



Effect of species, muscle location, food processing and refrigerated storage on the fish allergens, tropomyosin and parvalbumin

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ABSTRACT

Fish tropomyosin is a latest identified fish allergen without full understanding of its biochemical characteristics from the perspective of food allergen. Accordingly, the objective of this study was to investigate the effects of species, muscle location, food processing, and refrigerated storage on fish tropomyosin and compare with main fish allergen, parvalbumin. The result of mass spectrometry analysis revealed tropomyosin as the most abundant thermally stable protein in fish muscle. Fish tropomyosin was ubiquitous among all 28 edible fish species tested, abundant in fish skeletal muscle, resistant to common food processing, and resistant to refrigerated storage up to six days. By contrast, parvalbumin content varied between fish species and was not as thermally stable as tropomyosin under autoclaving. This study demonstrates the intrinsic and processing factors affecting fish allergens and provides valuable information for the presence of major fish allergens and practical consideration of fish allergen detection.

1. Introduction

Food allergy constitutes a crucial topic in food safety and public health. The World Allergy Organization indicated that 2–2.5 billion people have food allergies (Pawankar, 2014). The eight major food allergens, causing over 90 % of allergic reactions, are fish, shellfish, milk, eggs, wheat, peanuts, tree nuts, and soybeans (FDA, 2004). The prevalence of fish allergies in the United States is 0.48 % in adults and 0.22 % in children (Sicherer et al., 2004). In the European Union, the prevalence of fish allergies is 0.1 %–3% depending on the country (Stephen et al., 2017). Currently, avoiding the consumption of food allergens is the only means through which people with food allergies can avoid allergic reactions.

Parvalbumin, a 10–15 kDa sarcoplasmic protein with multiple isoforms, is the first fish allergen identified (Elsayed & Aas, 1971; Stephen et al., 2017). It has been identified as an allergen in Atlantic herring, grass carp, common carp, Baltic cod, Atlantic cod, barramundi, megrim, chum salmon, rainbow trout, Mozambique tilapia, striped catfish, Indian mackerel, Atlantic salmon, Pacific pilchard, Atlantic mackerel, ocean perch, yellowfin tuna, and swordfish (WHO/IUIS Allergen Nomenclature Sub-Committee, 2022). Fish collagen is another allergen

in muscle connective tissue (Hamada et al., 2001). However, fish collagen was degraded after heating, and lost its allergenicity (Hamada et al., 2001). Furthermore, multiple enzymes including creatine kinase, triosephosphate isomerase, glyceraldehyde-phosphate dehydrogenase, enolase, and aldolase recognized by IgE from the individuals with fish allergy (Kuehn et al., 2013; Liu et al., 2011; Nakamura et al., 2009; Ventel et al., 2011). Nevertheless, these enzymes are not thermally stable.

Tropomyosin is a myofibrillar protein and a major allergen in crustaceans and mollusks (Ruethers et al., 2018). Vertebrate tropomyosin was not considered to be allergenic. In recent years, fish tropomyosin has been identified as an allergen in tilapia (Liu et al., 2013) and then in cod, albacore, and swordfish (González-Fernández et al., 2018). Previous studies on fish tropomyosin have primarily focused on its physiological function in the regulation of muscle contraction and on the characteristics of its molecular structure (Reese et al., 1999). At present, fish tropomyosin was considered as a novel fish allergen and should be applied to fish allergy diagnosis along with fish parvalbumin (Ruethers et al., 2021). Therefore, study the characteristics of fish tropomyosin as a food allergen is warranted.

Immunoassays detect analytes through antibody–antigen interaction

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and has been widely used in industrials and research fields. Currently, the commercial enzyme-linked immunosorbent assay (ELISA) kits for the detection of fish have a detection rate of 26 % to 61 % (Ruethers et al., 2020). The main limitation of the development of immunoassay for fish detection could be a lack of understanding of the characteristics of fish allergens. Genetic differences across the wide variety of fish species lead to variation in the expression and characteristics of allergenic proteins. Studies have revealed that fish parvalbumin is low in specific fish species such as swordfish and tuna (Saptarshi et al., 2014). Although food allergenic proteins are often thermally stable, the thermal stability of fish allergens under different food processing needs further investigation, especially the processing with high temperature and pressure such as autoclaving for canned fish. In addition, the allergenic protein may not be equally distributed in the muscle. The study indicated that expression of parvalbumin decreases from anterior to posterior portions in tuna, mahi-mahi, catfish, and carp (Lee et al., 2012). Studies have reported the occurrence of muscle protein degradation during postmortem (Bao et al., 2020; Terova et al., 2011). However, the effect of refrigerated storage on fish allergenic proteins is still unknown.

The immunoreactivity of fish allergens in muscle could be affected by intrinsic factors such as species and muscle location. Degradation of fish allergens during food processing and refrigerated storage could also play an important role in the same matter. Information on the effects of these intrinsic and processing factors on fish allergens is critical for detecting fish and protecting sensitized individuals. Therefore, the objective of the study was to obtain information relevant for understanding how species, muscle location, food processing, and refrigerated storage affect the fish tropomyosin and compare with parvalbumin.

