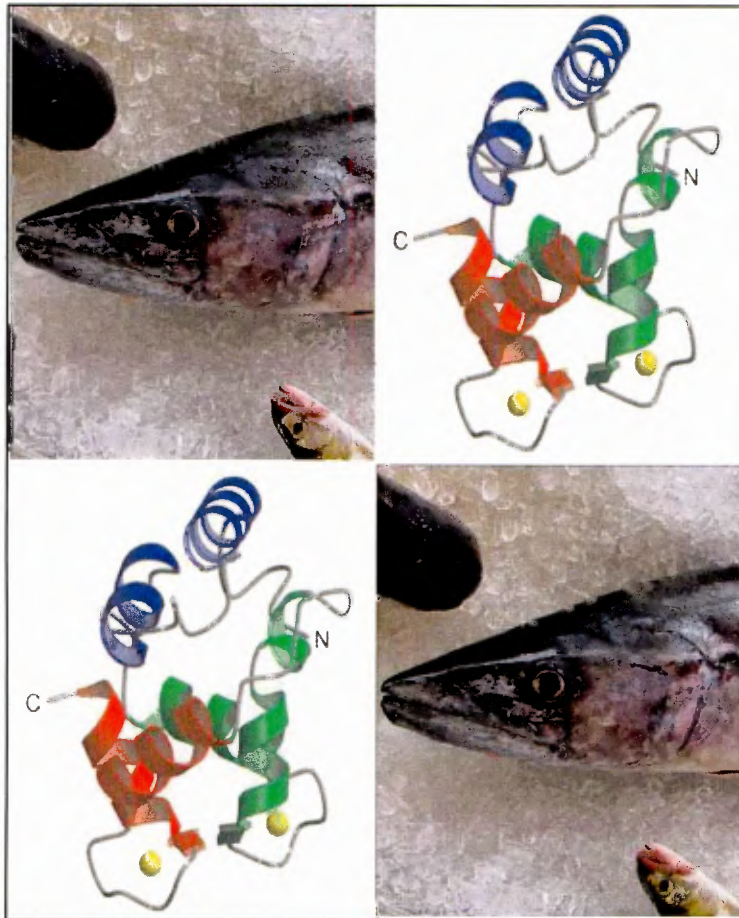


**Molecular characterisation of parvalbumin and
analysis of cross-reactivity in five fish species
using sera from fish-allergic consumers and
occupationally exposed workers.**



Janine Beale

Supervisor: Dr. Andreas Lopata

Co-supervisor: Professor Mohamed Jeebhay

**Presented for M.Sc. (Med) (Masters)
In the School of Biomedical Sciences
Faculty of Health Sciences
University of Cape Town
2008**

DECLARATION

I, Janine Beale, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise), and that neither the whole work or any part thereof has been, is or will be submitted for another degree in this or any other university.

I empower the University of Cape Town to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

.....
Janine Beale
March 2008

ACKNOWLEDGEMENTS

I would like to express my gratitude to the following people:

Dr Andreas Lopata, for your direction and intellectual input

Professor Mohamed Jeebhay for your input, guidance and assistance with editing

**Professor Gordon Brown, for your valued assistance and supervision with regard to
the molecular work**

Natalie Niewenhuizen, for your willingness to always help and assistance with editing

Sylva Schwager for all your assistance with regard to protein separation

Frank Brombacher, for allowing me the opportunity to work in your lab

My family, for their unconditional and unfailing support and encouragement

TABLE OF CONTENTS

Chapter 1: Introduction

| | |
|--|-----------|
| 1.1 An introduction to the immune system..... | 1 |
| 1.2 Immediate type I hypersensitivity or allergy..... | 3 |
| 1.2.1 Mechanisms of an allergic reaction..... | 4 |
| 1.2.2 Causative factors responsible for sensitisation in certain individuals..... | 5 |
| 1.3 Physical properties of an allergen..... | 6 |
| 1.3.1 Cross-reactivity between allergens..... | 7 |
| 1.4 Diagnosis of allergies..... | 9 |
| 1.4.1 Treatment of allergies..... | 10 |
| 1.4.2 Recombinant allergens..... | 11 |
| 1.5 Food allergens..... | 13 |
| 1.5.1 Fish allergies..... | 14 |
| 1.6 Parvalbumin..... | 15 |
| 1.6.1 Additional fish and fish-related allergens..... | 18 |
| 1.7 Occupational fish allergies..... | 19 |
| 1.8 Aims of the current study..... | 22 |
| 1.8.1 Significance of the current study..... | 23 |
| 1.8.2 Summarised objectives..... | 24 |

Chapter 2: Methods and Materials

| | |
|---|-----------|
| 2.1 Preparation of crude extract and purification of natural parvalbumin from five fish species..... | 25 |
|---|-----------|

| | | |
|---------|--|-----------|
| 2.1.1 | Preparation of crude fish extracts..... | 25 |
| 2.1.2 | Purification of parvalbumin..... | 26 |
| 2.1.3 | Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis..... | 27 |
| 2.1.3.1 | Standard SDS-PAGE procedure..... | 27 |
| 2.1.3.2 | Analysis of crude fish extract protein by SDS-PAGE..... | 28 |
| 2.1.3.3 | Analysis of loss of proteins during purification by SDS-PAGE..... | 28 |
| 2.1.3.4 | Assessment of parvalbumin purity by SDS-PAGE..... | 28 |
| 2.2 | Analysis of IgG-binding antigens and IgE-binding allergens..... | 28 |
| 2.2.1 | Immunoblot analysis..... | 28 |
| 2.2.2 | Standard protocol for immunoblotting..... | 29 |
| 2.2.3 | Confirmation of parvalbumin by immunoblot analysis..... | 29 |
| 2.2.4 | Human Sera..... | 30 |
| 2.2.5 | Assessment of sera from fish-processing factory workers..... | 30 |
| 2.2.6 | Assessment of sera from subjects sensitised via fish ingestion..... | 31 |
| 2.2.7 | IgG and IgE reactivity to crude fish extract and purified parvalbumin..... | 31 |
| 2.3 | Further characterisation of pilchard..... | 32 |
| 2.3.1 | Isoelectric focusing (IEF) analysis..... | 32 |
| 2.3.2 | Molecular sequencing of pilchard parvalbumin..... | 32 |
| 2.3.2.1 | Tryptic digestion of purified pilchard parvalbumin..... | 32 |
| 2.3.2.2 | Mass spectrometer (MS) analysis..... | 33 |
| 2.3.2.3 | Analysis of tryptic peptides..... | 34 |
| 2.3.2.4 | Total and messenger RNA Extraction and cDNA synthesis..... | 34 |
| 2.3.2.5 | Primer design..... | 35 |
| 2.3.2.6 | Polymerase chain reaction | |
| | i.) Amplification of partial parvalbumin sequence..... | 36 |
| | ii.) 5'- and 3'- Rapid Amplification of cDNA Ends (RACE)..... | 36 |
| 2.3.2.7 | Agarose gel electrophoresis..... | 37 |

| | |
|--|----|
| 2.3.2.9 Subcloning of insert DNA for sequencing..... | 37 |
| 2.3.2.10 Transformation and selection of colonies..... | 38 |
| 2.3.2.11 Confirmation of insert DNA..... | 39 |
| 2.3.2.12 Plasmid sequencing..... | 39 |

Chapter 3: Results

| | |
|---|-----------|
| 3.1 The investigation of natural parvalbumin from five fish species and its subsequent purification..... | 40 |
| 3.1.1 Isomers and oligomeric forms of parvalbumin were noted in 5 crude fish extracts..... | 40 |
| 3.1.2 Parvalbumin was purified from all 5 crude fish extracts..... | 42 |
| 3.2 Comparison of antibody binding to purified parvalbumin and crude fish extract using sera from fish-allergic patients..... | 49 |
| 3.2.1 Specific IgE levels were measured by Cap-RAST in both cohorts of fish-allergic subjects..... | 49 |
| 3.2.2 Sera from fish-processing factory workers was used to assess IgG and IgE reactivity to crude pilchard and anchovy proteins..... | 53 |
| 3.2.3 Sera from fish-allergic domestic consumers was used to assess IgG and IgE reactivity to crude fish proteins and purified parvalbumin..... | 56 |
| 3.2.3.1 A wide range of IgG specific antigens were recognised by all fish-allergic patients..... | 56 |
| 3.2.3.2 Parvalbumin was the dominant allergen in patients sensitised via fish ingestion..... | 58 |
| 3.2.4 Summary of cross-reactivity of parvalbumin from 5 fish species..... | 63 |
| 3.3 Further characterisation of pilchard parvalbumin..... | 68 |

| | |
|---|----|
| 3.3.1 Isoforms of pilchard parvalbumin were detected by two-dimensional electrophoresis and immunoblotting..... | 64 |
| 3.3.2 cDNA encoding parvalbumin from pilchard (<i>Sardinops sagax</i>)..... | 66 |
| 3.3.2.1 One tryptic peptide from parvalbumin was elucidated by MS/MS analysis for primer design..... | 66 |
| 3.3.2.2 Primers were used to amplify a portion of the pilchard parvalbumin gene.... | 69 |
| 3.3.2.3 The parvalbumin gene was partially deduced to attain sequence information..... | 72 |
| 3.3.2.4 The complete pilchard parvalbumin sequence was determined by 5' and 3' RACE..... | 72 |
| 3.3.2.5 Alignment of pilchard parvalbumin with 12 similar fish species..... | 75 |

Chapter 4: Discussion

| | |
|--|----|
| 4.1 Characterization of parvalbumin from five South African fish species..... | 79 |
| 4.1.1 Detection of parvalbumin using a commercial monoclonal anti-parvalbumin antibody..... | 79 |
| 4.2 Parvalbumin was purified from all five crude fish extracts..... | 80 |
| 4.3.1 Detection of allergens using sera from fish-processing workers..... | 81 |
| 4.3.2 Parvalbumin was not recognised as an allergen in fish-processing workers.... | 84 |
| 4.4.1 Cross-reactivity between parvalbumin from five fish species..... | 86 |
| 4.4.2 Applications of cross-reactivity and the relevance of findings in this study..... | 88 |
| 4.5.1 IgE binding to dimeric forms of parvalbumin using sera from allergic fish consumers..... | 89 |
| 4.5.2 IgE binding to additional fish allergens..... | 90 |
| 4.6.1 Further characterisation of isoforms from pilchard parvalbumin..... | 91 |
| 4.6.2 cDNA encoding parvalbumin from pilchard was determined..... | 92 |
| 4.7 Conclusion..... | 94 |

Chapter 5: Appendices

| | |
|--|------------|
| Appendix A: Solutions | 96 |
| Appendix B: Representation of fish taxonomy | 100 |

Chapter 6: References

| | |
|----------------------------|------------|
| 6. References | 101 |
|----------------------------|------------|

LIST OF FIGURES

Figure 1.1. A summary of the four populations of CD4⁺ T cells, namely T_{H2}, T_{H1}, T_{H17} and T_{Reg} lymphocytes.

Figure 1.2. Induction and effector processes of an IgE-mediated hypersensitivity reaction.

Figure 1.3. Representation of a conformational and sequential epitope of an allergen.

Figure 1.4. A flow diagram representing the steps carried out in the production of a recombinant allergen.

Figure 1.5 Three-dimensional structure of carp parvalbumin in a ribbon presentation.

Figure 1.6 Two common processes carried out in a fish-processing factory in factory in St. Helena Bay, Western Cape.

Figure 3.1.1 SDS-PAGE and immunoblot analysis of parvalbumin between different fish species.

Figure 3.1.2 DEAE-cellulose anion exchange chromatography profile of all 5 fish species

Figure 3.1.3 SDS-PAGE and immunoblot analysis of purified, partially purified and crude extracts of all 5 fish species.

Figure 3.1.4 Comparison of IgG and IgE binding to pilchard crude extract and as well IgG and IgE reactivity to anchovy crude extract.

Figure 3.1.5 Comparison of IgG reactivity to crude pilchard , yellowtail , hake, anchovy and snoek extract.

Figure 3.2.1 Two-dimensional electrophoresis map of nitrocellulose-blotted purified pilchard parvalbumin.

Figure 3.2.2 MS/MS spectrum of the peptide AFAIIDQDK present in parvalbumin.

Figure 3.2.3 A schematic representation of potential cleavage sites of trypsin in the chub mackeral parvalbumin amino sequence.

Figure 3.2.4 Primers were designed using the deduced amino acid sequence obtained by MS/MS analysis.

Figure 3.2.5 PCR reaction resolved on a 0.8% agarose gel using primers designed by MS/MS results and homology alignment.

Figure 3.2.6 Comparison of 2 cDNA clones (clone 1 and clone 2) obtained from sequencing a portion of the parvalbumin gene.

Figure 3.2.7 5' RACE and 3' RACE products were resolved on a 0.8% agarose gel.

Figure 3.2.8 Nucleotide and deduced amino acid sequence of the β form of pilchard parvalbumin which was designated Sar s 1.

Figure 3.2.9 Comparison of the deduced amino acid sequence of pilchard parvalbumin with 13 fish species.

LIST OF TABLES

Table 2.1 Primers used for sequencing of the parvalbumin gene

Table 3.1. Yield of total protein (mg/ml) and an indication of loss of protein after purification steps.

Table 3.2.1 Clinical and serological characterisation of fish-processing factory workers.

Table 3.2.2 Clinical and serological characterisation of individuals with allergic reactions after fish ingestion.

Table 3.2 Summary of cross-reactivity between parvalbumin from 5 fish species.

Table 3.4 The common and scientific names of the fish species represented by BLAST sequences, together with the lineage and percentage of homology to Sar s 1.

ABBREVIATIONS

| | |
|--------------------|--|
| aa | amino acid |
| Ab | antibody |
| Ag | antigen |
| AP | alkaline phosphatase |
| APC | antigen presenting cell |
| APDH | aldehyde phosphate dehydrogenase |
| B cell | lymphocytes established in bone marrow |
| BCIP/NBT | 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium |
| BSA | bovine serum albumin |
| BCA | bicinchoninic acid |
| Bp | base pairs |
| °C | degrees Celsius |
| CD | cluster of differentiation |
| CTL | cytotoxic T cell |
| DBPCFC | double-blind placebo-controlled food challenge |
| DC | dendritic cell |
| DEAE | diethyl aminoethyl |
| DEPC | diethyl pyrocarbonate |
| Der p 1 | house dust-mite (<i>Dermatophagoides pteronyssinus</i>) allergen 1 |
| DNA | deoxyribonucleic acid |
| ddH ₂ O | distilled deionised water |

| | |
|---|---|
| DTT | dithiothreitol |
| EDTA | ethylenediamine tetraacetic acid |
| Fc | fragment crystallisable portion |
| FcεR | Fc epsilon receptor. |
| Fel d 1 | cat (feline) allergen 1 |
| Fig. | figure |
| IEF | isoelectric focusing |
| IFN-γ | interferon gamma |
| Ig | immunoglobulin |
| IL | interleukin |
| kDa | units of molecular mass in kilo Dalton |
| mRNA | messenger RNA |
| MHC | major histocompatibility complex |
| MS | mass spectrometry |
| MW | molecular weight |
| (NH₄)₂SO₄ | ammonium sulphate |
| NK | natural killer cells |
| ON | overnight |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| RT | room temperature |
| RACE | rapid amplification of cDNA ends |
| RAST | radioallergosorbent test |
| RNA | ribonucleic acid |
| SDS | sodium dodecyl sulphate |
| SEM | standard error of the mean |

| | |
|------------------------|--|
| SPT | skin-prick test |
| TBS | transfer buffer solution |
| Tris | Tris (hydroxymethyl) aminomethane |
| T cell | T lymphocytes |
| T_c | cytotoxic T cell |
| T_H | T helper |
| T_{Reg} | T regulatory |
| TNF | tumour necrosis factor |
| Rpm | revolutions per minute |

ABSTRACT

Parvalbumin, the fish major allergen, accounts for over 95% of clinical symptoms in allergic fish consumers. Importantly, this allergen displays IgE cross-reactivity thus allergic sufferers can exhibit clinical symptoms after the ingestion of non-sensitising fish species. In an occupational setting, fish products have also been shown to cause allergic disease in fish-processing factory workers. Whether parvalbumin is a causative allergen in this occupational environment is unknown. The aim of this study was to evaluate IgE reactivity to parvalbumin and other fish fillet proteins using sera from domestic consumers with ingestion-induced fish allergies and sera from occupationally exposed allergic workers. In addition, cross-reactivity among parvalbumins from five highly consumed fish species in South Africa were assessed by immunoblotting and the most cross-reactive species was characterised further. Pilchard parvalbumin was identified as the most cross-reactive allergen in fish-allergic consumers. The cDNA sequence of the β form of pilchard parvalbumin was determined. This is the first time that parvalbumin from the fish order, *Clupeiformes*, has been characterised and represents a crucial primary step towards the generation of a recombinant form for potential diagnostic and therapeutic use in allergic individuals. Interestingly, sera IgE from fish-processing factory workers displayed no binding to parvalbumin, nor any other fish fillet proteins in immunoblotting. This result has raised several intriguing questions. Namely, does parvalbumin lack the intrinsic features required for eliciting allergic symptoms via inhalation and/or contact, as are primary routes of exposure in workers? Alternatively, could causative occupational allergens that appear to be absent in the fillet of fish occur in the enzyme-rich digestive tract or potentially the skin of fish species? Future studies aim to address these questions amongst others, which will contribute to preventative and therapeutic strategies of occupational allergies in workers.

Chapter 1: INTRODUCTION

1.1 An introduction to components of the immune system

The immune response is characterised by recognition of foreign elements and their subsequent elimination which is implemented by two arms of the immune system. Specifically, the innate response provides the first line of defence against foreign invaders while adaptive immunity is highly specific for invading antigens and capable of acquiring a memory for the foreign agent. The latter response can be further classified into a cell-mediated and humoral/antibody-mediated response ¹.

In innate immunity, phagocytic cells, including monocytes, polymorphonuclear neutrophils and macrophages act as a primary defence against infection ². Basophils, mast cells and platelets mediate inflammation with the purpose of attracting leukocytes and cytokines to the site of infection. In addition, mast cells and basophils are fundamental effector cells in hypersensitivity reactions and allergic conditions as well as a defence against parasites ³. Contributing to a protective response, natural killer (NK) cells carry out the role of termination of specific tumour cells and virus-infected cells while specialised leukocytes, known as eosinophils, are capable of breaking down large extracellular parasites ⁴.

Based on the magnitude and specificity of the response generated by components of innate immunity, the adaptive immune system is instructed to mount a response ^{5, 6}. This regulation is in the form of growth and differentiation factors termed cytokines as well as co-stimulatory molecules expressed on antigen presenting cells (APC) ⁷⁻⁹. Forming a bridge between the two immune responses, these phagocytic APCs, in particular dendritic cells (DC), recognise pathogens via pattern-recognition receptors (PRR) ¹⁰. Specifically, toll-like receptors (TLR) are crucial for identification of

microbial components and activation of the immune system ¹¹. The APCs engulf, process and present the foreign peptides bound to major histocompatibility complex (MHC) molecules which stimulate a highly specialised population of cells of adaptive immunity, namely T cells ^{1, 6}. Antigens that are processed through MHC class II molecules associate with the subset of T cells carrying the CD4⁺ marker, termed T helper (T_H) cells, responsible for activating and aiding other components of the immune system. The processing of antigen via class I MHCs results in stimulation of another subpopulation of T cells possessing CD8 markers. CD8 cells primarily have a killing function hence their title, cytotoxic T cells (CTLs) ¹².

The CD4 T cell population is further divided into T_H1 and T_H2 subtypes based on the cytokine environment created by APCs and PRR recognition ⁷. T_H1 cells are characterised by the secretion of interferon γ (IFN- γ), tumour necrosis factor (TNF), interleukin (IL)-2 and granulocyte-monocyte-colony-stimulating factor (GM-CSF). Favours cell-mediated immunity, the T_H1-type response is primarily responsible for defence against protozoa and intracellular bacteria, inflammation and macrophage stimulation. Differentiation into T_H2 type cells is encouraged by the presence of IL-4 secreted by APCs exposed to allergens and helminths components. T_H2 cells produce IL-4, IL-5, IL-9 and IL-13 and are central for antibody production and increased induction of mast cells and eosinophils ^{9, 12, 13}. Recently an additional T_H17 cell subset that produces IL-17 has been recognised and may be associated with early inflammation, autoimmunity and defense against parasites and extracellular bacteria ⁹. To counteract and control excessive immune responses, a CD4⁺ T cell population, termed regulatory T cells (T_{Reg} cells) serve to dampen responses through production of IL-10 and transforming growth factor β (TGF- β).

In addition to T cells, B cells are critical mediators of immunity. These antibody-secreting cells are stimulated by direct recognition of the antigen through B-cell

receptors (BCR) and can evolve into plasma cells and memory B cells ¹². The former cells are responsible for the production of large amounts of highly specific antibodies that initially target invading pathogens while the latter are high-affinity, long-lived lymphocytes that respond swiftly when restimulated with the same antigen ¹⁴. These antibodies, also termed immunoglobulins (Ig), are divided up into five major classes or isotypes, namely IgM, G, A, D and E ¹⁵.

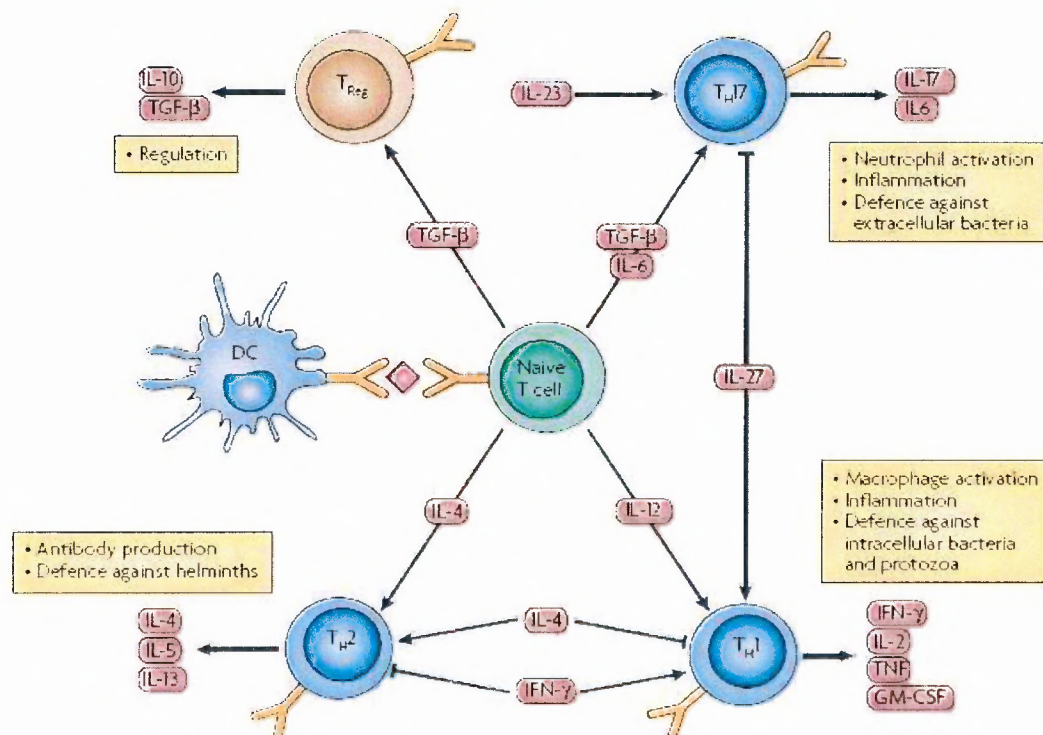


Figure 1.1: A summary of the four populations of CD4⁺ T lymphocytes. T_H1 lymphocytes are characterised by their production of IFN-γ and IL-2 and their subsequent influence on cellular immunity. The production of IL-4, IL-5, and IL-13 is a feature of T_H2 lymphocytes and play a role in humoral, hypersensitivity responses and defence against helminths. T_{Reg} cells dampen excessive immune responses via IL-10 and TGF-β while T_H17 cells produce IL-17 and may be involved in inflammation and defence against extracellular bacteria via neutrophil activation amongst other things (Adapted from Kaufmann *et al.*, 2007).

1.2 Immediate type I hypersensitivity or allergy

Certain immunological reactions can result in an exaggerated, inappropriate response, which is detrimental to the host, termed hypersensitivity. ¹⁶. The most frequent form of this response is Type I or immediate hypersensitivity, also commonly

referred to as allergy ¹⁷. This immunological response is characterised by the production of IgE antibodies against innocuous, environmental proteins, termed allergens ². Clinical symptoms of such a disorder manifest as IgE-mediated asthma, allergic rhinitis, conjunctivitis, IgE-mediated food allergy, atopic eczema and in extreme cases, anaphylaxis, which can potentially cause death ¹⁸. Those individuals that possess a personal and/or familial tendency to generate allergen-specific IgE are termed atopic, a condition that occurs in more than 25% of the global population ^{13, 19}.

1.2.1 Mechanisms of an allergic reaction

An allergen that comes into contact with the immune system is engulfed and processed by the APC prior to presentation to the naïve T cell. Sensitisation, or the primary response to an allergen, involves initial generation of allergen-specific T_H2 cells in the presence of IL-4 (Figure 1.2) ²⁰. The cytokines IL-4, IL-5, IL-9 and IL-13, generated by T_H2 cells facilitate the allergic response ²⁰. IL-4 and IL-13 stimulate B cells to produce IgE which bind to the high-affinity surface receptors (FcεRI) on mast cells and basophils, and to a lesser extent on DCs and monocytes ²¹. Subsequent exposure to the same allergen, or in some cases, a protein of a similar structure, results in cross-linking of the IgE-FcεRI complex ²². Signal transduction causes an influx of calcium which activates degranulation of the cells and the release of vasoactive amines, such as histamine, cysteinyl leukotrienes, prostaglandins, chemokines and cytokines ^{23, 24}. These mediators, predominantly released from mast cells, initiate symptoms associated with immediate hypersensitivity which begin minutes after allergen exposure ¹³. Such acute symptoms can include allergic rhinitis, conjunctivitis, urticaria, angioedema, IgE-mediated asthma, atopic eczema, gastrointestinal disorders and anaphylactic shock ²⁵. T_H2 related cytokines also mediate several effector functions such as increased eosinophil survival and recruitment, hypersecretion of mucus, hyperreactivity of the bronchi and mast cell

differentiation^{20, 23}. In some individuals a late-phase reaction (LPR) can occur, which is characterised by the return of clinical symptoms two or more hours after allergen exposure. This is due to the local influx of circulating eosinophils, basophils, mast cells and B cells in response to the mediators released during the early phase^{13, 24}. T_H1 components also play a role in this chronic phase by producing IFN- γ , TNF and Fas-ligand which contribute to the apoptosis of bronchial epithelial cells, keratinocytes and pulmonary smooth-muscle cells²⁶.

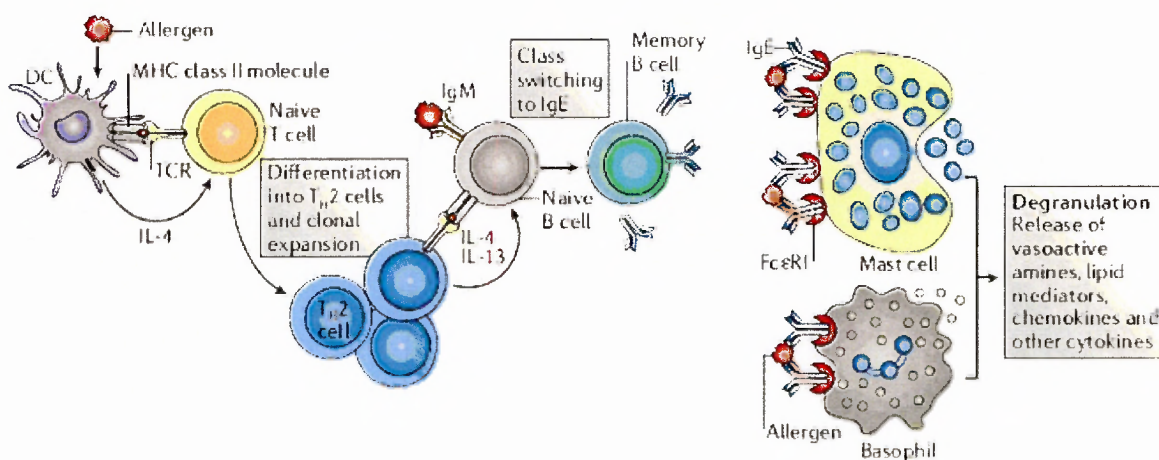


Figure 1.2 Mechanism of an allergic reaction. The allergen is taken up by the dendritic cell (DC) where it is processed and presented to the naïve T cell. Sensitisation involves the initial generation of allergen-specific T_H2 cells in the presence of IL-4. The cytokines, IL-4, IL-5, IL-9 and IL-13, generated by T_H2 cells facilitate the allergic response. IL-4 and IL-13 stimulate B cells to produce IgE (after class switching from IgM) which bind to the high-affinity surface receptors (Fc ϵ RI) on mast cells and basophils. Subsequent exposure to the same allergen or an allergen with a similar structure results in cross-linking of IgE and the degranulation of cells which release mediators responsible for the clinical symptoms of an allergic reaction (Adapted from Larche *et al.*, 2006).

