Food Control 118 (2020) 107427

Contents lists available at ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Immunochemical analysis of pepsin-digested fish tropomyosin

Behnam Keshavarz[a,b], Qinchun Rao[a,*], Xingyi Jiang[a], Yun-Hwa Peggy Hsieh[a]

[a] Department of Nutrition, Food and Exercise Sciences, Florida State University, Tallahassee, FL, USA
[b] Division of Allergy and Immunology, University of Virginia, Charlottesville, VA, USA

ARTICLE INFO

Keywords:
Epitope mapping
Fish allergen
Parvalbumin
Pepsin digestion
Tropomyosin

ABSTRACT

A critical step in determining the allergenicity of a food protein is to study the gastrointestinal digestion resistance, which is an essential characteristic of food allergens due to the preservation of their protein structure and immunoglobulin (Ig) E epitopes. In this study, the immunochemical properties of tropomyosin peptide fragments from Atlantic salmon (*Salmo salar*) and flathead grey mullet (*Mugil cephalus*) resulted from pepsin digestion were analyzed. To identify the allergens from salmon and mullet, their IgE- and IgG-binding proteins/peptides were studied using fish-allergic human sera and anti-fish protein monoclonal and polyclonal antibodies. From our results, as the major fish allergen, parvalbumin from both species was recognized in all tested human sera. Tropomyosin-bound IgE was identified in 71% of the tested sera. Antigenicity of intact tropomyosin decreased as a function of pepsin digestion time. The amino acid sequence of mullet tropomyosin was analyzed using a tandem mass spectrometer. Two IgE epitopes on Atlantic salmon tropomyosin alpha-1 were mapped. Overall, tropomyosin was identified as one of the major IgE-binding proteins in both fish species; however, further studies are required to determine the clinical significance of fish tropomyosin as an allergen due to its high homology with human tropomyosin.

1. Introduction

Among vertebrate fish species, it is well known that parvalbumin is the major food allergen (Van Do, Elsayed, Florvaag, Hordvik, & Endresen, 2005) and can react with the immunoglobulin E (IgE) in more than 90% of fish-allergic individuals (Bugajska-Schretter et al., 1998). Other fish allergens, such as collagen, gelatin, beta-enolase, and aldolase A, are also on the known list of fish allergens (Lopata & Lehrer, 2009).

It is well known that invertebrate tropomyosin is the major food allergen in different shellfish, including crustaceans (crab, shrimp, and lobster) (Leung et al., 1998) and mollusks (snail, oyster, and squid) (Ishikawa, Ishida, Shimakura, Nagashima, & Shiomi, 1998). Unlike invertebrate tropomyosin, vertebrate fish tropomyosin was not considered as a food allergen until its allergenicity was first reported in Mozambique tilapia (*Oreochromis mossambicus*) (FAO/WHO, 2001; R.; Liu, Holck, Yang, Liu, & Xue, 2013). Also, its allergenicity has been reported in Atlantic cod (*Gadus morhua*), albacore (*Thunnus alalunga*), and swordfish (*Xiphias gladius*) (González-Fernández, Alguacil-Guillén, Cuéllar, & Daschner, 2018). Recently, the WHO/IUIS Allergen Nomenclature Sub-committee officially recognized tropomyosin in three edible fish species (i.e., Atlantic salmon (*Salmo salar*), striped catfish (*Pangasianodon hypophthalmus*), and Mozambique tilapia) as a food allergen (WHO/IUIS Allergen Nomenclature Sub-Committee, 2020). It has been suggested that disparity in the allergenicity of tropomyosin from vertebrates and/or invertebrates is due to the difference in the resulted larger peptide fragments of invertebrates tropomyosin after pepsin digestion than those of vertebrate tropomyosin, leading to higher allergenicity of invertebrate tropomyosin (Mikita & Padlan, 2007).

It is important to identify food allergens for the development of standardized reference materials, which can be used for diagnostic purposes such as skin prick test and food challenge testing (Sicherer & Sampson, 2010). As recommended by the FAO/WHO (2001), one crucial step in determining the allergenicity of a food protein is to study the gastrointestinal digestion resistance, which is an essential characteristic of food allergens due to the preservation of their protein structure and IgE epitopes (Fu, 2002). In this study, antigenicity is defined as the ability to be specifically recognized by the antibodies generated as a result of the immune response to the given substance (Ilinskaya & Dobrovolskaia, 2016). The immunochemical properties of tropomyosin peptide fragments from Atlantic salmon and flathead grey mullet (*Mugil cephalus*) resulted from pepsin digestion were analyzed using fish-allergic human sera and anti-fish protein monoclonal (mAb)

* Corresponding author.
E-mail address: qrao@fsu.edu (Q. Rao).

https://doi.org/10.1016/j.foodcont.2020.107427
Received 3 March 2020; Received in revised form 18 May 2020; Accepted 12 June 2020
Available online 25 June 2020
0956-7135/ © 2020 Elsevier Ltd. All rights reserved.

Table 1
Amino acid (AA) sequence comparison of tropomyosin from fish, shellfish, and human[a].(The UniProt Consortium, 2017)

