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Evaluation and Comparison of the Species-Specificity of 3 Antiparvalbumin IgG Antibodies

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Abstract

Parvalbumin is a pan-allergen in fish and frogs that triggers IgE-mediated reactions in fish-allergic individuals. Previous studies demonstrated that antibodies raised against fish and frog parvalbumins displayed varying specificity for different fish species, and thus, the applicability of these antibodies for potential use in immunoassays to detect fish residues were limited. We aimed to determine the specificity of 3 IgG antibodies for various fish species. Indirect enzyme-linked immunosorbent assay (ELISA) and IgG-immunoblotting were used to compare the reactivity of the polyclonal anticod parvalbumin antibody and the commercially available, monoclonal antifrog and monoclonal anticarp parvalbumin antibodies against raw muscle extracts of 29 fish species. All antibodies demonstrated varying specificities for different fish species. Of the 3 antibodies, the polyclonal anticod parvalbumin antibody is the most suitable for the detection of fish parvalbumins, as it showed reactivity to the widest range of species, including herring, pilchard, carp, pike, cod, pollock, haddock, cusk, hake, bluegill, tilapia, bass, grouper, trout, catfish, and perch, although detection was still limited for several key fish species.

Keywords: fish allergy, IgG binding, fish parvalbumin, cross-reactivity, fish species

Fish is a general term that refers to a collection of taxonomic groups, including hagfish, lampreys, sharks, rays, and bony fish. At least 27,000 species of fish have been scientifically described.[1] Despite the enormous diversity of fish species, only a few orders of fish within the class Actinopterygii (ray-finned fish) are commonly consumed, namely, Salmoniformes, Perciformes, Gadiformes, Pleuronectiformes, Clupeiformes, and Cypriniformes.[2] Fish allergy limits the consumption of fish for some individuals. Fish is considered as a commonly allergenic food in the United States, Europe, Canada, Australia, New Zealand, and elsewhere. The prevalence of fish allergy is not precisely known but was estimated at 0.4% of the general population in the US on the basis of a random digit-dial telephone survey.[3] A metaanalysis showed the prevalence of fish allergy varied from 0% to 2%, depending on the type of diagnosis for fish allergy, including self-report, specific IgE measurement, skin prick test, symptoms combined with sensitization, and food challenge studies.[4] IgE-mediated hypersensitivity to fish can be induced through ingestion, direct contact, and inhalation of fish odors and fumes generated during cooking.[5–7] Typical symptoms of fish allergy range from skin, respiratory, and gastrointestinal symptoms to fatal anaphylaxis.[8,9] In contrast to milk and egg allergies that are commonly outgrown, fish allergy often persists throughout life once sensitized.[10,11]

Parvalbumin (Gad c 1) isolated from cod was the first major fish allergen described.[12] Later, homologous allergens from Atlantic salmon, carp, cod, Alaska pollock, horse mackerel, Japanese eel, bigeye tuna, mackerel, whiff, and swordfish were isolated and characterized.[13–22] These allergens displayed the ability to bind serum-specific IgE from fish-allergic individuals. Parvalbumin is a small, water-soluble, calcium-binding muscle protein involved in the muscle relaxation process.[23] Gad c 1 retains its allergenicity after heat treatment or exposure to extreme pH and denaturing chemicals.[24,25] Two separate phylogenetic lineages of parvalbumin, namely, α- and β-parvalbumin were identified.[26] β-Parvalbumin is responsible for the allergenicity of various fish species, but the allergenicity of frog α-parvalbumin has also been reported.[27,28]

