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# Measuring Parvalbumin Levels in Fish Muscle Tissue: Relevance of Muscle Locations and Storage Conditions

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**Abstract**

Fish is an allergenic food capable of provoking severe anaphylactic reactions. Parvalbumin is the major allergen identified in fish and frog muscles. Antibodies against fish and frog parvalbumin have been used to quantify parvalbumin levels from fish. However, these antibodies react variably with parvalbumin from different fish species. Several factors might be responsible for this variation including instability of parvalbumin in fish muscle as a result of frozen storage and differential parvalbumin expression in muscles from various locations within the whole fish. We aimed to investigate whether these factors contribute to the previously observed variable immunoreactivity of the anti-parvalbumin antibodies. Results showed the detection of parvalbumin by these antibodies was unaffected by frozen storage of muscles for 112 days. However, the parvalbumin content decreased in fish muscles from anterior to posterior positions. This factor may partially explain for the inconsistent reactivity of anti-parvalbumin antibodies to different fish species.

**Keywords:** parvalbumin detection, anti-parvalbumin IgG antibodies, frozen storage, muscle localization, fish allergy

## 1. Introduction

Fish is one of the eight most common allergenic foods—among peanuts, tree nuts, wheat, soybeans, crustacean shellfish, cow milk, and egg—that account for more than 90% of all documented food allergies (FAO, 1995). In the US, fish allergy affects approximately 0.4% of the population (Sicherer, Muñoz-Furlong, & Sampson, 2004). Fish can be a potent allergenic food; severe allergic reactions and even fatalities have occurred (Pumphrey & Gowland, 2007; Yunginger et al., 1988). Fish-allergic individuals can react to ingestion of low doses of fish; doses as low as 5 mg of either cod or herring have provoked allergic reactions in double-blind placebo-controlled food challenge tests, although relatively few patients have been evaluated with such low-dose challenges (Taylor et al., 2002).

The major allergen in fish is parvalbumin. Parvalbumin is an intracellular calcium-binding muscle protein that promotes relaxation in the fast-twitch muscle fibers (Rall, 1996). Parvalbumin belongs to the EF-hand protein family that contains other known allergens such as Bet v 4 from birch pollen (Ferreira et al., 1999) and the sacroplasmic calcium-binding protein from shrimp (Ayuso et al., 2009). In some studies, parvalbumin reacted with specific IgE from greater than 95% of the fish-allergic individuals (Bugajska-Schretter et al., 2000). However, in other studies, the percentage of fish-allergic individuals with parvalbumin-specific IgE is somewhat smaller (Griesmeier et al., 2010). Still, parvalbumin is often considered as a pan-allergen responsible for the cross-reactivity between various fish species among fish-allergic individuals (Hansen, Bindslev-Jensen, Skov, & Poulsen, 1997; Taylor, Kabourek, & Hefle, 2004). Accordingly, fish-allergic individuals are advised to strictly avoid all species of fish (Helbling et al., 1999). However, despite this advice, some fish-allergic patients are able to tolerate ingestion of some fish species in oral challenge studies (Bernhisel-Broadbent, Scanlon, & Sampson, 1992; de Martino et al., 1990). The basis for this variable reactivity to fish observed in some fish-allergic patients has never been delineated. One explanation could be that variable amounts of parvalbumin are expressed in different species of fish. A recent study by our group (Lee, Nordlee, Koppelman, Baumert, & Taylor, 2011) revealed variable binding of three anti-parvalbumin IgG antibodies to crude extracts of different fish species, perhaps indicating a variation in parvalbumin content between the muscle tissues of different fish species.

It is well recognized that fish undergo deterioration after death, including the degradation of muscle proteins, among others (Santos-Yap, 1996). The variation of parvalbumin content in fish muscles could perhaps be attributed to the denaturation of parvalbumin during frozen storage, but no studies have specifically evaluated the changes in parvalbumin content during frozen storage. On the other hand, studies have demonstrated that parvalbumin expression varied between muscles from different locations within whole fish (Coughlin, Solomon, & Wilwert, 2007; Lim, Neo, Goh, Shek, & Lee, 2005; Thys, Blank, Coughlin, & Schachat, 2001). Additionally, the parvalbumin content also varied with the muscle types; dark muscle is found to contain less parvalbumin than white muscle (Kobayashi et al., 2006). Hence, the muscles sampled from multiple parts of the fish body may differ in parvalbumin content, which could account for the differences in binding of the anti-parvalbumin antibodies to fish parvalbumin extracts.

Considering the possible influence of frozen storage and muscles sampling on the parvalbumin levels, the present study was undertaken to investigate whether these factors contribute to the variable immunoreactivity of the anti-parvalbumin antibodies.

