



Quantitative analysis of species specificity of two anti-parvalbumin antibodies for detecting southern hemisphere fish species demonstrating strong phylogenetic association



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ABSTRACT

This study aimed to develop a novel approach to determine the correlation between the parvalbumin (PAV) contents and their corresponding immunoreactivity (detectability) in southern hemisphere fish species. The immuno-detected PAV contents of the test fish species were estimated by a quantitative SDS-PAGE. A quantitative Enzyme-Linked ImmunoSorbent Assay (ELISA) was formatted to assess relative immunoreactivity of PAV. Sixteen species (forty-three percent) displayed a positive correlation with the anti-cod PAV polyclonal antibody, but no correlation with the anti-carp PAV monoclonal antibody. There was a strong phylogenetic association of the PAV immunoreactivity. Species from the order *Perciformes* showed strong binding with both antibodies; whereas species from *Salmoniformes*, *Ophidiiformes*, *Scombriformes*, *Scorpaeniformes*, and *Tetraodontiformes* showed weak or no binding. This approach showed for the first time a statistical correlation between the PAV content and the immunoreactivity and allowed to rank the relative species/order specificity of the two antibodies for the southern hemisphere fish PAV.

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1. Introduction

Fish is one of the “Big Eight” food allergens identified to cause more than ninety percent of IgE-mediated food allergies (Taylor & Hefle, 2001). Compared to other food allergies, fish allergy tends to show a high proportion of severe anaphylactic reactions, and has shown to be the primary cause of death related food allergy in emergency rooms in Australia (Schulkes et al., 2014; Sharp & Lopata, 2014). One major concern for fish allergic patients is cross-reactivity between fish species. The national telephone survey on fish allergy conducted in the United States (US) informs that sixty-seven percent of allergic consumers have reported reactions to multiple fish species (Sicherer, Muñoz-Furlong, & Sampson, 2004). Accordingly, strict avoidance of all fish species is the medical advice generally given to fish-allergic consumers to prevent recurrence. In order to help fish sensitive consumers to make appropriate food choices, and prevent recurrence from the consumption of processed foods, many developed countries, including U.S., Canada, European Union (EU), Australia, and New Zealand

(NZ), have established regulations that require the mandatory labelling of fish and the related products in processed foods (van Hengel, 2007).

Parvalbumin (PAV) is the major allergen identified in the majority of fish species (Lee, 2012), showing IgE recognition rate between sixty-seven and one hundred percent. They are a group of calcium-binding proteins belonging to the EF-hand protein family with a molecular weight (MW) of 10–15 kDa. Though their secondary and tertiary structures are relatively conserved, their primary structures vary drastically between species (Das Dores, Chopin, Villaume, Fleurence, & Guéant, 2002; Sharp & Lopata, 2014). Targeting PAV detection to support the allergen control plans, two quantitative sandwich immunoassays with broad specificity were developed, reporting high sensitivity and good assay precision (Fæste & Plassen, 2008; Shibahara, Uesaka, Wang, Yamada, & Shiomi, 2013). These assays, however, showed limitations in detecting commercially important species, such as tuna (*Thunnus* spp.), swordfish (*Xiphias gladius*), flounder (*Paralichthys* spp.), Japanese eel (*Anguilla japonica*) and European eel (*Anguilla anguilla*). In a recent study that compared the performance of four commercial ELISA kits, developed using purified PAV from cod or a crude protein extract from northern hemisphere fish (Bugyi, Baumert, & Taylor,

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2016), all four test kits showed poor detection of albacore tuna (*Thunnus alalunga*), swordfish (*Xiphias gladius*), Atlantic salmon (*Salmo salar*) and mahi-mahi (*Coryphaena hippurus*), while cod was detected very well (Bugyi et al., 2016). In addition, these test kits have not been applied to detect southern hemisphere fish species, particularly commercially important species, such as bight redfish (*Centroberyx gerrardi*), pink ling (*Genypterus blacodes*), and tiger flathead (*Neoplatycephalus richardsoni*). This highlights the importance of evaluating species specificity of anti-PAV antibodies to determine their capability of detecting fish residues from a wide range of species used in processed food globally.

Many studies had attempted to study the species specificity of anti-PAV antibodies, and most of these studies adopted the anti-frog PAV monoclonal antibody (PARV-19) as a model antibody. Most of the northern hemisphere fish species tested with immunoblot and indirect ELISAs showed good immunoreactivity to the PARV-19 antibody, except for swordfish (*Xiphias gladius*), Atlantic salmon (*Salmo salar*), and yellowfin tuna (*Thunnus albacares*), which showed weak or no reactivity (Chen, Hefle, Taylor, Swoboda, & Goodman, 2006; Gajewski & Hsieh, 2009; Lee, Nordlee, Koppelman, Baumert, & Taylor, 2011). Two other antibodies, anti-cod PAV polyclonal antibody (PoAb) and anti-carp PAV monoclonal antibody (MoAb), were compared with PARV-19 to investigate their species specificity among twenty-nine northern hemisphere fish species from US and Netherlands (Lee et al., 2011). The anti-cod PoAb was shown to be the better performer in detecting diverse fish species, though some species, such as albacore tuna (*Thunnus alalunga*) and mahi-mahi (*Coryphaena hippurus*), still showed weak reactivity. The variable amounts of PAV located in different muscle tissues could be the factor contributing to the observed variable immunoreactivity (Lee, Nordlee, Koppelman, Baumert, & Taylor, 2012).

The PARV-19 antibody was also evaluated for the detection of fish species from the southern hemisphere (Beale, Jeebhay, & Lopata, 2009; Saptarshi, Sharp, Kamath, & Lopata, 2014; Sharp et al., 2015). It was shown to recognize both monomeric and oligomeric PAV of pilchard (*Sardina pilchardus*), anchovy (*Engraulis encrasicolus*), yellowtail (*Seriola lalandi*), snoek (*Thyrsites atun*) and hake (*Merluccius merluccius*) from Southern Africa (Beale et al., 2009); but it showed limited detection to species from Indo-Pacific region (Saptarshi et al., 2014; Sharp et al., 2015). From the phylogenetic analysis of thirty-eight PAV amino acid sequences together with the inhibition ELISA, Saptarshi et al. (2014) concluded that immunoreactivity of different fish species with PARV-19 (the anti-frog parvalbumin antibody) was due to the molecular phylogenetic association of PAV.

Several studies have shown that PAV contents vary significantly between species, and correlate positively with their respective allergenicity (Griesmeier et al., 2010; Kobayashi, Kobayashi, & Shiomi, 2016; Kobayashi, Yang, et al. (2016), Kuehn, Scheuermann, Hilger, & Hentges, 2010; Van Do, Elsayed, Florvaag, Hordvik, & Endresen, 2005). For example, the PAV content from Atlantic cod (*Gadus morhua*) was twenty times higher than swordfish (*Xiphias gladius*) (Griesmeier et al., 2010); and the PAV content in yellowfin tuna (*Thunnus albacares*) was one hundred times lower than Atlantic herring (Kuehn et al., 2010), both of which were perceived to account for the low allergenicity of swordfish (*Xiphias gladius*) and yellowfin tuna (*Thunnus albacares*) (Griesmeier et al., 2010; Van Do et al., 2005).

