Identification and Characterization of Novel Group 5 and Group 21 Allergens from Dust Mite and IgE Binding Epitope Mapping of Blo t 5

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List of publications

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International Conference Abstracts

1. Gao YF, Wang DY, and Chew FT. Independent co-sensitization not due to cross-reactivity between paralogous of group 5 allergens from *Blomia tropicalis* and *Dermatophagoides farinae.* 2006. *J Allergy Clin Immunol*. Volume117, Issue 2, Supplement 1. *Page S119.*

2. Bi XZ, Gao YF and F.T. Chew. Blo t 5, the major allergen from dust mite *Blomia tropicalis*, is secreted from the mite stomach and gut epithelial and is associated with gut and fecal contents. 2005. *J Allergy Clin Immunol.* Volume 115, Issue 2, Supplement 1. *Page S91.*

3. Gao YF, Bi XZ, Shang HS, Wang DY and Chew FT. Molecular cloning and characterization of a group 5 paralogue from *Blomia tropicalis.* 2005. *J Allergy Clin Immunol.* Volume 115, Issue 2, Supplement 1. *Page S90.*

4. Reginald K, Gao YF, Siew YS, Shang HS and. Chew FT. Cross comparison of the IgE binding profiles to recombinant allergens from *Suidasia medanensis*, *Blomia tropicalis* and *Dermatophagoides farinae* using Sera from Blomia- and Dermatophagoides-Predominant Environments. In: The 61th American Academy of Allergy and Immunology Annual Meeting, 19 - 24 March 2004, San Francisco, USA. *J Allergy Clin Immunol* 113 (2): S228-9.

5. Tay ASL, Shang HS, Bi XZ, Reginald K, Gao YF, Angus AC, Ong ST, Wang WL, Kuay KT, Wang DY, Mari A, Chew FT (2005). Component-resolved diagnosis of house dust mite allergy with a large repertoire of purified natural and recombinant allergens from the major species of mites worldwide. In: The 62th American Academy of Allergy and Immunology Annual Meeting, March 2005, San Antonio, USA. *J Allergy Clin Immunology*, 115 (2): S164.

6. Gao YF, Tan X.J, Ong ST, Bi XZ, Shang HS, Wang DY, and Chew FT. Characterization of two paralogous genes showing identities to Group 5 allergens in house dust mite *Dermatophagoides farinae.* The XXIIIst Congress of the European Academy of Allergology and Clinical Immunology (EAACI 2004), Amsterdam RAI, Netherlands, 12-16 June 2004.

7. Reginald K, Gao YF, Lim YP, Chew FT (2004). The expressed sequence tag catalogue and allergens of dust mite, *Suidasia medanensis*. In: XXIIIth European Academy of Allergology and Clinical Immunology Annual Meeting (EAACI), June 2004, Amsterdam, The Netherlands.

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9. Gao YF, Tay SL and Chew FT. Identification of group 5 allergens from *Suidasia medanensis.* The 9th Biological Sciences Graduate Congress, Bangkok, Thailand, 16-18 December. 2004.

10. Loo AHB, Tan SPL, Angus AC, Kuay KT, Reginald K, Gao YF and Chew FT. Genetic relationship between allergy-causing dust mites: phylogenetic inference from random amplified polymorphic DNA (RAPD) markers, housekeeping gene (18S rDNA) and group 2 Allergens. In: The 60th American Academy of Allergy and Immunology Annual Meeting, 7 - 12 March 2003, Denver, USA. J Allergy Clin Immunol 111 (2): S162.