## 2. Materials and methods

### *2.1. Sampling and extraction of fish muscles*

#### *2.1.1. Fish samples*

Fresh and non-frozen carp (*Cyprinus carpio*), catfish (*Ictalurus punctatus*), chub mackerel (*Scomber japonicus*), sardine (*Sardinops sagax*), chinook salmon (*Oncorhynchus tshawytscha*), albacore tuna (*Thunnus alalunga*), and mahi-mahi (*Coryphaena hippurus*) were obtained from different fish and seafood distributors in the US. Upon receipt, the whole fish were skinned, gutted, rinsed briefly with distilled water, and patted dry with absorbent liner. The species of the fish samples were identified by Eurofins GeneScan, Inc. (Metairie, Louisianna) using either the Food and Drug Administration (FDA)–validated DNA barcode analysis (Handy et al., 2011) or nucleotide sequence analysis of the cytochrome b and 16S genes.

#### *2.1.2. Sampling after frozen storage*

Several pieces of the fish fillets from each individual species, including carp, catfish, mackerel, sardine, and salmon were ground to a uniform consistency using a commercial food processor. The ground fish sample (in triplicate) was then extracted, and the supernatant solution was kept at –80°C until analyzed to minimize any changes in fish proteins. Subsequently, 40 g of the remaining ground fish samples was stored as a single batch in a bag and kept frozen at –20°C. Thawing at 4°C, followed by sampling and extraction of these ground fish samples, was repeated every 28 days for four consecutive months. After the sampling was completed, all supernatant solutions stored at –80°C were analyzed together in the indirect ELISA.

#### *2.1.3. Sampling from various muscle locations within whole fish*

Six white muscle samples of 2 cm in width and 1 cm in length were obtained from different locations in two whole carp and catfish, and one whole tuna and mahi-mahi (Fig. 1). The locations comprised three longitudinal positions, including anterior [25% of the total muscle length (TML), excluding head and tail], middle (50% TML), and posterior (75% TML). At each longitudinal position, muscle sample was obtained from the dorsal (located at 1 cm from the upper edge) and the ventral side (located at 1 cm from the lower edge). The muscle samples were then extracted and analyzed by indirect ELISA and SDS-PAGE.

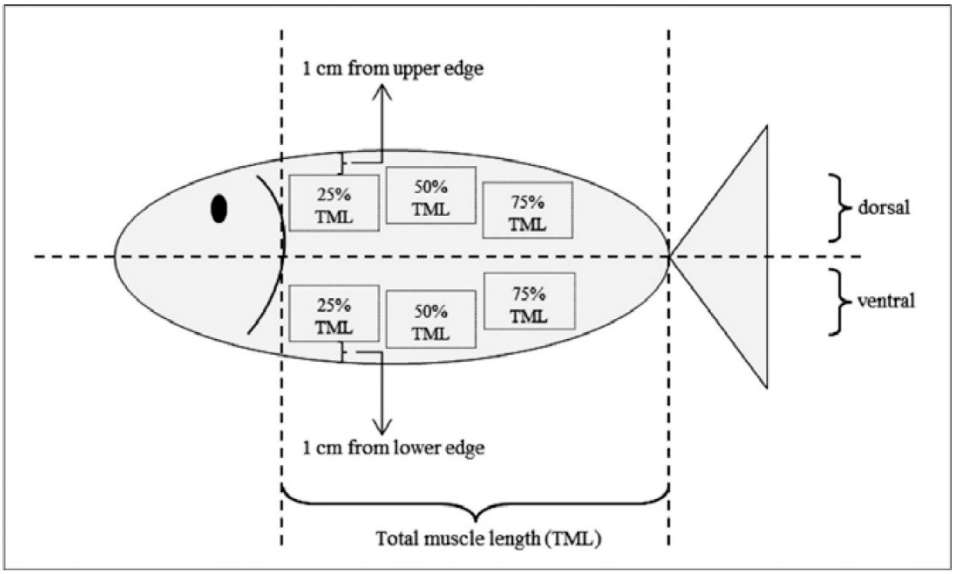


**Figure 1.** Diagram of muscle samples obtained from various locations within whole fish.

*2.1.4. 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 $NaH_2PO_4$, 0.85% NaCl, pH 7.4) overnight with gentle rocking at 4°C. Extracts were then centrifuged at 3612g in a tabletop centrifuge at 4°C for 30 min. Insoluble material was discarded and the supernatant solution was used for protein determination by the Lowry method as described previously (Lee et al., 2011).

### 2.2. *Indirect ELISA*

Indirect ELISA was performed according to the indirect ELISA methods as described elsewhere (Lee et al., 2011). Briefly, microtiter plates were coated by overnight incubation at 4°C with 1 μg/well of the fish extracts in sodium carbonate–bicarbonate buffer. Thereafter, all incubation steps were performed for 1 h at 37°C, except for the incubation after the addition of substrate. The plates were washed with PBS-Tween 20 (0.05%) between steps. Following blocking of the plates with PBS-gelatin (0.1%), monoclonal antifrog parvalbumin antibody (anti-frog MAb), monoclonal anti-carp parvalbumin antibody (anti-carp MAb), and polyclonal anti-cod parvalbumin antibody (anti-cod PAb) diluted 1:15 000 in PBS-bovine serum albumin (BSA; 0.1%) was added to the plates and incubated. The bound antibodies were detected by rabbit antimouse IgG (diluted 1:5000 and 1:1000 in PBS-BSA for anti-frog and anti-carp MAb, respectively) and goat anti-rabbit IgG (diluted 1:4500 in PBS-BSA for anti-cod PAb) labeled with alkaline phosphatase enzyme. Binding was visualized with *p*-nitrophenyl phosphate substrate and the color developed was measured at 405 nm. Each fish extract was analyzed in triplicate wells in two independent ELISA trials.