1.2.2 Factors responsible for allergic sensitisation in susceptible individuals

Sensitisation to an allergen is largely determined by an individual's genetic tendency to develop a T_H2 response^{24, 27}. A number of candidate genes with variations in the β chain of Fc ϵ RI, IL-4, IL-13 and the IL-4 receptor have been associated with atopy²⁷. However, no definitive genetic markers for IgE sensitisation have been identified, and

studies have predominantly shown allergic susceptibility to be a complex genetic state made up of multigenic determinants²⁸.

A large body of evidence suggests that chronic exposure to allergens typically presented to the immune system in low amounts is also a predictor of sensitisation²⁹. Studies demonstrate that priming of T_H2 cells occurs in the presence of low-dose allergen exposure while a T_H1 response is favoured by higher doses of antigen in genetically predisposed individuals^{30, 31}. These findings are in line with immunological responses generated by the allergen-specific treatment of allergic disease, namely immunotherapy²³. By administering increasing doses of the causative allergen to the patient, IgE antibody production is decreased accompanied by the production of protective specific IgG, resulting in desensitisation¹³.

1.3 Physical properties of allergens

Clinical symptoms of an allergic reaction may vary, partly depending on how the allergen is introduced into the body and its general properties^{27, 32, 33}. In the case of respiratory allergy, exposure to aeroallergens such as *Fel d 1* from the domestic cat and other indoor allergens are generally associated with asthma while exposure to *Phl p 1* and *V* from the pollen of grass are responsible for triggering seasonal rhinoconjunctivitis^{27, 33}. A likely contributing factor resulting in these distinctions include variations in the physical dimensions of the particles, as pollen allergens are comparatively larger thus cannot enter the lower bronchial tree of the lungs. Only a limited range of particle sizes are respirable and are able to reach low into the lungs rather than being deposited in the nose and throat²⁷.

In addition, many of the inhalant allergens exhibit enzymatic activities that can enhance allergenic potential, such as the common indoor allergen, *Der p 1*. This enzymatic protein crosses the epithelium and interacts with components of the immune system where it modifies certain molecules via proteolytic activity eliciting an allergic response³⁴. In an occupational environment, airborne enzymes act as potent allergens in many cases^{35, 36}. For example, the enzyme α -amylase is an important cause of baker's asthma³⁶. In addition, barrier disruption of the cutaneous surface via enzymatic activity has been shown to favour a T_H2 environment in atopic eczema. The reaction can then be exacerbated further by disruption of the skin caused by scratching due to the intensely itchy and erythematous inflammatory nature of this skin disease³⁷.

However, enzymatic activity is not a property that is present in all allergens²⁹. In fact, there are few structural features, if any, that are presently known to be common to all allergens. However, certain features are thought to play a role in allergenicity such as stability, solubility, size, the general fold and the extent of the posttranslational modification³².

Cross-reactivity between allergens

The property of cross-reactivity between allergens, or the sharing of antigenic features, has been demonstrated to affect allergenic potential^{2, 32, 38}. This phenomenon of pre-existing IgE binding to an allergen that did not elicit its formation is strongly related to structural similarities. Specifically, common features in the primary and tertiary structure between allergens, including comparable folds, have been noted. In most cases, over 70% amino acid identity is an indication that two allergens will display cross-reactivity, although additional knowledge of the three-

dimensional structure can ensure a more accurate prediction^{32, 39}. It is also important to take into account epitope-specific binding, i.e. the surface area of the allergen that interacts with the IgE antibody. This interaction can be sequential in nature, meaning the antibody reacts with a linear peptide usually 8-10 amino acids in length, or conformational, which involves simultaneous interaction with amino acids from different parts in the linear sequence^{32, 40} (Figure 1.3). Mapping of these allergenic epitopes have been carried out in numerous studies to determine specific IgE binding to many major food allergen which have revealed important information on clinical reactivity of the patient⁴¹⁻⁴⁴.

Importantly, this attribute of cross-reactivity provides a possible strategy for treatment of allergy, namely via immunotherapy. If desensitisation to an immunodominant allergen, that shares epitopes with a group of homologous allergens, could be utilised for immunotherapy then multiple desensitisation could be attained. This is a strategy that has been implemented for cat, ragweed and birch pollen allergy with promising results⁴⁵. In addition, similar allergens also hold potential as effective diagnostic tools. Specifically, it has been demonstrated that the use of two alike allergens, as opposed to using only one in a diagnostic panel, does not increase accuracy^{46, 47}. Thus, the clustering of cross-reactive allergens has the potential to greatly abridge diagnostic assays and therapeutic treatments³⁹.

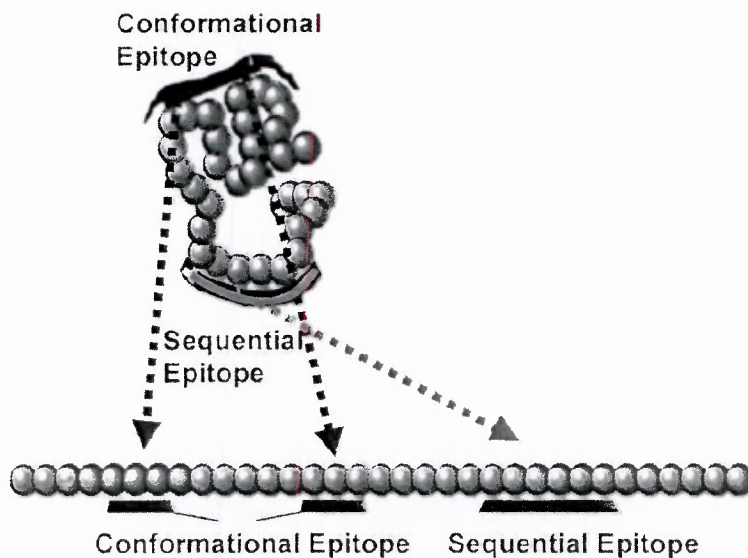


Figure 1.3 The surface area of the allergen that interacts with the IgE antibody is termed the epitope. Epitopes can be sequential in nature, meaning the antibody reacts with a linear peptide usually 8-10 amino acids in length, or conformational, which involves simultaneous interaction with amino acids from different parts in the linear sequence as illustrated. Conformational epitopes are destroyed when the structure of the allergen is altered, whereas sequential epitopes are not (Taken from Sampson, 2004).

1.4 Diagnosis of allergies

For the diagnosis of an allergic disease, a thorough clinical history of the patient as well as *in vivo* and/or *in vitro* tests to assess the presence of IgE antibodies, is standard practice⁴⁸⁻⁵⁰. Skin-prick tests (SPT) are the most widely used *in vivo* assays for indicating the presence of IgE⁴⁹. This protocol entails the application of the test allergen to the skin (usually the forearm), which is pricked with a lancet. A positive reaction is indicated by a wheal of 3mm or greater with surrounding erythema which is indicative of allergen specific IgE present on the surface of mast cells^{48, 49}.

In terms of commercially available *in vitro* immunoassays, the ImmunoCAP (Phadia) automated technology is frequently used as it provides quantitative, accurate and highly reproducible results^{48, 50}. The CAP refers to the cup shaped hydrophilic carrier polymer, which is covalently coupled to the test allergen. Any allergen-specific IgE

present in the serum will bind to this extract⁵⁰. Bound serum IgE is then detected with radioactive- or enzyme-labelled anti-human IgE antibodies⁵¹.

It should be noted, however, that the measurement of specific IgE in patient's serum as an isolated assay to diagnose allergic disease is controversial^{48, 50}. This is due to the fact that clinical symptoms of allergy do not always correspond with levels of specific IgE antibody⁵². Thus, additional laboratory-based methods such as the evaluation of eosinophil activation, mast cell activation and basophil degranulation have also been incorporated in some cases for diagnosis⁴⁸.

In addition, oral provocation assays can be carried out to assess clinical manifestations in patients. To date, the double-blind placebo-controlled food challenge (DBPCFC) is considered the benchmark for diagnosis of food allergy^{53, 54}. The suspected food is administered to the patient while disguising all taste and texture thus any subjective influence is eliminated. Carried out correctly, a positive DBPCFC is the most reliable indicator of a food-specific allergy⁵¹.

1.4.1 Treatment of allergies

In terms of food allergies, the only proven therapy is still the complete elimination of the causative allergen from a patient's diet or environment⁴⁰. However, allergen-specific immunotherapy has been proven to be a very effective clinical treatment that limits symptoms of allergy and reduces the use of medicine in individuals in certain cases⁵⁵. In addition, a reduction in the progression of allergic diseases has been noted⁵⁶. This procedure generally involves the administration of increasing doses of the causative allergen via subcutaneous injection but in recent times sublingual applications have been incorporated. The immunological mechanisms of immunotherapy are only partly defined, but it is known that treatment results in an

increase of T_H1 related cytokines as well as induction of T_{reg} cells. The resultant production of IL-10 and TGF- β is thought to contribute to immunoglobulin class switching to IgA, IgG₁ and IgG₄ which compete with IgE for allergen binding¹³. A resultant decrease in the amount of mast cells and the propensity to release mediators is also thought to occur⁵⁷.

The extensive implementation of immunotherapeutic treatment has largely been limited due to a lack of standardised parameters¹³. Specifically, the quality, amount and biological activity of the allergen extract used should be known without batch-to-batch variation⁴⁹. This is challenging when a natural allergen source is utilised¹³. However, in the last few decades, DNA technology has enabled the production of pure recombinant allergens of a standardised quality and of unconstrained quantities which have been utilised for diagnostic and therapeutic treatments⁵¹.

1.4.2 Recombinant allergens

The primary step in the production of a recombinant allergen is to elucidate the full cDNA sequence that encodes for the protein of interest. Once this has been completed, the cDNA is inserted into an expression plasmid which can, depending on the vector utilised, either produce a fusion or non-fusion recombinant protein (Figure 1.4). For protein expression, *Escherichia coli* is often utilised (particularly for non-glycosylated allergens) in the transformation of the engineered plasmid. Protein expression is then induced and the protein is purified and characterised in detail. This is particularly relevant if the recombinant allergen is destined to be used for a diagnostic and/or therapeutic function. Several *in vitro* and *in vivo* assays must be performed to characterise biophysical, biochemical, biological, and immunological features⁵⁸ (Figure 1.7). In particular, it is important to assess IgE reactivity with sera

from an assortment of populations to assess feasibility as a diagnostic or therapeutic tool⁴⁹.

The use of 'native' recombinant allergens that maintain the primary sequence and tertiary structure of the natural allergen can be implemented in diagnostic assays where exact amounts of the active compound can be administered. Conversely, these recombinants can potentially cause allergic symptoms particularly if utilised for immunotherapeutic treatment. For these uses, hypoallergens, or modified proteins with reduced IgE reactivity and/or increased immunogenicity have been generated. Such allergens have been developed for the peanut allergens Ara h 2 and Ara h 3, the major fish allergen, parvalbumin, from carp (Cyp c 1), group 2 mite allergens and the grass allergen, Phl p 5⁵⁰⁻⁶³.

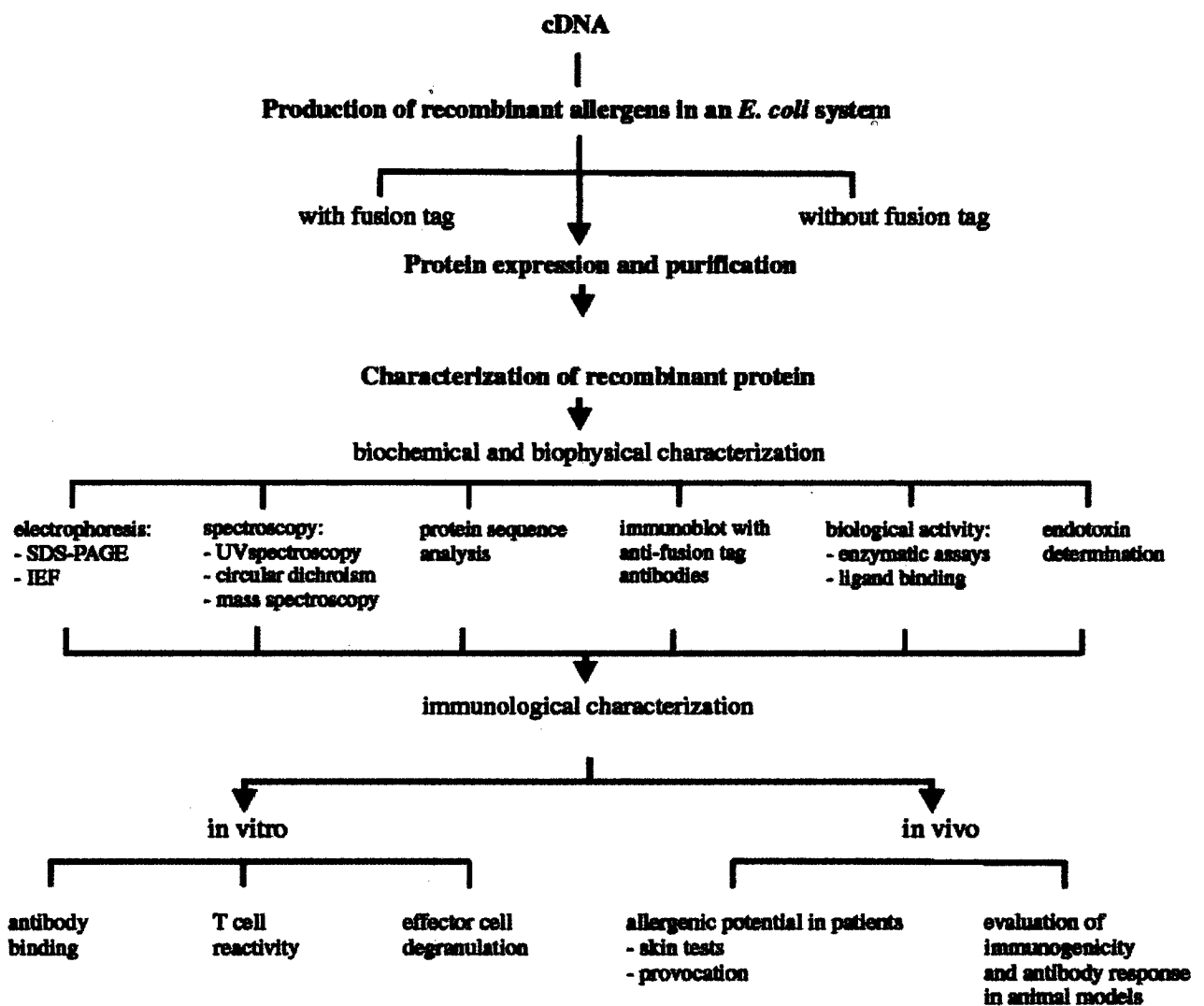


Figure 1.4 A flow diagram representing the steps carried out in the production of a recombinant allergen in *E.coli* as well as the characterisation required for its use as a diagnostic and/or therapeutic tool (Wallner, M., *et al.*, 2004)

1.5 Food allergens

The assortment of foods that make up the human diet is vast. However, a comparatively small range of foods are responsibly for eliciting a food-related allergic reaction ⁴⁰. Characteristically, food allergens are proteins or glycoproteins which vary between 3 and 90kDa in molecular weight and are typically very resistant to heat and extreme pH conditions ⁶⁴. Thus, conformation can be maintained during the harsh

conditions of food preparation as well as during gastrointestinal digestion which is important for IgE recognition and binding ⁴⁰.

Approximately 5-7% of children and 1-2% of adults suffer from food allergies. The main food types associated with triggering such a response include peanuts, tree nuts, cow's milk, egg, shellfish and fish ^{65, 66}. Milk, egg and peanut account for the majority of reactions in children, whereas allergic responses to fish, shellfish, tree nuts and peanut allergy are more prominent in adults ⁴⁰.

1.5.1 Fish allergy

Fish is an important source of protein, polyunsaturated fatty acids and fat-soluble vitamins, making it a very nutritional food group ⁶⁷⁻⁷⁰. A strong migration toward healthier eating habits has resulted in increased levels of production and consumption of fish products resulting in repeated reports of fish-associated allergy ⁷¹.

Fish-related allergic responses are most prevalent in coastal areas and fish-processing communities due to increased consumption characteristic of these areas as well as chronic exposure to fish products in an occupational setting ⁷². Allergy caused by ingestion of fish has been more widely studied and data exists for the prevalence of fish-specific allergy in several countries ^{40, 67, 71, 72}. For example, in the United States approximately 2% of the adult population have been shown to display allergic symptoms after fish ingestion ⁴⁰. In Norway, 1 in 1000 individuals are affected ⁶⁷ while in Japan, as many as 20% of food allergic adults suffer from allergy to fish ⁷². With regard to fish allergies in an occupational setting, comparatively less research has been carried out ⁷¹.

1.6.1 Parvalbumin

The major fish allergen, parvalbumin, has been shown to account for over 95% of immune hypersensitivities in people who consume fish ^{73 74-77}. This protein occurs in high amounts in the white muscle of lower vertebrates and in lower amounts in the skeletal muscle of higher vertebrates ^{78, 79}. However, only parvalbumin from fish and frog species have been identified as allergenic to human ⁷⁴.

Like many food allergens, parvalbumin has been demonstrated to be highly stable and resistant to heat, chemical denaturation and proteolytic enzymes ^{75, 80, 81}. The molecular weight of this protein varies between 10-13kDa in different species and has been shown to form oligomers via hydrophobic interactions in certain bird and fish species ⁸².

Parvalbumins can be divided into two distinct phylogenetic lineages, namely an α isoform with an isoelectric value of above 5 and a β isoform with a value of below 5 ⁸³. Other variations between lineages include sequence characteristics, metal ion binding affinities, crystal structures, chromosomal localisation and physiological roles ^{67, 84, 85}. Such polymorphisms appear to be species specific, age related and more common in lower vertebrates ⁸⁶. For example, three isoforms have been identified in silver hake muscle ⁸⁷ and four in frog muscle ⁸⁸. However, only singular isoforms have been identified in rat ⁸⁹, chicken ⁸⁸ and rabbit muscle ⁹⁰. The majority of parvalbumin sequences that have been deduced from fish species belong to the β lineage ⁸³, which are characterized by a shortened C-terminal end (commonly 109 amino acids or less) and 6 distinctive residues ⁹¹.

Parvalbumin is hypothesized to play an important role in muscle relaxation as well as to protect neurons and other non-muscle cells from excessive damage ^{92, 93}. This is

achieved by binding excess calcium via a calcium-binding domain or EF-hand motif composed of two perpendicularly placed α -helices of 8- to 10- residues and a 12-residue interhelical loop^{94, 94, 95}. Parvalbumin possesses three such EF-hands, two of which can chelate Ca^{2+} and Mg^{2+} while the non-functional N-terminal domain forms a cap that covers the hydrophobic surface of the protein (Figure 1.5)^{96, 97}. These calcium-binding regions have been identified as areas with a high degree of amino acid homology and have been proposed as sequential IgE binding epitopes^{81, 98-100}.

Importantly, IgE cross-recognition of parvalbumin from various fish species has been demonstrated^{76, 81, 101-103}. This accounts for why allergic patients can display clinical symptoms after ingestion of different species⁷³. As described previously, cross-reactivity of allergens is largely based on amino acid homology and ultimately conserved structure¹⁰⁴. Several primary sequences including cod, carp, salmon, mackerel and hake parvalbumin, as well as the three-dimensional structures of carp, hake and pike have been resolved and significant sequence homology has been confirmed as well as extensive cross-reactivity^{76, 87, 105}. However, it has also been demonstrated that some patients allergic to codfish are capable of consuming other fish with no resultant allergic symptoms¹⁰³. In addition, monosensitivity to single species such as swordfish and tropical sole have been illustrated^{106, 107}.

This phenomenon of cross-reactivity between fish species is not well characterised and, as illustrated, several conflicting reports exist¹⁰⁸. Further research of this subject is important due to the potential applications of cross-reactivity. Specifically, if a representative parvalbumin that displays extensive cross-recognition to IgE from various patients could be identified it could potentially be utilised as an effective diagnostic tool in a clinical setting. Alternatively, an altered hypoallergenic form could prove very effective in immunotherapy as a curative treatment⁷³. Recently, a hypoallergenic derivative of carp parvalbumin containing point mutations in both

functional calcium-binding domains was generated. This modified allergen displayed significantly reduced IgE-binding ability in a murine model as well as in a skin prick test of a fish-allergic patient⁶³. However, no data relating to the use of this hypoallergen in immunotherapeutic treatment is available. In addition, recombinant forms of cod, salmon and Alaska Pollack parvalbumin have been generated however no hypoallergenic forms of these proteins have been constructed^{67, 68, 109}. Use of these clones as diagnostics in a clinical setting has also not been achieved thus far. It is noteworthy that the majority of parvalbumins that have been investigated at this molecular level are from fish species predominantly consumed in Europe. Thus, it could be possible that these generated recombinants do not share extensive cross-reactivity to fish species commonly consumed in other populations, for example in South Africa^{49, 110, 111}.

Accurate knowledge of the extent of cross-reactivity between fish species could ensure less stringent elimination diets for allergic sufferers. For example, patients with allergic reactions to one fish species are often advised by practitioners to avoid all fish in general. A clearer understanding could lead to a re-evaluation of wholly eliminating a nutritional food source such as fish from a patient's diet¹⁰⁸.

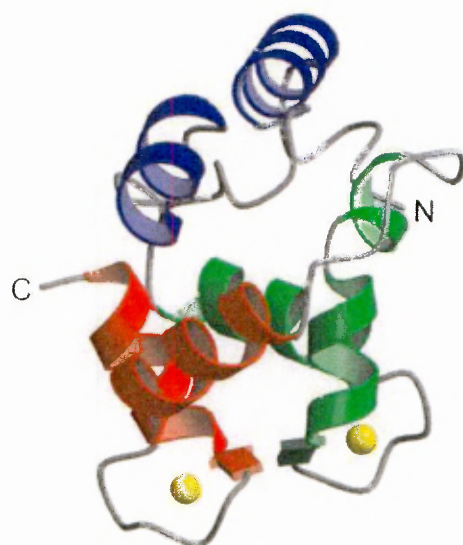


Figure 1.5 Three-dimensional structure of carp parvalbumin in a ribbon presentation. Represented in blue are the α -helices which form the non-functional N-terminal (represented as an 'N') EF-hand motif. The 2 functional EF-hand motifs are composed of α -helices and β -strands represented in red and green. The broad arrows illustrate the β -strands and the bound calcium ions are shown as yellow spheres. (Taken from Swoboda *et al.*, 2002)

1.6.2 Other fish and fish-related allergens

Several minor fish allergens have been identified^{72, 112-114}. Recently, collagen has been identified as a cross-reactive allergen in the skin of several fish species^{112, 115-117}. This protein is of a high molecular weight and occurs as two hetero α -chains in the range of 100-120kDa and as a dimer (β -chain) which has a molecular weight of approximately 210kDa¹¹². It has been noted that collagen is often absent in test extracts due to insolubility in water and buffer at lower temperatures and degradation at higher temperatures¹¹². As a result, analysis via SDS-PAGE and immunoblotting would not reveal the presence of collagen and has thus far resulted in inaccurate identification of this protein as a fish allergen¹¹².

Several additional allergens have been identified in cod extract under different conditions, namely pre-rigor mortis and post-rigor mortis¹¹⁴. Allergens with molecular weights of 104- and 130kDa were detected in fish before post-mortem rigidity, while

41- and 80kDa allergens were evident only after several days in storage¹¹⁴. Further analysis of the 41kDa protein revealed that it is homologous to an aldehyde phosphate dehydrogenase (APDH), which is a protein conserved in various other species of animal. This intracellular enzyme is thought to be released after cell death and thus increases in non-frozen fish that have been maintained in storage. An unglycosylated isoform of APDH of approximately 36kDa was also identified as a potential allergen¹¹⁸.

In addition, parasites that occur within fish species have been documented to cause hypersensitivity reactions⁷¹. In recent years the allergenic potential of the parasitic nematode *Anisakis* has been recognised¹¹⁹. This helminth can infect humans who consume raw fish resulting in gastric anisakiasis and can also cause hypersensitivity reactions in certain individuals¹¹⁹⁻¹²¹. Other non-seafood components such as bacteria within the fish (e.g. *Vibrio*), can result in a non-allergic adverse reaction, together with by-products caused by fish decomposition and bacterial and marine toxins such as scombroid toxin and histamine^{122, 123}. Adverse reactions to these non-seafood components have resulted in consumers wrongly self-diagnosing fish as the cause of their allergic reactions¹²⁴.

1.6 Occupational fish allergies

Occupational hypersensitivities to fish are more prevalent in large fish-processing communities¹⁰⁹. In South Africa, the fishing industry is lucrative and creates jobs for approximately 90 000 workers in 100 workplaces. Recently a study carried out in the Western Cape in over 570 fish-processing factory workers indicated that 6% suffered from allergies to bony fish¹²⁵.

It has been estimated that as many as one third of workers that handle food and suffer from occupational skin symptoms may be required to seek other positions due to the effects of their debilitating disease⁷¹. If an alternative job is not available due to unavoidable contact with the causative allergen, this can result in unemployment¹²⁶. In some cases, the removal of the causative allergens does not result in diminished allergic symptoms¹²⁷. In addition, job loss as a result of severe occupational asthma in workers has been reported³⁵. It has also been reported that workers with asthmatic reactions to crab displayed improvement in lung function only after two years of complete cessation of allergen exposure¹²⁸. Moreover, chronic exposure may lead to permanent lung damage and associated symptoms⁷¹.

The primary routes of sensitisation in such a fish-processing occupational setting include inhalation due to aerosolisation of fish products as well as dermal contact caused by unprotected handling^{129-131 129, 132}. The heading of fish, degutting and boiling in the presence of wet aerosol as well as dry particles produced by the mincing of fish meal have been shown to be primary sources of sensitisation via inhalation (Figure 1.6)⁷¹. In particular, dry aerosols appear to generate greater concentrations of particulate by-products¹³³. Insufficient ventilation by local exhaust systems have been shown to exacerbate these symptoms¹³¹. With regard to dermal contact, protective gloves may not be worn by the workers due to discomfort. Alternatively, if gloves are worn, by-products of the fish can potentially enter the interior of the gloves and subsequently rub against the skin resulting in an exacerbated allergic response⁷¹. In addition, the process of manual cutting and handling of sharp appendages of the fish can result in the mechanical damage of the skin which also leads to aggravated dermatological symptoms¹³⁴.

In such a work environment, allergic symptoms are commonly manifested as occupational asthma, rhinoconjunctivitis, urticaria, angioedema and dermatitis¹³¹. The level and extent of exposure to allergens have been shown to influence the frequency of sensitisation and allergic symptoms¹³⁵. Notably, the installation of a local exhaust ventilation system together with protective clothing significantly reduced the level of exposure and was shown to result in immediate cessation of clinical symptoms³⁵.