The current treatment for fish allergy is to strictly avoid all species of fish due to the cross-reactivity reported between various fish species.[29] Hence, the detection of allergenic fish residues in foods is necessary to protect the fish-allergic consumers and to ensure accurate labeling of food products. Compared to the methods available for detecting the allergenic proteins derived from the other commonly allergenic foods, there were fewer studies describing the detection of allergenic proteins in fish. Fæste and Plassen[30] developed a sandwich enzyme-linked immunosorbent assay (ELISA) for the quantification of fish in foods using polyclonal anticod parvalbumin antibody as the capture and detector antibody. The ELISA had a low detection limit for parvalbumin in foods, but the quantification capability of this method varied with different fish species because of the inconsistent binding of the anticod parvalbumin antibody. Similar observations on the variable binding of the antiparvalbumin IgG antibody to parvalbumin and crude extracts derived from different fish species have also been reported by others. Chen et al.[31] demonstrated variable immunoreactivity of the commercially available mouse monoclonal antifrog parvalbumin antibody (PARV-19) to the extracts from several fish species. A monoclonal antibody against the crude extracts of the cooked catfish muscle proteins was developed by Gajewski and Hsieh.[32] The comparisons of their antibody with the PARV-19 showed

further evidence of the variable specificity of both antibodies to the cooked extracts from different fish species. Recently, Weber et al.[33] developed a competitive ELISA using PARV-19 to detect fish parvalbumins in food grade fish gelatins and isinglass samples. Variable cross-reactivity of PARV-19 to cod, hake, tilapia, pollock, sturgeon, and haddock was also observed in that ELISA.

The aim of this study was to compare the polyclonal anticod parvalbumin antibody developed by our group to both commercially available, monoclonal anticarp, and antifrog parvalbumin antibodies with regard to their immunoreactivity to different fish species. This approach allowed us to determine the utility and possible applications of these antibodies for detecting parvalbumins derived from commercially important fish species.

Materials and Methods

Materials

Mouse monoclonal antifrog parvalbumin antibody (antifrog MoAb; clone PARV-19) was purchased from Sigma-Aldrich (St. Louis, Missouri), mouse monoclonal anticarp parvalbumin antibody (anticarp MoAb; clone PV 235) was from Swant, Inc. (Switzerland), and rabbit polyclonal anticod parvalbumin antibody (anticod PoAb) was developed using purified cod parvalbumin as the antigen with an immunization protocol that has been previously described.[34] Cod and carp parvalbumin were purified from the fish fillets by a combination of diafiltration and chromatography steps as described previously.[35,36]

Twenty-nine commonly consumed fish species and frog legs were obtained from different fish and seafood distributors in the US and Netherlands. Upon receipt, the raw fish fillets or whole fish were skinned, gutted, and rinsed briefly with distilled water. The fillets were ground to a uniform consistency using a commercial food processor and kept frozen at –20°C until used. The species of the fish samples were identified by Eurofins GeneScan, Inc. (Metairie, Louisiana) using either the Food and Drug Administration (FDA)–validated DNA barcode analysis[37] or nucleotide sequence analysis of the cytochrome *b* and 16S genes.

Extraction of Fish Proteins

Soluble proteins from the ground fish samples were extracted 1:10 (w/v) in 0.01 M phosphate buffered saline (PBS; 0.002 M NaH_2PO_4, 0.008 M Na_2HPO_4, and 0.85% NaCl, pH 7.4) overnight with gentle rocking at 4°C. Extracts were then centrifuged at 3612 *g* in a tabletop centrifuge at 4°C for 30 min. The clarified solutions were divided into aliquots and stored at –20°C until use. The protein content of the solutions was determined by the Lowry method.[38]

Indirect ELISA

Polystyrene microtiter plates (Nagle Nunc International, Rochester, New York) were coated with 100 μL/well of the crude fish extracts and purified parvalbumins at 10 μg/mL in coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, and 0.02% NaN_3, pH 9.6) and incubated overnight at 4°C. Thereafter, all incubation steps were performed for 1 h at 37°C, except for the incubation after the addition of substrate. Following incubation, the plates were washed with wash buffer (0.01 M PBS containing 0.05% Tween 20 and 0.02% NaN_3,

