

Matrix effect on food allergen detection – A case study of fish parvalbumin

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ARTICLE INFO

Keywords:

Allergen
Parvalbumin
Matrix effect
Extractability
Solubility
Thermostability
Immunodetection

ABSTRACT

Two fish parvalbumin models were established to study relationships among matrix effect, extractability, and thermostability during *in vitro* immunodetection using two parvalbumin-specific monoclonal antibodies (3E1 and PARV19). Our results illustrated that matrix-induced thermal instability of parvalbumin was due mainly to physical (hydrophobic effect) and chemical (thiol-disulfide interchange) interactions. The addition of sodium dodecyl sulfate (SDS, surfactant), β -mercaptoethanol (reducing agent) or ethylenediaminetetraacetic acid (EDTA, metal chelator) during sample preparation could not only increase the extractability of parvalbumin but also enhanced its immunodetection. Our findings demonstrated excess EDTA completely chelated Ca^{2+} in parvalbumin and rendered it undetectable using PARV19 (a Ca^{2+} -dependent antibody). Overall, our results showed that matrix effect on *in vitro* analyte quantification cannot be underestimated. Any false negative or positive results could lead to severe or life-threatening allergic reactions.

1. Introduction

To prevent the occurrence of undeclared food allergenic residues, monoclonal antibody (mAb) based immunoassays have been developed for the *in vitro* detection of allergenic protein residues in processed foods (Jayasena et al., 2015). One of the critical factors in evaluating the quality of an immunoassay is whether the target analyte detected in sample extracts correctly reflect its content in tested products. It should be noted that matrix effects on the *in vitro* target analyte quantification cannot be underestimated and should not be ignored. According to the International Union of Pure and Applied Chemistry (IUPAC) (Guilbault & Hjelm, 1991), matrix effect is defined as “the combined effect of all components of the sample other than the analyte on the measurement of the quantity.”

Currently, only a few studies have discussed matrix effects on the extractability of target analytes during *in vitro* immunodetection (Downs & Taylor, 2010; Gomma, Ribereau, & Boye, 2012; Montserrat et al., 2015). The weak extractability of target analytes can lead to false negatives, which may result in the incorrect conclusion. For example, there has been a controversy about the immunoreactivity of fish parvalbumin using a commercial anti-parvalbumin mAb, PARV19 (Sigma-Aldrich Co., St. Louis, MO, USA). As the major fish allergen (Van Do, Elsayed, Florvaag, Hordvik, & Endresen, 2005), parvalbumin reacts with immunoglobulin (Ig) E from more than 90% of fish allergic individuals (Bugajska-Schretter et al., 1998). Parvalbumin is a water-

soluble acidic intracellular Ca^{2+} binding protein, with a molecular weight (MW) of 10–13 kDa (Lopata & Lehrer, 2009). It is well known that parvalbumin content is different among various fish species (Kuehn, Swobode, Arumugam, Hilger, & Hentges, 2014). Even within the same species, parvalbumin is not evenly distributed in fish muscles (Lee, Nordlee, Koppelman, Baumert, & Taylor, 2012). It was reported that parvalbumin was immunodetected by Western blot (WB) using PARV19 in heated (95 °C for 15 min) Atlantic salmon, cod, orange roughy, and rainbow trout while it could not be detected in heated gummy shark (Saptarshi, Sharp, Kamath, & Lopata, 2014). However, another WB study showed that using the same sample preparation and immunodetection method, parvalbumin could not be detected in heated Atlantic salmon, cod, mahi-mahi, and orange roughy, but was immunodetectable in heated gummy shark and whiting (Sharp et al., 2015). When the heating conditions changed to 100 °C for 20 min, parvalbumin was not immunodetectable in cod, rainbow trout, and whiting; however, it was detected in mahi-mahi and orange roughy using PARV19-based indirect enzyme-linked immunosorbent assay (ELISA) (Gajewski & Hsieh, 2009). In order to explain this inconsistent phenomenon, two parvalbumin models were used in this study to demonstrate that matrix effect significantly impacts *in vitro* detection of food allergens. This study aims to investigate the relationship among matrix effect, extractability, and thermostability of fish parvalbumin during *in vitro* immunodetection. In this study, thermostability is defined as the capability of a target protein to retain its solubility,

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molecular integrity, and immunoreactivity after heat treatment.

2. Materials and methods

2.1. Materials

Fresh Atlantic salmon (*Salmo salar*) fillets were purchased from a local store (Tallahassee, FL, USA). Certified whole flathead grey mullet (*Mugil cephalus*) fishes were kindly provided by the Fish and Wildlife Research Institute (Eastpoint, FL, USA) at the Florida Fish and Wildlife Conservation Commission (FWC). Upon receiving, whole mullet fish was deboned. Boneless fish meats from both species were cut into small pieces, vacuum packed and stored at -80°C until use. One anti-parvalbumin mAb, 3E1, was previously developed in our laboratory (Gajewski & Hsieh, 2009). Two commercial antibodies, PARV19 (P3088) and goat anti-mouse IgG (Fc specific) horseradish peroxidase (HRP) conjugate (anti-IgG-HRP, A2554), were purchased from Sigma-Aldrich. All chemicals and reagents were of analytical grade. All solutions were prepared using deionized (DI) water from a NANOpure DIamond ultrapure water system (Barnstead International, Dubuque, IA, USA).

2.2. Model sample preparation

Two sample models were established to study the matrix effect on thermostability of parvalbumins from mullet and salmon (Fig. 1). *Model 1* was the crude fish protein extracts (PE). *Model 2* was the purified parvalbumin (PP). Unless otherwise specified, all subsequent operations were performed at 4°C . All samples were homogenized at 11,000 rpm for 2 min at room temperature (RT) using an ULTRA-TURRAX T25 basic homogenizer (IKA Works, Inc., Wilmington, NC, USA) and were centrifuged at $20,000g$ for 15 min. All heated samples were prepared at $100^{\circ}\text{C}/600$ rpm using a thermomixer (Eppendorf, Hamburg, Germany). Each heated sample was weighed to confirm whether its mass was lost during heat treatment. If so, it was reprepared. All samples were prepared and analyzed on the same day.

For *Model 1*, the PE samples were prepared according to Gajewski and Hsieh (2009) with modifications (Fig. 1). Half-thawed minced mullet and salmon fillets were thoroughly mashed by hand and mortar. 2 g of each meat was then mixed with 4 mL of ice-cold DI water. After homogenization and 2-h end-over-end rotation, the homogenate was centrifuged. The supernatant (i.e., unheated PE) was filtered through a filter paper (Whatman, Piscataway, NJ, USA) and a $0.8\text{-}\mu\text{m}$ syringe filter (Thermo Fisher Scientific Inc., Fair Lawn, NJ, USA). For heated