It is known that factors such as route of exposure, genetic factors of the employee, level and the extent of exposure to the allergen can contribute to the development of allergies in an occupational setting. However, these factors and the extent to which they influence allergic reactions are not well understood. It has been noted in several studies that further research of these factors is notable which could potentially contribute to the effective prevention and treatment of occupational allergies caused by seafood⁷¹.

The proteins that cause sensitisation in such an occupational setting have, thus far, not been characterised. In particular, very little is known about potential allergens present in aerosolised material in seafood processing working environments¹³⁶. Skin symptoms have been associated with exposure to fish juices which are composed of trypsin, pepsin, histamine, amines, compounds formed after post-mortem and high molecular weight proteins^{71, 134, 137, 138}. Specifically, denatured proteins broken down from larger fish muscle proteins by proteinases are postulated to be causative allergens¹³⁴. Additional proteins from *Anisakis* have been shown to cause allergic symptoms while other contaminants such as bacteria and bacterial toxins, indoor mould as well as exhaust fumes from indoor use of the machinery can also result in adverse reactions^{122, 123, 125}.

To date, it appears from the literature that while parvalbumin has been extensively well characterised as a food allergen among consumers, it has not been identified as a causative allergen in workers with occupational allergy and asthma due to fish processing activities^{64, 68, 72, 73, 103, 105, 109}.

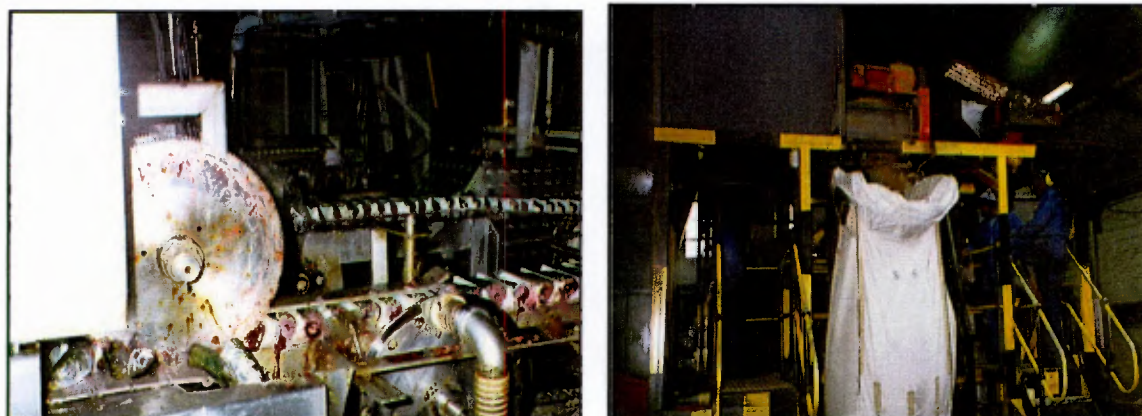


Figure 1.6 Two common processes carried out in a fish-processing factory in St. Helena Bay, Western Cape. The picture on the left indicates fish canning of pilchard and anchovy specifically. This process involves the gutting and heading of fish products. On the right, the process of fish-meal processing and 'bagging' is being carried out. Both procedures produce extensive aerosolisation.

1.8 Aims of the current study

This study aims to investigate IgE reactivity to the major allergen parvalbumin and other fish fillet allergens using sera from two groups of fish-allergic individuals:

- i.) Sera from factory workers primarily exposed to pilchard and anchovy and their by-products via inhalation and skin contact, and
- ii.) Sera from domestic consumers with allergies induced by the ingestion of fish.

IgE reactivity to proteins from fish fillet will be compared using sera from both cohorts by immunoblotting, and cross-reactivity among parvalbumin from different fish species will be investigated. Specifically, IgE reactivity to crude fillet extract and to purified parvalbumin from pilchard (*Sardinops sagax*), yellowtail (*Seriola lalandi*),

hake (*Merluccius merluccius*), anchovy (*Engraulis encrasicolus*) and snoek (*Thyrsites atun*) will be evaluated. These five fish species were selected based on their commercial importance and because they are highly consumed by the South African population. Parvalbumins from these species will be evaluated, and the most widely recognized parvalbumin will be characterized further by isoform separation using two-dimensional gel electrophoresis and by molecular sequencing. In addition, other potential allergens in the crude fillet extracts of all fish will be assessed.

1.8.1 Significance of the current study

Parvalbumin has been extensively documented as a major food allergen. However, whether this protein is a causative allergen in fish-processing factory workers is yet to be investigated. In addition the proteins that cause disease in these workers have been not been well characterised. Identification of the allergens involved in occupational disease could contribute to effective prevention and treatment in workers and others like them. In addition, subsequent characterisation could shed light on other undefined areas of research such as how the route of exposure and the dosage of allergen can influence occupational allergic disease

The detection of a cross-reactive, immunodominant parvalbumin has potential use as diagnostic tool of fish allergies. In addition, the identification of a parvalbumin with shared epitopes could be utilised for immunotherapy to attain multiple desensitisation. For these reasons, one of the aims of this study is to deduce the cDNA sequence of a cross-reactive form of parvalbumin as a primary step towards the generation of a recombinant allergen. In addition, due to the fact the all fish species included in this study are consumed in large quantities in South Africa the resultant recombinant parvalbumin would be specifically relevant to the South African population.

1.9 Summarised objectives

Specifically, this study aims to:

- Compare differences in molecular weight, oligomeric forms and the number of isoforms in parvalbumin between five major fish species using a monoclonal anti-parvalbumin antibody in immunoblot analysis.
- Purify parvalbumin from these five fish species to ensure that identical amounts of protein are used in serological immunoblot analyses and to confirm specific IgE binding to parvalbumin.
- Assess IgE reactivity to crude fish extract and purified parvalbumin from these five fish species using sera from fish-allergic subjects from two groups.
- Confirm or refute parvalbumin as a major allergen in both groups as well as assess variations in IgE reactivity to other allergens in crude extracts and purified parvalbumin from all five fish species.
- Characterise parvalbumin from a fish species that exhibits extensive cross-recognition by molecular sequencing as well as separate isoforms by two-dimensional gel electrophoresis.

2. Methods and Materials

2.1 PREPARATION OF CRUDE EXTRACT AND PURIFICATION OF NATURAL PARVALBUMIN FROM FIVE FISH SPECIES

2.1.1 Preparation of crude fish extracts

Five Osteichthyes (bony fish) species indigenous and commercially relevant to South Africa were studied to compare any varying allergenic potential of parvalbumin and other allergens. These fish species included pilchard (*Sardinops sagax*), anchovy (*Engraulis encrasicolus*), hake (*Merluccius merluccius*), snoek (*Thyrsites atun*) and yellowtail (*Seriola lalandi*). Pilchard and anchovy samples were kindly donated by St. Helena Bay Fisheries (St. Helena Bay, Western Cape) and the remaining fish samples were attained from a local supplier of fresh fish (Texies Seafood, Mowbray, Western Cape). Approximately 20g of raw muscle from each fish species was excised, homogenised in 500ml of phosphate-buffered saline (PBS) and extracted overnight (ON) at 4°C with constant gentle agitation. The crude extract was aliquoted into amounts of 25ml and centrifuged at 6000rpm for 30 minutes at 4°C. The supernatant was collected and placed at -20 °C ON (overnight) to separate the protein from the fat content. The supernatant was then thawed and centrifuged at 6000rpm for 30 minutes and the intermediate layer between the pellet and lipid layer was collected and centrifuged again under the same parameters. The supernatant was collected and sterile filtered through various filters of decreasing pore size including 8.0µm, 5.0µm, 1.2µm (Sartorius, Germany) and 0.45µm (Millipore, USA). Extracts were pooled and total protein concentration was estimated using a Bicinchoninic Acid (BCA) Assay Kit (Pierce, USA) according to manufacturer's instructions and Bovine Serum Albumin (BSA) (Pierce, USA) was used to prepare a

standard curve. The crude fish extracts were aliquoted into 10ml fractions and stored at -20°C until use.

2.1.2 Purification of parvalbumin

A 50ml volume of crude fish extract was aliquoted into quantities of 2ml and placed in a heating block set at 99°C for 25 minutes. Precipitated proteins were then removed by centrifugation at 4500rpm for 10 min at 4°C. The supernatant was collected and enriched by adding ammonium sulphate ((NH₄)₂SO₄) (Sigma, Germany) to 70% saturation at RT. Small amounts of the salt were added while continuously stirring. After dissolution, the mixture was stirred for another 2 hours to allow complete equilibration. Samples were then centrifuged at 13,000rpm for 15 min and approximately 30ml of supernatant was collected and dialysed (Pierce, USA) against 5 L of 10mM Tris, pH. 7,5 at 4 °C. After 12 hours the Tris dialysate was replaced with fresh Tris solution of the same volume and the sample was left to dialyse for a further 8 hours. An Amicon centrifugal filter device (molecular weight cut off of >5kDa) (Millipore, USA) was used to concentrate the supernatant and the protein concentration was again determined using a BCA Assay Kit and BSA standard curve.

Approximately 12mg of protein sample in 8ml of 10mM Tris, pH. 7,5 was then applied to a DEAE (Diethylaminoethanol) Sepharose column (2,5cm X 10cm) (Pharmacia). Fractions were eluted with a linear salt gradient of 10mM Tris pH 7.5 and 1M NaCl, pH 7.5. Constant flow rate was set at 1ml/min using a Minipuls 3 Gilson peristaltic pump and fractions of 5ml were collected mechanically (Foxy Jr. collector, ISCO, USA) over a total period of 5 hours. In total, a volume of 300ml was eluted. The elution profiles were detected by UV absorbance at 260nm simultaneously using a wavelength detector (DVW-10 Variable Wavelength Detector, D-Star Instruments) interfaced with a chromatographic data system (Ross Recorders) set at a chart

speed of 2cm/hr. Fractions that yielded a peak were collected and absorbance values were confirmed by analysing samples on a Nanodrop system (ND-1000 Spectrophotometer). The fractions containing protein from the same peak were pooled and concentrated using Amicon ultra centrifugal filter tubes to a volume in the range of 2.5ml. Concentration was then determined as previously stated and the presence of parvalbumin was confirmed by SDS-PAGE analysis and immunoblotting.

2.1.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis

Protein separation was carried out by SDS-PAGE to analyze the following:

- i.) total protein present in crude fish extract
- ii.) proteins precipitated out after a purification step and
- iii.) assessment of parvalbumin purity.

2.1.3.1 Standard SDS-PAGE procedure

The protein concentration of extracts was determined by BCA assay and diluted accordingly in 1XPBS and sample buffer (see Appendix A). Samples were then pulsed for 3 seconds prior to placing in a heating block set at 99°C for 10 min. Extracts were loaded onto a polyacrylamide stacking gel and separated on a 15% acrylamide gel alongside a molecular weight marker (Biorad Precision Plus Protein Standard, Dual Colour, USA). Gels were electrophoresed in 1X electrophoresis buffer (see Appendix A) at a constant voltage at 100-140V for 2-3 hours until the dye front had run off the resolving gel. Gels were analysed by staining with Coomassie Brilliant Blue (BDH Biochemical, England) (see Appendix A) for 1 hour at RT while gently agitating and destained in 5% methanol and 7.5% acetic acid O/N. Alternatively, gels were used in immunoblotting

2.1.3.2 Analysis of crude fish extract protein by SDS-PAGE

Crude extracts of all fish species containing 25µg protein were separated as described and visualised with Coomassie Brilliant Blue.

2.1.3.3 Analysis of loss of proteins during purification by SDS-PAGE

Samples were collected after cooking and salt precipitation and after ion-exchange chromatography and compared to crude extract to investigate loss of total protein. 25µg of protein was used for electrophoretic separation.

2.1.3.4 Assessment of parvalbumin purity by SDS-PAGE

A final purification product containing 20µg of protein was resolved and the absence of contaminating proteins was confirmed by developing with Coomassie Brilliant Blue.

2.2 ANALYSIS OF IgG-BINDING ANTIGENS AND IgE-BINDING ALLERGENS

2.2.1 Immunoblot analysis

Immunoblot analysis was used to:

- i.) confirm the presence of parvalbumin in all extracts, previously analysed by SDS-PAGE, using a monoclonal anti-frog parvalbumin.
- ii.) assess IgG and IgE reactivity to crude fish extract and purified parvalbumin using human sera.

2.2.2 Standard protocol for immunoblotting

Following electrophoresis, separated proteins were transferred to a HybondC+ nitrocellulose membrane (Amersham Biosciences, UK) pre-soaked in 1X transfer buffer (TB) (see Appendix A). Transfer was carried out in 1X TB by applying a constant voltage of 100V (400mA) for 1 hour. The temperature of the buffer was maintained below RT throughout. The membrane was blocked O/N at 4°C with 5% powder skim milk (see Appendix A) in 1X TBS/Tween with gentle agitation. Successful transfer was confirmed by Coomassie staining of the gel. The membrane was then incubated with test samples for 1 hour or 3 hours at RT and TBS/Tween was then used to wash the membrane for 45 minutes, changing the solution every 15 minutes. An alkaline phosphatase-labelled secondary antibody was added for 1 hour and the washing step was repeated. The membrane was developed with 5-bromo-4-chloro-3-indolyl phosphate-4-nitroblue tetrazolium (Sigma-Aldrich, Germany) and the reaction was stopped by placing the membrane in distilled water.

2.2.3 Confirmation of parvalbumin by immunoblot analysis

Crude extracts of all fish species, intermediate purification products as well as final purified products all containing 10µg protein were transferred to a membrane and parvalbumin was detected using a monoclonal mouse anti-frog parvalbumin IgG antibody (Clone Parv-19; Sigma, Germany) which was diluted 1:3000 in TBS and incubated for 1 hour at RT. An alkaline phosphatase-labelled goat anti-mouse IgG1 secondary antibody (Southern Biotech, USA), diluted 1:1000 in TBS, was then added and the membrane was developed.

2.2.4 Human Sera

The IgG and IgE binding to parvalbumin and other allergens present in the fillet of fish were investigated using sera from two groups of fish-allergic subjects.

2.2.5 Assessment of sera from fish-processing factory workers

In a previous study conducted by Professor Mohamed Jeebhay^{125, 136}, 578 workers employed at two industrial fish-processing factories in St. Helena Bay (Western Province, South Africa) that predominantly handled anchovy and pilchard were assessed for work-associated allergies related to bony fish-processing. Based on results obtained in this study, 13 workers were selected that possessed one of the following criteria:

- i.) a positive skin-prick test (SPT) result to fish
- ii.) a level of anchovy or pilchard specific IgE above 0.35kU/L measured using UniCAP RAST (Radioallergosorbent test) (ImmunoCAP InvitroSight, Phadia).
- iii.) typical clinical allergic symptoms manifested on the skin, chest or/and ocular/nasal

Fresh blood samples were taken from the 13 workers with informed consent and reassessed for levels of fish-specific IgE using the UniCAP RAST system. For analysis of IgE-binding capacity in immunoblotting, subjects that possessed specific IgE to pilchard and anchovy above 0.35kU/L and who displayed allergic symptoms were selected. Thus 6 out of the 13 subjects initially chosen were used in this study. Serum from a fish-processing worker that was non-atopic, had a negative SPT response as well as no detectable fish-specific IgE and displayed no clinical symptoms was used as a negative control for this group. All serum samples were aliquoted and stored at -20°C.

2.2.6 Assessment of sera from subjects sensitised via fish ingestion

Serum from ten patients with self-reported allergic reactions after the ingestion of fish that experienced one or more typical clinical symptom (dermatitis, urticaria, angioedema, diarrhoea, vomiting, rhinoconjunctivitis, wheezing or asthma) were obtained from Mrs. Bartha Fenemore, UCT Lung Institute and Dr. Ines Swoboda (Medical University of Vienna, Austria). The majority of patients were from South Africa (n=7) while three individuals were Austrian. The presence of fish-specific IgE was confirmed by measurement of pilchard, anchovy, hake, jack mackerel and tuna specific IgE using the UniCAP RAST system where sufficient amounts of sera were available. However, if serum samples were limited, as was the case for five patients, only level of pilchard and anchovy specific IgE were measured. Serum from a subject with no adverse reactions after food ingestion and no detectable fish-specific IgE was included as a negative control for this group.

2.2.7 IgG and IgE reactivity to crude fish extract and purified parvalbumin

Crude extracts of all fish species containing 100µg of protein and 50µg of purified parvalbumin were resolved and transferred to a membrane. Serum samples were either diluted 1:20 (for IgE detection) or 1:1000 (for IgG detection) in 1xTBS. All samples were then added to separate lanes in a slot blot and incubated for 3 hours at RT. To analyse subjects' IgE reactivity, an alkaline phosphatase labelled anti-human IgE antibody produced in mouse (Sigma, Germany), diluted 1:1000 in TBS, was incubated for 1 hour at RT. For detection of IgG-specific antigens, an alkaline phosphatase labelled anti-human IgG (γ-chain specific) antibody produced in goat (Sigma, Germany) was added under the same parameters.

2.3 FURTHER CHARACTERISATION OF PILCHARD PARVALBUMIN

2.3.1 Isoelectric focusing (IEF) analysis

To identify isoforms of pilchard parvalbumin, isoelectric focusing was carried out on an 11 cm nonlinear pH 3-10 Immobiline DryStrip gel (Amersham Biosciences) in a Multiphor II IEF system (Amersham Biosciences). The strip was rehydrated for 1 hour at RT in a solution of 320µl of rehydration buffer (see Appendix A) and 20µg of purified pilchard parvalbumin. Focusing conditions were controlled by a gradient program (Pharmacia, EPS 3500XL) under the following conditions: 150 V for 30 minutes, 300 V for 60 minutes, 500V for 5 hours and 3500V for 14 hours. After focusing, the strip was placed in equilibration buffer (see Appendix A) for ten minutes and then alkylated by incubation with 125mM iodoacetamide (Sigma, USA) containing bromophenol blue (Merck, South Africa) for ten minutes. The prepared strip was then separated in the second dimension based on size on a SDS-PAGE gel under conditions specific in section 2.2.2.

Proteins were then transferred to a nitrocellulose membrane and parvalbumin was detected using a monoclonal mouse anti-frog parvalbumin IgG antibody according to immunoblotting protocol outlined in section 2.3.1.

2.3.2 Molecular sequencing of pilchard parvalbumin

2.3.2.1 Tryptic digestion of purified pilchard parvalbumin

Tryptic digestion of parvalbumin was carried out using the ProteoExtract All-in-One Trypsin Digestion Kit (Calbiochem, Germany). Purified pilchard parvalbumin was excised from a Coomassie Blue-stained SDS/PAGE gel after electrophoresis and washed three times with wash buffer (supplied with the kit) at 37°C until colourless. The gels slice was then dried in a speed vacuum (Speed Vac SC110, Savant, USA)

for 20 minutes and digest buffer and reducing agent (both supplied with the kit) were added and incubated with the sample for 10 minutes at 37°C. The solution was cooled to RT and blocking agent was added and incubated at RT for 10 minutes. Trypsin was added to the sample to attain a final concentration of 8ng/μl and incubated for 2 hours and 30 minutes at 37 °C with constant shaking. After centrifugation at 13,000rpm for 15 minutes the supernatant containing tryptic peptides was collected and stored at -20 °C prior to mass spectrometry analysis.

2.3.2.2 Mass spectrometer (MS) analysis

Liquid chromatography (LC)-MS/MS experiments were performed on an electron spray ionization (ESI)-Quad (Q)- Time of Flight (TOF) (Waters API Q-TOF Ultima) equipped with nano-high-pressure liquid chromatography by Dr. Marietjie Stander, University of Stellenbosch. Capillary liquid chromatography of parvalbumin tryptic peptides was performed with a CapLC system equipped with a Waters Atlantis dC18 (3μm, 100μm X 150mm) reverse phase column. A sample injection of 5μl was loaded onto the column and peptides were eluted over 1 hour at 1.8μl/min.

An MS survey between the range of 400m/z to 1995m/z were acquired and peptides with peak intensities above 70 counts/second were selected for MS/MS analysis. The mass spectrometer was operated in V-mode. Full scan mass spectra were attained in continuum mode at a scan speed of 0.5s/scan. MS/MS analyses were attained using identical conditions on the same apparatus.

The acquired MS/MS spectra were analysed using the online search algorithm MASCOT at www.matrixscience.com for peptide identification against all taxonomy entries. The enzyme, trypsin, was specified and 1 missed cleavage was permitted. The peptide and MS/MS tolerances were both set to ±0.5 Da and peptide charge was

recorded at 2+ and 3+. A Micromass (.PKL) data format was also selected and the top 20 hits were detected.

2.3.2.3 Analysis of tryptic peptide

Correct tryptic cleavage of the 10 amino acid peptide fingerprint was confirmed using the "PeptideCutter" function on ExPaSy programme (www.expasy.ch) and compared to others parvalbumin protein sequences deposited in GenBank using the program BLASTp via the BLAST server (www.ncbi.nlm.nih.gov/BLAST).

2.3.2.4 Total and messenger RNA Extraction and cDNA synthesis

Total RNA was extracted utilising 2ml TRizol reagent (Invitrogen, USA) which was added to 200mg of pilchard muscle tissue and homogenised. The solution was left at RT for 5 minutes before adding 4ml of chloroform (BDH, England) and shook by hand for 15 seconds. The solution was left at RT for 3 minutes and centrifugation was carried out at 13,000rpm for 15 minutes at 4°C. The aqueous phase containing the RNA was collected and 1ml of isopropyl alcohol (BDH, England) was added. After incubation at RT for 10 minutes, the sample was centrifuged at 13,000rpm for 10 minutes at 4°C. The supernatant was discarded and the RNA pellet was washed with 1ml of 70% ethanol (Merck, South Africa) and centrifuged at 7,500rpm for 5 minutes at 4°C. The supernatant was removed and the pellet was partially dried and resuspended in 100µl of Rnase-free water (Qiagen, Germany). The concentration and 260/280 ratio were determined using a Nanodrop system (ND-1000 Spectrophotometer). Messenger RNA was extracted from 70µg of total RNA using the PolyATtract mRNA Isolation System III (Promega, USA) according to the manufacturer's instructions.

First-strand synthesis of cDNA was performed using the Improm-II Reverse Transcription System (Promega, USA) using 5µg of total RNA. This cDNA was used in initial PCR reactions to attain partial sequence information of parvalbumin. For 5'- and 3'-RACE PCR reactions, first-strand cDNA was synthesised from 1µg of total RNA and 1µg of mRNA using the SMART RACE cDNA Amplification Kit (Clontech) per manufacturer's instructions. In parallel, cDNA was synthesised from 1µg of human placental total RNA supplied with the kit as a positive control.

2.3.2.5 Primer design

Using the deduced amino acid sequence obtained by MS/MS analysis, a 5' primer was designed by cDNA alignment of 5 parvalbumin sequences that had a 100% homology to the tryptic peptide of interest. A degenerate 3' primer was designed based on a highly conserved region (23 nucleotides) of 5 aligned parvalbumin cDNA sequences. Sequences were aligned and primers were designed using Blast, Clustal W (www.ebi.ac.uk/clustalw/), DNAMAN and tools listed on the ExPasy home page. These primers were then used to amplify a portion of parvalbumin cDNA (180bp) which was ultimately used to design nested primers for 5'- and 3'- rapid amplification of cDNA ends (RACE). The primers designed were in accordance with parameters specified in the SMART RACE cDNA Amplification Kit.

Table 2.1 Primers used for sequencing of the parvalbumin gene

| | |
|--------------------------------------|---|
| 1a.) Forward primer (1st run PCR) | 5'-GGCTTTCGCCATCATTGACCAGGAC -3' |
| b.) Reverse primer (1st run PCR) | 5'-AACTCATCAA(TC)(AT)CC(GAT)ATCTTGCC-3' |
| 2a.) Forward Nested Primer (3' RACE) | 5'-GGCAAGGGCACTGACTGATGGCGAGACC-3' |
| b.) Reverse Nested Primer (5' RACE) | 5'-GCCATCGCCATCAGTGTCGCCAGC-3' |

2.3.2.6 Polymerase chain reaction

i.) Amplification of partial parvalbumin sequence

For cDNA amplification of partial parvalbumin sequence, 100ng of first-strand synthesis product and 0.5 μ M of primers 1a and 1b (Table 2.1) were added to a 20 μ l reaction volume of Fermentas 2X PCR Master mix (containing 0.05 units/ μ l of *Taq* DNA polymerase, 4mM of MgCl₂ and 0.4mM of dNTPs) and nuclease free water (Fermentas, USA). The PCR products were amplified in a hot-lid MJ thermocycler (Biozym, Hessisch Oldendorf, Germany) under the following conditions: 94°C for 1 minute; 35 cycles of 94°C for 30 seconds, 60 °C for 30 seconds and 72 °C for 1 minute; 72 °C for 4 minutes. Aliquots of 5 μ l of the PCR products were analysed by agarose gel electrophoresis or stored at -20°C until use.

ii.) 5'- and 3'- Rapid Amplification of cDNA Ends (RACE)

For amplification of the entire pilchard parvalbumin gene, nested primers (2a and b; Table 2.1) were used in a 5'- and 3' RACE reaction using the SMART RACE cDNA Amplification Kit. Amplification of experimental 5'- and 3'-RACE product cDNA and control human placental cDNA (supplied with the kit) were carried out according to manufacturer's protocol. The Advantage 2 Polymerase Mix (Clontech) was used for all PCR reactions and the following conditions for amplification of parvalbumin were applied: 5 cycles of 94 °C for 30 seconds and 72 °C for 3 minutes; 5 cycles of 94 °C for 30 seconds, 72 °C for 30 seconds and 72 °C for 3 minutes; 25 cycles of 94 °C for 30 seconds, 68 °C for 30 seconds and 72 °C for 3 minutes. For 5'- and 3'-RACE PCR reactions, the two nested primers and appropriate cDNA were used to amplify the overlapping 79bp RACE fragment between the primers which served as an internal positive control. Aliquots of 5 μ l-10 μ l of the PCR products were analysed by agarose gel electrophoresis or stored at -20°C until use

2.3.2.7 Agarose gel electrophoresis

Gels were prepared by dissolving PCR-grade agarose (Hispanagas, Spain) in 0.5X TBE buffer to a final concentration of 0.8-1.2%. Ethidium bromide (0.2µg/µl) was added to the agarose solution and left to set. DNA samples were prepared by adding 5µl of 6X loading dye and were loaded into the precast wells of the gel alongside a DNA ladder which indicated fragment size. The gels were resolved at a constant voltage of 100V until adequate separation was attained and visualised and captured using a UV transilluminator (Biorad, Gel Doc 1000, Single Wavelength Mini-Transilluminator)

RNA integrity was also assessed by agarose gel electrophoresis. Approximately 3µg of isolated total RNA was analysed using the protocol outlined above taking the necessary precautions to eliminate contamination and degradation of RNA.

2.3.2.8 Purification of DNA from agarose gel

Products of the correct size were excised from the gel and purified using the Wizard SV Gel PCR Clean-Up System (Promega, USA) according to manufacturer's instructions. DNA was eluted in 20µl-50µl of nuclease free water supplied with the kit.

2.3.2.9 Subcloning of insert DNA for sequencing

Subcloning was performed using the TOPO TA Cloning Kit for Sequencing (Invitrogen) with pCR4-TOPO vector. 4µl of purified PCR product was ligated into 0.5µl of TOPO vector (5ng/µl) together with 1µl of manufacturer's supplied salt solution and 1µl of sterile water. The reagents were mixed gently and incubated at

RT for 5 minutes. The reaction was then placed on ice and transformation was carried out.

2.3.2.10 Transformation and selection of colonies

Competent *Escherichia coli* (*E.coli*) Top10 cells (Invitrogen) were used for transformation. A volume of 2µl of the ligation mix was added to 15 µl of Top10 cells and incubated on ice for 10 minutes. Transformation was carried out by heat-shocking the cells for 30 seconds and then immediately placing the reaction on ice. A volume of 250µl of SOC media (Invitrogen) was added to the cells followed by incubation for 1 hour at 37°C under constant shaking. The total reaction volume was spread onto Luria Bertani (LB) (Merck, South Africa) agar plates containing 100µg/ml of ampicillin and incubated at 37°C overnight.