pH 7.4), then incubated with 350 μL/well of blocking buffer consisting of 0.1% porcine skin gelatin (Sigma-Aldrich, St. Louis, Missouri) in 0.01 M PBS, pH 7.4. After the plates were washed, 100 μL/well of the 3 antiparvalbumin antibodies diluted 1:15,000 in conjugate buffer [0.01 M PBS containing 0.1% bovine serum albumin (BSA; Affymetrix-USB, Cleveland, Ohio), pH 7.4] was added to the plates and incubated. Next, the plates were washed and incubated with 100 μL/well of rabbit antimouse IgG (diluted 1:5,000 and 1:1,000 in conjugate buffer for antifrog and anticarp MoAb, respectively) and goat antirabbit IgG (diluted 1:4,500 in conjugate buffer for anticod PoAb) labeled with alkaline phosphatase enzyme (Pierce Biotechnology, Inc., Rockford, Illinois). Binding was visualized with *p*-nitrophenyl phosphate substrate (Sigma Fast, Sigma-Aldrich, St. Louis, Missouri), and the color formed was measured at 405 nm.The dilutions of the 3 antiparvalbumin antibodies in the indirect ELISA were selected based on the statistically similar absorbance values (Dunnet's test, SAS programs, SAS Institute Inc., Cary, New York) for the northern pike. Each of the fish samples was extracted in triplicate, and each extract was analyzed in triplicate in 2 independent ELISA trials.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

The protein separation by SDS–PAGE was carried out with a Bio-Rad Mini Protean II electrophoresis cell (Bio-Rad Laboratories, Hercules, California). Five micrograms of crude cod extract and 1 μg of the purified cod and carp parvalbumin were boiled in Laemmli sample buffer containing 5.4% dithiothreitol (w/v) and separated on a 15% Tris-HCl precast gel (Bio-Rad Laboratories, Hercules, California) at 200 V (constant voltage) for 35 min. After the electrophoretic transfer, the gel was fixed and stained with Brilliant Blue G–Colloidal Stain (Sigma-Aldrich, St. Louis, Missouri). The gel image was captured using a Kodak Gel Logic 440 Imaging System (Eastman Kodak, Rochester, New York) equipped with Kodak 1D v. 3.6.5 software (Kodak Scientific Imaging Systems, New Haven, Connecticut).

IgG-Immunoblotting of Antiparvalbumin Antibodies

One microgram of soluble fish proteins and 0.1 μg of purified cod and carp parvalbumin were separated by SDS–PAGE using the conditions described above. After electrophoresis, the proteins were transferred onto a polyvinyl difluoride (PVDF) membrane (Millipore Corporation, Billerica, Massachusetts) at 65 V (constant voltage) for 80 min. The membrane was then blocked by incubation with 0.01 M PBS, pH 7.4, containing 0.05% Tween 20 (PBS-T) and 0.2% BSA (Affymetrix-USB, Cleveland, Ohio) for 2 h at room temperature. The antifrog, anticarp, and anticod parvalbumin antibodies were diluted 1:20,000, 1:12,500, and 1:75,000, respectively, in PBS-T containing 0.2% BSA. After washing the membrane with PBS-T, the diluted antiparvalbumin antibodies were added and incubated for 1 h at room temperature, followed by washing and incubation with rabbit antimouse IgG (diluted 1:80,000 and 1:100,000 in PBS-T containing 0.2% BSA for antifrog and anticarp MoAb, respectively) and goat antirabbit IgG (diluted 1:80,000 in PBS-T containing 0.2% BSA for anticod PoAb) labeled with alkaline phosphatase (Pierce Biotechnology, Inc., Rockford, Illinois) for 1 h. The bound antibodies was visualized with 1-Step NBT/BCIP substrate solution (Pierce Biotechnology, Inc., Rockford, Illinois) diluted 1:1 with distilled water. The membrane was photographed using the Kodak Gel Logic 440 Imaging System (Eastman

Kodak, Rochester, New York) equipped with Kodak 1D v. 3.6.5 software (Kodak Scientific Imaging Systems, New Haven, Connecticut).

Results and Discussion

Identification of Fish Species

Since misidentification of fish is a frequently encountered problem,[39,40] it was essential to identify all fish samples used in this study obtained from different seafood distributors. Fish species identification based on morphological characteristics was impossible because several fish samples were received in the forms of fillets and steaks. Hence, DNA-based methods were used to authenticate the fish samples to the species levels. The methods confirmed that the fish samples were accurately labeled by the suppliers, and the scientific names of the fish used in the study are indicated in Table 1.

