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# Parvalbumin: Characterisation of the cross-reactive major fish allergen

Thesis submitted by

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## STATEMENT ON THE CONTRIBUTION OF OTHERS

Nature of Contribution	Names, and affiliations of Co-Contributors
Intellectual support	Research Conceptualizing  Shruti Saptarshi (only chapter 2) and Assoc/Prof. Andreas Lopata <sup>1</sup>  Data Analysis  Shruti Saptarshi (only chapter 2) and Assoc/Prof. Andreas Lopata  Editorial Assistance  Shruti Saptarshi (only chapter 2) and Assoc/Prof. Andreas Lopata  Kasturi Reddy-Lopata
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Data Collection	Research Assistance  Shruti Saptarshi (only chapter 2) and Sandip Kamath (only chapter 4)

## **DECLARATION ON ETHICS**

This research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council (NHMRC) National Statement on Ethical Conduct in Human Research, 2007. The proposed research study received human research ethics approval from the JCU Human Research Ethics Committee Approval Number H4313

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## PUBLICATIONS ARISING FROM THESIS

At the time of this thesis submission, four journal manuscripts describing the research findings from Chapters 1, 2, 3 and 4 were published as well as one book chapter (Chapter 4). A manuscript for chapter 5 is in preparation.

- **Sharp M. F.** and Lopata A. L., 2013. Fish Allergy: In Review. Clin. Rev. Allergy Immunol. (Chapter 1)
- **Sharp M. F.** and Lopata A. L., 2013. Chapter 12: Muscle Proteins in Asian Seabass – Parvalbumin’s Role as a Physiological Protein and Fish Allergen. In Jerry D. R. (Ed.), CRC Press, pp. 293-307. (Chapter 4)
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- **Sharp M. F.**, Kamath S. D., Koeberl M., Jerry D. R., O’Hehir R. E., Campbell D. E. and Lopata A. L., 2014. Differential IgE binding to isoallergens from Asian seabass (*Lates calcarifer*) in children and adults. Mol. Immunol. 62, 77-85. (Chapter 4)
- **Sharp M. F.**, Stephen J. N., Kraft L., Weiss T., Kamath S. D., Lopata A. L., 2015. Immunological cross-reactivity between four distant parvalbumins— Impact on allergen detection and diagnostics. Mol Immunol. 63(2), 437-448. (Chapter 3)

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## LIST OF ABBREVIATIONS

μg	Microgram
μL	Microlitre
μm	Micrometre
aa	Amino acid
ab	Antibody
ag	Antigen
Amp	Ampicillin
BSA	Bovine serum albumin
°C	Degrees centigrade
C-terminal	Carboxyl terminus domain of an amino acid sequence/protein
Ca <sup>2+</sup>	Calcium ions
Da	Dalton
dH <sub>2</sub> O	Distilled water
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
g	Gram
h	Hour
H <sub>2</sub> O	Water
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IMAC	Immobilised metal affinity chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	One thousand Daltons
L	Litre
M	Molarity
mAb	Monoclonal antibody
Mg <sup>2+</sup>	Magnesium ion
Mg	Milligram
mM	Millimolar
MW	Molecular weight
N-terminal	Amino terminal domain of an amino acid sequence/protein

NaCl	Sodium chloride
NaOH	Sodium hydroxide
nm	Nanometre
OD	Optical density
pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	PBS with 0.05% (v/v) Tween 20
PCR	Polymerase chain reaction
pH	Negative algorithm of hydrogen ion concentration
SDS	Sodium dodecyl sulphate
TBS	Tris-buffered saline
TBS-T	TBS with 0.05% (v/v) Tween 20
TEMED	N,N,N',N'-tetramethylethylenediamine
TH2	Effector T-cells involved in the initiation of humoral immune responses
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
V	Voltage
v/v	Volume per volume
w/v	Weight per volume
WHO	World Health Organisation

## **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 and fish allergy diagnostics are however limited due to the great diversity of fish species, despite fish allergy and its major allergen parvalbumin being well documented. Currently the best treatment strategy for fish allergy is avoidance. The most commonly studied fish are cod, carp and Atlantic salmon as they 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.

The major fish allergen is parvalbumin, a small calcium binding protein found in the muscles of vertebrates which are the biggest group of animal derived food allergens, part of the EF Hand domain protein family. Fish can express multiple parvalbumin isoforms which may differ greatly in amino acid sequence that further complicates the diagnosis of fish allergy and the detection of these allergens. In this PhD thesis, fish allergen detection methods will be evaluated in addition to the characterisation of novel fish allergens from the Australasian region to improve current diagnostic and detection methods and future development of immunotherapies for fish allergy sufferers.

Firstly, the evaluation of the cross-reactivity of parvalbumin from a variety of bony and cartilaginous fish, from the Asia-Pacific region was performed with the monoclonal anti-parvalbumin antibody PARV-19. The presence of monomeric and oligomeric parvalbumin was demonstrated in all fish analysed, except for gummy shark, which is a cartilaginous fish. Heat processing of this allergen greatly affected its antibody reactivity. While heating caused a reduction in antibody reactivity to multimeric forms of parvalbumins for most bony fish, a complete loss of reactivity was observed for all cartilaginous fish except for the elephant shark. Molecular analysis demonstrated that

the observed cross-reactivity between parvalbumin from diverse fish species is due to the molecular phylogenetic association of this major fish allergen.

A more comprehensive phylogenetic analysis was performed with all currently known parvalbumin sequences to determine possible candidate antigens for new cross-reactive antibodies. 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, including raw, heated and canned fish. Anti-barramundi parvalbumin proved to be the most cross-reactive antibody followed by anti-pilchard and anti-basa antibody. In contrast the anti-salmon antibody was very specific and only reacted to salmonidae and very few other fish. All analysed fish species, except mahi mahi, swordfish, yellowfin tuna and all five canned fish had parvalbumin detected in raw extracts. However antibody reactivity to many species was heat liable or susceptible to denaturation, demonstrating that these parvalbumins have most likely conformational epitopes, which lose antibody reactivity after heat treatment.

Frequent allergic reactions to ingested barramundi among adults and children initiated further characterisation of allergenic proteins from this frequently ingested barramundi. Serum samples from 17 fish allergic adults and children from Australia were analysed by ELISA and immunoblotting for IgE antibody reactivity to raw and heated barramundi proteins. The molecular analysis of the identified allergens included mass spectrometric analysis, genetic sequencing and generation of recombinant allergens. Two novel parvalbumin isoforms (isoallergens) of the  $\beta$ -type were identified as the only allergens in barramundi and subsequently designated as Lat c 1.0101 and Lat c 1.0201 after registration with the International Union of Immunological Societies (IUIS). These two isoallergens are differentially expressed in barramundi tissue but bind IgE from the

same patients. However these heat stable parvalbumin allergens from barramundi seem to have differential IgE binding capacity between adults and paediatric patients. IgE and IgG<sub>4</sub> epitopic regions of Lat c 1.0101 were elucidated by using 7 overlapping peptides, which were analysed by immunoarray with serum of fish allergic patients. IgE and IgG<sub>4</sub> binding epitopes were compared to assess to the possibility of designing future novel immunotherapeutics for fish allergy.

In conclusion this dissertation has 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. These cross-reactivity studies also highlight the limitations of using antibodies for parvalbumin detection in processed fish. The in depth molecular and immunological characterisation of novel isoallergens from barramundi has widened the knowledge of fish allergy in the Asian Pacific region and improves current diagnostic approaches and the information gained from elucidating antibody epitopes can be used in the future development of specific immunotherapies for fish allergy sufferers.

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# CHAPTER 1

## Introduction to fish allergy

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<b>Details of publication on which this chapter is based</b>	<b>Nature and extent of the intellectual input of each author, including the candidate</b>
Sharp M. F. and Lopata A. L., 2014. Fish allergy: in review. Clin. Rev. Allergy Immunol. 46, 258-71.	Sharp wrote the first draft of the paper which was revised with editorial input from Lopata. Sharp developed the figures and tables.

## **1.1 General introduction**

Seafood plays an important role in human nutrition and health, but can provoke serious IgE-antibody mediated adverse reactions in susceptible individuals. A marked increase in allergic diseases is occurring in most major industrialized countries. The World Allergy Organization reports that in 2008 20-30% of the world population was affected by allergy of some type. The seafood allergy and anaphylaxis epidemic is particularly serious. Seafood allergy, including shellfish and fish, is typically life-long affecting up to 5% of all children and 2% of all adults. While shellfish and fish allergy are often discussed concurrently, likely due to culinary habits, the allergenic proteins causing allergic sensitization are entirely different and shellfish allergens are not discussed in this study. Prevalence rates specifically to fish vary considerably between regions and among children and adults. This chapter compares the different prevalence rates of fish allergy and explores the possible underlying molecular and immunological causes, resulting in better diagnostic approaches for improved management of this life-long food allergy.

## **1.2 Demographics and prevalence**

### ***1.2.1 Children***

Fish allergy has a significant adverse effect on anxiety and stress in the families of affected children. Parental recall of dietary advice is variable and many tend to impose more stringent dietary avoidance than that recommended. Despite this, subsequent accidental reactions are common and demonstrated in over 20% of diagnosed children [3]. Thus, the avoidance of fish in children may be more difficult than often presumed.

In Europe most of the populations based prevalence studies come from Spain, Portugal and the Scandinavian countries. In Norway, adverse food reactions were reported in a population based study among 3623 children and nearly 3% of all reactions were attributed to fish by the age of 2 years [4]. Thus fish allergy in Norway is almost as common as allergy to egg among children, while fish allergy is more common in children from Finland [5]. In Spain, a study among 355 children with diagnosed IgE mediated food allergy reported that fish allergy began predominantly before the second year of life [6].

In the USA allergy to seafood was reported by about 5.9% of 14,948 individuals, with about 0.4% accounting for fish and 0.2% for both, shellfish and fish allergy [7]. The major species reported causing allergic reactions are salmon, tuna, catfish and cod followed by flounder, halibut, trout and bass. The majority of allergic subjects reacted to multiple fish species (67%).

In Australia a retrospective study in a tertiary clinic among 2999 children with food allergy demonstrated the prevalence of fish allergy (5.6%), with white fish, tuna and salmon being the most implicated fish species [8].

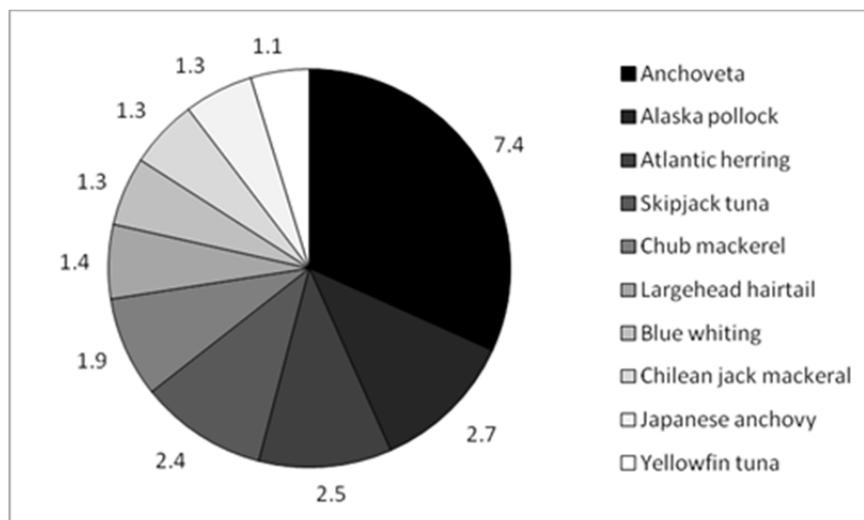
Fish allergy is common, not only in the Western civilization, but also in Asian countries where allergic reactions to fish are significant among children and adults [9] (Table 1.1). A study from Singapore of 227 children with food hypersensitivity confirmed that fish are significant sensitizers in approximately 13% of children. Interestingly, the first intake of fish seems to be very early in life in the Asian diet, with an average age of exposure as low as 7 months. A subsequent prevalence study in the Southeast Asia region used a survey previously developed by Sicherer *et al.* [7] to compare the occurrence of fish allergy among school children. The population-based study among

11,434 Filipino, 6,498 Singaporean and 2,034 Thai established that 2.29%, 0.26% and 0.29% of the children suffered from allergic sensitization to fish, respectively [10]. While the prevalence of fish allergy differed among these three Asian countries, females were overall more likely to be sensitized compared to males for all children combined. Nevertheless, most allergies appeared to be of mild nature as less than one third actually sought medical consultation. In most cases allergic symptoms occurred on first exposure and usually in later childhood. The majority of sensitized Filipino (>50%) were 11-16 years at the time of their first reaction. The most frequently reported fish to cause allergic reactions were anchovy and mackerel scad. Over one-third of sensitized children reported multiple-fish allergy, most probably due to the major cross-reactive fish allergen parvalbumin [11-13]. However, the majority of children demonstrated mono-sensitivity to one or the other fish species. Interestingly anchovy and mackerel scad are the 1<sup>st</sup> and 5<sup>th</sup> most common marine fish captured worldwide (Figure 1.1), highlighting that other populations with high consumption of these species might be of increased risk of developing fish allergy. There are considerable country specific differences, which give insights into the impact of cultural behaviors on developing a specific food allergy. While anchovies are used in all three countries to prepare fish sauce, in the Philippines these fish are prepared by drying and salting. This increased immunological reactivity of heated food allergens has previously been described for peanut [14] and also for the fish pilchard [15]. The molecular impact of heating fish allergens is discussed further below. Importantly, children with fish allergy, similar to peanut allergy, will predominately remain clinically reactive throughout their life. A follow-up study by Priftis *et al.* [16] reported that 65.5% of fish-sensitized children maintained their sensitization into school age and are at increased risk for wheezing illness and hyperactive airways.

**Table 1.1** Epidemiological studies of fish allergy from different continents.

Continent	Age	Study subjects	Sample number	Self report %	IgE sensitization %	Fish studied	Ref.
<b>Asia</b>							
China	<1	population based	477		0.21	Finfish, conger, whelk, tuna, mackerel, fish roe, anchovy.	[10, 17-22]
Hong Kong	2-7	population based	3,677	0.32			
Philippines	14-16	population based	11,434	2.29			
Singapore	14-16	population based	6,498	0.26			
Thailand	14-16	population based	2,034	0.29			
<b>North America</b>							
Canada	Children Adult All ages	population based	9,667			Salmon, catfish, tuna, cod, flounder, halibut, bass, trout	[7, 23]
USA	0-17	population based	3,607				
	18-67 all ages		8,816 14,948				
<b>Europe</b>							
Denmark	0.1-22	Birth-cohort plus relatives	936		0.52	Fish, codfish	[4, 22, 24-26]
	22-60		898		0.67		
	all ages		1,834		0.60		
France	2-14	population based	2,716	0.70			
Norway	0-2	birth cohort	2,803	3.0			
Sweden	0-4	birth cohort	2,614		0.69		
Turkey	6-9	population based	2,739	0.33	0.18		
<b>Africa</b>							
South Africa	25-46	Process workers	594		6.00	Hake, yellow tail, Salmon, anisakis in fish, canned fish and fish meal, pilchard, anchovy, snoek	[27-29]
<b>Australia</b>							
Australia	Children	allergy patients	clinic	2999	0.56	Barramundi, basa, bream, cod, tuna, salmon, white fish	[3, 8]

It is to note that prevalence data generated using a survey of self-reported fish allergy are usually higher as when confirmed by specific fish IgE tests. The diagnostic problems and improved approaches are discussed below under ‘diagnosis and management of fish allergy’.



**Figure 1.1** The top ten marine fish captured as of 2008, displayed in million tonnes [30]

### 1.2.2 Adults

A recent study by Vierk et al. [31] provided population-based prevalence data for American adults from a Food Safety Survey of over 4,400 individuals. The prevalence of fish allergy was found to be 0.7% and 0.6% among respondents with self-reported fish allergy and self-reported doctor diagnosed fish allergy, respectively. Overall, there was no difference in the prevalence of fish allergy between age or race/ethnic groups. However, significantly more black than white respondents reported a fish allergy. A similar observation was made by Sicherer *et al.* [7] in a telephone survey of 14,948 individuals with a prevalence of fish allergy of approximately 0.4%. The reasons for these unexpected observations are not apparent and require further studies among this ethnic group. A recent comparable survey in Canada among 9,667 individuals demonstrated a similar prevalence of fish allergy of 0.51% [23].

In Asia fish allergy seems to be also high as documented by a study from Singapore among 74 adults with IgE mediated food allergy, where fish allergy was 4.1 % however less common than crustacean allergy (33.8%) [18].

A study in South Africa determined from a questionnaire of 105 subjects with convincing history of seafood allergy found that the four most common bony fish species causing IgE mediated allergic reactions were hake (24.8%), yellowtail (21.9%), salmon (15.2%) and mackerel (15.2%) [32]. Clinical symptoms reported included gastrointestinal, respiratory and dermatological related allergic symptoms. Subsequently the allergenicity of five fish species was investigated among ten fish-allergic consumers [11]. Pilchard displayed the strongest IgE reactivity, followed by anchovy, snoek, hake and yellowtail. Interestingly most of these IgE reactivities increased after heat treatment [11]. These findings confirmed previous observations on the heat stability and activity of fish allergens[33].

Among adults, exposure to high concentrations of fish allergens and in particular heat-processed fish is observed in various working environments. Occupational sensitization to fish was first reported in 1937 by De Besche in a fisherman who developed allergic symptoms when handling codfish [34]. Since then various other fish species have been reported to cause occupational allergy and asthma including trout, salmon, pilchard, anchovy, plaice, hake, tuna, haddock, cod and pollock [35]. Various studies from South Africa and Norway report the prevalence of occupational asthma between 7-8% [28, 35-37] and protein contact dermatitis from 3-11% [35]. The Food and Agriculture Organization reports that over 45 million people are directly involved in fishery and aquaculture production worldwide (Food and Agriculture Organisation, 2010), making work-related reactions to fish allergens in various contexts an important consideration.

### 1.3 Clinical features, exposure routes and mechanisms of fish allergy

The main clinical manifestations of allergic reactions to fish include vomiting and diarrhea whilst the most extreme form of reaction is life-threatening anaphylactic shock (Table 1.2). Patients with fish allergy can however also react to aerosolized proteins generated by cooking or processing of fish resulting in dyspnea, wheezing, tightness of the throat, urticaria, edema and light-headedness [6, 28, 38-42]. Asthma appears to be a risk factor for fatal anaphylaxis to food [43], and conversely, food allergy is a risk factor for life-threatening asthma [16, 44].

The major route of sensitization to fish is however through the gastrointestinal tract. This mechanism was confirmed for codfish allergens in animal [45] and human studies [46]. The use of antacid medication that increased stomach pH can result in incomplete digestion and thereby increase exposure to and uptake of allergenic fish proteins or peptides. Challenge experiments on patients, without clinical sensitivity, demonstrated absorption of biologically active fish allergens within 10 min of ingestion. Fish digested at pH 3.0, as compared to normal stomach pH 2.0, revealed comparable reactivity patterns as undigested extracts. However, the nature of the allergen or allergen fragment was not identified in this study. These experiments confirm not only the very high biochemical stability of fish allergens, but also their rapid uptake through the gastrointestinal tract. If patients require antacid medication, this rapid uptake of fish allergens could be of concern and should be discussed with the patient.

**Table 1.2** Different routes and environments of exposures to fish species and allergens

Route of exposure	Allergen exposure	Domestic	Occupational	Clinical symptoms	Fish Species implicated	Ref.
Ingestion	Ingestion of - raw - cooked - processed fish	✓ ✓ ✓		- angiodema - rhinitis - oral allergy syndrome - urticaria - anaphylaxis - nausea - gastrointestinal	sea bream, eel, pilchard, salmon, cod	[47-49]
Skin	Dermal contact from - unprotected handling	✓	✓	- urticaria - angiodema	cod, herring, sardine, swordfish	[50-52]
	- preparation	✓	✓			
Inhalation	Inhalation of  wet aerosols from - fish heading - degutting - boiling			- asthma  - rhinitis - skin rash	plaice,  salmon, hake, pilchard, anchovy, tuna, trout, sole, pomfret, yellowfin, salmon	[6, 29, 35, 53]

In addition to uptake via the gastrointestinal tract, reactions to inhaled proteins are an important aspect of fish allergy in both the domestic and occupational environment. In domestic settings, a Spanish study reported 11% of children from a group of 197 allergic children experienced repeated allergic reactions upon incidental inhalation of fish odors or vapors, even while on strict fish avoidance. In most cases, these episodes occurred at home when other people were eating fish [40]. Similarly, a South African study of 105 individuals with self-reported seafood allergy, reported 30% of individuals with allergic symptoms after handling or inhaling seafood in the domestic home environment [54].

In the workplace environment, occupational allergy and asthma is reported among workers processing a variety of fish species including trout, salmon, pilchard, anchovy,

plaice, hake, tuna, haddock, cod and pollock [35, 55, 56]. Symptoms manifest mainly as upper and lower airway respiratory symptoms and dermatitis, whereas anaphylaxis is rarely seen with this type of exposure. Various studies from South Africa and Norway report the prevalence of occupational asthma between 7-36% [28, 35-37] and for occupational protein contact dermatitis, from 3-11% [35, 57]. Therefore work-related reactions to fish allergens in various contexts are an important consideration particularly as it is estimated that up to 15% of the asthmatic population in the USA and Europe have occupational asthma [58, 59]. Atopy, smoking and level of exposure are significant risk factors for allergic sensitization and the development of occupational asthma. Fish antigen exposure levels of more than 30 ng/m<sup>3</sup> have shown significant correlation with sensitization and work-related asthma symptoms [28]. A similar study quantified raw fish allergens from an open-air fish market and detected allergen concentrations ranging from 2-25 ng/m<sup>3</sup>, very similar to the levels identified in the occupational setting causing allergic sensitization [60]. From data on allergen exposure available so far it can be expected that extended exposure to aerosolized fish allergens can generate sensitization also in the domestic environment and probably also in children. In general it is accepted that breaching of oral tolerance leads to food allergy, however why adults develop *de novo* food allergy is as yet unknown, and inhalation of fish allergens might be a relevant route of sensitization to consider [61, 62].

A number of fish allergens have been purified and characterised (Table 1.3) for ingestion related sensitization. In contrast the fish proteins in aerosol responsible for allergic sensitization have not yet been fully described [55]. IgE reactive proteins in fresh, frozen and canned pilchard range from 12-250 kDa. Some of these proteins are identified as monomeric (12 kDa) and oligomeric (36, 48 and 60kDa) forms of parvalbumin, the major fish allergen in ingestion related allergy. Other fish allergens of

importance through the inhalational route might include glyceraldehyde-3-phosphate dehydrogenase, which was recently identified in an exposed worker and in a murine model of inhalational fish allergy [15]. In addition to allergens deriving directly from fish tissue, other contaminants such as the fish parasite *Anisakis* have been implicated in occupational sensitization [12, 28, 63, 64]. The major allergen seems to be tropomyosin, which demonstrates cross-reactivity to other invertebrates but not to fish [65, 66]. Future studies need to focus on the molecular characterisation of the aerosolized fish allergen causing allergic sensitization and symptoms in the occupational and domestic environment.

It is well recognized that food allergens are in general very heat stable. In addition it seems that food processing and in particular heating can even increase allergenicity as demonstrated for peanuts [67, 68]. Also the major fish allergen parvalbumin seems to increase its allergenicity as demonstrated in a recent study by Beale et al [11], where several IgE binding allergen variants of the major fish allergen parvalbumin were identified in different fish species. This increased IgE reactivity seems also to be related to stronger allergenicity of this allergen as shown in the subsequent development of the first murine model for inhaled fish allergens [15]. Heat-treated pilchard allergens significantly increased Th2 cytokines and specific IgE responses as compared to untreated allergens. In contrast, raw pilchard allergens initiated a specific IgE response to a novel fish allergen, glyceraldehyde-3-phosphate dehydrogenase. Interestingly sensitized fish processing workers also recognized this IgE reactive allergen. This murine model of inhalational fish allergy demonstrated for the first time that inhalation exposure to fish allergens can generate a strong IgE mediated allergic sensitization to parvalbumin.

This deep insight into the mechanism of inhaled fish allergy and the enhanced response to heat treated parvalbumin is supported by recent studies on human cells. Enhanced internalization of glycated allergens, such as ovalbumin, was recently studied in human dendritic cells, which led to increased CD4<sup>+</sup> T-cell immunogenicity of this protein [69, 70]. Heating of proteins in the presence of sugars such as glucose, result in the so-called ‘advanced glycation endproducts’ (AGEs), through the Maillard reaction. These AGEs seem to stimulate the uptake of allergens by antigen-presenting cells through the binding to scavenger receptors. In summary these studies give strong indications that heated fish allergens are more allergenic than their un-heated counterparts and this could be of considerable importance for better diagnostics but also the development of novel therapeutics for this type of food allergy.

#### **1.4 Classification of fish**

Fish species can be divided into two main groups; the bony fish and cartilaginous fish. Most edible fish belong to the bony fish (Osteichthyes), whereas sharks and rays are cartilaginous and belong to a different class; Chondrichthyes. Most studies on fish allergens have focused on cod, carp and salmon [71-77]. Although there are more than 32,400 different species of fish described [78], consumption depends heavily on regional availability and can include under investigated fish such as basa, barramundi and elephant shark.

The class of bony fish can be further divided into 45 orders. The most commonly consumed bony fish belong to the orders Clupeiformes (herrings and sardines), Salmoniformes (salmons and trouts), Cypriniformes (carps), Gadiformes (cods, hakes and whiting), Siluriformes (catfish) and Perciformes (perches, mackerels and tunas). The later order Perciformes itself comprises of 156 diverse families and is the largest order of vertebrates with over 9,300 species [78]. The top marine fish species captured

include representatives from most of these orders (Figure 1.1) [30]. However, less than 0.5% of all known fish species have been analysed for their allergens on molecular level and demonstrates unexpected large diversities as detailed below.

## 1.5 Fish Allergens

### 1.5.1 *The major fish allergen parvalbumin*

The Baltic cod was the first food source in the early 1970s to be ever analysed for the molecular nature of the offending allergen. The major allergen identified was subsequently named Gad c 1, a parvalbumin protein that regulates calcium switching in muscular skeletal cells [79-81]. Parvalbumin represents the major clinical cross-reactive fish allergen with 90% of fish allergic patients reacting to this protein [72, 82, 83]. Furthermore, this allergen forms the biggest group of animal derived food allergens, the EF Hand domain family (<http://www.meduniwien.ac.at/allergens/allfam/>), with over 63 allergens currently reported.

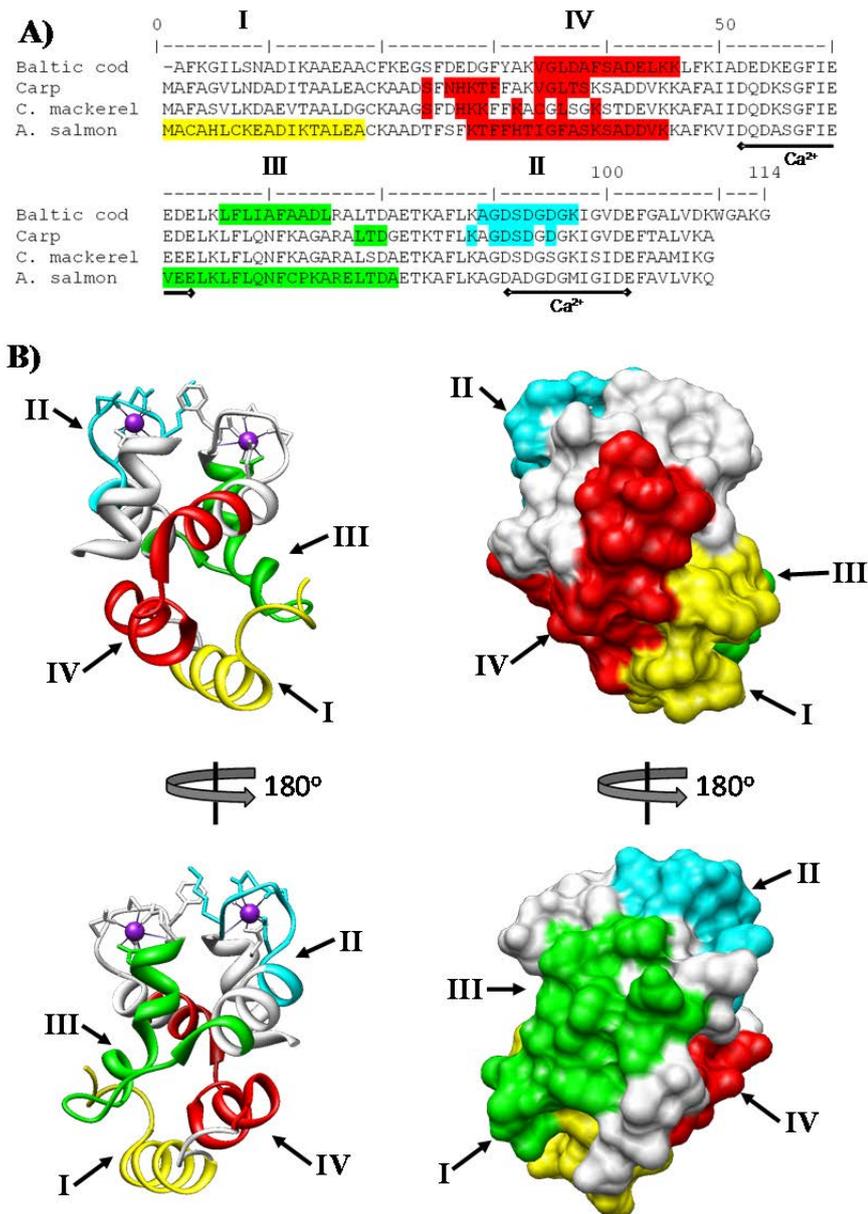
Parvalbumin is not only present in lower vertebrates such as fish and frog, where it can be an allergen [84, 85], but is also found in higher vertebrates including humans, demonstrating that parvalbumin plays a vital role in basic vertebrate calcium physiology [86]. Parvalbumins can be found as one of two distinct isoform lineages;  $\alpha$  and  $\beta$ . Fish often contain both  $\alpha$  and  $\beta$  parvalbumin, however the majority of allergenic parvalbumins reported belong to the  $\beta$  lineage (Table 1.1). Furthermore, most fish express 2 or more different  $\beta$  parvalbumin isoforms, which are subsequently named  $\beta 1$ ,  $\beta 2$  and so forth [71]. These  $\beta$  isoforms can differ significantly in amino acid sequence as demonstrated for Atlantic salmon (*Salmo salar*) where their  $\beta 1$  and  $\beta 2$  isoforms have only 64% identity. The differences in  $\beta$  parvalbumin isoforms in one species can result in a fish allergic patient reacting to one isoform more than another, which adds to the

complexity of diagnosing fish allergy and detecting allergenic parvalbumin [76]. In addition dimeric as well as polymeric forms of parvalbumin have also been reported to bind IgE antibody and these allergens form higher molecular weight aggregates of approximately 24 kDa and 48 kDa [87, 88]. The allergenicity of parvalbumin has been studied in a number of fish species and as of 2012 the allergome database ([www.allergome.org](http://www.allergome.org)) has 218 allergenic isoforms of fish parvalbumin listed, while only 27 of these isoforms are actually registered with the World Health Organization (WHO) or International Union of Immunological Societies (IUIS). This registration substantiates the prevalence and specific molecular nature of this allergen according to specific guidelines by WHO and IUIS and has only been achieved for just over 10% of all current studies. More detailed molecular studies on fish allergens will assist in the development of better diagnostics and potential immunotherapeutics.

### ***1.5.2 IgE Epitopes and Cross-reactivity***

Thus far there have been four attempts to identify the IgE epitopes of allergenic parvalbumins (Figure 1.2A). Parvalbumin from Baltic cod (Gad c 1), carp (Cyp c 1), chub mackerel (Sco j 1) and Atlantic salmon (Sal s 1) were analysed for their specific IgE epitopes. Allergic patient IgE was used in various techniques including phage display library, overlapping immunogenic peptides and tryptic digests of parvalbumin to map out these epitopes [73, 75, 76, 89]. These four fish parvalbumin display both linear and conformational epitopes, however do not share identical residues. This may be due to the polyclonal nature of IgE antibodies from different patients as well as the varying techniques utilized to identify these epitopes. In summary, the four parvalbumin allergens currently analysed on molecular level demonstrate very different IgE binding epitopes [12, 75, 76, 82]. While the secondary and tertiary structures of parvalbumins are highly conserved among fish, their primary structure, or amino acid sequence,

differs substantially. Epitope alignment of these four fish parvalbumins, using two different computer models, allows the identification of highly antigenic (region IV) in contrast to species-specific proteins regions (region I). Indeed the later can be confirmed by reports of mono-sensitivity to salmonids [90, 91]. This phenomenon could account for fish allergy sufferers having only about a 50% chance of being cross-reactive to another fish species [92] and is significantly lower than the rate of shellfish cross-reactivity which is up to 75% [92, 93]. Further studies need to confirm that the identified protein region IV is responsible for sensitivity to multiple fish species, and would be of great importance for improved diagnostics. The most recent parvalbumin epitope study was of Atlantic cod parvalbumin (Gad m 1), the study concluded that IgE epitopes to Gad m1 are highly variable and may be patient specific [94].