The TOPO plasmid utilised for subcloning possesses a lethal *E.coli* gene, *ccdB* fused to the C-terminus of LacZα fragment. Thus, ligation of a PCR insert disrupts the expression of the *lacZα-ccdB* gene fusion allowing only positive recombinants to grow. All bacteria lacking successfully ligated plasmids would not grow due to the ampicillin present.

Depending on the number of colonies that grew per plate, between 2-8 were selected and inoculated into 10ml of LB broth containing 100µg/ml of ampicillin and incubated at 37 °C overnight under constant shaking. Broth that appeared turbid was then centrifuged at 3500rpm for 15 minutes at RT. The supernatant was discarded and plasmid DNA was extracted from the cells using the SV Miniprep DNA Purification Kit (Promega, USA) as per manufacturer's instructions.

2.3.2.11 Confirmation of insert DNA

The presence of the correct size of insert DNA was confirmed by restriction enzyme (R.E) digest or, if the concentration of plasmid DNA was low, PCR was carried out using the isolated plasmid DNA. For a single RE digestion, 1µl of EcoRI (10X buffer with BSA) (Fermentas) was added to 5µl of plasmid DNA, 2 µl of SuRE/Cut Buffer H (10X concentration buffer (Roche) and 12 µl of nuclease free water. A negative control was included replacing the plasmid DNA with nuclease free water. The reactions were placed at 37°C for 1 and a half hours and the reaction was stopped by incubation at 65°C for 20 minutes. 10µl of the reaction volume was then analysed by agarose gel electrophoresis.

Alternatively, 1µl of plasmid DNA was added to 0.5µM of the primers used to amplify up the DNA insert of interest together with 20µl reaction volume of Fermentas 2X PCR Master mix and nuclease free water. The PCR products were amplified under the following conditions: 94°C for 1 minute; 35 cycles of 94°C for 30 seconds, 60 °C for 30 seconds and 72 °C for 1 minute; 72 °C for 4 minutes. Aliquots of 5µl of the PCR products were analyzed by agarose gel electrophoresis to confirm the presence of the correct insert.

2.3.2.12 Plasmid sequencing

Automated sequencing based on the dye-terminator sequencing method was performed at the Molecular and Cell Biology Department, UCT. Results were analyzed using the BlastX program and clones were aligned to assess homology using DNAMAN. Where differences were noted between the clones, chromatographs were viewed to assess the accuracy of the sequencing.

3. Results

3.1 THE INVESTIGATION OF NATURAL PARVALBUMIN FROM FIVE FISH SPECIES AND SUBSEQUENT PURIFICATION

3.1.1 Isomers and oligomeric forms of parvalbumin were noted in five crude fish extracts

The fillets from five fish species were collected, homogenised and assessed by SDS-PAGE and immunoblotting to compare any variations of parvalbumin between species (Figure 3.1.1).

It was noted that parvalbumin from each fish species varied slightly in molecular weight. Snoek (lane 3) and hake (lane 4) parvalbumin had a lower molecular weight of approximately 11kDa while anchovy (lane 1), yellowtail (lane 2) and pilchard parvalbumin (lane 5) were in the region of 12kDa as noted by immunoblot results (Figure 3.1.1 B). With regard to other proteins observed in SDS-PAGE analysis, binding patterns were similar in all 5 fish species above a molecular weight of approximately 24kDa although it appeared that there was less protein present in the crude snoek extract despite the fact that identical amounts were resolved.

Using an anti-parvalbumin antibody in immunoblot analysis, isoforms of parvalbumin from pilchard (lane 5) and yellowtail (lane 2) were detected as indicated by the presence of additional proteins above and/or below the prominent band present at 12kDa. Oligomeric forms of parvalbumin at 24kDa (dimer) were also noted in nitrocellulose-blotted anchovy (lane 1) and pilchard (lane 5) and a 48kDa tetrameric form was very faintly detected in all fish species with the exception of anchovy under reducing conditions.

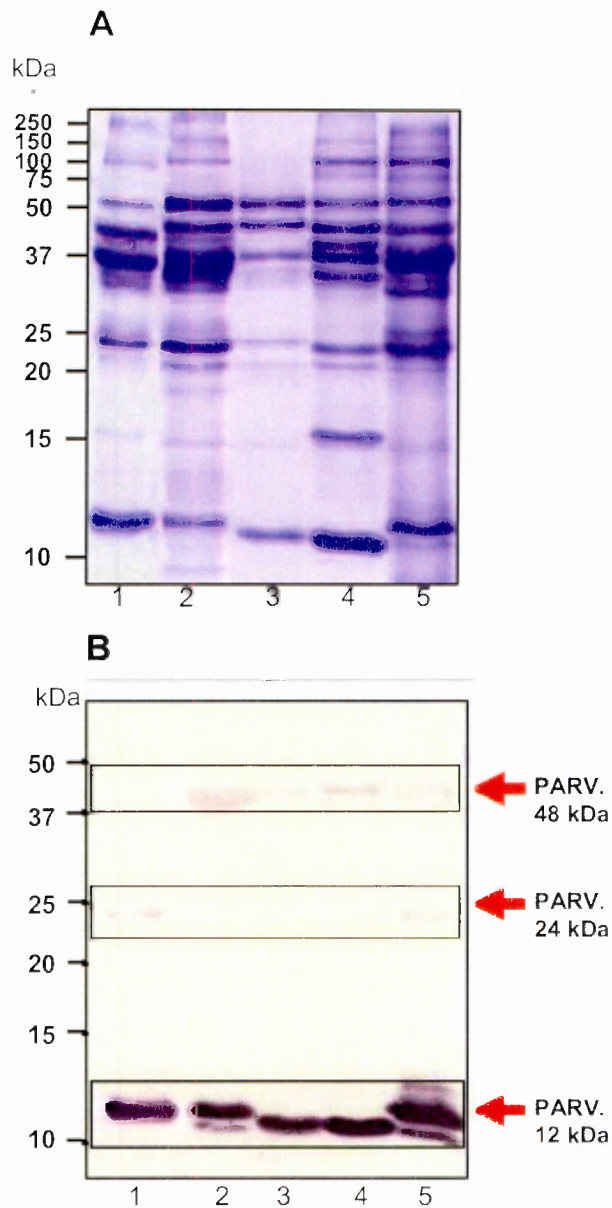


Figure 3.1.1 SDS-PAGE and immunoblot analysis revealed variations in molecular weight of parvalbumin between different fish species and the presence of isomers and oligomers. For SDS-PAGE analysis (A), crude protein extracts were separated a 15% polyacrylamide gel under reducing conditions and visualised with Coomassie blue staining. For parvalbumin detection in immunoblot analysis (B), a monoclonal anti-parvalbumin antibody was utilised with nitrocellulose-blotted crude fish extracts. The bold red arrows in B represent the monomeric and oligomeric forms of parvalbumin. Lane: 1, anchovy extract; 2, yellowtail extract; 3, snoek extract; 4, hake extract; 5, pilchard extract. The values of the standard markers are indicated on the far left.

3.1.2 Parvalbumin was purified from all five crude fish extracts

Pilchard, yellowtail, hake, anchovy and snoek parvalbumin were purified by cooking, ammonium sulphate precipitation and DEAE-ion exchange chromatography to confirm specific binding to this major allergen and to ensure equal amounts of protein was assessed using sera obtained from individuals with known fish allergies.

After cooking and ammonium sulphate precipitation, samples were applied separately to a DEAE column in order to elute concentrated native parvalbumin. Parvalbumin was eluted between fractions 20-35 as indicated by sharp peaks in absorbance at OD readings in the range of 0.102 and 0.132 (Figure. 3.1.2 A-C, E) in all species except hake (Figure. 3.1.2, D). The partially purified hake extract displayed two separate peaks in absorbance: an initial peak at a maximum OD of 0.102 and a second peak at a maximum OD of 0.08. Approximately 100ml of each peak from the elution profile of all five fish species was collected, pooled and concentrated.

Eluted fractions were analysed by Coomassie staining and immunoblotting to confirm the presence of purified parvalbumin (Figure 3.1.3). Protein collected from the eluted peaks were compared against partially purified products (Lane 2) that had been cooked and subjected to ammonium sulphate precipitation as well as crude fish extracts (lane 3). Parvalbumin was detected using a monoclonal anti-parvalbumin antibody in immunoblotting. Interestingly, both peaks noted in the elution profile of hake consisted of parvalbumin with no additional contaminating proteins. Monomeric (12kDa), dimeric (24kDa) and tetrameric (48kDa) forms of parvalbumin were detected in all the purified, partially purified and/or crude extracts from all fish species using this monoclonal anti-parvalbumin antibody.

The final concentration of parvalbumin after ion-exchange chromatography of eluted fractions varied between 1.6 mg (pilchard) and 4.9 mg (yellowtail) (Table 3.1). On average, 0.94% of parvalbumin was yielded from the initial crude extract of fish fillets.

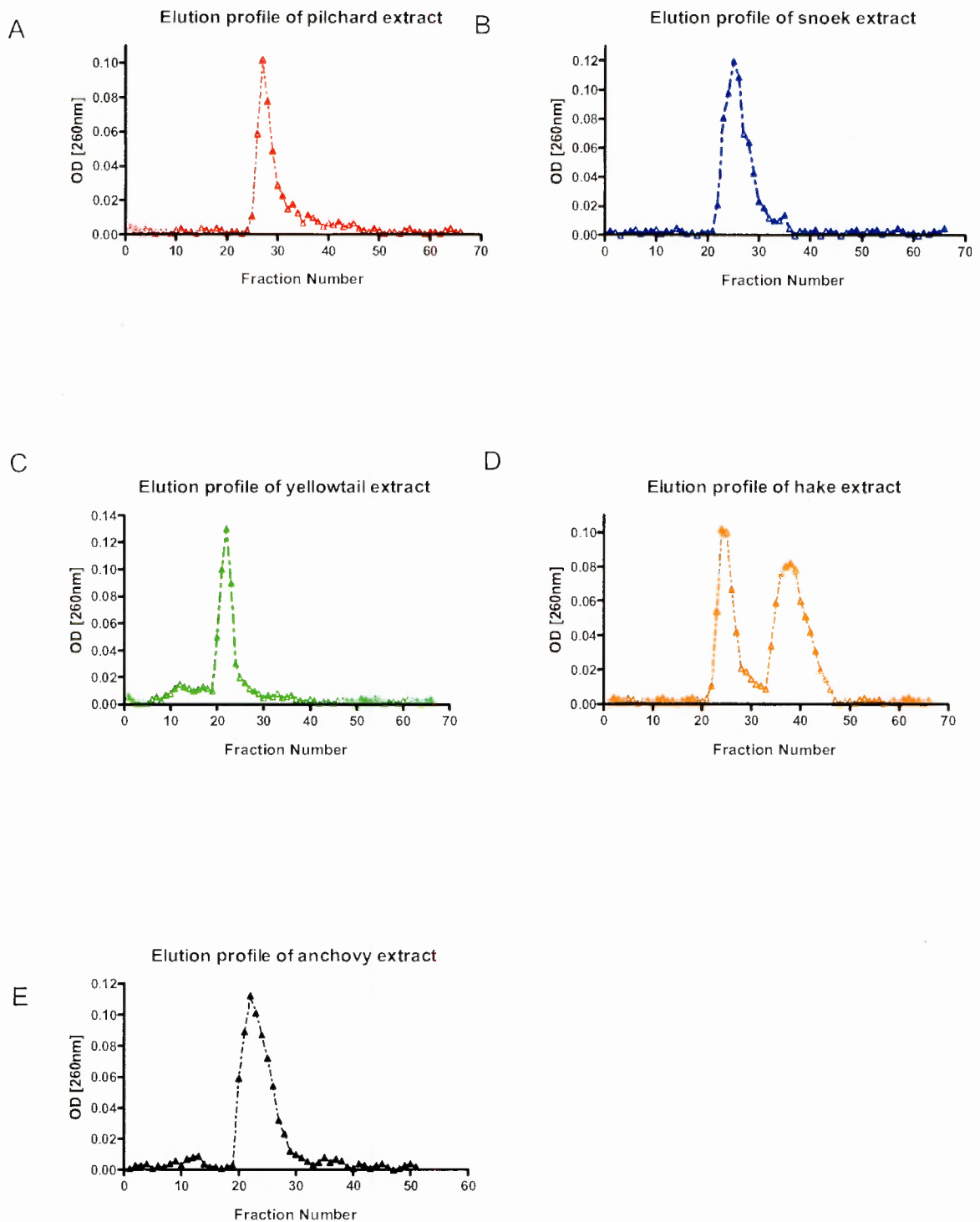
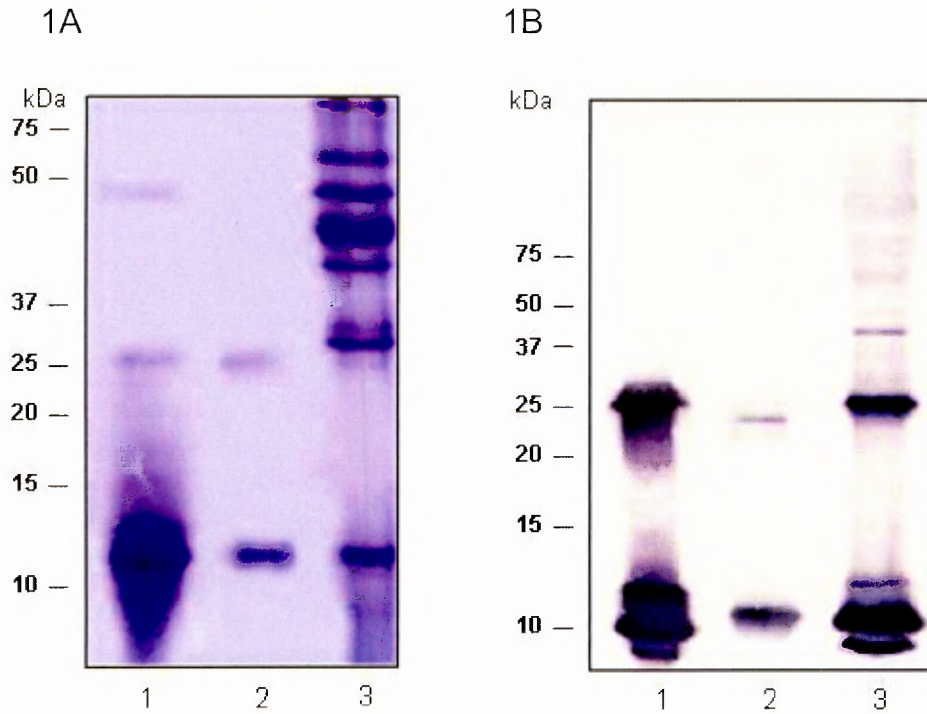
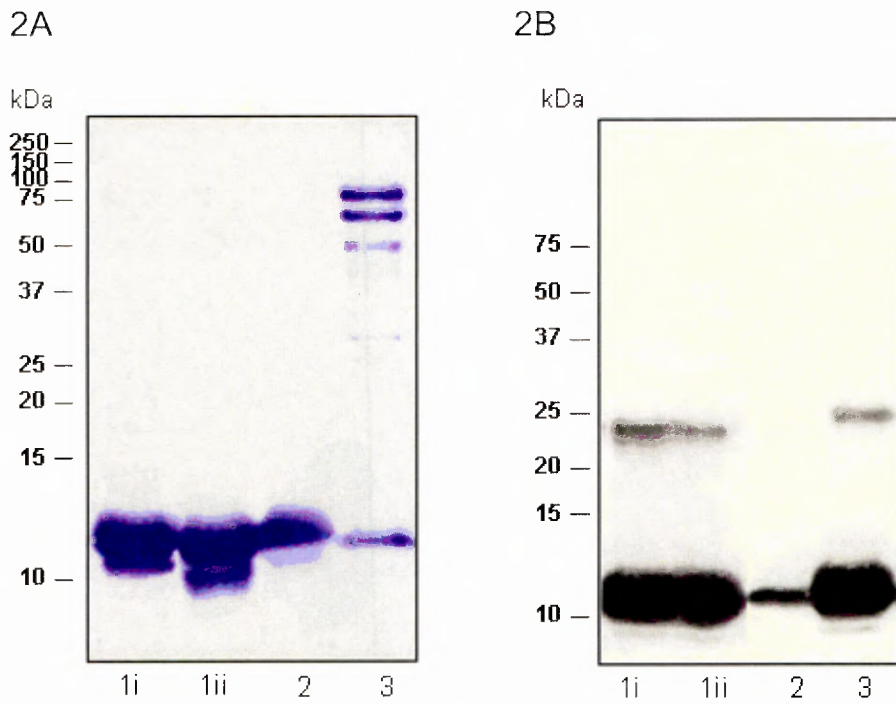


Figure 3.1.2 All eluted peaks yielded purified parvalbumin. Figures A-E represent the DEAE-cellulose anion exchange chromatography profile of all 5 fish species labelled appropriately. Approximately 12mg of protein sample in 8ml of 10mM Tris (pH=7.5) was applied to a 2.5 X 10cm DEAE-cellulose column and parvalbumin was eluted by establishing a linear Cl⁻ ion gradient. All fractions yielded a single peak containing solely parvalbumin, with the exception of hake which yielded 2, both of which also contained purified parvalbumin.

Pilchard

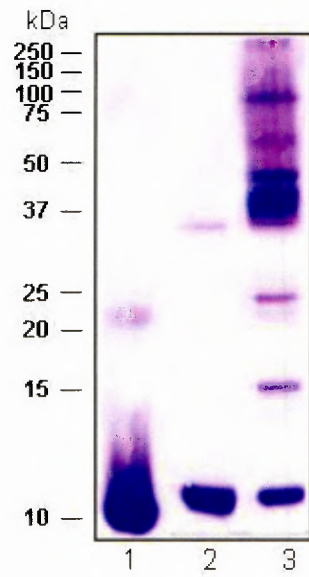


Hake

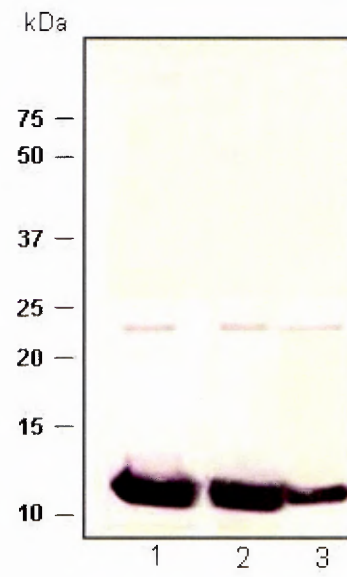


Snoek

3A

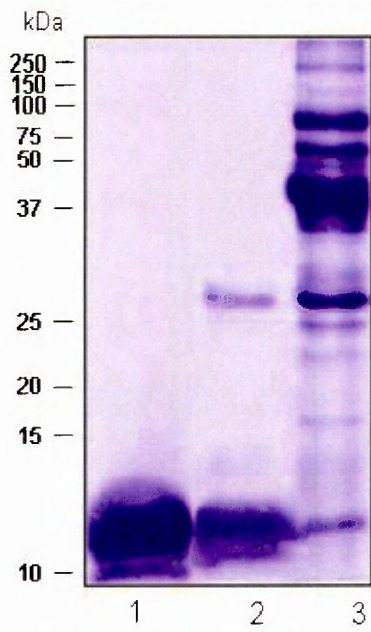


3B

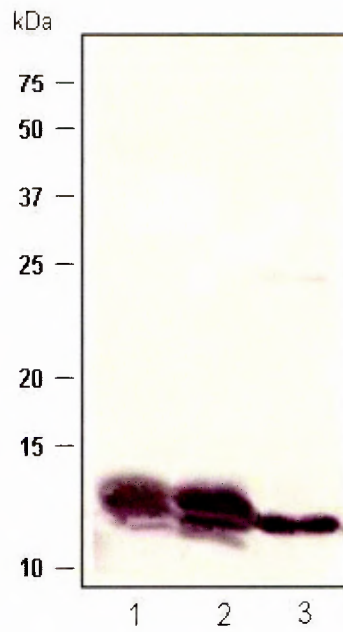


Yellowtail

4A



4B



Anchovy

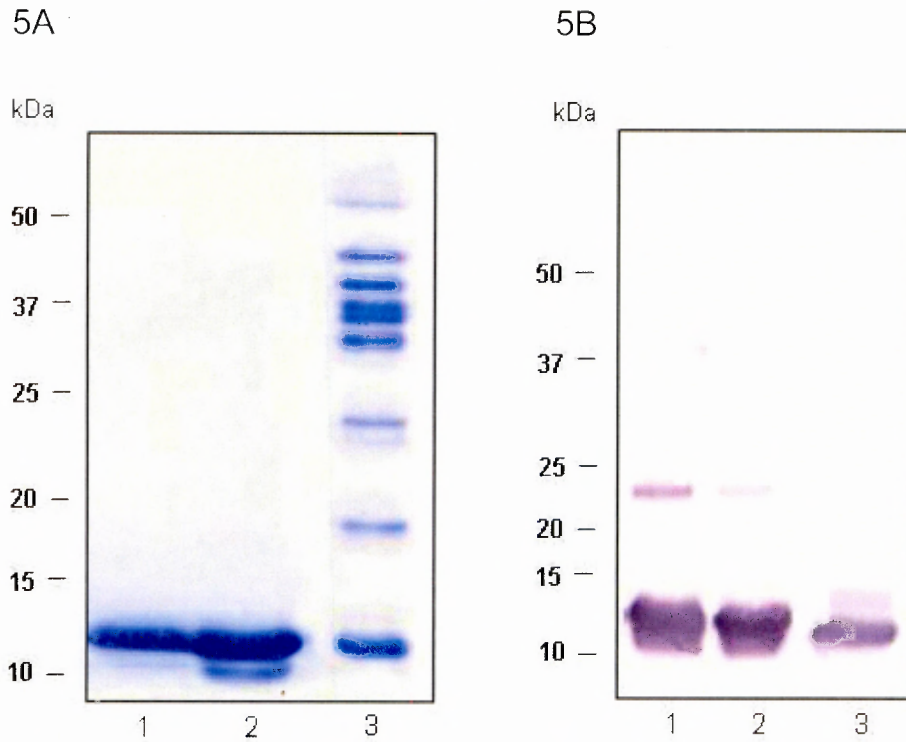


Figure 3.1.3 Parvalbumin was purified from all 5 fish species and concentrated compared to that of the crude fish extract. Purified parvalbumin (lane 1), partially purified fractions (lane 2) and crude fish extracts (lane 3) were compared in both SDS-PAGE (A) and immunoblotting (B) analysis. The two purified parvalbumin fractions represented by a dual peak in the elution profile of hake are represented in lane 1i and lane 1ii of figure 2A and 2B. A monoclonal anti-parvalbumin was used to confirm the presence of parvalbumin in all nitrocellulose-blotted extracts which detected both monomeric and oligomeric forms of parvalbumin in purified and crude fish extracts. The values of the standard markers are indicated on the left side of each gel or blot.

Table 3.1. Yield of total protein (mg/ml) and an indication of loss of protein after purification steps measured by BCA Assay kit and a BSA standard.

| PILCHARD | Volume(ml) | Protein (mg/ml) | Total protein (mg) | Yield (%) |
|---|------------|-----------------|--------------------|-----------|
| CRUDE EXTRACT | 50 | 9.8 | 489.5 | 100 |
| After cooking and (NH ₄) ₂ SO ₄ precipitation | 8 | 1.7 | 13.4 | 2.7 |
| After ion-exchange chromatography | 1.2 | 1.3 | 1.6 | 0.3 |

| HAKE | Volume(ml) | Protein (mg/ml) | Total protein (mg) | Yield (%) |
|---|------------|-----------------|--------------------|-----------|
| CRUDE EXTRACT | 50 | 5.7 | 285.5 | 100 |
| After cooking and (NH ₄) ₂ SO ₄ precipitation | 8.1 | 1.5 | 12 | 4.2 |
| After ion-exchange chromatography | 2.4 | 0.9 | 2.2 | 0.7 |

| SNOEK | Volume(ml) | Protein (mg/ml) | Total protein (mg) | Yield (%) |
|---|------------|-----------------|--------------------|-----------|
| CRUDE EXTRACT | 50 | 5.2 | 260 | 100 |
| After cooking and (NH ₄) ₂ SO ₄ precipitation | 8 | 1.5 | 11.7 | 4.5 |
| After ion-exchange chromatography | 2.2 | 1.8 | 4 | 1.5 |

| YELLOWTAIL | Volume(ml) | Protein (mg/ml) | Total protein (mg) | Yield (%) |
|---|------------|-----------------|--------------------|-----------|
| CRUDE EXTRACT | 50 | 7.7 | 382.7 | 100 |
| After cooking and (NH ₄) ₂ SO ₄ precipitation | 8.6 | 1.5 | 12.6 | 3.3 |
| After ion-exchange chromatography | 2.5 | 1.9 | 4.9 | 1.2 |

| ANCHOVY | Volume(ml) | Protein (mg/ml) | Total protein (mg) | Yield (%) |
|---|------------|-----------------|--------------------|-----------|
| CRUDE EXTRACT | 50 | 4.192 | 209.6 | 100 |
| After cooking and (NH ₄) ₂ SO ₄ precipitation | 7.5 | 1.2 | 9 | 4.3 |
| After ion-exchange chromatography | 2.2 | 1 | 2.2 | 1 |

3.2 COMPARISON OF ANTIBODY BINDING TO PURIFIED PARVALBUMIN AND CRUDE FISH EXTRACT USING SERA FROM FISH-ALLERGIC PATIENTS

Sera from two groups of fish-allergic subjects were used to assess antibody reactivity in immunoblotting. The first group comprised fish-allergic workers who were primarily exposed via airborne and skin contact with anchovy and pilchard by-products in an occupational setting. The second group comprised serum samples from individuals with food allergies caused by fish ingestion. Using sera from both groups, IgG and IgE binding to crude fish extracts and to purified parvalbumin was carried out. Any variations in IgE binding to parvalbumin as well as other fish fillet proteins were recorded for both groups. If parvalbumin was confirmed as a dominant allergen in either cohort, the extent of cross-reactivity to this allergen in five fish species was then assessed.

3.2.1 Specific IgE levels were measured by Cap-RAST in both cohorts of fish-allergic subjects

Fish-specific IgE levels were measured in sera from six allergic fish-processing factory workers (Table 3.2.1) and ten individuals with allergies caused by fish ingestion (Table 3.2.2) using the UniCAP RAST system. In addition, the age, sex, clinical symptoms, atopic status and skin-prick test results of individuals in both cohorts were recorded where information was obtainable (NA indicates where information was unavailable). The predominant symptoms noted in workers included urticaria, dermatitis, rhinoconjunctivitis, wheezing and asthma. Individuals with allergies caused by fish ingestion experienced one or more following clinical

symptom: dermatitis, urticaria, angioedema, diarrhoea, vomiting, rhinoconjunctivitis, wheezing and asthma.

IgE specific to pilchard and anchovy was measured in all fish-processing workers while pilchard, anchovy, hake, jack mackerel and tuna specific IgE was measured in individuals with allergies caused by fish ingestion (levels of IgE specific to yellowtail and snoek could not be assessed as ImmunoCAP diagnostic extracts of these fish species are not manufactured). Where serum samples were limited, as was the case in patients 6-10 in Table 3.2.2 only levels of pilchard and anchovy specific IgE were measured.

In fish-processing factory workers, the mean value of pilchard specific IgE levels (1.09 kUA/L) was higher than that of anchovy specific IgE (0.68 kUA/L). These values were considerably lower than the mean values of pilchard (14.93 kUA/L) and anchovy specific IgE (8.33 kUA/L) levels measured in sera from individuals with allergies caused by fish ingestion. Due to the lack of sera from patients 6-10 with ingestion-induced allergies, an accurate comparison of specific IgE levels in the remaining three fish species could not be performed.