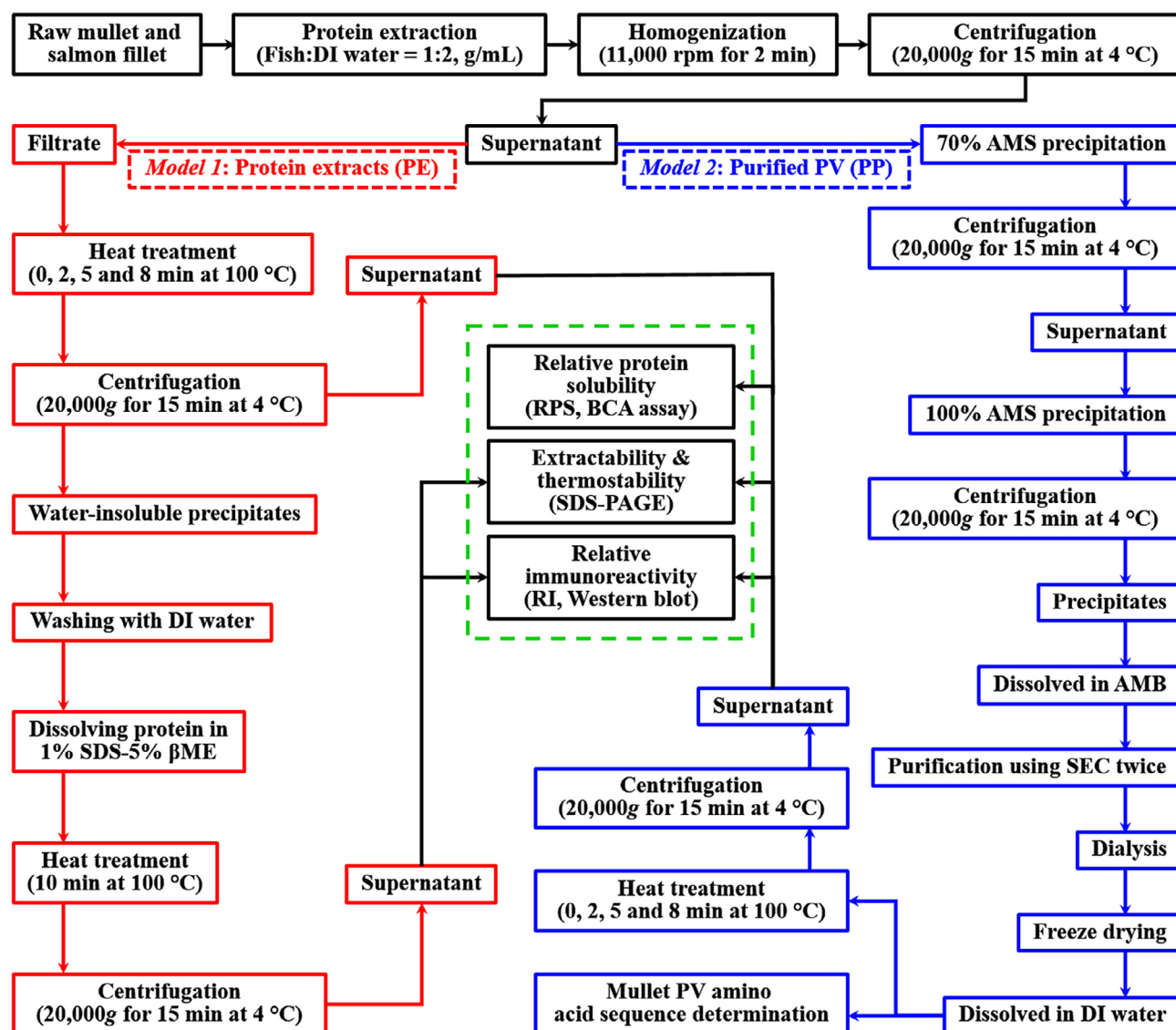


Fig. 1. Schema of sample preparation using DI water in two models.

PE, three 0.2-mL properly diluted PE extracts (1 mg/mL) were heated for 2, 5 and 8 min, respectively. The heated samples were centrifuged, and their supernatants were collected.

Simultaneously, heat-induced precipitates containing water-insoluble proteins were washed with 0.5 mL of DI water three times. After each wash, the mixture was thoroughly vortexed and centrifuged. The washed precipitates were then mixed with 0.2 mL of DI water containing 1% sodium dodecyl sulfate (SDS, g/mL) and 5% β -mercaptoethanol (mL/mL, SDS- β ME). The mixture was heated for 5 min, centrifuged and the supernatant was collected. It should be noted that no precipitates were visible to the naked eye in heated PE precipitate samples after centrifugation.

To study the effect of ethylenediaminetetraacetic acid (EDTA) on the extractability and thermostability of parvalbumin, DI water containing 5 mM EDTA was used to prepare another set of samples using the same procedure from Model 1.

For Model 2, a two-step purification of parvalbumin from each species was performed according to Ross, Hevener, Clark, Hartmann, and Mari (1998) with modifications (Fig. 1). First, parvalbumin was isolated from crude fish PE by ammonium sulfate (AMS) precipitation. 50 g of minced mullet and salmon meats were mixed with 100 mL of ice-cold DI water, respectively. After homogenization and centrifugation, the supernatant was filtered through glass wool and a filter paper (Whatman), respectively. The filtrate was taken to 70% (g/mL) AMS saturation, and the resulting precipitates were discarded by centrifugation. The remaining supernatant was further taken to 100% (g/mL) AMS saturation. The resulting precipitates were collected by centrifugation and suspended in 3 mL of 0.05 M ammonium bicarbonate (AMB, pH 7.8). The suspended proteins were dialyzed for 24 h in dialysis tubing (6–8 kDa MWCO, Spectrum Laboratories, Inc. Rancho Dominguez, CA, USA) with four changes against AMB. The dialyzed proteins (i.e., isolated parvalbumin) were lyophilized.

Second, the isolated parvalbumins from both species were purified twice by size exclusion chromatography (SEC) using the Econo System (Bio-Rad Laboratories Inc., Hercules, CA, USA). About 25 mg of isolated parvalbumin powder from each species was rehydrated in 1 mL of AMB. After filtered through a 0.8- μ m syringe filter (Thermo Fisher Scientific), it was loaded at a rate of 0.2 mL/min onto a 50-mL column (30 cm $\times$ 1.5 cm) packed with Sephadex G-75 (Pharmacia Fine Chemicals, Piscataway, NJ, USA). The loaded proteins were eluted from the column by AMB at 0.2 mL/min and collected in 2-mL fractions. The

absorbance of each fraction was monitored at 260 nm using a spectrophotometer (Bio-Rad). After analyzing the selected fractions using reducing SDS-polyacrylamide gel electrophoresis (SDS-PAGE, Section 2.6), the fractions containing parvalbumins were combined and concentrated using an Amicon Ultra-4 centrifugal filter unit (10 kDa MWCO, Millipore, Billerica, MA, USA). The concentrate was repurified by SEC under the same conditions. After verified by reducing SDS-PAGE and WB (Section 2.6), the fractions containing parvalbumins were combined and dialyzed for 24 h with four changes against DI water using dialysis tubing (6–8 kDa MWCO, Spectrum Laboratories) and then lyophilized. The PP powder was stored at -80°C until use. The purity of PP from both species and their isoelectric points (pI) were further analyzed using two-dimensional gel electrophoresis (2-DE, Supplementary document) and WB.

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.foodchem.2018.08.138>.

For unheated PP, lyophilized mullet and salmon PP (1 mg/mL) was dissolved in DI water, respectively. For heated PP, three 0.2-mL PP samples were heated for 2, 5 and 8 min, respectively. After centrifugation, supernatants were collected. It should be noted that unlike heated PE in Model 1, no precipitates were visible to the naked eye in heated PP after centrifugation.