Organism	Protein (allergen) name	Accession number		AA sequence[b]		Molecular weight (Da)	% AA similarity
Salmo salar (Atlantic salmon)	Tropomyosin alpha-1 chain (Sal s 4)	Q91472	1	MDAIKKKMQMLKLDKENALDRAEGAEGDKKAAEDKSKQLEDDLVALQKKLKGTEDELDKY	60	32,492	100
			61	SESLKDAQEKLEVAEKTATDAEADVASLNRRIQLVEEELDRAQERLATALTKLEEAEKAA	120		
			121	DESERGMKVIENRASKDEEKMELQDIQLKEAKHIAEEADRKYEEVARKLVIIESDLERTE	180		
			181	ERAELSEGKCSELEEELKTVTNNLKSLEAQAEKYSQKEDKYEEEIKVLTDKLKEAETRAE	240		
			241	FAERSVAKLEKTIDDLEDELYAQKLKYKAISEELDNALNDMTSI	284		
Mugil cephalus (Flathead grey mullet)[c]	Tropomyosin	Not available	1	----------SKQLEDDLVALQKKLKGTEDELDKY	60	Not available	Not available
			61	SEALKDAQEKLELAEKKATDAEGDVASLNRRIQLVEEELDRLEE----------AEKAA	120		
			121	DE----------SERHIAEEADRKYEEVARKLVIIEGDLER-	180		
			181	--------CSELEEEL--------KYSQKEDKYEEEIK----------	240		
			241	-------LEKTIDDLEDELYAQK----AISEELDHALNDMTSI	284		
Chelon auratus (Golden grey mullet)	Tropomyosin alpha-1 chain	P84335	1	MDAIKKKMQMLKLDKENALDRAEQAESDKKASEDRSKQLEDDLVALQKKLKGTEDELDKY	60	32,730	95
			61	SEALKDAQEKLELAEKKATDAEGDVASLNRRIQLVEEELDRAQERLATALTKLEEAEKAA	120		
			121	DESERGMKVIENRAMKDEEKMELQEIQLKEAKHIAEEADRKYEEVARKLVIIEGDLERTE	180		
			181	ERAELSESKCSELEEELKTVTNNLKSLEAQAEKYSQKEDKYEEEIKVLTDKLKEAETRAE	240		
			241	FAERSVAKLEKTIDDLEDELYAQKLKYKAISEELDHALNDMTSI	284		
Oreochromis mossambicus (Mozambique tilapia)	Tropomyosin (Ore m 4)	K4PEK4	1	MDAIKKKMQMLKLDKENALDRAEQAESDKKAAEDRSKQLEDDLVALQKKLKGTEDELDKY	60	32,655	95
			61	SEALKDAQEKLELAEKIATDAEGEVASLNRRIQLVEEELDRAQERLATALTKLEEAEKAA	120		
			121	DESERGMKVIENRAMKDEEKMELQEIQLKEAKHIAEEADRKYEEVARKLVIIEGDLERTE	180		
			181	ERAELSEGKCSELEEELKTVTNNLKSLEAQAEKYSQKEDKYEEEIKALTDKLKEAETRAE	240		
			241	FAERSVAKLEKTIDDLEDELYAQKLKYKAISEELDHALNDMTSI	284		
Pangasianodon hypophthalmus (Striped catfish)	Tropomyosin alpha-1 chain-like isoform X1 (Pen h 4)	XP_026781482	1	MEAIKKKMQMLKGDKENAFDRAEQAELDKKVSEDKCKQLEDELLGLQKKLKGTEDEVDKY	60	32,693	80
			61	SEALKDAQEKLELSEKKATDAEGEVAALNRRIQLIEEELDRAQGRLGTALQKLEEAEKQA	120		
			121	DESERGMKVIENRAMKDEEKMEIQELQLKEAKQIAEDSDRKYDEVARKLVVIEGELERAE	180		
			181	ERAEVSELKSGDLEEELKNVTNTLKSLEAQAEKYSDKEDKYEEEINLLNEKLKEAETRAE	240		
			241	FAEKTLVSKLEKTIDDLEDELYNQKLKVKAICEELDNALNDMTTL	284		
Penaeus aztecus (Brown shrimp)	Tropomyosin (Pen a 1)	Q3Y8M6	1	MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQV	60	32,849	56
			61	QESLLKANIQLVEKDKALSNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAEASQAA	120		
			121	DESERMRKVLENRSLSDEERMDALENQLKEARFLAEEADRKYDEVARKLAMVEADLERAE	180		
			181	ERAETGESKIVELEEELRVVGNNLKSLEVSEEKANQREEAYKEQIKTLTNKLKAAEARAE	240		
			241	FAERSVQKLQKEVDRLEDELVNEKEKYKSITDELDQTFSELSGY	284		
Penaeus monodon (Giant tiger prawn)	Tropomyosin (Pen m 1)	A1KYZ2	1	MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANNRAEKSEEEVHNLQKRMQQLENDLDQV	60	32,849	56
			61	QESLLKANIQLVEKDKALSNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAEASQAA	120		
			121	DESERMRKVLENRSLSDEERMDALENQLKEARFLAEEADRKYDEVARKLAMVEADLERAE	180		
			181	ERAETGESKIVELEEELRVVGNNLKSLEVSEEKANQREEAYKEQIKTLTNKLKAAEARAE	240		
			241	FAERSVQKLQKEVDRLEDELVNEKEKYKSITDELDQTFSELSGY	284		
Charybdis feriata (Crucifix crab)	Tropomyosin (Cha f 1)	Q9N2R3	1	MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANLRAEKTEEEIRATQKKMQQVENELDQA	60	30,436	55
			61	QEQLSAANTKLDEKEKALQNAEGEVAALNRRIQLPEEDLERSEERLNTATTKLAEASQAA	120		
			121	DESERMRKVLENRSLSDEERMDALENQLKEARFLAEEADRKYDEVARKLAMVEADLERAE	180		
			181	ERAESGESKIVELEEELRVVGNNLKSLEVSEEKANQREETYKEQIKTLANKLKAAEARAE	240		
			241	FAERSVQKLQKEVDRLEDELVNEK	264		
Metapenaeus ensis (Greasyback shrimp)	Tropomyosin (Met e 1)	Q25456	1	MEAIKNKMQAMKLEKDNAIDRAEIAEQKARDANLRAEKSEEEVRALQKKIQQIENELDQV	60	31,705	53
			61	QEQLSAANTKLEEKEKALQTAEGDVAALNRRIQLIEEDLERSEERLKIATAKLEEASQSA	120		
			121	DESERMRKMLEHRSITDEERMEGLENQLKEARMMAEDADRKYDEVARKLAMVEADLERAE	180		
			181	ERAETGESKIVELEEELRVVGNNLKSLEVSEEKAQQREEAHEQQIRIMTTKLKEAEARAE	240		
			241	FAERSVQKLQKEVGRLEDELVHEKEKYKSISDELDQTFAELTGY	274		
Homarus americanus (American lobster)	Tropomyosin (Hom a 1)	O44119	1	MDAIKKKMQAMKLEKDNAMDRADTLEQQNKEANIRAEKTEEEIRITHKKMQQVENELDQV	60	32,907	57
			61	QEQLSLANTKLEEKEKALQNAEGEVAALNRRIQLLEEDLERSEERLNTATTKLAEASQAA	120		
			121	DESERMRKVLENRSLSDEERMDALENQLKEARFLAEEADRKYDEVARKLAMVEADLERAE	180		
			181	ERAETGESKIVELEEELRVVGNNLKSLEVSEEKANQREEAYKEQIKTLANKLKAAEARAE	240		
			241	FAERSVQKLQKEVDRLEDELVNEKEKYKSITDELDQTFSELSGY	284		
Homo sapiens (Human)	Tropomyosin beta chain	P07951	1	MDAIKKKMQMLKLDKENAIDRAEQAEADKKQAEDRCKQLEEEQQALQKKLKGTEDEVEKY	60	32,851	86
			61	SESVKEAQEKLEQAEKKATDAEADVASLNRRIQLVEEELDRAQERLATALQKLEEAEKAA	120		
			121	DESERGMKVIENRAMKDEEKMELQEMQLKEAKHIAEDSDRKYEEVARKLVILEGELERSE	180		
			181	ERAEVAESKCGDLEEELKIVTNNLKSLEAQADKYSTKEDKYEEEIKLLEEKLKEAETRAE	240		
			241	FAERSVAKLEKTIDDLEDEVYAQKMKYKAISEELDNALNDITSL	284		
Homo sapiens (Human)	Tropomyosin alpha-1 chain	P09493	1	MDAIKKKMQMLKLDKENALDRAEQAEADKKAAEDRSKQLEDELVSLQKKLKGTEDELDKY	60	32,709	93
			61	SEALKDAQEKLELAEKKATDAEADVASLNRRIQLVEEELDRAQERLATALQKLEEAEKAA	120		
			121	DESERGMKVIESRAQKDEEKMEIQEIQLKEAKHIAEDADRKYEEVARKLVIISDLERAE	180		
			181	ERAELSEGKCAELEEELKTVTNNLKSLEAQAEKYSQKEDRYEEEIKVLSDKLKEAETRAE	240		
			241	FAERSVTKLEKSIDDLEDELYAQKLKYKAISEELDHALNDMTSI	284		

and polyclonal antibodies (pAb). In general, tropomyosin is a highly conserved protein with two major isoforms (alpha and beta) in both vertebrates and invertebrates (González-Fernández, Veleiro, Daschner, & Cuéllar, 2016). Interestingly, except for tuna (*Thunnus thynnus*) (M.-C. Huang, Ochiai, & Watabe, 2004), fish tropomyosin consists of only alpha isoform (Ochiai, Ozawa, Huang, & Watabe, 2010). In this study, the IgE epitopes on the Atlantic salmon tropomyosin alpha-1 chain were mapped.

2. Materials and methods

2.1. Materials

Fresh Atlantic salmon fillets were purchased from a local store (Tallahassee, FL, USA). Certified whole flathead grey mullet fish was kindly provided by the Fish and Wildlife Research Institute (St. Petersburg, FL, USA). After receiving, the whole mullet was immediately deboned. Boneless fillets from both fish species were cut into small pieces, vacuum packed, and stored at −80 °C until use.

Two mouse mAbs, anti-parvalbumin mAb3E1 (Gajewski & Hsieh, 2009) and anti-tropomyosin mAb8F5 (Chen, 2012), and a rabbit anti-tropomyosin pAb (Chen & Hsieh, 2014), previously developed in our laboratory were used in this study. Three antibodies, mouse anti-parvalbumin mAbPARV19 (P3088), goat anti-mouse IgG (Fc specific) horseradish peroxidase (HRP) conjugate (A2554), and goat anti-rabbit IgG (whole molecule) HRP conjugate (A0545), were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Eight human sera (seven fish-allergic and one non-fish-allergic) were purchased from PlasmaLab International (Everett, WA, USA). Total and specific IgE antibodies to fish as well as other foods were measured using the ImmunoCAP instrument (Thermo Fisher/Phadia, Portage, MI, USA), and the results are presented as IU/mL (1 IU ≈ 2.4 ng of IgE, Supplementary Document Table S1). Mouse anti-human IgE (Fc specific) HRP conjugate (9160-05) was purchased from

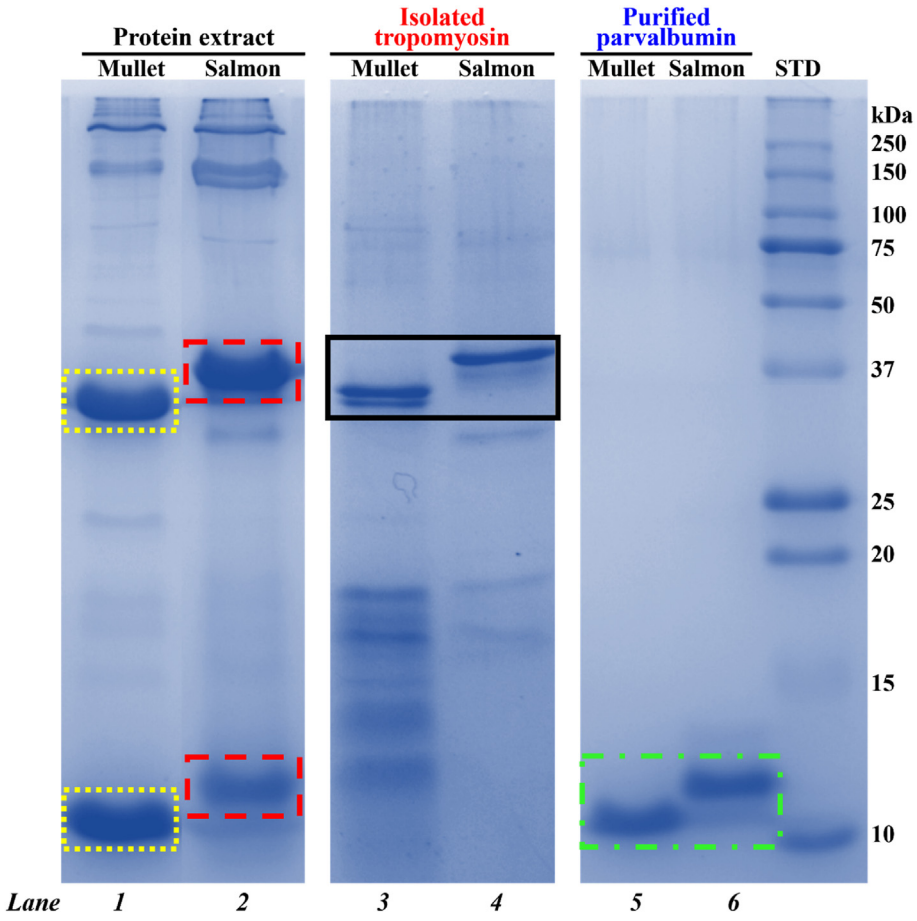


Fig. 1. Protein profile of heated mullet and salmon samples using reducing glycine-SDS-PAGE. Two major thermostable protein bands in mullet and salmon protein extracts (10 μg/lane) are indicated using a yellow dotted rectangle and a red dashed rectangle, respectively. The isolated tropomyosin (mullet: 1.5 μg/lane; salmon: 0.75 μg/lane) and purified parvalbumin (1 μg/lane) from mullet and salmon are indicated using a black solid-line rectangle and a green dash-dot-line rectangle, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

SouthernBiotech (Birmingham, AL, USA).