Table 3.2.1 Clinical and serological characterisation of fish-processing factory workers

| 1. Demographic characteristics, clinical symptoms and serological data of subjects in a 2 fish-processing factories | | | | | | | | | |
|---|-------------|-----|---|-------|-------------|------------------------------|------|---------|------|
| Subjects | Age (years) | Sex | Clinical symptoms | Atopy | SPT results | Specific IgE CAP-RAST (kU/l) | | | |
| | | | | | | Pilchard | | Anchovy | |
| | | | | | | Class | kU/L | Class | kU/L |
| W1 | 43 | M | Urticaria and dermatitis | Yes | Positive | 2 | 1.9 | 2 | 1.38 |
| W2 | 50 | M | NA | No | Positive | 2 | 0.9 | 1 | 0.42 |
| W3 | 25 | F | Urticaria and dermatitis | NA | NA | 1 | 0.64 | 1 | 0.66 |
| W4 | 37 | F | Wheezing | Yes | Negative | 1 | 0.69 | 1 | 0.66 |
| W5 | 37 | F | Rhinoconjunctivitis | Yes | Positive | 1 | 0.57 | 1 | 0.46 |
| W6 | 31 | M | Asthma, rhinoconjunctivitis, urticaria and dermatitis | Yes | Positive | 2 | 1.82 | 1 | 0.52 |

NA = not available

Table 3.2.2 Clinical and serological characterisation of allergic reactions after fish ingestion

| 2. Demographic characteristics, clinical symptoms & serological data of subjects with allergic responses to fish-ingestion | | | | | | | | | | |
|--|-------------|-----|---|-------|-------------|------------------------------|-----------------|--------------|-----------------------|--------------|
| Subjects | Age (years) | Sex | Clinical symptoms | Atopy | SPT results | Specific IgE CAP-RAST (kU/l) | | | | |
| | | | | | | Pilchard kU/L | Anchovy kU/L | Tuna kU/L | Jack mackerel kU/L | Hake kU/L |
| 1 | 25 | F | Asthma, rhinoconjunctivitis, dermatitis, urticaria, vomiting and angioedema | Yes | Positive | 44.1 | 24.3 | 28.3 | 46.8 | 62.5 |
| 2 | 28 | F | Rhinoconjunctivitis, urticaria and angioedema | Yes | Positive | 1.15 | 1.31 | 0.8 | 1.8 | 1 |
| 3 | 43 | F | Urticaria, diarrhoea, rhinoconjunctivitis, angioedema and asthma | Yes | Positive | 3.21 | 2.5 | 2.4 | 4.9 | 6.5 |
| 4 | 19 | M | Urticaria, asthma, and angioedema | Yes | NA | 25 | 17.7 | 12.4 | 7 | 7.8 |
| 5 | 24 | M | Asthma, dermatitis, urticaria, vomiting and rhinoconjunctivitis | Yes | Positive | 1.82 | 1.54 | 1.8 | 2 | 2.8 |
| 6 | NA | M | NA | NA | NA | 2.02 | <0.35 | NA | NA | NA |
| 7 | NA | M | NA | NA | NA | 13.2 | 10.3 | NA | NA | NA |
| 8 | 59 | F | Wheezing, dermatitis | Yes | Positive | 1.06 | 0.85 | NA | NA | NA |
| 9 | 10 | F | Wheezing and vomiting | Yes | Positive | 56.3 | 15.6 | NA | NA | NA |
| 10 | 5 | M | Vomiting and urticaria | Yes | Positive | 1.47 | 0.85 | NA | NA | NA |

NA = not available

3.2.2 Sera from fish-processing factory workers was used to assess IgG and IgE reactivity to crude pilchard and anchovy proteins

IgE and IgG reactivity to nitrocellulose-blotted pilchard and anchovy crude fillet extracts was assessed using sera from six fish-allergic workers (Figure 3.1.4). A monoclonal anti-parvalbumin antibody was used to confirm the presence of parvalbumin in all extracts and served as a positive control (lane P). Serum from a worker who displayed no allergic symptoms and no detectable levels of fish-specific IgE was utilised as a negative control (lane N). The order of the patients (labelled 1-6) was maintained in all 4 blots.

Extensive IgG reactivity to proteins from pilchard fillet were noted in all six serum samples (Figure 3.1.4 A). Antigens were detected in the range 12kDa to 150kDa. IgG recognition in all six serum samples including the negative control displayed no regular detection pattern. However, a common antigen in the range of 55kDa was observed in sera from all six patients and the negative serum sample. Serum IgG from one patient (lane 6) recognised a protein which corresponded in size with parvalbumin.

Assessment of IgE binding revealed that sera from all seven workers failed to recognise parvalbumin or any other allergen in the pilchard fillet (Figure 3.1.4 B) when compared to the positive parvalbumin control.

Assessment of IgG and IgE binding to nitrocellulose-blotted crude anchovy extract revealed similar findings to that of pilchard. IgG reactivity was diverse, however fewer antigens were recognised in the range of 12kDa to 100kDa compared to pilchard specific binding (Figure 3.1.4 C). Serum IgG from one worker (lane 1) recognised an antigen which corresponded in size to parvalbumin, while sera from two other

workers (lane 2 and 5) displayed IgG binding to a protein slightly heavier than that of parvalbumin (approximately 13kDa).

Analysis of IgE binding to anchovy fillet proteins showed that sera from one worker (lane 2) displayed slight recognition of a 37kDa allergen (Figure 3.1.4 D). Serum IgE from the remaining allergic workers did not bind any allergens in anchovy fillet.

The fish-processing workers that were assessed for IgG and IgE binding in immunoblotting predominantly handled pilchard and anchovy in an occupational environment. Due to the fact that no extensive IgE binding to these sensitising fish species was noted, assessment of antibody binding to the additional three fish species was not carried out.

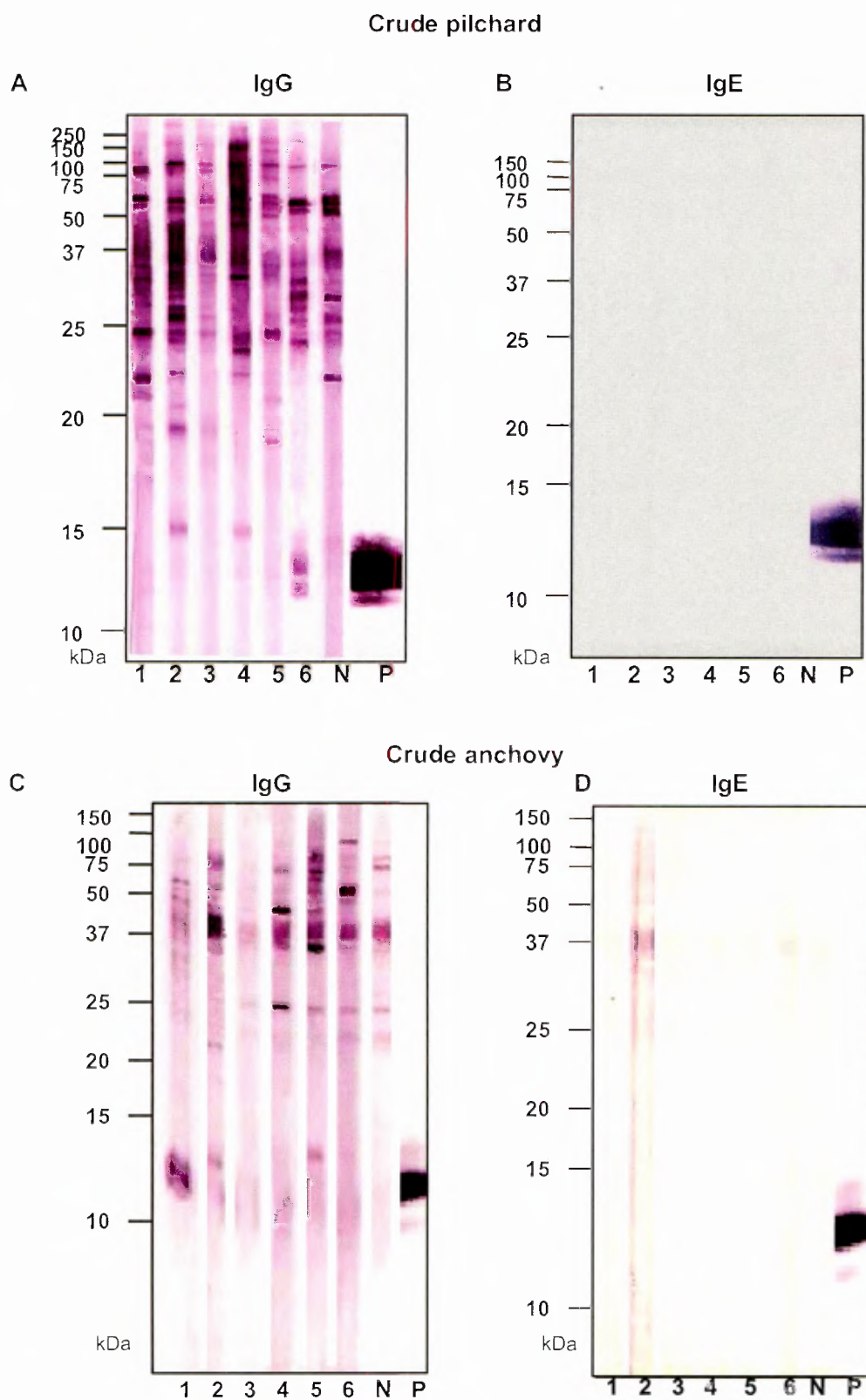


Figure 3.1.4 Comparison of IgG (A) and IgE binding (B) to pilchard crude extract and as well IgG (C) and IgE (D) reactivity to anchovy crude extract. Sera from 6 fish-factory workers with fish allergy (lanes 1-6) were probed with nitrocellulose-blotted fish extracts prepared under reducing conditions. Serum from a non-atopic individual (lane N) was used as a negative control while a monoclonal anti-parvalbumin antibody was used as a positive control (lane P) to confirm the presence of parvalbumin

3.2.3 Sera from fish-allergic domestic consumers was used to assess IgG and IgE reactivity to crude fish proteins and purified parvalbumin

Sera from ten patients with allergic reactions induced by fish consumption were tested for IgG and IgE reactivity to purified parvalbumin and crude fish fillet extracts from five fish species by immunoblotting. Again, a monoclonal anti-parvalbumin antibody was used to confirm the presence of parvalbumin in all extracts as a positive control (lane P), while serum from a non-allergic individual was utilised as a negative control (lane N).

3.2.3.1 A wide range of IgG specific antigens were recognised by all fish-allergic patients

All sera contained IgG antibodies that displayed reactivity to a wide range of antigens from all five fish species (Figure 3.1.5 A-E). The antigen recognition pattern to proteins greatly varied and no antigens common to all fish species were evident. In nitrocellulose-blotted pilchard crude fillet extract, a 12kDa protein was recognised by all ten fish-allergic sera which corresponded with parvalbumin based on the similar position of the positive control. Serum IgG from patients 1 and 7-10 bound two bands in the range of 12kDa that were in close proximity, which are likely to be different isoforms of pilchard parvalbumin (Figure 3.1.5 A). Similar results were noted in IgG binding to anchovy (Figure 3.1.5 D) and snoek extract (Figure 3.1.5 E). Specifically, serum IgG from patients 9 and 10 recognised two distinct anchovy-specific antigens in the range of 12kDa (Figure 3.1.5 D), while sera from patients 1,2,7 and 8 also displayed binding to two bands also in the range of 12kDa in snoek (Figure 3.1.5 E) again indicative of isoforms. Sera from four patients (1, 2, 7 and 8) also recognised a 12kDa antigen in blotted hake extract (Figure 3.1.5 C) as well as one patient (8) to yellowtail extract (Figure 3.1.5 B).

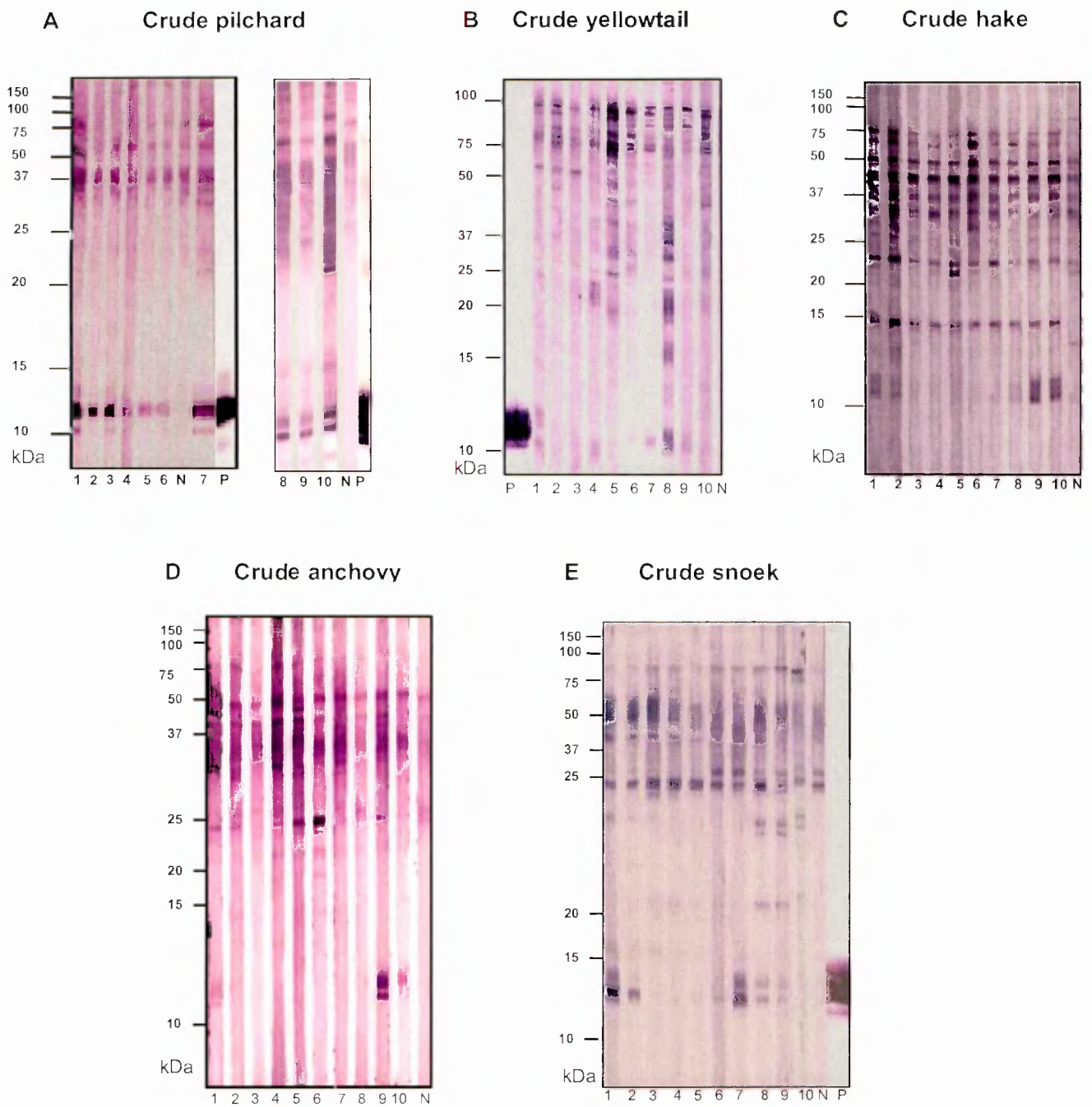


Figure 3.1.5 Comparison of IgG reactivity to crude pilchard (A), yellowtail (B), hake (C), anchovy (D) and snoek (E) extract. Sera from 10 fish-allergic patients (lane 1-10) sensitised via fish ingestion were probed with nitrocellulose-blotted extract under reducing conditions. Serum from a non-atopic individual (lane N) was used as a negative control while a monoclonal anti-parvalbumin antibody was used as a positive control (lane P) to confirm the presence of parvalbumin. The values of the standard markers are indicated on the left of all blots.

3.2.3.2 Parvalbumin was the dominant allergen in patients sensitised via fish ingestion

Pilchard specific IgE binding

Analysis of IgE binding to crude pilchard extract revealed that eight patients (patients 1-4, 7 and 8-10) recognised parvalbumin as an allergen (Figure 3.1.6 A) as confirmed by the same serum samples binding purified parvalbumin from pilchard (Figure 3.1.6 D). In addition, serum IgE from patients 1, 2 and 3 recognised an allergen of approximately 45kDa, although comparatively weaker binding was noted in patient 3. IgE-binding to two or three isoforms of pilchard were noted to both the blotted crude extract and purified parvalbumin. Patient 8 reacted strongly to three isoforms indicated by three bands that were closely situated next to each other (Figure 3.1.6 A and D). The remaining 6 patients that displayed IgE binding to parvalbumin recognised two isoforms of pilchard with comparatively weaker binding

Yellowtail specific IgE binding

IgE-binding to several allergens was detected in the crude extract, particularly in patients 3 and 10 (Figure 3.1.6 B). Serum IgE from both these patients bound parvalbumin, however serum from patient 3 displayed more intense IgE binding. In addition, serum from patient 3 recognised allergens at 24kDa, 37kDa, 110kDa and 160kDa. Serum IgE from patient 10 also displayed reactivity to a 24kDa as well as a 40kDa allergen. Due to the similar position of the parvalbumin dimer noted in the positive control, the 24kDa allergen detected in patient 3 and 10 could possibly be an oligomer, although no binding to oligomeric forms in purified yellowtail parvalbumin was noted by the anti-parvalbumin antibody nor patient's sera (Figure 3.1.6 E). In total, 7 out of the 10 allergic subjects recognised parvalbumin as an allergen, which was confirmed by the same patient's sera recognising nitrocellulose-blotted purified parvalbumin (Figure 3.1.6 E).

Hake specific IgE binding

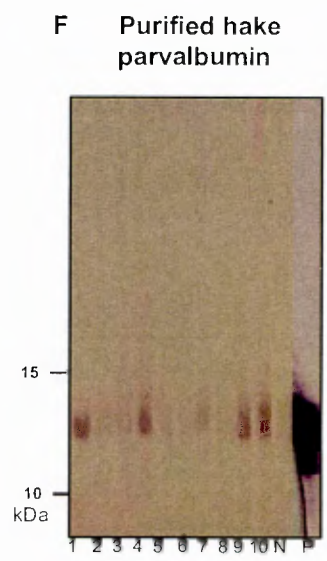
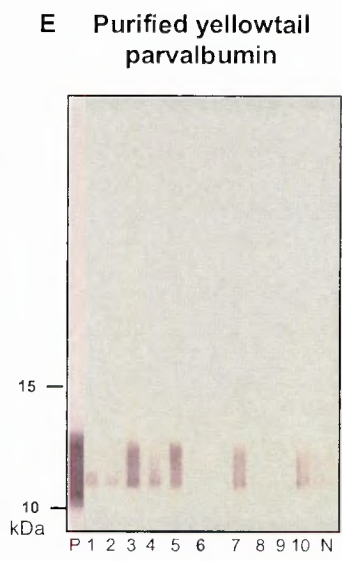
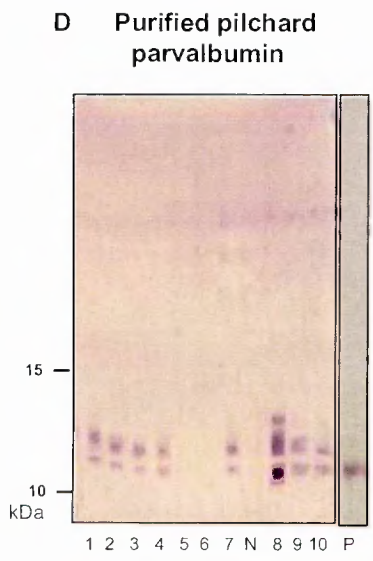
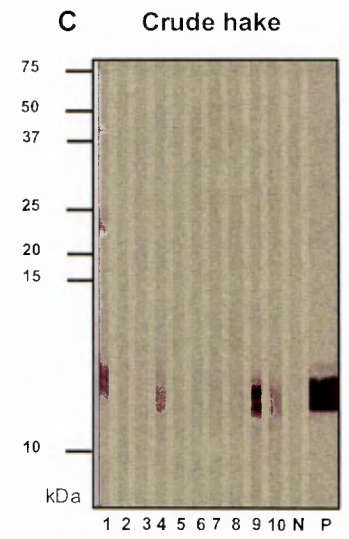
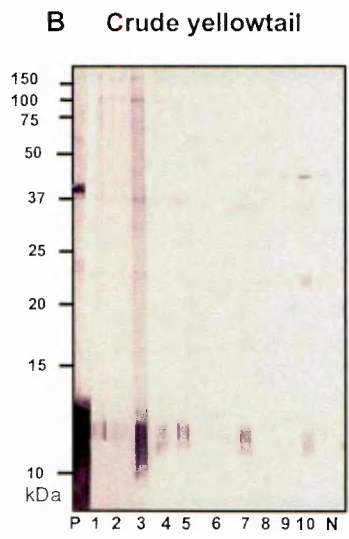
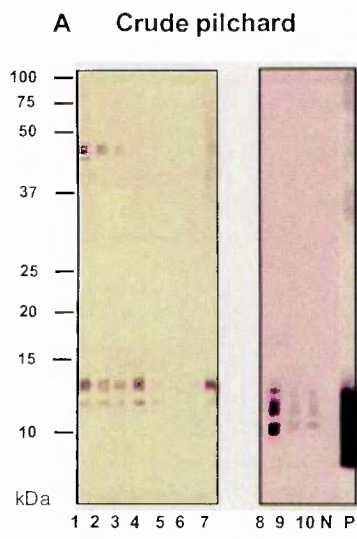
Four out of the ten serum samples possessed IgE that bound to parvalbumin in the crude fillet extract of hake (Figure 3.1.6 C). These included patients 1, 4, 9 and 10. Serum IgE from these same patients recognised purified parvalbumin, thus confirming specific IgE binding to this 12kDa allergen (Figure 3.1.7 F). Serum IgE from patient 1 recognised a 24kDa allergen, which again due to the position of the dimer noted in the positive control, was assumed to be an oligomer of parvalbumin (Figure 3.1.6 C). Similar results with regard to oligomeric binding were not noted in IgE reactivity to purified hake parvalbumin (Figure 3.1.7 F).

Anchovy specific IgE binding

IgE reactivity to parvalbumin present in crude anchovy extract was noted in 4 out of the ten sera, namely from patients 1, 4, 7 and 9 (Figure 3.1.6 G). Corresponding IgE binding to purified parvalbumin was noted in the same patients (Figure 3.1.6 I). Serum from patient 9 displayed repeated IgE detection of two isoforms as did sera from patient 4. Again, a 24kDa allergen was bound by serum IgE from patient 9 and by an anti-parvalbumin antibody in the positive control.

Snoek specific IgE binding

IgE reactivity to snoek parvalbumin was noted in sera from patients 1, 4, 7 and 9 which was identical to that of anchovy (Figure 3.1.6 H). Again, IgE-binding to two separate isoforms was evident in patient 9. A similar IgE reactivity pattern was noted in the purified snoek parvalbumin (Figure 3.1.9 J). Sera from patients 1, 4 and 7 appeared to detect one isoform, indicated by binding to a single band while serum from patient 9 again bound two IgE-specific isoforms in the purified extract.



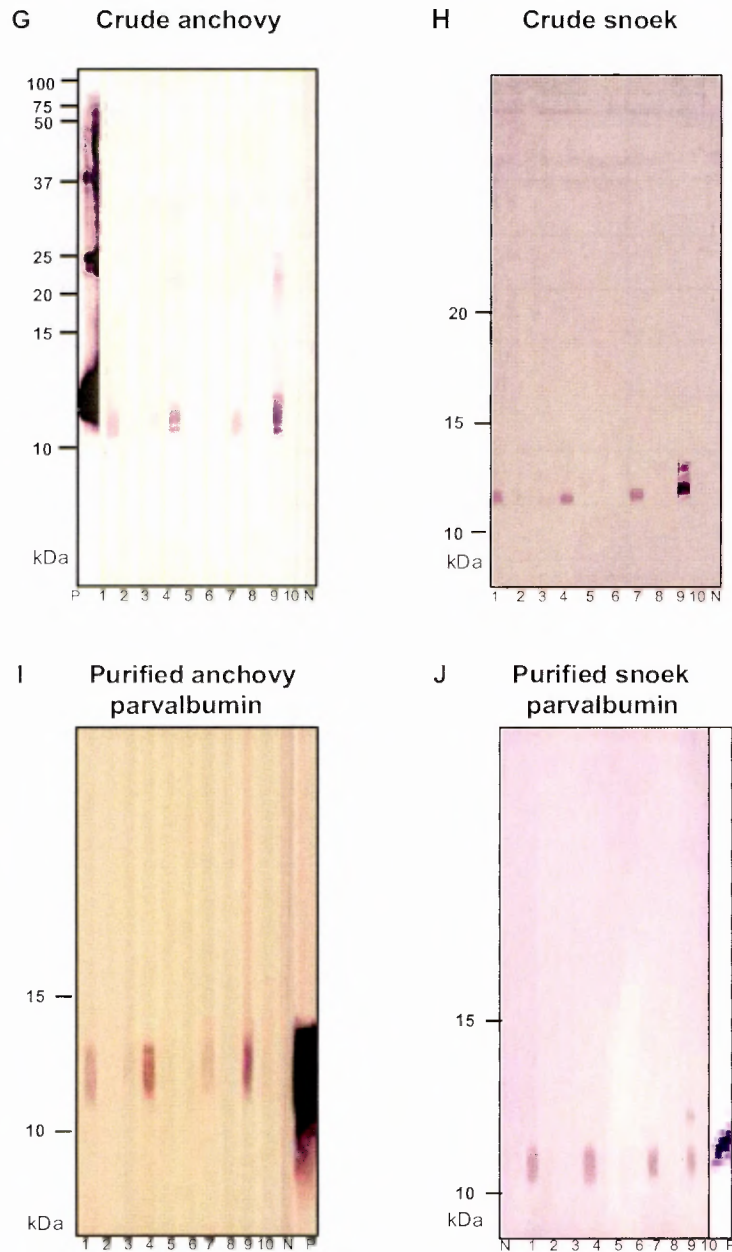


Figure 3.1.6 Comparison of **IgE reactivity** to the crude pilchard (A), yellowtail (B), hake (C), anchovy (G) and snoek (H) extract. IgE binding to purified parvalbumin from pilchard (D), yellowtail (E), hake (F), anchovy (I) and snoek (J) was also assessed by immunoblotting. Sera from 10 fish-allergic patients (lane 1-10) sensitised via fish ingestion were probed with nitrocellulose-blotted extract under reducing conditions. Serum from a non-atopic individual (lane N) was used as a negative control while a monoclonal anti-parvalbumin antibody was used as a positive control (lane P) to confirm the presence of parvalbumin. The values of the standard markers are indicated on the left of all blots.

3.2.4 Summary of cross-reactivity of parvalbumin from five fish species

From the work carried out here it is demonstrated that parvalbumin was the major allergen in patients with allergies caused by fish consumption, however this was not the case in the group of fish-processing factory workers.

Subjects sensitised via ingestion displayed the widest IgE reactivity to pilchard parvalbumin (80% of patients) indicated by the '•' markings and highlighted column (Table 3.2). Yellowtail parvalbumin bound IgE from 70% of patients, while parvalbumin from the remaining three fish species was recognised as an allergen by 40% of allergic patients. All serum IgE reacted to at least one species of parvalbumin with the exception of serum IgE from patient 6 that did not display any reactivity. IgE in sera from patients 4 and 7 recognised parvalbumin from all five fish species.

Based on pilchard parvalbumin's extensive IgE-binding abilities, as well as the commercial relevance and global distribution of this fish species, molecular sequencing as well as classification of isoforms was carried out.

3.3 FURTHER CHARACTERISATION OF PILCHARD PARVALBUMIN

3.3.1 Isoforms of pilchard parvalbumin were detected by two-dimensional gel electrophoresis and immunoblotting

In order to identify the number of isoforms and their respective lineage, purified pilchard parvalbumin was subjected to analysis by two-dimensional gel electrophoresis. Previously unidentified isoforms of pilchard parvalbumin were separated according to pH and then resolved based on size by SDS-PAGE (Figure 3.2.1). These isoforms of parvalbumin were then transferred to a nitrocellulose membrane and detected using a monoclonal anti-parvalbumin antibody.