### *2.3. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)*

SDS-PAGE was performed according to the methods as described elsewhere (Lee et al., 2011). Briefly, 5 μg of the crude fish extract was boiled in Laemmli sample buffer-dithiothreitol (5.4%) and separated on a 15% TRIS-HCL precast gel at 200 V for 35 min. After the electrophoretic separation, the gels were fixed and stained with Brilliant Blue G-Colloidal Stain (Sigma Chemical Co., St. Louis, Missouri) overnight at room temperature. Gels were then photographed 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). The band intensity of parvalbumin relative to total fish proteins on gel was performed by densitometry analysis using the Kodak 1D v. 3.6.5 software.

### *2.4. Statistical analysis*

Differences between the mean absorbance values obtained during frozen storage were statistically evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's test (SAS programs, SAS Institute Inc., Cary, New York).

## 3. Results and discussion

### *3.1. Parvalbumin variation due to frozen storage*

Protein denaturation is one of the chemical reactions that occur in fish muscle during frozen storage. It was postulated that the protein denaturation during frozen storage is caused by several factors, including dehydration and an increase in solute concentrations due to freezing out of water. Such changes disrupt the protein-water interaction and the native conformation of the proteins, leading to the exposure of the buried hydrophobic groups. Consequently, intermolecular cross-linkages form in proteins, causing aggregation and the formation of higher molecular weight polymers (Santos-Yap, 1996). Additionally, the interaction between proteins and lipids or formaldehyde in the frozen-stored fish was found to correlate with the decrease in protein solubility and extractability (Shenouda, 1980). The stability of fish proteins during frozen storage vary for different types of muscle proteins. Proteins in the myofibrillar groups are more susceptible to denaturation than the sarcoplasmic proteins (Sikorski, Olley, & Kostuch, 1976). Most studies have examined the biochemical changes of myofibrillar proteins in frozen stored fish muscles (del Mazo, Torrejón, Careche, & Tejada, 1999; Jiang & Lee, 1985; Tejada et al., 1996), but no research on the alteration of parvalbumin during frozen storage has been reported.

In this study, the detectability of parvalbumin from several fish species during frozen storage was evaluated by the three anti-parvalbumin antibodies by indirect ELISA. In general, statistically significant changes in the parvalbumin content of several fish species were observed, but these changes were minimal and the parvalbumin remained detectable throughout 112 days of frozen storage (Fig. 2). Among the species tested, sardine had significantly lower reactivity with the three anti-parvalbumin antibodies after 28, 56, 84, and 112 days of frozen storage, compared to that obtained in fresh sardine at day 0. Similar to the sardine, the reactivity of anti-cod PAb to chinook salmon was significantly lower throughout 112 days of frozen storage in comparison to the reactivity in fresh salmon. For carp, the

detectable parvalbumin by both anti-frog and anti-carp MAb was significantly lower after 84 and 112 days of frozen storage, as compared to fresh carp. After 112 days of frozen storage, catfish had significantly lower parvalbumin when analyzed by the anti-frog MAb. Although the decline in immunoreactivity was statistically significant, the decrease was considered minimal.