**Figure 1.2** Amino acid sequence alignment of Baltic cod (Gad c 1.01 UniProtKB Accession number: P02622), carp (Cyp c 1.01 UniProtKB Accession number: E0WD92), chub mackerel (Sco j 1.01 UniProtKB Accession number: P59747) and Atlantic salmon (Sal S 1.01 UniProtKB Accession number: B5DH15) with their IgE binding epitopes shaded in grey. Epitopes have been split into four regions colored yellow, blue, green and red, and labeled I, II, III and IV, respectively. The two calcium-binding sites, of this muscle protein are underlined. B) Ribbon and space fill carp parvalbumin models (PDB ID: 4cpv) with the four epitope regions colored and labeled according to ‘A’. Bound calcium ions are colored in purple.

### 1.5.3 Other Fish Allergens

In addition to parvalbumin, other fish allergens have been characterised such as the hormone vitellogenin from Beluga caviar [95, 96] and collagen and gelatin isolated from skin [97, 98] and muscle tissues of fish [99]. The allergenicity of isinglass derived from fish swim bladder used for filtering beer has also been investigated, demonstrating that the gelatin content of isinglass to be harmless to fish allergic subjects. However, small amount of allergenic parvalbumins were detected in isinglass at levels up to 414.7 mg/kg which might be of importance for very sensitive patients [100]. In addition, enzymes such  $\beta$ -enolase and aldolase from cod, Atlantic salmon and tuna have been submitted to WHO and IUIS as fish allergens. It is to note that there seems to be no cross-reactive allergens between fish and shellfish [12, 93]. In addition to these allergens derived from fish themselves, contaminants such as the parasite *Anisakis* can cause allergic reactions [12, 101]. Exposure to proteins from live or dead *Anisakis* can cause allergic reactions. The thirteen allergens characterised in *Anisakis* include tropomyosin, as well as paramyosin and protease inhibitors. Allergens from *Anisakis* appear not to be destroyed by heat or cooking and so allergic reactions may be triggered by dead parasites in fish that have been well cooked. A recent study demonstrated that these parasites can also cause considerable allergic sensitization among fish processing workers [64, 102]. While the identified allergens seen not to cross-react to fish allergens, possible allergic reactions to ingested fish could be directed to the contaminating parasite *Anisakis* and be falsely diagnosed as fish allergy [12].

**Table 1.3** Selection of allergenic proteins characterised in 24 fish species representing 8 different orders and their biochemical characteristics (PV = parvalbumin)

Common Name	Scientific Name	Order	Allergen identified Isoform	MW (kDa)	Ref.
Atlantic mackerel	<i>Scomber Scombrus</i>	Perciformes	PV $\beta$	11.5	[103]
Big eye tuna	<i>Thunnus obesus</i>	Perciformes	Collagen	120-240	[97]
Chub mackerel	<i>Scomber japonicus</i>	Perciformes	PV $\beta$	11.5	[103]
Japanese jack mackerel	<i>Trachurus Japonicus</i>	Perciformes	PV $\beta$	11.3	[104]
Skipjack tuna	<i>Katsuwonus pelamis</i>	Perciformes	PV $\beta$	11.4	[89]
Swordfish	<i>Xiphias gladius</i>	Perciformes	PV $\beta$	11.5	[105]
Yellowfin tuna	<i>Thunnus albacares</i>	Perciformes	PV $\beta$ Enolase $\beta$ Aldolase	11.5 47.1	[13]
Alaska pollock	<i>Theragra charlcoogramma</i>	Gadiformes	PV $\beta$	11.5	[74]
Atlantic cod	<i>Gadus morhua</i>	Gadiformes	PV $\beta$ 1 PV $\beta$ 2 PV $\beta$ 3 PV $\beta$ 4 Enolase $\beta$ Aldolase	11.5 11.5 11.5 11.5	[71] [71] [106] [106]
Baltic cod	<i>Gadus callarias</i>	Gadiformes	PV $\beta$	12.1	[73]
European hake	<i>Merluccius merluccius</i>	Gadiformes	PV $\beta$	11.3	[11, 107]
Atlantic herring	<i>Clupea harengus</i>	Clupeiformes	PV $\beta$ 1 PV $\beta$ 2 PV $\beta$ 3	11.7 11.7 11.8	[108] [108] [108]
Japanese sardine	<i>Sardinops melanostictus</i>	Clupeiformes	PV $\beta$	11.9	[33, 89]
Pacific pilchard	<i>Sardinops sagax</i>	Clupeiformes	PV $\beta$	11.9	[11]
Atlantic salmon	<i>Salmo salar</i>	Salmoniformes	PV $\beta$ 1 Enolase $\beta$ Aldolase	11.9 47.3	[77]
Rainbow trout	<i>Oncorhynchus mykiss</i>	Salmoniformes	PV $\beta$ 1 PV $\beta$ 1 Vitellogenin	11.8 11.3 -	[109]
Japanese flounder	<i>Paralichthys olivaceus</i>	Pleuronectiformes	PV $\beta$	11.6	[89, 104]
Whiff	<i>Lepidorhombus whiffiagonis</i>	Pleuronectiformes	PV $\beta$	11.7	[110]

Beluga	<i>Huso huso</i>	Acipenseriformes	Vitellogenin	118	[96]
Carp	<i>Cyprinus carpio</i>	Cypriniformes	PV $\beta$	11.5	[72, 82, 111]
Japanese eel	<i>Anguilla Japonicus</i>	Anguilliformes	PV $\beta$	11.7	[89]
Rose fish	<i>Sebastes marinus</i>	Scorpaeniformes	PV $\beta$ 1 PV $\beta$ 2	11.4 11.7	[112]

## 1.6 Non-IgE mediated reactions to fish

Adverse reactions to fish can also be mediated by non-immunological reactions in contrast to true food allergy [113, 114]. These reactions can result from exposure to fish itself or various non-fish components in the product. Non-immunological reactions to fish can be triggered by contaminants such as bacteria, viruses, marine toxins, parasites and biogenic amines. The latter is mostly found in ‘spoiled’ fish (scombroid poisoning) [115, 116]. Marine biotoxins, generated by algae, can be detected in fish [12, 93, 117] and also in filter feeders such as mussels and oysters. Eating fish that has been contaminated by algae-derived toxins in particular causes ciguatera poisoning. Ciguatera toxins are only present in fish, particularly large reef fish in the tropics. These toxins interfere with the function of nerve endings with symptoms occurring within 2 to 3 hours of eating contaminated fish, and consist of tingling of the lips, tongue and throat and sometimes change in blood pressure and heart rhythm. Most people recover within a few days or weeks with supportive treatment.

Contamination of fish with parasites can also cause severe adverse reactions as in the case of *Anisakis simplex*, a parasitic nematode that is found in most parts of the world [118, 119]. *Anisakis* can cause two major problems in humans: Infections with live *Anisakis* (anisakiasis) can result from eating raw, pickled or undercooked fish. Infection may cause nausea, vomiting, stomach pain, and sometimes appendicitis, bowel blockage or bleeding.

Finally ingredients, such as spices and monosodium glutamate, added during processing and canning of fish can also cause adverse reactions. Importantly all of these substances can trigger clinical symptoms, which are similar to true allergic reactions including respiratory symptoms, urticaria and headache. Due to this similarity in clinical reactions of affected consumer and worker, it is of critical importance to differentiate adverse reactions from true fish allergy and comprehend the underlying mechanisms of allergic reactions and molecular nature of these allergens. Adverse reactions to fish are however too manifold to be discussed in detail in this review and referred to other articles [12, 120, 121].

### 1.7 Diagnosis and management of fish allergy

*In vitro* diagnostic methods of fish allergy include *in vivo* skin prick test (SPT) as well as *in vitro* quantification of specific IgE antibodies using assays such as the ImmunoCAP (Thermo Fisher) and immunoblotting to identify the specific IgE binding allergens. One example of commercial *in vitro* assays to quantify specific IgE to allergens is the ImmunoCAP system, which offers currently 27 different fish species and two recombinant fish allergens from carp and cod. However, a direct comparison of all these fish species for their IgE reactivity has not been conducted. While these types of assays contain the majority of possible allergens found in the individual fish species, possible variations of parvalbumin concentrations cannot be taken into account. These parvalbumin variations have recently being analysed in seven fish species by Kuehn et al [108] and demonstrated over ten-fold lower concentrations of the major fish allergen in tuna compared to herring, which could impact on the sensitivity of various diagnostic tests.

It is well accepted that the level of serum IgE antibodies is directly related to the severity of allergic reactions and previous studies by Sampson et al [122] tried to

predict clinical reactivity based on specific IgE levels. For cod-fish, a diagnostic level of IgE that can predict clinical reactivity in a US population, with >95% certainty, was identified as 20 KUA/l. It is however questionable if this seemingly high value can be extrapolated to other fish species and other populations as IgE values as low as 1 kU/l could be determined in patients with anaphylactic reactions to pilchard and anchovy [11].

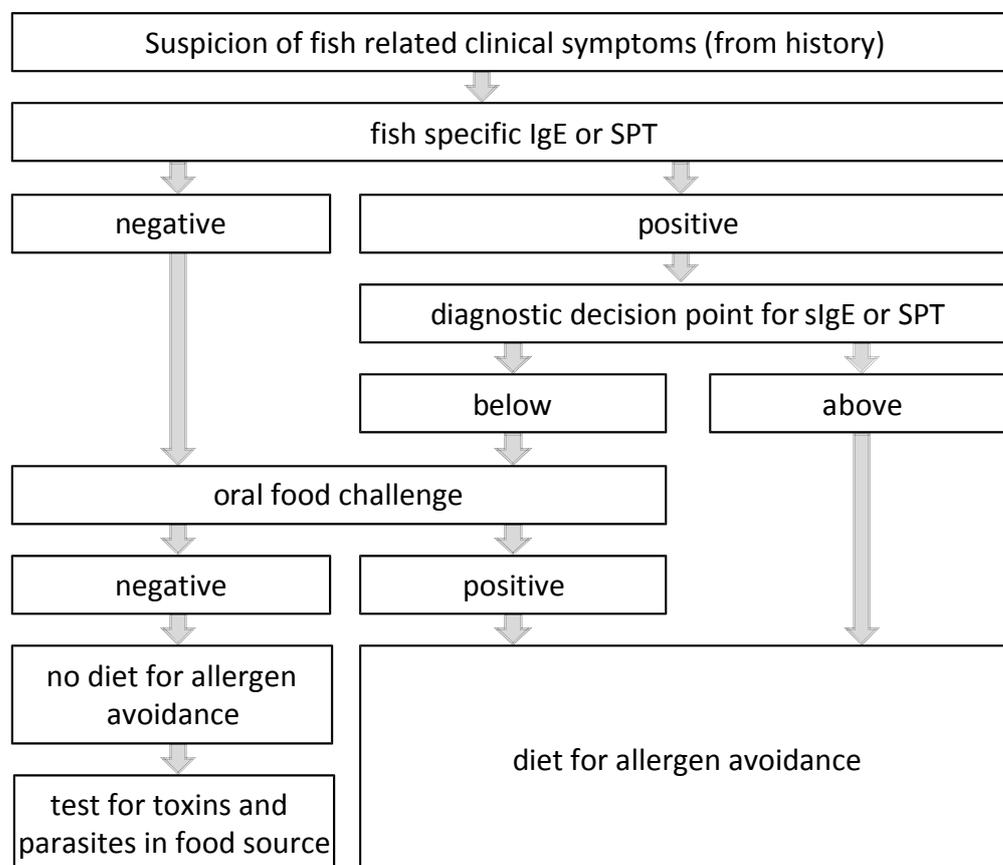
Patients who generate IgE antibodies to one parvalbumin often react to parvalbumin of other fish species, demonstrating the importance of parvalbumin as a cross-reactive major fish allergen [82]. Approximately one third of children and two thirds of adults appear to react to multiple types of fish [7, 83, 123, 124]. Van Do et al [13] demonstrated in 10 patients, using a combination of SPT, ImmunoCAP and immunoblotting, that Gad c 1, Sal s 1, The c 1, herring, and wolfish contained the most potent cross-reacting allergens, whereas halibut, flounder, tuna, and mackerel were the least allergenic in the current study. It is suggested that the latter fish species could probably be tolerated by some of the tested patients. However, allergic reactions to only one specific type of fish have been reported such as to salmonids where patients react to trout and salmon but not to cod, carp, herring or redfish [90, 91]. Asero *et al.* [125] described a patient that was monosensitive to tropical sole but did not react to lemon sole, cod, salmon, tuna and swordfish. Fish monosensitivity has also been seen in a patient who reacted to tilapia and basa, but not to cod. Subsequent analysis showed that the patient reacted to an unknown allergen but not to parvalbumin [126]. These few studies demonstrate that monosensitivity to fish is not uncommon and most prominent in children; however the molecular nature of responsible allergens is yet to be fully elucidated. Recent findings by Gill *et al.* [127] indicate that reactivity to specific

allergens is associated with disease risk, confirming the importance of molecular identification of causative allergens.

Skin prick testing (SPT) is frequently used as a first test to confirm or refute allergic reactions to fish as it provides a rapid, safe and inexpensive method for screening patients. Nevertheless, these types of tests are considered to be not very specific with a positive predictive value often below 50% [128]. Skin prick tests are of particular challenge for fish allergy due to the large variety of fish species being implicated and the fact that the majority of patients seem to demonstrate monosensitivity to specific species. To improve the specificity and sensitivity of this test Van do [13] compared the SPT reactivity of the recombinant with the natural parvalbumins from salmon, cod and pollock. Surprisingly only one of the ten patients recognized the recombinant versions of the natural parvalbumins, which were in contrast recognized by nine of the patients. The poor response obtained in using recombinant parvalbumin in SPT is possible due to conformational masking of high-affinity IgE-binding motifs (Figure 1.2B). These studies suggest that the IgE reactivity to recombinant parvalbumin has to be investigated in more detail in future studies to use these allergens in *in vitro* and *in vivo* tests.

The gold standard for diagnosing food allergy is still the double-blind, placebo-controlled food challenge. A recent review by Niggemann and Beyer [1] proposes various decision trees to approach food challenges including seafood-allergic patients. A similar decision tree is suggested for fish allergy, which includes non-immunological adverse reactions triggered by toxins and parasites (Figure 1.3). There is limited information on the establishment of threshold values for elucidating allergic reactions to fish. A recent study [46] demonstrated that for codfish, very small amounts of less than 3 mg protein could trigger allergic reactions, which is less than previously reported

[129]. A similar quantity was used to confirm allergy to yellowtail in a study on seven fish allergic patients from South Africa [32].



**Figure 1.3** Diagnostic decision tree on how to proceed from the suspicion of fish-related allergic symptoms to confirmation using laboratory based and clinical approaches, modified from Niggemann et al [1] and Mehl et al [2]

One target for the development of immunotherapeutics for fish allergy is the muscle protein parvalbumin, which is the major allergen recognized by over 90% of patients with fish allergies [12, 82, 130-132]. The major IgE-binding epitopes of parvalbumin are considered to be conformational epitopes as detailed in the studies above [75]. These antibody epitopes appear to be dependent on the functional reactivity of the binding sites for  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ . Conformational changes in these protein regions using recombinant technologies can result in hypoallergenic parvalbumin as has been recently

demonstrated for carp [131]. Although still immunogenic, as demonstrated through specific IgG responses in mice, the reactivity measured by SPT in patients was markedly reduced. This novel hypoallergenic protein forms the basis for safer novel forms of future vaccination against fish allergy. Nevertheless, it has to be highlighted that the immunological reactivity of recombinant allergens are not necessarily identical to native allergens. Van der Ventel *et al.* [15] demonstrated in an inhalant murine model that the recombinant parvalbumin from carp is not as reactive as parvalbumin from pilchard. In addition, heated parvalbumin was much more allergenic than raw parvalbumin and other allergens, in addition to parvalbumin, seem to be relevant.

While heating appears to increase allergenicity of some of the fish allergens, commercial heat processes, used to generate canned fish, seem to have a different effect. A recent descriptive study from Australia demonstrated that more than 20% of children allergic to salmon or tuna were able to tolerate the fish in canned form. Importantly this was associated with a reduction in SPT size in most patients, implying that the consumption of canned fish may have resulted in the induction of tolerance in these patients [133].

One molecular indicator of a successful allergen immunotherapy is an increase in allergen specific IgG<sub>4</sub> serum levels. IgG<sub>4</sub> can indicate one or both of two scenarios; (1) a blocking antibody which out-competes allergen specific IgE and (2) a marker of tolerance as studies have shown that successful immunotherapy will often lead to elevated IgG<sub>4</sub> serum levels [134-136]. However, the role of IgG<sub>4</sub> in allergy and specific immunotherapy is not completely understood due to IgG<sub>4</sub> being a highly variable antibody which has many roles in the immune system, many which are yet to be defined [134, 137].

While immunotherapy for fish allergy is still in development, management of fish allergy is generally directed at avoidance of the offending foods and prompt recognition and treatment of acute allergic reactions. In addition, reactions to hidden food allergens through inhalation of the fish allergens or via skin contact can also pose problems [38, 40]. In a recent study, 22.7% of 530 food-related reactions were due to hidden allergens, with 35% of fish allergic patients having reacted to fish proteins hidden in other foods or to fish vapors [38] .

In general, management of food allergies, including fish allergy, still primarily relies on avoidance. The labeling of foods containing materials derived from fish has already become mandatory in some countries such as the USA, Europe (EU) and Japan. While *in vitro* assays for currently 14 food allergens in the EU are available, the detection of parvalbumin is much more problematic as these allergens show very high biochemical and immunological variability among the different fish species as detailed above [138, 139]. Currently there is only one commercial test available to detect the presence of fish DNA, but is limited to twelve fish species ([www.r-biopharm.com](http://www.r-biopharm.com)). Labeling regulations have limitations because of accidental cross-contamination with allergens through shared equipment in production lines or the unknown presence of a hidden fish allergen such as clarification agents derived from fish bladders used in wine and beer [140].

## 1.8 Conclusion

Fish allergy can cause serious health effects and in addition has a significant adverse effect on anxiety and stress among adults but also in families with allergic children. There seems to be strong geographical differences in the prevalence of fish allergy, possible due to different cultural dietary habits and type of food processing. The later might even enhance allergenicity of fish allergens due to advanced glycation end-products as demonstrated *in vitro* and utilizing murine models. More detailed

immunological studies are needed to characterize the impact of heating on fish allergens to develop better food processing technologies to reduce their allergenicity.

The majority of allergic reactions to fish are caused by the major allergen parvalbumin. Immunological cross-reactivity between the vast varieties of fish species seems to be determined by the degree of amino acid homology and in addition number of allergen isoforms and variants present in some of the highly allergenic species. In addition the concentration of this major allergen varies significantly among the different fish species and might impact on patients' sensitivity to one or multiple species. Future comparative studies need to investigate the molecular and immunological similarity of parvalbumins among the different fish groups and families, with focus on B- and T-cell epitopes, to allow the generation of group-specific recombinant allergens for better identification of patients with multiple fish reactivity.

The route of sensitization to fish allergens seems to initiate differential immunological reactions to additional allergens as demonstrated in the occupational environment, which needs to be addressed for a complete diagnosis of fish allergy.

The current diagnosis and management of fish allergy are hampered by the lack of detailed information of the molecular nature of these allergens, the enormous variety of allergenic fish species consumed and the subsequent lack of suitable tests to detect specific allergens in food products. In the absence of commercial IgE or SPT's assays to a specific fish species it is suggested to quantify specific IgE to important fish species, including Atlantic cod, Atlantic salmon, Pacific pilchard, European hake, basa and barramundi as these species cover the broad molecular spectrum of the major allergen parvalbumin. In addition lipopolysaccharide free protein extracts, preferably raw and heat-treated, of the specific fish species could be used for SPT.

Future comparative studies on the clinical reactivity to different fish species among different populations will improve diagnosis and management of this life-long allergy. The development of better recombinant and hypoallergenic parvalbumins is an important basis for more sensitive and specific *in vivo* and *in vitro* diagnostics and safer novel forms of vaccination against fish allergy.

### **1.9 Research objectives**

Detailed review of current fish allergy literature has highlighted the lack of effective detection tools for fish allergen. Most of the literature is derived from studies on species from Europe and North America. Therefore in this thesis there will be a focus of detection and characterisation of fish allergens from the Asia-Pacific region.

The first objective is to evaluate the most common fish allergen detection tool, the monoclonal PARV-19 antibody, directed against frog parvalbumin antibody, using a collection of regional fish species.

The second objective is to generate more effective antibodies and assess their cross-reactive properties against an extensive range of fish species from the Asia-pacific region.

The third objective is to characterize the important novel allergens from the Australian iconic fish species, barramundi (*Lates calcarifer*).

The final objective is to characterize antibody interactions on a molecular scale of the barramundi major allergen parvalbumin by mapping its antibody epitopes.

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## CHAPTER 2

Antibody reactivity to the major fish allergen parvalbumin is determined by isoforms and impact of thermal processing

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<b>Details of publication on which this chapter is based</b>	<b>Nature and extent of the intellectual input of each author, including the candidate</b>
Saptarshi S. R. <sup>1</sup> , Sharp M. F. <sup>1</sup> , Kamath S. D. and Lopata A. L., 2014. Antibody reactivity to the major fish allergen parvalbumin is determined by isoforms and impact of thermal processing. <i>Food Chem.</i> 148, 321-328	Saptarshi, Sharp and Lopata co-developed the research question. Saptarshi and Sharp collected the data and performed the data analyses with assistance from Kamath. Saptarshi wrote the first draft of the paper which was revised with editorial input from Sharp and Lopata. Saptarshi and Sharp developed the figures and tables. Both Saptarshi and Sharp have been noted as equal contributors to this publication

## 2.1 Introduction

Fish is one of the eight prominent foods known to cause allergy [12]. Being an excellent source of proteins and omega fatty acids, the consumption of fish has increased greatly. However, allergy to fish is also increasing, affecting up to 0.2% of the general population. Fish allergy is also an important concern in the seafood processing environment. A recent study reported that prevalence of fish allergies can be as high as 8% among fish processing workers [28]. Along with consumption and handling of fish allergens, patients can also react to aerosolized fish proteins generated during cooking or processing [42, 141]. Clinical manifestations of fish allergy may include symptoms ranging from wheezing, tightness of the throat, urticaria, vomiting, diarrhea etc. to the life threatening reaction called anaphylaxis. The major fish allergen has been identified as parvalbumin, an EF hand calcium binding protein [11, 82]. Parvalbumins are globular proteins about 12kDa in size and are abundant in lower vertebrates such as amphibians and fish [142]. These are water soluble and remarkably stable over a broad temperature and pH range [75, 143]. Parvalbumins are abundant in the white muscle tissue of fish, however lower concentrations have also been reported in fish dark muscle tissue [33, 144]. Fish exhibit differences in their environmental habitats and their overall muscle composition. Multiple isoforms of parvalbumin can be expressed in a single fish species during its different developmental stages. [71, 145, 146] For example, fresh-water carp has been reported to express up to eight isoforms of parvalbumin, differing slightly in molecular weight and isoelectric properties [145]. The detection of fish parvalbumin is challenging compared to other food allergens; this can be attributed to the high biochemical and immunological variability among the different fish species [112, 141]. Fish consumption strongly depends on regional availability. Most studies on characterisation of parvalbumins have been conducted on fish

commonly consumed in the northern hemisphere. Several fish species such as barramundi, flathead, gummy shark are indigenous to the Asia-pacific region. However data on fish allergens in these species is limited. Moreover, not much research has been conducted on the comparison of the diversity of parvalbumin isoforms across different orders of fish or impact of heat processing on their antibody reactivity.

### ***2.1.1 Aims***

The aim of this chapter was to compare parvalbumin distribution profiles, specific antibody reactivity and cross-species recognition across 12 different orders of fish and study the impact of heat-processing on the mono and polymeric forms of these parvalbumins. Bioinformatic tools were used to show that molecular phylogenetic classification of fish based on the amino acid sequence of parvalbumin, can be linked to the immunological cross-reactivity of this allergen. Furthermore the deduction of different antibody binding sites of parvalbumin for bony and cartilaginous fish was investigated which can aid in designing specific antibodies for better detection of parvalbumin against different orders of fish species.

## 2.2 Materials and methods

### 2.2.1 Fish samples

Nineteen species of fish commonly consumed in the Asian-Pacific region (see Table 2.1) were analysed. Fresh fillets of each species were purchased from the local fish market and transported on ice to the laboratory. All samples were stored at -80 °C prior to processing.

### 2.2.2 Preparation of protein extracts

Fifty grams of fish white muscle was homogenized in 100 mL of phosphate buffered saline (PBS, 10mM, pH 7.2) using an Ultra Turrax blender (IKA, Staufen, Germany) and extracted overnight with gentle tumbling at 4° C. The crude extract was centrifuged at 5000g for 30 min at 4° C and filter sterilized using 0.2 µm cellulose acetate filter membranes (Sartorius, Germany) is referred to as 'raw extract'. To standardize the heat-processing an aliquot of raw extracts were heated at 95 °C for 15 min in a water bath. Precipitated proteins were removed by centrifugation at 5000 g for 15 minutes and the resulting referred to as 'heated extract'. The prepared protein extracts were stored at -80 °C until further analysis.

### 2.2.3 Protein quantification

Protein concentrations were determined for the raw and heated extracts using the Quick Start™ Bradford assay kit (Bio-Rad, USA). Readymade bovine serum albumin standards (Bio-Rad, USA, 0.125- 2.00 mg/ml) were used and the absorbance was determined at 595nm using a Multiskan Ascent®, (Pathtech, Australia) micro plate reader.

#### **2.2.4 SDS-PAGE analysis**

Protein profiles for raw and heated fish extracts were obtained using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Fish proteins (25µg) were diluted in 5X Laemmli sample buffer containing 2- mercaptoethanol, heated for 5min and loaded on a 12% Tris-Glycine 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 170V. Proteins were visualized by Coomassie Brilliant Blue R-250 (Bio-Rad, USA) staining.

#### **2.2.5 Immunoblotting**

The presence of different parvalbumins in raw and heated samples was determined by immunoblotting. Fish protein extracts (5µg) were separated using SDS-PAGE and then transferred to activated PVDF membrane (Bio-Rad, USA) using the Semi-dry Trans Blot electrophoretic transfer system (Bio-Rad, USA) for 10min at 10V. After blocking with 5% (w/v) skim milk (in TBS) for 1 h at room temperature, membranes were incubated for 1hr with the primary monoclonal anti-parvalbumin antibody (PARV-19; Sigma, USA) diluted 1:3000 in 1% skim milk and TBS. Membranes were subsequently washed in TBS with 0.5% Tween -20 (TBS-T) and incubated with secondary anti-mouse IgG HRP labelled antibody (Sigma, USA). The protein-antigen interaction was visualized using Enhanced Chemiluminescence substrate (Sigma, USA) followed by exposure to photographic film (GE Healthcare, Australia).

#### **2.2.6 Inhibition-ELISA**

To analyze the cross-species antibody reactivity for the different fish parvalbumins an in house developed inhibition ELISA was performed. Briefly, 10 µg/ml of heated carp extract was coated on a 96-well high binding plate (Costar, USA) and incubated

overnight at 4°C and subsequently blocked with 5% skim milk in TBS for 1 h at room temperature. The Primary antibody PARV-19 (diluted 1:1000) was mixed with increasing concentrations of inhibitors, ranging from 0-1000µg/ml for 1 h at 37°C in separate microcentrifuge tubes. This antibody-antigen mixture was then added to the 96-well plate and incubated for a further 1 h at 37°C. Following three washing steps with TBS-T plates were incubated with the secondary antibody (Rabbit anti-mouse monoclonal antibody; Sigma, USA) for 1 h at room temperature and washed three times and binding visualized using 3,3', 5,5'-tetramethylbenzidine (TMB) substrate for HRP (BD Biosciences, USA). The reaction was stopped using 2 M sulphuric 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) x 100].

**Table 2.1** Biological classification and scientific names of fish species analysed in this study.

Common name	Order	Family	Scientific name
<b>Bony fish</b>			
Snapper	Perciformes	Sparidae	<i>Pagrus auratus</i>
Silver bream	Perciformes	Sparidae	<i>Acanthopagrus australis</i>
Yellowtail kingfish	Perciformes	Carangidae	<i>Seriola lalandi</i>
Barramundi	Perciformes	Latidae	<i>Lates calcarifer</i>
Blue fin tuna	Perciformes	Scombridae	<i>Thunnus maccoyii</i>
Slimy mackerel	Perciformes	Scombridae	<i>Scomber australasicus</i>
Orange roughy	Beryciformes	Trachichthyidae	<i>Hoplostethus atlanticus</i>
Tiger flathead	Beryciformes	Platycephalidae	<i>Neoplatycephalus richardsoni</i>
Atlantic salmon	Salmoniformes	Salmonidae	<i>Salmo salar</i>
Rainbow trout	Salmoniformes	Salmonidae	<i>Onchorynchus mykiss</i>
Carp	Cypriniformes	Cyprinidae	<i>Cyprinus carpio</i>
Pilchard	Clupeiformes	Clupeidae	<i>Sardinops neopilchardus</i>
Rock ling	Ophidiiformes	Ophidiidae	<i>Genypterus blacodes</i>
Atlantic cod	Gadiformes	Gadidae	<i>Gadus morhua</i>
<b>Cartilaginous fish</b>			
Skate	Rajiformes	Rajidae	<i>Raja cerva</i>
Gummy shark	Carchariniformes	Triakidae	<i>Mustelus antarcticus</i>
Sparsely spotted stingaree	Myliobatiformes	Urolophidae	<i>Urolophus paucimaculatus</i>
Blacktip shark	Carchariniformes	Carcharhinidae	<i>Carcharias limbatus</i>
Elephant shark	Chimaeriformes	Callorhynchidae	<i>Callorhynchus milii</i>

### ***2.2.7 Amino acid sequence alignment of parvalbumin and epitope analysis***

Two multiple alignments of parvalbumin amino acid sequences were performed using Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). The first alignment consisted of 9 bony fish from 4 different orders (Gadiformes, Perciformes, Cypriniformes and Salmoniformes), which generated a bony fish consensus sequence. The second alignment used the two known cartilaginous fish parvalbumin amino acid sequences that also generated a consensus sequence. The difference between the two consensus sequences was calculated using BLOSUM 62 scoring. Possible antigenic sites in the bony fish consensus sequence were estimated using the epitope-predicting tool Antigenic (<http://emboss.bioinformatics.nl/cgi-bin/emboss>).