Kobayashi, Kobayashi, et al. (2016), Kobayashi, Yang, et al. (2016) developed a quantitative SDS-PAGE method to quantify the content of parvalbumins which were detected by a rabbit anti-Pacific mackerel (*Scomber japonicus*) PAV antibody in twenty-two commonly consumed fish species from Japan. Their results revealed a positive correlation between estimated PAV content and IgE reactivity with the Spearman's rank order correlation

coefficients (Rho) ranging between 0.63 and 0.88. Another study by the same authors (Kobayashi, Kobayashi, et al., 2016) further proposed that allergenicity of nine fish species correlated better with PAV content in fish meat than molecular difference determined by IgE ELISAs using an immobilized PAV at a fixed amount. This raised an important question about the immunoreactivity of antibodies raised in host animals against PAV, in that PAV content might be an important factor contributing to the observed inconsistent immunochemical detection.

Species specificity is typically studied qualitatively by comparing assay absorbance or binding intensity of an immunoblot of the crude protein extracts (Beale et al., 2009; Chen et al., 2006; Gajewski & Hsieh, 2009; Lee et al., 2011, 2012; Saptarshi et al., 2014; Sharp et al., 2015). The potential variations of PAV contents within the crude protein extracts of individual species, as well as non-linear dose-response nature of the affinity interaction between an antibody and an antigen in an ELISA, have not been considered. In order to evaluate analytical performance of two anti-PAV antibodies for detection of southern hemisphere fish residues in processed foods, we investigated a new analytical approach to evaluate species specificity of the antibodies as a function of PAV content. Therefore, this study presents the evaluation of immunoreactivity of PAV from thirty-seven southern hemisphere fish species by correlating results from a quantitative indirect ELISA and a quantitative SDS-PAGE method.

2. Materials and methods

2.1. Materials

Thirty-seven fish species from twelve orders were collected from the Sydney Fish Market and the local market in Townsville, Queensland (Table 1). The fish species were selected based on fish production, consumption and local availability (ABARES., 2014). Southern hemisphere fish species are defined as fish species that are harvested and commercially available in southern hemisphere.

The two anti-PAV antibodies selected for this study were rabbit anti-cod PAV PoAb developed by Food Allergy Research and Resource Program (FARRP) of the University of Nebraska-Lincoln (Lee et al., 2011), and mouse anti-carp PAV MoAb (PV 235, Swant Inc, Marly, Switzerland). The anti-cod PoAb was a serum produced by immunizing a rabbit with the purified cod PAV (Lee et al., 2011), and the anti-carp MoAb was a reconstituted lyophilized ascites fluid produced by the mouse hybridoma cells generated from a mouse immunized with the purified carp PAV (Celio, Baier, Schärer, De Viragh, & Gerday, 1988). Among the thirty-seven fish species, only four species (cod, swordfish, rainbow trout, and Atlantic salmon) had previously been tested with the anti-cod PAV PoAb and the anti-carp PAV MoAb (Lee et al., 2011).

The following buffer solutions were used and their abbreviation as follows will be used thereafter: coating buffer (50 mM carbonate buffer, pH 9.6); PBS buffer (0.01 M phosphate buffered saline, pH 7.4); blocking buffer 1 (1% BSA in 0.05 M Tris-HCl-0.9% NaCl, with 0.05% Tween-20 (1% BSA-TBS-T), pH 7.5); blocking buffer 2 (1% BSA in PBS buffer (1% BSA-PBS), pH 7.4); diluent buffer (1% BSA-PBS with 0.05% Tween-20 (1% BSA-PBS-T), pH 7.4); stop solution (1.25 M sulphuric acid); and washing buffer (0.05% Tween-20 in reverse osmosis water).

2.2. Methods

2.2.1. Identification of fish species

Identification of species was done by comparing the corresponding whole fish images, cross-section images of fish tissue, protein profiles and distribution information with a previous study

Table 1

The list of fish species analyzed in this study.

Common Name	Scientific Name	Family	Order	Distribution
Monkfish	<i>Squatina australis</i> ^[1]	Squatinae	Squatiniiformes	S
Australian Pilchard/Australian Sardine	<i>Sardinops neophilchardus</i> / <i>Sardinops sagax</i> ^[2]	Clupeidae	Clupeiformes	N & S
Atlantic Salmon	<i>Salmo salar</i> ^[1]	Salmonidae	Salmoniformes	N & S
Rainbow Trout	<i>Oncorhynchus mykiss</i> ^[1]	Salmonidae	Salmoniformes	H & S
Cod	<i>Gadus morhua</i> ^[1]	Gadidae	Gadiformes	N & S
Dory	<i>Cyttus</i> sp. ^[1]	Cyttidae	Zeiformes	N & S
Bight Redfish	<i>Centroberyx gerrardi</i> ^[3]	Berycidae	Beryciformes	S
Sea Mullet	<i>Mugil cephalus</i> ^[3]	Mugilidae	Mugiliformes	N & S
Garfish	<i>Arrhamphus sclerolepis</i> / <i>Hyporhamphus quoyi</i> ^[3]	Hemiramphidae	Beloniformes	S
Pink Ling	<i>Genypterus blacodes</i> ^[3]	Ophidiidae	Ophidiiformes	S
Barramundi	<i>Lates calcarifer</i> ^[4]	Latidae	Perciformes	N & S
Blue Threadfin	<i>Eleutheronema tetradactylum</i> ^[3]	Polynemidae	Perciformes	N & S
Blue Trevally	<i>Carangoides ferdau</i> / <i>Carangoides orthogrammus</i> ^[4]	Carangidae	Perciformes	N & S
Cobia/Black Kingfish	<i>Rachycentron canadum</i> ^[5]	Rachycentridae	Perciformes	N & S
Coral Trout	<i>Plectropomus leopardus</i> / <i>Plectropomus maculatus</i> / <i>Variola louti</i> ^[3]	Serranidae	Perciformes	N & S
Crimson Snapper/Crimson Seaperch	<i>Lutjanus erythropterus</i> ^[3]	Lutjanidae	Perciformes	N & S
Eastern School Whiting	<i>Sillago flindersi</i> ^[3]	Sillaginidae	Perciformes	S
Flame Snapper/Ruby Snapper	<i>Etelis coruscans</i> ^[4]	Lutjanidae	Perciformes	N & S
Grass Empero	<i>Lethrinus laticaudis</i> ^[3]	Lethrinidae	Perciformes	S
Grass Sweetlip				
Grunter Bream	<i>Pomadasys kaakan</i> / <i>Pomadasys argenteus</i> ^[4]	Haemulidae	Perciformes	N & S
Jewfish	<i>Argyrosomus japonicus</i> / <i>Tandanus tandanus</i> ^[4]	Sciaenidae	Perciformes	N & S
Pink Snapper	<i>Pagrus auratus</i> / <i>Chrysophrys auratus</i> ^[2]	Sparidae	Perciformes	N & S
Pumpkin Head Trevally/Snub-nosed dart	<i>Trachinotus blochii</i> ^[4]	Carangidae	Perciformes	N & S
Red Emperor/Redthroat Emperor	<i>Lethrinus miniatus</i> ^[1]	Lethrinidae	Perciformes	N & S
Sweetlip Emperor	<i>Lethrinus</i> sp. ^[1]	Lethrinidae	Perciformes	N & S
Saddletail Snapper/Saddletail Seaperch	<i>Lutjanus malabaricus</i> ^[3]	Lutjanidae	Perciformes	N & S
Sand Whiting	<i>Sillago ciliata</i> ^[3]	Sillaginidae	Perciformes	S
Spanish Mackerel	<i>Scomberomorus commerson</i> / <i>Scomberomorus semifasciatus</i> ^[3]	Scombridae	Perciformes	N & S
Stripped Snapper	<i>Lutjanus kasmira</i> ^[1]	Lutjanidae	Perciformes	N & S
Yellowfin Bream	<i>Acanthopagrus australis</i> ^[3]	Sparidae	Perciformes	S
Yellowtail Scad	<i>Trachurus novaezelandiae</i> ^[3]	Carangidae	Perciformes	S
Swordfish	<i>Xiphias gladius</i> ^[3]	Xiphiidae	Scombriformes	N & S
Yellowfin Tuna	<i>Thunnus albacares</i> ^[3]	Scombridae	Scombriformes	N & S
Northern Sand Flathead	<i>Platycephalus endrachtensis</i> / <i>Platycephalus arenarius</i> ^[3]	Platycephalidae	Scorpaeniformes	N & S
Red Gurnard	<i>Chelidonichthys kumu</i>	Triglidae	Scorpaeniformes	N & S
Tiger Flathead	<i>Neoplatycephalus richardsoni</i> / <i>Neoplatycephalus aurimaculatus</i> ^[3]	Platycephalidae	Scorpaeniformes	S
Mosaic Leatherjacket	<i>Eubalichthys mosaicus</i> ^[4]	Monacanthidae	Tetraodontiformes	S