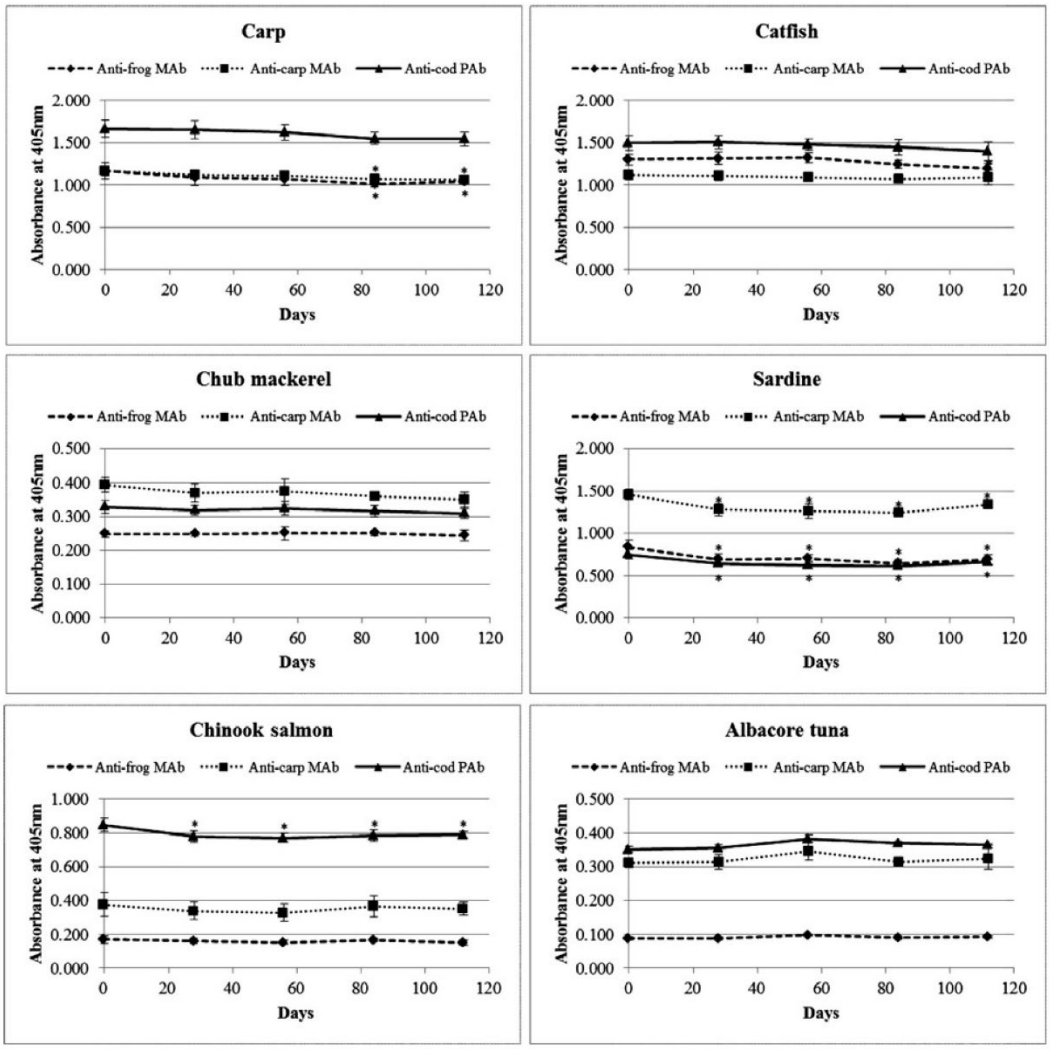


**Figure 2.** Stability of parvalbumin during frozen storage of carp, catfish, chub mackerel, sardine, chinook salmon, and albacore tuna, as determined by the indirect ELISA using anti-frog MAb, anti-carp MAb, and anti-cod PAb. Each data point and error bar represents the mean absorbance value and standard deviation of 18 observations, respectively. Asterisk indicates statistical difference from the mean absorbance value at day 0 ($p < 0.05$).

The results suggested that the parvalbumin in fish muscles was relatively stable to frozen storage at –20°C. Furthermore, the extractability of parvalbumin and the subsequent

binding of the antibodies to parvalbumin in the indirect ELISA was unaffected by freezing the fish muscles. Babbitt, Crawford, and Duncan (1972) demonstrated that there is only a slight decrease in the extractable sarcoplasmic proteins during frozen storage of intact or minced hake at –20°C, and thus our findings were in accordance with their observation as parvalbumin is a sarcoplasmic protein. Babbitt et al. (1972) also found that the denaturation of fish muscle proteins induced by frozen storage is predominantly due to the alteration of the myofibrillar proteins.

The stability of parvalbumin during frozen storage of fish muscles, as shown in this study, could not explain the previously observed variation in the parvalbumin detection among fish species. It should be noted, however, that fish protein denaturation induced by freezing appeared to be less pronounced in intact muscle than in the extracted protein in the form of solutions or suspensions (Sikorski & Kotakowska, 1994). Therefore, the influence of freezing should be considered when developing an immunoassay method based on antibody reactivity for fish proteins which is intended for detecting trace residues of fish that might contaminate other foods and pose a potential risk to fish-allergic consumers.

### *3.2. Parvalbumin variation due to muscle locations*

To examine the expression of parvalbumin in various muscle locations of carp, catfish, albacore tuna, and mahi-mahi, the muscle proteins were separated by SDS-PAGE, followed by densitometry analysis of the intensity of the parvalbumin bands with molecular weights ranging from 11 to 12 kDa (Figs. 3 and 4). Variations in the sample loading and/or pipetting errors that might occur during SDS-PAGE were normalized by computing the band intensity ratio of parvalbumin to total fish proteins. Compared to the muscles at the anterior and middle position, muscles at the posterior positions had a lower band intensity ratio regardless of fish species, indicating that muscles located near the tail contained lower amounts of parvalbumin than muscles near the head and the middle portions of the fish body. The effect of muscle locations on the expression of parvalbumin was more pronounced in tuna and mahi-mahi when compared to that observed in carp and catfish.