Table 1. Scientific Names and Sources of Fish and Frog Samples

Common name	Scientific name	Supplier
American bullfrog	*Rana catesbeiana*	Little Saigon (L[a])
Unsalted Atlantic herring	*Clupea harengus*	Local fresh fish store, The Netherlands (K[a])
Salted Atlantic herring	*Clupea harengus*	Local fresh fish store, The Netherlands
Pilchard or sardine	*Sardina pilchardus*	All Fresh Seafood Inc. (A[a])
Pacific sardine	*Sardinops sagax*	Dr. Yi-Cheng Su, Oregon State (O[a])
Carp	*Cyprinus carpio*	Joe Tess Live Fish Market (J[a])
Northern pike	*Esox lucius*	Julie Nordlee, Wisconsin (W[a])
Atlantic cod	*Gadus morhua*	Norland Products Inc. (N[a]), All Fresh Seafood Inc.
Pollock	*Pollachius virens*	Norland Products Inc.
Alaska pollock	*Theragra chalcogramma*	Gorton's, Inc. (G[a])
Haddock	*Melanogrammus aeglefinus*	Norland Products Inc., Gorton's, Inc.
Cusk	*Brosme brosme*	Norland Products Inc.
Hake	*Urophycis tenuis*	Norland Products Inc., All Fresh Seafood Inc.
Bluegill	*Lepomis macrochirus*	Julie Nordlee and Tony Korth, Nebraska (NE[a])
Tilapia	*Oreochromis niloticus*	Surf and Turf Food Co. (S[a])
Mahi-mahi	*Coryphaena hippurus*	All Fresh Seafood Inc.
Snapper	*Lutjanus guttatus/synagris*	All Fresh Seafood Inc.
Hybrid striped bass	*Morone chrysops* × *saxatilis*	All Fresh Seafood Inc.
(Red) grouper	*Epinephelus morio*	All Fresh Seafood Inc.
Albacore tuna	*Thunnus alalunga*	Surf and Turf Food Co.
Chub mackerel	*Scomber japonicas*	Dr. Yi-Cheng Su, Oregon State
Swordfish	*Xiphias gladius*	All Fresh Seafood Inc.
Pacific halibut	*Hippoglossus stenolepis*	All Fresh Seafood Inc.
American plaice or sole	*Hippoglossoides platessoides*	Norland Products Inc.
Yellowtail flounder	*Limanda ferruginea*	Gorton's, Inc.
Steelhead or rainbow trout	*Oncorhynchus mykiss*	Surf and Turf Food Co.
Atlantic salmon	*Salmo salar*	Midwest Seafood (M[a])
Chinook salmon	*Oncorhynchus tshawytscha*	Dr. Yi-Cheng Su, Oregon State
Catfish	*Ictalurus punctatus*	Joe Tess Live Fish Market
Ocean perch	*Sebastes fasciatus*	Norland Products Inc., All Fresh Seafood Inc.

[a]Letters represent the different suppliers and are used in the subsequent figures.