N = northern hemisphere; S = southern hemisphere; ^[1]Sharp et al. (2015); ^[2]DPI NSW (2014); ^[3]Yearsley, Last, and Ward (1999); ^[4]Pauly and Froese (2016); ^[5]Fisheries, 2016.

(Sharp et al., 2015), species handbook (Yearsley, Last, & Ward, 1999) and online databases (DPI, 2014; Fisheries, 2016; Pauly & Froese, 2016).

2.2.2. Soluble fish proteins extraction

The optimized fish protein extraction method was conducted as follows: approximately 1.5 g of fish tissue taken from the dorsal side of the anterior position of the fish body was ground with 20 ml of PBS using a food processor for 30 s (Lee et al., 2011). Fish proteins were extracted overnight at 4 °C with gentle rolling on a rotating mixer. Supernatants were collected as raw protein extracts after centrifugation at 12,000g for 15 min at 4 °C. The protein content of the extract was determined by the bicinchoninic acid (BCA) protein kit (Sigma-Aldrich, St Louis, USA). Extraction was performed in duplicate samples, and the samples were stored at −20 °C until further use.

2.2.3. Sodium Dodecyl Sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated by SDS-PAGE using the Bio-Rad Mini PROTEAN Tetra cell (Bio-Rad, Hercules, USA). 10 µg of raw protein extract with Bio-Rad sample buffer containing 6% dithiothreitol (DTT, w/v) was heated at 95 °C for 5 min and separated in 15% Tris-HCl hand-casting gels at a constant voltage of 150 V for 75 min. Proteins bands were stained with Coomassie Brilliant Blue R250 (Sigma-Aldrich, St Louis, USA). The gel images were captured by the Bio-Rad ChemiDoc™ MP imaging system equipped with Bio-Rad Image Lab™ software (Bio-Rad, Hercules, USA).

2.2.4. Immunoblotting analysis

Protein extracts (5 µg) were separated by SDS-PAGE using the conditions above. After the electrophoresis, the proteins were transferred onto a 0.2 µm nitrocellulose membrane (Bio-Rad software (Bio-Rad, Hercules, USA) using Bio-Rad trans-blot system (Bio-Rad software (Bio-Rad, Hercules, USA). After washing the membrane 3 times, with the washing buffer, the membrane was blocked with the blocking buffer 1. After blocking and washing, the anti-cod PoAb or anti-carp MoAb diluted in blocking buffer 1 was incubated with the membrane. After incubation and washing the membrane again, the membrane was incubated with a secondary anti-rabbit or anti-mouse MoAb conjugated with horseradish peroxidase (HRP) (Abcam, Cambridge, UK). The bound antibodies were visualized with 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Sigma-Aldrich, St Louis, USA). The images were captured using Bio-Rad ChemiDoc™ MP imaging system. All blocking and incubation steps were performed for 1 h at room temperature.

2.2.5. PAV identification and relative quantification based on SDS-PAGE

PAV bands were identified based on the specific bindings with the two anti-parvalbumin antibodies (anti-cod PAV PoAb and anti-carp PAV MoAb) on the immunoblot and the corresponding MWs were calculated using the Bio-Rad Image Lab™ software.

A quantitative SDS-PAGE, based on the method of Kobayashi, Kobayashi, et al. (2016), Kobayashi, Yang, et al. (2016) with modification, was conducted to estimate immune-recognized PAV content

from SDS-PAGE. 3 µg of purified cod PAV (the middle point of the calibration curve) was chosen as a reference to run with 3 µg per lane of all the fish protein extracts on the SDS-PAGE. A correction factor C was applied to normalize all the band intensity unit of test PAV bands.

$$C = \frac{d_{3\mu g}}{D_{3\mu g}}$$

Where $d_{3\mu g}$ is the band intensity unit of the reference cod PAV obtained from the individual gel; and $D_{3\mu g}$ is the normalized band intensity obtained from the calibration curve.

Then estimated PAV content was expressed as a quantification index Q relative to the reference cod PAV:

$$Q = \frac{P \times C}{D_{3\mu g}}$$

where Q is the relative quantity index; P is the band intensity unit of test PAV bands estimated by the Image Lab™ software.

2.2.6. Quantitative indirect ELISA

Microtiter plates (Thermo Fisher Scientific) were immobilized with the purified cod PAV in a range of 0.001–3.3 µg/ml as the calibration standards. The fish protein extracts of each fish species prepared in three concentrations of 0.11, 0.33, 1 µg/ml in the coating buffer were also immobilized in the same manner. Thereafter, all incubation steps, except for the blocking and incubation with the substrate solution, were performed for 30 min. After the immobilization step, the microwell plates were washed, 3 times, with washing buffer and incubated with 200 µl of blocking buffer 2 for 1 h. After washing the plate, 100 µl per well of the anti-PAV antibodies diluted in the diluent buffer was added to respective wells for incubation. Next, the plates were washed again and incubated with 100 µl per well of HRP conjugated anti-species antibodies in the diluent buffer. In the final washing, the plates were washed at least five times and 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (100 µl per well) (Sigma-Aldrich, St Louis, USA) was added to all the wells and incubated for 20 min for colour development. The stop solution (50 µl per well) was added to stop the reaction and the absorbance was measured at 450 nm using Optima microplate reader (BMG LABTECH, Ortenberg, Germany). All assays were performed in duplicate at room temperature.