All chemicals and reagents were of analytical grade. All solutions were prepared using deionized water from a NANOpure DIamond ultrapure water system (Barnstead International, Dubuque, IA, USA).

2.2. Sample preparation

During sample preparation, unless otherwise specified, (1) all subsequent manipulations were performed at 4 °C; (2) heated samples were treated at 100 °C/600 rpm for 8 min using a thermomixer (Eppendorf, Hamburg, Germany); (3) dialysis was performed for 24 h in dialysis tubing (6–8 kDa MWCO, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA); (4) centrifugation was performed at 20,000 *g* for 15 min at 4 °C; and (5) the Pierce BCA (bicinchoninic acid) Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) was used to determine protein concentration, in which bovine serum albumin (BSA) was the protein standard (working range: 25-2,000 μg/mL).

Crude protein extracts from both fish species were prepared according to our previous study (Keshavarz, Jiang, Hsieh, & Rao, 2019) with modifications. Briefly, 5 g of half-thawed minced mullet and salmon fillets were thoroughly mashed by pestle and mortar, placed in a beaker covered with aluminum foil, and heated in a boiling water bath for 8 min, respectively. After cooled to room temperature (RT), heated samples were added 10 mL of 0.15 M NaCl and homogenized at 11,000 rpm for 1 min using the ULTRA-TURRAX T-25 basic homogenizer (IKA Works, Inc., Wilmington, NC, USA), homogenates were rotated head-to-head at RT for 2 h. After centrifugation, each supernatant was sequentially filtered through filter paper (Whatman, Piscataway, NJ, USA) and a 0.8-μm syringe filter (Thermo Fisher Scientific, Waltham, MA, USA). The protein concentration of each fish filtrate was determined.

Parvalbumin from both species was purified according to our previous study (Keshavarz et al., 2019). Briefly, parvalbumin was isolated from crude fish protein extracts by ammonium sulfate (AMS) precipitation and purified twice by size exclusion chromatography using the Econo System (Bio-Rad Laboratories Inc., Hercules, CA, USA) at RT. After verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot, the parvalbumin-containing fractions were combined and dialyzed against water. The lyophilized parvalbumin from mullet and salmon was dissolved in 0.15 M NaCl to obtain 1 mg/mL concentration. After heat treatment and centrifugation, the supernatants were collected. It should be noted that no precipitates were visible to the naked eye in heated parvalbumin samples after centrifugation.

Tropomyosin from both species was isolated according to Huang and Ochiai (2005) with modifications. Briefly, 50 g of half-thawed minced mullet and salmon fillets were washed twice using 50 mM KCl containing 2 mM $NaHCO_3$, and the supernatant was discarded after centrifugation at 3,000 *g* for 5 min. Samples were then washed twice with acetone and dried overnight. The proteins in dried samples were extracted overnight with 20 mM Tris-HCl (pH 7.5) containing 1 M KCl and 10 mM β-mercaptoethanol. After centrifugation, supernatants were subjected to isoelectric precipitation at pH 4.5 using 1 N HCl. After centrifugation, precipitates were dissolved in 20 mM Tris-HCl and subjected to AMS fractionation to obtain a 100% (g/mL) saturated fraction. The resulting precipitates were collected by centrifugation and then dissolved in 20 mM Tris-HCl. After dialysis against 2 mM $NaHCO_3$ and lyophilization, crude tropomyosin was further purified using a Bio-Scale Mini UNOsphere Q Cartridge (Bio-Rad) (Hayley et al., 2008). The isolated tropomyosin was dialyzed against 0.15 M NaCl. The dialyzed tropomyosin from both species was heated and centrifuged. The protein concentration of the supernatants was determined.

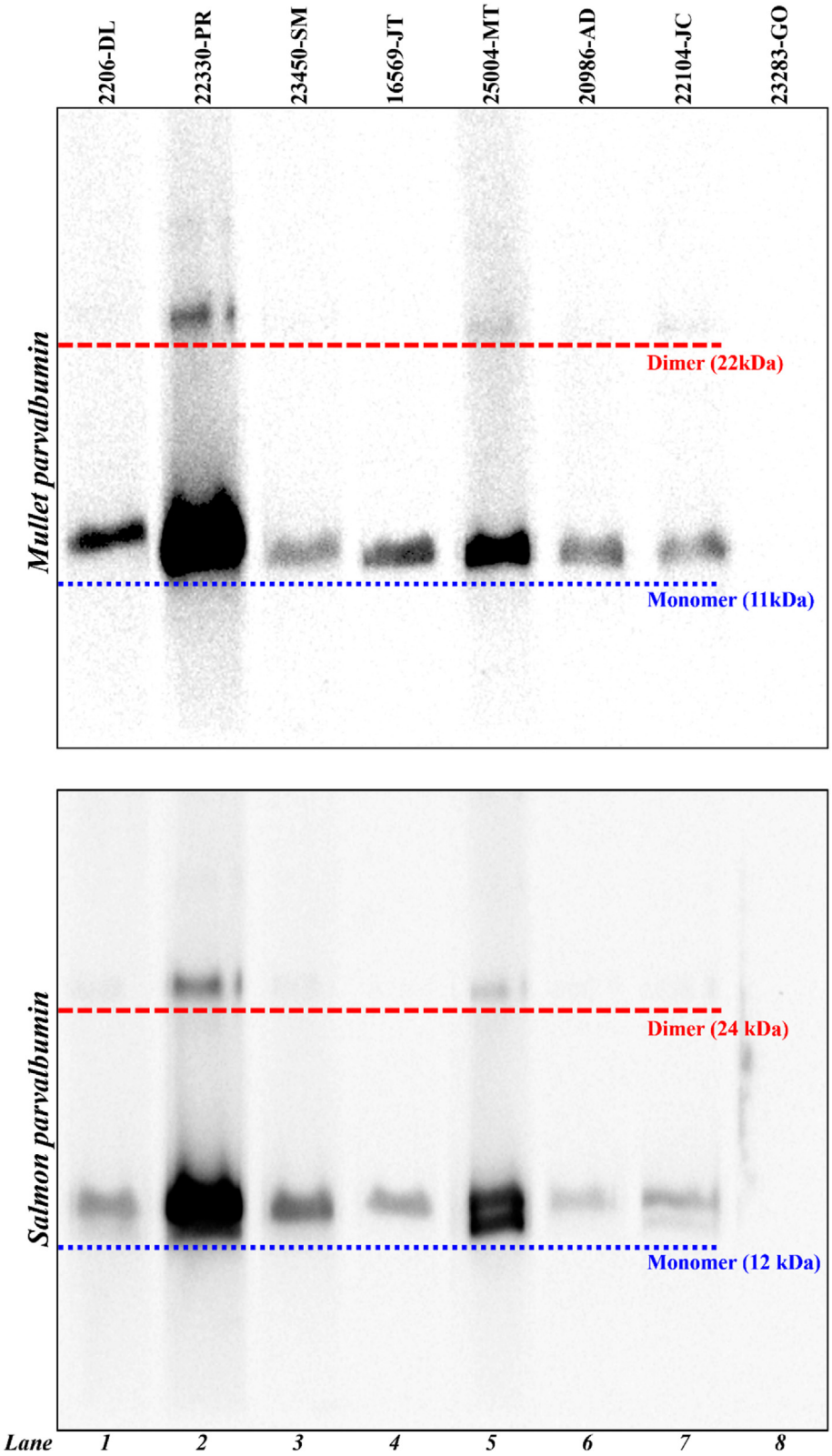


Fig. 2. Antigenicity of mullet and salmon parvalbumin (1 μg/lane) shown by Western blot using fish-allergic human sera. Total and specific IgE test results of these sera are provided in Supplementary Document Table S1.