2. Materials and methods

2.1. Materials

The following fish species were used in this study: great barracuda (*Sphyrna barracuda*), orange spotted grouper (*Epinephelus coioides*), basa (*Pangasius bocourti*), yellow back sea bream (*Dentex hypselosomus*), Spanish mackerel (*Scomberomorus commerson*), fourfinger threadfin (*Eleutheronema tetradactylum*), milk fish (*Chanos chanos*), seabass (*Lateolabrax japonicus*), Pacific mackerel (*Scomber australasicus*), bonito (*Sarda orientalis*), Pacific rudderfish (*Psenopsis anomala*), eel (*Anguilla japonica*), Nile tilapia (*Oreochromis niloticus*), flying fish (*Exocoetus volitans*), red-speckled parrotfish (*Cetoscarus ocellatus*), amberjack (*Seriola dumerili*), Pacific saury (*Cololabis saira*), beltfish (*Trichiurus lepturus*), rabbitfish (*Siganus fuscescens*), Atlantic mackerel (*Scomber scombrus*), yellowfin tuna (*Thunnus albacares*), Atlantic cod (*Gadus morhua*), Atlantic halibut (*Hippoglossus hippoglossus*), swordfish (*Xiphias gladius*), sweetfish (*Plecoglossus altivelis*), blue shark (*Prionace glauca*), Atlantic salmon (*Salmo salar*), rough leather jacket (*Aluterus monoceros*). The fish muscle samples used in the study were from deceased fishes that were purchased from local seafood markets or supermarkets. The fish muscle samples were stored at -20°C until use.

2.2. Preparation of heated fish extracts

For the preparation of the heated fish extract, 5 g of fresh fish muscle was placed in a beaker covered by aluminum foil and heated for 10 min in a boiling water bath. After heat treatment, 0.15 M NaCl in 1:5 (v/w) ratio was added as an extraction buffer, and then homogenized at 30,000 rpm by using T-10 homogenizer (IKA, Staufen, Germany). The homogenized extract was kept at 4°C for 2 h and centrifuged at $10,000 \times g$ for 30 min at 4°C . The supernatant was filtered using Grade No. 4 filter paper (Whatman, Maidstone, United Kingdom) and stored at -20°C until analysis.

2.3. Two dimensional gel electrophoresis

Heated fish muscle extracts were prepared from Atlantic cod (*Gadus morhua*). The extraction buffer used was 8 M urea with 0.5 % Triton X-100 and followed the same manner as described above. A 500- μg sample of protein extract was applied to a 13-cm Immobiline DryStrip pH 3–10 nonlinear strip gel (GE Healthcare Life Science, Chicago, IL), and the proteins were separated through isoelectric focusing for 45,000 Vhr using an Ettan IPGphor 3 IEF system (GE Healthcare Life Science, Chicago, IL). After equilibrating with 65 mM dithiothreitol and 135 mM iodoacetamide, the strip gel was loaded on a 12 % SDS-PAGE gel for second-dimensional separation at 350 V by Hoefer SE600 (GE Healthcare Life Science, Chicago, IL). The gel was visualized with Bio-Safe™ Coomassie Stain (Bio-Rad, Hercules, CA).

2.4. Protein identification

The protein identification was performed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer system (Waters Corp, Mailford, MA). The major protein spots were excised and then digested with sequencing grade modified porcine trypsin (Promega, Madison, WI) at 37°C for 16 h. The digested peptides were analyzed using a quadrupole time-of-flight mass spectrometer (Waters Corp, Mailford, MA). The identification analyses were performed using ProteinLynx Global SERVICETM 3.0.3 (Waters Corp, Mailford, MA). The identified tandem mass spectrometry (MS/MS) spectra were searched against protein sequences in UniProt database.

2.5. Sampling of fish muscle in different locations

The fish species, Nile tilapia (*Oreochromis niloticus*) and orange spotted grouper (*Epinephelus coioides*), were selected as samples. The whole fish body was divided into eight parts for comparing muscle protein profiles in different locations. The fish body was equally separated into four parts from anterior to posterior. These four parts were further separated into dorsal and ventral sides based on anteroposterior axis.

2.6. Sample preparation through different food processing

To study effects of different food processing, the muscle samples from the same muscle location (D2) of Nile tilapia (*Oreochromis niloticus*) and orange spotted grouper (*Epinephelus coioides*) were cut into 5-g chunks. Heat treatments included boiling, baking, frying, steaming, and autoclaving. Boiled fish samples were treated in 100°C boiling water for 5, 10, 20 and 40 min. Steamed fish chunks were heated over a boiling water bath in a covered beaker for 5 and 10 min. Baked fish samples were heated in a commercial oven at 200°C for 6 and 12 min. Fried fish samples were heated in 140°C vegetable oil for 1.5 and 3 min. The autoclaving process was performed at 121°C and 18 psi for 20 min. The heat treatments were followed by protein extraction as described in Section 2.2.

2.7. Sample preparation for studying effect of refrigerated storage

The deceased Nile tilapia (*Oreochromis niloticus*) were purchased from the local seafood market that sold daily slaughtered fishes from the seafood processing plant. The muscle samples (10 g) from same muscle location were vacuum sealed in plastic bags by vacuum sealer system (Foodsaver, Atlanta GA) and refrigerated at 4°C up to 6 days. The samples were divided into 7 groups to be refrigerated from Day 0 to Day 6. Protein extraction was performed for each group as described in Section 2.2 in day 6. The protein extracts were stored at -20°C until further analysis.