2.3. Relative protein solubility (RPS)

The water-soluble protein concentration of all samples prepared in both models was determined using the Pierce BCA (bicinchoninic acid) Protein Assay Kit (Thermo Fisher Scientific). Bovine serum albumin (BSA, Thermo Fisher Scientific) was the protein standard (working range: 20–2000 $\mu\text{g/mL}$). For the same species in the same model, the RPS (%) of three heated samples compared with their unheated one was calculated based on their total protein concentration.

2.4. Mullet parvalbumin amino acid (AA) sequencing

AA sequence of mullet PP was analyzed in the Translational Science Laboratory at Florida State University (Tallahassee, FL, USA). Briefly, in-solution partial tryptic digestion was performed using ProteoExtract All-in-One Trypsin Digestion Kit (Calbiochem, Darmstadt, Germany) according to manufacturer's instructions. About 20 μg of lyophilized mullet parvalbumin was resuspended in 25 μL of extraction buffer by

Table 1
Amino acid (AA) sequence comparison of parvalbumins from mullet, salmon, and catfish.[†]

Organism	Protein name	Accession number	AA sequence [*]	Molecular weight (Da)	Theoretical isoelectric point (pI)
<i>Mugil cephalus</i> (flathead grey mullet)	Parvalbumin	Not available	1 –AFAGLLSDADITAAIAAQAADSFYKDFFAKVGLAAKSADDKKKAFVVIDQDK 55 SGFIEDELKFLQNFASAGARALTDKETKFLKAGDSGDGKIGVDEFAAMVK-	54 107	11,264 4.51
<i>Salmo salar</i> (Atlantic salmon)	Parvalbumin beta 1	Q91482	1 MA [*] CAHLCKEADIKTALEACKAADTFSEKTFHTIGFASKSADDVKKAKKVIDQDA 56 SGFIEVEELKFLQNF [*] CPKARELTDKETKFLKAGDADGDMIGIDEFAVLVKQ	55 109	11,889 4.95
	Parvalbumin beta 2	Q91483	1 MSFAG-LNDADVAAALAACTAADSFNHKAFFAKVGLASKSSDDVKKATYVIDQDK 55 SGFIEDELKFLQNFASASARALTDKETKFLADGDKDGMIGVDEFAAMIKG	54 108	11,383 4.41
	Parvalbumin alpha	COHAT9	1 MAMNSILNAADIKKALDAFAAADSFDHKKFFEMVGLAKSAEDVKKATLVLDADA 55 SGFIEEELKFLVKGFSADGRDLTDKETKFLNEADKDGDMIGIDEFVALVHE	54 109	11,893 4.53
<i>Ictalurus punctatus</i> (channel catfish)	Parvalbumin 2	E3TGD0	1 MAFAGILKDEDVAAISA [*] CAADSFNKTFFAKVGLSAKSADEIKKATFVIDQDK 56 SGFIEDELKFLQNFASAGARALTDKETKFLAAGDSGDGKIGVDEFAALVKA	55 109	11,470 4.45
<i>Ictalurus furcatus</i> (blue catfish)	Parvalbumin beta	E3TBW7	1 MAFAGVLNDADITAAALDA [*] KADGSFDHKSFFTKVGLTGKSADDVKKATAIIDQDK 56 SGFIEDELKFLQNFKSSARALTDDETETKFLKAGDGDGKIGVDEFAALVKA	55 109	11,632 4.56

^{*} Cysteine residues are highlighted in yellow with a single underline. The epitope-containing peptides of 3E1 are highlighted in green with a double underline.

[†] All information is obtained from The UniProt Consortium (2017).

vigorous vortexing. The sample was centrifuged at 21,000g for 10 min, and the supernatant was transferred to a fresh tube. About 25 μ L of digestion buffer was added along with 1 μ L of reducing agent and

incubated at 37 °C for 10 min. Samples were cooled to RT and then blocked using blocking reagent for 10 min at RT. Trypsin at a final concentration of 2 ng/ μ L was added and incubated for 1 h at 37 °C with