2.3. In vitro pepsin digestion of tropomyosin

According to the procedure from FAO/WHO (2001), 500 μg of isolated tropomyosin from each species were added 200 μL of 0.32% (g/mL) pepsin (P53-100, Fisher Scientific, Hampton, NH, USA) dissolved in 30 mM NaCl (pH 2.0), and maintained at 37 °C for a designated time interval (0, 0.25, 0.5, 1, 2, 4, 8, 15, and 60 min). At each digestion time, two control samples, mullet and salmon tropomyosin without pepsin, were prepared. After digestion, each sample was neutralized with an adequate amount of 200 mM Na_2CO_3 and ice-cooled

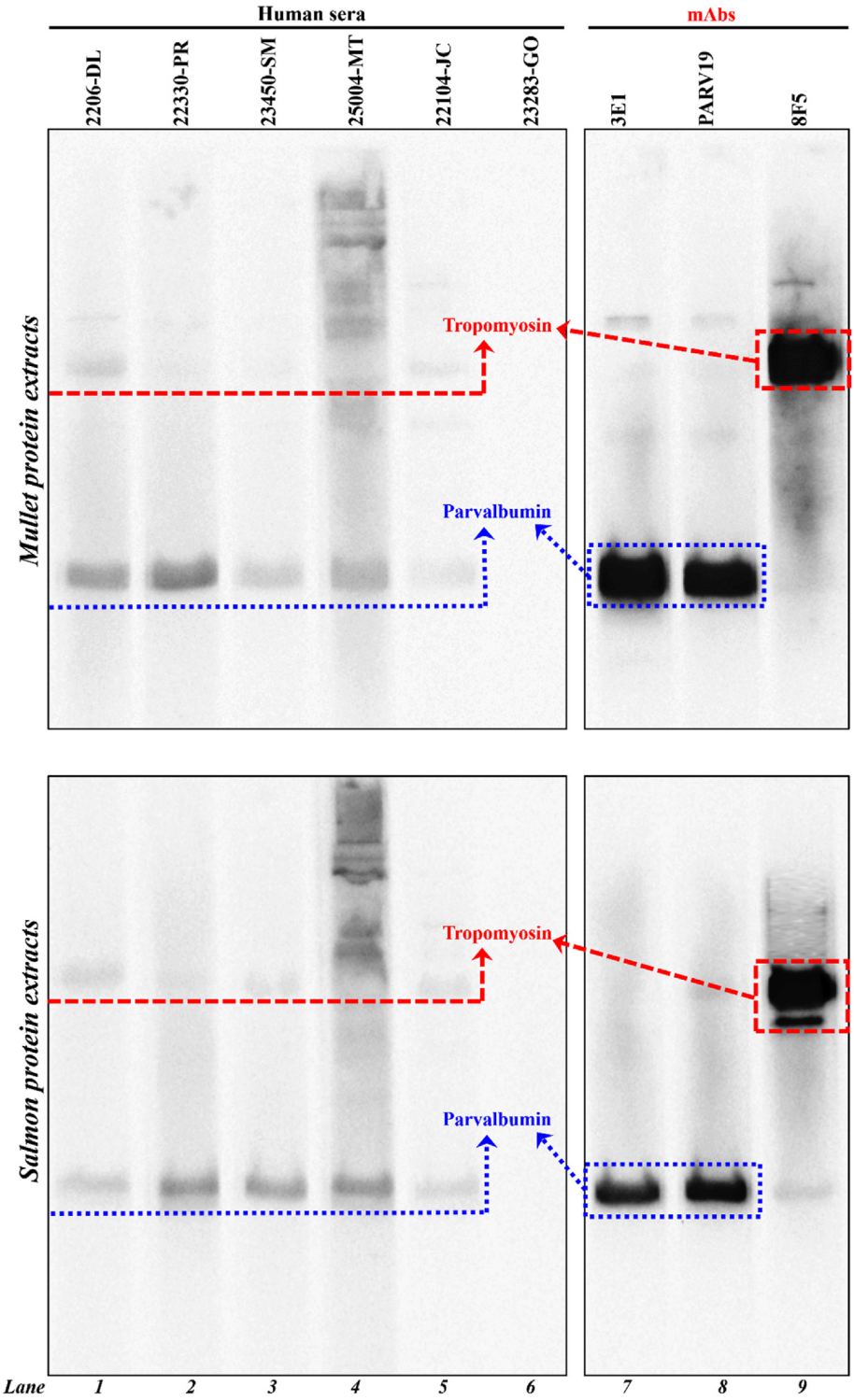


Fig. 3. In vitro identification of IgE- and IgG-binding proteins in mullet and salmon protein extracts (10 μg/lane) revealed by Western blot using fish-allergic human sera and mAbs, respectively.

immediately.

2.4. SDS-PAGE

Reducing glycine-SDS-PAGE (4% stacking gel and 15% separating gel) was used to study the protein profile of heated samples (*i.e.*, protein extracts, parvalbumin, and isolated tropomyosin) from both species (Laemmli, 1970). Briefly, all diluted samples from both species were mixed with the 2 × Laemmli sample buffer containing β-mercaptoethanol (reducing agent), and heated at 96 °C for 5 min. After gel separation using a Mini-PROTEAN Tetra Cell (Bio-Rad), the protein bands were visualized by the Coomassie blue staining. An unstained protein ladder (1610363, Bio-Rad) was used for band sizing.

10–20% tricine-SDS-PAGE gels (Jule, Inc., Milford, CT, USA) were used to identify tropomyosin and its peptides after *in vitro* pepsin digestion (Schagger, 2006). Briefly, all digested samples from both

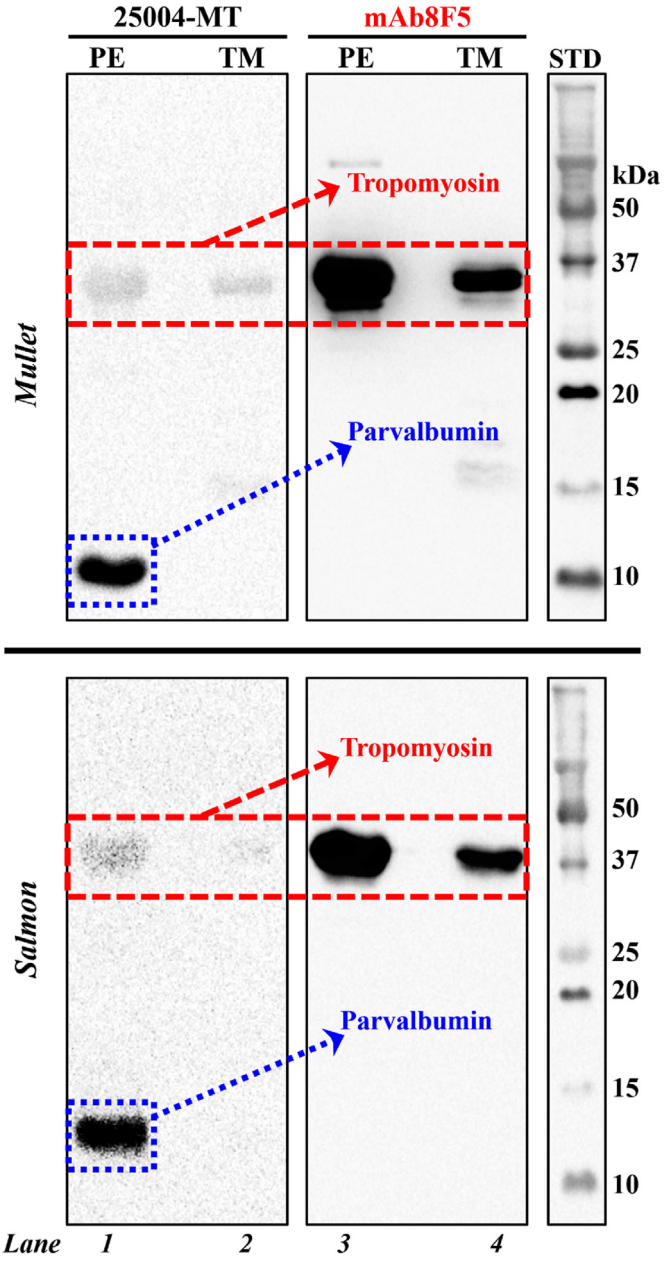


Fig. 4. Antigenicity of mullet and salmon tropomyosin revealed by Western blot using a fish-allergic human serum (25004-MT) and mAb8F5, respectively. PE: protein extracts (10 μg/lane); TM: isolated tropomyosin (mullet: 2 μg/lane; salmon: 1 μg/lane).

species were mixed with the 2 × tricine gel sample buffer with and without β-mercaptoethanol, and heated for 30 min at 40 °C. After separation, gels were fixed with 5% (mL/mL) glutaraldehyde solution for 30 min and visualized by the Coomassie blue staining. A low range protein ladder (26628, Thermo Fisher Scientific) was used for band sizing. In addition, the protein profile of two control samples (i.e., isolated tropomyosin from mullet and salmon) without pepsin was analyzed using reducing glycine-SDS-PAGE.

2.5. Chemiluminescent Western blot

Chemiluminescent Western blot was performed to identify (1) IgE-binding proteins using different human sera, (2) parvalbumin using two mAbs (3E1 and PARV19), and (3) tropomyosin using mAb8F5, respectively, according to our previous study (Keshavarz et al., 2019) with modifications. Electrophoretically separated protein bands were transferred onto a 0.45-μm nitrocellulose membrane (Bio-Rad) using a Trans-Blot Turbo Transfer System (Bio-Rad).

Also, chemiluminescent Western blot was used to identify tropomyosin and its pepsin-digested peptides using pooled human sera (subjects No.: 22206-DL, 22330-PR, 23450-SM, 25004-MT, and 22104-JC, Supplementary Document Table S1) and anti-tropomyosin pAb. Electrophoretically separated samples were transferred onto a 0.2-μm nitrocellulose membrane (GE Healthcare Life Science, Freiburg, Germany).