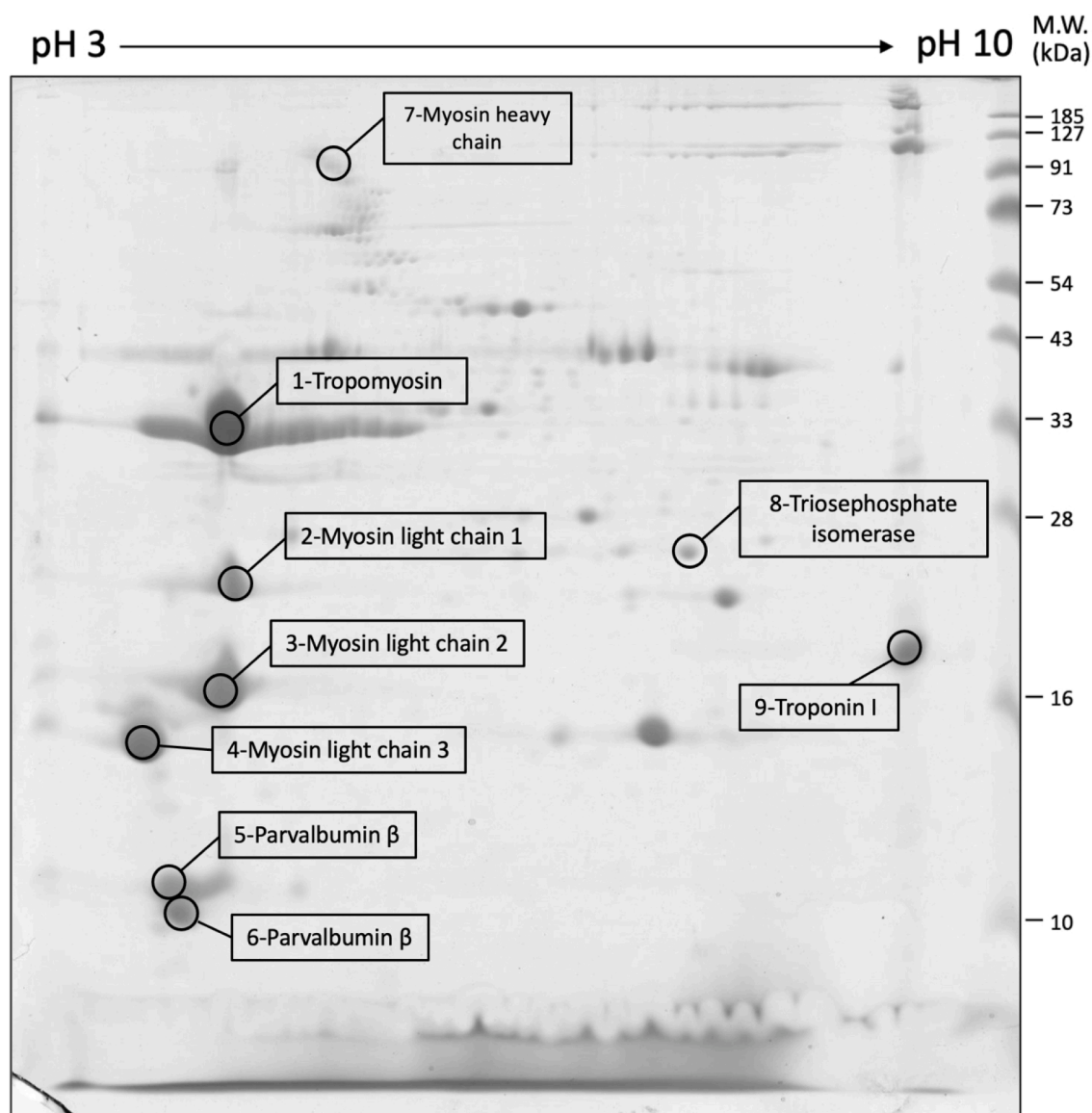


Fig. 1. Two dimensional gel electrophoresis of heated Atlantic cod (*Gadus morhua*) muscle extract. The circled spots were identified proteins and the identities.

2.8. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blot

A fixed amount (6 µg/lane) of protein extract was loaded on a 12 % SDS-PAGE gel and separated at 100 V for 1 h. The gel was stained by Bio-Safe™ Coomassie Stain (Bio-Rad, Hercules, CA). The transfer was performed at 300 mA for 1 h. After transfer, the 0.45 µm PVDF membrane (Merk, Darmstadt, Germany) with proteins was blocked by Tris-based saline (TBS) with 5 % bovine serum albumin (BSA) fraction V (Sigma, St. Louis, MO) at room temperature (RT) for 1 h. The anti-tropomyosin antibody (TM311, Abcam, Cambridge, United Kingdom) and anti-parvalbumin antibody (Parv-19, Sigma, St. Louis, MO) was diluted 1:2,000 and 1:1,000 in TBS with 1 % BSA respectively, and applied to membranes at RT for 1 h. After washing three times by TBS with 0.05 % Tween 20 (TBST), the goat anti-mouse alkaline phosphatase conjugated antibody (Abcam, Cambridge, United Kingdom) diluted 1: 5,000 in TBS with 1 % BSA was applied to the membrane at RT for 1 h. After washing three times by TBST, color development was performed using an Immun-blot AP kit (Bio-Rad, Hercules, CA).