2.2.7. Data analysis

A standard calibration curve was fitted to a sigmoidal dose-response (with a variable slope) model with the equation shown as follows using GraphPad Prism; where A is the Y value at the bottom plateau, B is the Y value at the top plateau, C is an EC_{50} (effective concentration), the concentration of analyte giving 50% of the maximum response, and D is hillslope describing the slope of the curve (in the most linear part of the curve):

$$Y = A + \left[\frac{B - A}{1 + 10^{(\log C - x) \cdot D}} \right]$$

All of the ELISA results were calculated and expressed as cod-PAV equivalent (CPQ) concentrations. A correlation index (CI) was determined using the equation below to assess the correlation between immunoreactivity of PAV and the SDS-PAGE estimated PAV content.

$$CI = \frac{E}{Q}$$

E is the fish protein concentration determined by ELISA and Q is the quantity index of PAV bands relative to the reference cod PAV. Cut-off points of PAV quantity was chosen to be $\pm 50\%$ (50–150% or CI of 0.5–1.5) for the evaluation based on the recommended recovery of

an ELISA for fish allergen detection (Shibahara et al., 2013). Species with correlation index within the range from 0.5 to 1.5 were considered having a positive correlation between immunoreactivity of PAV and PAV content.

3. Results and discussion

3.1. Protein profile by SDS-PAGE and immunoblot analysis

PAV contents are more abundant in the dorsal side than in ventral side of the fish body and decrease from anterior to posterior locations (Kobayashi, Kobayashi, et al., 2016; Kobayashi, Yang, et al., 2016; Lee et al., 2012). To minimize variation in the PAV contents in the extracts, tissues used for this study were taken only from the dorsal side of the anterior position of the fish body (Supplementary Fig. 1). Prior to conducting a series of experiments, the consistency of our optimized extraction protocol was tested, which had confirmed no significant differences in the intensity of PAV bands run in replicates from each of the twenty-four selected species (data not shown). As raw protein extracts were susceptible to degradation and cryo-precipitation, fresh aliquots were used in all of the experiments. The protein profiles (Fig. 1a) show the most abundant proteins in the soluble fish protein extracts between 37 and 50 kDa; and twenty fish species had more than one band between 10 and 15 kDa where (monomeric) parvalbumin bands were (Saptarshi et al., 2014). Our protein profiles agreed well with the results of the same species reported by Saptarshi et al. (2014) and Sharp et al. (2015), except for Spanish mackerel and striped snapper. Unlike Sharp et al. (2015) who did not observe the PAV band in the raw extract, but did observe a band in the heated extract, we identified a distinct band in the raw extract of Spanish mackerel. Also, their SDS-PAGE image of striped snapper showed one extra distinct band between 10 and 15 kDa, compared to our results. The extra protein band may be prone to degradation or oligomerisation, therefore inconsistently showing in different extracts.

The immunoblots of thirty-seven southern hemisphere fish species with anti-cod PAV PoAb and anti-carp PAV MoAb are illustrated in Fig. 1b and c, respectively. For ease of comparison, the immunoblot images had been arranged according to the phylogenetic distance of the twelve fish orders (Nelson, 2006; Wiley & Johnson, 2010). The yellowfin tuna extract showed only one distinct band at 14 kDa on the SDS-PAGE, which agreed with Chen et al. (Chen et al., 2006). However, the MWs of IgE-reactive proteins identified in tuna were 12, 32, 46, 40 kDa (aldolase A) and 50 kDa (β -enolase) proteins (BerNisel-Broadbent, Scanlon, & Sampson, 1992; Kuehn et al., 2013; Yamada, Nolte, & Zychlinsky, 1999), but it was not the band corresponding to 14 kDa. Considering the 14 kDa protein was not recognized by the antibodies in our immunoblots and in other previous studies (Chen et al., 2006; Sharp et al., 2015), we concluded that it may not be relevant to PAV or that tuna PAV bound very poorly with anti-cod or anti-carp PAV antibodies. As shown in Fig. 1b and c, there were two weakly binding bands in swordfish extract by both antibodies in the immunoblots, but there were no corresponding bands on the SDS-PAGE (Fig. 1a), suggesting that these may be low abundant proteins. Interestingly, despite that the rainbow trout PAV has more than ninety-five percent homology with the Atlantic salmon PAVs (Peñas, Uberti, Baviera, Di Lorenzo, & Restani, 2014), the rainbow trout showed stronger immunoreactivity with both antibodies than Atlantic salmon.

The two antibodies showed a similar binding trend, but with varying degree of binding intensity corresponding to their immunoreactivity. Our immunoblot analysis agreed well with Saptarshi et al. (2014) in that immunoreactivity among the test fish