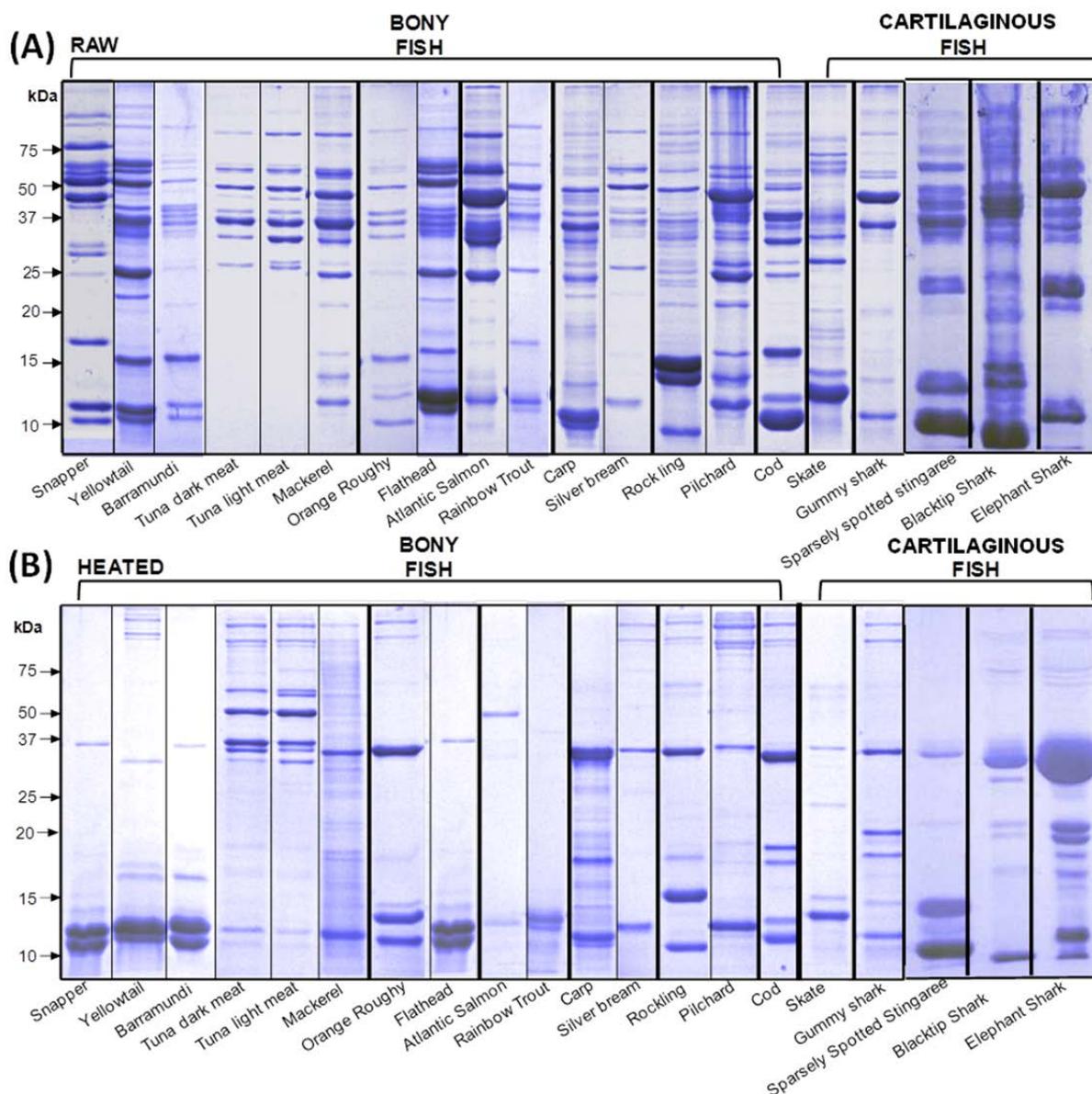
### ***2.2.8 Phylogenetic analysis of fish parvalbumin amino acid sequences.***

Phylogenetic analysis of 38 fish parvalbumin isoforms was conducted using the Neighbour- Joining method using the software MEGA5. The bootstrap consensus tree inferred from 10000 replicates was taken to represent the evolutionary history of fish parvalbumins.

## 2.3 Results

### 2.3.1 *Protein profiles of fish extracts and effect of heat treatment*

Proteins present in raw and heat-processed extracts of 19 fish species (see Table 1) were separated effectively using SDS-PAGE (F). Most raw fish extracts showed presence of two or more protein bands in the molecular weight range of 10-15kDa except silver bream and elephant shark extracts which displayed a single band. Both light and dark tuna extracts showed no bands. The profile for the higher molecular weight protein bands in the range of 37-75kDa was similar for most fish species. To analyze the effects of heat-processing, the fish protein extraction parameters such as osmolality, pH extraction buffer volume, specimen weight, heating temperature and heating time were kept constant. This allowed direct comparison of the effect of heating on different shellfish species without any bias. Heating of the fish extracts denatured many heat labile proteins although all fish extracts showed presence of heat stable proteins around 37kDa and multiple heat stable proteins at 10-15kDa. However, mackerel, Atlantic salmon, silver bream and blacktip shark displayed only one heat stable protein band in the 10-15 kDa weight range. Interestingly, heated dark and light tuna protein extracts had two distinct bands at 12-13 kDa, which were not present in the raw extracts.

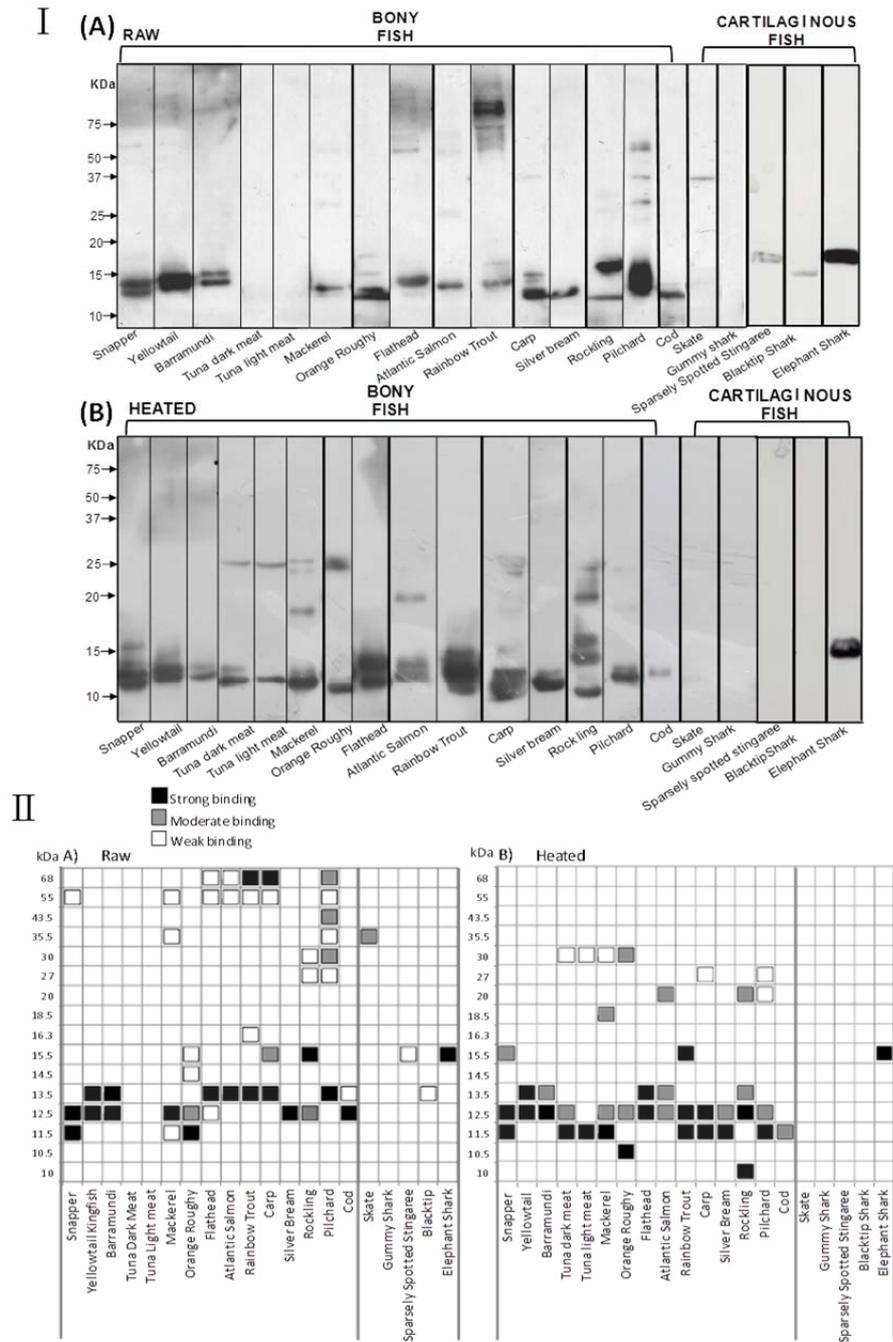


**Figure 2.1** Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of raw (A) and heat-processed (B) protein extracts of bony and cartilaginous fish. The common names are depicted under each lane. See Table 2.1 for complete list of scientific names.

### 2.3.2 Monoclonal antibody reactivity to parvalbumin in raw and heated fish extracts

Immunoblotting with the monoclonal antibody against the raw extracts of the various fish showed several bands in the molecular weight range of 10-15kDa, demonstrating the presence of parvalbumin isoforms (Figure 2.2A). Some fish species, such as

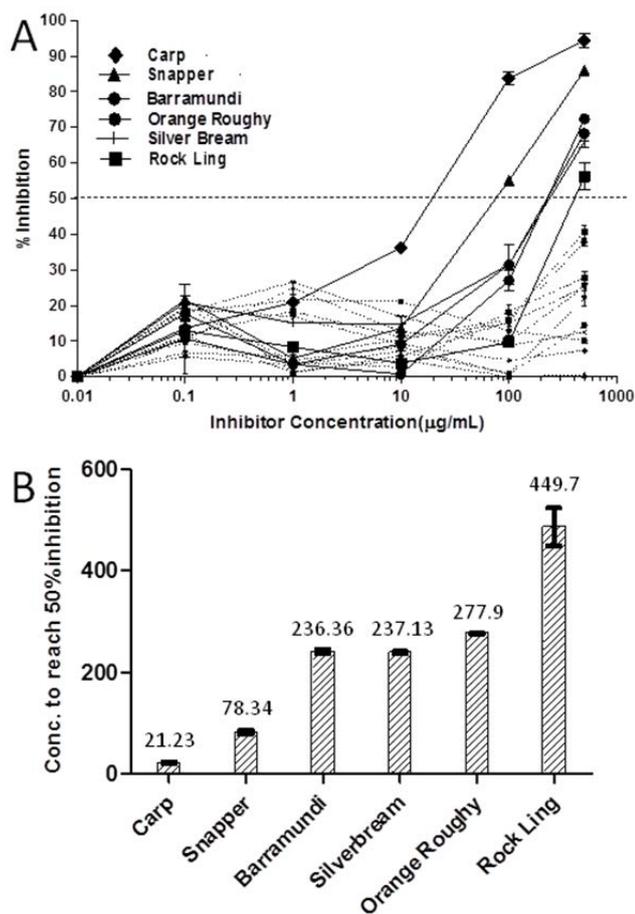
snapper, yellowtail, barramundi and carp demonstrated parvalbumin isoforms with very similar molecular weights, while other species seemed to have only one dominant isoform, as observed for mackerel, flathead, Atlantic salmon and cod. Few species, such as rainbow trout and pilchard showed presence of additional higher molecular weight parvalbumin isoforms. In contrast, no bands were observed for Yellow fin tuna (light meat and dark meat) and gummy shark. The heated fish extracts demonstrated prominent monoclonal antibody binding to most; lower molecular weight parvalbumin forms (Figure 2.2B). Heating had the most profound effect on yellow fin tuna, which demonstrated strong bands. In contrast, cartilaginous fish lost all antibody reactivity after heating and none of the five species showed parvalbumin bands except elephant shark that showed presence of one band. Comparison of the location and band intensity of the different parvalbumin forms by densitometric analysis resulted in a detailed allergogram, allowing the specific evaluation of impact of heat-processing on antibody recognition. The higher molecular weight antibody reactivity, particularly in raw extracts, was most likely due to the presence of polymeric forms of monomeric parvalbumins, in molecular weight range of 11.5 kDa to 13.5 kDa



**Figure 2.2** Immunoblot analysis of raw (A) and heat-processed (B) protein extracts from bony and cartilaginous fish using monoclonal anti-parvalbumin antibody (I). The common names of each fish species are shown under each lane. See Table 2.1 for complete list of scientific names. Calculated molecular weights of monomeric and polymeric forms of parvalbumin are presented in the allergogram (II) for raw (A) and heat-processed (B) fish, facilitating the analysis of parvalbumin. Antibody binding is graded as mild, moderate or intense.

### ***2.3.3 Monoclonal antibody cross-reactivity of carp parvalbumin against heated fish extracts***

The cross-reactivity of 19 fish species was evaluated by performing a quantitative inhibition ELISA using heated carp extract as coating antigen (Figure 2.3A). The decrease in reactivity of monoclonal antibody to the immobilized carp allergen 231 extract, comprising of two strong parvalbumin forms, was used as a measure of immunological cross-reactivity against other heated fish extracts used as inhibitors. Carp extract as expected, was able to completely abolish antibody reactivity at about 500  $\mu\text{g/ml}$ , while only 5 of the 19 fish species were able to inhibit the antibody reactivity by over 50% in a dose depended manner. The concentrations needed to reach 50% inhibition varied significantly from about 20  $\mu\text{g/ml}$  for carp to about 450  $\mu\text{g/ml}$  for rockling (Figure 3.2B). In contrast none of the other 14 fish extracts were able to achieve inhibitions above 40%, even at the highest inhibitor concentration.

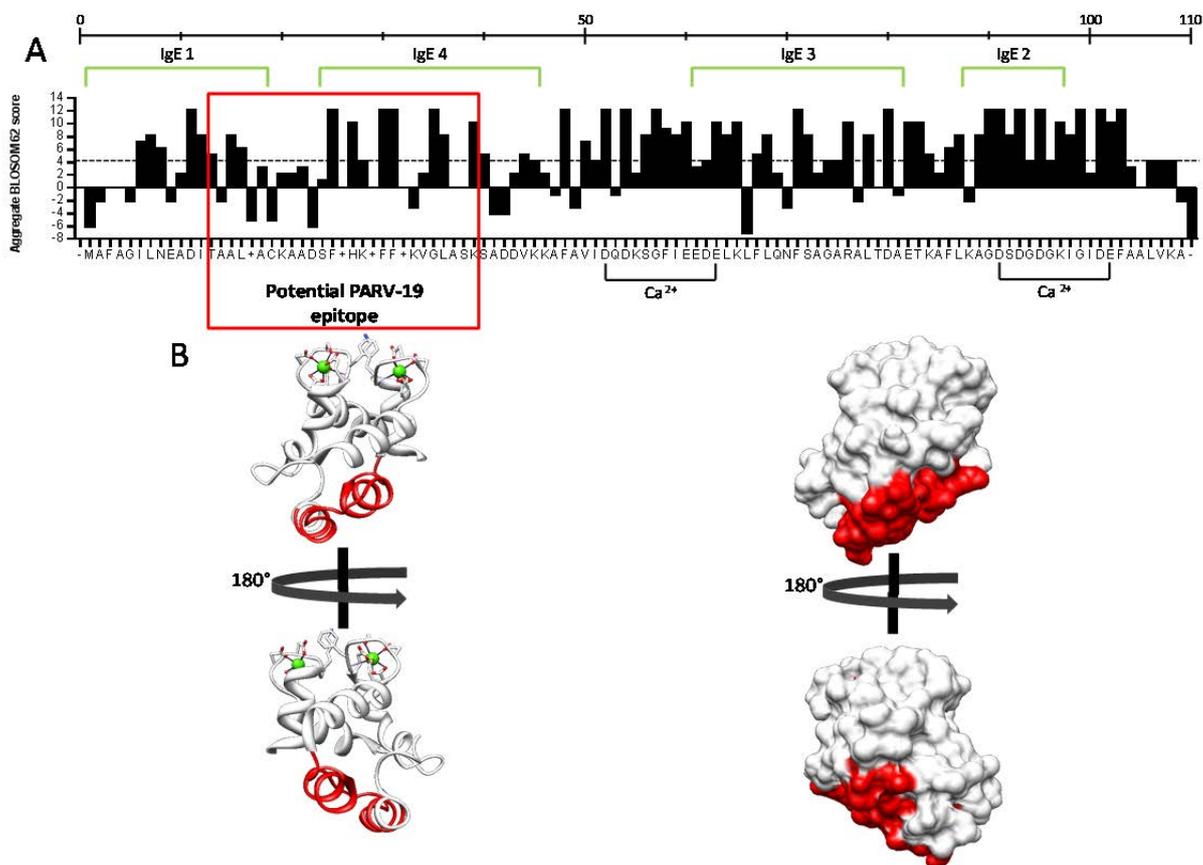


**Figure 2.3** Inhibition ELISA for the quantification of the monoclonal anti-parvalbumin antibody cross-reactivity between carp parvalbumin (coating antigen) and 19 different fish parvalbumins (A). The inhibition dose response curves for the 19 fish demonstrate that only 5 species achieved over 50% inhibition, with homologous carp parvalbumin being the strongest inhibitor. The protein concentrations to reach 50% inhibition show great differences with rock ling requiring over 20 times more parvalbumin compared to homologues carp (B).

#### 2.3.4 Epitope analysis of monoclonal antibody between bony and cartilaginous fish

The multiple alignments of fish parvalbumin amino acid sequences demonstrated regions of high consensus in bony and cartilaginous fish, situated at the two calcium binding sites. However, the amino acid sequences are less conserved at the N-terminal region, demonstrated by values under 4 in the BLOSUM 62 score graph of (Figure 2.4).

The program 'Antigenic' predicted the most antigenic site, the most likely target for this monoclonal antibody to be between positions T13, 39 and K39.

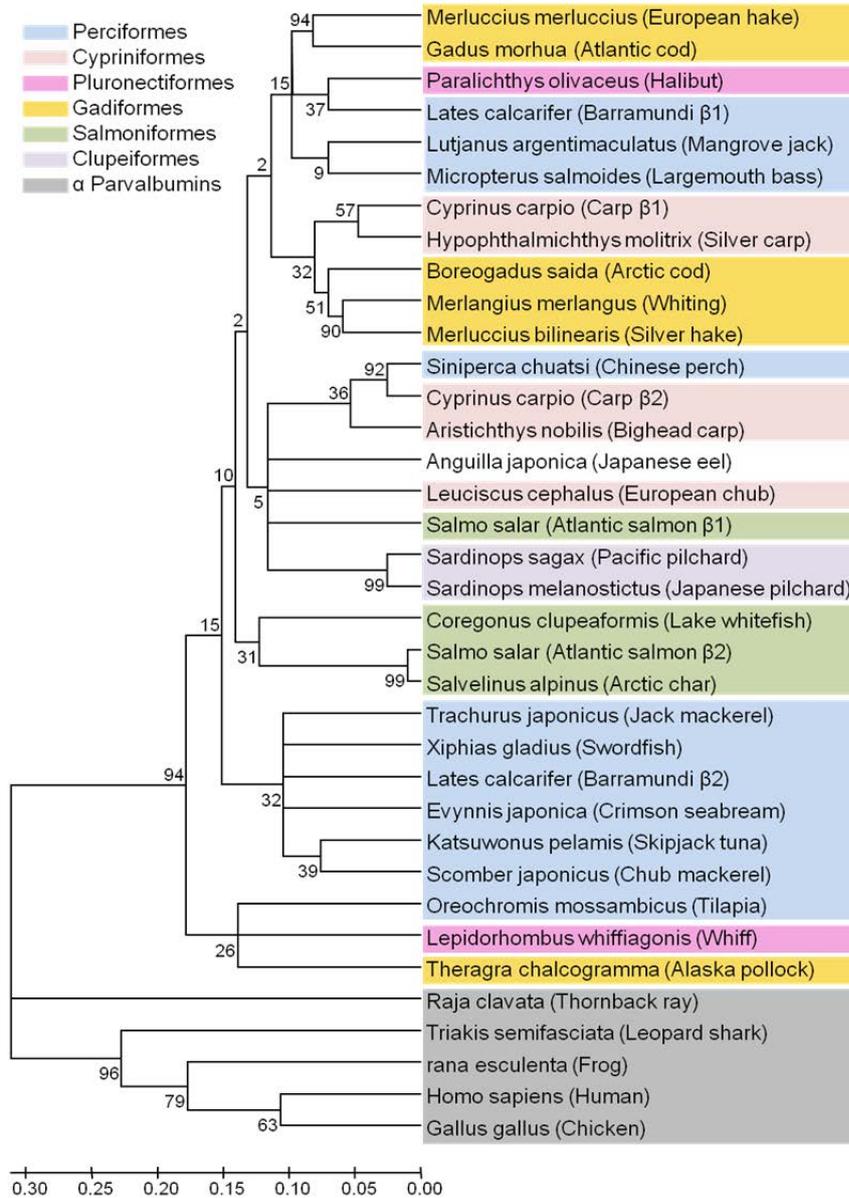


**Figure 2.4** (A) The potential PARV-19 epitope predicted using 'Antigenic' analysis is boxed in red. The aggregate BLOSUM 62 score was calculated from the two consensus sequences generated for bony and cartilaginous fish. Bony fish consensus is shown under the chart with the four known IgE epitopes highlighted in green. (B) Ribbon and space fill models of carp parvalbumin (PDB ID: 4cpv), with the predicted epitope coloured in red. Bound calcium ions are shaded in green.

### 2.3.5 Phylogenetic relationships of fish parvalbumins

The phylogenetic analysis of fish parvalbumin demonstrated relationships between isoforms from specific taxonomic orders. For example, Gadiformes, Perciformes and Cypriniformes form two distinct clades each and three out of four Salmoniformes form another clade (Figure 2.5). All the  $\alpha$ -parvalbumins, which also include the only two

cartilaginous fish parvalbumins with known amino acid sequences, grouped close to the higher vertebrate parvalbumins.



**Figure 2.5** The evolutionary history of a variety of parvalbumins is inferred using the Neighbour-Joining method. The bootstrap consensus tree inferred from 10000 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary

distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 38 parvalbumin amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 99 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. For convenience each order is shaded a separate colour.

## 2.4 Discussion

The major fish allergen parvalbumin is a highly diverse protein and exists as multiple isoforms in different fish species [141]. The present study analyses for the first time, parvalbumins in a comprehensive range of fish from the Asia-Pacific region. This study has made use of anti-frog parvalbumin antibody PARV-19, widely used for the identification of this major allergen [100, 112]. Immunoblotting experiments confirmed the presence of multiple bands of parvalbumin isoforms in the molecular weight range of 10-15 kDa for most raw and heated fish extracts. Characteristic parvalbumin oligomers were also detected by the monoclonal antibody in raw extracts of eight fish including mackerel, flathead, trout, carp, rock ling, pilchard and skate. Multimeric formation of parvalbumin can be attributed to the presence of readily reducible cysteine residues in the protein [147]. Heat processing of the fish extracts affected the antibody recognition of parvalbumins for some fish species. For example, while no bands were observed in raw extracts of tuna, monomeric as well as oligomeric parvalbumin was detected in the heated extracts. Similarly heating also led to oligomer formation of parvalbumin in orange roughy extract. Oligomeric parvalbumin from flathead, rainbow trout and skate was found to be heat labile. These results highlight the difference in thermal stability of parvalbumin across different genera of fish and confirm that, heat processing has profound effect on antibody reactivity of different parvalbumins.

The 32,400 different types of fish can be largely grouped as bony (Osteichthyes) which comprises of most edible fish and cartilaginous (Chondrichthyes) which include sharks and rays [78]. Parvalbumin has been described as a pan-allergen in a majority of bony fish varieties such as cod, carp and salmon [11, 71-73, 75-77]. Interestingly reports on allergenic parvalbumin from cartilaginous fish are limited [148, 149]. Parvalbumins from sharks have been suggested to be structurally different to those of bony fish [150].

The monoclonal antibody reactivity to the cartilaginous fish extracts used in our study was found to be minimal with no binding detected for gummy shark. This reactivity was completely lost for the heated extracts demonstrating that shark parvalbumin may be heat labile. The inhibition assay further confirmed the low cross reactivity of this allergen between cartilaginous and bony fish. Interestingly, the antibody was capable of binding to parvalbumins in raw as well as heated extract of the elephant shark. The elephant shark is a chimera fish. A recent study suggested that parvalbumin from elephant shark may be more similar to bony fish parvalbumin [151]. The inability of the monoclonal antibody to detect shark parvalbumin may be because of the dissimilarity in the amino acid sequence homology of parvalbumins from bony and cartilaginous fish.

The cross-reactivity among fish is most likely due to the molecular phylogenetic association of parvalbumin, though Fæste & Plassen [138] in their study with anti-cod antibodies have suggested that cross reactivity may be based on habitat (fresh-water or saltwater) of fish. Detailed molecular analysis of parvalbumins from fish used in the present study, and phylogenetic grouping of thirty eight available parvalbumin amino acid sequences was used to understand the cross-reactive nature of parvalbumin in different fish. For example, amino acid sequences of the order Perciformes group together (Figure 2.5) correlating with the three species of fish (barramundi, snapper, silver bream) that cross-reacted with carp parvalbumin in the inhibition assay. These fish showed up to 50% inhibition of the monoclonal antibody to carp parvalbumin. Currently there is a lack of parvalbumin sequences available from the orders Ophidiiformes (rockling) and Beryciformes (orange roughy), and this made it difficult to analyse the molecular relationships and cross-reactivity of these species with the Perciformes or carp. Interestingly the two known cartilaginous fish parvalbumins (leopard shark and thornback ray) clustered close to the  $\alpha$ -parvalbumins of higher

vertebrates such as humans and chicken away from other bony fish. This not only explains the lack of binding of the monoclonal antibody to cartilaginous fish parvalbumin in our study, but may also explain the current reports on their low allergenicity [152].

In summary, the monoclonal antibody used in the present study reacted to all fish species except gummy shark, demonstrating that the parvalbumin epitope for this antibody must be in a relatively conserved region of this allergenic protein. The effect of heating on fish allergens must be taken into account when designing future diagnostics. This study also identified a specific antibody target at the N-terminal region of parvalbumin, allowing the distinction of bony from cartilaginous fish.

#### ***2.4.1 Future direction***

Knowledge gained from this chapter can be used to aid in predicting immunological cross-reactivity by performing parvalbumin phylogenetic analysis. This could be used to generate fish order-specific detection tools to further our resources in detecting this major fish allergen in foods. This chapter has also highlighted the cross reactive limitations of monoclonal antibodies as they are very specific to distinct protein regions. In chapter 3 polyclonal antibodies will be generated against multiple phylogenetically diverse parvalbumins and analysed against a large panel of Asian-pacific fish.

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## CHAPTER 3

Analysis of immunological cross reactivity between parvalbumins using four polyclonal anti-fish parvalbumin

IgG antibodies

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<b>Details of publication on which this chapter is based</b>	<b>Nature and extent of the intellectual input of each author, including the candidate</b>
Sharp MF, Stephen JN, Kraft L, Weiss T, Kamath SD, Lopata AL. Immunological cross-reactivity between four distant parvalbumins—Impact on allergen detection and diagnostics. <i>Mol Immunol</i> 2015, 63(2): 437-448.	The authors Sharp and Lopata co-developed the research question. Sharp and Stephen collected the data and performed the data analyses. Sharp wrote the first draft of the paper, which was revised with editorial input from Lopata, Kamath, Kraft and Weiss. Sharp developed the figures and tables.

### 3.1 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 [92]. Despite fish allergy being common and well documented, detection of fish allergens and allergic sensitization is difficult due to species diversity [141].

Parvalbumin, a small calcium binding protein, is the major allergen identified in most fish species [84, 141]. 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 [153, 154]. Although differences in parvalbumin primary structure, they have highly conserved tertiary structures and calcium binding residues located on paired  $\alpha$ -helices (EF-Hand motif) [84]. Current methods of detecting fish allergens include real-time PCR [155] and antibody based assays that detect either fish proteins or the allergens themselves [138, 139, 156]. The most investigated antibody against parvalbumin is the commercial monoclonal anti-frog parvalbumin, PARV-19 [112, 154]. 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 [153, 157, 158]. PARV-19 has also been compared to other monoclonal and polyclonal antibodies generated against Atlantic cod, carp, pilchard and anchovy [139, 157]. Lee *et al.* [157] demonstrates recently that polyclonal anti-cod antibody seemed to be the most cross-reactive, most likely due to its polyclonal nature, however this antibody still could not detect all fish including mahi mahi, swordfish and albacore tuna.

Despite structural and functional similarities between fish parvalbumins their amino acid sequence differ greatly, for example, two different parvalbumin isoforms are expressed in both Atlantic salmon (*Salmo salar*) and barramundi (*Lates calcarifer*) each, which the former only shares 64% and the later 67% amino acid sequence identity [77, 153]. 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 [108].

Parvalbumin cross-reactivity has been linked with their phylogenetic relationships [138, 154]. In this study, four polyclonal antibodies are generated against parvalbumin from four phylogenetically diverse fish species; Atlantic salmon (Salmoniformes), barramundi (Perciformes), basa/catfish (Siluriformes) and pilchard (Clupeiformes). Cross-reactivity of these antibodies is analysed among 40 different fish species across 17 orders and their ability to detect the major allergen parvalbumin evaluated across commonly consumed fish species.

### **3.1.1 Aims**

The aim of this chapter is to generate four polyclonal antibodies against phylogenetically diverse parvalbumin from four diverse fish species, Atlantic salmon (Salmoniformes), barramundi (Perciformes), basa (Siluriformes) and pilchard (Clupeiformes). Cross-reactivity of these antibodies will be analysed against a large panel of Asian-pacific fish to evaluate the possibility of these being used in combination as detection tools.

## 3.2 Materials and methods

### 3.2.1 *Fish protein extraction and parvalbumin purification*

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

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 Figure 3.2 as well as popular consumption. These extracts were dialysed against 25 mM Tris pH 8.0 and 1mL of the dialysed extract loaded on to a 5mL DEAE sepharose anion exchange column (BioRad Laboratories, Hercules, USA). Fractions were eluted with a linear salt gradient of 25mM Tris and 1M NaCl pH 8.0. A constant flow rate was set at 1.5mL per minute using BioRad DuoFlow system. Elution profile was generated using UV214 with BioRad QuadTech UV spectrophotometer.

**Table 3.1** List of fish species used in this study

Common Name	Species	Family	Order
Sturgeon	<i>Acipenser persicus</i>	Acipenseridae	Acipenseriformes
Orange Roughy	<i>Hoplostethus atlanticus</i>	Trachichthyidae	Beryciformes
Blacktip Shark	<i>Carcharhinus limbatus</i>	Carcharhinidae	Carcharhiniformes
Gummy Shark	<i>Mustelus antarcticus</i>	Triakidae	Carcharhiniformes
Elephant Shark	<i>Callorhynchus milii</i>	Callorhynchidae	Chimeraformes
Pacific Pilchard	<i>Sardinops sagax</i>	Clupeidae	Clupeiformes
Canned Sardine	<i>Sardinops Sp.</i>	Clupeidae	Clupeiformes
Cod	<i>Gadus morhua</i>	Gadidae	Gadiformes
Blue Grenadier	<i>Macruronus novaezelandiae</i>	Gadidae	Gadiformes
Milk fish	<i>Chanos chanos</i>	Chanidae	Gonorynchiformes
Sparsely Spotted Stingaree	<i>Urolophus paucimaculatus</i>	Urophidae	Myliobatiformes
Ling	<i>Genypterus blacodes</i>	Ophidiidae	Ophidiiformes
Barramundi	<i>Lates calcarifer</i>	Latidae	Perciformes
Blue Eye Trevella	<i>Hyperoglyphe antarctica</i>	Centrolophidae	Perciformes
Blue Theadfin	<i>Eleutheronema tetradactylum</i>	Polynemidae	Perciformes
Coral Trout	<i>Plectropomus leopardus</i>	Serranidae	Perciformes
Gemfish	<i>Rexea solandri</i>	Gempylidae	Perciformes
Grunter Bream	<i>Pomadasyd sp.</i>	Haemulinae	Perciformes
Jewel	<i>Argyrosomus japonicus</i>	Sciaenidae	Perciformes
Mahi Mahi	<i>Coryphaena hippurus</i>	Coryphaenidae	Perciformes
Nile Perch	<i>Lates niloticus</i>	Latidae	Perciformes
Red Mullet	<i>Mullus barbatus</i>	Mullidae	Perciformes
Red Throat Emperor	<i>Lethrinus miniatus</i>	Lethrinidae	Perciformes
Scad	<i>Atule mate</i>	Carangidae	Perciformes
Silver Bream	<i>Acanthopagrus australis</i>	Sparidae	Perciformes
Sweetlip	<i>Lethrinus sp.</i>	Lethrinidae	Perciformes
Spanish Mackerel	<i>Scomberomorus maculatus</i>	Scombridae	Perciformes
Stripped Snapper	<i>Lutjanus kasmira</i>	Lutjanidae	Perciformes
Swordfish	<i>Xiphias gladius</i>	Xiphiidae	Perciformes
Trevally	<i>Caranx sp.</i>	Carangidae	Perciformes
Whiting	<i>Sillago sp.</i>	Sillaginidae	Perciformes
Yellow Fin Tuna	<i>Thunnus albacares</i>	Scombridae	Perciformes
Canned Tuna	<i>Thunnus albacares</i>	Scombridae	Perciformes
Flounder	<i>Paralichthys olivaceus</i>	Paralichthyidae	Pleuronectiformes
Sole	<i>Paraplagusia bilineata</i>	Cynoglossidae	Pleuronectiformes
Atlantic Salmon	<i>Salmo Salar</i>	Salmonidae	Salmoniformes
Rainbow trout	<i>Oncorhynchus mykiss</i>	Salmonidae	Salmoniformes
Canned Pink Salmon	<i>Salmo Salar</i>	Salmonidae	Salmoniformes
Canned Red Salmon	<i>Salmo Salar</i>	Salmonidae	Salmoniformes
Canned Tasmanian Salmon	<i>Salmo Salar</i>	Salmonidae	Salmoniformes
Gurnard	<i>Chelidonichthys cuculus</i>	Triglidae	Scorpaeniformes
Basa/catfish	<i>Pangasius bocourti</i>	Pangasiidae	Siluriformes
Monkfish	<i>Squatina australis</i>	Squatinae	Squatinae
Leather Jacket	<i>Eubalichthys mosaicus</i>	Monacanthidae	Tetraodontiformes
Dory	<i>Cyttus sp.</i>	Cyttidae	Zeiformes
Black Tiger Prawn			
Chicken		<i>Non-fish controls</i>	
Pork			

Fractions were collected for all peaks throughout the gradient and subsequently analysed by electrophoresis and immunoblotting. 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 Figure 3.2 as well as popular consumption. These extracts were dialysed against 25 mM Tris pH 8.0 and 1mL of the dialysed extract loaded on to a 5 mL DEAE sepharose anion exchange column (BioRad Laboratories, Hercules, USA). Fractions were eluted with a linear salt gradient of 25mM Tris and 1M NaCl pH 8.0. A constant flow rate was set at 1.5 mL per minute using BioRad DuoFlow system. Elution profile was generated using UV214 with BioRad QuadTech UV spectrophotometer. Fractions were collected for all peaks throughout the gradient and subsequently analysed by electrophoresis and immunoblotting. Parvalbumin containing peaks were pooled, dialysed against 100 mM ammonium bicarbonate, lyophilized and stored at -20°C until further use.