2.9. Indirect ELISA

Two micrograms of protein in carbonate buffer was coated on a 96-well microplate (Corning, Corning, NJ) at 37 °C for 1 h and blocked by phosphate-buffered saline (PBS) with 5 % BSA (Sigma, St. Louis, MO) at 37 °C for 1 h. The anti-tropomyosin antibody (TM311, Abcam, Cambridge, United Kingdom) and anti-parvalbumin antibody (Parv-19, Sigma, St. Louis, MO) was diluted 1:10000 and 1:1000 in PBS with 0.05 % Tween 20 (PBST), respectively. One hundred microliters of diluted primary antibody was added to the wells and incubated at 37 °C for 1 h. After washing three times with PBST, the goat anti-mouse horseradish peroxidase conjugated antibody (Abcam, Cambridge, United Kingdom) diluted 1:20,000 in PBST was added and incubated at 37 °C for 1 h. After incubation and washing, the results were developed by Turbo TMB (Thermal Fisher, Waltham, MA) and read at 415 nm using a spectrophotometer (BMG Labtech, Ortenberg, Germany). All absorbance data were obtained in triplicate.

Table 1

Summary of the identification of thermally stable proteins in Atlantic cod (*Gadus morhua*) muscle extract.

Spot No.	Accession No.	Protein Name	Theoretical Molecular Weight (kDa)	Theoretical pI value
1	Q8AV86	Tropomyosin	32.618	4.551
2	Q9IB19	Myosin light chain 1	21.240	4.506
3	Q9DEA8	Myosin light chain 2	19.038	4.437
4	Q9DEA9	Myosin light chain 3	17.216	4.143
5	A5I873	Parvalbumin beta	11.406	4.377
6	P86739	Parvalbumin beta	10.341	4.248
7	P87344	Myosin heavy chain	128.547	5.045
8	A0A0R4IGG3	Triosephosphate isomerase	26.809	7.124
9	Q8QG69	Fast skeletal muscle troponin I	18.939	9.117

Table 2

Analysis of tropomyosin and parvalbumin by indirect ELISA with heated fish muscle extracts from 28 common edible fish species.

Fish species		Immunoreactivity ^a	
Market name	Scientific name	Tropomyosin	Parvalbumin
Great barracuda	<i>Sphyrna barracuda</i>	+++	+++
Orange spotted grouper	<i>Epinephelus coioides</i>	+++	+++
Basa	<i>Pangasius bocourti</i>	+++	+++
Yellow back sea bream	<i>Dentex. hypselosomus</i>	+++	+++
Spanish mackerel	<i>Scomberomorus commerson</i>	+++	+++
Fourfinger threadfin	<i>Eleutheronema tetradactylum</i>	+++	+++
Milk fish	<i>Chanos chanos</i>	++	+++
Seabass	<i>Lateolabrax japonicus</i>	+++	+++
Pacific mackerel	<i>Scomber australasicus</i>	++	+++
Bonito	<i>Sarda orientalis</i>	+++	+++
Pacific rudderfish	<i>Psenopsis anomala</i>	+++	++
Eel	<i>Anguilla japonica</i>	+++	++
Nile tilapia	<i>Oreochromis niloticus</i>	+++	++
Flyingfish	<i>Exocoetus volitans</i>	+++	++
Red-speckled parrotfish	<i>Cetoscarus ocellatus</i>	+++	++
Amberjack	<i>Seriola dumerili</i>	+++	++
Pacific saury	<i>Cololabis saira</i>	+++	++
Beltfish	<i>Trichiurus lepturus</i>	+++	++
Rabbitfish	<i>Siganus fuscescens</i>	+++	+
Atlantic mackerel	<i>Scomber scombrus</i>	+++	+
Yellowfin tuna	<i>Thunnus albacares</i>	+++	+
Atlantic cod	<i>Gadus morhua</i>	+++	-
Atlantic halibut	<i>Hippoglossus hippoglossus</i>	+++	-
Swordfish	<i>Xiphias gladius</i>	+++	-
Sweetfish	<i>Plecoglossus altivelis</i>	+++	-
Blue shark	<i>Prionace glauca</i>	+++	-
Atlantic salmon	<i>Salmo salar</i>	+++	-
Rough leather jacket	<i>Aluterus monoceros</i>	+++	-

^aImmunoreactivity was examined by indirect ELISA using the anti-tropomyosin antibody (TM-311) and anti-parvalbumin (Parv-19). Heated fish Sample extracts were coated at 2 µg/well in microplates. Absorbance readings over 0.2 were considered "positive"; those <0.2 were considered negative. The absorbance reading: <0.2: "-"; 0.2–0.6: "+"; 0.6–1.0: "++"; ≥1.0: "+++".

3. Results and discussion

3.1. Identification of thermally stable muscle proteins

Muscle is the main edible portion of fish and often heated before

consumption. In the study, the proteomics analysis was applied to reveal the identities of major thermally stable proteins. Two-dimensional gel electrophoresis and MALDI-TOF were performed to reveal the identities of thermally stable proteins and allergen composition of heated fish muscle. The main challenge of fish muscle protein identification is the limited availability of protein sequences. Atlantic cod (*Gadus morhua*) were selected, because Atlantic cod is common edible fish species and has more protein sequences available in database for identification. The 2D gel electrophoresis and protein identification results indicated that the dominant thermally stable protein (spot 1) around 36 kDa is tropomyosin (Fig. 1 & Table 1). Other thermally stable proteins were myosin light chain (spot 2–4), parvalbumin (spot 5–6), myosin heavy chain (spot 7), triosephosphate isomerase (spot 8), and troponin I (spot 9) (Fig. 1 & Table 1).