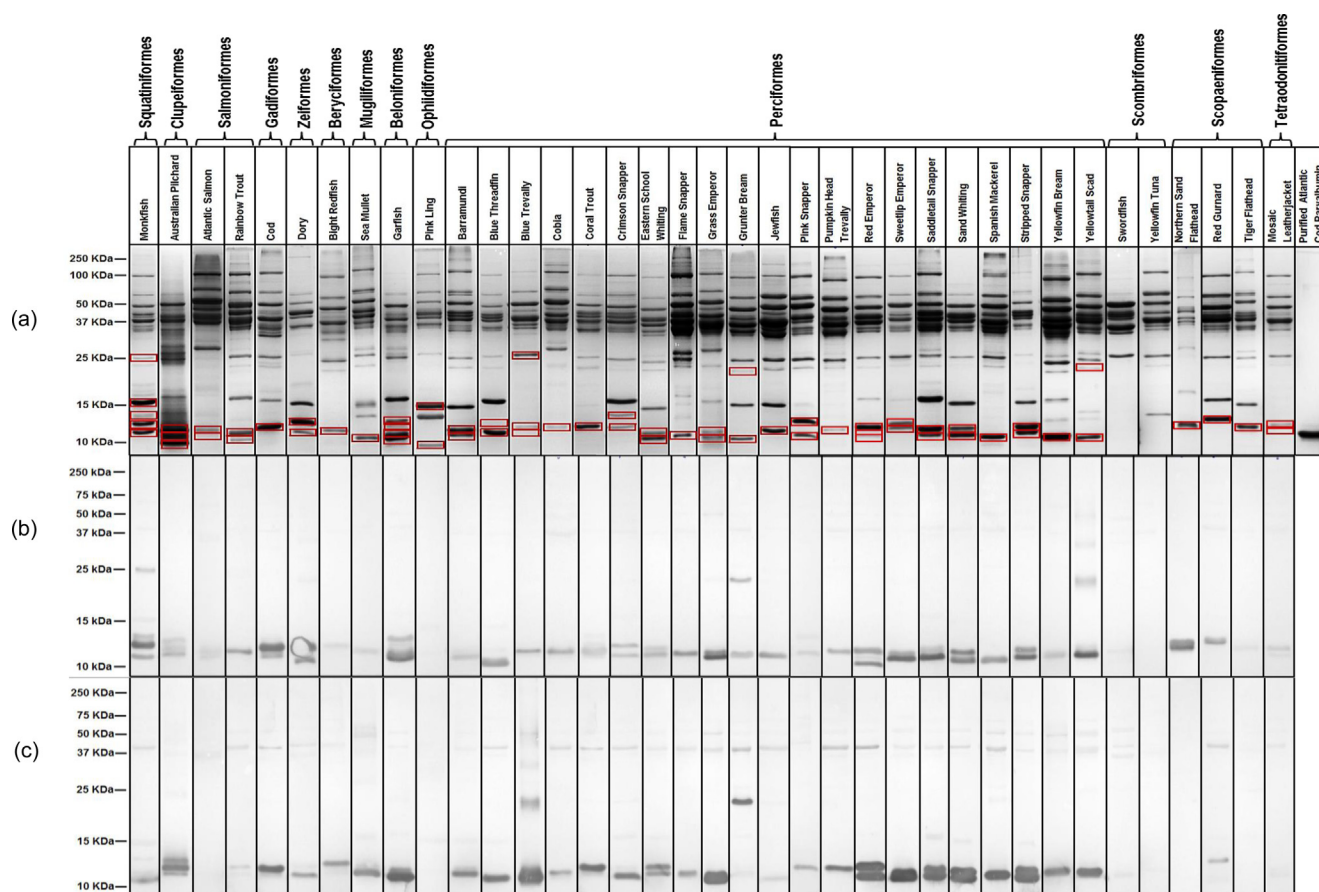


Fig. 1. (a) The protein profiles of fish protein extracts separated by SDS-PAGE with Coomassie brilliant blue staining; (b) immunoblot analysis of fish protein extracts with the anti-cod polyclonal antibody; (c) immunoblot analysis of fish protein extracts with the anti-carp monoclonal antibody. The red squares indicate the protein bands in the region of 10–15 kDa that were recognized by the two anti-PAV antibodies collectively in the immunoblot. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

species demonstrated correlation with molecular phylogeny of PAV. For example, fish species from the order of *Perciformes* showed strong immunoreactivity with both antibodies; whereas species from *Salmoniformes*, *Ophidiiformes*, *Scombriformes*, *Scorpaeniformes*, *Tetraodontiformes*, including salmon, pink ling, swordfish, yellowfin tuna, red gurnard, tiger flathead, and mosaic leatherjacket, showed weak or no immunoreactivity. Lee et al. (2011) and Sharp et al. (2015) also observed similar order specificity in their immunoblot analysis. Lee et al. (2011) showed that all of the test northern hemisphere fish species from *Perciformes* were recognized strongly by the anti-cod PAV antibody and the anti-carp PAV antibody. Mahi-mahi, swordfish and albacore tuna and the species from *Salmoniformes* showed low antibody binding. Sharp et al. (2015) demonstrated that most southern hemisphere fish species from *Perciformes* showed bindings to the PARV-19 antibody; while species from *Salmoniformes* and *Ophidiiformes* showed no binding.

Our immunoblot results showed that the anti-cod PoAb showed broader specificity than that of the anti-carp MoAb for the southern hemisphere species. This was expected, as polyclonal antibodies are likely to bind more epitopes on the same protein, while monoclonal antibodies can only bind one single epitope. Interestingly, the anti-carp MoAb showed binding with 36 kDa protein for most fish species in our study. The identification of PAV bands in the SDS-PAGE (Fig. 1a) was guided by the binding of PAV specific antibodies in the immunoblots (Celio et al., 1988; Chen et al., 2006; Hemmi, Yamashita, & Nishioka, 1997; Kuehn et al., 2010; Lee et al., 2011, 2012). Table 2 summarizes the calculated molecular weight

of monomeric PAV bands that were immunochemically detected by the two antibodies. Of the thirty-seven species in this study, only nine species (Australian pilchard, barramundi, cod, cobia, swordfish, yellowfin bream, yellowfin tuna, Atlantic salmon and rainbow trout) have PAV amino acid sequences and molecular characteristics documented in the Allergome database (<http://www.allergome.org>), and five among them (cod, swordfish, yellowfin tuna, Atlantic salmon and rainbow trout) have records in the database of WHO/IUIS Allergen Nomenclature (<http://www.allergen.org>) and AllergenOnline (<http://www.allergenonline.com>). Considering there are still many species in our list with uncharacterized PAVs, we decided to mainly focus on monomeric PAV immunologically recognized by the two antibodies as identified by the immunoblots. Several proteins between 22 and 25 kDa in monkfish, blue trevally, grunter bream and yellowtail scad showing a strong immunoreactivity could be PAV oligomers and they were included in the estimation of PAV content.

3.2. Indirect ELISA

3.2.1. Qualitative indirect ELISA

Species specificity of the two antibodies for the thirty-seven test fish species was evaluated using two analytical approaches. The first was a conventional approach by performing a qualitative indirect ELISA based on immobilized fish proteins, and examining the correlation between the absorbance values and the estimated PAV content as described in the method section (Fig. 2). As shown in Fig. 2a, the anti-cod polyclonal antibody showed some trend of

Table 2

The calculated molecular weight of PAV bands detected by anti-cod polyclonal antibody and anti-carp monoclonal antibody in fish protein extracts.

Fish samples	Molecular weight (kDa) of PAV				
	1	2	3	4	5
Monkfish	25.2	15.5	13.5	12.4	11.4
Australian Pilchard	11.7	10.8	10.3		
Atlantic Salmon	13.4	11.8			
Rainbow Trout	11.8	11.0			
Cod	11.9				
Dory	12.5	11.1			
Bight Redfish	11.8				
Sea Mullet	10.8				
Garfish	12.8	12.2	11.4	10.8	
Pink Ling	14.8	10.5			
Barramundi	11.8	11.1			
Blue Threadfin	12.8	11.4			
Blue Trevally	26.1	11.9	11.4		
Cobia	11.9				
Coral trout	12.2				
Crimson Snapper	12.3	11.4			
Eastern School Whiting	11.2	10.7			
Flame Snapper	11.3				
Grass Emperor	11.6	11.1			
Grunter Bream	21.8	10.9			
Jewfish	11.9				
Pink Snapper	13.2	11.1			
Pumpkin Head Trevally	11.5				
Red Emperor	11.7	11.2	10.7		
Sweetlip Emperor	12.2	11.6			
Saddletail Snapper	11.4	10.8			
Sand Whiting	11.7	10.9			
Spanish Mackerel	10.6				
Stripped Snapper	11.9	11.3			
Yellowfin Bream	10.8				
Yellowtail Scad	22.6	11.4			
Swordfish					
Yellowfin Tuna					
Northern Sand Flathead	11.6				
Red Gurnard	12.7				
Tiger Flathead	12.2				

MW of PAV detected only by anti-cod polyclonal antibody was shaded as light grey; MW of PAV detected only by anti-carp monoclonal antibody was shaded as medium grey; MW of PAV detected by both the two antibodies was shaded as dark grey.

non-linear correlation, but many of the species showed binding saturation, therefore not allowing clear differentiation between low immunoreactive and high immunoreactive species. While the anti-carp antibody, as shown in Fig. 2b, did not show any correlation between absorbance values and the estimated parvalbumin content with this approach.

