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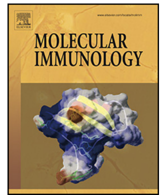

Immunological cross-reactivity between four distant parvalbumins—Impact on allergen detection and diagnostics

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ABSTRACT

Fish are the largest and most diverse group of vertebrates. Fish are also a part of the eight food groups that cause the majority of IgE mediated food reactions. Detection tools for fish allergens are however limited due to the great diversity of fish species, despite fish allergy and its major allergen parvalbumin being well documented. The most commonly studied fish are frequently consumed in North America and Europe. However, much less is known about fish allergens in the Australasian region although fish is widely consumed in this region.

A comprehensive phylogenetic analysis was performed of known parvalbumin amino acid sequences to determine possible candidate antigens for new cross-reactive antibodies to be used to detect most fish parvalbumins. Polyclonal rabbit antibodies were raised against parvalbumins from frequently consumed barramundi (*Lates calcarifer*), basa (*Pangasius bocourti*), pilchard (*Sardinops sagax*) and Atlantic salmon (*Salmo salar*). These were evaluated for cross-reactivity against a panel of 45 fish extracts (raw, heated and canned fish). Anti-barramundi parvalbumin proved to be the most cross-reactive antibody, detecting 87.5% of the 40 species analyzed, followed by anti-pilchard and anti-basa antibody. In contrast the anti-salmon antibody was very specific and only reacted to salmonidae and a few other fish. All analyzed fish species, except mahi mahi, swordfish, yellowfin tuna and all 5 canned fish had parvalbumin detected in raw extracts. However antibody reactivity to many fish was heat liable or susceptible to denaturation, demonstrating that some parvalbumins have most likely conformational epitopes, which lose antibody reactivity after heat treatment.

We have demonstrated the generation of highly cross-reactive anti-parvalbumin antibodies that could be used for the detection of allergenic fish parvalbumin in contaminated food products. This cross-reactivity study thus shows processing of fish, especially canning, can have on impact on antibody recognition by ELISA, possibly similar to IgE-binding in vivo.

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1. Introduction

Fish are the largest and most diverse group of vertebrates. Fish are also a part of the eight food groups that cause the majority of IgE mediated food reactions (Sicherer and Sampson, 2010). Despite fish allergy being common and well documented, detection of fish allergens and allergic sensitization is difficult due to species diversity (Sharp and Lopata, 2014).

Parvalbumin, a small calcium binding protein, is the major allergen identified in most fish species (Arif, 2009; Kuehn et al., 2014; Sharp and Lopata, 2014). Parvalbumins range between 10 and 15 kDa in size and have low isoelectric points ranging from 3.5 to 5. Fish may express multiple parvalbumins (isoforms), which can be seen as monomeric, dimeric or oligomeric forms and may differ significantly in primary structure (Saptarshi et al., 2014; Sharp et al., 2014). Although different in parvalbumin primary structure, they have highly conserved tertiary structures and calcium binding residues located on paired α-helices (EF-Hand motif) (Arif, 2009). Current methods of detecting fish allergens include real-time PCR (Sun et al., 2009) and antibody based assays that detect either fish proteins or the allergens themselves (Fæste and Plassen, 2008; Lopata et al., 2005; Shibahara et al., 2013). The most investigated

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antibody against parvalbumin is the commercial monoclonal anti-frog parvalbumin, PARV-19 (Gajewski and Hsieh, 2009; Saptarshi et al., 2014). This antibody has been evaluated against fish parvalbumins from North America, Europe and Australasia and has been found to cross-react to most but not all parvalbumins (Chen et al., 2006; Lee et al., 2011; Sharp et al., 2014). PARV-19 has also been compared to other monoclonal and polyclonal antibodies generated against Atlantic cod, carp, pilchard and anchovy (Lee et al., 2011; Lopata et al., 2005). Lee et al. (2011) demonstrated recently that polyclonal anti-cod antibody seemed to be very cross-reactive, most likely due to its polyclonal nature, however this antibody still could not detect all fish species including mahi mahi, swordfish and albacore tuna.

Despite structural and functional similarities between fish parvalbumins, their amino acid sequences differ greatly, for example, two different parvalbumin isoforms are expressed in both Atlantic salmon (*Salmo salar*) and Barramundi (*Lates calcarifer*) which only share 64% and 67% amino acid sequence identity, respectively (Lindstrom et al., 1996; Sharp et al., 2014). This verifies that is not only difficult to detect interspecific parvalbumin forms using antibodies, but also intraspecific parvalbumins as they can differ greatly. Detection of fish parvalbumins is further complicated by the impact of food processing such as cooking and canning which may degrade or alter tertiary and quaternary structures of parvalbumin, inhibiting the detection by antibodies (Kuehn et al., 2010).

Parvalbumin cross-reactivity has been linked with their phylogenetic relationships (Fæste and Plassen, 2008; Saptarshi et al., 2014). In this study, four polyclonal antibodies are generated against parvalbumin from four phylogentically diverse fish species; Atlantic salmon (Samoniformes), barramundi (Perciformes), basa/catfish (Siluriformes) and pilchard (Clupeoformes). Cross-reactivity of these antibodies is analyzed among 40 different fish species across 17 orders and their ability to detect the major allergen parvalbumin evaluated across commonly consumed fish species.