### ***3.2.2 Production and purification of polyclonal anti-parvalbumin antibodies***

Polyclonal antiserum was raised against the purified parvalbumins by injecting two rabbits per antigen subcutaneously with 500µg of antigen in Freund's complete adjuvant. Further doses of 500µg in Freund's incomplete adjuvant were given at week 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.

### 3.2.3 *Polyclonal antibody inter-antigen 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 barramundi, 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 1h 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 mins. Wells were 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 1M Hydrochloric acid and the absorbance measured at 450 nm. The percent inhibition was calculated as  $100 + \left[ \frac{\text{O.D. 450 nm of antibody with inhibitor}}{\text{O.D. 450 nm of antibody without inhibitor}} \right] + 100$ .

### 3.2.4 *Antibody screening of fish protein extracts*

Protein profiles for raw and heated fish extracts were obtained using SDS-PAGE. Fish proteins (10 µg) were diluted in 5X sample buffer containing Dithiothreitol 14 mM (DTT), heated for 5 mins 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 mins. 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 (BioRad) at 11 V for 15 mins. 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 (Antibodies were pooled according to their respective antigen as there were two rabbit per antibody). Membranes were washed 3x with PBS-T and incubated with anti-rabbit IgG HRP conjugate diluted 1:20,000 in 1% skim milk (Promega, USA). Membranes were washed a final 3x with PBS-T and antibody reactivity was visualized using ECL method.

Further screening of the fish protein extracts involved an indirect ELISA. Wells of ELISA plates (96 well) were coated with 5  $\mu$ g of each protein extract in triplicates in carbonate buffer, pH 9.6, overnight at 4°C. Plates were then blocked with 5% skim milk in PBS-T and washed 3 times with PBS-T. Plates were then incubated with incubated with the rabbit anti-fish parvalbumin polyclonal antibodies diluted in 1% skim in PBS-T at a concentration of 200 ng/mL for 1 h at room temperature and washed another 3x. Plates were finally incubated with anti-rabbit IgG HRP conjugate diluted 1:20,000 in 1% skim milk (Promega, USA) for 30 mins then subsequently washed 5x with PBS-T and the assays were 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. Cut-off values were determined using the mean of 3 non-fish extracts plus 2 times the standard deviation.

### 3.2.5 *Immunoblot Analysis*

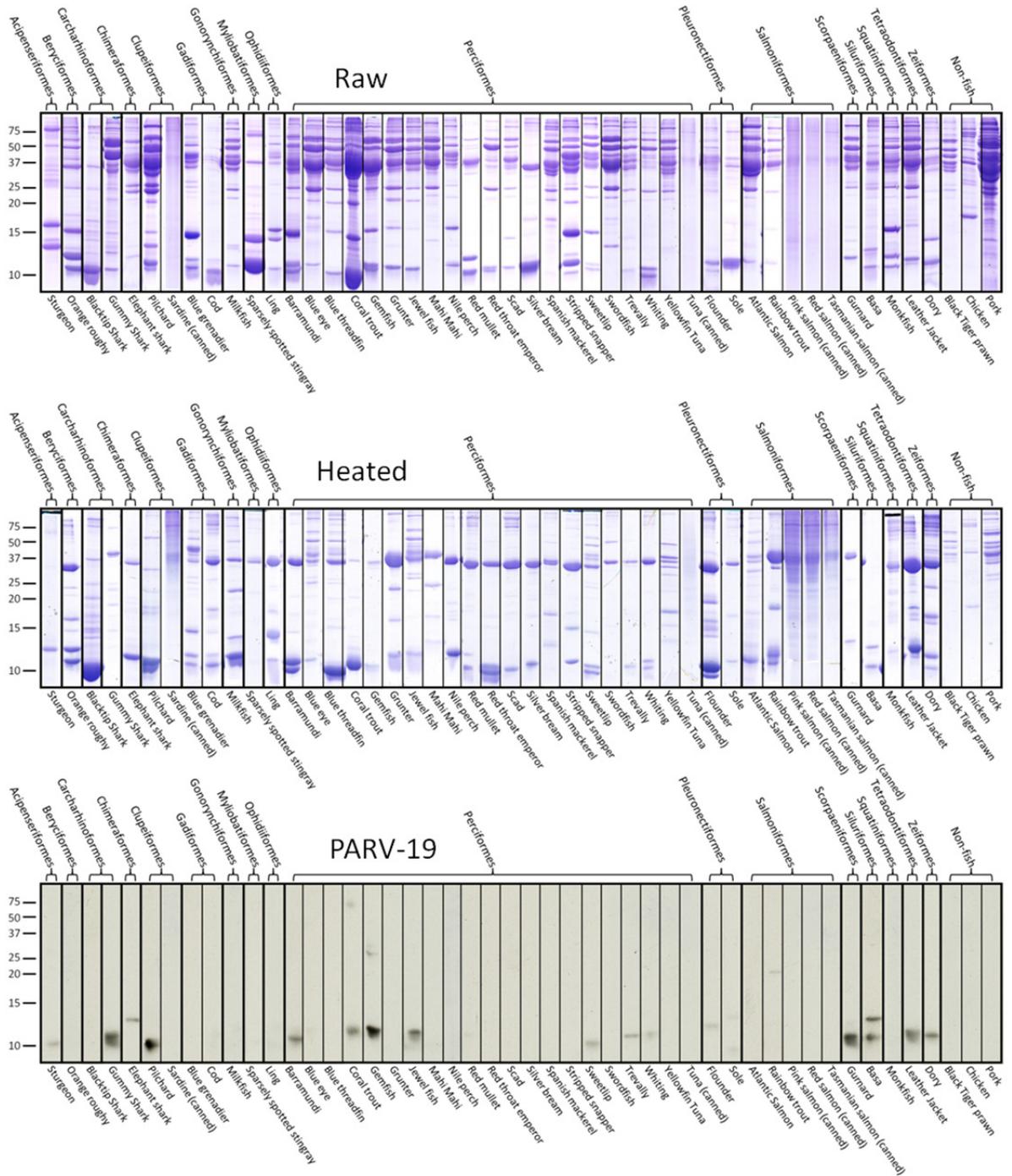
The binding capacity of the polyclonal antibodies on immunoblots was determined by comparing densitometric values of each band against the antigen homologous to the

antibody. Densitometric analysis was performed using TotalLab Quant. Strong, moderate and weak binding were determined by values greater than 80%, greater than 40% and less than 40% of the homologous antigen value, respectively.

### 3.3 Results

#### 3.3.1 *Fish extract analysis*

Proteins present in raw and heat-processed extracts from 45 diverse fish sources local to the Asia-Pacific region (see Table 1) were separated using SDS-PAGE (Figure 3.1). Protein profiles differ between each raw fish extract, in particular the 10-15 kDa region. Twenty-eight raw extracts have two or more bands in this region, whereas 11 show only one band. All canned fish show no distinct bands and Spanish mackerel is the only other fish to have no bands in this region even in the raw extract. The profile for the higher molecular weight protein bands in the range of 37-75 kDa was similar for most fish species. Heating of the fish extracts denatured many heat labile proteins although all fish extracts showed the presence of heat stable proteins of about 37 kDa and most showed heat stable proteins at 10-15 kDa. Eight protein extracts showed no bands in the 10-15 kDa region including the sparsely spotted stingaree, yellowfin tuna and swordfish, all of which had bands in this region in the raw extracts. Interestingly a band was present in heated Spanish mackerel extract. This band may be visible now due to the concentrating effect of heat treatment on heat stable proteins. No distinct bands can be seen in any of the canned extracts. Furthermore, only 18 fish species had parvalbumin detected by PARV-19, with basa being the only species with multiple (two) parvalbumin isoforms. The PARV-19 immunoblot also detected oligomeric parvalbumin in gemfish and rainbow trout. Complete amino acid sequences are only known for 10 out of the 40 fish species analysed, demonstrating the diversity of the fish analysed in this study.



**Figure 3.1** Coomassie stained SDS-PAGE profile of all 45 raw (A) and heated (B) fish muscle extracts from 17 different fish orders. C) PARV-19 anti-parvalbumin immunoblots against all raw extracts. Three non-fish (black tiger prawn, chicken and pork) were used as negative controls.

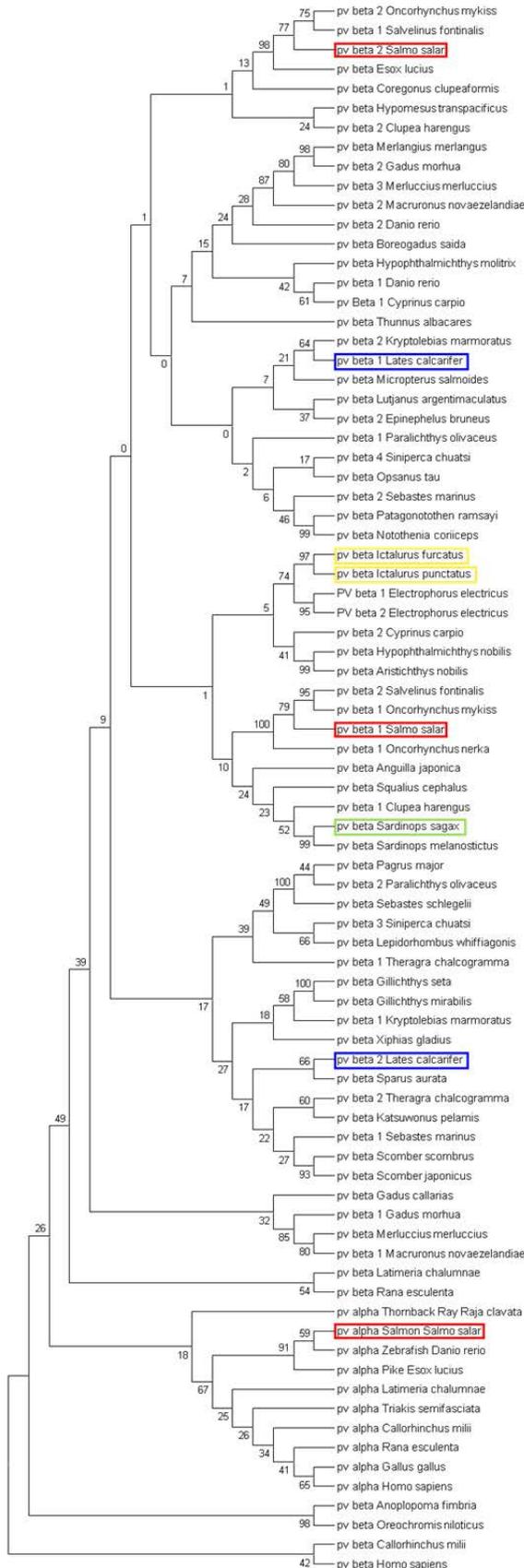
### 3.3.2 *Antigen selection and purification*

The selection of antigens for the generation of antibodies was performed using multiple criteria. Firstly, there must be distant phylogenetic relationships between fish parvalbumins as seen in Figure 3.2, to cover even distantly related groups. Secondly, the fish must be commonly consumed in the Asia-pacific region. Lastly the fish had to be from different taxonomic orders. The optimum species fulfilling these criteria are barramundi (*Lates calcarifer*), basa (Catfish; *Pangasius bocourti*), pilchard (*Sardinops sagax*) and Atlantic salmon (*Salmo salar*). The purification of these parvalbumins was performed using anion exchange chromatography. Eluted barramundi and basa parvalbumin isoforms showed similar ionic properties as they were the first proteins eluted along the salt gradient (Figure 3.3. Ai and ii). This may be due to pilchard parvalbumin having a higher isoelectric point of 6.0, greater than most other known beta-parvalbumins. In contrast, Atlantic salmon parvalbumin was the only heated protein from the salmon extract to bind to the column. Two clear parvalbumin isoforms were purified from barramundi and basa and only one visible parvalbumin from both pilchard and Atlantic salmon (Figure 3.3 B).

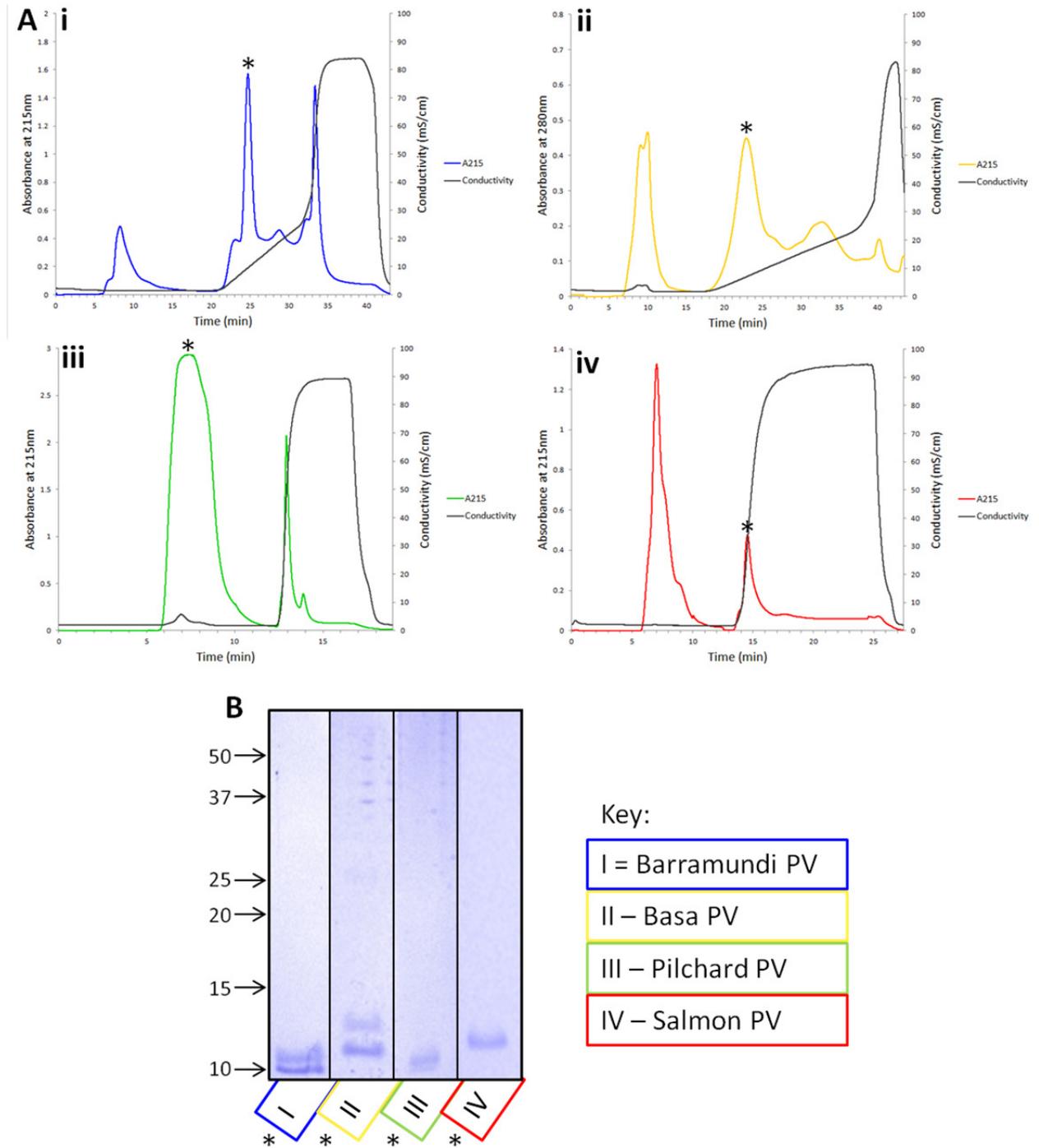
### 3.3.3 *Cross reactivity of generated polyclonal antibodies*

Polyclonal antibodies were generated in rabbits against parvalbumin from barramundi, basa, pilchard and salmon. Inhibition ELISA's were performed using these antibodies to evaluate their cross-reactivity between the four purified antigens (Figure 3.4). The anti-barramundi antibodies appear to be the most cross-reactive as all the antigens reached 50% inhibition in the assays. The next most cross-reactive were the anti-basa antibodies which were inhibited by pilchard and salmon parvalbumin, however barramundi parvalbumin was unable to reach 50% inhibition Only salmon parvalbumin reached

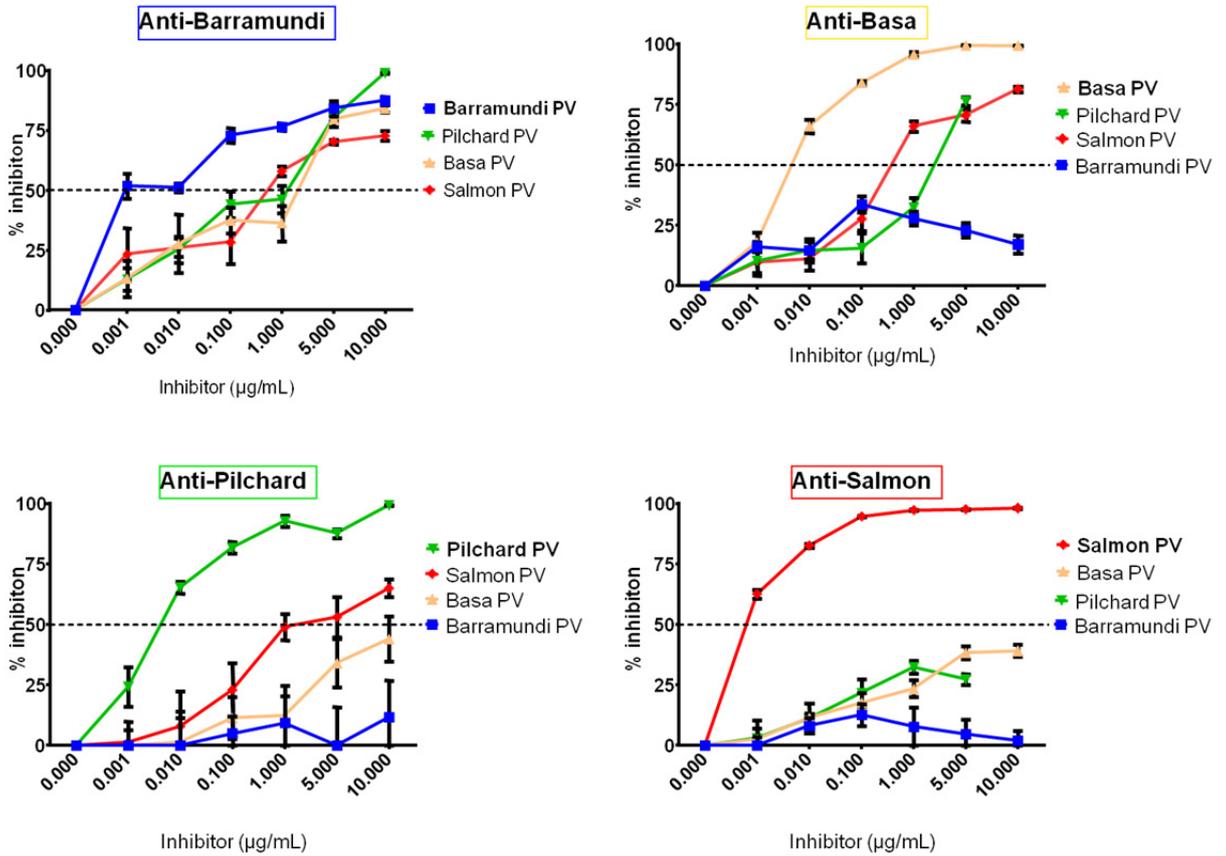
50% inhibition against the anti-pilchard antibodies. Interestingly no heterologous parvalbumin was able to inhibit the anti-salmon antibodies.



**Figure 3.2.** The evolutionary history of a selection of fish parvalbumin was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 8.16467625 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10000 replicates) is shown next to the branches. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 77 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 100 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. Isoforms used to raise antibodies boxed. Barramundi = blue, Pilchard = green, Salmon = red and the two closest relatives to Basa, blue and channel catfish = yellow.



**Figure 3.3** A Purification chromatograms of parvalbumin from barramundi (i), basa (ii), pilchard (iii) and Atlantic salmon (iv) heated extracts using anion exchange chromatography. B Peaks containing pure parvalbumin (\*) were separated by SDS-PAGE and stained with Coomassie blue.



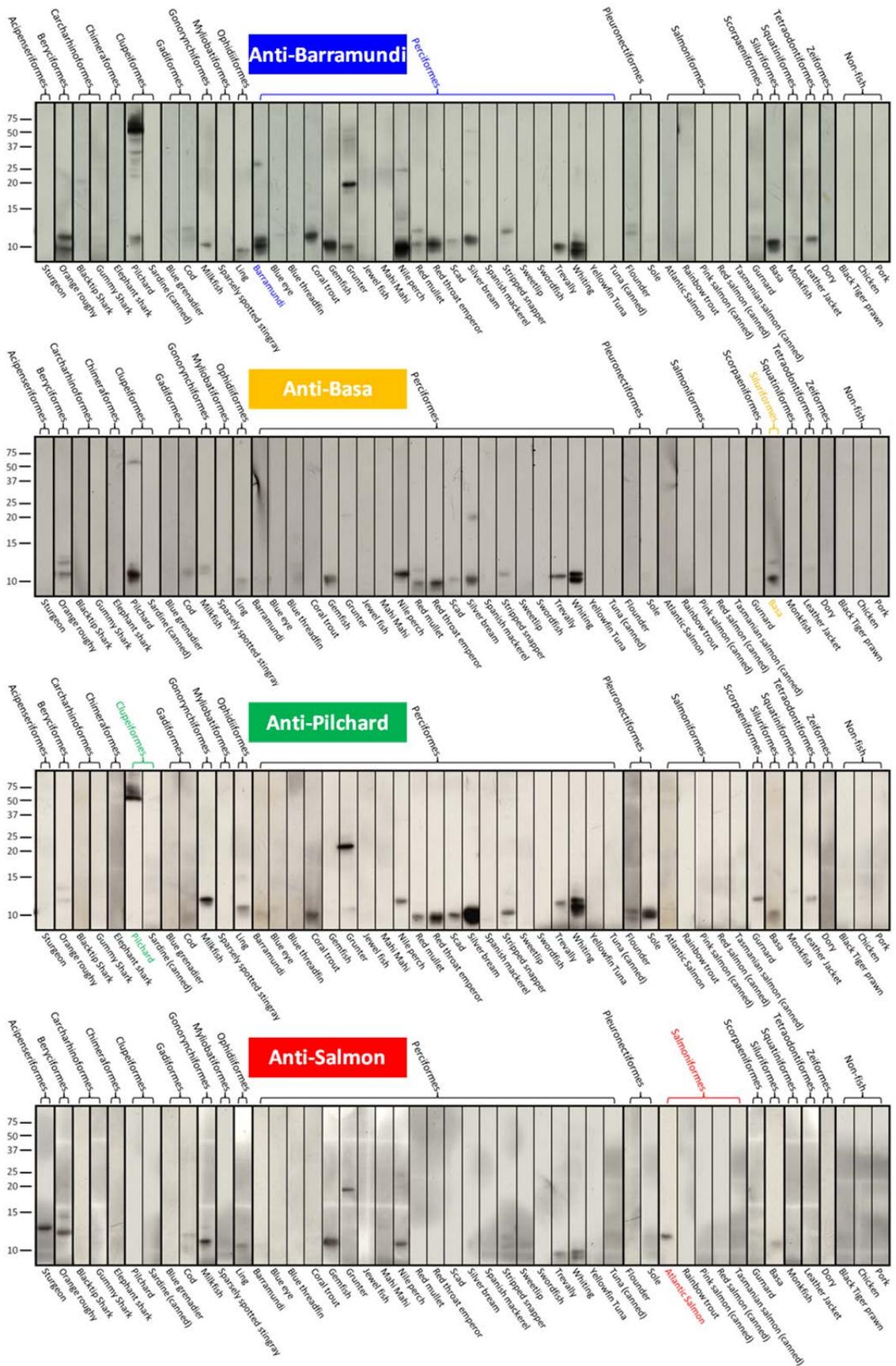
**Figure 3.4** Inhibition ELISA’s for the evaluation of the generated anti-parvalbumin polyclonal antibodies cross-reactivity against the four antigens they were raised against. Error bars demonstrate the standard error of the mean of the three replicates from the two rabbit immunized per antigen.

### 3.3.4 Antibody reactivity against fish extract panel

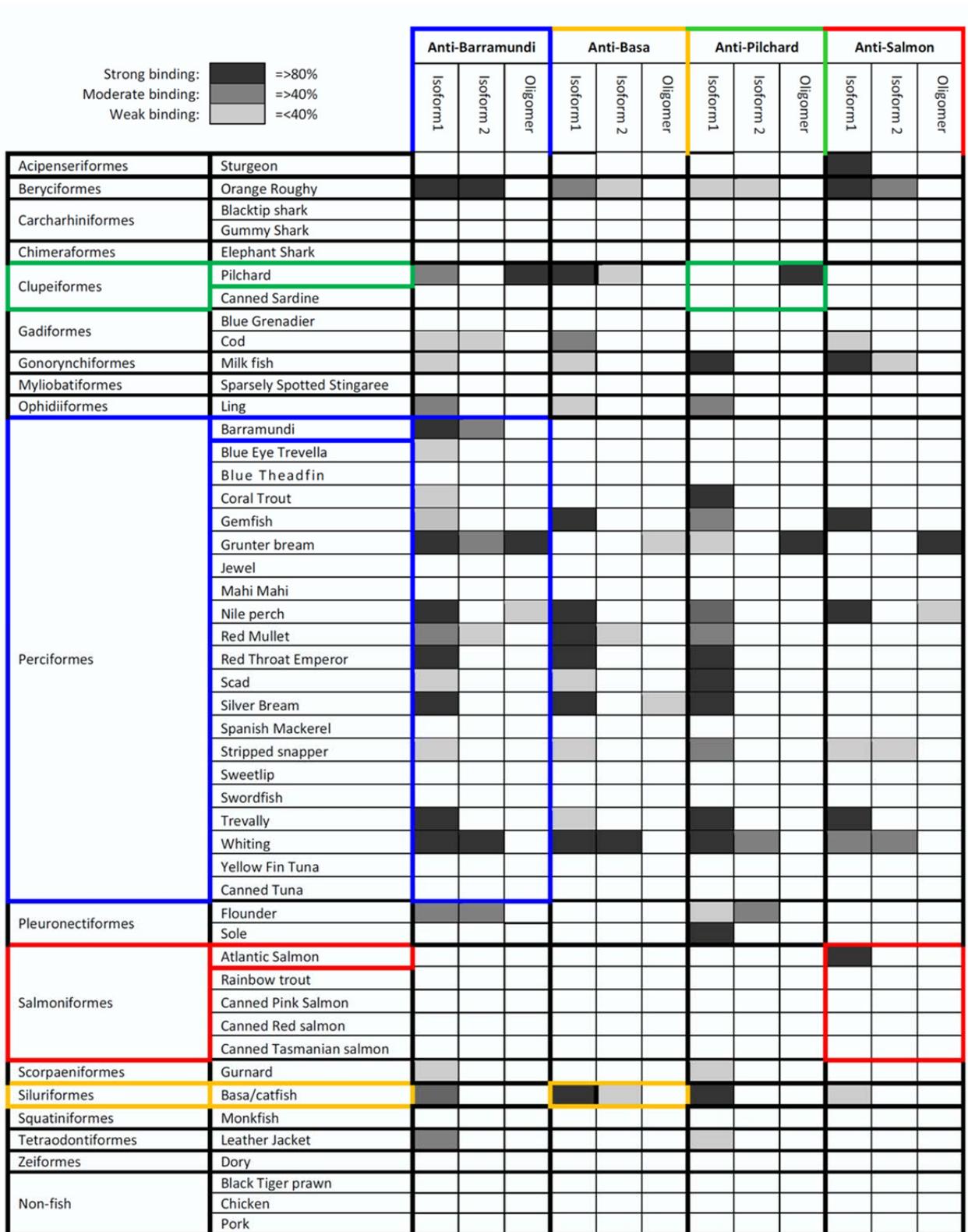
Specific binding strengths between the antibodies and different fish extracts are summarized in Figure 3.6. The anti-barramundi antibody was the most reactive as it detected 22/45 extracts followed by anti-pilchard (20/45), anti-basa (16/45) and salmon (12/45). Predictably, anti-barramundi parvalbumin detects the most Perciformes parvalbumin (13/21), as barramundi is a Perciformes. Only six fish were detected by all the antibodies including orange roughy, milkfish, grunter bream, stripped snapper, whiting and basa. Inversely, 20 fish extracts were not detected by any of the antibodies, including the five canned fish extracts, large Perciformes (mahi mahi, Spanish

mackerel, swordfish and yellowfin tuna) as well as the five cartilaginous fish (blacktip shark, gummy shark, elephant shark, sparsely spotted stingaree and monkfish). The anti-barramundi and anti-pilchard antibodies demonstrated similar binding profiles, with both reacting synonymously to 18 fish species and the only antibodies to detect parvalbumin from gurnard, flounder, and leather jacket. The anti-barramundi antibody demonstrated exclusive specificity by detecting parvalbumin from barramundi and blue eye trevella extract by immunoblotting. In addition the anti-salmon antibody was the only one to detect Atlantic salmon and sturgeon and the anti-pilchard antibody the only one to detect sole.

Interestingly the generated antibodies detected multiple parvalbumin isoforms (2 bands in the 10-15 kDa range) in 10 of the 45 fish extracts analysed (Figure 3.6) Orange roughy and whiting had both of their parvalbumin isoforms detected by all the antibodies. In contrast basa parvalbumin isoform 2 was only detected by anti-basa antibodies. In addition higher molecular weight oligomeric forms of parvalbumin were also present, with parvalbumin dimers seen in grunter, Nile perch and silver bream and a tetramer present in pilchard. Interestingly the anti-pilchard antibody only recognizes tetrameric parvalbumin from raw pilchard extract, however only dimeric and monomeric parvalbumin is detected in the heated pilchard extract (Figure 3.7).

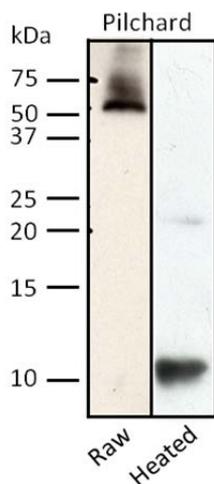


**Figure 3.5** Immunoblots using anti-barramundi (blue), anti-basa (yellow), anti-pilchard (green) and anti-salmon (red) polyclonal antibodies against all raw fish extracts. Three non-fish (black tiger prawn, chicken and pork) were used as negative controls.