Tropomyosin was observed to be the most abundant thermally stable protein in 2D gel. Fish tropomyosin is a 36–38-kDa myofibrillar protein that involves in the regulation of muscle contraction (Huang & Ochiai, 2005). Fish tropomyosin from tilapia was first reported as an allergen (Liu et al., 2013). Recent research further reported that fish tropomyosin from cod, albacore, swordfish, salmon and catfish reacted with IgE obtained from patients with fish allergies and more tropomyosins from different fish species have been registered as allergens in WHO/IUIS database (González-Fernández et al., 2018; Ruethers et al., 2021; Sudhanson et al., 2021). In the present study, all identified thermally stable proteins in the 2D gel have been recognized as fish allergens including tropomyosin, parvalbumin, myosin light chain, myosin heavy chain, triosephosphate isomerase, and troponin I (Ruethers et al., 2018). Unlike other well studied fish allergens, fish tropomyosin is the latest identified fish allergen and its biochemical characteristics remains to be elucidated. In addition, the study of IgE-binding capacity of fish proteins showed that parvalbumin is primary protein binding to IgE from fish allergic patients and tropomyosin is secondary (Ruethers et al., 2021). The next step was therefore to characterize fish tropomyosin along with the main fish allergen, parvalbumin.

3.2. Comparison of tropomyosin and parvalbumin among fish species

The presence of tropomyosin and parvalbumin in common edible fish species was investigated by indirect ELISA. The ELISA results demonstrated that fish tropomyosin showed high absorbance values (>0.6) for all of the 28 fish species tested (Table 2). The absorbance values of parvalbumin were negative (<0.2) in 7 of the 28 fish species tested (Table 2). The seven fish species determined to be negative are Atlantic cod (*Gadus morhua*), Atlantic halibut (*Hippoglossus hippoglossus*), swordfish (*Xiphias gladius*), sweetfish (*Plecoglossus altivelis*), blue shark (*Prionace glauca*), Atlantic salmon (*Salmo salar*) and rough leather jacket (*Aluterus monoceros*) (Table 2).

The high absorbance values of the ELISA results indicated that tropomyosin was abundant and ubiquitous in all 28 saltwater and freshwater fish species tested. By contrast, the presence of parvalbumin was varied. The expression of parvalbumin has been reported to be low in large fish such as tuna and swordfish (Kobayashi et al., 2016; Liang et al., 2021). Parvalbumin has been used as an analyte in the immunoassays for fish detection (Fæste & Plassen, 2008; Shibahara et al., 2013). However, these immunoassays could not detect all fish species tested. On the basis of our results, this detection failure could be explained by the varied expression of parvalbumin. By contrast, tropomyosin is a ubiquitous and abundant muscle protein in all fish species tested. The previously developed immunoassay based on recognizing 36 kDa protein was able to detect 63 fish species tested (Chen & Hsieh, 2014). In comparison with parvalbumin, fish tropomyosin is a suitable target analyte for the detection of fish in food products. Furthermore, tropomyosin may play more important role of triggering allergic reactions than parvalbumin in the fish species with extremely low amount of parvalbumin.