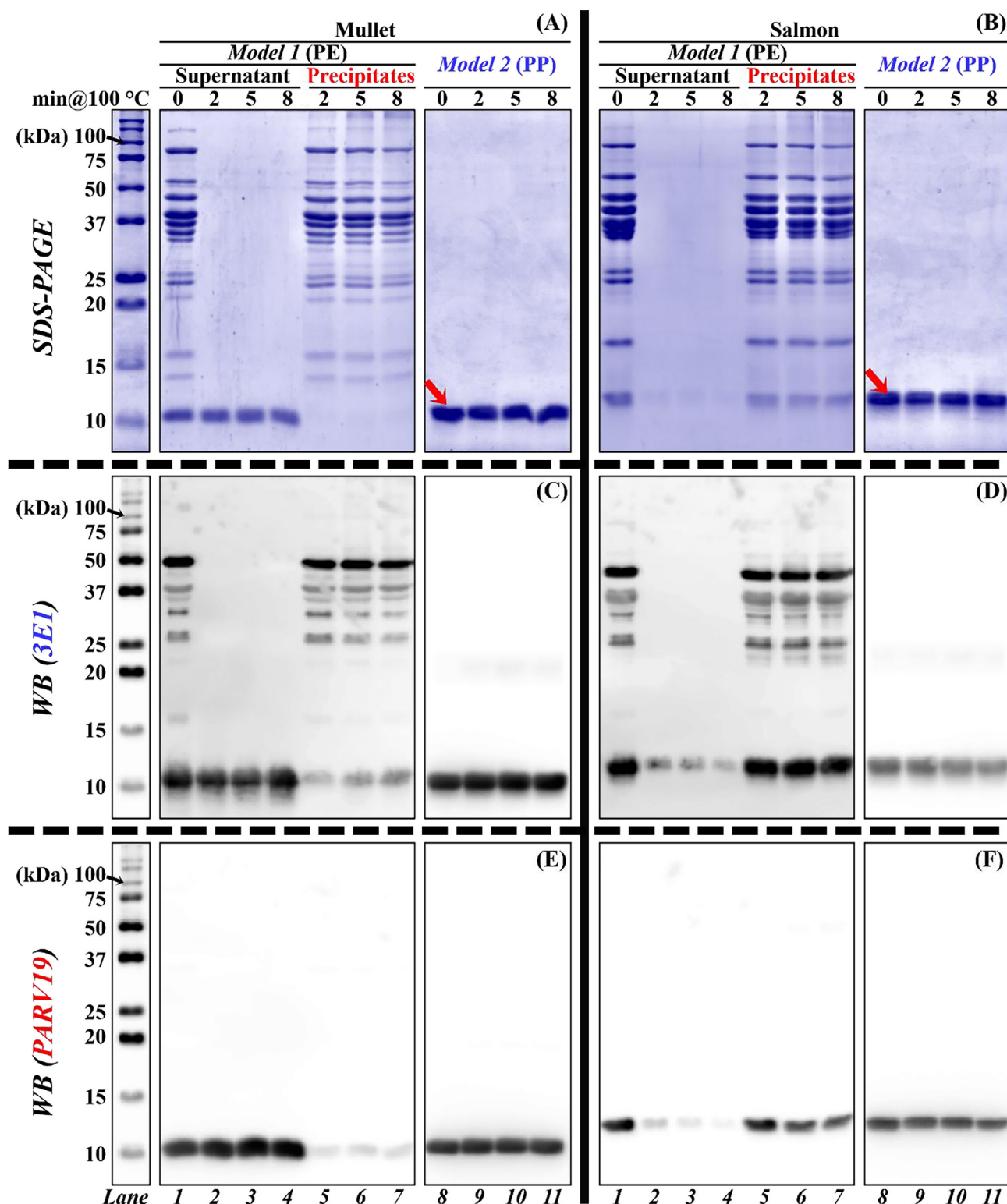


Fig. 2. Effect of heating time at 100 °C on the extractability and immunodetection of water-extracted parvalbumins in two models using reducing SDS-PAGE (A and B) and WB (3E1: C and D; PARV19: E and F). Mullet: A, C, and E; Salmon: B, D, and F. The loaded protein mass for unheated PE and PP samples were 5 μ g/lane and 0.5 μ g/lane, respectively. Each heated sample was prepared with the same dilution ratio as its unheated one without determining protein concentration. Both 3E1 (1.36 μ g/mL) and PARV19 (1:3000, μ L/ μ L) were diluted in the antibody buffer (BSA-PBST). The Precision Plus Protein WesternC standard (Bio-Rad) was used to indicate the molecular weights. The purified parvalbumins (PP) from mullet and salmon are indicated using a red arrow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

shaking. Peptides were eluted in 40 μ L 0.1% formic acid and run on liquid chromatography-mass spectrometry analysis (LC/MS), as described below.

For bottom-up sequencing, an externally calibrated Thermo Q Exactive HF (high-resolution electrospray tandem mass spectrometer) was used in conjunction with Dionex UltiMate3000 RSLCnano System (Thermo Fisher Scientific). About 0.5 μ g/ μ L sample was aspirated into a 50 μ L loop and loaded onto the trap column (Thermo μ -Precolumn 5 mm). The flow rate was set to 0.3 μ L/min for separation on the analytical column (Acclaim pepmap RSLC 75 μ m $\times$ 15 cm). Mobile phase A was composed of 99.9% water (EMD Omni Solvent), and 0.1% formic acid and mobile phase B was composed of 99.9% acetonitrile and 0.1% formic acid. A 1-h linear gradient from 3% to 45% B was performed. The LC eluent was directly nanosprayed into the mass spectrometer. During the chromatographic separation, the mass spectrometer was operating in a data-dependent mode and under the direct control of the Xcalibur 3.1.66 instrument software (Thermo Fisher Scientific). The MS data were acquired using the following parameters: 20 data-dependent MS/MS scans per full scan (350–1700 m/z) at 60,000 resolution. MS2 was acquired in centroid mode at 15,000 resolution. Ions with single charge or charges more than seven as well as unassigned charge were excluded. A 15-second dynamic exclusion window was used. All measurements were performed at RT. Resultant raw files were searched with PEAKS Studio 7.5 (Bioinformatics Solutions, Inc., Waterloo, ON, USA).

2.5. Epitope mapping

The epitope of 3E1 was mapped using the entire AA sequence of mullet parvalbumin (Table 1). Through using the SPOT synthesis technique, 32 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 nM peptide. To increase the stability, the peptides were N-acetylated. The membrane was stored at -20 °C until use. Before mapping, the membrane was incubated with 25 mL of methanol for 5 min to prevent precipitation of hydrophobic peptides during the washing step. All subsequent manipulations were the same as those for WB (Section 2.6) except that (1) the buffers containing 10 mM phosphate buffered saline (PBS, pH 7.2) were replaced by 10 mM Tris-buffered saline (TBS, pH 8.0); and (2) the Precision Protein StrepTactin-HRP conjugate (Bio-Rad) was not used.

2.6. Reducing SDS-PAGE and Western blot (WB)

Reducing SDS-PAGE was used to evaluate the effect of matrix on the extractability and thermostability of parvalbumin after different heat treatments. Briefly, after appropriately diluted in DI water and then mixed 1:1 (mL/mL) with SDS sample buffer, unheated and heated samples from both models were separated by SDS-PAGE (4% stacking gel and 15% separating gel) using a Mini-PROTEAN Tetra Cell (Bio-Rad). The loaded protein mass for unheated PE and PP samples were 5 μ g/lane and 0.5 μ g/lane, respectively. Each heated sample was prepared using the same dilution ratio as its unheated one without determining protein concentration. The protein profiles on the gel were visualized by the Coomassie blue staining.

WB was performed (1) to evaluate the effect of heating time on the antigenicity of parvalbumin, and (2) to study the effect of different chemicals (SDS, β ME, EDTA, and CaCl_2) on the immunodetection of parvalbumin. Briefly, electrophoretically separated protein bands on the unstained gel were transferred onto a 0.45- μ m nitrocellulose membrane (Bio-Rad) using a Trans-Blot Turbo Transfer system (Bio-Rad). The transferred proteins on the membrane were verified using the Ponceau S staining. The membrane was then blocked using the antibody buffer (BSA-PBST: 1% BSA (g/mL) in 0.05% (mL/mL) Tween 20 in

PBS (PBST)) with or without 10 mM EDTA overnight at 4 °C. After incubated with either 3E1 (1.36 μ g/mL) or PARV19 (1:3000, μ L/ μ L), diluted in the antibody buffer with or without EDTA, the membrane was further incubated with anti-IgG-HRP conjugate (167 ng/mL) and the Precision Protein StrepTactin-HRP conjugate (1:50,000, μ L/ μ L, Bio-Rad) dissolved in the antibody buffer. The blotted antigens were detected using the luminol chemiluminescence method (Rao, Richt, & Hsieh, 2016).

After color development, the membrane incubated with PARV19 in the antibody buffer with EDTA was reblocked with the antibody buffer containing 5 mM CaCl_2 . After incubation with PARV19 (1:3000, μ L/ μ L), diluted in the antibody buffer with CaCl_2 , the membrane was treated with the same steps as previously mentioned in this section. During WB, the incubation time of each step was at least 1 h at RT. Between each step, the membrane was washed several times with PBST. The Precision Plus Protein WesternC standards (Bio-Rad) were used for accurate MW estimation on gels and blots. For the same model, the relative immunoreactivity (RI, %) of parvalbumin was calculated based on the parvalbumin band color intensity in three heated samples compared with their unheated one from the same species. All images from SDS-PAGE and WB were captured by the Azure C600 Imaging System (Azure Biosystems Inc., Dublin, CA, USA) and were analyzed using the AzureSpot software (version 14.2).

2.7. Statistical analysis

For the same model, only intraspecies comparison of the matrix effect on RPS and RI among different heat treatments was performed using two-way analysis of variance (ANOVA) with Tukey post-test (GraphPad Prism, version 7.03 for Windows, GraphPad Software, Inc., La Jolla, CA, USA). All experiments were independently repeated at least twice. $P < 0.05$ was considered as statistically significant.