**Figure 3.6** Allergogram of all raw extract immunoblots grading the strength of binding to isoform 1 (lowest molecular weight), isoform 2 (higher molecular weight) and oligomer (weight above 20 kDa). Binding grading was determined by comparing

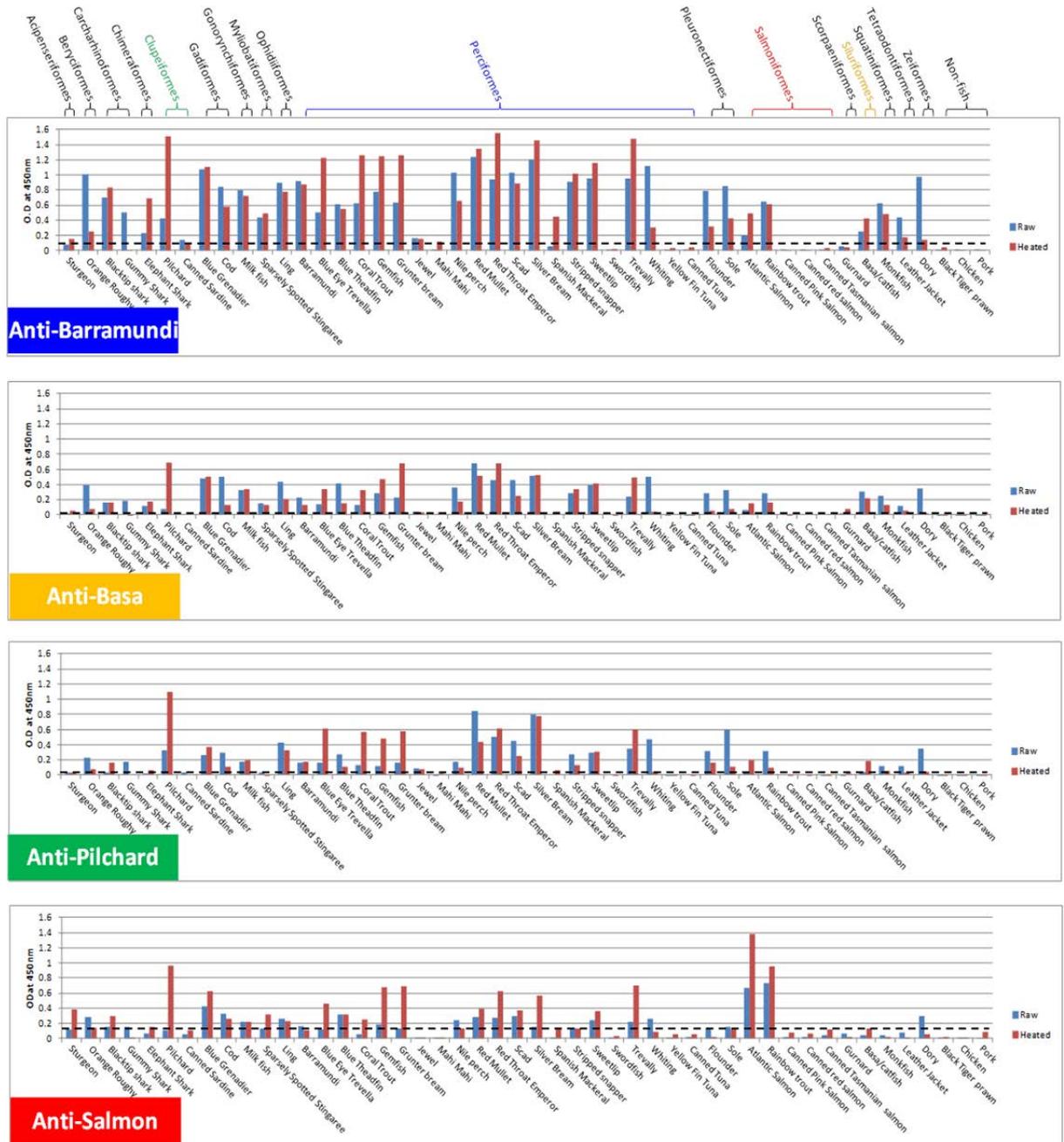
densitometric values of each band against the antigen homologous to the antibody. Strong, moderate and weak binding were determined by values greater than 80%, greater than 40% and less than 40% of the homologous antigen value, and displayed in black, grey and light grey, respectively. Boxes are colour coded according to the respective orders of the antibodies homologous antigen source.



**Figure 3.7** Immunoblot with anti-pilchard parvalbumin antibody against both raw and heated pilchard extract.

The indirect ELISA's were performed to evaluate the cross-reactive properties of the generated anti-parvalbumin antibodies against native protein extracts from all the fish analysed (Figure 3.8). The anti-barramundi parvalbumin antibodies were the most cross-reactive out of the four antibodies, detecting parvalbumin in 35 and 34 out of 45 raw and heated extracts, respectively. The next most cross reactive antibody was anti-basa with 33 raw and 28 heated detected, followed by the anti-pilchard antibody with 29 raw and heated and the anti-salmon reacting with 17 raw and 21 heated extracts. Based on these findings we cannot conclude that heat treatment will decrease or increase the four antibodies reactivity to parvalbumin in ELISAs. However heating did affect the reactivity to specific species. For example, heated pilchard, gemfish, grunter bream, coral trout, trevally, blue eye trevella and Atlantic salmon were detected by all the antibodies and demonstrated stronger binding to heated than raw (Table 3.2 Figure 3.8). In contrast, binding was lost after heating orange roughy, whiting and dory. The anti-salmon antibody was far more specific than the other antibodies, with only strongly

binding to Atlantic salmon, rainbow trout (both are salmoniformes) and heated pilchard. Interestingly, this antibody did not bind to rainbow trout by immunoblotting, indicating conformational changes impacting on antibody reactivity. Other fish extracts demonstrated similarly increased reactivity in the ELISA analysis. For instance, raw and heated extracts were exclusively detected from blacktip shark, elephant shark, blue grenadier, sparsely spotted stingaree, blue threadfin, jewel, sweet lip, rainbow trout, monkfish, gummy shark (raw only), Dory (raw only) and Spanish mackerel (heat only). In contrast, Gurnard was not detected by ELISA, however detected weakly by anti-barramundi and anti-pilchard antibody in the immunoblot analysis.



**Figure 3.8** Indirect ELISA’s using anti-barramundi, anti-basa, anti-pilchard and anti-salmon polyclonal antibodies against all raw and heated fish extracts. The cut-off is derived from two standard deviations above the mean reactivity of 3 non-fish extracts and indicated by the dotted line (O.D.: Anti-barramundi=0.08, Anti-basa=0.00, Anti-pilchard=0.00 and anti-salmon=0.11). n=1.

**Table 3.2** Analysis of raw and heated extracts from the indirect ELISA. Ticks represent greater binding to raw, heated, no difference (neutral) or negative binding.

	Anti-barramundi				Anti-basa				Anti-pilchard				Anti-salmon			
	raw	heated	neutral	negative	raw	heated	neutral	negative	raw	heated	neutral	negative	raw	heated	neutral	negative
Sturgeon		✓						✓				✓		✓		
Orange roughy	✓						✓	✓			✓	✓			✓	
Blacktip shark			✓				✓	✓			✓	✓			✓	
Gummy shark	✓							✓	✓							
Elephant shark			✓				✓	✓			✓	✓				✓
Pilchard		✓				✓				✓				✓		
Canned sardine				✓				✓				✓				✓
Blue grenadier			✓				✓	✓			✓	✓				
Cod			✓				✓	✓			✓	✓				
Milk fish			✓				✓	✓			✓	✓				
Sparsely spotted stingaree			✓				✓	✓				✓				
Ling			✓				✓	✓			✓	✓				
Barramundi			✓				✓	✓			✓	✓	✓			
Blue eye trevella		✓					✓	✓			✓	✓		✓		
Blue threadfin			✓				✓	✓			✓	✓			✓	
Coral trout		✓				✓				✓	✓				✓	
Gemfish			✓				✓	✓			✓	✓			✓	
Grunter bream		✓				✓					✓	✓			✓	
Jewel			✓					✓			✓	✓				✓
Mahi mahi				✓				✓				✓				✓
Nile perch			✓				✓	✓			✓	✓			✓	
Red mullet			✓				✓	✓			✓	✓			✓	
Red throat emperor		✓					✓	✓			✓	✓		✓		
Scad			✓				✓	✓			✓	✓			✓	
Silver bream			✓				✓	✓			✓	✓			✓	
Spanish mackerel		✓						✓			✓	✓				✓
Stripped snapper			✓				✓	✓			✓	✓				✓
Sweetlip			✓				✓	✓			✓	✓			✓	
Swordfish				✓				✓			✓	✓				✓
Trevally		✓				✓					✓	✓		✓		
Whiting	✓				✓					✓			✓			
Yellow fin tuna				✓				✓				✓				✓
Canned tuna				✓				✓				✓				✓
Flounder			✓				✓	✓			✓	✓				✓
Sole			✓				✓	✓			✓	✓				✓
Atlantic salmon		✓				✓				✓	✓			✓		
Rainbow trout			✓				✓	✓			✓	✓			✓	
Canned pink salmon				✓				✓				✓				✓
Canned red salmon				✓				✓				✓				✓
Canned Tasmanian salmon				✓				✓				✓				✓
Gurnard				✓			✓	✓			✓	✓				✓
Basa/catfish			✓				✓	✓			✓	✓				✓
Monkfish			✓				✓	✓			✓	✓				✓
Leather jacket			✓				✓	✓			✓	✓				✓
Dory	✓						✓	✓			✓	✓	✓			
Black tiger prawn				✓				✓				✓				✓
Chicken				✓				✓				✓				✓
Pork				✓				✓				✓				✓

### 3.4 Discussion

Parvalbumin, the major allergen from fish is a diverse protein that is expressed in many forms in different fish species [12, 141, 159]. The diversity of these highly allergenic parvalbumin proteins has proven it difficult to be detected by any one single cross-reactive antibody. In the present study, four polyclonal antibodies were generated against purified parvalbumin from barramundi, basa, pilchard and Atlantic salmon and were evaluated for their cross-reactivity against a diverse collection of fish species.

Immunoblotting analysis using PARV-19 against the present group of fish extract demonstrated the need of multiple anti-parvalbumin antibodies as PARV-19 detected only 18 out of the 45 raw extracts. The selection of the four antigens for the antibody production was based on the molecular phylogenetic tree. These antigens coming from four distinct orders of fish, Perciformes (barramundi), Siluriformes (catfish), Clupeiformes (pilchard) and Salmoniformes (Atlantic salmon). Parvalbumins from Atlantic salmon (2 isoforms), pilchard (1 isoform) and barramundi (2 isoforms) have previously been characterised as allergens [11, 77, 153] and SDS-PAGE profiles demonstrate the presence of 2 isoforms from basa. Inter-antigen cross-reactivity analysis demonstrated that the anti-barramundi antibodies were the most cross-reactive. The phylogenetic tree shows the 2 barramundi isoforms being the most distantly related from each other, suggesting that the specific polyclonal antibodies raised may have the greatest cross-reactivity. The next most diverse antibody should be the Atlantic salmon isoforms, however the anti-salmon antibodies were surprisingly the least cross-reactive. These findings are supported by clinical studies where fish allergic patients can be monosensitive to Salmonids [90, 91, 160], which could attributed to salmon parvalbumin having a different IgE epitope at the N-terminal of parvalbumin [76, 141, 160]. This phenomenon may also be occurring for the rabbit antibodies analysed in this

study. The most antigenic region of salmon parvalbumin could be the N-terminal region, which is known to be the least conserved region as it does not contain any calcium binding sites and subsequently possible generating a very specific antibody [154]. The anti-salmon antibody reacts therefore strongly to heated pilchard extract in the indirect ELISA, correlating with the close phylogenetic relationship between  $\beta 1$  salmon parvalbumin and pilchard parvalbumin.

Analyzing the fish extracts by ELISA using the generated polyclonal antibodies demonstrated binding to nearly all species, excluding mahi mahi, swordfish, yellowfin tuna and the five canned fish extracts. Parvalbumin from Spanish mackerel was only detected by the indirect ELISA method using the anti-barramundi antibody against heat extract. Mahi mahi, Spanish mackerel, swordfish and yellowfin tuna are all large perciformes and as demonstrated by SDS-electrophoresis, they express little or no visible proteins in the 10-15 kDa region. This suggests that the lack of antibody binding to these fish may be due to low expression levels rather than a lack of cross-reactivity. It has been previously demonstrated that swordfish, mackerel and tuna express less parvalbumin than other fish [105, 108] and is due to their high content of dark muscle which contains less parvalbumin [33]. A recent study by Lee et al [157] validated our findings for mahi mahi as the polyclonal antibodies was also unable to detect mahi mahi parvalbumin, probably due to low expression levels.

Some fish seem to have heat labile parvalbumin, including Orange roughy, gummy shark, whiting, dory, sole and flounder, as their ELISA signals were all decreased after heat treatment.

In addition some parvalbumin proteins seem to have conformational epitopes, demonstrated by the lack of immunoblot binding despite having good signals for raw

and heated extract by ELISA and included blacktip shark, elephant shark, sparsely spotted stingaree, blue grenadier, blue threadfin, Spanish mackerel, sweetlip, rainbow trout and monkfish.

Raw pilchard extract demonstrated high molecular weight parvalbumin oligomers (possibly a tetramer) and is detected by anti-barramundi, anti-basa and anti-pilchard antibodies. Interestingly, after heating tetrameric pilchard parvalbumin was reduced to the monomeric form and a low binding dimer at about 24 kDa. Pilchard parvalbumin has previously been shown by Beale *et al.* [11] and Saptarshi *et al.* [154] (Chapter 2) to have oligomeric parvalbumins that were reduced to dimers and monomers after heating. Some fish species were not detected by any antibodies in ELISA or by immunoblotting and included mahi mahi, swordfish, yellowfin tuna and the five canned fish.

It is well known that the process of canning subjects the fish to extreme heating and pressure, which leads to degradation of protein [161, 162]. This is evident by the SDS-PAGE profiles of the five canned fish extracts, which demonstrated no distinct protein bands. The canning process has been previously shown to decrease also patient IgE antibody binding due to protein degradation [162], which is also evident in our experiments where all four polyclonal IgG antibodies are unable to detect parvalbumin in the canned extracts, even though two of the antibodies were raised against the specific fish species, pilchard and salmon. A different approach must be used to detect allergens from canned fish with antibodies, such as described in Lopata *et al* [139], to be raised against canned pilchard which in turn reacted to canned pilchard extract. Heated treated pilchard parvalbumin retains its allergenicity as demonstrated in the subsequent murine model developed [15].

While most edible fish species belong to the group of bony fish, several cartilaginous species are consumed in the Asian-Pacific region including the analysed Blacktip shark, elephant shark, gummy shark, sparsely spotted stingaree and monkfish. However reports on allergenic parvalbumin from cartilaginous fish are limited [148, 149] and attempts to detect parvalbumin from cartilaginous fish with cross-reacting antibodies not being very successful [154]. As expected all five cartilaginous fish species were not detected by immunoblotting, however all were detected by the indirect ELISA method, demonstrating that antibody binding to parvalbumin was lost under denaturing conditions. Cartilaginous fish parvalbumin, which mostly is of the alpha-lineage and is considered to have significantly different biophysical properties to bony fish parvalbumin, may have led to the lower antibody binding observed [141, 150]. Excluding cartilaginous fish and the canned fish extracts leaves only the bony fish extracts. All antibodies were effective in detecting parvalbumin from bony fish. In particular, the anti-barramundi antibodies which detected 32 out of 35 bony fish, these were followed by anti-basa (28/35), anti-pilchard (28/35) and the least cross-reactive anti-salmon (24/35). This demonstrates that these antibodies greatest application may be to detect bony-fish parvalbumin.

In this study, it has been demonstrated that the generation of highly cross-reactive anti-parvalbumin antibodies can be used for the characterisation allergenic fish parvalbumin and its detection in contaminated food products. All analysed 40 fish species across 17 orders, except mahi mahi, swordfish and yellowfin tuna had parvalbumin detected in raw extracts (92.5% of all species analysed). Furthermore, antibody reactivity to several fish seems to be susceptible to denaturation of allergens during heating, demonstrating that these parvalbumins have most likely conformational epitopes, which lose antibody reactivity after heat treatment. The lack of detecting parvalbumins in some species is

probably due to the low expression of this protein or being of the alpha-parvalbumin lineage as for most cartilaginous fish. This cross-reactivity study also shows that processing of fish, especially canning, can have an impact on antibody recognition in ELISA, possibly similar to IgE binding *in vivo*.

### **3.4.1 Future directions**

In this chapter, 40 Australasian fish parvalbumins were screened for cross-reactivity with four anti-parvalbumin antibodies. The allergens in many of these fish have not been characterised, even though some are commonly consumed in the region. One fish in particular, barramundi (*L. calcarifer*), is commonly eaten in South East Asia and Australia and is known to have IgE reactive proteins that are yet to be characterised [3]. This chapter also demonstrated that antibodies against barramundi parvalbumin were highly cross reactive, furthering the interest in allergens from barramundi. In chapter 4, allergens from barramundi will be characterised using a fish allergic cohort of paediatric and adults patients.

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## CHAPTER 4

### Characterisation and identification of two different novel allergenic parvalbumin isoforms from Barramundi

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<b>Details of publication on which this chapter is based</b>	<b>Nature and extent of the intellectual input of each author, including the candidate</b>
Sharp M. F., Kamath S. D., Koeberl M., Jerry D. R., O’Hehir R. E., Campbell D. E. and Lopata A. L., 2014. Differential IgE binding to isoallergens from Asian seabass ( <i>Lates calcarifer</i> ) in children and adults. Mol. Immunol. 62, 77-85	The authors Sharp, Jerry, Campbell and Lopata co-developed the research question. Sharp collected the data and performed the data analyses with assistance from Kamath and Koeberl. Serum samples were collected by Campbell and O’Hehir. Sharp wrote the first draft of the paper, which was revised with editorial input from Campbell and Lopata. Sharp developed the figures and tables.

## 4.1 Introduction

Consumption of fish and its products is growing globally due to its nutritional value and global cultural diversity. This has however contributed to an increased incidence of allergic reactions to fish [141] on the background of an overall significant increase in food allergy prevalence over the past two decades [163]. Fish Allergy is one of the eight most common food allergies with prevalence rates in the general population ranging between 0.2% and 2.3% [7, 10]. Unlike food allergy to milk and egg, fish allergy is rarely outgrown and may be a more common food allergy in adults than children [7, 22, 23]. Additionally, fish allergy, and allergen avoidance impacts negatively on the quality of life of fish allergic individuals and has been shown to result in anxiety and stress in families of affected children [3].

Individuals with fish allergy may be allergic to a defined species or to many species of fish, and it is therefore of significant clinical importance to be able to define and characterize major allergens amongst and within specific fish species. Comparative IgE reactivity between adults and children to selected fish allergens has not been previously investigated.

The major allergens characterised in bony fish are the calcium binding muscle protein parvalbumins, which are small, heat stable proteins with molecular weights ranging from 10-15 kDa. Parvalbumins appear to occur as two genetically distinct lineages, alpha and beta [84], which can differ up to 36% in amino acid sequence, such as described in Atlantic salmon [77]. How these differences might influence allergenicity has not been investigated. Moreover, most allergenic parvalbumins have been characterised in only European fish species, and very little is known about allergenic fish in the Asian-Pacific region [154].

The Asian seabass (*Lates calcarifer*), also known as Barramundi, is one of the most valued and consumed fish species in the Asian-Pacific region, and increasingly exported to Europe and North-America, due largely to successful aquaculture methodology [164, 165]. It is also a fish associated with clinical allergy in the Australian setting. A recent retrospective study in Australia among 2,999 children with food allergy demonstrated a prevalence of 5.6% for fish allergy, listing 'Barramundi' as one of the most frequent species implicated [133].

Barramundi parvalbumin is highly cross-reactive against parvalbumin-specific antibodies, as seen in Chapter 2 and 3. Expression of parvalbumin in barramundi has previously been investigated as a possible indicator of fish growth and it has been established that two parvalbumin isoforms are present in the muscle tissue ( $\beta 1$  and  $\beta 2$ ) [166]. Beta 1 parvalbumin is also present in other tissue such as the kidney, small intestine, skin, spleen, liver, heart, gill, eye and in high concentration in the brain [167], while  $\beta 2$  expression is limited to skeletal muscle.

The aim of this study was to identify and characterize allergenic proteins from barramundi and to compare the IgE reactivity to these major allergens in paediatric and adult fish allergic patients. Identified allergens were characterised on molecular level using proteomic and gene technology approaches and generation of recombinant isoallergens. Molecular characterisation of important food allergens is essential to improving *in vivo* and *in vitro* diagnostics approaches and for the management of individuals with fish allergy.

#### **4.1.1 Aims**

The aim of the chapter is characterize allergens from barramundi (*Lates calcarifer*) using a fish allergic patient cohort of children and adults. Fish allergic patient IgE will

be used in immunoblot and ELISA's in tandem with mass spectrometry will be used to identify allergens from barramundi. Recombinant allergens will then be generated for isolation and further analysis of the allergens.

## 4.2 Materials and methods

### 4.2.1 Patient sera

Subjects were selected on the basis on a convincing recent history of an IgE mediated reaction (including urticaria, angioedema, stridor, cough, wheeze, hypotension and/ or gastrointestinal symptoms of vomiting and/or diarrhoea) to any Australian- Asian Pacific fish which occurred within 2 hours of ingestion and confirmation of fish reactivity by prick-to-prick testing or specific IgE to tuna, salmon cod, and trout. Human ethics for this study was approved by the Sydney Children's Hospital Network ethics committee (HREC) (Table 4.1).

### 4.2.2 Fish collection

Barramundi (*Lates calcarifer*) was collected from the Aquaculture Genetics research group of the School of Marine and Tropical Biology, James Cook University, QLD Australia and subsequently filleted and stored at -80°C until further use.

### 4.2.3 Protein extraction

Raw extract was produced by homogenizing 50 g of the barramundi white muscle fillet in 100 mL of phosphate buffered saline (PBS, 10mM, pH 7.4) and extracted over 3 h with gentle tumbling at 4°C. The crude extract was 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 extract was produced by heating 50 g of white muscle at 95°C for 15 min in PBS followed by gentle tumbling at 4°C for 3 h. The crude heated extract was subjected to centrifugation at 5000 g for 30 min at 4°C and filter sterilized using 0.2 µm cellulose acetate filter membrane. Protein concentration of each extract was estimated by using the Pierce<sup>®</sup> 660 protein assay (Thermo Scientific, Rockford, USA) and extracts were aliquoted and stored at -80°C.

#### ***4.2.4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis***

Raw and heated protein extracts and purified recombinant parvalbumin isoforms were separated on a 15% polyacrylamide gel, and subsequently stained with Coomassie brilliant blue R-250 (BioRad laboratories, Hercules, USA).

Immunoblots were performed with the protein samples using anti-frog parvalbumin monoclonal antibody (mAb), PARV-19 (Sigma-Aldrich) and anti-barramundi parvalbumin polyclonal antibody (pAb) generated in rabbit (in-house). Proteins samples were separated on 0.75 mm thick 15% polyacrylamide gel and transferred to PVDF membrane using semidry electrophoresis and blocked with casein blocking buffer for 1 h at room temperature. Membranes were incubated for 1h at room temperature with the mAb and pAb in dilutions of 1:3,000 and 1:40,000 in 20% casein blocking buffer in PBS/0.05% tween (PBS-T), respectively. Membranes were washed 3-times with PBS-T and subsequently incubated for 30 min with corresponding HRP tagged anti-mouse (1:80,000) and anti-rabbit (1:40,000) IgG antibodies. Membranes were washed a further 3-times in PBS-T, blots were then incubated for 5 min with 1 mL of Pierce<sup>®</sup> Western blotting enhanced chemiluminescence (ECL) substrate (Thermo Scientific, Rockford, USA) Bands were visualized through exposure to ECL Hyperfilm (GE Healthcare Biosciences, Little Chalfont, UK) in the dark room and developed using standard X-ray film developing techniques.

Immunoblots using IgE from allergic patient sera against raw and heated protein extract and recombinant  $\beta$ 1 and  $\beta$ 2 parvalbumin isoforms were performed by separating the proteins on a 15% polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane (BioRad laboratories, Hercules, USA) using semidry electrophoresis and blocked in casein blocking buffer (Sigma-Aldrich, St. Louis, USA) for 1 h at room

temperature. Sera were diluted 1:20 in 20% casein blocking buffer in PBS-T. Membranes were placed into slot blot manifold and the diluted serum aliquoted into the slots. Membranes were incubated with sera for 16 h at 4°C and subsequently washed 6-times with 4% casein blocking buffer in PBS-T at room temperature. Membranes were then incubated with a horseradish peroxidase labeled goat anti-human IgE antibody (Genetex, Irvine, USA) diluted 1:20,000 in 20% casein blocking buffer in PBS-T for 1 h at room temperature. Membranes were washed 5-times with 4% casein blocking buffer in PBS-T at room temperature and IgE binding visualized by performing ECL technique as described above.

#### ***4.2.5 Identification of allergens by mass spectrometry***

The two IgE reactive bands were excised from the SDS gel and were destained with ammonium bicarbonate:acetonitrile, reduced with 10 mM Dithiothreitol and alkylated with 20 mM Iodoacetamide. Gels bands were washed, dried then underwent trypsin digestion (100 ng) overnight. Peptides were extracted from gel with acetonitrile:formic acid, then concentrated prior to analysis. Peptides were analysed using NanoLC system (Eksigent Ultra nanoLC system) coupled with ESI Triple TOF 5600 MS system (AB Sciex). Peptide were separate with Halo C18 column using a linear solvent gradient, with steps, from H<sub>2</sub>O:CH<sub>3</sub>CN (95:5; + 0.1% formic acid) to H<sub>2</sub>O:CH<sub>3</sub>CN (5:95; + 0.1% formic acid) with independent flow (300-400 nL/min) over an 80 min period. Analysis of LC/MS/MS data was generated using ProteinPilot v4.2 searched with Paragon method. The data was searched against *Lates* proteins (taken from SwissProt). This work was undertaken at APAF the infrastructure provided by the Australian Government through the National Collaborative Research Infrastructure Strategy (NCRIS).

#### ***4.2.6 Two-dimensional electrophoresis of raw extract***

Two-dimensional electrophoresis analysis experiment was conducted with PARV-19 mAb anti-parvalbumin immunoblotting, 160 µg of desalted and lyophilized fish protein was resuspended in 150 µL of IPG rehydration buffer (8M Urea, 2% CHAPS, 50mM Dithiothreitol (DTT), 0.2% (w/v) Biolyte 3/10 ampholytes (BioRad) and bromophenol blue. Samples were actively rehydrated into 7 cm pH 3-10 IPG strips (Bio-Rad) at room temperature for 12 h. Isoelectric focusing (IEF) was performed using a Protean IEF cell (Bio-Rad) for a total of 10,000V hours with a rapid voltage ramp. Following IEF, the IPG strips were incubated in equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol) supplemented with 2% DTT for 15 min at room temperature followed by incubation with 2.5% iodoacetamide in equilibration buffer for another 15 min at room temperature. IPG strips were placed onto a 15% polyacrylamide SDS gel, covered 0.5% agarose and separated by size at 200 V for 1 h. The protein matrix was visualized using Coomassie blue and parvalbumin was identified by immunoblotting using PARV-19 anti-parvalbumin mAb's as described in 4.2.4.

#### ***4.2.7 RNA isolation and cDNA synthesis***

RNA was extracted from 100 mg of barramundi muscle tissue according to the TRIzol® reagent protocol (Invitrogen, Carlsbad, USA). RNA extracted was quantified using the NanoDrop ND 1000 spectrophotometer (Thermo Scientific, Waltham, USA) and the first strand cDNA synthesized using the Bioline cDNA synthesis kit (Bioline, London, UK).

#### ***4.2.8 PCR amplification and sequencing***

Isoform specific forward and reverse primers were designed from previously sequenced Barramundi beta-1 and beta-2 parvalbumin with the addition of the restriction sites BamH1 and EcoR1 at placed at the sense 5' and 3' ends, respectively (beta-1 forward

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5'-GCGGGATCCGCATTCGCCGGAATCCTGA-3',      beta-1      reverse      5'-  
GCCGAATTCTTAAACCTTGACCAAGGCAGCA-3',      beta-2      forward      5'-  
CCGGGATCCGCCTTCTCAAATGTACTG-3',      beta-2      reverse      5'-  
GCGGAATTCTTATGGCTTAACCAATGC-3'). Fragments of Barramundi cDNA encoding both beta-1 and beta-2 parvalbumin were amplified by PCR using the specific primers created with GoTaq® Hot Start polymerase (Promega, Madison, USA). PCR products were gel-purified, cloned into the pCR2.1- TOPO vector (Invitrogen, Carlsbad, USA) and sequenced (Macrogen, Seoul, South Korea) for subsequent deduction of amino acid sequence.

**Table 4.1** Summary of clinical histories and laboratory data of children ‘C’ and adult ‘A’ patients used in the study.

Subject number	Gender	ImmunoCap (kU/L)			Skin Prick Test			Symptoms	Contact	Reactive history to fish
		Cod (f3)	Tuna (f40)	Salmon (f41)	Cod	Tuna	Salmon			
A1	M	3(6.45)	1(0.41)	1(0.43)	10mm	7mm	8mm	R, OAS	Oral	salmon trevally, blue grenadier,
A2	F	2(1.40)	-	2(1.35)	-	-	-	A, GIS, U	Oral, handling	sea perch, fish
A3	F	2(0.91)	3(4.46)	3(5.75)	-	6mm	10mm	A, OAS	Oral	fish, trout
A4	M	2 (1.14)	2 (0.91)	3 (4.10)	-	4mm	5mm	OAS	Oral	tuna, salmon, trout
A5	F	3(7.62)	3(5.67)	3(13.6)	-	-	-	A, R, OAS, AE, U	Oral	fish
A6	M	3(4.23)	3(7.75)	3(7.14)	-	-	-	A, R, AE, U	Oral	fish
A7	F	2(3.36)	2(2.17)	2(3.20)	-	-	-	A, R, OAS, U	Oral	fish
A8	F	0(0.01)	0(0.01)	0(0.01)	-	-	-	R, OAS	Oral	white fish, anchovies
A9	F	-	0(<0.01)	-	-	-	-	-	-	-
A10	-	-	-	-	-	-	-	-	-	-
A11	-	-	-	-	-	-	-	-	-	-
C1	F	-	-	-	11mm	5mm	6mm	AN,R, U	Oral	milk fish, tilapia
C2	F	-	-	-	5mm	5mm	6mm	R, OAS, AE	Oral	red mowong, whiting
C3	M	-	-	-	11.5mm	8mm	8mm	AE, GIS	OAS, Oral, inhalation	Salmon, silver perch, flake ling, whiting, barramundi
C4	F	-	-	-	11mm	3.5mm	5mm	U	Oral	
C5	F	-	-	-	8.5mm	5.5mm	0mm	AE, U	-	-
C6	F	-	-	-	14mm	4mm	7.5mm	E	-	cod, whitefish
N1	M	0(<0.01)	-	-	-	-	-	-	-	None
N2	M	0(<0.01)	-	-	-	-	-	-	-	None
N3	F	0(<0.01)	-	-	-	-	-	-	-	None
N4	F	0(<0.01)	-	-	-	-	-	-	-	None
N5	F	0(<0.01)	-	-	-	-	-	-	-	None

A, asthma; AN, anaphylaxis; AE, angioedema; E, eczema; GIS, gastrointestinal syndrome; OAS, oral allergy syndrome; R, rhinitis; U, Urticaria. '-' =Not performed

#### 4.2.9 Recombinant protein expression and purification

The expression vector used was pPROEX HTb which contains ampicillin resistance selectivity and N-terminal His(6) tag. The pCR2.1- TOPO vector containing the parvalbumin inserts and the expression vector pPROEX HTb underwent double restriction digest with BamH1 and EcoR1 according to manufacturer's instructions (Promega Co., Madison, USA). Cleaved parvalbumin insert and expression vector were separated by electrophoresis on 1% agarose gel and purified using DNA Wizard purification kit (Promega Co., Madison, USA). Insert was ligated into expression plasmid using T4 ligase according to manufacturer's instructions (Invitrogen, Carlsbad, USA). Chemically competent NM522 *E. coli* cells were transformed with the ligated recombinant plasmid by heat shock for 1 min at 42°C. Successfully transformed cells were selected by their ampicillin resistance and cultured in Luria Broth (LB) overnight at 37°C as a starter culture. Fresh LB media was inoculated with the overnight starter culture and incubated shaking at 37°C until turbidity had given an absorbance of 0.5 at 600 nm. Recombinant protein expression was then induced with a final concentration of 0.06 µM Isopropyl β-D-thiogalactoside (IPTG) and cultured for a further 3 h. Cultures were centrifuged and cells were washed in 20 mM sodium phosphate, 0.5 M sodium chloride, pH 7.4. Cells were subsequently centrifuged and supernatant discarded and pellet resuspended in immobilized metal affinity chromatography (IMAC) binding buffer (20 mM sodium phosphate, 0.5 M sodium chloride, 5 mM imidazole, pH 7.4). Cells were lysed using 3 freeze-thaw cycles and by a French pressure cell and lysate centrifuged and supernatant loaded onto HisTrap HP column (GE Healthcare Biosciences, Little Chalfont, UK) washed with IMAC binding buffer and his-tagged proteins were eluted with IMAC elution buffer (20mM sodium phosphate, 0.5 M sodium chloride, 250 mM imidazole, pH 7.4) and purity confirmed on 15%

polyacrylamide SDS gel. Recombinant allergens were also analysed by immunoblotting with anti-parvalbumin IgG's and patient IgE as described in 4.2.4.