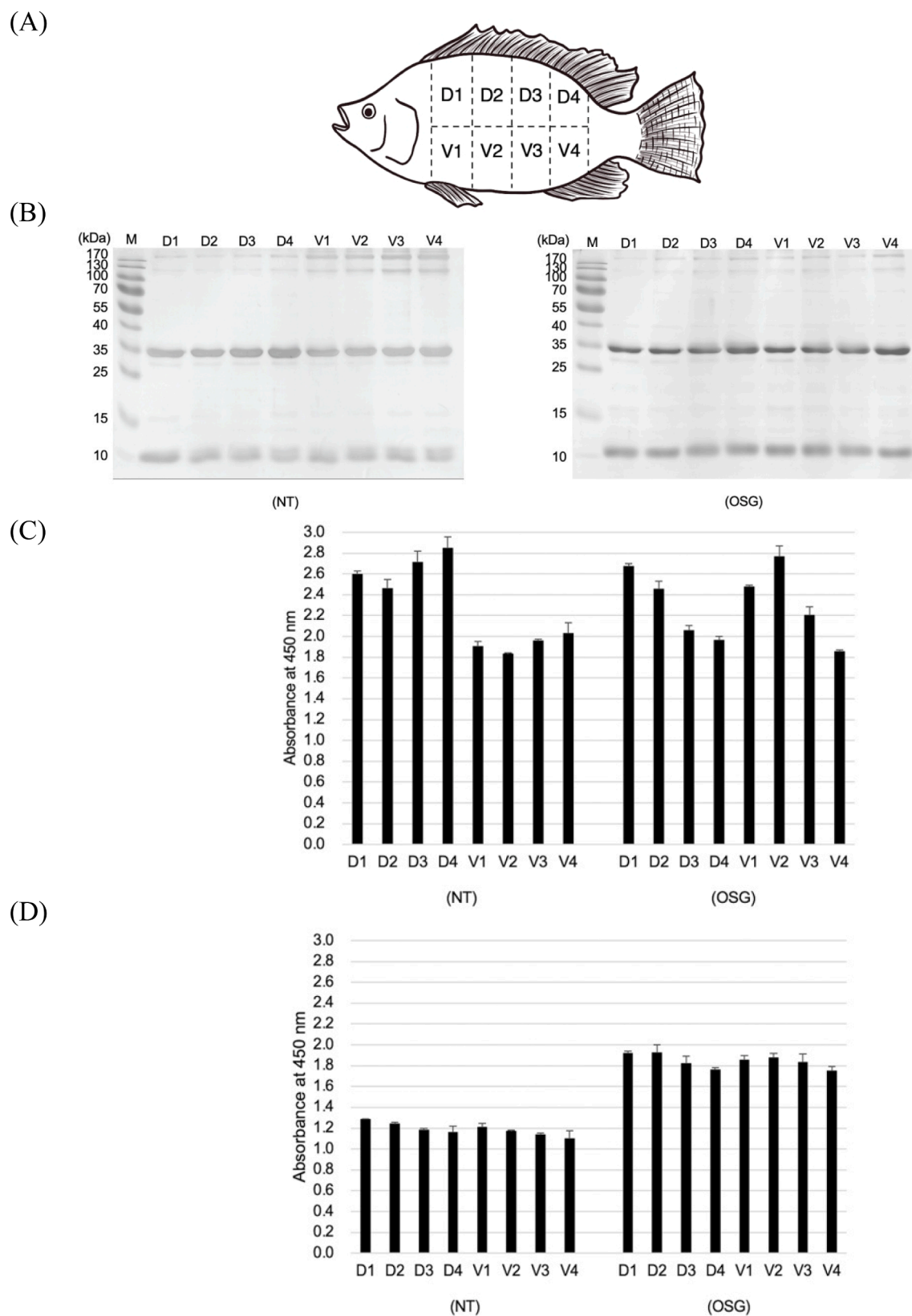


Fig. 2. Analysis of the presence of tropomyosin and parvalbumin of Nile tilapia (*Oreochromis niloticus*) and orange spotted grouper (*Epinephelus coioides*) in different muscle locations. (A) Diagram of fish muscle sampling. (B) SDS-PAGE (C) indirect ELISA of tropomyosin. (D) indirect ELISA of parvalbumin. The loading amount was 6 μ g per lane. M: molecular weight marker. NT: Nile Tilapia. OSG: orange spotted grouper.

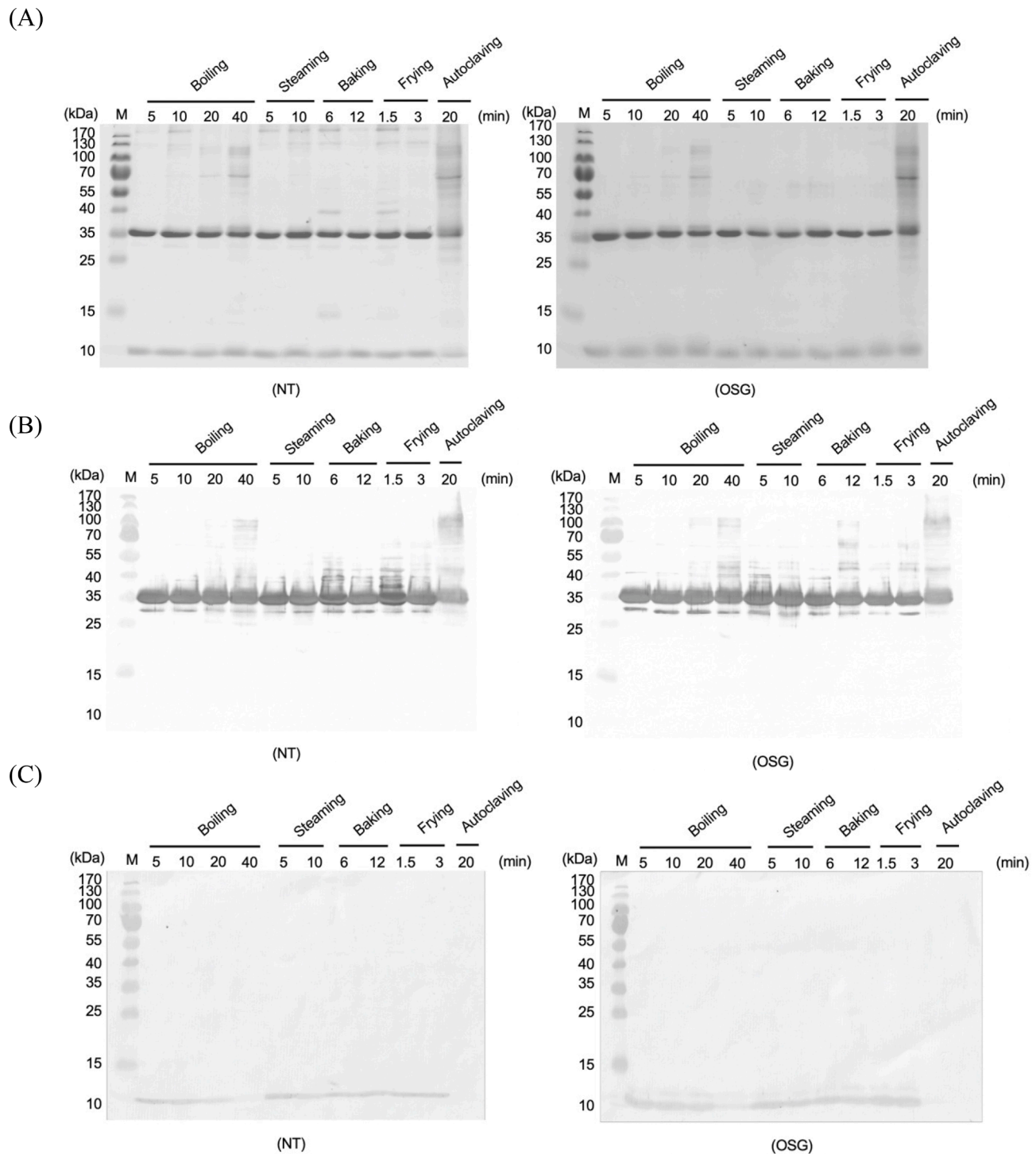


Fig. 3. SDS-PAGE (A) and western blot of tropomyosin (B) and parvalbumin (C) of Nile tilapia (*Oreochromis niloticus*) and orange spotted grouper (*Epinephelus coioides*) muscle samples with different food processing. The loading amount was 6 μ g per lane. M: molecular weight marker. NT: Nile Tilapia. OSG: orange spotted grouper.

