable assay for detecting the presence of fish protein is not available. In this study, a sandwich enzyme-linked immunosorbent assay (sELISA) was developed using polyclonal antibodies raised against a 36 kDa thermal-stable fish muscle protein. The sELISA was able to detect both raw and cooked (100 °C, 8 min) fish samples of all 63 common species tested without any cross-reaction with non-fish samples. The assay can also detect fish products with different processing (salting, smoking and canning). The detection limit of the assay was 0.1 ppm for both raw and cooked fish (whiting, pollock and basa) in crab meat. The assay exhibited low intra- (CV $\leq$ 8.9%) and inter-assay variability (CV $\leq$ 9.3%). The rates of false-positive and false-negative were 0%. This fish-specific sELISA is, therefore, an effective assay for the detection of fish muscle protein in food for the protection of fish-allergic individuals.

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1. Introduction

Fish is known to be one of the major allergenic foods that cause allergic reactions. In the Unites States, fish were responsible for 0.1% and 0.4% of food allergies in children and adults, respectively (Sicherer, Muñoz-Furlong, & Sampson, 2004). One in 32 deaths listed in a national registry of food-induced fatal anaphylaxis was caused by fish (Bock, Muñoz-Furlong, & Sampson, 2001). To protect sensitized people from the severe consequences of their food allergy, the Food Allergen Labeling and Consumer Protection Act (FALCPA) requires any protein-containing ingredient derived from the eight major allergen foods (fish, crustacean shellfish, eggs, milk, tree nuts, peanuts, soybeans, and wheat) to be declared in the ingredient statement on the label of foods (FALCPA, 2004). However, no effective method has been developed to detect the presence of fish in food products. Besides the allergy concerns related to fish in human food, fishmeal, a common animal feed ingredient, has been banned for use in ruminant feed in Australia, and European Union to reduce the risk of prion disease, bovine spongiform encephalopathy (BSE) (EC 999/2001, 2001). Although no evidence has been found to indicate that fishmeal could cause BSE, during processing and handling fishmeal could be contaminated by a low amount of banned ruminant protein which was identified as the culprit to spread the BSE in animal feed (Andreoletti et al., 2007).

At present, the only officially approved method for fish detection in animal feed is microscopy examining muscle fibers and bones, which requires specially trained personnel and expensive equipment (2003/126/EC, 2003). Although a number of DNA-based techniques (Rasmussen & Morrissey, 2008) and protein-based immunoassays using antibodies for the identification of specific fish species such as grouper, wreck fish, Nile perch (Asensio et al., 2003), red snapper (Huang, Marshall, Kao, Otwell, & Wei, 1995) and pangasius catfish (Gajewski, Chen, & Hsieh, 2009) have been reported, none are able to cover all food fish species for the purpose of reducing fish allergy and/or BSE risks. Fæste and Plassen (2008) developed a polyclonal antibody (pAb) based sandwich enzyme-linked immunosorbent assay (sELISA) to detect a major fish allergenic protein, parvalbumin, in food but the results showed non-specific reactivity and the assay was not able to detect parvalbumin in all the fish samples tested. Shibahara, Uesaka, Wang, Yamada, and Shiomi (2013) also developed another pAb-based sELISA for the detection of fish parvalbumin in processed food. However, their results showed non-specific cross-reactivity to cephalopod samples. At present, no effective rapid immunoassays have yet been developed that are capable of detecting fish protein in either food or feedstuffs. Since a reliable convenient assay for the detection of all common fish in food or feed is urgently needed for the protection of fish allergic individuals and for the animal health, the present study was designed to address this lack by developing a user-friendly sELISA capable of detecting all commonly consumed fish in raw or processed food and feed materials.

All reported immunoassays developed for the fish allergy concerns targeted the major fish allergen, parvalbumin, as the marker protein to represent the presence of fish tissue in food. The major

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Abreactions:

ABTS	2, 2-azino-di-[3-ehyl-benothiazoline-6-sulfonic acid]
BCIP/NBT	5-bromo-4-chloro-3-indolyl phosphate/p-nitroblue tetrazolium chloride
BSE	bovine spongiform encephalopathy
CV	coefficient of variation
FALCPA	Food Allergen Labeling and Consumer Protection Act
HRP	horseradish peroxidase
Ig	immunoglobulin
LOD	limit of detection
pAb	polyclonal antibody
PBS	phosphate buffered saline
PBST	PBS containing 0.05% Tween 20
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sELISA	sandwich enzyme-linked immunosorbent assay
TBS	Tris buffered saline
TBST	Tris buffered saline containing 0.05% Tween 20

drawback was that the amount of parvalbumin varies significantly among different fish species and in different parts of the muscle (Kobayashi et al., 2006; Kuehn, Scheuermann, Hilger, & Hentges, 2010; Rehbein & Kündiger, 1984; Van Do, Elsayed, Florvaag, Hordvik, & Endresen, 2005). In this study, we alternatively developed a sELISA targeting a 36 kDa fish muscle protein. This 36 kDa protein was previously identified as a major thermal-stable and antigenic protein recognized by two cross reactive-antifish monoclonal antibodies (F1G11 and F7B8) in cooked fish muscle (Gajewski et al., 2009).