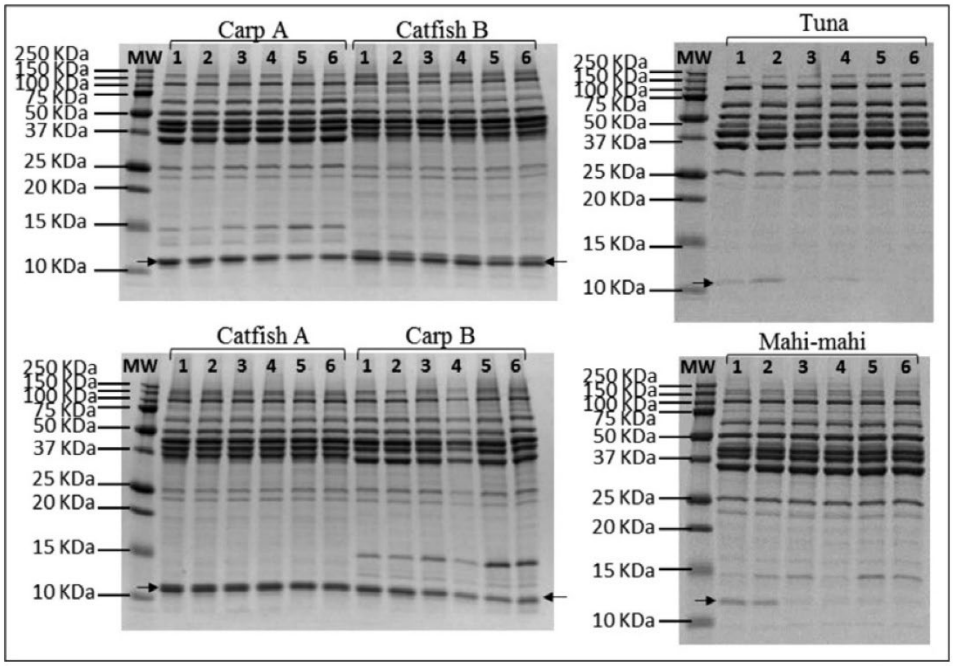


**Figure 3.** SDS-PAGE profiles of the raw muscles obtained from 6 different body positions of 2 carp (Carp A and B), 2 catfish (Catfish A and B), one albacore tuna, and one mahi-mahi. The body positions were represented by numbers: 1 = 25% TML, dorsal; 2 = 25% TML, ventral; 3 = 50% TML, dorsal; 4 = 50% TML, ventral; 5 = 75% TML, dorsal; 6 = 75% TML, ventral. The arrow represents the expected position of the parvalbumin band.

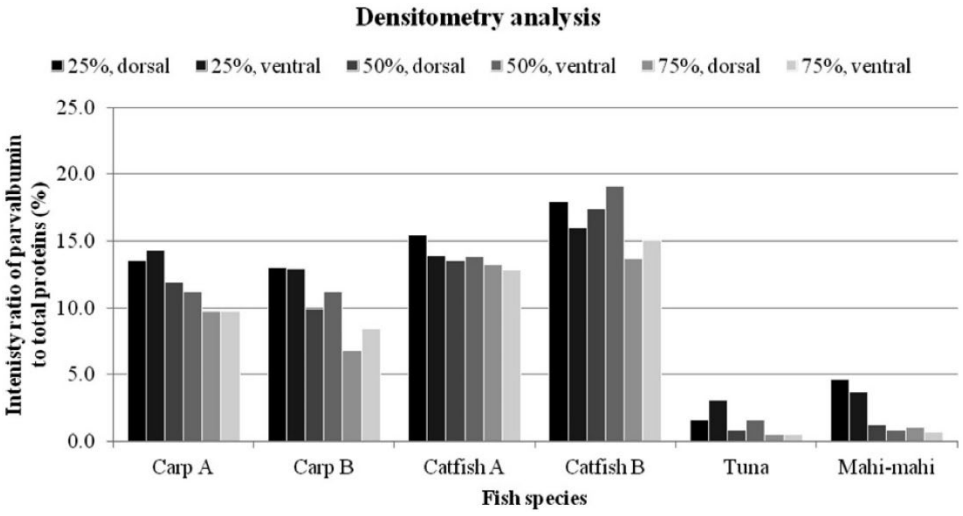


**Figure 4.** Band intensity ratio of parvalbumin to total proteins (expressed in percentage) in six body positions of two carp (Carp A and B), two catfish (Catfish A and B), one albacore tuna, and one mahi-mahi, as determined by the densitometry analysis of stained SDS-PAGE gels.