The transferred proteins and/or peptides on the membranes were verified using the Ponceau S staining. All membranes were blocked using BSA-PBS (10 mM phosphate-buffered saline (PBS) containing 1% BSA (g/mL), pH 7.2). The blocked membranes were incubated overnight at 4 °C with mAb3E1 (1.36 μg IgG/mL), mAbPARV19 (6.3 μg IgG/mL), 1:20 (mL/mL) diluted mAb8F5 supernatant, pAb (1.16 μg IgG/mL), or 1:5 (mL/mL) diluted human sera (individual or pooled). The membranes were then incubated with the corresponding HRP-conjugated secondary antibodies (1:50,000, μL/μL). All antibodies and human sera were diluted using BSA-PBST (BSA-PBS containing 0.05% (mL/mL) Tween 20). The blotted antigens were detected using the luminol chemiluminescence method. Between each step, the membrane was washed with PBST several times. All images from SDS-PAGE and Western blot were captured by the Azure c600 Imaging System and were analyzed using the AzureSpot software (version 14.2, Azure Biosystems Inc., Dublin, CA, USA).

2.6. Mullet tropomyosin amino acid (AA) sequencing

AA sequence of isolated mullet tropomyosin was analyzed using a tandem mass spectrometer in the Translational Science Laboratory at Florida State University (Tallahassee, FL, USA) according to our previous study (Keshavarz et al., 2019) with modifications. Briefly, in-gel digestion of mullet tropomyosin was performed using ProteoExtract All-in-One Trypsin Digestion Kit (Calbiochem, Darmstadt, Germany) according to the manufacturer's instructions. Peptides were eluted in 0.1% (mL/mL) formic acid and run on an externally calibrated Thermo Scientific Q Exactive HF Hybrid Quadrupole-Orbitrap Mass Spectrometer in conjunction with the UltiMate3000 RSLCnano System (Thermo Fisher Scientific). Resultant raw files were searched with the Proteome Discoverer software (version 1.4, Thermo Fisher Scientific) using Sequest HT as the search engine, with the modified mouse FASTA database and percolator as the peptide validator. The resulting msf files were further analyzed by the Scaffold software (version 4, Proteome Software, Inc., Portland, OR, USA).

2.7. IgE epitope mapping

The IgE epitopes were mapped using the entire AA sequence of Atlantic salmon tropomyosin alpha-1 chain (Table 1) according to our previous study (Keshavarz et al., 2019) with modifications. Through using the SPOT synthesis technique, 91 peptides were synthesized as overlapping linear peptides and were covalently bound at their C-terminus to the cellulose-β-alanine membrane as described by the manufacturer (JPT Peptide Technologies, Berlin, Germany). Each peptide length was 15 AAs. The AA offset was three AAs. Each spot carried about 5 nmol peptides. The peptides were N-acetylated to increase stability. The membrane was blocked with BSA-TBST (50 mM tris-buffered saline (TBS) containing 1% BSA (g/mL) and 0.05% (mL/mL) Tween 20, pH 8.0). The blocked membrane was incubated with 1:3 (mL/mL) diluted pooled human sera from individuals with relatively high titer different fish species, including salmon (subjects No.: 22330-PR, 23450-SM, 25004-MT, and 22104-JC, Supplementary Document Table S1) overnight at 4 °C. The membrane was incubated with 1:50,000 (μL/μL) diluted anti-human IgE HRP conjugate. Both pooled human sera and labeled secondary antibody were diluted using BSA-

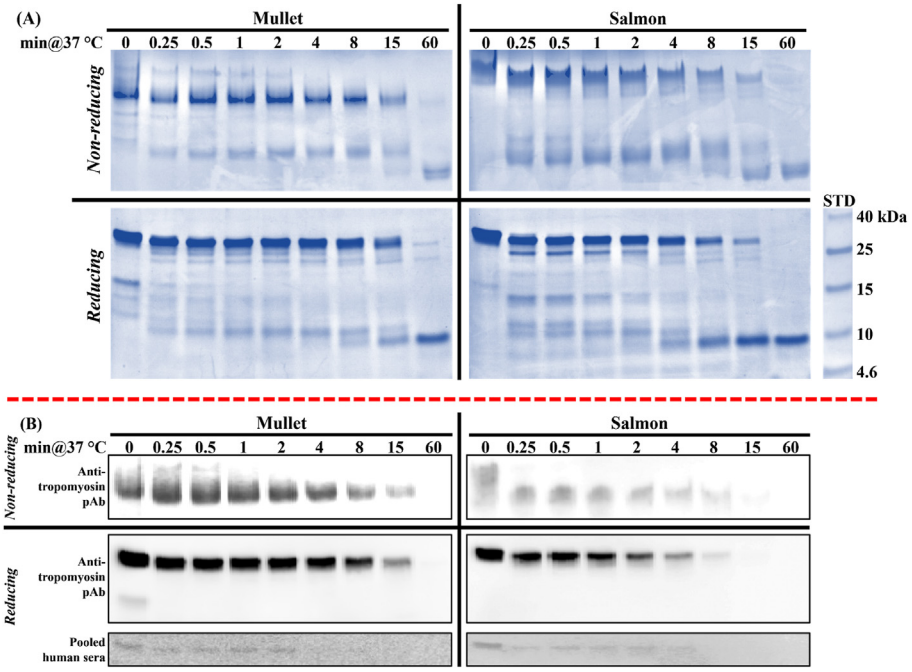


Fig. 5. (A) Protein/peptide profile of digested mullet and salmon tropomyosin (3 μg/lane) using tricine-SDS-PAGE and (B) Antigenicity of digested mullet and salmon tropomyosin revealed by Western blot using pooled fish-allergic human sera (subjects No.: 22206-DL, 22330-PR, 23450-SM, 25004-MT, and 22104-JC), and anti-tropomyosin pAb, respectively.

TBST. Between each step, the membrane was washed with TBST several times. The images were then captured and analyzed using the same methods as those for chemiluminescent Western blot.

3. Results and discussion

3.1. Identification of IgE-binding proteins

The protein profile of heated samples (*i.e.*, protein extracts, isolated tropomyosin, and purified parvalbumin from mullet and salmon) is shown in Fig. 1. As to protein extracts, two major thermostable protein bands were observed in mullet (yellow dotted rectangle, lane 1) and salmon (red dashed rectangle, lane 2), respectively (Fig. 1). In both species, the band patterns of the high molecular weight (MW) bands (about 37 kDa) and the low MW bands (about 12 kDa) are similar to those of isolated tropomyosin (black solid-line rectangle, lanes 3 and 4) and purified parvalbumin (green dash-dot-line rectangle, lanes 5 and 6), respectively (Fig. 1).

As the major fish allergen, the IgE-binding ability of parvalbumin was confirmed using two sample models. In the purified parvalbumin model (Fig. 2), IgE from seven fish-allergic human sera could react with the monomer form from mullet and salmon (blue dotted line, lanes 1–7). Several sera were also positive with parvalbumin dimer (red dashed line) in mullet (lanes 2, 5, and 7) and salmon (lanes 1, 2, 3, 5, and 7), respectively. It has been reported that heat-induced oligomerization of parvalbumin could occur during sample preparation (de Jongh, de los Reyes Jimenez, Baumert, Taylor, & Koppelman, 2015; Keshavarz et al., 2019). As a negative control, the human serum with no history of food allergy (23283-GO), was negative to mullet and salmon parvalbumin (lane 8, Fig. 2). It should be noted that this is the first study that confirmed mullet parvalbumin antigenicity using human sera.

In the model of protein extracts (Fig. 3), the presence of parvalbumin monomer (blue dotted rectangle) from both species was verified using two anti-parvalbumin mAbs, 3E1 (lane 7) and PARV19 (lane 8). Also, parvalbumin monomer was recognized as the major IgE-binding protein in both species using five fish-allergic human sera (blue dotted line, lanes 1–5, Fig. 3).

As shown in Fig. 3, besides parvalbumin, another IgE-binding protein (about 37 kDa, red dashed line, lanes 1–5) was observed in both mullet and salmon protein extracts. Its MW was similar to that of tropomyosin, in which using anti-tropomyosin mAb8F5 (red dashed rectangle, lane 9, Fig. 3), we confirmed its presence in protein extracts from both species.

To verify the antigenicity of isolated tropomyosin from both species, their IgE- and IgG-binding capacities were confirmed (red dashed rectangle, Fig. 4) using a human serum (25004-MT with a class 5 IgE titer to salmon, lane 2) and mAb8F5 (lane 4), respectively. Combining the results from protein extracts (Fig. 3), sera from five subjects (lanes 1–5) were positive to tropomyosin (red dashed line), indicating that tropomyosin from both species is another major IgE-binding protein.

It is noted that for the same allergen, different levels of antigenicity was observed among different human sera (Figs. 2 and 3) which can be explained by the following reasons: (1) differences in IgE titer to fish species from different subjects; (2) differences in IgE epitopes in different sera; and (3) difference in allergen content among various fish species (Kuehn, Swoboda, Arumugam, Hilger, & Hentges, 2014). It should be noted that allergens are also not evenly distributed in the fish muscles of the same species (Lee, Nordlee, Koppelman, Baumert, & Taylor, 2012).