3.2.2. Quantitative indirect ELISA indicating correlation between PAV content and the immunoreactivity

As the dose-response relationship of an ELISA is sigmoidal, we took a second approach of constructing a quantitative ELISA to better estimate the quantifiable immunological responses of different species, and subsequently, correlate the immunoreactivity with the SDS-PAGE estimated PAV content. As purified cod PAV was used as a reference to quantify PAV contents on SDS-PAGE, it was also used as a standard in our quantitative ELISA with both antibodies (calibration curves see Supplementary Figs. 1 and 2), in order to establish a correlation between ELISA (i.e., immunoreactivity) and SDS-PAGE (i.e., relative quantity). The quantification range of the quantitative ELISA with anti-cod PoAb was established between 0.2 and 3.4 $\mu\text{g/ml}$ and the quantification range with anti-cod PoAb was between 0.8 and 2.8 $\mu\text{g/ml}$.

According to the anti-cod PoAb ELISA (Table 3 and Fig. 3a and b), twenty-five out of thirty-seven fish samples showed

quantifiable responses (i.e., within the linear quantification range), and sixteen of the twenty-five samples (forty-three percent of total samples), shown as grey in Table 3, had a correlation index within the cut-off points between 0.5 and 1.5, showing a positive correlation between the parvalbumin content and the immunoreactivity ($R^2 = 0.74$). The correlation index of yellowfin tuna and swordfish is 1.00, because the absence of PAV bands on SDS-PAGE positively correlates with the “undetected” in the ELISA. Interestingly, there were no observable bands on the yellowfin tuna immunoblot, while there were two weakly visible bands on the swordfish immunoblot. For species that had responses below the limit of detection of the ELISA ($<0.2 \mu\text{g/ml}$), an arbitrary concentration of 0.1 $\mu\text{g/ml}$ was used only for the purpose of calculating the corresponding correlation index. Noticeably, Atlantic salmon and pink ling had low reactivity ($<0.2 \mu\text{g/ml}$) with the anti-cod PoAb, which

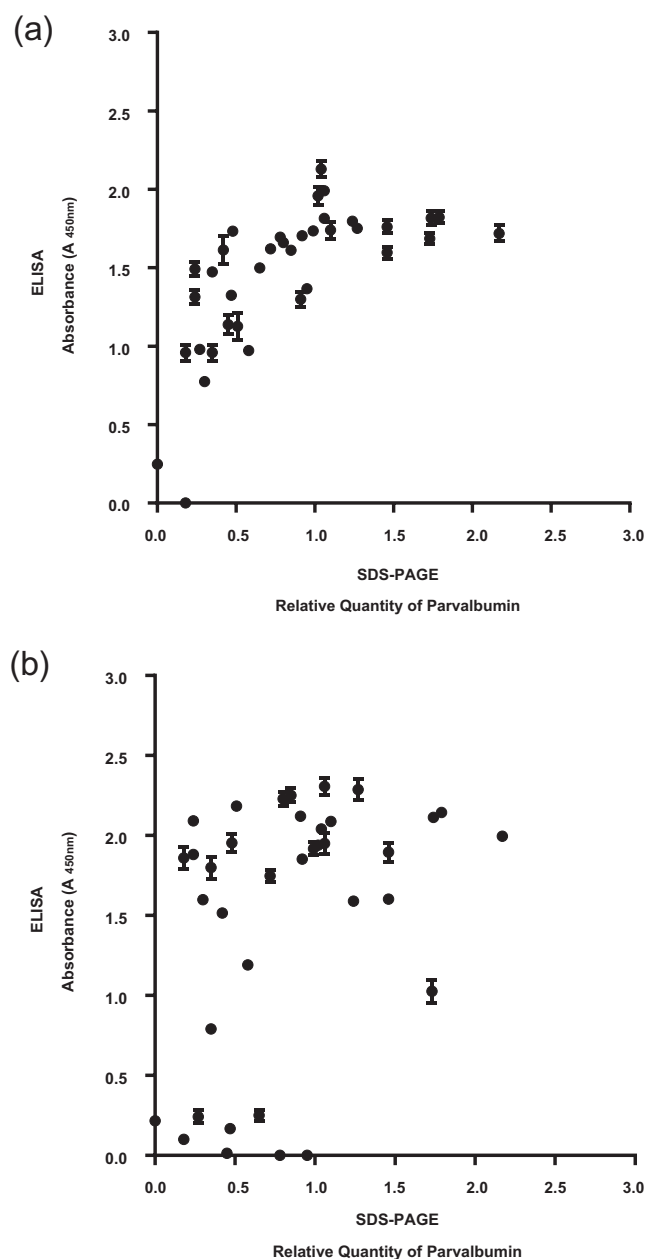


Fig. 2. The correlation between relative quantity of PAV estimated from SDS-PAGE and absorbance values of ELISA for 1 μg crude protein using (a) anti-cod polyclonal antibody and (b) anti-carp monoclonal antibody.

Table 3

The relative quantity of PAV estimated by SDS-PAGE and quantitative ELISA analysis of fish protein extracts using anti-cod polyclonal antibody (ELISA 1) and using anti-carp monoclonal antibody (ELISA 2). Fish species shaded in grey were readily quantifiable fish species, having a positive correlation between estimated PAV contents and ELISA responses.

Fish species	SDS-PAGE RPQ	ELISA 1 CPQ (µg/ml)	CI-1*	ELISA 2 CPQ (µg/ml)	CI-2*
Monkfish	1.8	0.7	0.39	<0.8	0.39
Australian Pilchard	2.2	<0.2	0.05	0.9	0.41
Atlantic Salmon	0.4	<0.2	0.25	<0.8	1.75
Rainbow Trout	0.7	<0.2	0.14	<0.8	1.00
Cod	0.7	1.1	1.47	1.2	1.68
Dory	1.5	2.2	1.50	<0.8	0.47
Bight Redfish	0.4	0.2	0.57	3.5	9.97
Sea Mullet	0.5	0.3	0.64	3.5	7.33
Garfish	1.7	0.8	0.44	2.9	1.66
Pink Ling	1.0	<0.2	0.10	0.0	0.00
Barramundi	1.0	0.8	0.79	3.4	3.42
Blue Threadfin	1.3	0.5	0.37	0.8	0.64
Blue Trevally	0.9	0.2	0.22	0.9	1.00
Cobia	0.3	<0.2	0.33	<0.8	2.33
Coral Trout	0.6	0.5	0.93	<0.8	1.17
Crimson Snapper	0.2	<0.2	0.50	<0.8	3.50
Eastern School Whiting	1.1	1.1	1.00	2.1	2.01
Flame Snapper	0.2	<0.2	0.50	<0.8	3.50
Grass Emperor	0.8	1.5	1.83	5.3	6.59
Grunter Bream	0.5	0.3	0.57	2.4	4.63
Jewfish	0.5	<0.2	0.20	<0.8	1.40
Pink Snapper	0.9	0.5	0.48	1.0	1.12
Pumpkin Head Trevally	0.2	<0.2	0.50	<0.8	3.50
Red Emperor	1.2	1.2	0.95	3.9	3.10
Sweetlip Emperor	1.1	1.5	1.38	2.9	2.69
Saddletail Snapper	0.9	0.8	0.97	3.0	3.50
Sand Whiting	1.8	1.4	0.77	2.8	1.59
Spanish Mackerel	1.0	1.1	1.03	2.7	2.63
Stripped Snapper	1.5	2.4	1.66	6.3	4.31
Yellowfin Bream	1.0	1.3	1.26	5.7	5.60
Yellowtail scad	1.2	1.9	1.58	6.1	5.08
Swordfish	0.0	0.0	1.00	0.0	1.00
Yellowfin Tuna	0.0	0.0	1.00	<0.8	0.00
Northern Sand Flathead	0.5	0.4	0.86	0.0	0.00
Red Gurnard	0.9	<0.2	0.11	<0.8	0.77
Tiger Flathead	0.8	0.2	0.26	0.0	0.00
Mosaic Leatherjacket	0.3	0.3	1.07	0.0	0.00

RPQ – Relative Quantity of PAV; CPQ – Cod-parvalbumin Equivalent.