3. Results and discussion

3.1. Sample model comparison

In this study, two sample models (PE and PP) were established for each fish species (Fig. 1). There were two major differences between two models. First, the unheated sample matrix complexity of *Model 1* (PE) was greater than that of *Model 2* (PP). As shown in the SDS-PAGE (Fig. 2A), different water-soluble protein bands were observed in the unheated mullet PE (*Model 1*, lane 1), while only one major protein band (~ 11 kDa) was observed in the unheated mullet PP (*Model 2*, lane 8). This 11-kDa band was further confirmed as parvalbumin by WB using two parvalbumin-specific mAbs, 3E1 (lane 8, Fig. 2C) and PARV19 (lane 8, Fig. 2E). Similar findings were also observed in two salmon models (Fig. 2B, D, and F). According to SDS-PAGE and WB (lane 8, Fig. 2B, D, and F), the MW of salmon parvalbumin (~ 12 kDa) is slightly larger than that of mullet parvalbumin. The detailed AA sequence comparison of parvalbumins from both species is shown in Table 1. It should be noted that the complete AA sequence of mullet parvalbumin is reported for the first time, which has 67% and 79% sequence identity to salmon parvalbumin $\beta 1$ and $\beta 2$ isoforms, respectively.

Second, the quantity of the target analyte (i.e., parvalbumin) in *Model 1* was unknown due to the sample matrix complexity while it was known in *Model 2* due to the sample purity. However, both models had a common ground that the maximum quantity of parvalbumin in all heated samples (*Model 1*: lanes 2–4; *Model 2*: lanes 9–11, Fig. 2A and B) was fixed, which was no more than that in their unheated extracts because each heated sample was directly prepared from its unheated one (Fig. 1). This is crucial to this study since it is helpful in quantitative analysis of parvalbumin among different heating times within the models. Furthermore, protein extractability can be affected by many internal and external factors (Efsa NDA Panel (EFSA Panel on Dietetic

Table 2

Comparison of (1) the relative protein solubility (RPS) and (2) the relative immunoreactivity (RI) of parvalbumins from mullet and salmon as a function of heating time at 100 °C.

RPS study		Mullet (mean ± SEM ^a , %)				Salmon (mean ± SEM, %)			
Time (min) at 100 °C		0	2	5	8	0	2	5	8
Model 1 (PE)									
DI water		100.0 ± 0.0 ^a	17.5 ± 1.5 ^b	17.4 ± 1.5 ^b	16.8 ± 1.4 ^b	100 ± 0.0 ^a	8.6 ± 0.6 ^b	8.0 ± 0.6 ^b	8.5 ± 0.7 ^b
5 mM EDTA		100.0 ± 0.0 ^a	22.6 ± 0.7 ^b	22.7 ± 0.4 ^b	22.9 ± 1.2 ^b	100 ± 0.0 ^a	12.4 ± 0.2 ^b	12.6 ± 0.1 ^{bc}	13.0 ± 0.1 ^c
Model 2 (PP)									
DI water		100.0 ± 0.0 ^a	97.6 ± 2.4 ^a	97.4 ± 3.2 ^a	97.2 ± 2.6 ^a	100.0 ± 0.0 ^a	98.1 ± 4.8 ^a	92.5 ± 2.4 ^a	97.4 ± 1.8 ^a
RI study									
3E1	Model 1 (PE)								
	DI water	100.0 ± 0.0 ^a	52.9 ± 0.3 ^b	50.9 ± 1.1 ^b	57.6 ± 1.1 ^b	100.0 ± 0.0 ^a	16.0 ± 1.8 ^b	11.6 ± 1.2 ^{bc}	7.5 ± 0.1 ^c
	5 mM EDTA	100.0 ± 0.0 ^a	68.2 ± 0.4 ^b	72.9 ± 3.5 ^b	70.4 ± 3.3 ^b	100.0 ± 0.0 ^a	35.8 ± 3.6 ^b	35.1 ± 1.9 ^b	38.5 ± 0.8 ^b
	Model 2 (PP)								
	DI water	100.0 ± 0.0 ^a	108.7 ± 6.2 ^a	108.9 ± 8.7 ^a	101.9 ± 4.4 ^a	100.0 ± 0.0 ^a	95.9 ± 2.9 ^a	88.9 ± 1.4 ^a	86.3 ± 2.0 ^a
	Model 1 (PE)								
	DI water	100.0 ± 0.0 ^a	115.1 ± 0.4 ^b	124.5 ± 0.9 ^c	128.4 ± 3.8 ^c	100.0 ± 0.0 ^a	15.8 ± 0.2 ^b	7.7 ± 0.3 ^c	4.1 ± 0.3 ^c
PARV19	5 mM EDTA	100.0 ± 0.0 ^a	125.5 ± 4.5 ^b	126.3 ± 4.5 ^b	125.3 ± 1.2 ^b	100.0 ± 0.0 ^a	82.6 ± 0.7 ^b	72.9 ± 1.1 ^c	78.0 ± 1.0 ^{bc}
	Model 2 (PP)								
	DI water	100.0 ± 0.0 ^a	104.2 ± 0.3 ^a	104.5 ± 2.5 ^a	103.4 ± 0.2 ^a	100.0 ± 0.0 ^a	89.7 ± 2.0 ^b	87.3 ± 2.1 ^b	75.3 ± 2.7 ^c

^a SEM: the standard error of the mean.

^{a,b,c,d} For the same species, values in the same row with different letters are significantly different within the model ($P < 0.05$).

Products Nutrition and Allergies) (2014)). The major internal factors include the properties of the target proteins such as pIs and polar/apolar nature. The major external factors are food processes, food/sample matrix, and characteristics of the extraction methods such as the pH and the extraction temperature.

3.2. Relative protein solubility (RPS) of model samples

In *Model 1*, the RPS of both mullet and salmon water soluble PE samples decreased significantly after 2 min of heating by about 82% and 91%, respectively, while there was no significant difference in RPS among different heating times for each heated PE water samples within the species (*Model 1*, Table 2). No significant difference in RPS was observed between any PP samples in each species (*Model 2*, Table 2). The protein profiles of these samples were shown in reducing SDS-PAGE (Fig. 2A and B). In both models from two species (Fig. 2A and B), no other protein bands, except parvalbumin monomer, were observed in any heated samples (*Model 1*: lanes 2–4; *Model 2*: 9–11). However, in *Model 2*, there was no significant difference in protein profile between unheated (lane 8) and heated (lanes 9–11) samples from both species (Fig. 2A and B). These results indicated that heat-induced water-insoluble protein aggregates (i.e., protein precipitates) were formed in *Model 1* but not in *Model 2*.

To further study the effect of matrix-induced thermal instability of parvalbumin, heat-induced protein precipitates in *Model 1* were dissolved in SDS-βME. From SDS-PAGE, it was shown that after heat treatment, water-soluble proteins turned into water-insoluble but could be resolubilized in SDS-βME (lanes 5–7, Fig. 2A and B). SDS is an anionic detergent, which can disrupt noncovalent bonds such as the hydrophobic interaction in the proteins and impart a negative charge to the proteins. βME is a strong reductant whose redox potential is –260 mV at pH 7 and can cleave the inter- and intra-molecular disulfide bonds because the disulfide redox potentials in proteins vary from –122 to –270 mV (Pulgisi, Aitken, & Marshall, 2008; Winther, Ostergaard, Henriksen, & Hansen, 2001).