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List of Abbreviations

Chemical and reagents

AP	alkaline phosphatase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
dH_20	distilled water
EDTA	ethylenediaminetetraacetic acid
IPTG	isopropyl-β-thiogalactopyranoside
NBT	nitroblue tetrazolium
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline with 0.05% Tween 20 (v/v)
PVDF	polyvinyldiflouride
SDS	Sodium dodecylsulphate
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
TE	tris-EDTA
Tris	Tris (hydroxymethyl)-aminomenthane

Units and Measurements

bp	base pair
kb	kilo base pair

kDa	kilo Dalton
IU	international unit
M	Molar
min	minute
OD	optical density
pH	abbreviation of "potential of hydrogen"
rpm	round per minute
sec	second
V	Volt
(v/v)	volume: volume ratio
(w/v)	weight: volume ratio
µg	microgram/s
µl	microliter/s

Others

AAAAI	American Academy of Allergy, Asthma & Immunology, Inc
APC	Antigen Presenting Cell
BLAST	Basic Local Alignment Search Tool
CD spectra	circular dichroism spectra
cDNA	complementary deoxyribonucleic acid
Dc	dendritic cell
DNA	deoxyribonucleic acid
EST	expressed sequence tag

IgE	immunoglobulin E
IgG_1	immunoglobulin G, class 1
IgG_4	immunoglobulin G, class 4
IgM	immunoglobulin M
IL	interleukin
IUIS/WHO	International Union of Immunologic Societies Subcommittee/ World Health Organization
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization- Time of Flight
mRNA	messenger ribonucleic acid
MHC	major histocompatibility complex
MW	molecular weight
NCBI	National Center for Biotechnology Information
NMR	Nuclear Magnetic Resonance Spectroscopy
ORF	open reading frame
PCR	polymerase chain reaction
PDB	Protein Data Bank
pET	expression vector (Novagen)
pI	isoelectric point
r	recombinant
RACE	Rapid amplification cDNA ends
RAST	radioallergosorbent Test
RNA	ribodeoxyribonucleic acid
RT-PCR	reverse transcription- polymerase chain reaction

SPT	Skin prick test
spp.	Speciese
TCR	T cell receptor
T_{reg}	regulatory T cell
TCR	T cell receptor
TH	T-helper cell
WHO	World Health Organization

Summary

The aim of this thesis is to have a deeper insight and more comprehensive understanding of mite allergy, focusing on Group 5 and its homologue Group 21 allergens. The study started with characterization of Blo t 21 allergen as *B. tropicalis* is a predominant mite species in Singapore as well as in many other regions of the world. Group 5 and Group 21 allergens in other mite species were also characterized, and finally IgE-binding areas of Blo t 5, a representative Group 5 allergen, were mapped.

Blo t 21 sensitization is strongly associated with allergic rhinitis. It is a product of a single-copy gene mainly with α-helical secondary structure, sharing 39% identity to Blo t 5 protein. When it was evaluated in 43 adult patients, 93% positive response was obtained by ELISA and 95% by skin prick test. Blo t 21 was found to be the third most prevalent allergen among 19 *B. tropicalis* allergens through studies in 97 *B. tropicalis* positive individuals. In addition, the majority (>75%) of the Blo t 21 sensitized individuals were co-sensitized to Blo t 5. Blo t 21 and Blo t 5 were identically distributed in house dusts, implying co-exposure of these allergens in the population that leads to co-sensitization of both allergens. Low to moderate degrees of cross-reactivity among Blo t 21 and other Group 5 allergens observed from inhibition studies indicates that Blo t 21 can induce specific hypersensitive responses. Hence, Blo t 21 is a novel clinically important allergen of *B. tropicalis.*

The IgE-binding activities of both Blo t 5 and Blo t 21 are stable. Blo t 21 and Blo t 5 are resistant to heat treatment (up to 90 °C), extreme pH conditions (pH 2 and pH 12) and chemical denaturation with 6 M urea. The high stability of IgE-binding activites of Blo t 21 and Blo t 5 explains why these two molecules are so allergenic in *B. tropicalis*.