It is noted that tropomyosin MW from both species on the gel (lanes 3–4, Fig. 1) was larger than those UniProt predicted (Table 1). This may be due to different types of tropomyosin in the same species may have different MW. As Fine and Blitz (1975) reported, calf skeletal tropomyosin had a 5-kDa larger MW than its non-skeletal tropomyosin. It was observed that salmon tropomyosin had a bigger MW than mullet tropomyosin (red dashed rectangle, Fig. 4). This may be caused by different protein mobility in the gel. A similar mechanism has been proposed in the study of Heeley and Hong (1994), in which salmon fast muscle tropomyosin exhibited a larger MW than other fish species such as Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*) and pollack (*Pollachius virens*).

To the best of our knowledge, this is the first study that reports the immunoreactivity of human sera with mullet tropomyosin, which is similar to the report of the binding of salmon tropomyosin to human IgE (WHO/IUIS Allergen Nomenclature Sub-Committee, 2020). The immunoreaction between fish tropomyosin and human IgE was also reported by González-Fernández et al. (2018), in which around 59% (10/17) of fish-allergic patients sera showed immunoreactive tropomyosin band from Atlantic cod, swordfish (*Xiphias gladius*), or albacore (*Thunnus alalunga*). In another study, the IgE-binding ability to tilapia

tropomyosin from all ten patients was confirmed using immunoblotting and enzyme-linked immunosorbent assay (R. Liu et al., 2013). The human IgE also recognized tropomyosin from hake (*Merluccius merluccius*), monkfish (*Lophius piscatorius*), swordfish, and albacore (González-Fernández et al., 2016). Other clinical data suggested that 34% (27/80) and 76% (61/80) of the patients showed IgE binding to striped catfish tropomyosin and positive skin-prick test, respectively (WHO/IUIS Allergen Nomenclature Sub-Committee, 2020). However, it should be noted that none of these published studies elaborated on the digestion stability and the IgE-epitopes of fish tropomyosin.

3.2. Comparison of tropomyosin AA sequences

Tropomyosin is an actin-binding myofibrillar protein that regulates muscle contraction and is highly expressed in the muscle cells of vertebrates (Perry, 2001). As a major allergen in shellfish (Lopata, O'Hehir, & Lehrer, 2010), its AA sequence comparison among fish, shellfish, and human is shown in Table 1. It should be noted that the AA sequence of flathead grey mullet tropomyosin was partially determined in this study, which is identical to that of golden grey mullet (Table 1). Atlantic salmon tropomyosin alpha-1 chain has a similarity of about 95% compared with that from fish, which decreases to about 55% compared with shellfish (Table 1). Atlantic salmon tropomyosin alpha-1 chain also has a high AA sequence similarity of 86% and 93% with human tropomyosin beta and alpha-1 chains, respectively. It is noted that the AA similarity between α and β tropomyosin ranges from 73 to 83% (M.-C. Huang et al., 2004). It has been suggested that protein allergenicity is inversely related to its homology to human proteins (Aalberse, Akkerdaas, & van Ree, 2001), and the allergenicity of animal food proteins depends on its evolutionary distance from the human homologs (Jenkins, Breiteneder, & Mills, 2007). Tropomyosin from species with a homology of greater than 55% is rarely allergenic (Reese, Ayuso, & Lehrer, 1999). In our study, a high homology between fish and human tropomyosin was observed. Hence, further studies are required to investigate whether fish tropomyosin has any clinical significance, despite having high AA sequence similarity with its human protein counterpart.

3.3. In vitro pepsin digestion of tropomyosin

It has been proposed that the capability of a food allergen to pass through the gastrointestinal tract and reach the intestinal mucosa is a prerequisite for allergenicity (Astwood, Leach, & Fuchs, 1996). The *in vitro* pepsin digestion stability assay is considered as a useful method to predict the potential allergenicity and relative stability of a food protein in the extreme conditions of low pH and pepsin protease (Fu, 2002). The digestion stability of fish parvalbumin has been reported in many publications (de Jongh et al., 2013; Tao, Liang, Xiao, Liu, & Guan, 2016; Untersmayr et al., 2005). In this study, two major findings of fish tropomyosin were observed as a function of digestion time. First, the concentration of intact tropomyosin from both species decreased while the concentration of pepsin-digested peptides increased (Fig. 5A). Second, the antigenicity of intact tropomyosin from both species gradually decreased when the digestion time increased (Fig. 5B), indicating that IgE- and IgG-epitopes on intact tropomyosin were destroyed as a function of digestion time. Antigenicity of intact tropomyosin from salmon and mullet was completely diminished after 8-min and 60-min pepsin digestion, respectively (Fig. 5B). The findings are consistent with Mikita and Padlan's study (Mikita & Padlan, 2007), suggesting that fish tropomyosin digestion led to peptide fragments which were not long enough to bind with the antibody. The digestion stability of tropomyosin can be affected by the species difference, type of enzyme, protein-enzyme ratio, and digestion conditions. For example, after 60-min pepsin digestion, tropomyosin from brown shrimp (*Farfantepenaeus aztecus*) (Gamez et al., 2015) was not able to activate basophils and induce positive reaction during *in vivo* skin prick test. Shen et al. (2014) found that intact tropomyosin from octopus (*Octopus fangsiao*) was still visible after 60 min pepsin digestion but disappeared after 60 min of trypsin digestion. Similarly, tropomyosin from mud crab (*Scylla serrata*) (Y. Y. Huang et al., 2010) and Pacific white shrimp (*Litopenaeus vannamei*) (G. M. Liu et al., 2011) was resistant to pepsin but not to trypsin digestion.

On the other hand, two control samples, *i.e.*, mullet and salmon tropomyosin without pepsin, were acid-stable at pH 2.0 for 60 min (Supplementary Document Fig. S1), indicating that those tropomyosin peptide fragments were mainly generated by enzymatic hydrolysis not by acid hydrolysis during *in vitro* pepsin digestion. It is worth noting that, in some individuals who take anti-acid medication, proteins may not be fully digested, and food allergens including tropomyosin may preserve their IgE epitopes which could sensitize susceptible individuals and lead to allergy (Untersmayr & Jensen-Jarolim, 2008). This may also lower the threshold levels required for allergenicity reactions in sensitized individuals (Untersmayr et al., 2005).

3.4. Epitope mapping

Through epitope mapping of the synthetic Atlantic salmon tropomyosin alpha-1 chain peptides, two IgE epitope-containing peptides, i.e., LVALQKKLKGTEDEL (AA 43–57) and AETRAEFAERSVAKLEKT (AA 235–252), were identified. Both peptides are identical to the tropomyosin AAs from flathead grey mullet and Mozambique tilapia (Table 1). These two identified peptides also shared 80% and 83% similarity with those from striped catfish, respectively. It should be noted that one IgE epitope-containing peptide (AETRAEFAERSV, AA 235–246) is observed in tropomyosin AAs from shellfish and human (Table 1). Another IgE epitope-containing peptide (LQKKLKGTEDE, AA 46–56) is shown in human tropomyosin (Table 1). This is an important observation since IgE antibodies in tested human sera can be considered as functional, auto-reactive tropomyosin-specific IgE. The presence of autoreactive human milk-specific IgE was reported in infants with a history of cow's milk allergy, where some patients had IgEs positive to both bovine and human α-lactalbumin, β-casein, and κ-casein (Jarvinen, Geller, Bencharitiwong, & Sampson, 2012). Generally, a crucial phase in B cell maturation is inactivation or elimination of autoreactive B cells that express autoreactive receptors. Due to the high homology between fish and human tropomyosins, it is not expected to detect IgE antibodies that can bind to food allergen epitopes (*e.g.*, fish tropomyosin) as well as their counterpart proteins in human (*e.g.*, human tropomyosin). It might be because around 25% of adults with atopic dermatitis (AD) have reactive IgE antibodies against self-proteins (Bieber, 2008); and even the infants with AD and autoreactive IgE antibodies were observed to be frequently sensitized to food allergens (Mothes et al., 2005). Tsakok et al. (2016) have shown that there was a strong and dose-dependent association between AD, food sensitization, and food allergy. In this study, there was no direct contact with the patients, and no information on the status of the patients, whether they were diagnosed with AD, was available.

In addition, protein antigenicity is affected by immunoglobulin protective isotypes, food processing methods, protein stability, and digestibility (González-Fernández, Daschner, & Cuéllar, 2017). Modifications such as glycosylation or phosphorylation can significantly affect the three-dimensional structure of the protein which impacts the antibody binding (González-Fernández et al., 2017). Although fish are closer to the invertebrates in the phylogenetic tree, it is the molecular uniqueness rather than the molecular similarity between allergens and human proteins that will cause the allergic response (González-Fernández et al., 2017). The clinical significance of fish tropomyosin remains to be determined. Also, further studies are needed to assess the cross-immunoreactivity among fish, shellfish, and human tropomyosin.

4. Conclusions

In this study, fish-allergic sera from seven individuals were used to identify IgE-binding proteins from mullet and salmon. Based on our findings, parvalbumin was confirmed as the major cross-reactive allergen in both fishes. Also, tropomyosin-bound IgE was identified in five of seven human sera (about 71%). The antigenicity of intact tropomyosin from mullet and salmon gradually decreased as a function of pepsin digestion time. In addition, two IgE epitopes on the Atlantic salmon tropomyosin alpha-1 chain were mapped. Overall, to the best of our knowledge, this is the first report of fish tropomyosin as an IgE-binding protein from mullet. It should be noted that although fish tropomyosin showed the *in vitro* IgE-binding ability, it may not be clinically significant or cause an allergic reaction due to the high homology with human tropomyosin, which still needs to be clinically evaluated.