Heat-induced protein aggregation/precipitation was presumably due to the coaction of two factors. First, due to the increased exposure of the hydrophobic regions, protein aggregates can form by lowering the unfavorable exposure environment. Second, sulphhydryl groups and disulfide bonds that are not accessible in the native state can become available and may react heterogeneously and homogeneously to form

inter- and intramolecular cross-links, respectively. It is noted that mullet parvalbumin contains only one cysteine group at position 18, while salmon parvalbumin contains five cysteine groups (β1 isoform: position 18; β2 isoform: positions 3, 7, 19, and 72, Table 1). This implies that salmon parvalbumin, compared with mullet parvalbumin, more easily undergoes protein-protein interactions through thiol-disulfide interchange reactions during heat treatment. Based on the color intensity of the parvalbumin-bands in SDS-PAGE (Fig. 2A and B), after 8-min heat treatment, the remaining water-soluble mullet parvalbumin monomer was about 94% (lane 4, Fig. 2A), while it was only about 7% for salmon parvalbumin monomer (lane 4, Fig. 2B).

3.3. Epitope mapping of 3E1

Through epitope mapping of the synthetic mullet parvalbumin peptides, one epitope-containing peptide for 3E1 (IKKAFDAVID) was identified (Table 1). This mAb was developed against heated catfish sarcoplasmic protein extract (Gajewski & Hsieh, 2009). Based on the results of 2-DE (Supplementary Fig. 1), parvalbumins from mullet and salmon had one and three major isoforms with different acidic pIs (4–5, Table 1), respectively, which needs to be further confirmed experimentally. All of them could react with 3E1 (Supplementary Fig. 1), indicating that they have the same epitope for 3E1. Based on the AA sequence comparison (Table 1), one peptide segment, KKAF, was common in all parvalbumin AA sequences of mullet, salmon, and catfish, indicating that it should be a unique linear epitope for 3E1.

3.4. Relative immunoreactivity (RI) of parvalbumin

In the 3E1 WB (Fig. 2C and D), after 2 min of heating, the RI of parvalbumins in mullet and salmon PE water samples (*Model 1*) decreased about 47% and 84%, respectively (Table 2). No significant differences were observed in the PP water samples from both species as a function of heating time (*Model 2*, Table 2). In the PARV19 WB (Fig. 2E and F), the RI of parvalbumin in mullet water soluble PE (*Model 1*) increased as a function of heating time, while it was exactly the opposite in both salmon PE and PP water samples (Table 2). However, no significant differences were observed in the mullet PP water samples over the heating time (*Model 2*, Table 2).

In addition, the WB analysis revealed three other findings. First, oligomerization of parvalbumin was observed. From the 3E1 WB,

several parvalbumin oligomer bands between 25 kDa and 50 kDa were identified in unheated PE samples from both species (lane 1, Fig. 2C and D), which may correspond to parvalbumin polymers such as dimer, trimer, and tetramer. This indicates that the reducing conditions of SDS-

PAGE did not completely dissociate these oligomers. Similar results were observed in parvalbumins from Atlantic cod (de Jongh, de los Reyes Jimenez, Baumert, Taylor, & Koppelman, 2015) and wolf herring (Mohammadi et al., 2016). Interestingly, in the 3E1 WB, no

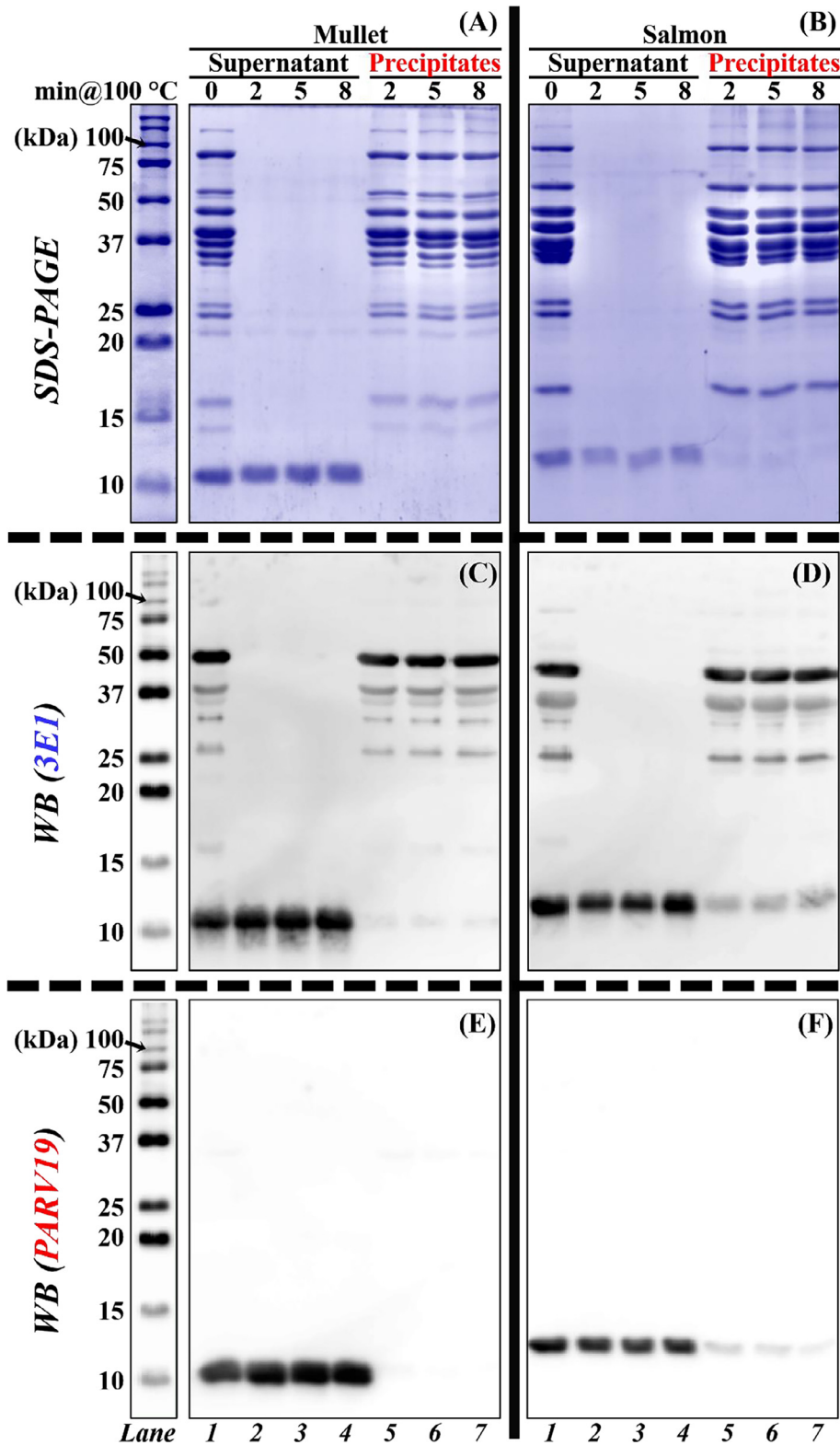


Fig. 3. Effect of heating time at 100 °C on the extractability and immunodetection of EDTA-extracted parvalbumins in Model 1 using reducing SDS-PAGE (A and B) and WB (3E1: C and D; PARV19: E and F). Mullet: A, C, and E; Salmon: B, D, and F. The loaded protein mass for unheated PE samples were 5 µg/lane. Each heated sample was prepared with the same dilution ratio as its unheated one without determining protein concentration. Both 3E1 (1.36 µg/mL) and PARV19 (1:3000, µL/µL) were diluted in the antibody buffer (BSA-PBST). The Precision Plus Protein WesternC standards (Bio-Rad) were used to indicate the molecular weights.