Parallel studies on Der f 21, Sui m 5.01, Sui m 5.02 and Sui m 5.03 consistently show that Group 5 and Group 21 allergens are important in both predominant mite *D. farinae* and less commonly occurring mite *S. medanensis*. When eleven Group 5 and Group 21 allergens from seven mite species were evaluated in 118 local mite positive atopic subjects, Blo t 5 was found the most important allergen. Cross-inhibition studies revealed the predominant sensitization of Group 5 and Group 21 allergens of *B. tropicalis* and *D. farinae* species and partial cross-reaction to Der p 5, Lep d 5 and Ale o 5 in atopic individuals.

IgE-binding epitopes of Blo t 5 mapped by both site-directed mutagenesis and systematic overlapping peptide mapping approaches demonstrate that multiple IgE-binding epitopes exist throughout Blo t 5 molecule, including N-terminal, Center and C-terminal of the protein. Majority of the patients respond to multiple epitopes of Blo t 5, but different individuals react to different epitopes. DLNILERF (98-105) is found to be a common IgE-binding epitope of Blo t 5.

Chapter 1: Literature Review

The immune system is a host defense system protecting our body against potentially life threatening infectious microorganisms, foreign harmful substances and abnormal cells like cancerous cells in our body. The response of the immune system to the introduction of harmful or foreign substances is called immune response. This process is a carefully coordinated and controlled interaction between immune cells with the ultimate goal to eliminate the invader by pathogen-specific mechanisms.

The immune responses fall into two broad categories, the innate and adaptive immune responses. Innate immunity is mediated by the phagocytic cells and it prevents organisms' entry in a non-specific recognition manner. Adaptive immunity has two main features: specificity and memory. The specificity enables the adaptive immune system to recognize 'self' and 'non-self', and one particular antigen and other different antigens from invaded microorganisms using major histocompatibility complex (MHC) molecules as markers and subsequently remove harmful substances from our bodies. The memory of adaptive immunity enables host to recognize the antigens that it had encountered before. Therefore, the bodies can rapidly respond to the repeated challenge with similar foreign substances. Disorder of immune system can be either from lack of immune response or over reaction.

1.1 Hypersensitivity

Hypersensitivity refers to the excessive immune response produced by the normal immune system. Hypersensitivity reactions require a pre-sensitization of the host and re-exposure of the similar antigens. According to Gell and Coombs's study (1963),

hypersensitivity was classified into four types, Type I to Type IV hypersensitivity based on the mechanisms involved and time taken for the reaction. Usually, a particular clinical disease may involve more than one type of reactions. Type I hypersensitivity is the immediate or anaphylactic hypersensitivity. The reaction is mediated by IgE antibodies. Type II hypersensitivity is the cytotoxic hypersensitivity and may affect a variety of organs and tissues. The reaction is primarily mediated by IgM or IgG antibodies and complement. Type III hypersensitivity is known as the immune complex hypersensitivity**.** The reaction may be general or may involve individual organs. Type IV hypersensitivity is often called the delayed type hypersensitivity. This reaction is a type of cell-mediated response. Recently, Type V hypersensitivity driven by the innate immunity was added to modify the Gell-Coombs classification (Rajan, 2003).

1.2 Allergy-type I hypersensitivity

The word "allergy" originated from Greek, meaning "altered reactivity" (Arshad, 2002). The concept of allergy was coined by Clemens von Pirquet in 1906 who observed the symptoms caused by harmful immune reactions to dust, pollen and certain foods (Roecken *et al*., 2004). Allergy refers to an acquired potential to develop immunologically medicated adverse reactions to normally innocuous substances which may induce tolerance in normal people (2000, the American Academy of Allergy, Asthma and Immunology). The substances provoking the allergic response are named allergens. Most of so called "Allergy" is type I hypersensitivity and mediated by IgE antibody. The reaction may be either local or systemic. Symptoms vary from allergic rhinitis, allergic asthma, atopic dermatitis, etc., to sudden death from anaphylactic shock.

Death in anaphylaxis is due to systemic release of vasoactive mediators leading to general vasodilation and smooth muscle contraction resulting in sudden loss of blood pressure, angio-oedema and severe bronchiole constriction (systemic anaphylaxis).