2. Methods

2.1. Fish protein extraction and parvalbumin purification

Fish samples were collected from seafood retailers in Townsville and Melbourne, Australia. Samples were stored at −80 °C until further use. Details of fish species collected can be seen in Table 3.1. Raw extracts were produced by homogenizing 50 g of muscle fillet in 100 mL of phosphate buffered saline (PBS, 10 mM, pH 7.4) and extracted over 3 h with gentle tumbling at 4 °C. The crude extracts were subjected to centrifugation at 5000 × *g* for 30 min at 4 °C and filter sterilized using 0.2 μm cellulose acetate filter membrane (Sartorius, Germany). Heated extracts were produced by heating 50 g of heating white muscle at 95 °C for 15 min in PBS followed by gentle tumbling at 4 °C for 3 h. The crude heated extracts were subjected to centrifugation at 5000 × *g* for 30 min at 4 °C and filter sterilized using 0.2 μm cellulose acetate filter membrane. Protein concentrations for each extract was estimated by using the Pierce® 660 protein assay (Thermo Scientific, Rockford, USA) and extracts were aliquoted and stored at −80 °C.

Parvalbumin was purified from heated barramundi, basa, pilchard and Atlantic salmon extracts using anion exchange chromatography. The selection of these antigens is based on their phylogenetic diversity as seen in Fig. 2 as well as popular consumption. These extracts were dialysed against 25 mM Tris pH 8.0 and 1 mL of the dialysed extract loaded on to a 5 mL DEAE sepharose anion exchange column (Bio-Rad Laboratories, Hercules, USA). Fractions were eluted with a linear salt gradient of 25 mM Tris and 1 M NaCl pH 8.0. A constant flow rate was set at 1.5 mL per minute using Bio-Rad DuoFlow system. Elution profile was generated using UV214 with Bio-Rad QuadTech UV spectrophotometer. Fractions were collected for all peaks throughout the gradient and subsequently analyzed by electrophoresis and immunoblotting. Parvalbumin containing peaks were pooled, dialysed against 100 mM ammonium bicarbonate, lyophilized and stored at −20 °C until further use.

2.2. Production and purification of polyclonal anti-parvalbumin antibodies

Polyclonal antiserum was raised against the purified parvalbumins by injecting two rabbits per antigen subcutaneously with 500 μg of antigen in Freund's complete adjuvant. Further doses of 500 μg in Freund's incomplete adjuvant were given at weeks 3, 6 and 9 and final bled performed at week 10. Antiserum production was performed at the South Australian health and medical research institute (SAHMRI). The immunoglobulin G fraction of the rabbit sera was purified using Pierce Protein A Agarose resin (Thermo Fisher Scientific, USA). Both antibodies to each fish species demonstrated very similar reactivity by ELISA and therefore were pooled for subsequent experiments.

2.3. Polyclonal antibody cross-reactivity

An inhibition ELISA was performed using the four purified fish parvalbumins against the four generated antibodies. In brief, four ELISA plates were coated overnight at 4 °C with 0.1 μg per well of pure parvalbumins from Asian seabass, basa, pilchard and Atlantic salmon in coating buffer (50 mM carbonate buffer, pH 9.6). Wells were blocked with 5% skim milk in phosphate buffered saline + 0.05% Tween-20 (PBS-T) for 1 h at room temperature and subsequently washed 3 times with PBS-T. Polyclonal anti-parvalbumin antibodies were preabsorbed with inhibitors for 2 h at room temperature. Antibody concentration was 200 ng/mL and inhibitor concentrations were 0.01, 0.1, 1, 10, 50 and 100 μg/mL. This mixture was then added to the wells (100 μL) and incubated at room temp for 1 h. Wells were washed with PBS-T and 100 μL of Anti-Rabbit IgG (H + L), HRP Conjugate (Promega, USA) diluted 1:20,000 in 1% skim milk was added to each well and incubated at room temperature for 30 min. Wells where washed with PBS-T and assay was visualized using 3,3′,5,5′-tetramethylbenzidine (TMB) substrate for HRP (BD Biosciences, USA). The reaction was stopped using 1 M Hydrochloric acid and the absorbance measured at 450 nm. The percent inhibition was calculated as 100 + [O.D. 450 nm of antibody with inhibitor/O.D. 450 nm of antibody without inhibitor] + 100.

2.4. Antibody analysis of 45 fish protein extracts

Protein profiles for raw and heated fish extracts were obtained using SDS-PAGE. Fish proteins (10 μg) were diluted in 5× sample buffer containing Dithiothreitol 14 mM (DTT), heated for 5 min at 100 °C and loaded on a 15% polyacrylamide SDS gel. Precision Plus protein standards (Bio-Rad, USA) were used to estimate the molecular weights of individual proteins, using the Mini-PROTEAN® Tetra Cell (Bio-Rad, USA) system at 170 V for 70 min. Proteins were visualized by Coomassie Brilliant Blue R-250 (Bio-Rad, USA) staining.

Immunoblotting process involved the transfer of SDS-PAGE separated proteins onto PVDF membrane using semidry blot system (Bio-Rad) at 11 V for 15 min. Membranes were blocked with 5% skim milk in PBS-T for 1 h, and then incubated with the rabbit anti-fish parvalbumin polyclonal antibodies diluted in 1% skim in PBS-T at a concentration of 200 ng/mL. Membranes were washed 3× with PBS-T and incubated with anti-rabbit IgG HRP conjugate diluted 1:20,000 in 1% skim milk (Promega, USA). Membranes were washed

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