SDS–PAGE Analysis of Protein Profiles in Fish Extracts

The SDS–PAGE profiles of the crude muscle extracts of 29 fish species are shown in Figure 1. The fish species, whether within the same orders or from different orders, displayed heterogeneity in the protein-banding patterns. Our analysis focused primarily on the protein bands with a molecular weight range of 10–13 kDa where parvalbumin is known to migrate. The bands for purified cod and carp parvalbumin did migrate at 10–13 kDa as expected. All frog and fish species contained either one or two protein bands between 10 to 13 kDa at different intensities, with the exception of mahi-mahi, albacore tuna, and swordfish. Gajeswski et al.[32] indicated that the multiple parvalbumin bands may represent the presence of isoforms, as some fish species have been shown to express from two to five parvalbumin isotypes.[15] The absence of parvalbumin bands in tuna was in agreement with the observation made by Chen et al.[31] and Van Do et al.,[41] even though a different species of tuna (albacore) was analyzed here. Kuehn et al.[42] recently reported that tuna contained between 40 to 110 times less parvalbumin in the raw muscles compared to herring, carp, redfish, trout, salmon, and cod, according to quantitative determination by ELISA. This is in line with the clinical observation of Sampson that tuna is less often causing allergic reactions than other fish.[43] Lim et al.[44] stated that the muscles sampled from different parts of tuna may affect the parvalbumin content due to the differential distribution of parvalbumin in various muscle types and locations within whole tuna. This finding may provide an explanation for the lack of visible parvalbumin bands in tuna in our current study. Shiomi et al.[18] were able to purify parvalbumin from bigeye tuna. Similar to tuna, no 10–13 kDa band was observed with swordfish in our study (Fig. 1). Griesmeier et al.[22] also stated that the swordfish expressed low levels of parvalbumin in muscles when compared to those of cod and whiff. Although SDS–PAGE suggested that mahi-mahi, swordfish, and tuna contained no or low amounts of parvalbumins, additional research is necessary to confirm this finding because SDS–PAGE allows only for an approximate estimation of the parvalbumin content, as dye-binding differs among proteins.

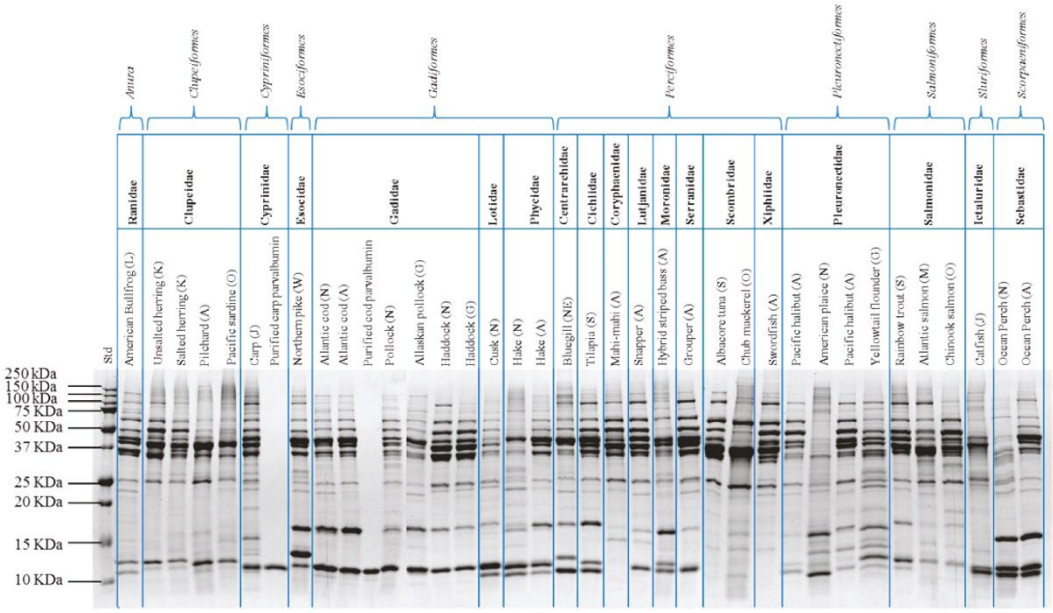

Figure 1. SDS–PAGE profiles of the raw muscle protein extracts of frog and fish species. The family and order of the species are represented by bold and italic characters, respectively.