Monomeric and dimeric isoforms of pilchard parvalbumin with isoelectric (pI) values between approximately 4 - 5 and 5.5-5.9 were detected as indicated by the red arrows. Thus, both evolutionary lineages of isoforms were visualised, namely the α group (pI>5) and the β group (pI<5). The high isoelectric values of the β and α isoforms represent unusually basic pH values when compared with other characterised parvalbumin isoforms. The length of the protein smear along with the lower pI indicated the presence of more than one isoform that was not resolved sufficiently. The monomeric isoforms were clearly visible compared to the dimeric forms at 24kDa. This data is therefore indicative of the presence of at least two distinct isoforms of parvalbumin though potentially three isoforms may be present.

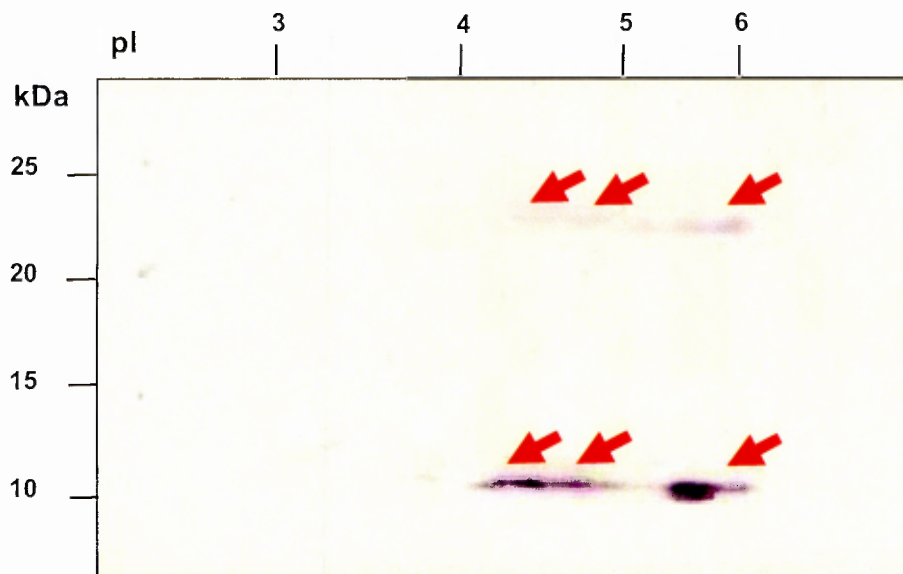


Figure 3.2.1 Two-dimensional gel electrophoresis map of nitrocellulose-blotted purified pilchard parvalbumin. Isoforms were detected using a monoclonal anti-parvalbumin antibody after separation according to charge (by isoelectric focusing) and size (by SDS-PAGE). Monomeric (12kDa) and dimeric (24kDa) isoforms of parvalbumin are indicated by red arrows. Values of molecular weight measured in kilodaltons are represented on the left hand side while pI values are indicated along the top of the blot.

3.3.2 cDNA encoding parvalbumin from pilchard (*Sardinops sagax*)

The pilchard parvalbumin gene was sequenced to attain information from the primary amino acid sequence such as the lineage (ie. α or β isoform) and to compare homology with previously characterised parvalbumins. In addition, the elucidation of the cDNA sequence is the primary step towards the generation of a pilchard recombinant parvalbumin protein which could potentially be used for diagnosis and treatment of fish allergies.

3.3.2.1 One tryptic peptide from parvalbumin was elucidated by MS/MS analysis for primer design

Purified pilchard parvalbumin was digested with trypsin and the resulting peptides were subjected to LC, MS and MS/MS analysis (Figure 3.2.2). The acquired raw MS/MS data was analysed using the on-line search algorithm MASCOT for peptide identification. This amino acid information was used to design a primer for the amplification of parvalbumin cDNA.

A tryptic peptide of 9 amino acids (AFAIIDQDK) was identified which possessed an individual ion score of 45 indicating a positive peptide match (Ion score is defined as $-10 \cdot \log(P)$ where P is the probability that the observed match is random). The query peptide corresponded with 8 other amino acid residues of parvalbumin from the following fish species: chub mackerel (*Scomber japonicus*), common carp (*Cyprinus carpio*), jack mackerel (*Trachurus japonicus*), Alaska pollack (*Theragra chalcogramma*), zebrafish (*Danio rerio*), channel catfish (*Ictalurus punctatus*), barramundi (*Lates calcirifer*) and mangrove rivulus (*Rivulus marmoratus*). A

calculated mass residue (Mr) of 1019.5287 was recorded and compared with a calculated Mr of 1019.4578 ($\Delta = -0.0709$).

Using the PeptideCutter function on ExPasy Tools, the tryptic fragment identified was verified as having the correct cleavage sites using the amino acid sequence for chub mackerel, which contained the matching peptide. The approximate position of this fingerprint was 47-55 in the amino acid sequence of parvalbumin (Figure 3.2.3).

3.3.2.2 Primers were used to amplify a portion of the pilchard parvalbumin gene

Primers were designed using the amino acid information deduced by MS/MS analysis and alignment of previously sequenced parvalbumin genes. All parvalbumin cDNA sequences with 100% homology to the translated peptide were aligned and a 25 bp consensus sequence with the appropriate GC content was utilised as a 5' primer (Figure 3.2.4 A). The 3' primer was designed by alignment of the same 5 cDNA sequences and the most conserved region of the sequence was used to design a degenerate primer (Figure 3.2.4 B). This primer consisted of 23 nucleotides and is composed of base pairs 291-312 of the parvalbumin gene (position 97-104 in the amino acid sequence).

The 5' and 3' primers were used to amplify a portion of the parvalbumin sequence from pilchard cDNA and a product of approximately 180bp (lane 1; Figure 3.2.5) was detected which correlated with the position of the 2 primers in the parvalbumin gene. No amplification products were detected in the negative control (lane 2; Figure 3.2.5).

A

| | A | F | A | I | I | D | Q | D |
|-----------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Cyprinus carpio | GCT | TTC | CCA | ATC | ATT | GAT | CAG | GAC |
| Scomber japonicus | GCT | TTC | CCC | ATC | ATT | GAC | CAG | GAC |
| Trachurus japonicus | GCC | TTC | CCA | ATC | ATC | GAC | CAG | GAC |
| Trachurus japonicus | GCC | TTC | CCA | ATC | ATC | GAC | CAG | GAC |
| Theragra chalcogramma | GCC | TTC | GGC | ATC | ATT | GAC | CAG | GAC |
| Consensus | gc | ttc | g | atc | at | ga | cag | gac |

B

| | G | K | I | G | V | D | E | F |
|-----------------------|-----|-----|-----|-----|-----|-----|-----|---------|
| Cyprinus carpio | GGC | AAG | ATT | GGG | GTT | GAT | GAG | TTT.... |
| Scomber japonicus | GGC | AAG | ATC | GGT | ATT | GAT | GAG | TTT.... |
| Theragra chalcogramma | GGC | AAG | ATA | GGG | GTT | GAT | GAG | TTT.... |
| Trachurus japonicus | GGC | AAG | ATT | GGG | GTT | GAT | GAG | TTT.... |
| Trachurus japonicus | GGC | AAG | ATT | GGG | GTT | GAT | GAG | TTT.... |
| Consensus | ggc | aag | at | gg | tt | gat | gag | tt |

Figure 3.2.4 Primers were designed using the deduced amino acid sequence obtained by MS/MS analysis (A). Five parvalbumin cDNA sequences that had a 100% homology to the tryptic peptide of interest (listed in blue print) were aligned and the consensus sequence (listed underneath the alignment) was used as a 5' primer. A degenerate 3' primer 23 nucleotides long was also designed based on a conserved region identified in the alignment of the same 5 cDNA parvalbumin sequences (B). The consensus sequence was used as a degenerate primer. The 5 cDNA sequences of parvalbumin that were aligned in both A and B were from the fish species *Cyprinus carpio* (common carp), *Scomber japonicus* (chub mackerel), 2 isoforms of *Trachurus japonicus* (jack mackerel) and *Theragra chalcogramma* (Alaska pollack).

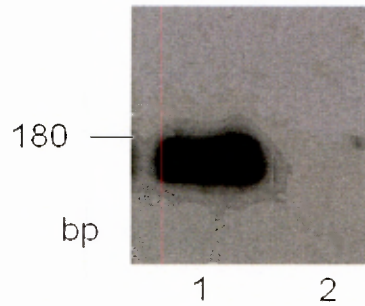


Figure 3.2.5 PCR reaction resolved on a 0.8% agarose gel using primers designed by MS/MS results and homology alignment. A product of approximately 180bp was detected as noted in lane 1. The negative control yielded no product as illustrated in lane 2.

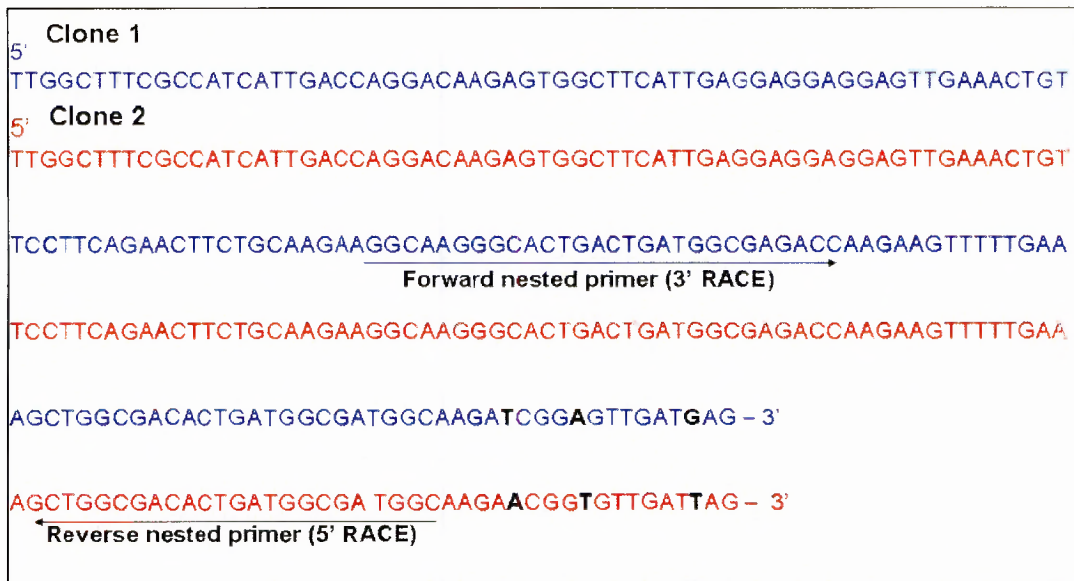


Figure 3.2.6 Comparison of 2 cDNA clones (clone 1 and clone 2) obtained from sequencing a portion of the parvalbumin gene. Both clones displayed significant homology to the β forms of parvalbumin noted in BLASTX. Alignment of the clones revealed 99% similarity. Three base pairs differed between the clones which are highlighted in black print. The position of the nested primers designed for 5' (indicated by the reverse-directional arrow) and 3' RACE (indicated by the forward-directional arrow) are illustrated.

3.3.2.3 The parvalbumin gene was partially deduced to attain cDNA sequence information

The 180bp fragments (lane 1 in Figure 3.2.5) were sequenced and results were interpreted using the BLASTX program. Two out of six clones sequenced displayed significant homology to parvalbumin beta from common carp (*Cyprinus carpio*) and northern pike (*Esox lucius*), both with 82% identity. Numerous additional matches to parvalbumin beta from various fish species were also noted with identity ranging from 79% (Cod) to 62% (Atlantic salmon). Clones were then aligned to confirm sequencing accuracy. It was noted that the two clones displayed 99% similarity (Figure 3.2.6). Using this sequence information, nested primers were designed for 5' and 3' RACE. The positions of these primers in relation to the partially sequenced parvalbumin gene are indicated by directional arrows in Figure 3.2.6. Primers were positioned to create an overlap of 79bp in the sequence which served as an internal control.

3.3.2.4 The complete pilchard parvalbumin sequence was determined by 5' and 3' RACE

Using the 5' and 3' nested primers and cDNA synthesized using the SMART RACE cDNA Amplification Kit, the 5'-ends as well as the 3'-ends of the parvalbumin gene were determined.

In 5' and 3' RACE, products of approximately 400bp and 500bp respectively were amplified (Figure 3.2.7). All products were sequenced and results were again interpreted using the BLASTX program. An overlapping region of approximately 70bp was noted which confirmed that both the start of the 5' and 3' regions were accurately sequenced. Seven clones of each RACE product were obtained. Two

clones of the 3' end revealed 69% identity with the β form of common carp parvalbumin which is in accordance with results of the partial parvalbumin sequence. Identity to the β isoforms of northern pike (66%), cod (65%), hake (63%) and coelacanth (63%) were also noted. Comparison of the clones showed 98% similarity.

With reference to the 5' products, two clones displayed a high degree of homology to common carp (89%) as well as zebra fish (89%), jack mackerel (89%), channel catfish (89%) and mangrove rivulus (85%). Alignment of the clones exhibited 97% similarity.

Due to the overlapping regions of the 5' and 3' sequences, the complete cDNA sequence of the parvalbumin gene could be accurately deduced. The resultant sequence was identified as a 327 bp open reading frame, encoding 109 amino acids. Pilchard parvalbumin was designated Sar s 1 (submitted on the 23/06/2008 to the EMBL/GenBank/DDBJ database under accession number FM177701) according to the International Nomenclature of Allergens ¹³⁹ and revealed to have a theoretical molecular weight of 11916.66 Da. This protein was identified as a β -type parvalbumin by its chain length ie. 109 residues as well as by the positions of the following 6 amino acids: Ala-14, Leu-16, Cys-19, Phe-67, Gln-69, and Thr-79 (Figure 3.2.8).

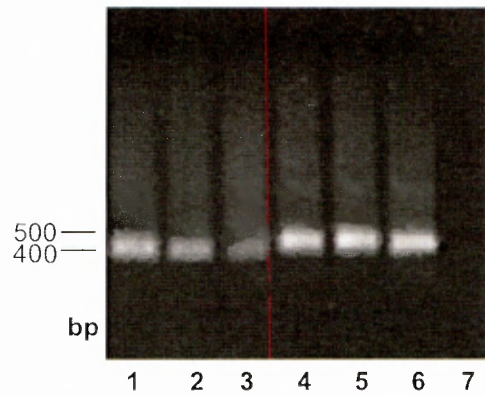


Figure 3.2.7 5' RACE and 3' RACE products were amplified using nested gene-specific primers and universal primers provided in the SMART RACE cDNA Amplification Kit and resolved on a 0.8% agarose gel. Amplification of 5' ends resulted in products of approximately 400bp (lanes 1-3) while 3' end amplification yielded products in the region of 500bp (lanes 4-6). The negative control yielded no product as illustrated in lane 7.

Sar s 1 (β isoform)

```

gggactncagttgctcgagtccttctcatccaacctaacccaaagacaactgagctaaa 60
M A L A G L V K E A D I T A A L E A C K 20
atggcactcgccggcttgggtcaaggaagcggacatcactgcagcactggaggcctgcaaa 120
A A D S F D H K A F F H K V G M S G K S 40
gctgctgactcattcgaccacaaggctttctccacaagggttggaaatgtcaggaaaatct 180
A D E L K K A F A I I D Q D K S G F I E 60
gctgatgagctgaagaaggcctttgccatcattgatcaggacaagagtggcttcattgag 240
E E E L K L F L Q N F C K K A R A L T D 80
gaggaggagttgaaactgttccttcagaacttctgcaagaaggcaagggcactgactgat 300
G E T K K F L K A G D N V G D G K I G I 100
ggcgagaccaagaagtttttgaaagctggcgacaatggttggcgatggcaagattggaatt 360
D E F N H L V K H * 109
gacgagttcaaccatttgggtaagcactaaactgcaactgaccaacagatettgcccgga 420
aggaacaacattgccaagacttcccattttcatttaataaacattatattttatatttg 480
cttatggagagattcctcccacgcaccactttgccc aaaatgatgattgCGAATGTAGTC 540
ggttgcgcctcatgtcttaaatatgaattgtaaaaggtaaaatcttcattgcacttttcg 600
ggcaggacctaccatatagtgagtcatatagaaggaaag 639

```

| | | |
|--------|--------|--------|
| Ala-14 | Cys-19 | Gln-69 |
| Leu-16 | Phe-67 | Thr-79 |

Figure 3.2.8 Nucleotide and deduced amino acid sequence of the β form of pilchard parvalbumin which was designated Sar s 1. The amino acid sequence is listed above the nucleotide sequence and the stop codon is marked with an asterisk. The defining characteristics that indicate pilchard parvalbumin is from a β lineage are indicated by red outlines i.e. the position of 6 amino acids (Ala-14, Leu-16, Cys-19, Phe-67, Gln-69, and Thr-79) as well as the chain length of 109 residues which are all outlined

3.3.2.5 Alignment of pilchard parvalbumin with 12 similar fish species

Fish species that displayed the highest similarities with translated pilchard parvalbumin were aligned as indicated in Figure 3.2.9. The boxes surrounding stretches of amino acids in the alignment indicate the two calcium-binding sites or functional EF-hand motifs of parvalbumin.

All 12 fish species in the sequence alignment were β isoforms of parvalbumin with the exception of the form of carp parvalbumin. This confirmed an unambiguous relationship to the β lineage. Sar s 1 displayed the highest homology to the β form of parvalbumin from common carp as well as to zebra fish of the same lineage. Other fish species which displayed 70% homology or above included Atlantic salmon, chub mackerel, mangrove rivulus, dark-banded rock fish and the α isoform of common carp (Table 3.4). The scientific and common names of the fish species listed in the alignment are recorded next to their BLAST sequence identity codes, as are the corresponding lineages and percentage homology with Sar s 1 (Table 3.4).

| | | | |
|---------------------------|----|--|-----|
| SAR S 1 | 1 | MALAGLVKREADITAALBACKAADSFDHKAFPHKVMGMSCKSADELKKAFAIIDQDKSCFIE | 60 |
| CAC83659 | 1 | ..F.VLND.....N.T.A.LTS...DV..... | 60 |
| NP_997948 | 1 | ..F.VLND...S.....N.S.A.LAS...V..... | 60 |
| Q91482 | 1 | ..C.H.C...KT.....T.SF.T...TI.FAS...DV...KV...A..... | 60 |
| P59747 | 1 | ..F.SVL.D.EV...DG...G...K.KAC.L...T.V..... | 60 |
| AAT44428 | 1 | ..F.VL.D.EVK...DG.S...NY.S.KAC.L...S.V.....N..... | 60 |
| P05939 | 4 | ..L.....N...A...A...GDV...E...F..... | 57 |
| BAE46762 | 1 | ..FK.VLND..V...DG..S...KAC.LAA...DI..... | 58 |
| AAT90497 | 1 | ..F.VLND...K.DE.G...Y.K.KTC.LA...DV..... | 60 |
| Q90YK7 | 1 | ..F.IL.D.EVA.....S.G...TK.KSC.LA...S.DV...G...Q.D... | 60 |
| ABD24011 | 1 | ..F.FLSGT.K.AG.S...S.T.KAC.LAS.....N.AY... | 60 |
| AAT45383 | 1 | ..FSNVLSDS.VA...DG.D.GT...K.SAC.L.N.TS.DV..... | 60 |
| AAZ52553 | 1 | ..F.VLSD.VK...DG.A...Y.R.KAS.LAA.T.DV...K...N..... | 60 |
| IB8R-A | 1 | ..F.VLND...A.....N...A.LTS...DV..... | 59 |
| SAR S 1 | 61 | EEELKLFQNFCKKAPALTDGETKKFLKAGDMVGDGKIGIDEPNHLVKH | 109 |
| CAC83659 | 61 | .D.....KAG.....T.....SD.....V...TA... 108 | |
| NP_997948 | 61 |KAD.....T.....SD.....AA... 108 | |
| Q91482 | 61 | V.....P...E...A...A...AD...M...AV...Q 109 | |
| P59747 | 61 |KAG...S.A.A...SD.....AAMI... 108 | |
| AAT44428 | 61 | .D.....VAS...K.A.A.A...SD...V...AA... 108 | |
| P05939 | 58 |KAG...A.I...AD...AA... 105 | |
| BAE46762 | 59 | .D.....AG...S.A.A...SD...V...AAM... 107 | |
| AAT90497 | 61 | .D.....SGG...A.A...SD...A...AVM... 108 | |
| Q90YK7 | 61 |SAS...S.A.A...SD...V...AAM... 108 | |
| ABD24011 | 61 |AAG...K.T.A...SD...V...TA... 108 | |
| AAT45383 | 61 |KAD.V.V.ST...TD...A...TA... 108 | |
| AAZ52553 | 61 |SAG...K.A.S...SD...AA... 108 | |
| IB8R-A | 60 | .D.....KAD.....T.....SD.....V...WTA... 107 | |

Figure 3.2.9 Comparison of the deduced amino acid sequence of pilchard parvalbumin with twelve fish species that displayed high homology. Identical amino acids to Sar s 1, represented in full at the top, are denoted by dots while gaps are indicated by dashes. The two calcium-binding motifs are outlined and the length of sequences are indicated on the far right. Each sequence is preceded by its BLAST sequence identity code.

Table 3.4 The common and scientific names of the fish species are represented alongside their BLAST sequence identity codes in table format. Also illustrated are the lineages of the parvalbumins (i.e. α or β) together with the percentage of homology to Sar s 1 on the far right.

| Blast sequence ID code | Fish species | Isoform type | % homology with Sar s 1 |
|------------------------|---|--------------|-------------------------|
| CAC83659 | Common carp (<i>Cyprinus carpio</i>) | β | 78% |
| NP 997948 | Zebra fish (<i>Danio rerio</i>) | β | 78% |
| 18R-A | Common carp (<i>Cyprinus carpio</i>) | α | 77% |
| PO5939 | European chub (<i>Leuciscus cephalus</i>) | β | 75% |
| P59747 | Chub mackerel (<i>Scomber japonicus</i>) | β | 73% |
| Q91482 | Atlantic salmon (<i>Salmo salar</i>) | β | 72% |
| AAT90497 | Mangrove rivulus (<i>Rivulus marmoratus</i>) | β | 72% |
| ABD24011 | Dark-banded rock fish (<i>Sebastes inermis</i>) | β | 70% |
| BAE46762 | Jack mackerel (<i>Trachurus japonicus</i>) | β | 70% |
| AAT44428 | Gilthead seabream (<i>Sparus aurata</i>) | β | 70% |
| Q90YK7 | Alaska Pollack (<i>Theragra chalcogramma</i>) | β | 68% |
| AAZ52553 | Mozambique tilapia (<i>Oreochromis mossambicus</i>) | β | 69% |
| AAT45383 | Barramundi perch (<i>Lates calcarifer</i>) | β | 65% |

4. Discussion

Parvalbumin has been recognized as a major food allergen and has been shown to account for over 95% of immune hypersensitivities in people who consume fish ^{73 74-77}. It has been demonstrated that parvalbumin from various fish species display IgE cross reactivity and this accounts for the common finding that fish allergic patients can exhibit allergic symptoms after the ingestion of different species ^{73, 76, 81, 101-103}.

Hypersensitivities to fish have also been reported in an occupational setting in fish-processing factory workers exposed predominantly via inhalation and skin contact with fish products. Whether parvalbumin is an occupational allergen in these allergic fish-processing factory workers remains unknown. Furthermore, the proteins that cause disease in these workers have been poorly investigated^{71, 129, 131, 136}.

The purpose of this study was to identify allergens in the fillet of fish that could cause either or both occupational allergic disease and ingestion-related allergic reactions. We also aimed to identify a widely cross-reactive parvalbumin from a fish species that is consumed in large amounts by the South African population and consequently carry out molecular sequencing of this protein as a primary step towards generating a recombinant protein for diagnosis and treatment.

IgE binding was assessed using sera from fish-allergic processing factory workers as well as sera from individuals with allergies caused by the ingestion of fish in immunoblotting. The degree to which serum IgE cross-reacted to parvalbumin was evaluated using crude fish extract and purified parvalbumin from pilchard, yellowtail, hake, anchovy and snoek. Consequently, parvalbumin from the fish species that displayed the most extensive cross-reactivity was then further characterised. In addition, parvalbumin from these fish species were compared using a monoclonal anti-

parvalbumin antibody and variations in molecular weight and the tendencies to form isomers and/or oligomers were evaluated.

4.1 Characterization of parvalbumin from five South African fish species

4.1.1 Detection of parvalbumin using a commercial monoclonal anti-parvalbumin antibody

Using a parvalbumin-specific antibody in immunoblotting, oligomers of parvalbumin were detected in the semi-purified and purified extracts of all five fish species but not in all crude fish extracts. These disparities are most likely due to the relative increase in the concentration of parvalbumin in the more purified extracts. Oligomeric forms have previously been observed in common snook parvalbumin and the related avian thymic parvalbumin under non-denaturing conditions. However, after reductive treatment these oligomers were shown to completely dissociate.^{140, 141} Therefore, the fact that denaturing conditions did not result in the dissociation into monomeric forms in this case was surprising. This phenomenon has previously been reported exclusively in Atlantic cod parvalbumin, Gad m1^{140, 141}. In this case, it was hypothesized that hydrophobic interactions may occur between these proteins depending on parvalbumin's calcium-bound or calcium-free state and could potentially facilitate these oligomeric forms^{82, 142}. The formation of disulphide bridges can occur, however the reductive conditions of SDS-PAGE in this instance should exclude this possibility.

Isoforms of parvalbumin were distinguished using the same monoclonal antibody in immunoblot analysis. Three isoforms of pilchard and two isoforms of yellowtail, hake and anchovy were detected in the semi-concentrated and concentrated extracts. However, no isoforms of snoek were detected in all three of these prepared extracts. It is interesting to note that serum IgE from a patient with fish allergy strongly recognised two allergenic isoforms of snoek parvalbumin. This indicates that the

commercial monoclonal anti-parvalbumin used is not effective in detecting all isoforms that may be present in fish samples. In a previous study, Chen *et al.* failed to detect the two previously documented isoforms of cod parvalbumin using this same antibody, which only displayed binding to a singular isoallergen ⁷⁹.

Conversely, the commercial monoclonal antibody detected isoforms of parvalbumin, specifically in hake and yellowtail that were not bound by serum IgE from any fish-allergic patients. These differences in antibody binding suggest significant sequence discrepancies and consequently epitope variations between isoforms. Such distinctions would ultimately influence IgE binding which implies that certain isoforms may be more allergenic than others ^{79, 109}.

4.2 Parvalbumin was purified from all five crude fish extracts

Parvalbumin from all five fish were purified by cooking, salt precipitation and ion exchange chromatography to confirm specific IgE binding to parvalbumin and to ensure that identical amounts of protein were used in serological immunoblot analyses.

SDS-PAGE and immunoblot analysis of extracts subjected to cooking and salt precipitation revealed that these processes effectively eliminated the large majority of contaminating proteins. This was confirmed by the fact that ion exchange elution profiles of all extracts displayed peaks that contained parvalbumin exclusively. Singular peaks were noted in all fish species except hake, which displayed a dual peak. Both analytes from the hake elution profile contained parvalbumin, which possibly indicates that isoforms of significant varying net charges were eluted at different ionic strengths ^{143, 144}. Thus, an isoform with a lower pI (i.e. more positively charged) would have been eluted in the first peak. Similarly, a more negatively

charged protein would have been eluted in the second peak as increased competition of buffer ions in the form of a rising sodium chloride gradient was required to reduce interactions with the positively charged DEAE-cellulose resin¹⁴⁴. Other fish species exhibited parvalbumin isoforms, but it is possible the difference in overall charges between these proteins were not as pronounced and were eluted as a single peak¹⁴³. To confirm these premises, isoelectric focusing of hake parvalbumin could be performed to identify differences in overall net charge of the isoforms⁷⁹. SDS-PAGE and immunoblot analysis has already confirmed that both eluted hake parvalbumin isoforms were of a similar molecular weight.

With regard to the slight variations noted in the molecular weight of parvalbumin between all five fish species, this is a commonly noted phenomenon. Several previous studies have documented parvalbumins from different fish species in the range of 11-12kDa, which correlates with the results obtained in this study^{68, 72, 73, 79, 105, 109}.