3.3. Comparison of tropomyosin and parvalbumin in different muscle locations

Distribution of allergenic proteins in fish muscle is crucial for the sampling of allergen testing. A freshwater fish species, Nile tilapia (*Oreochromis niloticus*) and a saltwater fish species, orange spotted grouper (*Epinephelus coioides*) were selected as samples. The muscle of fishes were divided into eight parts to examine the distribution of tropomyosin and parvalbumin (Fig. 2A). Based on visual observation of

protein bands in the gels, tropomyosin (36 kDa) and parvalbumin (10 kDa) are equally distributed in Nile tilapia and orange spotted grouper (Fig. 2B). Thys et al. (2001) analyzed muscle protein expression in different locations of largemouth bass (*Micropterus salmoides*) muscle by gel electrophoresis and indicated no variations in tropomyosin from anterior to posterior (Thys et al., 2001). Our result of tropomyosin in SDS-PAGE was similar to previous study. However, SDS-PAGE is not able to present the presence of specific protein precisely. A single protein band in SDS-PAGE gel may contain more than one protein.

The indirect ELISA with anti-tropomyosin antibody or anti-parvalbumin antibody was applied to investigate the presence of fish tropomyosin (Fig. 2C) and parvalbumin (Fig. 2D) in different muscle locations. Nile tilapia has more tropomyosin in dorsal muscle than ventral muscle and a gradual increase of tropomyosin from anterior to posterior (Fig. 2C). The distribution of tropomyosin in orange spotted grouper does not have similar pattern as Nile tilapia (Fig. 2C). In contrast, the distribution pattern of parvalbumin in Nile tilapia and orange spotted grouper is similar. The anterior portion of both two fish species contained slightly more parvalbumin than the posterior portion (Fig. 2D). Our results of parvalbumin distribution agrees with previous observations by using ELISA in carp, catfish, tuna, mahi-mahi (Lee et al., 2012) and SDS-PAGE analysis in 12 fish species (Kobayashi et al., 2016). Tropomyosin and parvalbumin are muscle proteins involving fish movement. Tropomyosin regulates muscle contraction with troponin and actin (Ochiai & Ozawa, 2020). Parvalbumin plays a role in the enhancement of muscle relaxation through buffering myoplasmic Ca^{2+} (Radauer et al., 2008) and its content is correlated with the rate of relaxation in fish muscle (Coughlin et al., 2007). The swimming types, body shape and other intrinsic factors may relate to specific protein expression in different muscle locations. Our results demonstrated that the presence of fish allergens in different muscle locations could be varied among species like tropomyosin or have similar distribution pattern like parvalbumin. In addition, it is worth to be noted that the presence of tropomyosin is more abundant than parvalbumin in every muscle location in both Nile tilapia and orange spotted grouper (Fig. 2C & 2D).

3.4. Effect of food processing on fish allergens

Most fish muscle proteins are heat labile and degrade under heat treatment (Chen & Hsieh, 2022). Food processing methods including boiling, steaming, baking, frying and autoclaving are commonly applied to fish for consumption and production of fish products. Although majority of allergenic proteins are thermally stable, the effects of common thermal processing on fish tropomyosin and parvalbumin are unclear. Fish tropomyosin (36 kDa) and parvalbumin (10 kDa) of two fish species were the major thermally stable proteins and maintained the integrity in the most of samples with different heat treatments (Fig. 3A). The smeared protein bands appeared in the samples of boiling for 40 min and autoclaving, but tropomyosin in the Nile tilapia and orange spotted grouper samples still maintained certain integrity as a protein band (Fig. 3A). The western blot results further revealed that the immunoreactivity of tropomyosin and parvalbumin in both fish species was not greatly affected by boiling for up to 20 min, steaming for up to 10 min, baking at 200 °C for 12 min, or frying at 160 °C for 3 min (Fig. 3B & 3C). The minor differences of thermal stability were observed between Nile tilapia and orange spotted grouper. The immunoreactivity of orange spotted grouper tropomyosin was not affected by prolonged steaming, baking and frying, while the immunoreactivity of Nile tilapia tropomyosin, in contrast, was slightly reduced (Fig. 3B). It suggested that the thermal stability of tropomyosin may be different among fish species. The boiling for 40 min and autoclaving partially reduced the immunoreactivity of tropomyosin (Fig. 3B). By contrast, immunoreactivity of parvalbumin greatly reduced in boiling for 40 min and was fully lost after autoclaving (Fig. 3C). Differential scanning calorimetry and circular dichroism indicated that the structure of fish tropomyosin unfolded during heat treatment but refolded to its original form during cooling (Huang & Ochiai, 2005), explaining the high thermal tolerance of tropomyosin. Based on the ability to resist common food processing, fish tropomyosin is a suitable marker protein for detecting the presence of fish. In addition, tropomyosin would be the main allergen in autoclaved food products such as canned fish. On the other hand, autoclaving produced a severe effect on the integrity and immunoreactivity of parvalbumin. The potential of autoclaving on the reduction allergenicity of fish parvalbumin is worth further investigation.