1.3 Mechanism of allergy

Foreign antigens/allergens enter the body through respiratory mucosa, gastrointestinal mucosa and skin. Figure 1.1 illustrates that the first step of allergic immune response is the uptake and presentation of allergen by professional antigen presenting cells (APCs) like dendritic cells, macrophages and B lymphocytes. One of the most potent types of APC is the dendritic cell (DC). Dendritic cells digest antigen into short peptides associated with the major histocompatibility complex (MHC) molecules and present them on the cell surface (Clancy, 2000). In the mean time, dendritic cells migrate to the draining lymphoid organ to interact with naïve CD4+ T-cells.

After interacting with the antigen-MHC complex through specific T-cell receptors (TCR), Naïve CD4+ T-cells (TH0) are then activated, with the help of other co-stimulatory molecules such as CD80 and CD86, to secrete regulatory cytokines that determine the polarization of T helper responses (Lambrecht, 2001). Table 1.1 lists the factors that affect the polarization of T helper cells by dendritic cells.

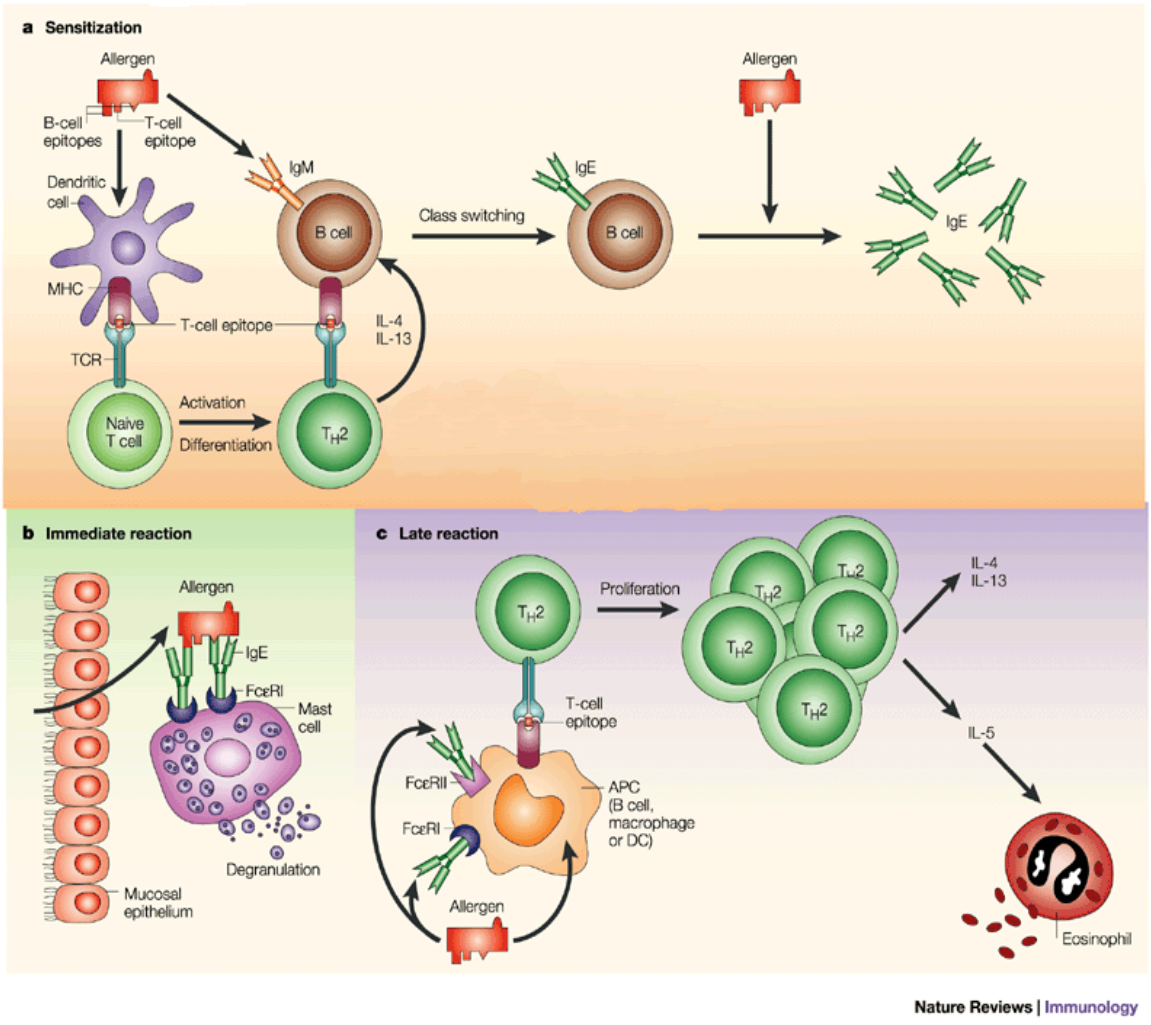

Figure 1.1 Allergy mechanism: (a) sensitization; (b) immediate reaction; and (c) late reaction (Adapted from Valenta, 1999).

Table 1.1 Factors influencing T helper cell polarization by dendritic cells (Adapted from Lambrecht, 2001).

Type of variable	Favouring Th1	Favouring Th2
Antigen		
dose	high dose	low dose
characteristics	*Candida albicans* yeasts	*Candida albicans* hyphae
	Toxoplasma Ag	Filarial nematode products
		Allergens ?
adjuvant factors	CpG oligodesoxynucleotides	