All species, with the exception of tuna, revealed no difference in the parvalbumin content between the dorsal and ventral side of the muscles. It was observed that tuna muscles located at both the anterior and middle positions demonstrated higher levels of parvalbumin at the ventral side, as compared to that obtained from the dorsal side. Furthermore, tuna muscles showed a gradual decrease in parvalbumin content from the anterior to the posterior positions. A study by Lim et al. (2005) investigated the parvalbumin content in the rostral (anterior), middle, and caudal (posterior) portions of tuna, *Thunnus tonggol*. For each of these portions, muscles were sampled from three different parts, including the dorsal and ventral white muscle, and the middle red muscle. According to the immunoblotting analysis of the muscle extracts using the antifrog MAb, the parvalbumin content decreased from the rostral and caudal regions. Moreover, the ventral white muscles contained higher amounts of parvalbumin than dorsal white muscles. Our observations with a different species of tuna confirm these earlier findings. A similar trend of parvalbumin expression was observed in rainbow trout parr and smolts, and largemouth bass, according to the relative intensity of the parvalbumin bands on the stained SDS-PAGE gels (Coughlin et al., 2007; Thys et al., 2001).

Parvalbumin has been proposed to act as an intracellular calcium buffer and facilitate relaxation in fast-contracting muscle. During muscle contraction, the calcium released from sarcoplasmic recticulum binds to troponin C, causing movements of the tropomyosin and subsequent interaction between myosin and actin. The contractile activity ceases when parvalbumin sequesters calcium from the troponin C into the sarcoplasmic recticulum via a calcium pump (Ca-ATPase), allowing muscle relaxation to occur (Arif, 2009; Rall, 1996). Studies have demonstrated that the higher concentration of parvalbumin in rostral muscle is correlated with a faster rate of relaxation, whereas caudal muscle relaxes at a slower rate due to the lower concentration of parvalbumin (Coughlin et al., 2007; Thys et al., 2001).

The parvalbumin expression in different muscle locations were further analyzed by determining the reactivity of the three antiparvalbumin antibodies to the muscle extracts in the indirect ELISA (Fig. 5). The reactivity, measured via absorbance values, was directly proportional to the parvalbumin content within individual species as these antibodies specifically recognized parvalbumin. Overall, the results obtained from the indirect ELISA supported the observation made in the densitometry analysis of the parvalbumin band intensity. However, the variation in the antibodies reactivity to mahi-mahi appeared to be less prominent when detected by both the anti-frog and anti-carp MAb. This observation might be due to the exceptionally low reactivity of these antibodies with parvalbumin from mahi-mahi, which thus impairs the ability of these antibodies to detect the parvalbumin variations.

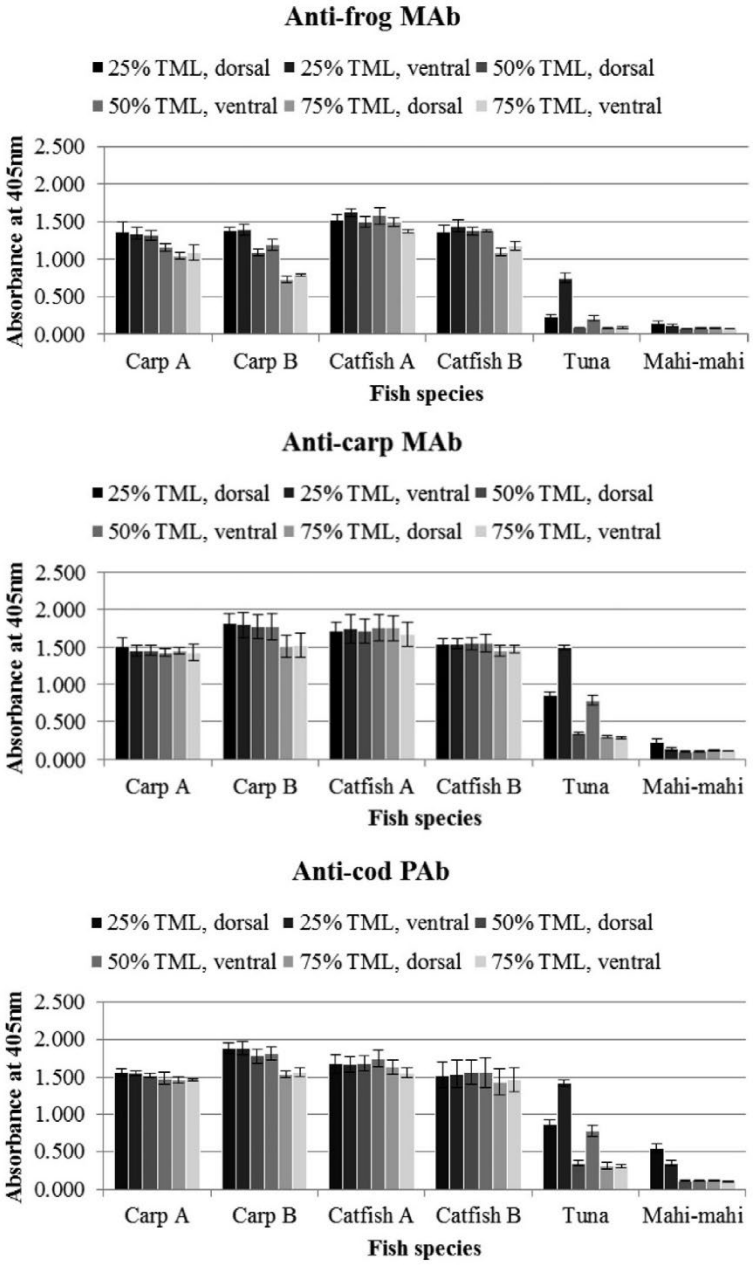


**Figure 5.** Reactivity of anti-frog MAb, anti-carp MAb, and anti-cod PAb with the raw muscle extracts obtained from six different body positions of two carp (Carp A and B), two catfish (Catfish A and B), one albacore tuna, and one mahi-mahi, as determined by the indirect ELISA. Each column and error bars represents the mean absorbance values and standard deviation of six observations, respectively.