CI (Correlation Index) = $\frac{\text{ELISA parvalbumin concentration}}{\text{Parvalbumin relative quantity}}$; *0.1 µg/ml was used to calculate CI of fish species which had ELISA response lower than 0.2 µg/ml (lower quantification limit) for ELISA 1; *0.7 µg/ml was used to calculate CI of fish species which had ELISA response lower than 0.8 µg/ml (lower quantification limit) for ELISA 2

agreed with the weak bands in the immunoblot. Conversely, although strong binding was observed in the immunoblot of rainbow trout, cobia, crimson snapper, flame snapper, jewfish, pumpkin head trevally and red gurnard, these fish showed low ELISA responses (<0.2 µg/ml). Examining the species from the order of *Perciformes* with low ELISA responses, it was noted that they had relatively low PAV, which contributed to the low ELISA responses. Australian pilchard, which was reported to have the greatest IgE reactivity among 5 fish species by 10 fish-allergic patients (Beale et al., 2009), showed three distinct parvalbumin isoforms in the immunoblot, but it showed very low immunoreactivity with the anti-cod PoAb.

With respect to the anti-carp MoAb (Table 3 and Fig. 3c), twenty fish samples showed quantifiable results. For species that had results below the limit of detection of the ELISA (<0.8 µg/ml), 0.7 µg/ml was used to calculate the corresponding correlation

index. Interestingly, the quantitative ELISA responses did not show any correlation with the PAV content, even though the overall binding pattern of the anti-carp MoAb shared close similarity with that of the anti-cod PoAb. Except for cobia, crimson snapper, flame snapper, jewfish and, pumpkin head trevally, all of the other species from the order of *Perciformes* presented strong ELISA responses. The species from the order of *Salmoniformes*, *Ophidiiformes*, *Scombriformes*, *Scorpaeniformes*, *Tetraodontiformes*, including Atlantic salmon, rainbow trout, pink ling, swordfish, yellowfin tuna, sand flathead, red gurnard, tiger flathead and mosaic leatherjacket, showed either low or no reactivity.

The low immunoreactivity of swordfish observed in our study and in other studies (Lee et al., 2011; Sharp et al., 2015) is likely due to low PAV content. The absence of 12 kDa tuna PAV, which have been reported previously (Kawase, Ushio, Ohshima, Yamanaka, & Fukuda, 2001; Kuehn et al., 2010; Shiomi, Hamada,

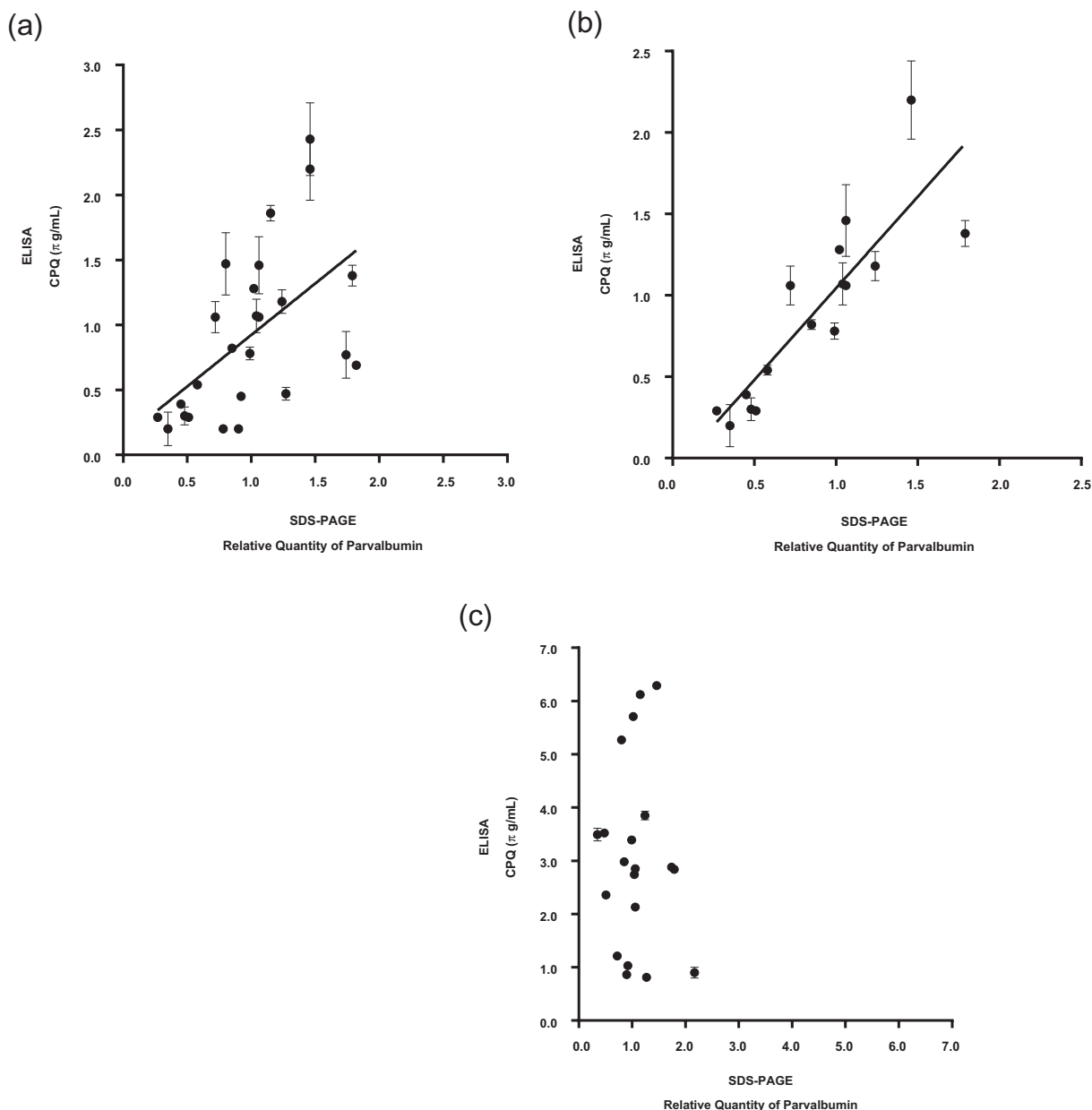


Fig. 3. The correlation between relative quantity of PAV estimated by SDS-PAGE and cod-PAV equivalent concentrations by ELISA using the two antibodies for (a) quantifiable samples with the anti-cod polyclonal antibody ($R^2 = 0.30$, $y = 0.80x + 0.13$); (b) quantity-related samples with the anti-cod polyclonal antibody ($R^2 = 0.74$, $y = 1.13x - 0.08$) and (c) quantifiable samples with the anti-carp monoclonal antibody ($R^2 = 0$).

