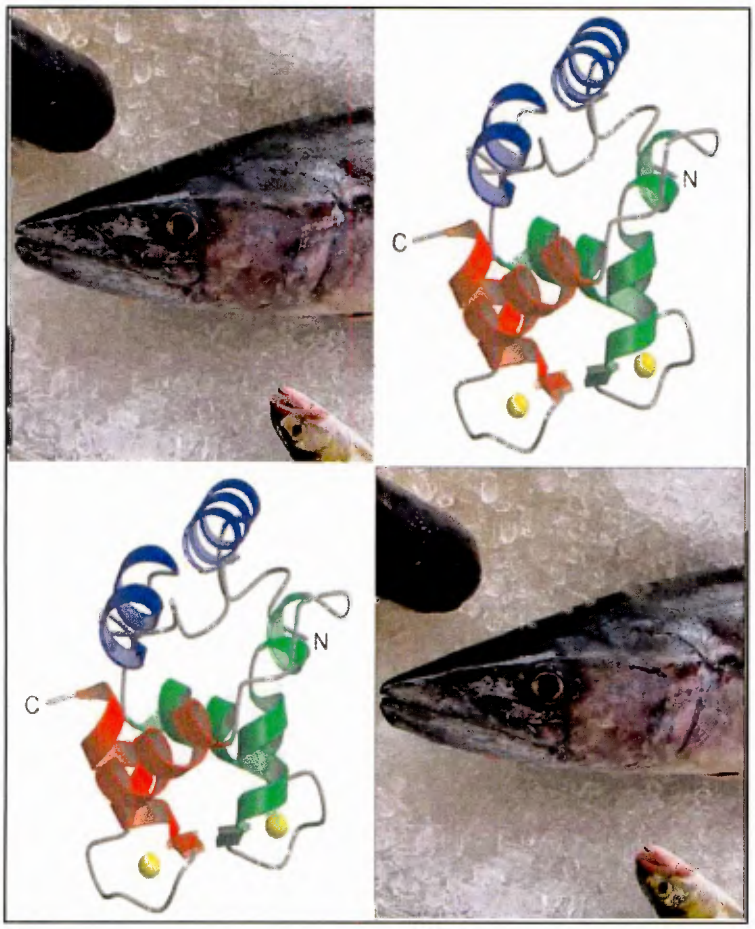
Molecular characterisation of parvalbumin and analysis of cross-reactivity in five fish species using sera from fish-allergic consumers and occupationally exposed workers.



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

LIST OF FIGURES

LIST OF TABLES

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 (*Dermatophagoides pteronyssinus*) allergen 1
DNA	deoxyribonucleic acid
ddH_2O	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	messanger RNA
MHC	major histocompatibility complex
MS	mass spectrometry
MW	molecular weight
$(NH_4)_2SO_4$	ammonium sulphate
NK	natural killer cells
ON	overnight
PAGE	polyacrylamide gel electriphoresis
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). Favouring 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].