#### ***4.2.10 IgE enzyme-linked immunosorbent assay (ELISA)***

The wells of a 96-well EIA/RIA plate (Costar, St. Louis, USA) were coated with 100  $\mu$ L fish extract (1 $\mu$ g/mL in sodium carbonate/bicarbonate buffer pH 9.6), and incubated overnight at 4°C. All of the following incubations were performed for 1 h unless otherwise stated and plates washed 4-times in PBS/0.05% Tween 20 (PBS-T) between steps. Blocking was performed using 5% skim milk diluted in PBS-T. Serum, 100  $\mu$ L diluted 1:10 in 1% skim milk/PBS-T, was added to wells and incubated for 3 h at room temperature while shaking (45 rpm). Rabbit anti-human IgE antibody (1:4000; Dako, Glostrup, Denmark) and goat anti-rabbit IgG-HRP (1:1000; Promega, Madison, USA) were added sequentially to wells and plates incubated at room temperature for 1 h with gentle shaking. Plates were then washed 5-times in PBS-T, followed by 3 washes in PBS (no Tween). IgE binding was detected using TMB (3,3',5,5'-Tetramethylbenzidine) substrate (BD, Franklin Lakes, USA) and reaction terminated using 1 M HCl and the absorbance (O.D.) at 450 nm measured by spectrophotometry (Molecular Devices, Sunnyvale, USA). Three non-fish allergic subjects were analysed to determine the extent of non-specific binding. Experiment was performed once.

#### ***4.2.11 3D modeling of parvalbumin isoforms***

Beta 1 and 2 parvalbumin isoforms were aligned to a carp parvalbumin template (PDB ID: 4cpv) using SWISS-MODEL and protein modeled in UCSF Chimera version 1.7.

#### ***4.2.12 Data analysis***

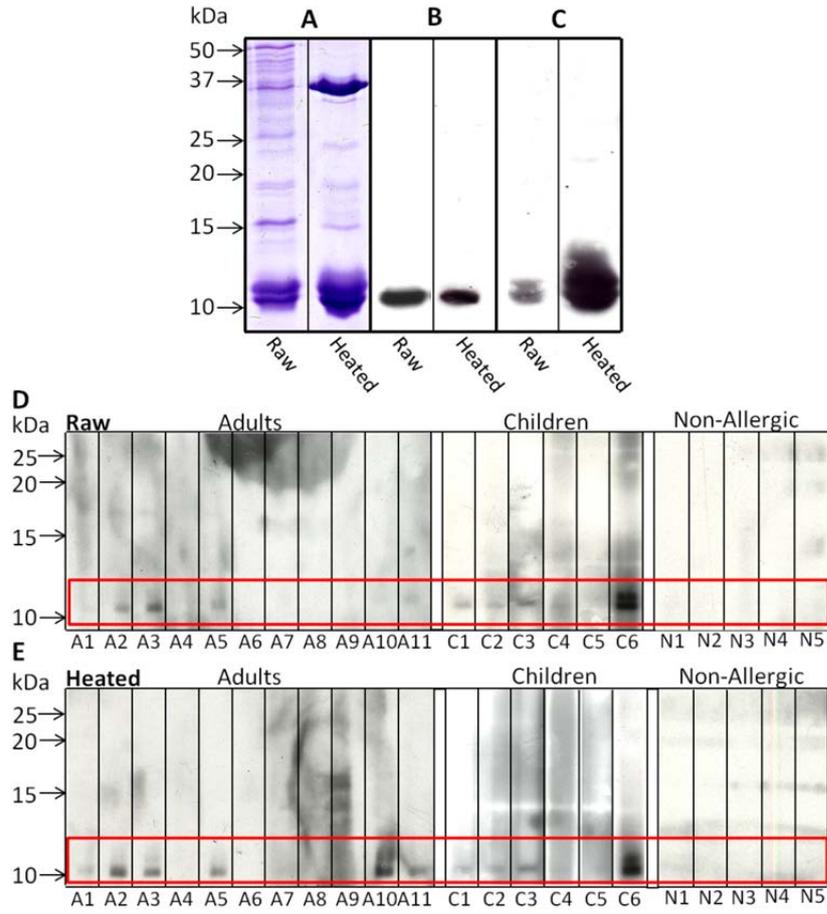
Cut-off values in indirect IgE ELISA were determined using the mean of three non-fish allergic patients plus 2 times the standard deviation. For direct comparison between

protein samples each value from the IgE ELISA was divided by their corresponding cut-off value and recorded as 'fold increase' in Figure 4.6. The Wilcoxon matched-pairs signed rank test was used to compare overall serum IgE reactivity between raw and heated extracts and recombinant parvalbumin isoforms. Analyses were performed using GraphPad Prism version 5.04 for Windows (GraphPad, San Diego, CA).

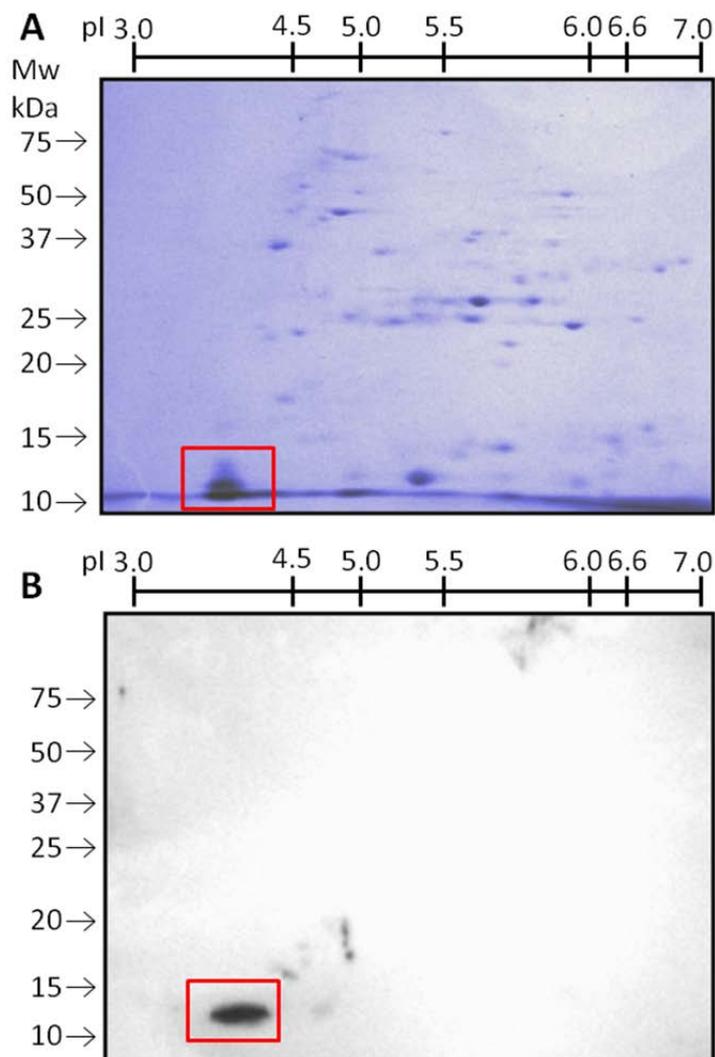
### 4.3 Results

#### 4.3.1 SDS-PAGE and immunoblot analysis of IgE binding proteins.

Raw, heated barramundi protein extracts and both  $\beta 1$  and  $\beta 2$  recombinant parvalbumin isoforms were separated by SDS-PAGE and stained with Coomassie brilliant blue (Figure 4.1). The two parvalbumin isoforms are highly expressed in barramundi as seen in between 10 and 15 kDa in the raw extract. These isoforms were confirmed as allergenic as they bind fish allergic patient serum IgE as seen in the immunoblot in Figure 4.1D. Three adults (A2, 3 and 5) and three children (C1-3) show moderate binding to parvalbumin in the raw extract, while patient A1, A10, A11 and C4 show weak binding and patient C6 clearly binds strongly to both raw parvalbumin isoforms. These isoforms are highly heat stable as there are only three strong bands in the heat extract, two of them are suspected parvalbumin as their molecular weights is between 10 and 15 kDa. Heating does also not affect the allergenicity of parvalbumin as a near identical binding pattern can be seen between the patients with the exception of patient A10, who appears to bind stronger to heated than raw parvalbumin. Purified recombinant  $\beta 1$  and  $\beta 2$  parvalbumins have calculated molecular weights of 14.83 and 14.95 kDa, respectively. This is higher when compared to their natural counterparts due to their His-tag not being cleaved after purification. The IgE immunoblot again show a similar binding pattern to the raw protein extract with the same patients demonstrating binding. However patient A7 appears to bind to both recombinant parvalbumins, but interestingly showed no binding to raw or heated extract. Furthermore, the r $\beta 1$  isoform seems to assemble dimers with a molecular weight of about 30 kDa and patient C6 is the only patient who reacts to this oligomer (Figure 4.4D). It confirmed the presence of the two different barramundi parvalbumin allergens by mass spectrometry, with 3 peptides for both  $\beta 1$  and  $\beta 2$  (Figure 4.3 B).



**Figure 4.1** The extracted barramundi proteins are separated by SDS-PAGE and stained with coomassie brilliant blue (A) and have been detected via immunoblot with a commercial anti-frog parvalbumin mAb, PARV-19 (B) and an in house rabbit anti-barramundi parvalbumin pAb (C). Immunoblots using fish allergic patient sera against raw (D) and heated barramundi extracts (E).

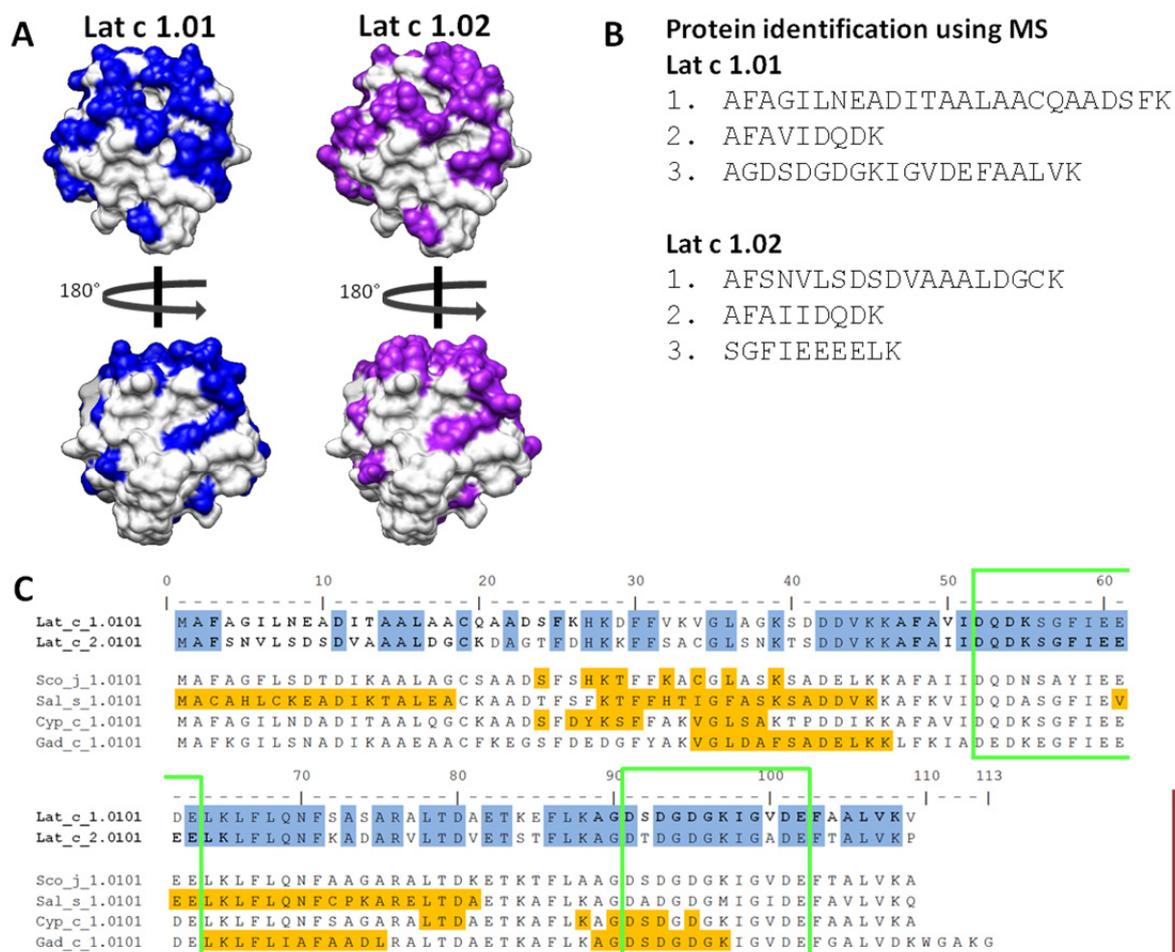


**Figure 4.2** Two-dimensional electrophoresis analysis of raw barramundi extract. A) Coomassie blue stained gel of 2D electrophoresis. B) Two-dimensional immunoblot with a commercial anti frog parvalbumin mAb, PARV-19. Parvalbumin isoforms are boxed in red.

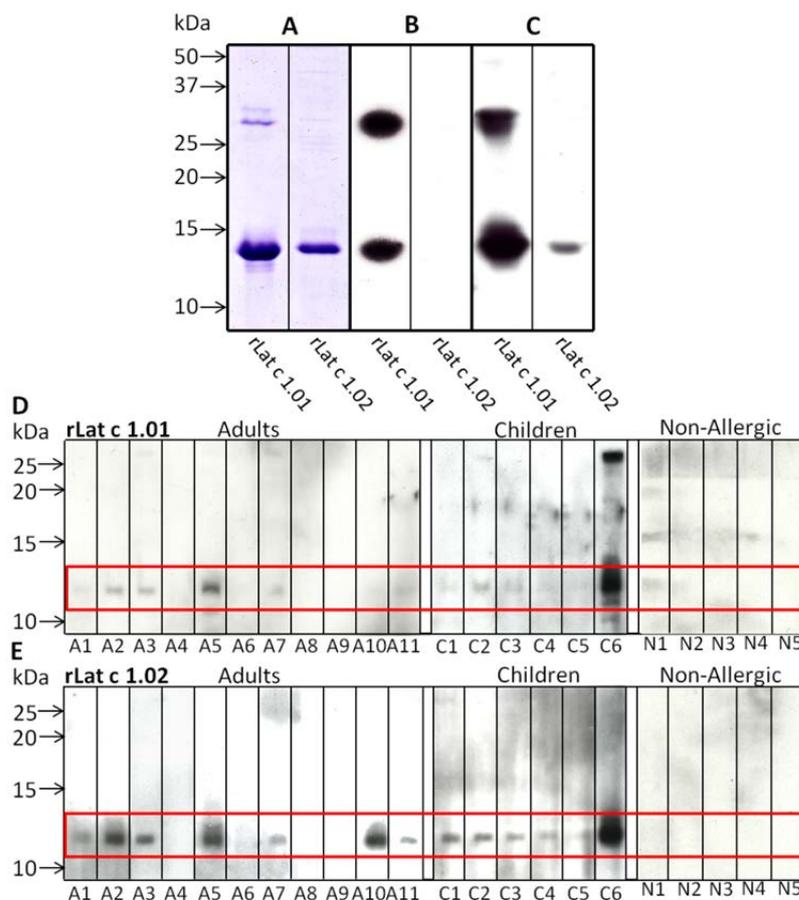
#### 4.3.2 Analysis of parvalbumin isoforms amino acid sequences translated from cDNA.

PCR primers were designed from previously sequenced Asian barramundi parvalbumin [15] to amplify parvalbumin genes from a cDNA library generated from the muscle of Australian barramundi. Amplicons were cloned into the sequencing vector and demonstrated that the barramundi expresses two different beta parvalbumin isoforms,  $\beta 1$  and  $\beta 2$ . These two isoforms have identical calcium binding EF-hand residues. Beta-1

and beta-2 share similar physical properties such as their respective molecular weights with 11.54 and 11.71 kDa, and theoretical isoelectric points of 4.50 and 4.48 respectively. Two-dimensional electrophoresis demonstrated that the parvalbumin isoforms had a practical pI of 4 (Figure 4.2). Despite these similarities between the two isoforms they share only 67% of their amino acid sequences. The space filling model (Figure 4.3) demonstrates that despite differing by 33%, the two isoforms form similar theoretical 3D structures. Sequences were submitted to GenBank and assigned accession numbers KF021278 ( $\beta$ 1) and KF021279 ( $\beta$ 2).



**Figure 4.3** Space filling models of both Lat c 1.0101 and Lat c 1.0201 based on the template 4cpv. Residues that differ between the isoforms are shaded. B) Amino acid sequence alignment of the  $\beta$ 1 (Lat c 1.0101) and  $\beta$ 2 (Lat c 1.0201) parvalbumin isoforms from *L. calcarifer* compared to allergenic parvalbumin from *Scomber japonicus*, *Salmo salar*, *Cyprinus carpio* and *Gadus callarias* with confirmed IgE epitopes shaded in light. Identical amino acid residues between the two *L. calcarifer* isoforms are shaded in dark. EF-hand calcium binding residues are boxed in green.



**Figure 4.4** Generated recombinant parvalbumin isoforms are separated by SDS-PAGE and stained with coomassie brilliant blue (A) and have been detected via immunoblot with a commercial anti frog parvalbumin mAb, PARV-19 (B) and an in house rabbit anti-barramundi parvalbumin pAb (C). Immunoblots using fish allergic patient sera against generated rLat c 1.01 (D) and rLat c 1.02 (E).

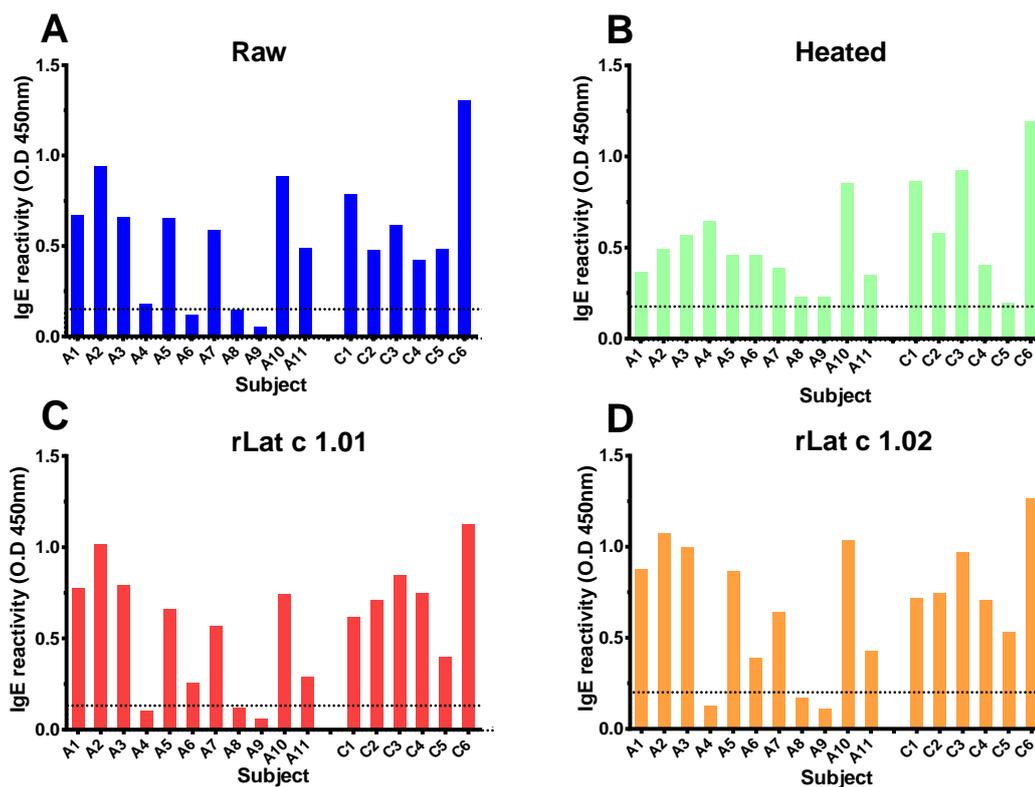
#### 4.3.3 Comparison of IgE binding from adults and children against barramundi extracts.

The direct ELISA for barramundi IgE demonstrates that eight out of 12 adults and all six children react to raw barramundi extract (Figure 4.5 A and as summarized in Table 4.2). In contrast, only nine adults and five children appear to react to heated extract (Figure 4.5B). Patients A1, A2, A3, A5, A7, A10, A11 and C6 demonstrate greater binding to raw than heated protein extract. Three patients (A4, A6 and C3) show greater binding to heated than raw protein extracts. Interestingly patient A4 does not show any

significant binding to raw extract however mounts a considerable response to heated extract. Patients A8 and A9 did not show binding above the cut-off to raw or and only marginally to the heated protein extract. Overall there is no significant difference between IgE reactivity to raw and heated protein extracts. IgE reactivity between recombinant  $\beta 1$  and  $\beta 2$  isoforms (Lat c 1.01 and Lat c 1.02, respectively) shows similar ELISA profiles (Figure 4.5 C, D), with nine adult patients (A1-3, 5-7, 10-12) and all six children resulting in OD values above the cut-off values. All patients with significantly reactivity to both recombinant isoforms, excluding A6, seem to mount a marginally but significant ( $p=0.0001$ ) greater IgE response to rLat c 1.01 than Lat c 1.02. However, there is no significant difference between the IgE reactivity of the children and adults sample, despite all of the children reacting to all the extracts tested.

**Table 4.2** Summary of immunoblot IgE reactivity between adult and paediatric patients.

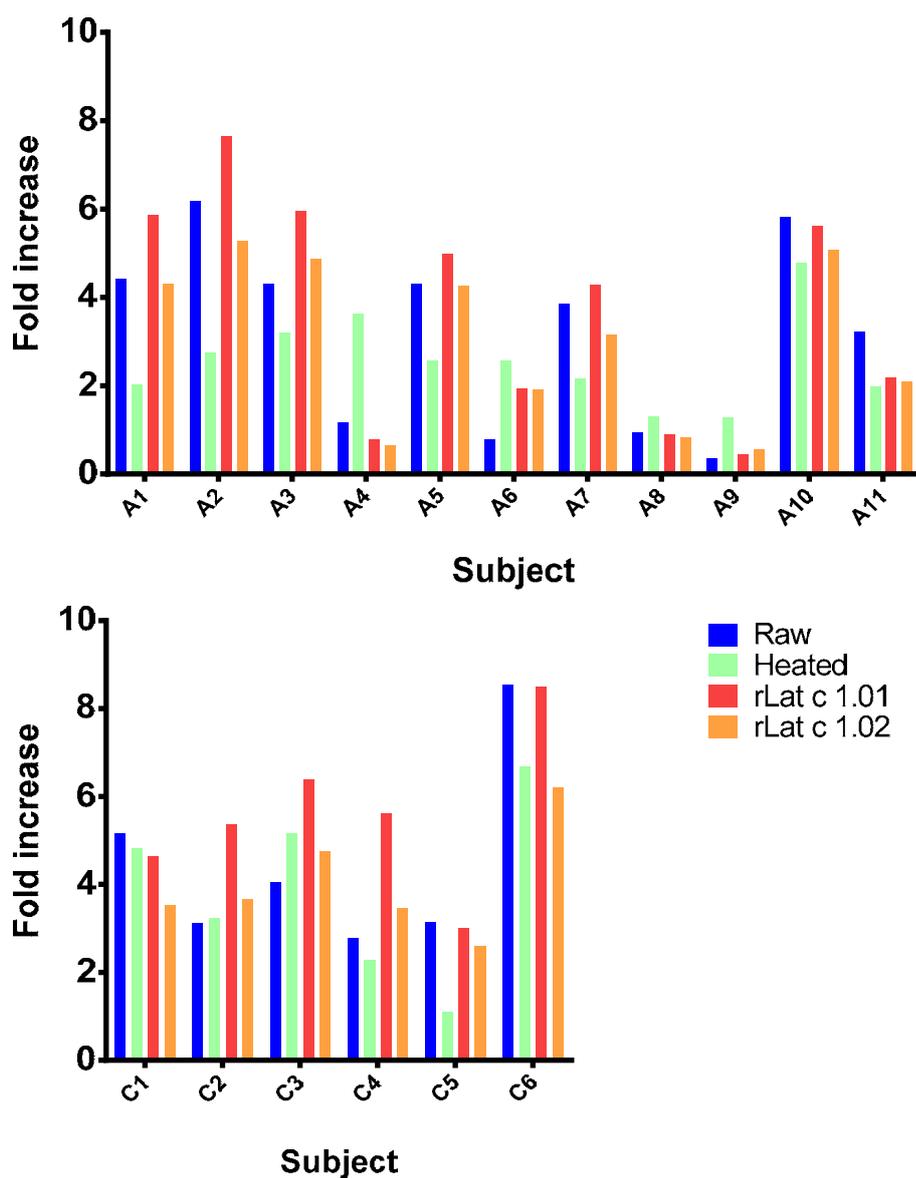
Protein Sample	Reactive Patient (out of 11)	Adult	Reactive Paediatric Patient (out of 6)
Raw	7		6
Heated	8		5
rBeta-1	8		6
rBeta-2	8		6



**Figure 4.5** ELISA for serum IgE reactivity to (A) raw and (B) heated *Lates calcarifer* muscle extracts and purified recombinant (C) Lat c 1.01 and (D) Lat c 1.02 isoforms for fish-allergic patient sera from 11 adults (A1-11) and six children (C1-6) labeled ‘A’ and ‘C’ respectively. The cut-off derived from two standard deviations above the mean reactivity of three non-allergic subjects to each of the extracts is indicated by the dotted line (O.D.: Raw=0.18, Heated=0.15, r $\beta$ 1=0.13 and r $\beta$ 2=0.20). n=1.

The results of the ELISA were compared and displayed for each individual patient as fold increase above negative patient cut-off (Figure 4.6). The raw extracts (blue) seem to mount in general a stronger IgE response than heated extracts. However, patient A4, A6 and A9 displayed a much stronger response to heated protein extract, which is particularly importantly as the latter two patients were negative to the raw extract. Furthermore, the IgE response to rLat c 1.01 seems to be for almost all patients stronger than the response to rLat c 1.02, however none of the patients demonstrated selective

reactivity. However, the response of patient A4, A6 and A9 are negative to these recombinant parvalbumins. These three patients are the same patients who demonstrate higher reactivity to heated extract (see above), indicating the possible presence of other yet unidentified allergens in the heated barramundi protein extract. Following the IgE immunoblot and ELISA experiments with these novel parvalbumin isoforms, the results were submitted to the I.U.I.S. Allergen Nomenclature Sub-Committee as isoallergens.  $\beta$ 1 parvalbumin was subsequently designated Lat c 1.0101 and  $\beta$ 2 parvalbumin was designated Lat c 1.0201.



**Figure 4.6** Comparison of the IgE ELISAs of raw and heated protein extract and both  $\beta 1$  and  $\beta 2$  recombinant parvalbumins against 11 adults and 6 children fish allergic patients. The graph was generated by dividing the O.D values with the cut-off O.D value for each experiment.

#### 4.4 Discussion

Fish allergy in Australia and the Pacific region is an important and increasing problem, and there are currently limited diagnostic tools available to provide accurate specific species diagnosis [141]. Barramundi is an important emerging aquaculture species and is now commonly consumed throughout South East Asia and Australia, however prior to this study specific barramundi allergens had not been characterised [165].

In this current study two novel allergenic parvalbumin isoallergens have been identified in Barramundi. The two novel isoallergens Lat c 1.0101 and Lat c 1.0201 have been registered with the IUIS. It was also demonstrated that the allergenicity to raw and heated barramundi proteins and to recombinant forms of barramundi parvalbumin isoallergens, as measured by IgE reactivity, differs between the  $\beta$ 1 isoform and  $\beta$ 2 isoform and between fish allergic adults and children.

In our study, heating of Barramundi resulted in a loss of higher molecular proteins and stronger banding pattern in the 10 to 12 kDa region. Subsequent immunoblotting of both raw and heated barramundi protein extracts with 17 fish allergic patients showed two IgE binding proteins with molecular weights of about 12 kDa. The majority of adults and children demonstrated IgE reactivity to these proteins. Previous studies on various fish species, but not barramundi, identified parvalbumin as a major allergen [11] and in addition other allergenic proteins including high molecular weight vitellogenin and collagen [97] as well as the enzymes enolase and aldolase [168]. Interestingly our study did not demonstrate any IgE binding to other proteins in raw and heated Barramundi.

Further analysis of Barramundi extract using parvalbumin specific monoclonal and polyclonal antibodies confirmed that these two 12 kDa proteins to be parvalbumin. The

monoclonal antibody in our study has been previously comprehensively investigated for its ability to bind to fish parvalbumin and is known to detect most  $\beta$  isoforms of this protein, but not  $\alpha$ -parvalbumin, which is mostly present in sharks and rays [154] [157, 158, 169]. Importantly, this antibody only detects the  $\beta 1$  isoform of barramundi and might have restricted the identification of parvalbumin isoforms in other studies [157]. In addition an in-house generated anti-barramundi parvalbumin polyclonal antibody raised in rabbits was able to detect both parvalbumins in raw and heated Barramundi. Parvalbumin is a very heat-stable protein and abundant in the white muscle tissue of fish as previously described [84]. Several different isoforms can be expressed in a single fish species as demonstrated for fresh-water carp during physiological development [145].

Previous studies demonstrated IgE reactivity to multiple parvalbumins, however their differential IgE binding capacity has not been investigated. This include Atlantic cod [71], rainbow trout, redfish, carp [72], Atlantic herring, and Atlantic salmon [77], with the two isoforms from the later differing the greatest from each other, sharing only 64% of their primary structure. However Atlantic salmon parvalbumin isoforms differ little in their biophysical properties such as size isoelectric point, where they only differ by 0.51 kDa and 0.54 pI units, respectively.

Sequence analysis of barramundi parvalbumins in our current study using recombinant gene technology demonstrated that the novel  $\beta 1$  and  $\beta 2$  isoforms, share only 67% amino acid identity, similar to salmon, but have near identical physical properties such as molecular weight and isoelectric point (differing only by 0.12 kDa and 0.02 pI units). The amino acid substitutions were mostly situated in the N-terminal region of the parvalbumins, while the two calcium-binding sites remained over 90% identical in their sequence. The generation of space-filling models for both isoforms showed that these

proteins were structurally very similar, even while over 30% of the amino acids were different. This suggests that any differences in allergenicity between the two isoforms most likely result from the variance in primary structure of the proteins, rather than secondary or tertiary structure.

Previous gene expression studies on barramundi have demonstrated that the two parvalbumin isoforms are differentially expressed throughout the body of this fish [167]. The  $\beta 1$  parvalbumin is present not just in the muscle but also in other tissues including kidney, small intestine, skin, spleen, liver, heart, gill and eye, with the highest concentration in the brain [167]. In contrast the  $\beta 2$  isoform is exclusively expressed in the skeletal muscle. This study demonstrated that two heat-stable parvalbumin isoforms are present in high concentration in barramundi muscle tissue.

Generation of recombinant proteins of the  $\beta 1$  and  $\beta 2$  parvalbumins enables the comparison of the allergenicity of each discrete isoallergen, as physical separation of the natural counterparts is extremely difficult, due to their similar biophysical properties. Interestingly, the anti-frog parvalbumin monoclonal antibody appeared to only detect the  $\beta 1$  recombinant isoform in the immunoblot, as mono- as well as dimeric forms. The formation of  $\beta 1$  dimers was confirmed by the polyclonal antibody raised against heated barramundi. Patient's IgE binding did not show this selective recognition, but demonstrated stronger reactivity to the  $\beta 1$  compared to  $\beta 2$  isoform of parvalbumin in eight of 11 adults and in all children. The ELISA experiments confirmed a significant increase in IgE reactivity to the  $\beta 1$  isoform. This increased IgE reactivity might be explained by the differential expression of both isoforms, with the  $\beta 1$  being ubiquitously expressed throughout all tissues, resulting in higher dietary exposure in consumers and therefore higher sensitization.

Overall the immunogenicity of these isoforms did not appear to be affected by heating, however eight fish allergic patients recognized raw extract marginally more than heated extract. The effect of heating has been known to increase allergenicity of some food allergens including peanut and shrimp [68, 170] and also for the fish pilchard [11]. However, in this study only three patients had greater IgE reactivity to heated protein than raw. These results suggest that some patients may have antibodies to heat labile antibody binding regions (conformational epitopes) of this major allergenic protein, such as has been well described in egg allergens [171]. Furthermore, these three patients are the same demonstrating no response to raw and the two recombinant parvalbumin allergens, indicating the possible presence of other yet unidentified allergens in the heated barramundi protein extract. Other potential allergens have been characterised in fish including collagen [97], aldolase and enolase [168] which might contribute to IgE binding in this in-solution assay, but are not necessarily detectable in the denaturing immunoblot. To date, five fish parvalbumins have been investigated for their IgE binding epitopes including carp (Cyp c 1.0101), Atlantic cod (Gad m 1.0101), Baltic cod (Gad C 1.01), Atlantic salmon (Sal s 1.0101) and pacific mackerel (Sco j 1.0101) [73, 75, 76, 89, 94]. From these studies one can conclude that parvalbumin may have up to four different IgE binding epitopes, and that some have high homology and others are very specific as seen in Atlantic salmon parvalbumin [141].