4.3.1 Detection of allergens using sera from fish-processing workers

In this study, sera from fish-processing factory workers displayed negligible IgE binding to proteins present in pilchard and anchovy fillet. In contrast, serum IgE from individuals suffering from allergies caused by fish ingestion recognized parvalbumin as well as other allergens in the fillet of pilchard, anchovy and the remaining three fish species. Possible contributing factors for the overall lack of IgE reactivity in the population of workers are discussed below.

Lack of antibody detection in immunoblotting, in this case IgE binding, could be an indication of experimental error¹⁴⁵. However, the presence of parvalbumin was confirmed in all worker-specific blots using a commercial monoclonal anti-

parvalbumin antibody, indicating adequate transferral of proteins from gel to membrane. Similarly, specific IgG binding was clearly detected in all worker blots and both IgG and IgE binding occurred in the group of people sensitised via ingestion, again validating adequate transferral of proteins and successful antibody binding. In addition immunoblot analyses were repeated four times with workers' serum to confirm these results.

Measurement of specific IgE levels using the UniCAP RAST system revealed that workers had lower fish-specific IgE levels than patients with ingestion-induced allergies. Therefore, it could be argued that insufficient IgE levels in workers' sera were available for the detection of allergens using immunoblotting^{145, 146}. However, two individuals with allergies induced by ingestion recognised pilchard parvalbumin as an allergen despite the fact they had lower specific IgE levels than fish-processing factory workers. Moreover, serum from one patient with ingestion-related allergy contained lower levels of pilchard-specific IgE but displayed strong reactivity to three isoforms of pilchard parvalbumin. This observation implies that the specific IgE levels of workers were sufficient for binding and subsequent detection of allergens. However, in order to confirm this potential lack of antibodies to fish allergens a more sensitive detection system should be tested. For example immunoblot analysis using secondary antibodies labelled with radioactive ¹²⁵I for detection and subsequent quantification by gamma counting could be carried out^{48, 50}.

In this study only proteins in the fillet of fish were assessed for antibody binding by immunoblotting. Therefore, it is plausible that proteins present in other components of these fish species are responsible for eliciting allergies in these workers. Serum IgE from workers displayed binding to pilchard and anchovy specific ImmunoCAP diagnostic extracts which are listed to contain 'whole fish' and not the fillet exclusively¹⁴⁷. Thus, components present in these diagnostic extracts such as proteins in the

skin or gut of the fish could be responsible for fish specific IgE observed in these workers. Potential sensitising agents include the protein collagen, which has recently been recognised as an allergen present in the skin of fish that is otherwise absent in the fillet^{112, 115-117}. Furthermore, the gut of the fish contains a multitude of enzymes with the purpose of degrading dietary proteins^{71, 137, 138}. As previously reported, airborne enzymes act as potent allergens in occupational environments in many cases³⁵. Perhaps the most well documented occupational enzyme is the inhalant allergen α -amylase, responsible for bakers' asthma¹⁴⁸⁻¹⁵⁰. Aerosolised allergenic enzymes have also been identified in the following industries: pharmacy, dairy, catering, bakery, feed, paper, detergent and textiles¹⁵¹. As mentioned previously, a primary means of sensitisation in fish-processing workers is the inhalation of aerosolised proteins generated during the gutting and the bagging of fish-meal.^{71, 129-131, 129, 132} Therefore, enzymatic proteins from the digestive tract of fish are possible candidates for sensitising components. In addition, certain enzymes disrupt the epithelial junctions of the skin or lung, favouring a T_H2 environment that can lead to allergic conditions^{34, 37}. These reactions correlate with the predominant allergic symptoms noted in these workers in an occupational setting, namely dermatitis and urticaria as well as asthma and rhinoconjunctivitis. It is therefore suggested that future research aim to characterise potential allergens present in the skin and digestive gut of pilchard, anchovy and other commercially important fish species.

To date, the proteins that cause sensitisation in fish processing workers have yet to be thoroughly investigated, particularly allergens present in aerosolised extracts⁷¹. Subsequent identification and characterisation of these allergens could lead to more tailored and effective methods of limiting exposure of workers. For example, the implementation of local exhaust ventilation where specific allergen exposure is most abundant could decrease exposure³⁵. Knowledge of occupational allergens and the properties that favour sensitisation via inhalation and skin contact could be gained by

carrying out detailed structural analyses of identified allergens via molecular characterisation¹⁵²⁻¹⁵⁴.

4.3.2 Parvalbumin was not recognised as an allergen in fish-processing workers

It is feasible that other components of pilchard and anchovy are responsible for the allergic symptoms observed in this group of fish-processing factory workers. However, the question remains as to why parvalbumin was not recognised as an allergen in this group, as was the case in individuals sensitised via fish ingestion.

The specific route of entry of an allergen into the body can determine whether exposure results in the clinical manifestations of allergy. In addition, allergenicity is also largely dependant on the intrinsic features of the allergen such as structure, resistance to degradation and proteolytic activity²⁹. For example, α -amylase is used routinely in the production of bread and has been characterised principally as an inhalant allergen in bakers¹⁴⁸⁻¹⁵⁰. However, ingestion of this protein in the form of bread does not cause an allergic reaction in the majority of cases. This is largely due to a conformational change during the heating process^{149, 150, 155}. Some aeroallergens possess proteolytic activity and can interact with components in the airways, such as protease-activated receptors resulting in an infiltration of leukocytes and the production of pro-inflammatory cytokines, chemokines and other components. These attributes contribute to eliciting the symptoms of allergic disease¹⁵⁶. Thus, it could be that intrinsic features, which can facilitate sensitisation via inhalation or contact in certain allergens, may be absent in parvalbumin.

The conformation of an allergen is an important determinant of allergenicity, and subsequent alterations of this structure can result in the loss of IgE binding

epitopes^{29, 32, 155}. Therefore, it is also important to note that the native structure of parvalbumin is likely to have been altered by the denaturing and reducing conditions of SDS gel electrophoresis¹⁴⁵. This is significant as workers would predominantly be exposed to raw fish where potential allergens would occur in their natural structure in initial processing⁷¹. Destruction of IgE epitopes of parvalbumin during SDS gel electrophoresis could have resulted in lack of recognition and loss of IgE binding in workers. This would suggest that in an occupational setting, IgE from the sera of workers are directed against conformational epitopes of parvalbumin, and not sequential epitopes³².

With regard to individuals with ingestion-induced allergies, it is possible that the structure of parvalbumin to which their immune systems were exposed may have been altered by the harsh denaturing conditions of the digestive tract⁴⁰. A variety of proteases and peptidases perform the task of breaking dietary proteins into smaller peptides that can then be absorbed⁶⁴. Perhaps parvalbumin, subjected to this route of exposure, would more closely mimic the denatured test extract used in immunoblotting, and therefore have similar IgE binding epitopes. In support of this, previous studies that have investigated antibody epitopes of parvalbumin using sera from individuals who experienced allergic reactions after fish ingestion have shown that many IgE reactive domains are linear or sequential in nature^{67, 72, 81}. In addition, it has recently been documented that parvalbumin, together with other common food allergens, can be broken down within a number of seconds by the digestive enzyme pepsin under acidic conditions⁶⁴. This is very interesting as parvalbumin like many food allergens has been reported in the literature to be a highly resistant protein^{63, 72, 73}.

Future research should attempt to carry out identical immunoassays under non-denaturing conditions to assess if sera IgE from workers recognize the native form of parvalbumin. It would also be important to compare any differences in IgE binding to

the native form of parvalbumin as well as to the denatured structure using sera from individuals with ingestion-induced allergies to confirm this hypothesis.

4.4.1 Cross-reactivity between parvalbumin from five fish species

The majority of individuals with allergic reactions caused by fish consumption recognised parvalbumin as a dominant allergen. Based on this finding, this study aimed to assess the extent of cross-reactivity between parvalbumin from five fish species that are commercially relevant and highly consumed by the South African population^{157, 158}.

All patients from this population, with the exception of one, recognised parvalbumin as an allergen from at least one fish species. Parvalbumin from pilchard displayed the widest IgE binding ability, with 80% (8 out of 10 individuals) of subjects displaying reactivity while 70% (7 out of 10) of patients' IgE antibodies bound yellowtail parvalbumin. The least allergenic fish in this group were hake, anchovy and snoek, with only 40% of all individuals recognising parvalbumin of these species as an allergen. The observed cross-reactivity suggests that parvalbumin from some of these fish species share identical IgE-binding epitopes due to similar structural features^{32, 39, 48, 104}. It has been documented that fish species which are more closely related on a phylogenetic basis are more likely to have comparable IgE-binding profiles due to higher degrees of homology in the primary structure of proteins^{48, 103}. Surprisingly, contrasting results were found in our study, where two sets of related fish species displayed diverse IgE reactivity patterns. Specifically, pilchard and anchovy are grouped under the same order, *Clupeiformes*, while snoek and yellowtail both fall under the order, *Perciformes* (See Appendix B)¹⁵⁹. In this population, pilchard parvalbumin was twice as allergenic as parvalbumin from anchovy. Similarly,

30% more individuals recognised yellowtail parvalbumin as an allergen compared with the phylogenetically related snoek parvalbumin.

Interestingly, patients with the highest fish-specific IgE levels displayed the widest cross-reactivity amongst parvalbumins from all fish species. Specifically, parvalbumin was recognised as an allergen in at least four fish species in these patients. It is possible that the IgE antibodies produced by some of these patients are specific to a particularly conserved region of parvalbumin, thus a greater degree of cross-reactivity exists¹⁶⁰. The higher levels of fish-specific IgE could also indicate greater exposure to parvalbumin resulting in increased polyclonality, which would result in more varied antibody binding. This could ultimately result in an increased probability of cross-reactivity⁴⁸.

In contrast, monosensitivity to yellowtail parvalbumin was displayed in one patient while another individual exclusively displayed IgE reactivity to pilchard parvalbumin. In these cases, IgE antibodies could be specific to an epitope of parvalbumin that is present only in these particular fish species¹⁰⁵. This information is of clinical relevance as these patients could, instead of wholly eliminating fish from their diet as is commonly advised by practitioners, simply avoid particular species of fish¹⁰⁸. However, these facts would have to be confirmed by a double-blind placebo-controlled food challenge (DBPCFC).

In previous studies the extent of IgE reactivity to a certain fish species has been attributed to its degree of consumption. For example, Pascual *et al.* demonstrated that out of the six fish species that they investigated, the two most allergenic were among the most frequently consumed by the Spanish population^{161, 162}. This was not a trend noted in this study. Hake is amongst the most consumed fish in South Africa, together with pilchard and anchovy^{157, 158}. Although pilchard parvalbumin displayed

the widest IgE reactivity, hake and anchovy were among the least allergenic in this population. In addition, sera from three Austrian patients were attained and included in this group. Interestingly, some of these individuals were allergic to parvalbumin from fish species that are indigenous to the Southern hemisphere, such as snoek and yellowtail^{163, 164}. Thus, common consumption of such fish species by these three patients would be improbable. In addition, all three individuals recognised pilchard parvalbumin as an allergen. Although pilchard is consumed in Austria, it is unlikely that the species frequently consumed in South Africa, *Sardinops sagax*, would be readily available^{165, 166}. These findings imply that although certain individuals were not initially exposed and sensitised to parvalbumin from these fish species, IgE reactivity occurred because of the presence of shared IgE epitopes in parvalbumin from other fish species¹⁰⁴.

4.4.2 Applications of cross-reactivity and the relevance of findings in this study

The identification of a highly cross-reactive form of parvalbumin has potential use as a diagnostic tool^{73, 103, 167}. The application of a single cross-reactive allergen could replace several parvalbumin types in a diagnostic test panel, which would be more cost-effective^{46, 47}. In addition, the benefits of using an immunodominant form to attain desensitisation to multiple fish species in immunotherapy is clear^{13, 45, 73}. Furthermore, a greater understanding of the phenomenon of cross-reactivity could facilitate ways of controlling initial sensitisation to certain allergens in prone individuals. Lastly, the elucidation of cross-reactive epitopes could also prevent their inadvertent integration into novel foods such as genetically modified products^{48, 103}.

A number of studies have carried out the molecular sequencing of parvalbumin from various fish species, and in several cases, have generated recombinant forms with the aim of producing a diagnostic and/or therapeutic tool. However, prior

investigation of the degree of cross-reactivity of these allergens is often not performed^{67, 68, 72, 73, 82, 109, 142}. As illustrated in this study, cross-recognition can vary to large degree. Thus, in terms of generating a recombinant parvalbumin with clinical relevance, confirmation of a high degree of cross-reactivity prior to extensive molecular characterisation, cloning and expression is valuable.

In this study, pilchard parvalbumin was the most cross-reactive allergen in a specific population. It should be noted that a larger group of fish-allergic patients' sera would be useful to strengthen these novel findings¹⁶⁰. However, based on these results as well as the commercial relevance and global distribution of the pilchard species *Sardinops sagax*, molecular sequencing of this allergen was performed^{158, 165}. The majority of parvalbumin types that have been previously characterised are from fish that are consumed in large quantities in Europe^{68, 73, 109, 118, 142}. The fish species investigated in this body of research are highly consumed in South Africa and thus may be of greater relevance to the South African population and perhaps to other areas of the Southern Hemisphere.

4.5.1 IgE binding to dimeric forms of parvalbumin using sera from fish allergic consumers

Sera from four patients with allergic reactions caused by fish consumption displayed IgE binding to dimeric forms of yellowtail, hake and anchovy parvalbumin. This was confirmed by the similar binding position of monoclonal anti-parvalbumin, which served as a positive control. However, IgE reactivity to dimers occurred exclusively in crude fish extracts, and not in the purified samples. In addition, specific binding of monoclonal anti-parvalbumin antibodies to nitrocellulose-blotted purified parvalbumin was also absent. This could indicate that dimeric parvalbumin dissociated into its

monomeric form as a result of the harsh purification procedure, which has been observed in previous studies^{140, 168}.

The allergenicity of oligomeric parvalbumin has previously been documented in cod but the effect that such associations have on IgE reactivity is unknown⁸². This is of interest as some dimeric and oligomeric forms of proteins have been shown to be more allergenic than their monomeric forms such as the major birch pollen allergen Bet v 1¹⁶⁹.

4.5.2 IgE binding to additional fish allergens

Several minor allergens were identified in yellowtail and pilchard crude extracts using sera from allergic fish consumers. Specifically, IgE binding proteins of 110kDa and 160kDa in yellowtail and a 45kDa allergen in pilchard were recognised. Assessment of the literature reveals that proteins of similar molecular weights have not been identified. This fact, together with the observation that these allergens were absent in the other fish extracts investigated, indicates that they may be specific to these fish species. Other minor, species-specific allergens have also been identified in extracts of swordfish, cod and tropical sole^{106, 107, 114}.

Allergens of 37kDa and 40kDa were also recognised in yellowtail crude extract, which closely correlates with the unglycosylated and glycosylated isoforms of the documented allergen, aldehyde phosphate dehydrogenase (APDH) from cod¹¹⁸. Interestingly, a 37kDa anchovy allergen was also recognized weakly by serum IgE from one fish processing factory worker. APDH has been characterised in cod and displays homology in diverse animal species. Therefore, it is probable that it may be conserved in these fish species. In addition, this protein is thought to be released after cell death and thus increases in non-frozen fish that have been maintained in storage

which would correlate with conditions of extracts of crude fish used in this study¹¹⁸. Molecular sequencing of these allergens would verify whether they were, in fact, isoforms of ADPH. If positive identification was confirmed, further characterisation could be worthwhile due to the likely cross-reactivity of this allergen amongst fish species¹¹⁸.

4.6.1 Further characterisation of isoforms from pilchard parvalbumin

Monomeric and dimeric isoforms of pilchard parvalbumin with isoelectric values in the range of 4 - 5 and 5.5-5.9 were detected using a monoclonal anti-parvalbumin following two-dimensional gel electrophoresis. Both evolutionary lineages that have previously been characterised were visualised, namely the α group ($pI > 5$) and the β group ($pI < 5$). However, the isoelectric points observed in this study were higher than average for both α and β lineages indicating the presence of more basic amino acid in these proteins. Typically, values for the β -parvalbumins range between 4-4.5 while α -parvalbumins are generally between 5-5.5^{67, 73, 79, 85}. This is the first study in which isoforms of pilchard parvalbumin have been investigated thus comparative results are not available.

Two predominant isoforms were observed after isoelectric separation, however isoforms below a pH value of 5 appeared to be insufficiently resolved as indicated by a smear rather than distinct spots. The fact that three isoforms were previously detected in one-dimensional separation using a monoclonal anti-parvalbumin and patient sera also suggest that more than two isoforms of pilchard parvalbumin may be present. Technical improvements to potentially improve resolution of isoelectric migration include using longer Immobiline DryStrips with a narrower pH range. For example, a 24cm strip with a pH interval between 4-7 would be optimal for separation in the first dimension in this instance¹⁷⁰.

It has been demonstrated by Lowenstein *et al.* that isoallergens can show marked differences in antibody reactivity and thus display differences in allergenicity¹⁷¹. In this study, 70% of individuals with allergies caused by fish ingestion were allergic to two isoforms of pilchard, while IgE from one patient displayed binding to three isoforms. The identification of the most allergenic isoform of pilchard parvalbumin may prove the most effective as potential use as a diagnostic tool. Similarly, recognition of the least allergenic isoform or hypoallergenic form may prove useful as a safer option to attain desensitisation in immunotherapeutic studies^{63, 73, 103, 167}. It would therefore be of interest to further investigate these isoforms using patient sera in isoelectric focusing and solve the cDNA sequences of all pilchard parvalbumin isoallergens.

4.6.2 cDNA encoding parvalbumin from pilchard was determined

In this study, the pilchard parvalbumin gene was sequenced to attain information from the primary amino acid sequence such as the lineage (i.e. α or β isoform) and to compare homology with previously characterised parvalbumins. Moreover, the cDNA sequence is the primary step towards the generation of recombinant form of pilchard parvalbumin⁶³. The merit of producing such a recombinant for potential diagnosis and treatment of fish allergies has been verified by the fact that pilchard parvalbumin displayed a high degree of cross-reactivity^{103, 158, 165}.

A cDNA sequence comprising a 327 bp open reading frame, encoding 109 amino acids was verified as pilchard parvalbumin. This protein was recognised as the dominant allergen in individuals with ingestion-induced fish allergies and was therefore designated Sar s 1 according to the International Nomenclature of Allergens¹³⁹.

Although more than one isoform of pilchard parvalbumin was previously identified in this study, only a single distinct parvalbumin cDNA sequence was detected. This isoform was identified as a β -type by the fact that it was 109 residues long and by the positions of the following 6 amino acids: Ala-14, Leu-16, Cys-19, Phe-67, Gln-69, and Thr-79^{72, 172, 173}. In addition the derived amino acid sequence displayed the highest identities to several previously sequenced β isoforms of parvalbumin with the exception of one α -type from common carp.

The amino acid sequence, Sar s 1, displayed the highest homology to the β form of parvalbumin from common carp as well as to zebrafish of the same lineage. Both carp and zebrafish belong to the same phylogenetic family, *Cyprinidae*, while pilchard falls under the order *Clupeiformes*^{159, 174}. No sequence information of parvalbumin from the phylogeny of pilchard is available in the database thus comparison is not viable. Similarities to the parvalbumin α -isoform from common carp was also noted as well as high identities (70-75%) with parvalbumin from European chub, chub mackerel, Atlantic salmon, mangrove rivulus, gilthead seabream, dark-banded rock fish and Mozambique tilapia.

The calcium-binding motifs of parvalbumin have been identified as areas with a high degree of amino acid homology and have been proposed as sequential IgE binding epitopes^{81, 98-100}. Thus it was surprising to note that the most conserved stretch of amino acids noted between all 12 aligned sequences was not within these regions but just outside the second EF-hand motif. Specifically, a 100% identity was noted in the segment 64-72. Previous studies of IgE-binding epitopes in the cod parvalbumin, Gad c 1, revealed that five different stretches displayed IgE reactivity, namely the segments 13-32, 33-44, 41-64, 65-74 and 88-96⁸¹. Interestingly, the segment, 65-74 coincides with the stretch of homologous amino acids noted in Sar s 1. A high degree of similarity was also noted in both EF-hand motifs as well as in the segment 44-53,

- i.) Are other proteins that are absent in the fillet but present in other bodily components of the fish responsible for the clinical manifestations noted in these workers?
- ii.) Does parvalbumin lack the intrinsic allergenic features potentially needed to elicit an allergic reaction via inhalation and/or contact, which are primary routes of sensitisation in workers in an occupational setting?
- iii.) Was a lack of IgE binding to parvalbumin due to the destruction of its native form in test extracts resulting in the loss of allergenic epitopes that might have usually elicited allergic reactions in the occupational environment?

Future work will address these questions by assessing proteins present primarily in the skin and digestive gut of pilchard and anchovy for their IgE reactivity using the sera from these fish-processing workers. The characterisation of these occupational allergens, which have in the past been poorly studied, will hopefully shed light on the extent to which other factors such as the route of exposure and the concentration of a specific allergen influence occupational allergic disease^{71, 129, 136}. Moreover, identification of these allergens may help in the effective prevention and treatment of fish-processing factory workers in Southern Africa and potentially worldwide.

5. Appendices

Appendix A: Solutions

General reagents

10X Phosphate Buffered Saline (PBS)

80g NaCl

2.4g KH_2PO_4

2.0g KCL

14.4g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$

pH 7.4

Dissolve in 1L ddH₂O

1M TRIS-HCl, pH 7.5

60.55g TRIS-HCl

Dissolve in 450ml ddH₂O.

Adjust pH to 7.5 and bring to a final volume of 500ml.

SDS-PAGE Reagents

2.5X separating gel buffer

1.875M Tris Cl

0.25% SDS

Adjust to pH 8.9

5X stacking gel buffer

0.3M Tris phosphate

0.5% SDS

Adjust to pH 6.7

5X running buffer

0.5M Tris base

1.92M glycine

0.5% SDS

Coomassie stain

500ml methanol

0.5g coomassie powder

100ml acetic acid

Make up to 1L with ddH₂O

Coomassie destain solution

75ml acetic acid

50ml methanol

Make up to 1L with ddH₂O

2X sample buffer

0.25M Tris (pH6.8)

4% SDS

20% glycerol

10% β-mercaptoethanol

0.001% bromophenol blue

10% Ammonium persulphate (AMPS)

10μg AMPS

Diluted in 100μl ddH₂O

Immunoblot reagents

Transfer Buffer

41.4mM Tris

320mM Glycine

400ml Methanol

Make up to 2L with ddH₂O

Blocking Buffer

2% Milk powder (SPAR instant)

Make up to 1L with 1XPBS

Wash buffer

0.05% Tween-20 in PBS

10X Tris Buffered Saline (TBS)

12g TRIS

88g NaCl

Dissolve in 900ml ddH₂O.

Adjust pH to 7.5 and bring to a final volume of 1L with ddH₂O .

Substrate

1 NBT/BCIP (Nitro blue tetrazolium chloride 5-Bromo-4Chloro-3-indoyl-phosphate, toluidine salt) tablet dissolved in 10ml ddH₂O

Isoelectric focusing (IEF) analysis

Rehydration buffer

8M Urea

2M Thiourea

4% CHAPS

2% pH 3-10 carrier ampholytes

20mM Tris base

30mM Dithiothreitol (DTT)

Equilibration buffer

20ml Tris-HCl stock

72g Urea

60ml Glycerol

4g SDS

Make up to 200ml with ddH₂O

Dithiothreitol (DTT)

150mg DTT

Add to 15ml of equilibration buffer

RNA extraction

DEPC-treated water

1L ddH₂O

1ml Diethylpyrocarbonate (DEPC)

Mix components together and incubate ON at 37°C with agitation. Autoclave and aliquot into RNase-free vessels.

70% Ethanol

70% Absolute ethanol

Add to 30ml DEPC-treated H₂O

Gel running marker (6x) for DNA agarose gels

50% (v/v) glycerol (diluted in DEPC-treated H₂O)

0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol FF

10mM EDTA (pH 8.0)

Chapter 6: References

1. Spellberg, B. & Edwards, J.E., Jr. Type 1/Type 2 immunity in infectious diseases. *Clin Infect Dis* **32**, 76-102 (2001).
2. Ivan Roitt, J.B., David Male. Immunology (ed. Loiuise Crowe, R.F.) (Mosby, Spain, 2001).
3. Wedemeyer, J., Tsai, M. & Galli, S.J. Roles of mast cells and basophils in innate and acquired immunity. *Curr Opin Immunol* **12**, 624-31 (2000).
4. Medzhitov, R. & Janeway, C. Innate immune recognition: mechanisms and pathways. *Immunological Reviews* **173**, 89-97 (2000).
5. Wittamer, V. et al. Neutrophil-mediated maturation of chemerin: a link between innate and adaptive immunity. *J Immunol* **175**, 487-93 (2005).
6. Steinman, R.M. & Hemmi, H. Dendritic cells: translating innate to adaptive immunity. *Curr Top Microbiol Immunol* **311**, 17-58 (2006).
7. Guy, B. The perfect mix: recent progress in adjuvant research. *Nat Rev Microbiol* **5**, 505-17 (2007).
8. Banchereau, J. & Steinman, R.M. Dendritic cells and the control of immunity. *Nature* **392**, 245-52 (1998).
9. Steinman, L. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. *Nat Med* **13**, 139-45 (2007).
10. Luster, A.D. The role of chemokines in linking innate and adaptive immunity. *Curr Opin Immunol* **14**, 129-35 (2002).
11. Medzhitov, R., Preston-Hurlburt, P. & Janeway, C.A., Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388**, 394-7 (1997).
12. McNeela, E.A. & Mills, K.H. Manipulating the immune system: humoral versus cell-mediated immunity. *Adv Drug Deliv Rev* **51**, 43-54 (2001).
13. Larche, M., Akdis, C.A. & Valenta, R. Immunological mechanisms of allergen-specific immunotherapy. *Nat Rev Immunol* **6**, 761-71 (2006).
14. Manz, R.A. et al. Humoral immunity and long-lived plasma cells. *Curr Opin Immunol* **14**, 517-21 (2002).
15. Meyer. (University of South Carolina, 2005).
16. Thomas, M.S., Kunkel, S.L. & Lukacs, N.W. Regulation of cockroach antigen-induced allergic airway hyperreactivity by the CXCR3 ligand CXCL9. *J Immunol* **173**, 615-23 (2004).
17. Asher, I. et al. World Allergy Organization guidelines for prevention of allergy and allergic asthma. *Int Arch Allergy Immunol* **135**, 83-92 (2004).
18. Sicherer, S.H. & Leung, D.Y. Advances in allergic skin disease, anaphylaxis, and hypersensitivity reactions to foods, drugs, and insects. *J Allergy Clin Immunol* **116**, 153-63 (2005).
19. Novak, N. & Bieber, T. Allergic and nonallergic forms of atopic diseases. *J Allergy Clin Immunol* **112**, 252-62 (2003).
20. Akdis, M. Healthy immune response to allergens: T regulatory cells and more. *Curr Opin Immunol* **18**, 738-44 (2006).
21. Bacharier, L.B., Jabara, H. & Geha, R.S. Molecular mechanisms of immunoglobulin E regulation. *Int Arch Allergy Immunol* **115**, 257-69 (1998).
22. Sellge, G. et al. Development of an in vitro system for the study of allergens and allergen-specific immunoglobulin E and immunoglobulin G: Fcepsilon receptor I supercross-linking is a possible new mechanism of immunoglobulin G-dependent enhancement of type I allergic reactions. *Clin Exp Allergy* **35**, 774-81 (2005).