CRediT authorship contribution statement

Behnam Keshavarz: Data curation, Formal analysis, Methodology, Writing - original draft. **Qinchun Rao:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Writing - review & editing. **Xingyi Jiang:** Writing - review & editing. **Yun-Hwa Peggy Hsieh:** Conceptualization, Supervision, Writing - review & editing.

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.

Acknowledgements

A part of this study was supported by the Betty M. Watts Professorship Fund at Florida State University, the Florida State University Dissertation Research Grant, and the National Institute of Food and Agriculture, U.S. Department of Agriculture (2018-70001-28759). The authors would like to thank the Fish and Wildlife Research Institute at the Florida Fish and Wildlife Conservation Commission (FWC) for providing the certified mullet samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2020.107427.

References

Aalberse, R. C., Akkerdaas, J., & van Ree, R. (2001). Cross-reactivity of IgE antibodies to allergens. *Allergy, 56*(6), 478–490. https://doi.org/10.1016/S0091-6749(85)80003-8.

Astwood, J. D., Leach, J. N., & Fuchs, R. L. (1996). Stability of food allergens to digestion *in vitro*. *Nature Biotechnology, 14*(10), 1269–1273. https://doi.org/10.1038/nbt1096-1269.

Bieber, T. (2008). Atopic dermatitis. *New England Journal of Medicine, 358*(14), 1483–1494. https://doi.org/10.5021/ad.2010.22.2.125.

Bugajska-Schretter, A., Elfman, L., Fuchs, T., Kapiotis, S., Rumpold, H., Valenta, R., et al. (1998). Parvalbumin, a cross-reactive fish allergen, contains IgE-binding epitopes sensitive to periodate treatment and Ca^{2+} depletion. *The Journal of Allergy and Clinical Immunology, 101*(1), 67–74. https://doi.org/10.1016/S0091-6749(98)70195-2.

Chen, Y.-T. (2012). *Characterization of a 36 KDa fish protein and its application to the development of an immunoassay for the detection of fish muscle*Doctoral Dissertation. Florida State University.

Chen, Y.-T., & Hsieh, Y.-H. P. (2014). A sandwich ELISA for the detection of fish and fish products. *Food Control, 40*, 265–273. https://doi.org/10.1016/j.foodcont.2013.12.010.

Fao/Who (2001). Evaluation of allergenicity of genetically modified foods: Report of a joint FAO/WHO expert consultation on allergenicity of foods derived from biotechnology. http://www.fao.org/fileadmin/templates/agns/pdf/topics/ec_jan2001.pdf, Accessed date: 13 May 2020.

Fine, R. E., & Blitz, A. L. (1975). A chemical comparison of tropomyosins from muscle and non-muscle tissues. *Journal of Molecular Biology, 95*(3), 447–454. https://doi.org/10.1016/0022-2836(75)90202-8.

Fu, T. J. (2002). Digestion stability as a criterion for protein allergenicity assessment. *Annals of the New York Academy of Sciences, 964*(1), 99–110. https://doi.org/10.1111/j.1749-6632.2002.tb04135.x.

Gajewski, K., & Hsieh, Y.-H. P. (2009). Monoclonal antibody specific to a major fish allergen: Parvalbumin. *Journal of Food Protection, 72*(4), 818–825. https://doi.org/10.4315/0362-028X-72.4.818.

Gamez, C., Zafra, M. P., Sanz, V., Mazzeo, C., Ibanez, M. D., Sastre, J., et al. (2015). Simulated gastrointestinal digestion reduces the allergic reactivity of shrimp extract proteins and tropomyosin. *Food Chemistry, 173*, 475–481. https://doi.org/10.1016/j.foodchem.2014.10.063.

González-Fernández, J., Alguacil-Guillén, M., Cuéllar, C., & Daschner, A. (2018). Possible allergenic role of tropomyosin in patients with adverse reactions after fish intake. *Immunological Investigations, 47*(4), 416–429. https://doi.org/10.1080/08820139.2018.1451882.

González-Fernández, J., Daschner, A., & Cuéllar, C. (2017). Allergenicity of vertebrate tropomyosins: Challenging an immunological dogma. *Allergologia et Immunopathologia, 45*(3), 297–304. https://doi.org/10.1016/j.aller.2016.08.002.

González-Fernández, J., Veleiro, B., Daschner, A., & Cuéllar, C. (2016). Are fish tropomyosins allergens? *Annals of Allergy, Asthma, & Immunology, 116*(1), https://doi.org/10.1016/j.anai.2015.09.017.

Hayley, M., Chevaldina, T., Mudalige, W. A., Jackman, D. M., Dobbin, A. D., & Heeley, D. H. (2008). Shark skeletal muscle tropomyosin is a phosphoprotein. *Journal of Muscle Research & Cell Motility, 29*(2-5), 101–107. https://doi.org/10.1007/s10974-008-9143-z.

Heeley, D. H., & Hong, C. (1994). Isolation and characterization of tropomyosin from fish muscle. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 108*(1), 95–106. https://doi.org/10.1016/0305-0491(94)90169-4.

Huang, Y. Y., Liu, G. M., Cai, Q. F., Weng, W. Y., Maleki, S. J., Su, W. J., et al. (2010). Stability of major allergen tropomyosin and other food proteins of mud crab (*Scylla serrata*) by *in vitro* gastrointestinal digestion. *Food and Chemical Toxicology, 48*(5), 1196–1201. https://doi.org/10.1016/j.fct.2010.02.010.

Huang, M.-C., & Ochiai, Y. (2005). Fish fast skeletal muscle tropomyosins show species-specific thermal stability. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 141*(4), 461–471. https://doi.org/10.1016/j.cbpc.2005.05.008.

Huang, M.-C., Ochiai, Y., & Watabe, S. (2004). Structural and thermodynamic characterization of tropomyosin from fast skeletal muscle of bluefin tuna. *Fisheries Science, 70*(4), 667–674. https://doi.org/10.1111/j.1444-2906.2004.00855.x.

Ilinskaya, A. N., & Dobrovolskaia, M. A. (2016). Understanding the immunogenicity and antigenicity of nanomaterials: Past, present and future. *Toxicology and Applied Pharmacology, 299*, 70–77. https://doi.org/10.1016/j.taap.2016.01.005.

Ishikawa, M., Ishida, M., Shimakura, K., Nagashima, Y., & Shiomi, K. (1998). Tropomyosin, the major oyster *Crassostrea gigas* allergen and its IgE-binding epitopes. *Journal of Food Science, 63*(1), 44–47. https://doi.org/10.1111/j.1365-2621.1998.tb15672.x.

Jarvinen, K. M., Geller, L., Bencharitiwong, R., & Sampson, H. A. (2012). Presence of functional, autoreactive human milk-specific IgE in infants with cow's milk allergy. *Clinical and Experimental Allergy, 42*(2), 238–247. https://doi.org/10.1111/j.1365-2222.2011.03864.x.

Jenkins, J. A., Breiteneder, H., & Mills, E. N. C. (2007). Evolutionary distance from human homologs reflects allergenicity of animal food proteins. *The Journal of Allergy and Clinical Immunology, 120*(6), 1399–1405. https://doi.org/10.1016/j.jaci.2007.08.019.

de Jongh, H. H. J., de los Reyes Jimenez, M., Baumert, J. L., Taylor, S. L., & Koppelman, S. J. (2015). Electrophoretic behavior in relation to the structural integrity of codfish parvalbumin upon heat treatment. *Journal of Agricultural and Food Chemistry, 63*(18), 4683–4689. https://doi.org/10.1021/jf505990h.

de Jongh, H. H. J., Robles, C. L., Timmerman, E., Nordlee, J. A., Lee, P. W., Baumert, J. L., et al. (2013). Digestibility and IgE-binding of glycosylated codfish parvalbumin. *BioMed Research International*. https://doi.org/10.1155/2013/756789.

Keshavarz, B., Jiang, X. Y., Hsieh, Y.-H. P., & Rao, Q. C. (2019). Matrix effect on food allergen detection – a case study of fish parvalbumin. *Food Chemistry, 274*, 526–534. https://doi.org/10.1016/j.foodchem.2018.08.138.

Kuehn, A., Swoboda, I., Arumugam, K., Hilger, C., & Hentges, F. (2014). Fish allergens at a glance: Variable allergenicity of parvalbumins, the major fish allergens. *Frontiers in Immunology, 5*, 179. https://doi.org/10.3389/fimmu.2014.00179.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680–685. https://doi.org/10.1038/227680a0.

Lee, P. W., Nordlee, J. A., Koppelman, S. J., Baumert, J. L., & Taylor, S. L. (2012). Measuring parvalbumin levels in fish muscle tissue: Relevance of muscle locations and storage conditions. *Food Chemistry, 135*(2), 502–507. https://doi.org/10.1016/j.foodchem.2012.05.030.

Leung, P. S. C., Chen, Y. C., Mykles, D. L., Chow, W. K., Li, C. P., & Chu, K. H. (1998). Molecular identification of the lobster muscle protein tropomyosin as a seafood allergen. *Molecular Marine Biology and Biotechnology, 7*(1), 12–20.

Liu, R., Holck, A. L., Yang, E., Liu, C., & Xue, W. (2013). Tropomyosin from tilapia (*Oreochromis mossambicus*) as an allergen. *Clinical and Experimental Allergy, 43*(3), 365–377. https://doi.org/10.1111/cea.12056.