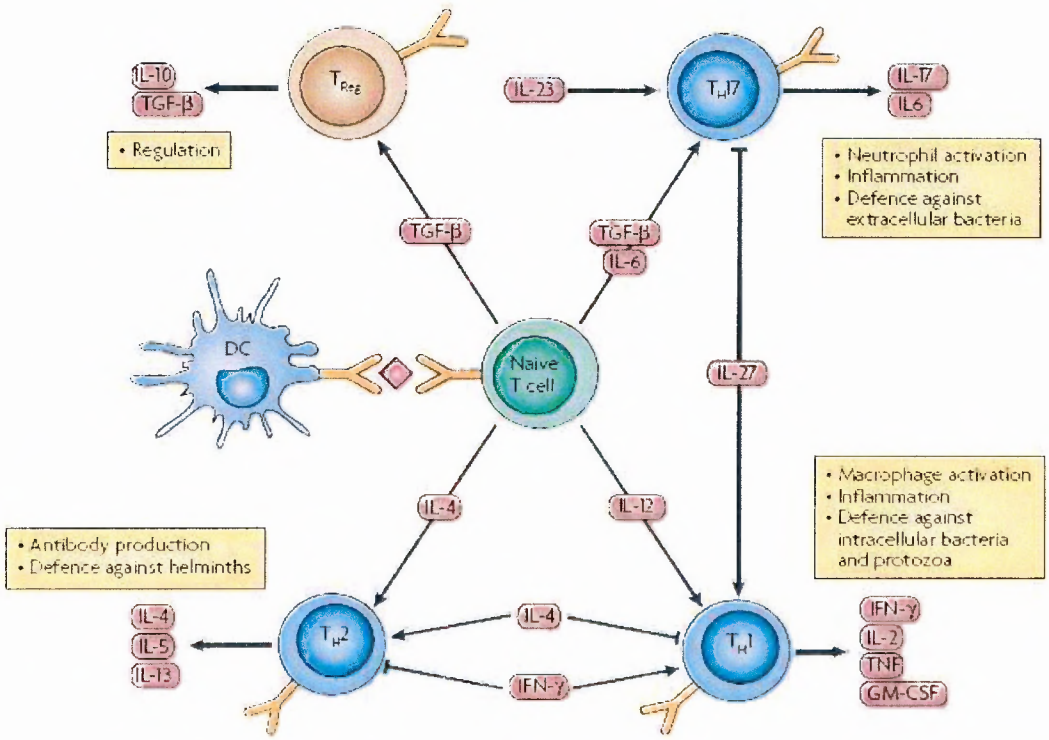


Figure 1.1: A summary of the four populations of $CD4^+$ T lymphocytes. T_H1 lymphocytes are characterised by their production of IFN-γ and IL-2 and their subsequent influence on cellular immunity. The production of IL-4, IL-5, and IL-13 is a feature of T_H2 lymphocytes and play a role in humoral, hypersensitivity 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 cells [26].

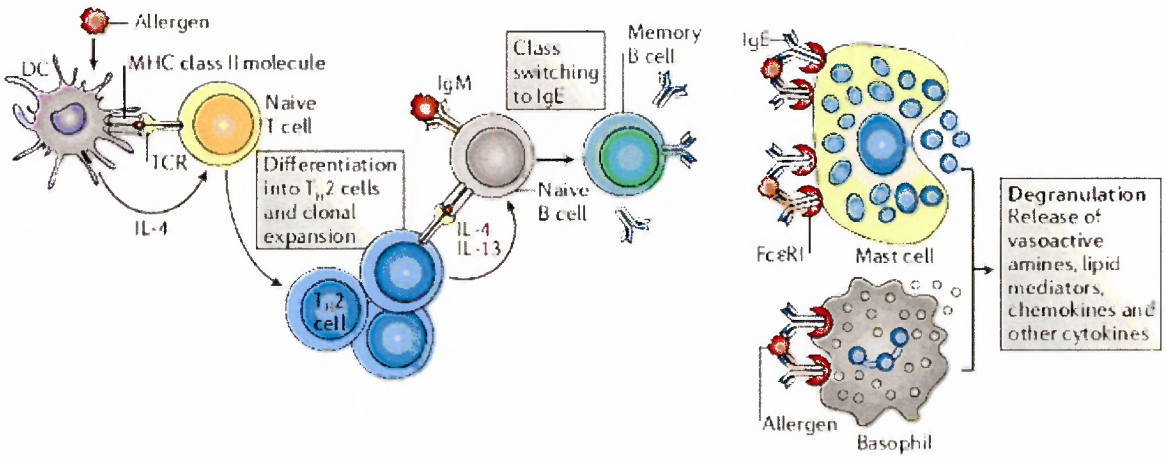


Figure 1.2 Mechanism of an allergic reaction. The allergen is taken up by the dendritic cell (DC) where it is processed and presented to the naïve T cell. Sensitisation involves the initial generation of allergen-specific T_H2 cells in the presence of IL-4. The cytokines, IL-4, IL-5, IL-9 and IL-13, 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 (FcεRI) 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 FcεRI, IL-4, IL-13 and the IL-4 receptor have been associated with atopy [27]. 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 determinants [28].

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 sensitisation [29]. 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 immunotherapy [23]. 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 desensitisation [13].

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* I from the domestic cat and other indoor allergens are generally associated with asthma while exposure to *Phl p* I 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 throat [27].

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 response [34]. 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 asthma [36]. 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 disease [37].

However, enzymatic activity is not a property that is present in all allergens [29]. 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 modification [32].

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-