## 4. Conclusions

Our current findings revealed no substantial change in the ability of the three anti-parvalbumin antibodies to detect parvalbumin from fish muscle that has been stored frozen at –20°C for 112 days. Investigation of the expression of parvalbumin in different muscle locations within whole fish demonstrated that muscles at the posterior position had lower parvalbumin content than the muscles at the anterior and middle position of the fish body, especially in albacore tuna and mahi-mahi. Hence, the immunoreactivity of anti-parvalbumin antibodies will be affected by the spatial variation of parvalbumin in fish. When using parvalbumin as a marker for detecting undeclared fish residues in foods, it is important to realize that the parts of fish muscles used in the food preparation could influence the detectable amounts of parvalbumin and/or fish residues in foods. More work is necessary to further elucidate the factors and variables responsible for the differences in the immunoreactivity of the anti-parvalbumin antibodies to fish species. These factors include, but are not limited to, the differential expression of parvalbumin among fish species and the differences in the sequential and conformational IgG-binding epitopes on the parvalbumin of various fish species.

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## References

Arif, S. H. (2009). A $Ca^{2+}$-binding protein with numerous roles and uses: Parvalbumin in molecular biology and physiology. *Bioessays, 31,* 410–421.

Ayuso, R., Grishna, G., Ibanez, M. D., Blanco, C., Carrillo, T., Bencharitiwong, R., Sinchez, S., Nowak-Wegrzyn, A., & Sampson, H. A. (2009). Sarcoplasmic calcium-binding protein is an EF-hand-type protein identified as a new shrimp allergen. *Journal of Allergy and Clinical Immunology, 124,* 114–120.

Babbitt, J. K., Crawford, D. L., & Duncan, K. L. (1972). Decomposition of trimethylamine oxide and changes in protein extractability during frozen storage of minced and intact hake (*Merluccius productus*) muscle. *Journal of Agricultural and Food Chemistry, 20,* 1052–1054.

Bernhisel-Broadbent, J., Scanlon, S. M., & Sampson, H. A. (1992). Fish hypersensitivity. I. In vitro and oral challenge results in fish-allergic patients. *Journal of Allergy and Clinical Immunology, 89,* 730–737.

Bugajska-Schretter, A., Grote, M., Vangelista, L., Valent, P., Sperr, W. R., Rumpold, H., Pastore, A., Reichelt, R., Valenta, R., & Spitzauer, S. (2000). Purification, biochemical, and immunological characterisation of a major food allergen: different immunoglobulin E recognition of the apo- and calcium-bound forms of carp parvalbumin. *Gut, 46,* 661–669.

Coughlin, D. K., Solomon, S., & Wilwert, J. L. (2007). Parvalbumin expression in trout swimming muscle correlates with relaxation rate. *Comparative Biochemistry and Physiology – Part A: Molecular & Integrative Physiology, 147,* 1074–1082.

de Martino, M., Novembre, E., Galli, L., de Marco, A., Botarelli, P., Marano, E., & Vierucci, A. (1990). Allergy to different fish species in cod-allergic children: In vivo and in vitro studies. *Journal of Allergy and Clinical Immunology, 86,* 909–914.

del Mazo, M. L., Torrejón, P., Careche, M., & Tejada, M. (1999). Characteristics of the salt-soluble fraction of hake (*Merluccius merluccius*) fillets stored at –20 and –30°C. *Journal of Agricultural and Food Chemistry, 47,* 1372–1377.

FAO: Food and Agriculture Organization of the United Nations. (1995). *Report of the FAO technical committee on food allergies.* Rome, Italy, November 13–14.

Ferreira, F., Engel, E., Briza, P., Richter, K., Ebner, C., & Breitenbach, M. (1999). Characterization of recombinant Bet v 4, a birch pollen allergen with two EF-hand calcium-binding domains. *International Archives of Allergy and Immunology, 118,* 304–305.

Griesmeier, U., Vázquez-Cortés, S., Bublin, M., Radauer, C., Ma, Y., Briza, P., Fernández-Rivas, M., & Breiteneder, H. (2010). Expression levels of parvalbumins determine allergenicity of fish species. *Allergy, 65,* 191–198.