Species-Specific Immunoreactivity by Indirect ELISA

The reactivities of antifrog MoAb, anticarp MoAb, and anticod PoAb to the parvalbumins in raw fish muscle extracts were compared using indirect ELISA (Fig. 2a and b). The mean absorbance value of wells containing all reagents except antiparvalbumin antibodies +3 standard deviations was used as an arbitrary cutoff point for the positive reactivity (~0.15). The antifrog MoAb was produced by using the parvalbumin purified from frog muscle as an immunogen, according to Sigma-Aldrich. The antifrog MoAb showed reactivity to the extracts of frog, pilchard, sardine, carp, pike, bluegill, tilapia, snapper, catfish, and ocean perch. No reactivity to herring, mahi-mahi, albacore tuna, swordfish, and all fish species in the order Gadiformes was observed. The antifrog MoAb showed consistently low reactivity to all fish species in the orders Pleuronectiformes and Salmoniformes. Furthermore, the antifrog MoAb showed variable reactivity to the fish species that belong to the same orders. As an example, the antifrog MoAb reacted to sardine but failed to react to the herring in the order Clupeiformes. Similarly, the antifrog MoAb bound strongly or moderately to bluegill, tilapia, and snapper but reacted weakly or not at all to the remaining species in the order Perciformes.

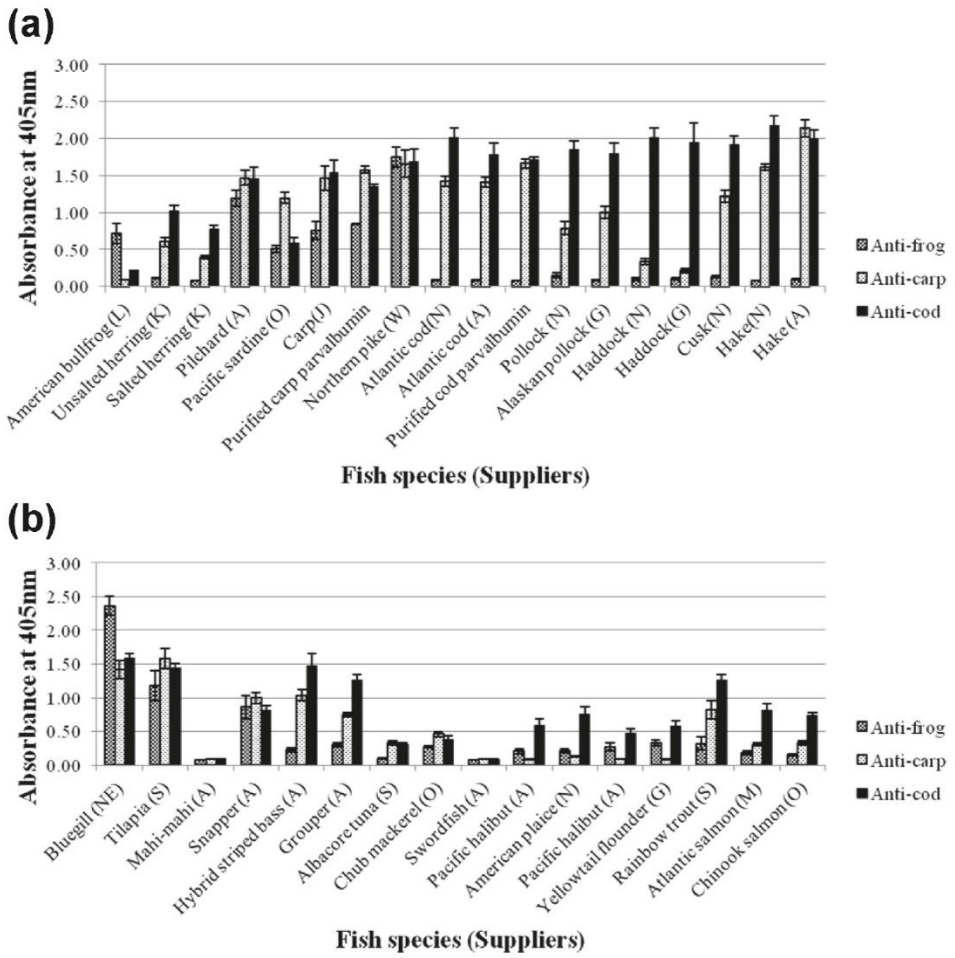

Figure 2. Reactivity of antifrog, anticarp, and anticod parvalbumin antibodies with the raw muscle extracts of frog and fish species, as determined by indirect ELISA. Each column and error bar represents the mean absorbance values and standard deviation of 18 observations, respectively.