Sekiguchi, Shimakura, & Nagashima, 1999), could be due to low abundance in our extract. The weak reactivity of Atlantic salmon observed in our study and in other studies (Lee et al., 2011; Sharp et al., 2015), suggests that the detection of salmon parvalbumin probably requires a separate highly specific antibody, as the antigenic region of salmon PAV is found to be the least conservative region of the protein (Saptarshi et al., 2014; Sharp et al., 2015). Though clear bands were observed for Australian pilchard in the immunoblot and high ELISA absorbance was observed, pilchard had very low quantitative responses with both antibodies.

The anti-carp MoAb is reported to be a Ca^{2+} dependent antibody that recognizes one of the two Ca^{2+} binding sites on PAV (Tinner, Oertle, Heizmann, & Bosshard, 1990). Even though Ca^{2+} binding sites are reported to be the most conservative regions of PAV (Chen et al., 2006), our study together with other studies (Chen et al., 2006; Lee et al., 2011) have demonstrated that conservative

protein structure could not guarantee similar binding capacity of anti-carp MoAb and PARV-19 with certain species. The possible binding to other less conservative sites might have led to the discrete detection in some cases. The binding of the anti-carp MoAb to the unknown 36 kDa protein band may be also contributing to the frequently observed inconsistent results. This, on the other hand, highlights the advantage of anti-cod PoAb's ability to recognize multiple epitopes of PAV in different species, allowing for better quantification.

4. Conclusion

The approach based on the quantitative ELISA and quantitative SDS-PAGE enables us to assess the correlation between the immunoreactivity of PAV and PAV content in a statistical manner and consequently evaluate species specificity of the two PAV

specific antibodies for southern hemisphere fish. By examining the correlation index, we could differentiate species showing positive correlation from those showing no correlation. Sixteen of thirty-seven species (forty-three percent) exhibited a positive correlation between the immunoreactivity of PAV and PAV content with the anti-cod PoAb, while no such correlation could be established with the anti-carp MoAb using this approach. The sixteen fish species showing positive correlation are cod, dory, bight redfish, sea mullet, barramundi, coral trout, eastern school whiting, grunter bream, red emperor, sweetlip emperor, saddletail snapper, sand whiting, Spanish mackerel, yellowtin bream, northern sand flathead, and mosaic leatherjacket.

In this study, the anti-cod PAV polyclonal antibody outperformed the anti-carp PAV monoclonal antibody, showing better species specificity for the thirty-seven commercially important fish from the southern hemisphere, as well as quantitatively detecting parvalbumin. Our results along with other related studies based on northern hemisphere species have confirmed that fish species from the order of *Perciformes* showed strong binding with anti-PAV antibodies; whereas species from *Salmoniformes*, *Ophidiiformes*, *Scombriformes*, *Scorpaeniformes*, and *Tetraodontiformes* showed weak or no binding. Order specificity in relation to PAV detection and potential for developing a new immunoassay with broad species specificity should be explored further.

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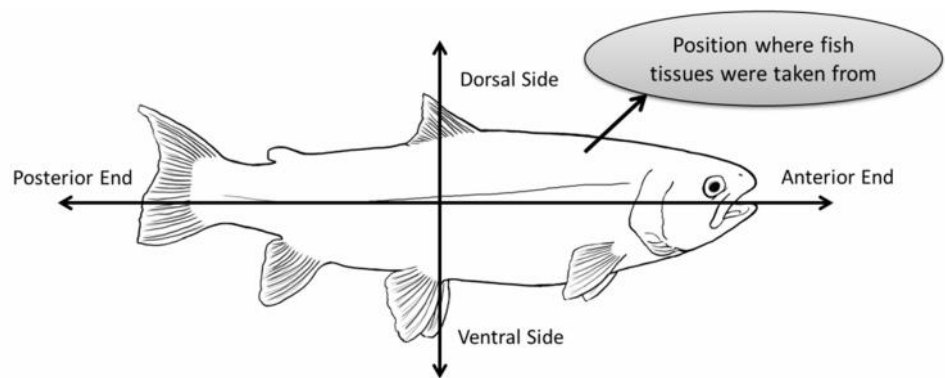
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2017.05.153>.

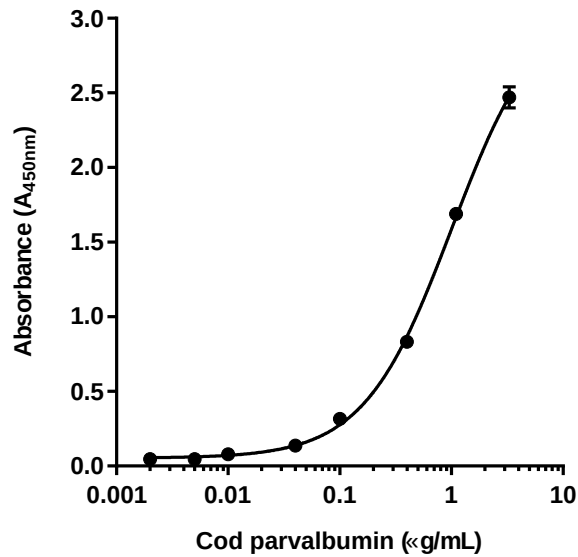
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1 **Supplementary Figures**



3 **Supplementary Fig. 1.** Illustration of tissue position obtained from fish samples

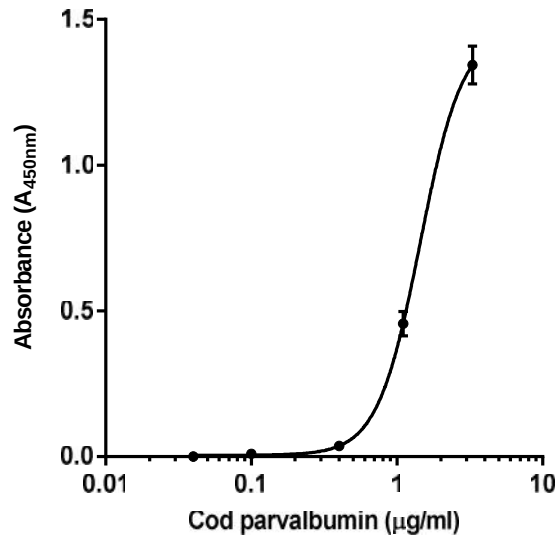


5 **Supplementary Fig. 2.** Calibration curve of quantitative ELISA using anti-cod polyclonal antibody. The calibration curve was fitted to the sigmoidal dose-response (variable slope) model using GraphPad Prism.

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Supplementary Fig. 3. Calibration curve of quantitative ELISA using anti-carp monoclonal antibody. The calibration curve was fitted to the sigmoidal dose-response (variable slope) model using GraphPad Prism.