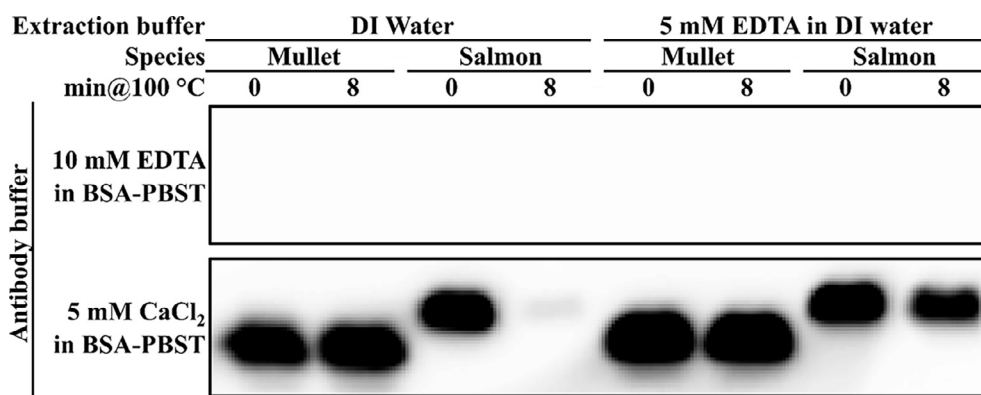


Fig. 4. Effect of EDTA on the immunoreactivity of parvalbumins from mullet and salmon (*Model 1*) in the WB using PARV19. The loaded protein mass for unheated PE samples was 5 µg/lane. Each heated sample was prepared with the same dilution ratio as its unheated one without determining protein concentration. PARV19 was 1:3000 (µL/µL) diluted in the antibody buffer (BSA-PBST) with 10 mM EDTA or 5 mM CaCl₂.

parvalbumin oligomer was immunodetectable in *Model 1* after any of heat treatments (lanes 2–4, Fig. 2C and D), while they were immunodetectable in the SDS-βME-soluble precipitates (lanes 5–7, Fig. 2C and D). Our SDS-PAGE and WB results suggest that mainly due to the matrix-induced thermal instability of parvalbumin, parvalbumin oligomers had two outcomes during heat treatment: dissociate into soluble parvalbumin monomer and/or aggregate into insoluble polymers. Second, the PP from both species (*Model 2*) was thermostable over the 8-min heat treatment at 100 °C since no significant matrix effect was observed in WBs using two mAbs, 3E1 (Fig. 2C and D) and PARV19 (Fig. 2E and F). It is noted that a faint parvalbumin dimer band was detected by both mAbs in all heated PP samples from both species (lanes 9–11, Fig. 2C–F). Third, although both mAbs could recognize parvalbumins from both species, their epitopes were different. Compared with 3E1 which could detect parvalbumin monomer and oligomers (Fig. 2C and D), PARV19 could only react with parvalbumin monomer (Fig. 2E and F).

3.5. Effects of EDTA on the extractability and thermostability of parvalbumin

Mullet and salmon PE samples (*Model 1*) prepared without (i.e., DI water only) or with 5 mM EDTA were used to study the effects of EDTA on the extractability and thermostability of parvalbumin. Overall, there are three major findings. First, the addition of 5 mM EDTA during sample preparation improved the total protein extractability. The SDS-PAGE protein profiles of samples prepared using the EDTA (Fig. 3A and B) were similar to those relevant water samples from the same species (Fig. 2A and B). However, for the same species, the RPS of the heated samples prepared with EDTA was slightly higher (4–5%) than their water counterparts (Table 2). Second, the addition of 5 mM EDTA during protein extraction enhanced the extractability of parvalbumin. For the same heat treatment, the quantity of parvalbumin monomer in the EDTA samples (lanes 2–4, Fig. 3A and B) increased compared with that in the water samples (lanes 2–4, Fig. 2A and B), especially in the heated salmon samples prepared with EDTA (lanes 2–4, Fig. 3B). For example, based on the relative band color intensity, after 8-min heat treatment, the quantity of salmon parvalbumin monomer in the EDTA extract (lane 4, Fig. 3B) increased about 15 fold compared with that in the water extract (lane 4, Fig. 2B). Correspondingly, the quantity of the parvalbumin in the heat-induced EDTA precipitates decreased (lanes 5–7, Fig. 3A and B) because the total amount of parvalbumin in each sample was fixed. It was reported that 10 mM EDTA effectively prevented lipid droplet aggregation in heated whey protein-stabilized emulsions (Keowmaneechai & McClements, 2006). Third, the addition of 5 mM EDTA during protein extraction increased the RI of parvalbumin in WBs using two mAbs, 3E1 (Fig. 3C and D) and PARV19 (Fig. 3E and F). In addition, both immunoblots showed that the effect of EDTA on the RI of salmon parvalbumin was stronger than that of mullet parvalbumin (Table 2). It was observed that in the PARV19 WB, after 8-

min heat treatment, the color intensity of salmon parvalbumin monomer in the EDTA extract (lane 4, Fig. 3F) increased about 22 fold compared with that in the water extract (lane 4, Fig. 2F), while it decreased about 13 fold in the EDTA precipitates (lane 7, Fig. 3F) compared with that in the water precipitates (lane 7, Fig. 2F).

As a food additive (USFDA, 1977), calcium disodium EDTA is one of the most commonly used food ingredients for sequestering multivalent ions, which can significantly contribute to stabilization of food color, aroma, and texture (Keowmaneechai & McClements, 2002). Additionally, EDTA is widely used for protein extraction to (1) inhibit proteases, (2) eliminate endotoxin, and (3) prevent oxidation by metals (Monico, Martinez-Senra, Canada, Zorrilla, & Perez-Sala, 2017). Both fish species contain four EDTA-chelatable metal minerals (i.e., Fe, Zn, Ca and Mg, Supplementary Table 1). A hierarchy for the relative binding affinities of EDTA for these metals is $\text{Fe}^{3+} > \text{Zn}^{2+} > \text{Fe}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+}$ (Waters, Bryden, Patterson, Veillon, & Anderson, 2001). It is easy to assume that depending on the accessibility of different metal ions in the sample matrix, EDTA should follow this order to chelate them in both models. Furthermore, based on the antibody manufacturer's instruction, PARV19 is a calcium-dependent antibody. Hence, our PARV19 WB findings indicated that (1) those parvalbumin oligomers detected by 3E1 were Ca^{2+} -free because they were not detected by PARV19 (Figs. 2 and 3); and (2) parvalbumin monomers from both species in both models (with or without 5 mM EDTA) contained Ca^{2+} ion because they were detected by PARV19 (Figs. 2 and 3). Addition of 5 mM EDTA during protein extraction did not completely chelate Ca^{2+} in the samples from both species (Fig. 3E and F), especially in mullet samples (Table 2). Although the contents of Fe^{2+} , Zn^{2+} and Mg^{2+} ions are similar in both species, the Ca^{2+} ion content in mullet is about four times higher than that of salmon (Supplementary Table 1).