A comparison of our study and the finding by Gajewski and Hsieh[32] revealed the similar reactivity of the antifrog MoAb with the majority of species, with the exception of mahi-mahi and striped bass. The dissimilarities in the reactivity may be due to the use of different species of mahi-mahi and striped bass or the use of different forms of antigens (raw versus cooked fish extracts) for reacting with the antifrog MoAb in the indirect ELISA. The present study confirms the finding by Gajewski and Hsieh[32] that the antifrog MoAb did not react with cod, hake, pollock, and haddock, but a recent study by Weber et al.[33] reported the contrary using a competitive ELISA. Weber et al.[33] attributed the differences to the use of fish extracts in Gajewski and Hsieh[32] rather than purified parvalbumin in their study. Compared to the purified parvalbumins, fish extracts did not contain standardized amounts of parvalbumins and thus resulted in the differential binding of the antifrog

MoAb to these species. Additionally, Weber et al.[33] discussed that the presence of other soluble, nonparvalbumin fish proteins in the extracts may affect the ability of parvalbumins to be coated effectively on the wells and thus the detectability of parvalbumins by the antifrog MoAb in the indirect ELISA. Nevertheless, this study showed that the antifrog MoAb did not react with the purified cod parvalbumin in indirect ELISA, suggesting that cod parvalbumin in its native form does not contain cross-reactive epitopes recognized by the antifrog MoAb.

The commercially available anticarp MoAb was produced by immunizing mice with parvalbumin purified from carp muscle.[45] To date, no study has extensively evaluated the specificity of this antibody to various fish species. This study revealed that the anticarp MoAb reacted equally well with the purified carp parvalbumin and the parvalbumin in raw carp extracts. Interestingly, the anticarp MoAb reacted strongly to all fish species in the order of Gadiformes, except for the haddock from both suppliers. Moreover, the anticarp MoAb reacted to the remaining fish species, with the exception of frog, mahi-mahi, swordfish, and all fish species in the order Pleuronectiformes and ocean perch.

Of the 3 antibodies, the anticod PoAb showed reactivity to the widest range of fish species but did not react with mahi-mahi and swordfish. The anticod PoAb reacted strongly or moderately to the majority of the fish species but weakly with frog, albacore tuna, and chub mackerel. Similar to the observation made with the antifrog MoAb, both the anticarp MoAb and the anticod PoAb showed variable reactivity to fish species that belong to the same orders. The inconsistent reactivity may possibly be due to the differences in plate-coating efficiency, quantity, and the primary or conformational structure of the parvalbumins among the fish species within the same orders. Faste et al.[30] published a sandwich ELISA for the quantification of fish in foods using a rabbit polyclonal anticod parvalbumin antibody that cross-reacted with other fish species to a different extent. Fish species that were optimally detected included cod, tilapia, herring, pollock, salmon, and carp, but the antibody reactivity to rainbow trout, tuna, swordfish, and northern pike, among others were quite low. This observation was in accordance with our finding, with the exception of trout and pike, which showed higher reactivity in our study.

Overall, the 3 antiparvalbumin antibodies showed reactivity rather similar to those of pilchard, northern pike, tilapia, snapper, and catfish but much more diverse reactivity to that of other fish species. Additionally, no reactivity to mahi-mahi and swordfish was noted for all 3 antibodies, probably owing to either the lack of detectable amounts of parvalbumins in the fish muscles or the lack of Ab-binding epitopes in the parvalbumins of these species. All fish samples were tested in the raw and unprocessed form, with the exception of salted herring, which is widely consumed in Europe. Salting of herring is a typical nonthermal process to preserve fish, and the immunoreactivity of both the anticarp MoAb and anticod PoAb to salted herring was shown to be unaffected as a result of the salting process.

Species-Specific Immunoreactivity by IgG-Immunoblotting

The IgG immunoblotting was performed to further investigate the binding of the antibodies to parvalbumins in the frog and fish species (Figs. 3–5). In general, all 3 antiparvalbumin antibodies showed a lack of reactivity to proteins in the mahi-mahi and swordfish

extracts in both the immunoblotting and the indirect ELISA even though the detection was conducted under both reducing (immunoblotting) and nonreducing (indirect ELISA) conditions. The reasons for this lack of reactivity are not yet clear.