2. Materials and methods

2.1. Materials

NHS-activated agarose resin and sulfo-NHS-biotin, were purchased from Thermo Fisher Scientific (Rockford, IL, USA). The horseradish peroxidase (HRP)-conjugate goat anti-rabbit immunoglobulin (Ig), 2, 2-azino-di-[3-ehyl-benothiazoline-6-sulfonic acid] (ABTS), EZ Blue™ Gel staining, and HRP-conjugated streptavidin were purchased from Sigma–Aldrich (St. Louis, MO, USA). The nitrocellulose membranes, goat anti-rabbit IgG alkaline phosphatase conjugated antibody, 5-bromo-4-chloro-3-indolyl phosphate/p-nitroblue tetrazolium chloride (BCIP/NBT), Protein Assay Kit II and Precision Plus Protein Kaleidoscope Standards were purchased from Bio-Rad (Hercules, CA, USA). All other chemicals were purchased from Fisher Scientific (Waltham, MA, USA). All chemicals and reagents used in this study were analytical grade.

2.2. Preparation of protein extracts

Fish samples used in the study were either provided by State government agencies or purchased from reliable seafood retail chains and domestic seafood markets. The shellfish and land animal samples and processed fish products were purchased from local food stores. To prepare the protein extracts of samples, fresh lean muscle tissue of selected animal species was minced and a portion of 5 g was weighted into each beaker. For the preparation of cooked samples, the minced raw muscle in the beaker was covered by aluminum foil and heated in a water bath (100 °C) for 8 min. Five vol. (v/w) 0.15 M NaCl solution was added to the beaker containing minced raw or cooked muscle samples. Both raw and cooked samples were homogenized 1 min at 13,000 rpm by ULTRA-TURRAX T25 basic homogenizer (IKA Works, Wilmington, NC, USA), placed at 4 °C for 2 h and then centrifuged (10,000 × *g* at 4 °C, 30 min). Supernatant was then filtered through Whatman No. 4 filter paper (Whatman, Piscataway, NJ, USA). The protein concentration was determined by Protein Assay Kit II. The clear protein extracts were stored at −20 °C until use.

2.3. Production of pAb for fish detection

The immunogen for the development of the pAb is partially purified fish 36 kDa protein. This 36 kDa protein for immunization was isolated as follows. Equal amount of fresh fish muscle from each species was taken from Atlantic salmon, yellowfin tuna, swordfish, black grouper, tilapia, red snapper, amberjack, basa, catfish, and perch. The fish muscle was minced together and placed in a beaker then heated in a water bath (100 °C) for 15 min. Cooked fish samples were homogenized for 1 min at 13,000 rpm with 5 vol. (v/w) 0.15 M sodium chloride solution, placed at 4 °C for 2 h and then centrifuged (20,000 × *g* at 4 °C, 30 min) and filtered through Whatman No. 4 filter paper. The clear supernatant was subjected to isoelectric precipitation at pH 4.5 with 1 N hydrogen chloride. After centrifugation at 20,000 × *g* for 20 min the pellet was brought to pH 7.6 with 1 N sodium hydroxide, and subjected to ammonium sulfate fractionation, yielding a 50–60% saturated fraction. These procedures were repeated twice, and the final fraction dialyzed against phosphate buffered saline (PBS) was used as the immunogen. A polyclonal antiserum was raised in New Zealand rabbits by immunizing the animal with 200 μg isolated fish 36 kDa protein in 200 μl PBS emulsified with Freund's complete adjuvant (1:1 v/v). After the second and third boosts with 100 μg fish 36 kDa protein in PBS mixed with Freund's incomplete adjuvant (1:1 v/v), blood from the rabbits was collected at the 7th week from initial immunization. The IgG in the pooled antisera was collected by Protein-A affinity chromatography and diluted in PBS. The total protein concentration of purified IgG was determined by the UV method using Smartspec™ 3000 UV spectrophotometer (Bio-Rad) at 280 nm and was stored in aliquots (5 mg/ml) at −80 °C until use.

2.4. Affinity depletion of the pAb IgG

To remove any non-specific cross-reactivity from the pAbs, crude protein extracts of cooked pork and chicken were prepared for the affinity depletion. The protein extracts of cooked pork and chicken was prepared as described previously. A 4-ml sample of the meat protein extract was then mixed with 330 mg of NHS-activated agarose resin in a tube at 4 °C overnight. The NHS-activated resin was packed in a spin column and washed 10 times by 4 ml PBS. After washing, the NHS-activated resin was quenched by 4 ml 1 M Tris buffer at room temperature for 20 min. After a further washing with 4 ml PBS, 2 ml purified pAb IgG (5 mg/ml) in PBS was loaded into the 10-ml column and mixed at 4 °C overnight. After mixing, the spin column was centrifuged at 1,000 × *g* for 1 min at 4 °C. The flow-through antibodies in the solution were then collected. The depletion procedures were repeated twice to adequately eliminate the non-fish species cross-reactivity. The concentration of depleted IgG was determined by Smartspec™ 3000 UV spectrophotometer (Bio-Rad) at 280 nm. The depleted IgG was stored in aliquots (1 mg/ml) at −80 °C until use.

2.5. Biotinylation of the depleted pAb

The depleted IgG was conjugated with biotin using sulfo-NHS-biotin according to the manufacture's protocol. A volume of 3.7 μl