	Gram positive cell wall	
	early after lipopolysaccharide stimulation	late after lipopolysaccharide stimulation
		Cholera toxin
MHC–TCR interaction		
	high affinity	low affinity
	short interaction	sustained interaction
	high stimulator/responder	low stimulator/responder
Costimulatory molecules		
	low expression CD80/86	
	ICAM-1	
	CD40	OX40-L
		ICOS-L (B7RP-1 or -2)
Dendritic cell heterogeneity		
maturation	mature	immature
lineage	monocyte-derived dendritic cell	lymphoplasmacytoid dendritic cell
	macrophage derived dendritic cell	
	$CD1a^+$ myeloid dendritic cell	$CD1a^-$ myeloid dendritic cell
localization		mucosal dendritic cell
Cytokine production		
	high IL-12	low IL-12 (PGE_2-exposed)
	low IL-10	high IL-10
		high IL-6

ICAM, intercellular adhesion molecule; ICOSL, inducible costimulator ligand; MHC, major histocompatibility complex; PGE, prostaglandin; TCR, T cell receptor.

When treated with allergen (Der p 1 and 2, Bet v 1) and cultured with autologous naive or memory T cells in vitro, dendritic cells induce both Th1 and Th2 cytokines - but predominantly Th2 when dendritic cells from atopic donors are used (Bellinghausen *et al.*, 2000; De *et al.*, 2000). Th2 cells then secrete cytokines IL-4 and IL-13. These cytokines promote antibody class switching to produce antigen specific IgE antibody in B cells (Pène *et al.*, 1988; Finkelman *et al.*, 1988; Punnonen *et al.*, 1993; Emson *et al.*, 1998). The IgE antibodies are circulated throughout the body and able to bind high-affinity receptors (FcεR1) and low-affinity receptors (FcεR2) on mast cells, eosinophils, macrophages and platelets (Roitt *et al.*, 1998).