Previous studies have shown that carp has the highest homology with barramundi parvalbumin, sharing 87% of their primary structures. Carp parvalbumin has three IgE epitopes, two of which are conformational, which may render these epitopes more heat labile [75]. In contrast linear epitopes are much less degraded by heat and has been shown for a varied of food allergens including egg, milk and a variety of fruits [172].

#### ***4.4.1 Conclusion and future direction***

In this study differential IgE binding patterns between raw and heated parvalbumin and the two isoallergens appeared to be patient specific. There may be up to four different IgE binding epitope as seen for other fish parvalbumins [141] and patients may detect any combination of these which may result in differential IgE binding as seen in this study. This finding merits further exploration in a larger population of Barramundi allergic patients to see whether any common pattern of IgE epitope binding emerges.

This study has identified two novel Barramundi parvalbumin isoallergens and has also demonstrated the importance of heat labile and stabile epitopes in barramundi parvalbumin and that the barramundi isoallergens have differential IgE reactivity. This suggests that the current limitations in diagnosing allergy to a specific fish species in Australia and the Pacific region (especially for patients presenting with a clinical history of reactivity to an unidentified or seemingly distantly related fish species) might be overcome through molecular characterisation of important Australian and Pacific fish allergens and exploration of more specific epitope binding by screening overlapping peptides.

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## CHAPTER 5

### Antibody epitope mapping of allergenic beta-1 parvalbumin isoform from Barramundi

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<b>Details of publication on which this chapter is based</b>	<b>Nature and extent of the intellectual input of each author, including the candidate</b>
Sharp, M. F., Stephen J. N., Kamath, S. D., Campbell D. E., Lopata, A. L., 2015. IgE antibody epitope mapping of the major food allergen parvalbumin. Manuscript in preparation.	The authors Sharp and Lopata co-developed the research question. Sharp collected the data and performed the data analyses with assistance from Stephen and Kamath. Serum samples were collected by Campbell. Sharp wrote the first draft of the paper, which was revised with editorial input from Lopata. Sharp developed the figures and tables.

## 5.1 Introduction

Consumption of barramundi in the Asia-Pacific region is growing due to increased availability from aquaculture industries and its culinary popularity [164, 165]. Unfortunately barramundi can cause allergic reactions in fish allergy sufferers [133]. Chapter 4 demonstrated that 88% of fish allergic patients in our cohort reacted to at least one of the major parvalbumins isoallergens[153]. Lat c 1.0101 appeared to have the greatest IgE reactivity and will be further investigated in this chapter with its antibody epitopes to be analysed.

Currently there have been five studies mapping the IgE antibody binding epitopes of parvalbumin from fish. Parvalbumin from Baltic cod (Gad c 1), carp (Cyp c 1), chub mackerel (Sco j 1), Atlantic salmon (Sal s 1) and Atlantic cod (Gad m 1) have been analysed for their specific IgE epitopes. Allergic patient IgE was used in various techniques including phage display library, overlapping synthetic peptides and tryptic digests of parvalbumin to map out these epitopes [73, 75, 76, 89, 94]. These five parvalbumins analysed display both linear and conformational epitopes, however do not share identical residues. Most recently, Perez-Gordo *et al* [94] reported only one unanimous IgE epitope for Gad m 1, however additional epitopes were identified, which were patient specific.

Identifying IgE epitopes may help develop and predict successful specific immunotherapies (sIT) for allergy sufferers [173, 174]. An increase in specific IgG<sub>4</sub> antibodies can also be an indicator of successful sIT due to this antibody being able to out-compete IgE binding for the allergen[134]. However, a comparative study of the IgE and IgG<sub>4</sub> epitopes of a fish allergen has never been performed.

**5.1.1 Aims**

In this chapter the IgE and IgG<sub>4</sub> reactivity to the  $\beta$ 1 parvalbumin isoform from barramundi (Lat c 1.01) will be investigated and compared using recombinant Lat c 1.01 and seven overlapping synthetic peptides by immunoblotting and inhibition ELISA.

## 5.2 Materials and methods

### 5.2.1 Patient sera

Subjects were selected on the basis on a convincing recent history of an IgE mediated reaction (including urticaria, angioedema, stridor, cough, wheeze, hypotension and/ or gastrointestinal symptoms of vomiting and/or diarrhoea) to any Australian- Asian Pacific fish which occurred within 2 hours of ingestion and confirmation of fish reactivity by prick-to-prick testing or specific IgE (ImmunoCAP, Thermo Fisher Scientific, Sweden) to tuna (*Thunnus albacares*), salmon (*Salmo salar*), and cod (*Gadus morhua*). Human ethics for this study was approved by the Sydney Children's Hospital Network ethics committee (HREC) (Table 5.1). Patients were selected based on positive IgE [153] reactivity to barramundi parvalbumin (rLat c 1.0101) (see Chapter 4)

### 5.2.2 Recombinant parvalbumin

Recombinant barramundi  $\beta$ 1 parvalbumin was generated as previously described in detail in Chapter 4 [153]. In brief, his-tagged  $\beta$ 1 parvalbumin gene was cloned into the expression vector pPROEX HTb that was used to transform NM522 *E. coli* cells. Transformed cells were grown at 37°C and expression was induced with IPTG. Recombinant parvalbumin was purified from cell lysate by IMAC using HisTrap HP column (GE lifesciences).

### 5.2.3 IgE and IgG<sub>4</sub> immunoblot analysis

Immunoblots were analysed for IgE and IgG<sub>4</sub> binding using sera from fish allergic patients against recombinant Lat c 1.01 by separating the allergen on a 15% polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane (BioRad laboratories, Hercules, USA) using semidry electrophoresis and blocked in TBS-T-0.3% Tween-20 for 2 h at room temperature. Sera were diluted 1:20 in 0.1%

BSA in TBS-T. Membranes were placed into the slot blot manifold and the diluted serum aliquoted into the slots. Membranes were incubated with sera for 16 h at 4°C and subsequently washed six-times with TBS-T at room temperature. The IgE immunoblots were incubated with a horseradish peroxidase labeled goat anti-human IgE antibody (Genetex, Irvine, USA) diluted 1:10,000 in 0.1% BSA in TBS-T for 1 h at room temperature. The IgG<sub>4</sub> immunoblots were incubated with monoclonal Anti-Human IgG<sub>4</sub> MAB1313 (Merck, USA) diluted 1:2000 for 1 h at room temperature. The IgE immunoblot was washed five-times with TBS-T at room temperature and IgE binding visualized by performing ECL technique as described in Chapter 4.2.4. The IgG<sub>4</sub> immunoblot was washed three-times with TBS-T and then incubated with anti-mouse IgG HRP labelled antibody (Sigma, USA) diluted 1:20,000 for 1 h. Immunoblots were washed five-times with TBS-T and IgG<sub>4</sub> binding was visualized by again performing ECL technique.

#### ***5.2.4 Peptide inhibition of IgE and IgG<sub>4</sub>***

Seven overlapping peptides, each 25 amino acids in length, with 16 amino acid overhang were synthesized and purified to 95% homogeneity by Mimotopes. Antibody binding to the peptides was measured by inhibition ELISA. The wells of a 96-well EIA/RIA plate (Costar, St. Louis, USA) were coated with 20 µL recombinant  $\beta$ 1 barramundi parvalbumin (1µg/mL in sodium carbonate/bicarbonate buffer pH 9.6), and incubated overnight at 4°C. All of the following incubations were performed for 1 h unless otherwise stated, plates washed three-times in TBS/0.05% Tween 20 (TBS-T) between steps and five times for final wash and antibodies were diluted in TBS-T 0.1% BSA. Blocking was performed using 5% skim milk diluted in TBS-T. Primary antibodies were preabsorbed with inhibitors for 2 h at room temperature. Patient serum was diluted 1:10 and the inhibitor concentrations were 12.5 µg/mL. This mixture was

then added to the wells (20  $\mu$ L) and incubated at room temperature. Wells were washed and then incubated with 40  $\mu$ L of Anti-Human IgE HRP conjugate (diluted 1:10,000, Genetex Inc., USA) for serum IgE or mAb Anti-Human IgG<sub>4</sub> MAB1313 (Merck, USA); diluted 1:2000 was added to each well and incubated at room temperature. The final well wash was performed for serum IgE plates. Serum IgG<sub>4</sub> plates were incubated with anti-mouse IgG HRP conjugate (Sigma-Aldrich, USA) diluted 1:20,000 at room temperature and then washed. Assays were colourimetrically developed with TMB substrate (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  $100 + [\text{O.D. 450 nm of antibody with inhibitor} / \text{O.D. 450 nm of antibody without inhibitor}] + 100$ .

### ***5.2.5 3D modeling of parvalbumin isoforms***

The allergens Gad c 1.01, Cyp c 1.01, Sal s 1.01, Sco j 1.01, Gad m 1.01 and Lat c 1.01 were aligned to the carp parvalbumin template (PDB ID: 4cpv) using SWISS-MODEL and protein structure modeled in UCSF Chimera version 1.7.

### ***5.2.6 Data analysis***

Cut-off values for the indirect IgE ELISA were determined using the mean of three non-fish allergic patients, plus two times the standard deviation. Multiple T-tests were used to compare overall serum IgE and IgG<sub>4</sub> reactivity to each peptide between the negative and fish allergic patients. Analyses were performed using GraphPad Prism version 5.04 for Windows (GraphPad, San Diego, CA).

**Table 5.1** Clinical history and laboratory data of paediatric and adult patients analysed.

Subject number	Gender	ImmunoCap (kU/L)			Skin Prick Test			Symptoms	Contact	Reactive history to fish
		Cod (f3)	Tuna (f40)	Salmon (f41)	Cod	Tuna	Salmon			
A1	M	3(6.45)	1(0.41)	1(0.43)	10mm	7mm	8mm	R, OAS	Oral	Salmon trevally, blue grenadier,
A2	F	2(1.40)	-	2(1.35)	-	-	-	A, GIS, U	Oral, handling	Sea perch, fish
A3	F	2(0.91)	3(4.46)	3(5.75)	-	6mm	10mm	A, OAS	Oral	Fish, trout
A4	F	3(7.62)	3(5.67)	3(13.6)	-	-	-	A, R, OAS, AE, U	Oral	Fish
A5	M	3(4.23)	3(7.75)	3(7.14)	-	-	-	A, R, AE, U	Oral	Fish
A6	M	2(2.74)	3 (3.78)	-	-	-	-	R, OAS	Oral	Snapper, tuna
A7	F	2(3.36)	2(2.17)	2(3.20)	-	-	-	A, R, OAS, U	Oral	Fish
A8	F	-	-	-	-	-	-	-	-	Fish
A9	F	-	13.9	25.8	-	-	-	A, AN, U, R, OAS	Oral	Trout
C1	M	-	-	-	6mm	3mm	4mm	OAS, GI	Oral	White fish
C2	F	-	-	-	5mm	6mm	3mm	U	Oral	Salmon
C3	M	-	-	-	5mm	3mm	4mm	AN, OAS	Oral	Trout, tuna, whitefish
C4	M	-	-	-	8mm	3mm	3mm	AN	Oral	Basa
C5	M	-	-	-	6mm	0mm	2mm	AE, U	Oral	Smoked salmon, fish finger
C6	M	-	-	-	0mm	4mm	0mm	AE, U	Oral	Bream
C7	F	-	-	-	11mm	5mm	6mm	AN,R, U	Oral	Milk fish, tilapia
C8	F	-	-	-	8.5mm	5.5mm	0mm	AE, U	-	-
C9	F	-	-	-	5mm	5mm	6mm	R, OAS, AE	Oral	Red mowong, whiting
C10	M	-	-	-	11.5mm	8mm	8mm	AE, OAS, GIS	Oral, inhalation	Salmon, silver Perch, flake
C11	F	-	-	-	11mm	3.5mm	5mm	U	Oral	Ling, whiting, Barramundi
C12	M	-	-	-	8mm	4.5mm	3.5mm	OAS	Oral	White fish
C13	F	-	-	-	4mm	5mm	5mm	U	Oral	Salmon
C14	F	-	-	-	10mm	5.5mm	3mm	AN	Oral	Basa
C15	M	-	-	-	0mm	0mm	0mm	AN, GIS, OAS	Oral	Basa, white fish
N1	M	0(<0.01)	-	-	-	-	-	-	-	None
N2	M	0(<0.01)	-	-	-	-	-	-	-	None
N3	F	0(<0.01)	-	-	-	-	-	-	-	None
N4	F	0(<0.01)	-	-	-	-	-	-	-	None
N5	F	0(<0.01)	-	-	-	-	-	-	-	None

A, asthma; AN, anaphylaxis; AE, angiodema; E, eczema; GIS, gastrointestinal syndrome; OAS, oral allergy syndrome; R, rhinitis; U, Urticaria. '-'=Not performed

## 5.3 Results

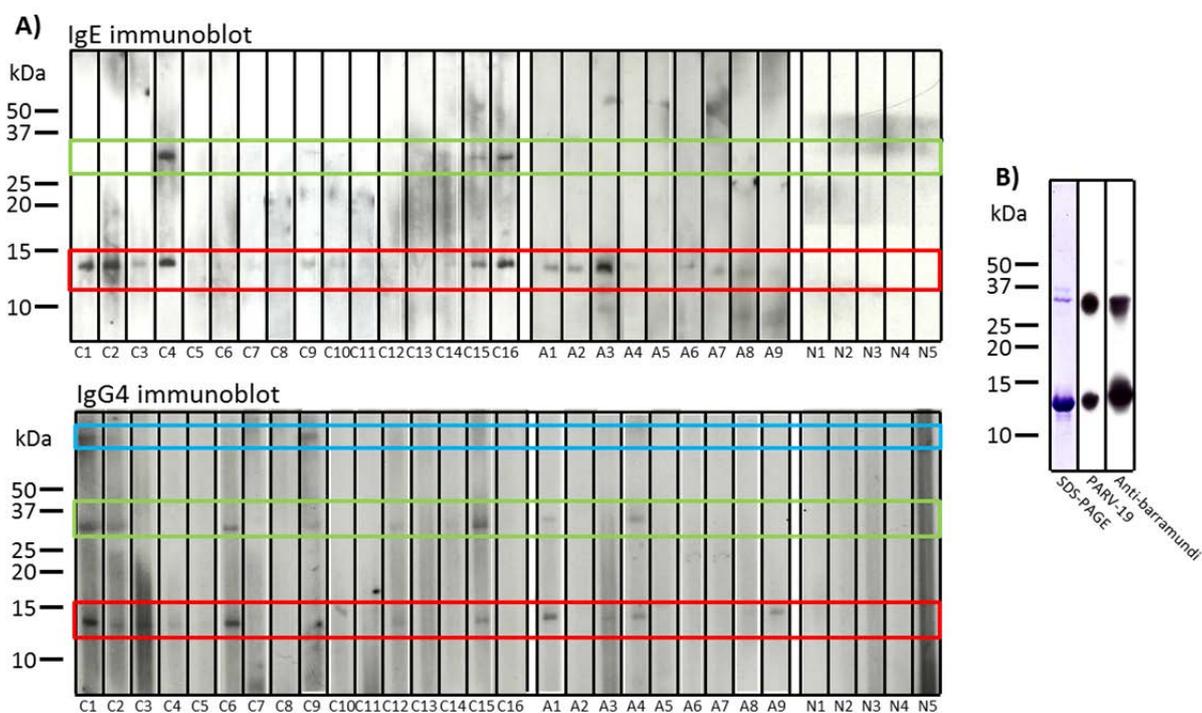
### 5.3.1 Fish allergic patient IgE and IgG<sub>4</sub> binding to rLat c 1.01

An analysis of IgE and IgG<sub>4</sub> binding to rLat c 1.01 was performed by immunoblotting as seen in Figure 5.1. All but one fish allergic paediatric (patient C13) and all nine fish allergic adult patients demonstrated IgE binding to rLat c 1.01 in Figure 5.1 A. Five paediatrics also bound to dimeric forms of Lat c 1.01 at 30 kDa (C4, C9 and C13-15), which is not surprising as both parvalbumin specific antibodies used in Figure 5.1B detect dimeric Lat c 1.01. Fewer patients demonstrated clear IgG<sub>4</sub> binding to Lat c 1.01 in the immunoblots, with nine out of fifteen paediatrics (C1-7, C12 and C13-14) and seven out of nine adults (A1-5 and A8-9). IgG<sub>4</sub> from patient C1-2, C6, C9, C12, C13-14, A1 and A4 were all also able to bind to the dimeric form of parvalbumin. Patients C1 and C9 also bound to an rLat c 1.01 oligomer over 50 kDa in size. All five control patients (N) demonstrated no IgE or IgG<sub>4</sub> binding to rLat c 1.01. However, a considerable amount of background can be seen in Figure 5.1B for patient N3 and N5.

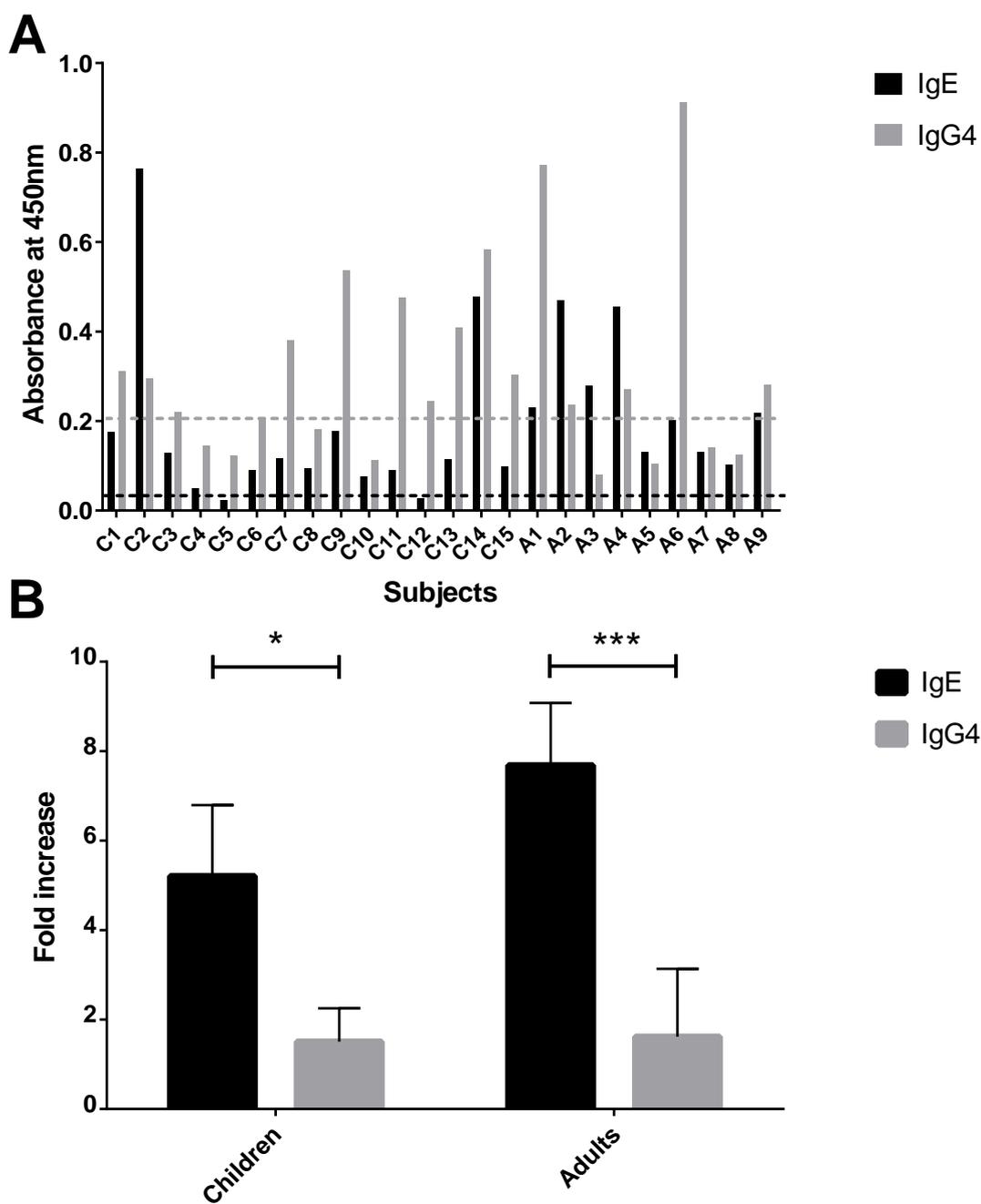
Further analysis of IgE and IgG<sub>4</sub> antibody binding to rLat c 1.01 was performed by indirect ELISA (Figure 5.2). Positive binding is identified by the patient having a higher OD value than the cut-off value for the experiment (dotted line). All paediatric and adult patients IgE bound to Lat c 1.01 except for patient C5, who coincidentally demonstrated low binding in the immunoblot. However, the IgG<sub>4</sub> antibody binding by ELISA presented different results compared to the immunoblots. There were ten out of sixteen children that demonstrated a positive reaction, including patients C1-3, C7, C12 and C13-14, however patients C11 and C15 were IgG<sub>4</sub> positive in the ELISA, but did not show binding in the immunoblot. In contrast, patients C4-6 were IgG<sub>4</sub> negative by

ELISA, but did bind in the blots. Lat c 1.01 positive adult IgG<sub>4</sub> in the ELISA experiment was only seen in patients A1, A4, A6 and A9.

Uniformly, patients C8-10 and A7 did not show IgG<sub>4</sub> binding in either of the experiments. Fewer patients IgG<sub>4</sub> bound to rLat c 1.01 in the ELISA, with 13 out of 24 compared to 17 out of 24 in the immunoblot. This may be due to the high cut-off value set in the ELISA due to high background readings of the negative control patients. A comparison of IgE and IgG<sub>4</sub> binding between the paediatric and adult cohort can be seen in Figure 5.2B. Mean IgE binding for both paediatric and adult cohorts was significantly higher than IgG<sub>4</sub>.



**Figure 5.1** A) IgE and IgG<sub>4</sub> immunoblots against barramundi recombinant  $\beta$ 1 parvalbumin with the sixteen paediatric (C) and nine adult (A) fish allergic patients. Five non-fish allergic patients were used as negative controls (N). B) Analysis of recombinant  $\beta$ 1 parvalbumin with coomassie stained SDS gel, immunoblots with PARV-19 mAb and anti-barramundi parvalbumin polyclonal antibody. Parvalbumin monomers, dimers and tetramers are boxed in red, green and blue, respectively.

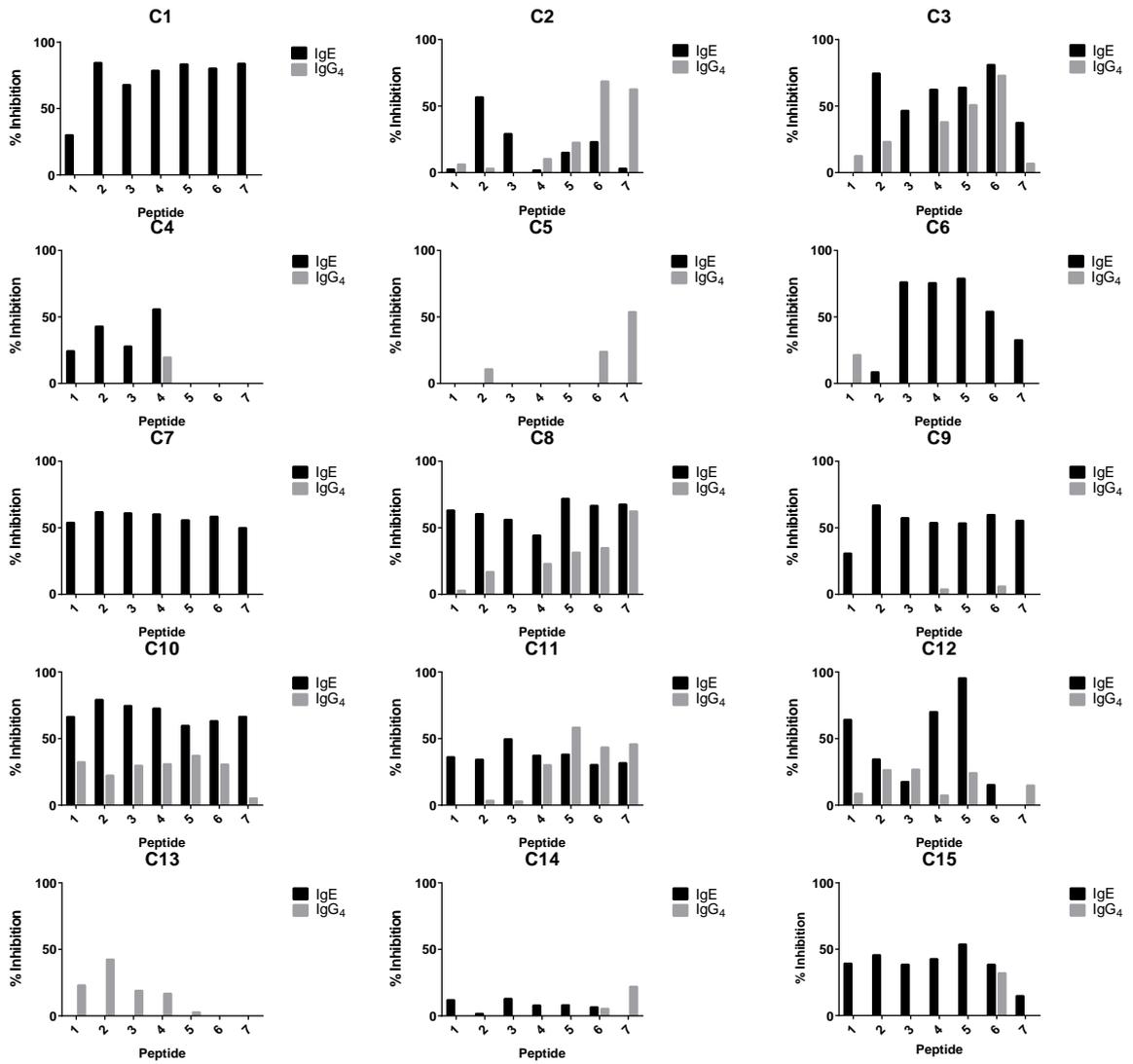


**Figure 5.2** ELISA for serum IgE (black) and IgG<sub>4</sub> (grey) reactivity to purified recombinant Lat c 1.01 for fish-allergic patient sera from fifteen children (C1-15) and nine adults (A1-9) labeled ‘C’ and ‘A’ respectively. The cut-off derived from two standard deviations above the mean reactivity of five non-allergic subjects to each of the extracts is indicated by the dotted line (IgE=0.03, IgG<sub>4</sub>=0.20). n=1. B) Mean fold increase values for all adult and children IgE and IgG<sub>4</sub> against rLat c 1.01 from the

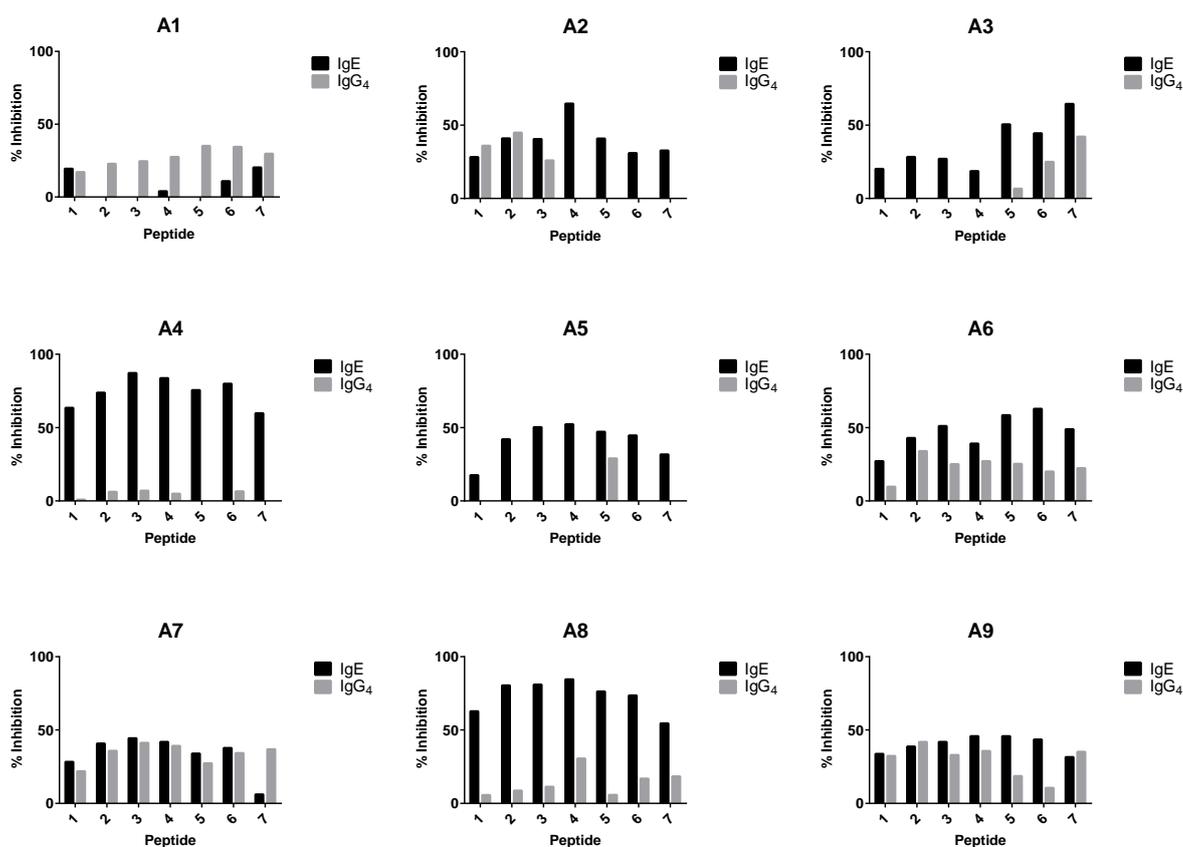
ELISA. Fold increase calculated by dividing O.D values by cut-off value. Error bars represent standard error of the mean. ‘\*’=P<0.05, ‘\*\*\*’=P<0.0001.

### ***5.3.2 IgE and IgG<sub>4</sub> epitope analysis of Lat c 1.01***

Further analysis of IgE and IgG<sub>4</sub> binding to Lat c 1.01 was performed using seven overlapping peptides to help identify their respective antibody binding epitopes. Inhibition ELISA's were performed using these peptides to inhibit IgE and IgG<sub>4</sub> binding to rLat c 1.01. Firstly, IgE and IgG<sub>4</sub> inhibition profiles for each of the fifteen paediatric patients and nine adult patients were compared as seen in Figure 5.3 and Figure 5.4, respectively. Epitope profiles seem to differ between the patients with no single peptide demonstrating outstanding inhibition of either IgE or IgG<sub>4</sub> antibodies. Interestingly, patient specific epitopes are present. Patients C5 and C13 showed no inhibition IgE from the peptides. No IgG<sub>4</sub> inhibition was seen for patients C1 and C6, low inhibition values were seen in patients C4, C6, C9, C14 and A4. Some patients demonstrated distinctly different IgE and IgG<sub>4</sub> epitopic regions. For example, peptide 2 and 3 was able to inhibit patient C2s IgE, where IgG<sub>4</sub> from this patient was strongly inhibited by peptide 6 and 7. Interestingly, all 7 peptides exhibited relatively uniform IgE inhibition from patients C8, C11, C15, A2-3 and A5, however in these patients inhibition of IgG<sub>4</sub> was seen with only 1-3 specific peptides.



**Figure 5.3** ELISA inhibition with the synthetic peptides and individual paediatric patient serum IgE (black) and IgG<sub>4</sub> (grey) labeled C1-C15. Y-axis represents inhibition of serum IgE and IgG<sub>4</sub> binding to 20 ng of rβ1 parvalbumin with 250 ng of the seven generated peptides (x-axis).