Figure 3. IgG-immunoblot analysis of the antifrog MoAb reactivity with the raw muscle protein extracts of frog and fish species.

Figure 4. IgG-immunoblot analysis of the anticarp MoAb reactivity with the raw muscle protein extracts of frog and fish species.

Figure 5. IgG-immunoblot analysis of the anticod PoAb reactivity with the raw muscle protein extracts of frog and fish species.

The results obtained from immunoblotting agreed with the ELISA analysis for the majority of the fish species. On the basis of the immunoblotting results, the antifrog MoAb did not bind to parvalbumins from species with an absorbance value ≤ 0.15 in the ELISA, including the unsalted and salted herring, all species in the order Gadiformes, mahi-mahi, albacore tuna, swordfish, and salmon. Besides, the antifrog MoAb also did not bind to all species in the order Pleuronectiformes in the immunoblot despite the occurrence of absorbance values > 0.15 in the ELISA, but these species reacted weakly with the antifrog MoAb in the ELISA (absorbance values of > 0.15 but < 0.3). On the basis of the immunoblotting results, the anticarp MoAb did not bind to species with an absorbance value ≤ 0.15 in the ELISA, including frog, mahi-mahi, swordfish, Pacific halibut, yellowtail flounder, and ocean perch. In addition, the anticarp MoAb did not bind to unsalted and salted herring, haddock, and salmon in the immunoblot despite the occurrence of absorbance values > 0.15 in the ELISA, but the reactivity of the anticarp MoAb to these species were also relatively low in the ELISA (absorbance values > 0.15 but < 0.6). The anticod PoAb bound to parvalbumins in all species but albacore tuna, mahi-mahi, and swordfish.

In conclusion, both the indirect ELISA and IgG immunoblotting consistently showed that the 3 antiparvalbumin antibodies had varying specificities for proteins in extracts of different fish species, which can probably be attributed to differences in the parvalbumin content or immunoreactivity among fish species. The polyclonal anticod parvalbumin antibody showed reactivity to the widest range of fish species probably due to the recognition of multiple epitopes based upon the polyclonal nature of the antisera. In comparison, the monoclonal antifrog parvalbumin antibody showed the least cross-reactivity due to the recognition of a single epitope and the frog parvalbumin being less homologous to fish than cod parvalbumin. The anticod parvalbumin antibody appeared to be more suitable

for the detection of parvalbumin derived from different fish species; however, limitations still exist regarding the inconsistent binding to different fish species. These 3 antiparvalbumin antibodies can potentially be applied to the standardization of the parvalbumin content in the fish extracts used for the skin prick test and radioallergosorbent test. In addition, the antibodies would be useful for monitoring the purification and localization of fish parvalbumins in research studies. Our study may serve as a guide when selecting the appropriate antibodies for detecting fish parvalbumins. However, the disadvantages associated with the use of any of the 3 antiparvalbumin antibodies in detecting allergenic fish residues in foods are that the antiparvalbumin antibody may fail to detect certain fish species that are possibly deficient in parvalbumin. Examples include tuna, mahi-mahi, and swordfish, as demonstrated in the SDS–PAGE, IgG immunoblotting, and indirect ELISA. Some may argue that the absence of parvalbumin allergens in food samples may result in a lower risk of eliciting an allergic reaction, but some fish-allergic subjects may be allergic to proteins that are not parvalbumins. Kelso et al.[46] and James et al.[47] reported 2 subjects with monospecific allergy who showed IgE-reactivity to only a protein band at 25 kDa in swordfish and 40 kDa in tuna. Therefore, even if parvalbumins are undetectable in foods, that does not necessarily indicate that the foods are safe for individuals with fish allergy. Efforts can be made in future research to produce antibodies that are targeted specifically to fish proteins that have equal abundance in all fish species for the development of an ELISA to detect allergenic fish residues in foods.

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