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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.



## 1. Introduction

Fish is one of the eight most common allergenic foods, amongst 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 at 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 fibres (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 recognised 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

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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, LA) 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 analysed to minimise 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 were repeated every 28 days for four consecutive months. After the sampling was completed, all supernatant solutions stored at −80 °C were analysed 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 of two whole carp and catfish, and one whole tuna and mahi-mahi (Fig. 1). The locations comprised of 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 analysed by indirect ELISA and SDS–PAGE.

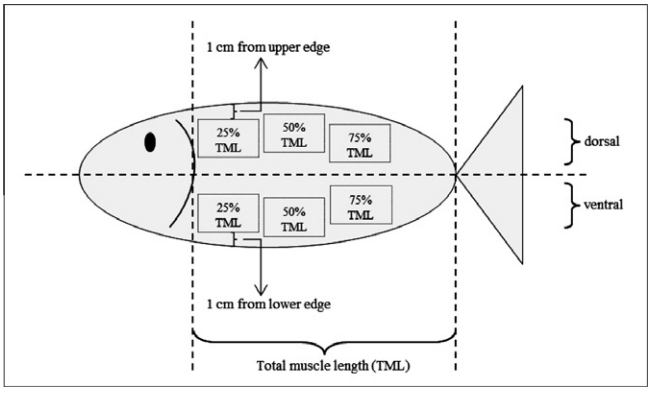


**Fig. 1.** Diagram of muscle samples obtained from various locations within whole fish.

#### 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 $Na_2HPO_4$, 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. 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 anti-frog parvalbumin antibody (anti-frog MAb), monoclonal anti-carp parvalbumin antibody (anti-carp MAb), and polyclonal anti-cod parvalbumin antibody (anti-cod PAb) diluted 1:15000 in PBS-bovine serum albumin (BSA; 0.1%) was added to the plates and incubated. The bound antibodies were detected by rabbit anti-mouse IgG (diluted 1:5,000 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 visualised with *p*-nitrophenyl phosphate substrate and the colour developed was measured at 405 nm. Each fish extract was analysed 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, MO) overnight at room temperature. Gels were then photographed using a Kodak Gel Logic 440 Imaging System (Eastman Kodak, Rochester, NY) equipped with Kodak 1D v. 3.6.5 software (Kodak Scientific Imaging Systems, New Haven, CT). 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, NY).