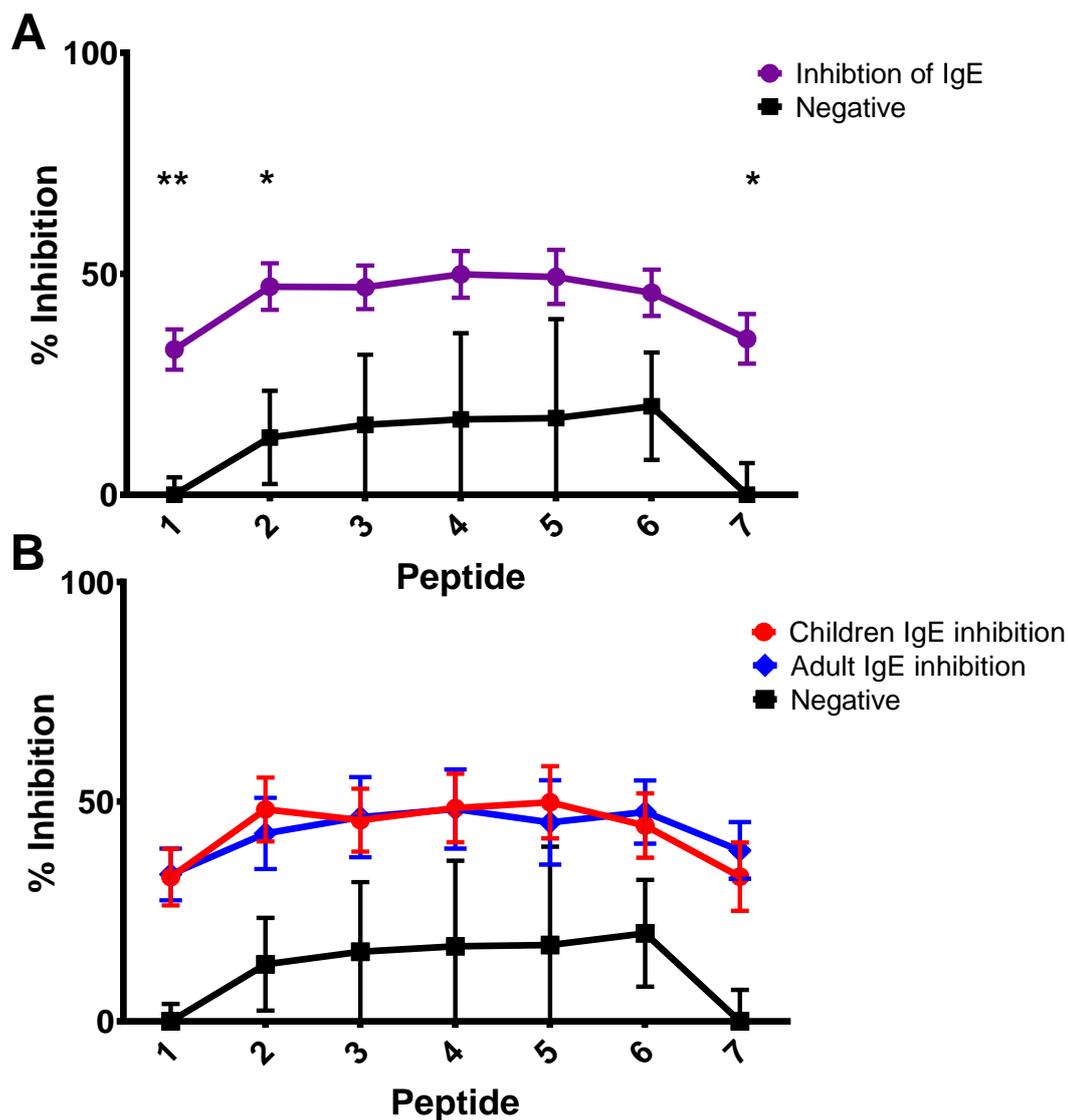


**Figure 5.4** ELISA inhibition with synthetic peptides and individual adult patient serum IgE (black) and IgG<sub>4</sub> (grey) labeled A1-A9. Y-axis represents inhibition of serum IgE and IgG<sub>4</sub> binding to 20 ng of rβ1 parvalbumin with 250 ng of the seven generated peptides (x-axis).

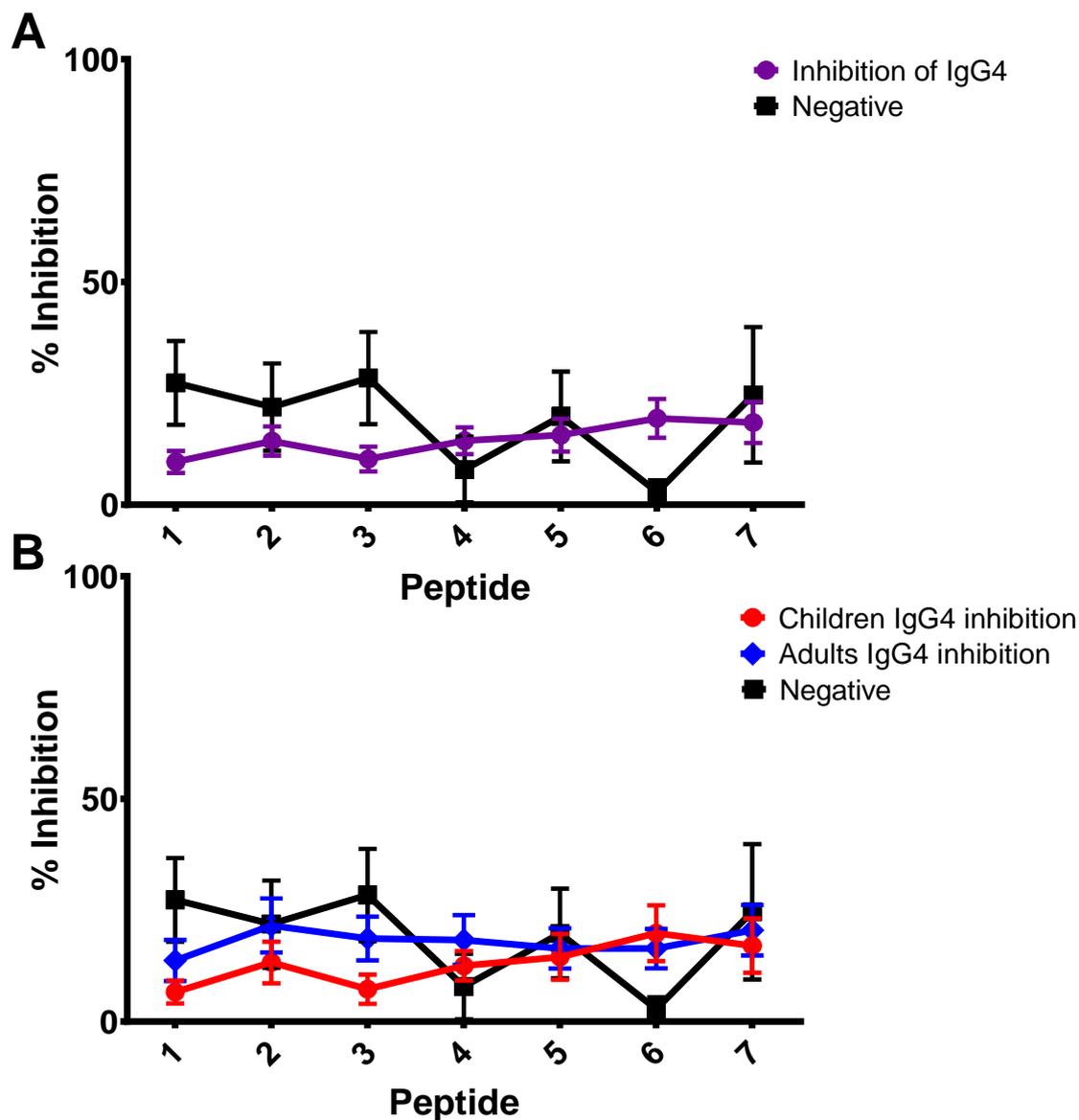
Overall analysis of these peptides inhibiting patient IgE can be seen in Figure 5.5A. Three peptides demonstrated inhibition of IgE binding significantly higher than the negative controls, which were; peptide 1, 2 and 3 with respective mean inhibitions of 31.7%, 45.0% and 34.1%, and p values of 0.006, 0.030 and 0.008. Peptides 4 and 5 had the two highest inhibition percentages with 46.93% and 46.94%, respectively; however these values were not significantly higher than the negatives controls. Furthermore, no peptide significantly inhibits IgE more than another of the peptides used. Figure 5.5B presents the peptide-IgE inhibition data with the adults and children separated in blue

and red, respectively. Interestingly, there is no significant difference between adult and children IgE inhibition for any peptide, with both subgroups following the same trend.

A similar analysis of these peptides inhibiting patient IgG<sub>4</sub> can be seen in Figure 5.6A. No peptide demonstrated inhibition IgG<sub>4</sub> significantly higher than the negative controls. Peptide 6 had the highest inhibition percentages with an average of 19.86. The IgG<sub>4</sub> inhibition experiment had higher background as seen with the negative controls, resulting in uniform low inhibition percentages. Additionally, no peptide significantly inhibits IgG<sub>4</sub> more than another of the peptides used. Figure 5.6B presents the peptide-IgG<sub>4</sub> inhibition data with the adults and children separated in blue and red, respectively. Again, there is no significant difference between adult and children IgG<sub>4</sub> inhibition for any peptide.



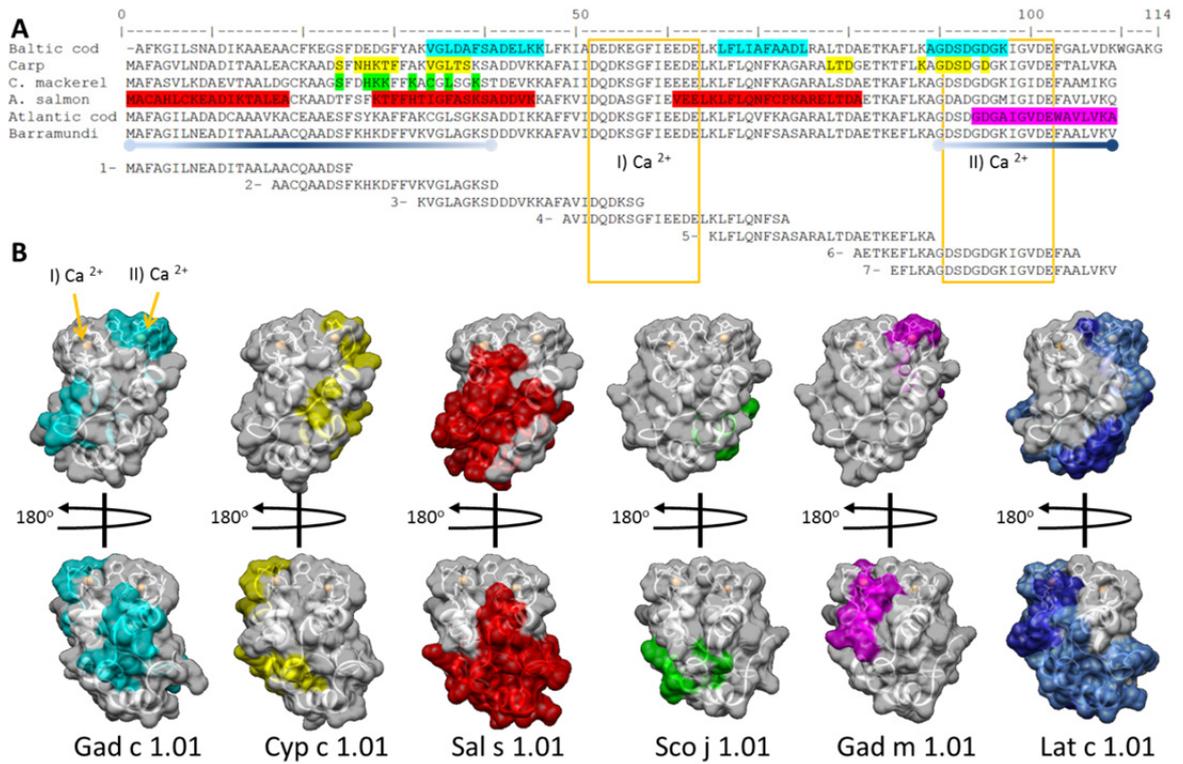
**Figure 5.5** A) IgE Inhibition ELISA using all 24 patients (purple) and 5 negative patients (black). B) IgE Inhibition ELISA data has been separated with 15 paediatric patients (red), the nine adult patients (blue) and negative patients in black. Y-axis represents inhibition of serum IgE (diluted 1:10) binding to 20 ng recombinant r $\beta$ 1 parvalbumin with 250 ng of the 7 generated peptides (x-axis). Error bars represent standard error of the mean. \* =  $P < .05$ , \*\* =  $P < .001$ .



**Figure 5.6** A) IgG<sub>4</sub> Inhibition ELISA using all 24 patients (purple) and five negative patients (black). B) IgG<sub>4</sub> Inhibition ELISA data has been separated with 15 paediatric patients (red), the nine adult patients (blue) and negative patients in black. Y-axis represents inhibition of serum IgG<sub>4</sub> (diluted 1:10) binding to 20 ng recombinant rβ1 parvalbumin with 250 ng of the seven generated peptides (x-axis). Error bars represent standard error of the mean.

To evaluate the proximity of potential IgE epitopes on Lat c 1.01, a multiple alignment of the amino acid sequences of parvalbumins with known IgE epitopes and Lat c 1.01

was performed, as well as 3D models generated for each protein (Figure 5.7). Peptides 1, 2 and 7 demonstrated significantly higher IgE inhibition than the negative serum and these epitope regions are underlined in Figure 5.7A and shaded in Figure 5.7B in navy blue. The dark shading represents the region where IgE most likely binds. The rationale behind the gradient shading is that peptide 1 and 2 share nine amino acids (AACQAADSF) and as these two peptides were able to inhibit IgE, it is more likely that an epitope is in this nine amino acid region. Peptides 6 and 7 share 21 amino acids, however peptide 7 was the only one which significantly inhibited IgE, leaving four amino acids at the c-terminal which it not shared with peptide 6, suggesting an IgE epitope at the c-terminal of the allergen. IgG<sub>4</sub> epitopes were unable to be modelled due to the peptides demonstrating no significant inhibition in these experiments.



**Figure 5.7** A) Multiple alignment of the five known parvalbumin sequences that have IgE epitopes elucidated. IgE epitopes are shaded in colour as follows; Baltic cod (Gad c 1.01) light blue, common carp (Cyp c 1.01) yellow, Atlantic salmon (Sal s 1.01) red, chub mackerel (Sco j 1.01) green and Atlantic cod (Gad m 1.01) pink. Additionally the  $\beta$ 1 barramundi parvalbumin (Lat c 1.01) sequence is present with suspected IgE epitope regions underlined in a navy blue gradient. Sequences of the overlapping peptides used in this study are aligned under the multiple alignment. The two calcium binding sites are boxed in orange. **B**) Protein models of the five parvalbumin allergens with their IgE epitopes highlighted in their respective colours, as well as the barramundi allergen analysed in this study.

## 5.4 Discussion

Understanding IgE epitopes is crucial for the development of future immunotherapies, simultaneously, IgG<sub>4</sub> antibodies play a key role in successful immunotherapy, however this role is yet to be defined. Lat c 1.01 is a major allergen from the fish barramundi [153], as it was characterised in the previous chapter. In this chapter binding characteristics of IgE and IgG<sub>4</sub> from fifteen paediatric and nine adult fish allergic patients to Lat c 1.01 was analysed.

It is known that IgG<sub>4</sub> producing B-cells can switch to an IgE B-cell, however not the other way around as a consequence of the sequence order of the antibody isotypes are situated on their chromosome [134, 136]. So it could be expected that allergen specific IgG<sub>4</sub> be present if there is IgE to the allergen, for example, the IgE negative controls sera were also IgG<sub>4</sub> negative. However immunoblot experiments in this chapter demonstrated that all but one of the fish allergic patients had IgE binding to rLat c 1.01, but only 17 out of 24 had specific IgG<sub>4</sub> to the allergen, implying this isotype switch may be the other way around. In some patients this may be explainable from their epitope binding profiles. For example, patients C8, C10 and A6-seven demonstrated similar epitope profiles, most with higher inhibition of IgE than IgG<sub>4</sub> suggesting that the serum IgE is out-competing the IgG<sub>4</sub> in the immunoblot as they share the same epitopes. Purification of both IgE and IgG<sub>4</sub> from the sera would help to confirm this hypothesis. Fewer patients demonstrated IgG<sub>4</sub> binding in the ELISA which suggests IgG<sub>4</sub> may recognize sequential epitopes that are hidden in the tertiary structure of rLat c 1.01 as the ELISA as performed under native conditions. The negative controls in the IgG<sub>4</sub> immunoassays presented a considerable amount of background, which may have resulted in false negatives in the ELISA. Fewer patients presented IgG<sub>4</sub> binding to rLat c 1.01 than IgE, demonstrating a dissociation between IgG<sub>4</sub> and IgE in fish allergic

patients as reported previously [72]. Despite IgG<sub>4</sub> being associated with allergic disease, these experiments further support that specific IgE is the antibody that directly correlates with allergic symptoms [134, 136].

Fish allergic patients in this study presented individual IgE and IgG<sub>4</sub> epitope profiles that differed between each patient. Specific IgE and IgG<sub>4</sub> epitope profiles could be used to predict the outcomes of specific immunotherapies. A study of milk allergy demonstrated that patients who share IgE and IgG<sub>4</sub> epitopes were more likely to positively respond to their specific immunotherapy using whole allergens due to the increase of serum IgG<sub>4</sub> which acts as a competitive blocking antibody [173]. For example, patients C3, C10, A6, A7 and A9 could successfully respond to this treatment using this hypothesis, however this has not been clinically proven for fish allergy. Patients who had different IgE and IgG<sub>4</sub> epitopes may respond to a specific immunotherapy that has been previously demonstrated to lower IgE reactivity to carp allergen. This involves mutating the calcium binding sites of parvalbumin, which in turn generated a hypoallergenic recombinant carp allergen that could lower the chance of carp allergic patient having an anaphylaxis after ingesting this fish. [72, 131]. The ability of recombinant carp allergen not to cause an allergic Th2 response via challenge has also been demonstrated in mice [15].

IgE and IgG<sub>4</sub> epitopes of Lat c 1.01 were analysed. Specific IgG<sub>4</sub> allergen epitopes could not be mapped and speculative IgE epitopes were identified in peptide 1, 2 and 7. An IgE epitope may be shared by peptide 1 and 2 as they overlap, and the epitope in peptide 7 must lay at the c-terminal as the first 21 amino acids of this peptide overlapped with peptide 6. A sole IgE epitope at the C-terminal was also identified in Gad m 1 in a study which concluded that most IgE epitopes are patient specific [94], which in large has also been presented in this chapter. The IgE epitopes present in

peptides 1 and 2 cover two previously identified epitopes regions of importance. The first is peptide 1 (N-terminal) which is situated in the least conserved region of fish parvalbumin [141]. The only epitope previously mapped in this region is in Sal s 1 from Atlantic salmon, which is possibly responsible for salmon specific fish allergy [76, 91, 175]. Patients who specifically identify this N-terminal epitope may have low cross-reactivity to other fish due to the low amino acid homology in this region. However in this study, no patient binds solely to this peptide. Peptide 2 aligns with a region that seems to contain the most epitopes mapped from fish, including Baltic cod, Atlantic salmon, chub mackerel and carp [141]. This is a highly cross-reactive region of parvalbumin and patients who recognise peptide 2 would most likely react to many other fish allergens. It is not surprising that this cross-reactive epitope has been identified in Lat c 1.01 as barramundi parvalbumin has previously been characterised as a highly cross-reactive antigen [175] (Chapter 3CHAPTER 3).

#### ***5.4.1 Conclusions and future directions***

In this chapter IgE and IgG4 reactivity to Lat c 1.01 was analysed, demonstrating that both of these antibodies isotypes from fish allergic patients detect barramundi allergen. IgE and IgG<sub>4</sub> epitopes of Lat c 1.01 are most likely patient specific. However two regions at the each of the protein terminals contain general IgE epitopes. Further analysis using more overlapping peptides and peptide alanine screening will increase the resolution of the mapped Lat c 1.01 IgE epitopes. Patient epitope specificity has demonstrated the importance of specific fish allergy diagnostics and immunotherapies, where once a patient is diagnosed with fish allergy, further diagnosis may be needed to identify which specific fish the patient is allergic to and the possible specific immunotherapies that could be tailored for the fish allergy sufferer. Further studies on

fish allergen epitopes and cross-reactivity are needed to advance diagnostics and immunotherapies for fish allergy.

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## CHAPTER 6

### General discussion

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Fish plays an important role in human nutrition and health, but can provoke serious IgE-antibody mediated adverse reactions in susceptible individuals. A marked increase in allergic diseases is occurring in most major industrialized countries. The fish allergy and anaphylaxis epidemic is particularly serious and is typically life-long, affecting up to 0.2% of all children and 0.5% of all adults [7]. Prevalence rates specifically to fish vary considerably between regions and among children and adults. Areas with high fish consumption, such as South East Asia and Scandinavia, have a high prevalence of fish allergy and up to 3% of children could be allergic to fish [141]. Greater than 65% of fish allergic infants will continue to be allergic throughout their life [16], and as more children becoming sensitized throughout childhood, most current prevalence studies report more adults with fish allergy than children [7, 92]. Unlike many food allergies, including peanut and egg where a patient is allergic to allergens from a specific species, fish allergy can be caused by any fish allergen across any of the diverse 32,400 species of fish. The major fish allergen is the muscle protein parvalbumin [82, 84], which is the focus point of this thesis. This protein was analysed and characterised to be applied to three key aspects of fish allergy; (1) detection of fish parvalbumin, (2) diagnosis of fish allergy involving parvalbumin and (3) help to advance immunotherapies for fish allergy sufferers.

The detection of this allergenic protein parvalbumin is often difficult due to its structural diversity among various fish species [141]. Fish can express multiple parvalbumin isoforms, which can differ greatly in primary structure. For example; Atlantic salmon and barramundi express two parvalbumin isoforms. The Atlantic salmon isoforms share only 64% of their primary structure and the barramundi isoforms share only 67% [77, 167]. However their biophysical properties differ little as all parvalbumins are small, soluble, acidic proteins, which can bind calcium ions. Nevertheless, this disparity in primary structure of parvalbumin has led to no commercial fish allergen detection tools currently available, such as fish parvalbumin specific antibodies.

Presently, the most commonly available anti-parvalbumin antibody is a monoclonal anti-frog parvalbumin antibody (PARV-19). This antibody has been extensively analysed for its cross-reactivity to different fish parvalbumins throughout Europe and North America [100, 112, 176]. However this antibody had not been analysed for fish from the Asia-Pacific region. In fact, no fish parvalbumin cross-reactivity studies were ever performed on Asian-Pacific fish. In Chapter 2, the cross-reactivity of parvalbumins from Asian-Pacific bony and cartilaginous fish was analysed. The monoclonal antibody, PARV-19, was evaluated as a detection tool and used in the cross-reactivity analysis. This chapter demonstrated the presence of monomeric and oligomeric parvalbumin in all fish analysed, except gummy shark, a cartilaginous fish. Heat processing of this allergen affected its antibody reactivity, despite parvalbumin, like many food allergens, being characterised as a heat stable allergen [143, 177]. Although, the differences in antibody reactivity to heated fish extracts were seen in a reduction in reactivity to multimeric forms of parvalbumins for most bony fish, a complete loss of reactivity was only observed for cartilaginous fish.

The cross reactivity inhibition ELISA profiles from Chapter 2, showed that three of the perciformes uniformly inhibited antibody binding to carp parvalbumin, suggesting parvalbumin cross-reactivity has a molecular and phylogenetic relationship. This prompted a phylogenetic analysis of fish parvalbumins that further demonstrated that parvalbumin cross-reactivity among fish species is due to a molecular phylogenetic association. This was an important finding for the generation of fish specific cross-reactive antibodies that could be used for the detection of fish allergens. Candidate antigens for new cross-reactive antibodies were selected using a more comprehensive parvalbumin phylogenetic tree. A recent clinical study demonstrated that fish allergy is quite specific, where most patients have IgE antibody against a small variety of fish [178]. So, to generate a more comprehensive antibody detection tool, multiple antigens were selected for the generation of polyclonal rabbit IgG antibodies. Previous attempts to generate highly cross-reactive anti-parvalbumin antibodies involved using an antigen from one single fish species, for example Atlantic cod or carp [157, 176]. In this study, the 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 analysed, 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 species. The low cross-reactivity of the anti-salmon antibody correlates with clinical studies which showed monosensitivity to Salmonids [90, 91, 160]. An IgE epitope analysis of salmon parvalbumin highlighted a specific epitope at the N-terminal of this protein. This region is the least conserved primary structure in fish parvalbumin and may be the cause of the

clinical nonsensitivity to salmonids and the specificity of the anti-salmon antibody generated in Chapter 2 [76, 141, 160]. Collectively, the antibodies generated were able to detect parvalbumin in raw extracts of all the analysed fish species, except mahi mahi, swordfish, yellowfin tuna and all 5 canned fish. Mahi mahi, swordfish and yellowfin tuna are all large fish that contain dark slow twitching muscle that has been shown to express lower levels of parvalbumin [105, 108]. The canned fish appeared to have no intact proteins which would result in the lack of antibody reactivity. The parvalbumin tertiary structure has been known to change under food processing techniques [161, 162]. Canned fish and the large fish may be seen less allergenic to patients with parvalbumin mediated fish allergy, however fish specific diagnostics would need to be developed to prove that a patients are not allergic to these specific fish sources.

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. Heat treatment of barramundi extracts seen in Chapter 4 exhibited varying allergenicity, where some patients had an increased IgE reaction and some had a decreased reaction. The importance of heat treated fish in relation to parvalbumin mediated fish allergy appears to be patient specific.

The cross-reactivity studies from Chapter 2 and 3 demonstrated parvalbumin from barramundi to be highly cross-reactive, however at that point in time little was known about the allergenicity of this parvalbumin, with only one fish allergy study involving this commonly eaten fish [133]. Fourteen of the 17 patients analysed in Chapter 4 reacted to both parvalbumin isoforms. Patients had been previously diagnosed against tuna, salmon or cod, further demonstrating how cross-reactive these barramundi allergens are. Subsequently the two novel parvalbumins were submitted as official allergens and designated Lat c 1.0101 and Lat c 1.0201 by the International Union of

Immunological Societies. These two isoallergens demonstrated different IgE reactivity, with IgE binding stronger to Lat c 1.01, however every patient who had IgE bind to Lat c 1.01 was also positive to Lat c 1.02.

The barramundi allergen Lat c 1.01 was further characterised by the evaluation of its IgE and IgG<sub>4</sub> binding abilities and the mapping of their respective antibody epitopes. The analysis showed that specific IgG<sub>4</sub> to Lat c 1.01 is only present when there is specific IgE to the allergen, despite being known that IgG<sub>4</sub> is expressed before IgE, due their respective chromosomal positions [134, 136]. This further reiterates that it is the specific IgE antibody that directly correlates with clinical symptoms, despite the known interaction of IgG<sub>4</sub> in allergy. The epitope analysis also demonstrated that IgE and IgG<sub>4</sub> epitopes are patient specific. IgE and IgG<sub>4</sub> shared epitopes were also patient specific; this could change the response to a specific immunotherapy. An in depth molecular diagnosis of fish allergy may be the future precursor to specific immunotherapies, where IgE and IgG<sub>4</sub> epitope profiles could help tailor a patient specific therapy. A patient who shares these epitopes would more likely respond to allergen immunotherapy, which has been previously demonstrated for milk [173]. A patient who has different IgE and IgG<sub>4</sub> epitopes may respond to a modified allergen immunotherapy [72, 131, 179].

Throughout Chapter 4 and 5, fish allergic patient cohorts were separated into children and adults. Consistently there were no significant differences in IgE binding between the two groups. The lack of difference in IgE binding between children and adults may be due to the fact that fish allergy, similar to shellfish and peanut allergy, is rarely outgrown and continues into adulthood [7, 92].

The data presented in this thesis have demonstrated how allergenic fish parvalbumin has very specific cross-reactive IgE binding profiles. In addition, fish can express multiple parvalbumin isoforms that can differ greatly, which further complicates the development of accurate detection and diagnostic methods. However, implications of the allergen cross-reactive species-specific reactivity and the importance of molecular characterisation of fish allergens outlined in this thesis are vital for the next generation of fish allergen detection tools, diagnostics tools and specific fish allergen immunotherapeutics.

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## APPENDIX A

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### **Buffer Formulations**

#### **PBS 1L:**

NaCl 8 g

KCl 0.2 g

Na<sub>2</sub>HPO<sub>4</sub> 1.44 g

KH<sub>2</sub>PO<sub>4</sub> 0.24 g

Adjust pH to 7.4

#### **TBS 1L:**

NaCl 8 g

Tris ((hydroxymethyl)aminomethane) 3 g

KCl 0.2 g

Adjust pH to 7.4

#### **PBS/TBS-Tween:**

PBS/TBS 1 L

Tween 20 0.5 mL

#### **ELISA coating buffer:**

#### **0.1M Sodium Carbonate Buffer 50mL:**

Na<sub>2</sub>CO<sub>3</sub> 0.53 g

Dissolve in de-ionised water 50mL

**0.1M Sodium Bicarbonate Buffer 50mL:**

NaHCO<sub>3</sub> 0.42g

Dissolve in de-ionised water 50mL

**Sodium Carbonate/Bicarbonate Coating Buffer pH 9.6 (25mL)**

0.1M Na<sub>2</sub>CO<sub>3</sub> 2 mL

0.1M NaHCO<sub>3</sub> 4.25 mL

De-ionised water 18.75 mL

**SDS-PAGE Buffers:****Resolving gel buffer (Buffer B):**

2M Tris-HCl pH 8.8 75 mL

10% SDS 4 mL

De-ionised water 21 mL

**Stacking gel buffer (Buffer C):**

1M Tris-HCl pH 6.8 50 mL

10% SDS 4 mL

de-ionised water 46 mL

**Acrylamide (Buffer A):**

40% Acylamide-bis (Merck, USA)

**Resolving gel 15% Acrylamide (x2)**

Buffer A 3.75 mL

Buffer B 2.5 mL

de-ionised water 3.75 mL

10% Ammonium Persulfate 200  $\mu$ L

TEMED (Tetramethylethylenediamine) 10  $\mu$ L

**SDS-PAGE Running buffer 1L:**

Tris 3 g

Glycine 14.4 g

SDS 1 g

Adjust to pH 8.3

**Transfer buffer:**

Tris 1.164 g

Glycine 0.58 g

10% SDS 750  $\mu$ L

De-ionised water 160 mL

Methanol 40 mL

**Two-dimensional electrophoresis**

**Rehydration/Sample buffer 10mL:**

8M Urea 4.8 g

2% CHAPS 0.2 g

50mM Dithiothreitol (DTT) 77 mg

0.2%(w/v) Biolyte 3/10 ampholytes 20 mg

Trace of Bromophenol blue

**Equilibration Buffer I 30mL:**

6M Urea 10.8 g

2% SDS 0.6 g

20% Glycerol 6 mL

0.375M Tris-HCl pH 8.8 1.37 g

2% (w/v) DTT 0.6 g (add and dissolve just before rehydration)

**Equilibration Buffer II 30mL:** 6 M Urea 10.8 g

2% SDS 0.6 g

20% Glycerol 6 mL

0.375 M Tris-HCl pH 8.8 1.37 g

0.75 g Iodoacetamide (add and dissolve just before rehydration)

**Overlay Agarose gel 50 mL:**

Low melting point agarose gel 0.25 g

25 mM Tris 0.15 g

192 mM Glycine 0.72 g

0.1% SDS 0.05

Trace of Bromophenol blue

**Chromatography buffers:**

**IMAC binding buffer 1 L**

0.5M NaCl 29.22 g

20 mM Sodium Phosphate

5 mM Imidazole 0.34 g

Adjust to pH 7.4 and filter through 0.22  $\mu$ m membrane

**IMAC elution buffer 1 L**

0.5M NaCl 29.22 g

20 mM Sodium Phosphate

250 mM Imidazole 17 g

De-ionised water 1 L

Adjust to pH 7.4 and filter through 0.22 µm membrane

**Tris binding buffer for Anion exchange chromatography 1 L:**

25 mM Tris 3.03 g

De-ionised water 1 L

Adjust pH for specific protein (pH 7-9) and filter through 0.22 µm membrane

**Tris elution buffer for Anion exchange chromatography 1 L:**

25 mM Tris 3.03 g

1 M NaCl 58.44 g

De-ionised water 1 L

Adjust pH for specific protein (pH 7-9) and filter through 0.22 µm membrane

**Bacterial broths and Agars:**

**Luria-Bertani (LB) Medium:**

Tryptone 10 g

Yeast extract 5 g

NaCl 10 g

dH<sub>2</sub>O to 1000 ml

Adjust to pH 7.4 and autoclave at 121°C for 15 min. Store at 4°C until use.

**LB Agar:**

LB Medium 500 ml

Agar 15 g

Antibiotics as required

Autoclave at 121°C for 15 min, cool to 50°C and aseptically add antibiotics before pouring into petri plates

**Ampicillin (100 mg/ml):**

Ampicillin 10 g

ddH<sub>2</sub>O 100 ml

Dissolve ampicillin in water. Filter sterilise (0.22 µm) and store at -20°C in 1 ml aliquots.