Liu, G. M., Huang, Y. Y., Cai, Q. F., Weng, W. Y., Su, W. J., & Cao, M. J. (2011). Comparative study of *in vitro* digestibility of major allergen, tropomyosin and other proteins between grass prawn (*Penaeus monodon*) and Pacific white shrimp

(*Litopenaeus vannamei*). *Journal of the Science of Food and Agriculture, 91*(1), 163–170. https://doi.org/10.1002/jsfa.4167.

Lopata, A. L., & Lehrer, S. B. (2009). New insights into seafood allergy. *Current Opinion in Allergy and Clinical Immunology, 9*(3), 270–277. https://doi.org/10.1097/Aci.0b013e32832b3e6f.

Lopata, A. L., O'Hehir, R. E., & Lehrer, S. B. (2010). Shellfish allergy. *Clinical and Experimental Allergy, 40*(6), 850–858. https://doi.org/10.1111/j.1365-2222.2010.03513.x.

Mikita, C. P., & Padlan, E. A. (2007). Why is there a greater incidence of allergy to the tropomyosin of certain animals than to that of others? *Medical Hypotheses, 69*(5), 1070–1073. https://doi.org/10.1016/j.mehy.2006.12.060.

Mothes, N., Niggemann, B., Jenneck, C., Hagemann, T., Weidinger, S., Bieber, T., et al. (2005). The cradle of IgE autoreactivity in atopic eczema lies in early infancy. *The Journal of Allergy and Clinical Immunology, 116*(3), 706–709. https://doi.org/10.1016/j.jaci.2005.06.025.

Ochiai, Y., Ozawa, H., Huang, M.-C., & Watabe, S. (2010). Characterization of two tropomyosin isoforms from the fast skeletal muscle of bluefin tuna *Thunnus thynnus orientalis*. *Archives of Biochemistry and Biophysics, 502*(2), 96–103. https://doi.org/10.1016/j.abb.2010.07.015.

Perry, S. V. (2001). Vertebrate tropomyosin: Distribution, properties and function. *Journal of Muscle Research & Cell Motility, 22*(1), 5–49. https://doi.org/10.1023/a:1010303732441.

Reese, G., Ayuso, R., & Lehrer, S. B. (1999). Tropomyosin: An invertebrate pan-allergen. *International Archives of Allergy and Immunology, 119*(4), 247–258. https://doi.org/10.1159/000024201.

Schagger, H. (2006). Tricine-SDS-PAGE. *Nature Protocols, 1*(1), 16–22. https://doi.org/10.1038/nprot.2006.4.

Shen, H. W., Liu, Y. Y., Chen, F., Hu, J. W., Cao, M. J., Su, W. J., et al. (2014). Purification, characterization and immunoreactivity of tropomyosin, the allergen in *Octopus fangsiao*. *Process Biochemistry, 49*(10), 1747–1756. https://doi.org/10.1016/j.procbio.2014.07.005.

Sicherer, S. H., & Sampson, H. A. (2010). Food allergy. *The Journal of Allergy and Clinical Immunology, 125*(2), S116–S125. https://doi.org/10.1016/j.jaci.2009.08.028.

Tao, M., Liang, T., Xiao, C., Liu, M., & Guan, R. (2016). Effect of parvalbumins on gastrointestinal digestion in freshwater fish with *in vitro* simulation. *Journal of Agricultural Biotechnology, 24*(2), 1908–1914. https://doi.org/10.3969/j.issn.1674-7968.2016.12.013.

The UniProt Consortium (2017). UniProt: The universal protein knowledgebase. *Nucleic Acids Research, 45*(D1), D158–D169. https://doi.org/10.1093/nar/gkw1099.

Tsakok, T., Marrs, T., Mohsin, M., Baron, S., du Toit, G., Till, S., et al. (2016). Does atopic dermatitis cause food allergy? A systematic review. *The Journal of Allergy and Clinical Immunology, 137*(4), 1071–1078. https://doi.org/10.1016/j.jaci.2015.10.049.

Untersmayr, E., & Jensen-Jarolim, E. (2008). The role of protein digestibility and antacids on food allergy outcomes. *The Journal of Allergy and Clinical Immunology, 121*(6), 1301–1308. https://doi.org/10.1016/j.jaci.2008.04.025.

Untersmayr, E., Poulsen, L. K., Platzer, M. H., Pedersen, M. H., Boltz-Nitulescu, G., Skov, P. S., et al. (2005). The effects of gastric digestion on codfish allergenicity. *The Journal of Allergy and Clinical Immunology, 115*(2), 377–382. https://doi.org/10.1016/j.jaci.2004.10.029.

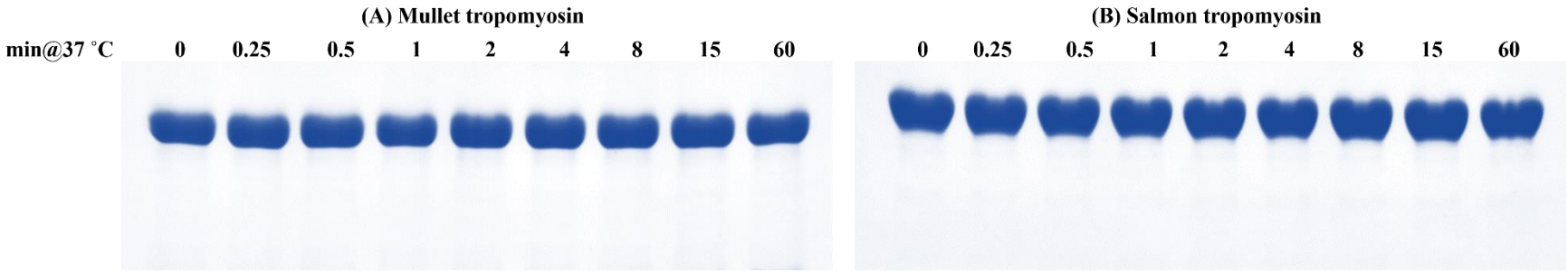
Van Do, T., Elsayed, S., Florvaag, E., Hordvik, I., & Endresen, C. (2005). Allergy to fish parvalbumins: Studies on the cross-reactivity of allergens from 9 commonly consumed fish. *The Journal of Allergy and Clinical Immunology, 116*(6), 1314–1320. https://doi.org/10.1016/j.jaci.2005.07.033.

Who/Iuis Allergen Nomenclature Sub-Committee (2020). Allergen nomenclature. Accessed http://www.allergen.org/search.php?allergenname=&allergensource=&TaxSource=&TaxOrder=&foodallerg=all&bioname=tropomyosin, Accessed date: 13 May 2020.

Supplementary Document Table S1. Specific IgE test results using ImmunoCAP

Subject No.	A/G/R*	Total IgE (IU/mL)	Reported fish allergy (IgE IU/mL)	Other reported food (non-fish) allergy (IgE > 0.35 IU/mL)
22206-DL	1981/F/Caucasian	4,105	Salmon (44.6), cod (68.4), tuna (11.4)	Egg white, crab, shrimp, apple, hazelnut, oat, strawberry, coconut, cherry, blue mussel, corn, tomato, milk, pork, baker's yeast, rye, peanut, barley, rice, onion, sesame seed, white bean, garlic, buckwheat, potato, wheat, almond, orange, kiwi fruit, brazil nut, carrot, soybean, banana, green pea, clam, gluten, peach, scallop, strawberry, egg yolk, chicken meat
22330-PR	1956/F/Caucasian	Not available	Salmon (68.8), cod (69.2), tuna (24.5), trout (85.9)	Egg white, peanut, soybean, hazelnut, almond, crab, shrimp, egg yolk, pecan nut, walnut
23450-SM	1988/M/Caucasian	Not available	Salmon (40.7), cod (26.7)	Peanut, soybean, hazelnut, milk, crab, shrimp, banana, lemon
16569-JT	1964/M/Caucasian	183	Salmon (4.51), cod (6.59), tuna (1.28), trout (6.41), plaice (3.43)	Peanut, hazelnut, Baker's yeast, chicken meat
25004-MT	1990/M/African American	2,432	Salmon (85), cod (84.4), tuna (40.4), trout (80.4), chub mackerel (32.1)	Crab, shrimp, apple, hazelnut, oat, strawberry, coconut, cherry, blue mussel, corn, tomato, pork, beef, chicken meat, rye, peanut, barley, rice, onion, sesame seed, white bean, garlic, buckwheat, potato, wheat, almond, orange, kiwi fruit, brazil nut, carrot, soybean, banana, celery, green pea, clam, gluten
20986-AD	1969/F/Caucasian	Not available	Salmon (9.86), trout (11.1), tuna (6.51), cod (12.2)	Brazil nut

22104-JC	1971/M/Caucasian	398	Salmon (39.2), cod (42.1), trout (46), sardine (38.7)	Crab, shrimp, blue mussel, clam, lobster, oyster, snail, scallop
23283-GO	1975/M/Caucasian	3.9	No allergen reported	No allergen reported

[*]A/G/R: Birthdate/Gender/Race; F: Female; M: Male

Supplementary Document Fig. S1. Protein profile of two control samples (mullet and salmon tropomyosin without pepsin, 3 µg/lane) at different designated time intervals using reducing glycine-SDS-PAGE. (A) Mullet tropomyosin; (B) Salmon tropomyosin.