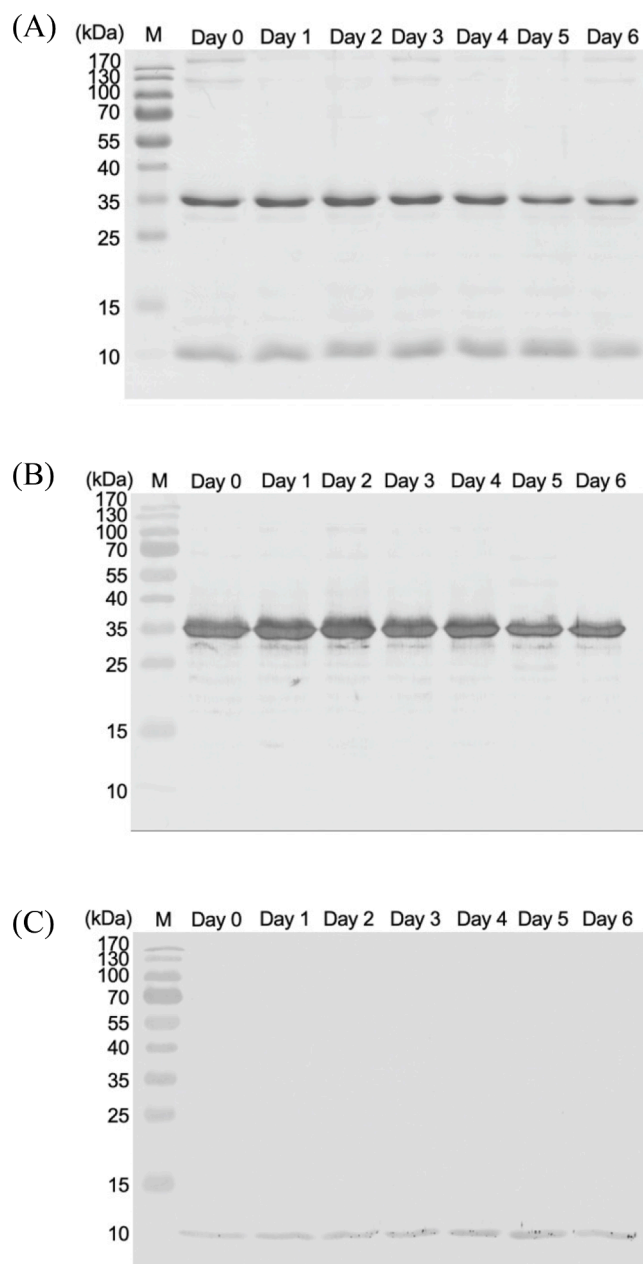


Fig. 4. SDS-PAGE (A) and western blot analysis of tropomyosin (B) and parvalbumin (C) of Nile tilapia (*Oreochromis niloticus*) muscle samples with refrigerated periods from Day 1 to Day 6. The loading amount was 6 µg per lane. M: molecular weight marker.

3.5. Effect of refrigerated storage on fish allergens

The conditions and duration of storage affect freshness and directly relate to the degradation of fish allergens. Fresh Nile tilapia muscle samples were refrigerated at 4 °C up to 6 days to study the effects of refrigerated storage on the allergens (Fig. 4). The SDS-PAGE and western blot revealed that the integrity and immunoreactivity of tropomyosin were consistent from day 0 to day 4 but slightly weaker on day 5 and 6 (Fig. 4A & 4B). In contrast to tropomyosin, the integrity and immunoreactivity of parvalbumin were not affected by refrigerated storage up to 6 days (Fig. 4A & 4C). Although tropomyosin and parvalbumin have different resistance to refrigerated storage, the integrity and immunoreactivity of these two fish allergens were not affected at 4 °C at least 4 days. Refrigeration is most common method for preserving fish for industries and consumers. However, cold storage can not fully prevent

protein degradation due to autolysis and microbiological growth (Verrez-Bagnis et al., 2001). In general, the shelf life of raw fish kept in refrigerator is 1–2 days (FDA, 2018). Our results showed that refrigerated storage does not affect the integrity and immunoreactivity of tropomyosin or parvalbumin while fish is fresh and edible for consumption.

4. Conclusions

In this study, we identified fish thermally stable proteins and revealed the effects of species, food processing, muscle location, and refrigerated storage on the integrity and immunoreactivity of two fish major allergens, tropomyosin and parvalbumin. This study provides essential and deeper understanding of the characteristics of fish tropomyosin and fish parvalbumin that is useful for the detection of fish allergens and the formulation of strategies for reducing allergenicity of parvalbumin.

CRediT authorship contribution statement

Chia-Lin Tsai: Investigation, Validation, Data curation, Visualization. **Kristy Perng:** Investigation, Writing – original draft. **Yu-Chen Hou:** Data curation, Methodology. **Cheng-Jou Shen:** Investigation, Visualization. **I-Ning Chen:** Investigation. **Yi-Tien Chen:** Conceptualization, Methodology, Supervision, Writing – original draft, Project administration, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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