Handy, S. M., Deeds, J. R., Ivanova, N. V., Hebert, P. D. N., Hanner, R., Ormos, A., Weigt, L. A., Moore, M. M., Hellberg, R. S., & Yancy, H. F. (2011). Single laboratory validated method for DNA-barcoding for the species identification of fish for FDA regulatory compliance. Retrieved from <http://www.fda.gov/Food/ ScienceResearch/LaboratoryMethods/ucm237391.htm>.

Hansen, T. K., Bindslev-Jensen, C., Skov, P., & Poulsen, L. K. (1997). Codfish allergy in adults: IgE cross-reactivity among fish species. *Annals of Allergy, Asthma, and Immunology, 78,* 187–194.

Helbling, A., Haydel, R., McCants, M. L., Musmand, J. J., El-Dahr, J., & Lehrer, S. B. (1999). Fish allergy: is cross-reactivity among fish species relevant? Double-blind placebo-controlled food challenge studies of fish allergic adults. *Annals of Allergy, Asthma and Immunology, 83,* 517–523.

Jiang, S.-T., & Lee, T.-C. (1985). Changes in free amino acids and protein denaturation of fish muscle during frozen storage. *Journal of Agricultural and Food Chemistry, 33,* 839–844.

Kobayashi, A., Tanaka, H., Hamada, Y., Ishizaki, S., Nagashima, Y., & Shiomi, K. (2006). Comparison of allergenicity and allergens between fish white and dark muscles. *Allergy, 61,* 357–363.

Lee, P.-W., Nordlee, J. A., Koppelman, S. J., Baumert, J. L., & Taylor, S. L. (2011). Evaluation and comparison of the species-specificity of three anti-parvalbumin IgG antibodies. *Journal of Agricultural and Food Chemistry, 59,* 12309–12316.

Lim, D. L., Neo, K. H., Goh, D. L., Shek, L. P., & Lee, B. W. (2005). Missing parvalbumin: Implications in diagnostic testing for tuna allergy. *The Journal of Allergy and Clinical Immunology, 115,* 874–875.

Pumphrey, R. S. H., & Gowland, H. (2007). Further fatal allergic reactions to food in the United Kingdom, 1999–2006. *Journal of Allergy and Clinical Immunology, 119,* 1018–1019.

Rall, J. A. (1996). Role of parvalbumin in skeletal muscle relaxation. *News in Physiological Sciences, 11,* 249–255.

Santos-Yap, E. E. M. (1996). Fish and seafood. In L. E. Jeremiah (Ed.), *Freezing effects on food quality* (pp. 109–133). New York: Marcel Dekker, Inc.

Shenouda, S. Y. K. (1980). Theories of protein denaturation during frozen storage of fish flesh. *Advances in Food Research, 26,* 275–311.

Sicherer, S. H., Muñoz-Furlong, A., & Sampson, H. A. (2004). Prevalence of seafood allergy in the United States determined by a random telephone survey. *Journal of Allergy and Clinical Immunology, 114,* 159–165.

Sikorski, Z. E., Olley, J., & Kostuch, S. (1976). Protein changes in frozen fish. *Critical Reviews in Food Science and Nutrition, 8,* 97–129.

Sikorski, Z. E., & Kotakowska, A. (1994). Changes in proteins in frozen stored fish. In Z. E. Sikorski, B. S. Pan, & F. Shahidi (Eds.), *Seafood proteins* (pp. 99–112). New York: Chapman & Hall, Inc.

Taylor, S. L., Hefle, S. L., Bindslev-Jensen, C., Bock, S. A., Burks, A. W., Christie, L., Hill, D. J., Host, A., Hourihane, J. O., Lack, G., Metcalfe, D. D., Moneret-Vautrin, D. A., Vadas, P. A., Rance, F., Skrypec, D. J., Trautman, T. A., Yman, I. M., & Zeiger, R. S. (2002). Factors affecting the determination of threshold doses for allergenic foods: How much is too much? *Journal of Allergy and Clinical Immunology, 109,* 24–30.

Taylor, S. L., Kabourek, J. L., & Hefle, S. L. (2004). Fish allergy: Fish and products thereof. *Journal of Food Science, 69,* R175–R180.

Tejada, M., Careche, M., Torrejón, P., del Mazo, M. L., Solas, M. T., García, M. L., & Barba, C. (1996). Protein extracts and aggregates forming in minced cod (*Gadus morhua*) during frozen storage. *Journal of Agricultural and Food Chemistry, 33,* 839–844.

Thys, T. M., Blank, J. M., Coughlin, D. J., & Schachat, F. (2001). Longitudinal variation in muscle protein expression and contraction kinetics of largemouth bass axial muscle. *The Journal of Experimental Biology, 204,* 4249–4257.

Yunginger, J. W., Sweeney, K. G., Sturner, W. Q., Giannandrea, L. A., Teigland, J. D., Bray, M., Benson, P. A., York, J. A., Biedrzycki, L., Squillace, D. L., & Helm, R. M. (1988). Fatal food-induced anaphylaxis. *The Journal of the American Medical Association, 260,* 1450–1452.