In addition, when the membrane containing mullet and salmon parvalbumin monomers (*Model 1*, with or without 5 mM EDTA) was incubated with 10 mM EDTA, parvalbumin monomers were no longer immunodetectable using PARV19 (Fig. 4), indicating that Ca^{2+} in all parvalbumin monomers had been completely chelated by the extra amount of EDTA. When the same membrane was further incubated with 5 mM CaCl₂, parvalbumin monomers in all samples were detected by PARV19 again (Fig. 4), indicating that PARV19 has a Ca^{2+} -dependant conformational epitope. It is noted that the antigenicity of 3E1 was not affected by the same experiment, which still produced the same results shown in Fig. 3C and D, denoting that 3E1 is calcium-independent.

In this study, the effects of three chemicals, SDS (surfactant), βME (reducing agent) and EDTA (metal chelator), on the extractability and thermostability of parvalbumins from mullet and salmon were compared. Our results clearly showed that addition of a suitable amount of these three chemicals during sample preparation could (1) effectively reduce the matrix effect on parvalbumins from both species, (2) greatly increase their thermostability, and (3) significantly improve their immunodetectability. This approach was more beneficial for the *in vitro*

detection of salmon parvalbumin compared with mullet parvalbumin. However, the dose-effect relationship between each chemical and the antibody should be carefully studied. For instance, as shown in Fig. 4, an excess amount of EDTA led to false negative results in the PARV19 WB. It has been reported that the antigen-antibody interaction could be completely inhibited when human IgG was premixed with SDS at concentrations above 0.06% due to extensive protein denaturation or SDS blocking of potential sites of epitope-paratope interaction (Lechtzier et al., 2002).

4. Conclusions

Overall, parvalbumins from mullet and salmon in two simple sample models were used in this study to demonstrate that matrix effect can significantly affect *in vitro* food allergen detection. In *Model 1*, matrix-induced thermal instability of parvalbumin was mainly due to physical (i.e., hydrophobic effect) and chemical (i.e., thiol-disulfide interchange) interactions. In *Model 2*, parvalbumins purified from both species were thermostable without matrix effect. Our results also showed that the addition of SDS (surfactant), β ME (reducing agent) and EDTA (metal chelator) during sample preparation not only increased the extractability of parvalbumin but also enhanced its immunodetection using two mAbs (3E1 and PARV19). On the other hand, our findings suggested that as a result of the excess amount of EDTA, parvalbumin monomer became Ca^{2+} -free and undetectable in the PARV19 WB. In summary, our findings illustrated the importance of extraction method selection. To extract target analytes efficiently, extraction buffer ingredients, as well as extraction conditions, should be carefully selected based on the antigen and antibody characteristics. Overall, due to potential life-threatening nature of allergic reactions, the importance of studying matrix effects during *in vitro* food allergen detection should not be underestimated and it should be studied carefully to avoid any false negative assay consequences.

5. Conflict of interests

The authors declare no competing financial interest.

Acknowledgements

A part of this study was supported in part by the Florida State University Dissertation Research Grant, and by the Florida State University Council on Research and Creativity (CRC) Planning Grant. 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.

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Supplementary document

Supplementary Table 1. Comparison of the mineral content (mg/100 g edible portion) between raw mullet and salmon¹

Minerals	EDTA-chelatable (pK-values) ²	Mullet 15055 [*]	Atlantic salmon	
			Wild 15076 [*]	Farmed 15236 [*]
Iron (Fe)	Yes (25.7 for Fe ³⁺ ; 14.3 for Fe ²⁺)	1.02	0.8	0.34
Zinc (Zn)	Yes (16.5)	0.52	0.64	0.36
Calcium (Ca)	Yes (10.7)	41	12	9
Magnesium (Mg)	Yes (8.7)	29	29	27
Potassium (K)	No	357	490	363
Sodium (Na)	No	65	44	59
Phosphorus (P)	No	221	200	240

¹ US Department of Agriculture (2015); ² Belitz, Grosch, and Schieberle (2009)

^{*} US Department of Agriculture National Nutrient Database number

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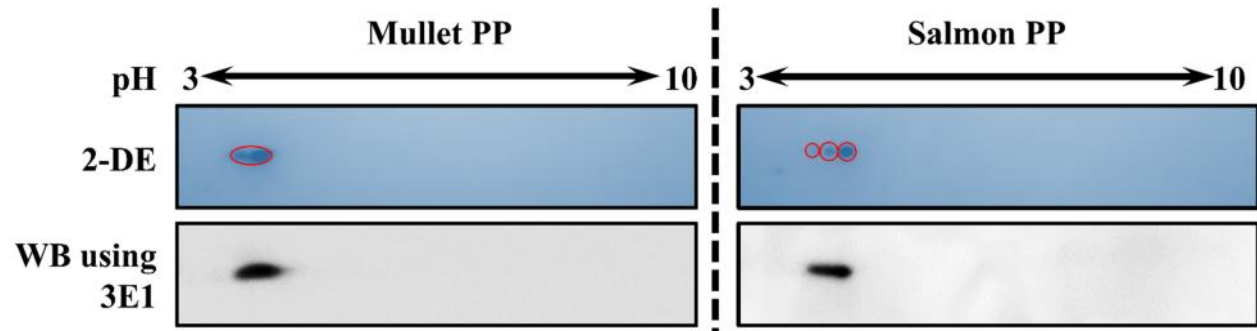
Supplementary document

Two-dimensional gel electrophoresis and Western blot for analysis of purified parvalbumins

Methods

Two-dimensional gel electrophoresis (2-DE) and Western blot (WB) were performed to analyze the purified parvalbumins (PP, *Model 2*) from mullet and salmon. 2-DE was carried out with isoelectric focusing (IEF) as the first dimension and SDS-PAGE as the second. For IEF, each ReadyStrip IPG strip (7 cm, pH 3-10 nonlinear, immobilized pH gradient (IPG), Bio-Rad) was rehydrated in the Bio-Rad rehydration buffer containing 1 μ g of mullet or salmon PP samples at 50 V for 12 h. The strips were then focused for 20 min at 250 V, 2 h at 4,000 V, and were held at 4,000 V until total voltage hour (Vh) reached 20,000 Vh using a PROTEAN IEF Cell (Bio-Rad). Before running SDS-PAGE, the strips were equilibrated in the Bio-Rad ReadyPrep equilibration buffer I, the Bio-Rad ReadyPrep equilibration buffer II, and the SDS running buffer for 10 min, respectively. The remaining steps of SDS-PAGE and WB using 3E1 were the same as previously mentioned in *Section 2.5*.

Results



Supplementary Figure 1. Two-dimensional gel electrophoresis (2-DE) and Western blot (WB) for analysis of purified parvalbumins (PP) from mullet and salmon. The IgG concentration of 3E1 was 1.36 μ g/mL. Different potential PV isoforms are indicated using a red circle.