23. Larche, M. & Wraith, D.C. Peptide-based therapeutic vaccines for allergic and autoimmune diseases. *Nat Med* **11**, S69-76 (2005).
24. Oettgen, H.C. & Geha, R.S. IgE in asthma and atopy: cellular and molecular connections. *J Clin Invest* **104**, 829-35 (1999).
25. Akdis, C.A. & Blaser, K. Histamine in the immune regulation of allergic inflammation. *J Allergy Clin Immunol* **112**, 15-22 (2003).
26. Akdis, M. et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. *J Exp Med* **199**, 1567-75 (2004).
27. Cookson, W. The alliance of genes and environment in asthma and allergy. *Nature* **402**, B5-11 (1999).
28. Finkelman, F.D. & Vercelli, D. Advances in asthma, allergy mechanisms, and genetics in 2006. *J Allergy Clin Immunol* **120**, 544-50 (2007).
29. Pomes, A. Intrinsic properties of allergens and environmental exposure as determinants of allergenicity. *Allergy* **57**, 673-9 (2002).
30. Rogers, P.R. & Croft, M. Peptide dose, affinity, and time of differentiation can contribute to the Th1/Th2 cytokine balance. *J Immunol* **163**, 1205-13 (1999).
31. Constant, S., Pfeiffer, C., Woodard, A., Pasqualini, T. & Bottomly, K. Extent of T cell receptor ligation can determine the functional differentiation of naive CD4+ T cells. *J Exp Med* **182**, 1591-6 (1995).
32. Aalberse, R.C. Structural biology of allergens. *J Allergy Clin Immunol* **106**, 228-38 (2000).
33. Holgate, S.T. The epidemic of allergy and asthma. *Nature* **402**, B2-4 (1999).
34. Schulz, O., Sewell, H.F. & Shakib, F. Proteolytic cleavage of CD25, the alpha subunit of the human T cell interleukin 2 receptor, by Der p 1, a major mite allergen with cysteine protease activity. *J Exp Med* **187**, 271-5 (1998).
35. Baur, X. Enzymes as occupational and environmental respiratory sensitizers. *Int Arch Occup Environ Health* **78**, 279-86 (2005).
36. Valdivieso, R. et al. Bakers' asthma caused by alpha amylase. *Ann Allergy* **73**, 337-42 (1994).
37. Kondo, H., Ichikawa, Y. & Imokawa, G. Percutaneous sensitization with allergens through barrier-disrupted skin elicits a Th2-dominant cytokine response. *Eur J Immunol* **28**, 769-79 (1998).
38. Dearman, R.J., Stone, S., Caddick, H.T., Basketter, D.A. & Kimber, I. Evaluation of protein allergenic potential in mice: dose-response analyses. *Clin Exp Allergy* **33**, 1586-94 (2003).
39. Aalberse, R.C., Akkerdaas, J. & van Ree, R. Cross-reactivity of IgE antibodies to allergens. *Allergy* **56**, 478-90 (2001).
40. Sampson, H.A. Update on food allergy. *J Allergy Clin Immunol* **113**, 805-19; quiz 820 (2004).
41. Cooke, S.K. & Sampson, H.A. Allergenic properties of ovomucoid in man. *J Immunol* **159**, 2026-32 (1997).
42. Burks, A.W. et al. Mapping and mutational analysis of the IgE-binding epitopes on Ara h 1, a legume vicilin protein and a major allergen in peanut hypersensitivity. *Eur J Biochem* **245**, 334-9 (1997).
43. Jarvinen, K.M., Chatchatee, P., Bardina, L., Beyer, K. & Sampson, H.A. IgE and IgG binding epitopes on alpha-lactalbumin and beta-lactoglobulin in cow's milk allergy. *Int Arch Allergy Immunol* **126**, 111-8 (2001).
44. Beyer, K. et al. Measurement of peptide-specific IgE as an additional tool in identifying patients with clinical reactivity to peanuts. *J Allergy Clin Immunol* **112**, 202-7 (2003).
45. Holt, P.G. & Thomas, W.R. Sensitization to airborne environmental allergens: unresolved issues. *Nat Immunol* **6**, 957-60 (2005).
46. van Ree, R., van Leeuwen, W.A. & Aalberse, R.C. How far can we simplify in vitro diagnostics for grass pollen allergy?: A study with 17 whole pollen

- extracts and purified natural and recombinant major allergens. *J Allergy Clin Immunol* **102**, 184-90 (1998).
47. Van Ree, R., Van Leeuwen, W.A., Akkerdaas, J.H. & Aalberse, R.C. How far can we simplify in vitro diagnostics for Fagales tree pollen allergy? A study with three whole pollen extracts and purified natural and recombinant allergens. *Clin Exp Allergy* **29**, 848-55 (1999).
 48. Plebani, M. Clinical value and measurement of specific IgE. *Clin Biochem* **36**, 453-69 (2003).
 49. van Hage-Hamsten, M. & Pauli, G. Provocation testing with recombinant allergens. *Methods* **32**, 281-91 (2004).
 50. Hamilton, R.G. & Franklin Adkinson, N., Jr. In vitro assays for the diagnosis of IgE-mediated disorders. *J Allergy Clin Immunol* **114**, 213-25; quiz 226 (2004).
 51. Bohle, B. & Vieths, S. Improving diagnostic tests for food allergy with recombinant allergens. *Methods* **32**, 292-9 (2004).
 52. Dolen, W.K. IgE antibody in the serum—detection and diagnostic significance. *Allergy* **58**, 717-23 (2003).
 53. Bindslev-Jensen, C. Standardization of double-blind, placebo-controlled food challenges. *Allergy* **56 Suppl 67**, 75-7 (2001).
 54. Bruijnzeel-Koomen, C. et al. Adverse reactions to food. European Academy of Allergology and Clinical Immunology Subcommittee. *Allergy* **50**, 623-35 (1995).
 55. Walker, S.M., Pajno, G.B., Lima, M.T., Wilson, D.R. & Durham, S.R. Grass pollen immunotherapy for seasonal rhinitis and asthma: a randomized, controlled trial. *J Allergy Clin Immunol* **107**, 87-93 (2001).
 56. Moller, C. et al. Pollen immunotherapy reduces the development of asthma in children with seasonal rhinoconjunctivitis (the PAT-study). *J Allergy Clin Immunol* **109**, 251-6 (2002).
 57. Durham, S.R. et al. Grass pollen immunotherapy decreases the number of mast cells in the skin. *Clin Exp Allergy* **29**, 1490-6 (1999).
 58. Wallner, M. et al. Lab scale and medium scale production of recombinant allergens in *Escherichia coli*. *Methods* **32**, 219-26 (2004).
 59. Burks, A.W., King, N. & Bannon, G.A. Modification of a major peanut allergen leads to loss of IgE binding. *Int Arch Allergy Immunol* **118**, 313-4 (1999).
 60. Smith, A.M. & Chapman, M.D. Reduction in IgE binding to allergen variants generated by site-directed mutagenesis: contribution of disulfide bonds to the antigenic structure of the major house dust mite allergen Der p 2. *Mol Immunol* **33**, 399-405 (1996).
 61. Takai, T. et al. Engineering of the major house dust mite allergen Der f 2 for allergen-specific immunotherapy. *Nat Biotechnol* **15**, 754-8 (1997).
 62. Schramm, G. et al. "Allergen engineering": variants of the timothy grass pollen allergen Phl p 5b with reduced IgE-binding capacity but conserved T cell reactivity. *J Immunol* **162**, 2406-14 (1999).
 63. Swoboda, I. et al. A recombinant hypoallergenic parvalbumin mutant for immunotherapy of IgE-mediated fish allergy. *J Immunol* **178**, 6290-6 (2007).
 64. Untersmayr, E. & Jensen-Jarolim, E. The effect of gastric digestion on food allergy. *Curr Opin Allergy Clin Immunol* **6**, 214-9 (2006).
 65. Bush, R.K. & Hefle, S.L. Food allergens. *Crit Rev Food Sci Nutr* **36 Suppl**, S119-63 (1996).
 66. Mills, E.N. & Breiteneder, H. Food allergy and its relevance to industrial food proteins. *Biotechnol Adv* **23**, 409-14 (2005).
 67. Van Do, T., Hordvik, I., Endresen, C. & Elsayed, S. Expression and analysis of recombinant salmon parvalbumin, the major allergen in Atlantic salmon (*Salmo salar*). *Scand J Immunol* **50**, 619-25 (1999).
 68. Van Do, T., Hordvik, I., Endresen, C. & Elsayed, S. The major allergen (parvalbumin) of codfish is encoded by at least two isotypic genes: cDNA

- cloning, expression and antibody binding of the recombinant allergens. *Mol Immunol* **39**, 595-602 (2003).
69. Aro, T.L., Larmo, P.S., Backman, C.H., Kallio, H.P. & Tahvonen, R.L. Fatty acids and fat-soluble vitamins in salted herring (*Clupea harengus*) products. *J Agric Food Chem* **53**, 1482-8 (2005).
 70. Antonijevic, B. et al. Simulated impact of a fish based shift in the population n-3 fatty acids intake on exposure to dioxins and dioxin-like compounds. *Food Chem Toxicol* **45**, 2279-86 (2007).
 71. Jeebhay, M.F., Robins, T.G., Lehrer, S.B. & Lopata, A.L. Occupational seafood allergy: a review. *Occup Environ Med* **58**, 553-62 (2001).
 72. Hamada, Y. et al. Purification, reactivity with IgE and cDNA cloning of parvalbumin as the major allergen of mackerels. *Food Chem Toxicol* **41**, 1149-56 (2003).
 73. Swoboda, I. et al. Recombinant carp parvalbumin, the major cross-reactive fish allergen: a tool for diagnosis and therapy of fish allergy. *J Immunol* **168**, 4576-84 (2002).
 74. Hilger, C., Grigioni, F., Thill, L., Mertens, L. & Hentges, F. Severe IgE-mediated anaphylaxis following consumption of fried frog legs: definition of alpha-parvalbumin as the allergen in cause. *Allergy* **57**, 1053-8 (2002).
 75. Aas, K. & Elsayed, S.M. Characterization of a major allergen (cod). Effect of enzymic hydrolysis on the allergenic activity. *J Allergy* **44**, 333-43 (1969).
 76. Elsayed, S. & Bennich, H. The primary structure of allergen M from cod. *Scand J Immunol* **4**, 203-8 (1975).
 77. Bernhisel-Broadbent, J., Scanlon, S.M. & Sampson, H.A. Fish hypersensitivity. I. In vitro and oral challenge results in fish-allergic patients. *J Allergy Clin Immunol* **89**, 730-7 (1992).
 78. Lehky, P., Blum, H.E., Stein, E.A. & Fischer, E.H. Isolation and characterization of parvalbumins from the skeletal muscle of higher vertebrates. *J Biol Chem* **249**, 4332-4 (1974).
 79. Chen, L., Hefle, S.L., Taylor, S.L., Swoboda, I. & Goodman, R.E. Detecting fish parvalbumin with commercial mouse monoclonal anti-frog parvalbumin IgG. *J Agric Food Chem* **54**, 5577-82 (2006).
 80. Elsayed, S. & Aas, K. Characterization of a major allergen (cod). Observations on effect of denaturation on the allergenic activity. *J Allergy* **47**, 283-91 (1971).
 81. Elsayed, S. & Apold, J. Immunochemical analysis of cod fish allergen M: locations of the immunoglobulin binding sites as demonstrated by the native and synthetic peptides. *Allergy* **38**, 449-59 (1983).
 82. Das Dores, S., Chopin, C., Villaume, C., Fleurence, J. & Gueant, J.L. A new oligomeric parvalbumin allergen of Atlantic cod (*Gad ml*) encoded by a gene distinct from that of *Gad cl*. *Allergy* **57 Suppl 72**, 79-83 (2002).
 83. Lindstrom, C.D., van Do, T., Hordvik, I., Endresen, C. & Elsayed, S. Cloning of two distinct cDNAs encoding parvalbumin, the major allergen of Atlantic salmon (*Salmo salar*). *Scand J Immunol* **44**, 335-44 (1996).
 84. Fohr, U.G. et al. Human alpha and beta parvalbumins. Structure and tissue-specific expression. *Eur J Biochem* **215**, 719-27 (1993).
 85. Laney, E.L., Shabanowitz, J., King, G., Hunt, D.F. & Nelson, D.J. The isolation of parvalbumin isoforms from the tail muscle of the American alligator (*Alligator mississippiensis*). *J Inorg Biochem* **66**, 67-76 (1997).
 86. Palmisano, W.A. & Henzl, M.T. Molecular cloning of the thymus-specific parvalbumin known as avian thymic hormone: isolation of a full length cDNA and expression of the recombinant protein in *Escherichia coli*. *Arch Biochem Biophys* **285**, 211-20 (1991).

87. Pechere, J.F., Capony, J.P. & Ryden, L. The primary structure of the major parvalbumin from hake muscle. Isolation and general properties of the protein. *Eur J Biochem* **23**, 421-8 (1971).
88. Simonides, W.S. & van Hardeveld, C. Identification and quantification in single muscle fibers of four isoforms of parvalbumin in the iliofibularis muscle of *Xenopus laevis*. *Biochim Biophys Acta* **998**, 137-44 (1989).
89. Berchtold, M.W., Heizmann, C.W. & Wilson, K.J. Primary structure of parvalbumin from rat skeletal muscle. *Eur J Biochem* **127**, 381-9 (1982).
90. Enfield, D.L., Ericsson, L.H., Blum, H.E., Fischer, E.H. & Neurath, H. Amino-acid sequence of parvalbumin from rabbit skeletal muscle. *Proc Natl Acad Sci U S A* **72**, 1309-13 (1975).
91. Kretsinger, R.H. Structure and evolution of calcium-modulated proteins. *CRC Crit Rev Biochem* **8**, 119-74 (1980).
92. Gillis, J.M., Thomason, D., Lefevre, J. & Kretsinger, R.H. Parvalbumins and muscle relaxation: a computer simulation study. *J Muscle Res Cell Motil* **3**, 377-98 (1982).
93. Pauls, T.L., Cox, J.A. & Berchtold, M.W. The Ca²⁺(-)-binding proteins parvalbumin and oncomodulin and their genes: new structural and functional findings. *Biochim Biophys Acta* **1306**, 39-54 (1996).
94. Kretsinger, R.H. & Nockolds, C.E. Carp muscle calcium-binding protein. II. Structure determination and general description. *J Biol Chem* **248**, 3313-26 (1973).
95. Heizmann, C.W. & Braun, K. Changes in Ca²⁺-binding proteins in human neurodegenerative disorders. *Trends Neurosci* **15**, 259-64 (1992).
96. Declercq, J.P., Tinant, B., Parello, J. & Rambaud, J. Ionic interactions with parvalbumins. Crystal structure determination of pike 4.10 parvalbumin in four different ionic environments. *J Mol Biol* **220**, 1017-39 (1991).
97. Kawasaki, H. & Kretsinger, R.H. Calcium-binding proteins. 1: EF-hands. *Protein Profile* **1**, 343-517 (1994).
98. Apold, J. & Elsayed, S. The immunochemical reactivity of regions encompassing Tyr-30 and Arg-75 of allergen M from cod. *Mol Immunol* **17**, 291-6 (1980).
99. Elsayed, S., Ragnarsson, U., Apold, J., Florvaag, E. & Vik, H. Allergenic synthetic peptide corresponding to the second calcium-binding loop of cod allergen M. *Scand J Immunol* **14**, 207-11 (1981).
100. Untersmayr, E. et al. Mimotopes identify conformational epitopes on parvalbumin, the major fish allergen. *Mol Immunol* **43**, 1454-61 (2006).
101. Aas, K. & Jebsen, J.W. Studies of hypersensitivity to fish. Partial purification and crystallization of a major allergenic component of cod. *Int Arch Allergy Appl Immunol* **32**, 1-20 (1967).
102. Helbling, A. et al. Fish allergy: is cross-reactivity among fish species relevant? Double-blind placebo-controlled food challenge studies of fish allergic adults. *Ann Allergy Asthma Immunol* **83**, 517-23 (1999).
103. Van Do, T., Elsayed, S., Florvaag, E., Hordvik, I. & Endresen, C. Allergy to fish parvalbumins: studies on the cross-reactivity of allergens from 9 commonly consumed fish. *J Allergy Clin Immunol* **116**, 1314-20 (2005).
104. Aalberse, R.C., Kleine Budde, I., Stapel, S.O. & van Ree, R. Structural aspects of cross-reactivity and its relation to antibody affinity. *Allergy* **56 Suppl 67**, 27-9 (2001).
105. Bugajska-Schretter, A. et al. Parvalbumin, a cross-reactive fish allergen, contains IgE-binding epitopes sensitive to periodate treatment and Ca²⁺ depletion. *J Allergy Clin Immunol* **101**, 67-74 (1998).
106. Kelso, J.M., Jones, R.T. & Yunginger, J.W. Monospecific allergy to swordfish. *Ann Allergy Asthma Immunol* **77**, 227-8 (1996).

107. Asero, R., Mistrello, G., Roncarolo, D., Casarini, M. & Falagiani, P. True monosensitivity to a tropical sole. *Allergy* **54**, 1228-9 (1999).
108. Sten, E. et al. Cross-reactivity to eel, eelpout and ocean pout in codfish-allergic patients. *Allergy* **59**, 1173-80 (2004).
109. Van Do, T., Hordvik, I., Endresen, C. & Elsayed, S. Characterization of parvalbumin, the major allergen in Alaska pollack, and comparison with codfish Allergen M. *Mol Immunol* **42**, 345-53 (2005).
110. Jeebhay, M.F., Lopata, A.L. & Robins, T.G. Seafood processing in South Africa: a study of working practices, occupational health services and allergic health problems in the industry. *Occup Med (Lond)* **50**, 406-13 (2000).
111. Zinn, C., Lopata, A., Visser, M. & Potter, P.C. The spectrum of allergy to South African bony fish (Teleostei). Evaluation by double-blind, placebo-controlled challenge. *S Afr Med J* **87**, 146-52 (1997).
112. Hamada, Y., Nagashima, Y. & Shiomi, K. Identification of collagen as a new fish allergen. *Biosci Biotechnol Biochem* **65**, 285-91 (2001).
113. Galland, A.V. et al. Purification of a 41 kDa cod-allergenic protein. *J Chromatogr B Biomed Sci Appl* **706**, 63-71 (1998).
114. Dory, D. et al. Recognition of an extensive range of IgE-reactive proteins in cod extract. *Allergy* **53**, 42-50 (1998).
115. Sakaguchi, M. et al. IgE antibody to fish gelatin (type I collagen) in patients with fish allergy. *J Allergy Clin Immunol* **106**, 579-84 (2000).
116. Andre, F., Cavagna, S. & Andre, C. Gelatin prepared from tuna skin: a risk factor for fish allergy or sensitization? *Int Arch Allergy Immunol* **130**, 17-24 (2003).
117. Wild, L.G. & Lehrer, S.B. Fish and shellfish allergy. *Curr Allergy Asthma Rep* **5**, 74-9 (2005).
118. Das Dores, S. et al. IgE-binding and cross-reactivity of a new 41 kDa allergen of codfish. *Allergy* **57 Suppl 72**, 84-7 (2002).
119. Daschner, A. & Pascual, C.Y. Anisakis simplex: sensitization and clinical allergy. *Curr Opin Allergy Clin Immunol* **5**, 281-5 (2005).
120. Audicana, L., Audicana, M.T., Fernandez de Corres, L. & Kennedy, M.W. Cooking and freezing may not protect against allergenic reactions to ingested Anisakis simplex antigens in humans. *Vet Rec* **140**, 235 (1997).
121. Audicana, M.T., Ansoategui, I.J., de Corres, L.F. & Kennedy, M.W. Anisakis simplex: dangerous—dead and alive? *Trends Parasitol* **18**, 20-5 (2002).
122. Durborow, R.M. Health and safety concerns in fisheries and aquaculture. *Occup Med* **14**, 373-406 (1999).
123. Harvima, R.J., Tuomisto, L. & Husman, T. Repeated hand urticaria due to contact with fishfood. *Scand J Work Environ Health* **25**, 151-2 (1999).
124. Anibarro, B., Seoane, F.J. & Mugica, M.V. Involvement of hidden allergens in food allergic reactions. *J Investig Allergol Clin Immunol* **17**, 168-72 (2007).
125. Nieuwenhuizen, N. et al. Exposure to the fish parasite Anisakis causes allergic airway hyperreactivity and dermatitis. *J Allergy Clin Immunol* **117**, 1098-105 (2006).
126. Hogan, D.J. The prognosis of occupational contact dermatitis. *Occup Med* **9**, 53-8 (1994).
127. Belsito, D.V. Occupational contact dermatitis: etiology, prevalence, and resultant impairment/disability. *J Am Acad Dermatol* **53**, 303-13 (2005).
128. Malo, J.L. et al. Patterns of improvement in spirometry, bronchial hyperresponsiveness, and specific IgE antibody levels after cessation of exposure in occupational asthma caused by snow-crab processing. *Am Rev Respir Dis* **138**, 807-12 (1988).
129. Jeebhay, M.F. et al. Environmental exposure characterization of fish processing workers. *Ann Occup Hyg* **49**, 423-37 (2005).

130. Rodriguez, J. et al. Occupational asthma caused by fish inhalation. *Allergy* **52**, 866-9 (1997).
131. Bang, B. et al. Exposure and airway effects of seafood industry workers in northern Norway. *J Occup Environ Med* **47**, 482-92 (2005).
132. Lopata, A.L., Fenemore, B., Jeebhay, M.F., Gade, G. & Potter, P.C. Occupational allergy in laboratory workers caused by the African migratory grasshopper *Locusta migratoria*. *Allergy* **60**, 200-5 (2005).
133. Gaddie, J., Legge, J.S., Friend, J.A. & Reid, T.M. Pulmonary hypersensitivity in prawn workers. *Lancet* **2**, 1350-3 (1980).
134. Halkier-Sorensen, L., Heickendorff, L., Dalsgaard, I. & Thestrup-Pedersen, K. Skin symptoms among workers in the fish processing industry are caused by high molecular weight compounds. *Contact Dermatitis* **24**, 94-100 (1991).
135. Baur, X., Chen, Z. & Liebers, V. Exposure-response relationships of occupational inhalative allergens. *Clin Exp Allergy* **28**, 537-44 (1998).
136. Lopata, A.L. et al. Detection of fish antigens aerosolized during fish processing using newly developed immunoassays. *Int Arch Allergy Immunol* **138**, 21-8 (2005).
137. Bjelland, S., Gildberg, A. & Volden, G. Degradation of human epidermal keratin by fish pepsin. *Arch Dermatol Res* **280**, 119-23 (1988).
138. Bjelland, S., Hjelmeland, K. & Volden, G. Degradation of human epidermal keratin by cod trypsin and extracts of fish intestines. *Arch Dermatol Res* **280**, 469-73 (1989).
139. King, T.P. et al. Allergen nomenclature. WHO/IUIS Allergen Nomenclature Subcommittee. *Int Arch Allergy Immunol* **105**, 224-33 (1994).
140. Henzl, M.T., Zhao, H. & Saez, C.T. Self-association of CPV3, an avian thymic parvalbumin. *FEBS Lett* **375**, 137-42 (1995).
141. Ross, C., Tilghman, R.W., Hartmann, J.X., Mari, F. Distribution of parvalbumin isotypes in adult snook and their potential applications as species-specific biomarkers. *Journal of Fish Biology* **51**, 561-572 (1997).
142. Bugajska-Schretter, A. et al. Purification, biochemical, and immunological characterisation of a major food allergen: different immunoglobulin E recognition of the apo- and calcium-bound forms of carp parvalbumin. *Gut* **46**, 661-9 (2000).
143. Yamamoto, S. & Ishihara, T. Ion-exchange chromatography of proteins near the isoelectric points. *J Chromatogr A* **852**, 31-6 (1999).
144. Amersham. (Amersham Pharmacia Biotech, Uppsala, Sweden, 2002).
145. Coligan, J.K., Shevach, E.M., Strober, W. in *Current Protocols in Immunology* (ed. John Wiley and Sons, I.) (USA, 2004).
146. Sampson, H.A. Utility of food-specific IgE concentrations in predicting symptomatic food allergy. *J Allergy Clin Immunol* **107**, 891-6 (2001).
147. Phadia. (ImmunoCAP InVitroSight, 2006).
148. Cullinan, P. et al. Clinical responses to ingested fungal alpha-amylase and hemicellulase in persons sensitized to *Aspergillus fumigatus*? *Allergy* **52**, 346-9 (1997).
149. Sander, I., Raulf-Heimsoth, M., Van Kampen, V. & Baur, X. Is fungal alpha-amylase in bread an allergen? *Clin Exp Allergy* **30**, 560-5 (2000).
150. Baur, X., Sander, I., Jansen, A. & Czuppon, A.B. [Are amylases in bakery products and flour potential food allergens?]. *Schweiz Med Wochenschr* **124**, 846-51 (1994).
151. AMFEP. (Brussels, 2001).
152. Kurup, V.P. et al. Alkaline serine proteinase from *Aspergillus fumigatus* has synergistic effects on Asp-f-2-induced immune response in mice. *Int Arch Allergy Immunol* **129**, 129-37 (2002).
153. Wan, H. et al. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J Clin Invest* **104**, 123-33 (1999).

154. Stewart, G.A. & McWilliam, A.S. Endogenous function and biological significance of aeroallergens: an update. *Curr Opin Allergy Clin Immunol* **1**, 95-103 (2001).
155. Baur, X., Czuppon, A.B. & Sander, I. Heating inactivates the enzymatic activity and partially inactivates the allergenic activity of Asp o 2. *Clin Exp Allergy* **26**, 232-4 (1996).
156. Zhu, Z. et al. Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. *Science* **304**, 1678-82 (2004).
157. Kashorte, M. Commercialization of South Africa's subsistence fisheries. *International Journal of Oceans and Oceanography* **1**, 45-65 (2006).
158. Fairweather, T.P., van der Lingen, C.D., Booth, A.J., Drapeau, L., van der Westhuizen, J.J. . Indicators of sustainable fishing for South African sardine *Sardinops sagax* and anchovy *Engraulis encrasicolus*. . *African Journal of Marine Science* **28**, 661-680 (2006).
159. Yman, L. (Pharmacia & Upjohn Diagnostics AB, 1999).
160. Aalberse, R.C. Assessment of allergen cross-reactivity. *Clin Mol Allergy* **5**, 2 (2007).
161. Pascual, C., Martin Esteban, M. & Crespo, J.F. Fish allergy: evaluation of the importance of cross-reactivity. *J Pediatr* **121**, S29-34 (1992).
162. Koyama, H., Kakami, M., Kawamura, M., Tokuda, R., Kondo, Y., Tsuge, I., Yamada, K., Yasuda T., and Urisu, A. Grades of 43 Fish Species in Japan Based on IgE-binding Activity. *Allergology International* **55**, 311-316 (2006).
163. FishBase. (Foese, R and Pauly, D., 2005).
164. FishBase. (2006).
165. FishBase. *Sardinops sagax*. (2006).
166. Failler, P. 1-243 (FAO Fish Synop., 2007).
167. Valenta, R. et al. Calcium-binding allergens: from plants to man. *Int Arch Allergy Immunol* **117**, 160-6 (1998).
168. Ross, C., Tilghman, R.W., Hartmann, J.X., Mari, F. Distribution of parvalbumin isotypes in adult snook and their potential applications as species-specific biomarkers. *Journal of Fish Biology*, 561-572 (1997).
169. Scholl, I. et al. Dimerization of the major birch pollen allergen Bet v 1 is important for its in vivo IgE-cross-linking potential in mice. *J Immunol* **175**, 6645-50 (2005).
170. Amersham. 1-6 (Amersham Biosciences AB, Uppsala, 2000).
171. Lowenstein, H., Sparholt, S.H., Klysner, S.S., Ipsen, H. & Larsen, J.N. The significance of isoallergenic variations in present and future specific immunotherapy. *Int Arch Allergy Immunol* **107**, 285-9 (1995).
172. Moncrief, N.D., Kretsinger, R.H. & Goodman, M. Evolution of EF-hand calcium-modulated proteins. I. Relationships based on amino acid sequences. *J Mol Evol* **30**, 522-62 (1990).
173. Goodman, M. & Pechere, J.F. The evolution of muscular parvalbumins investigated by the maximum parsimony method. *J Mol Evol* **9**, 131-58 (1977).
174. Morizot, D.C. et al. Xiphophorus genetic linkage map: beginnings of comparative gene mapping in fishes. *Mar Biotechnol (NY)* **3**, S153-61 (2001).