Upon re-exposure to allergen, cross-linking of allergen to specific IgE on mast cell FcεR1 receptor triggers mast cell degranulation and the secretion of mediators such as histamine, tryptase, heparin, prostaglandins, leukotrienes and bradykinin (Kinet, 1999). These mediators cause smooth muscle contraction and vasodilatation, increase capillary permeability and attract cells into the tissues, thus leading to inflammation. The symptoms of immediate hypersensitivity reactions include runny nose, watery eyes, sneezing, coughing, sinus congestion and constricted airways in the respiratory tract, cramping, diarrhea and vomiting in the gastrointestinal tract, erythema and urticaria on the skin. The reaction takes place within minutes.

Mast cells stimulated by antigen cross-linked to IgE-FcεRI complexes induce synthesis of another group of mediators leading to prolonged symptoms (late-phase response). Upon activation, eosinophils release pre-formed and newly synthesized mediators such as eosinophilic cationic protein (ECP), major basic protein (MBP), leukotrienes and prostaglandins to enhance inflammation and prolong epithelial damage (Dombrowicz and Capron, 2001; The Allergy Report, 2000). The late response takes place a few hours after the allergen exposure.

1.3.1 Dendritic cell

In the allergy response network, dendritic cells (DCs) play an important role in the orientation of immune response to inhaled allergens. Some clinically important allergens, such as Der p 1, a proteolytic enzyme of house dust mite, can directly activate dendritic cells or epithelial cells. However, other allergens, such as ovalbumin (OVA), do not have activating ability (de Wit *et al.*, 2000). How dendritic cells recognize natural

allergens as a danger signal and how they are activated by this signal are still under investigation.

Plasmacytoid dendritic cells play an essential role in preventing airway inflammation by inducing T cell unresponsiveness and the differentiation of regulatory T cells (T reg). Pulmonary dendritic cells from mice exposed to respiratory antigen transiently produce interleukin-10 (IL-10) and stimulate the development of CD4 (+) T regulatory 1-like cells, which subsequently suppress the inflammation reaction. In addition, after adoptive transfer of pulmonary DCs from IL-10(+/+) mice, the recipient mice can induce antigen-specific unresponsiveness upon exposure to respiratory antigen (Akbari *et al*., 2001). Hence, dendritic cell mediated T cell tolerance requires interleukin-10.

Dendritic cells are also crucial for the maintenance of allergic airway inflammation. Depletion of the dendritic cells at the time of allergen challenge abolishes the characteristic features of asthma, including eosinophilic inflammation, goblet cell hyperplasia and bronchial hyperreactivity in the mouse model. The airway hypersensitivity can be restored by intratracheal injection of dendritic cells (van Rijt *et al.*, 2005). Another study from America also showed that myeloid dendritic cells are important for airway inflammation and airway hyperresponsiveness (Koya *et al.*, 2006). Upon 11 challenges of ovalbumin in sensitized mice, the number of dendritic cells in the lung decreased. It was also observed that intratracheal instillation of bone marrow–derived dendritic cells restored airway hyperresponsiveness and airway eosinophilia. Thus, dendritic cells have many functions in airway hyperresponsiveness pathway—antigen uptake, tolerance induction and maintenance of airway hyperresponsiveness.

1.3.2 Regulatory T cell

There are two major categories of regulatory T cells: "naturally-occurring" CD4+, CD25+ regulatory T cells and antigen-specific regulatory T cells. "Naturally-occurring" regulatory T cells express the forkhead family transcription factor FOXP3 (forkhead box p3) that is required for the regulatory T cell development and function (Hori *et al.*, 2003; Shevach, 2004; Ramsdell, 2003). Antigen-specific regulatory T cells produce IL-10 and/or TGFβ and regulate immune reaction (Hawrylowicz *et al.*, 2005; Maloy *et al.*, 2001). Activation of regulatory T cells results in suppression of Th2 cells, Th1 cells, mast cells, eosinophils and basophils, and subsequent prevention of allergic airway hyperresponsiveness. Figure 1.2 illustrates the suppression of allergic response by the regulatory T cell. Application of recent knowledge of the peripheral T-cell tolerance mechanism will help us develop more rational and safer protocols to control allergic diseases.

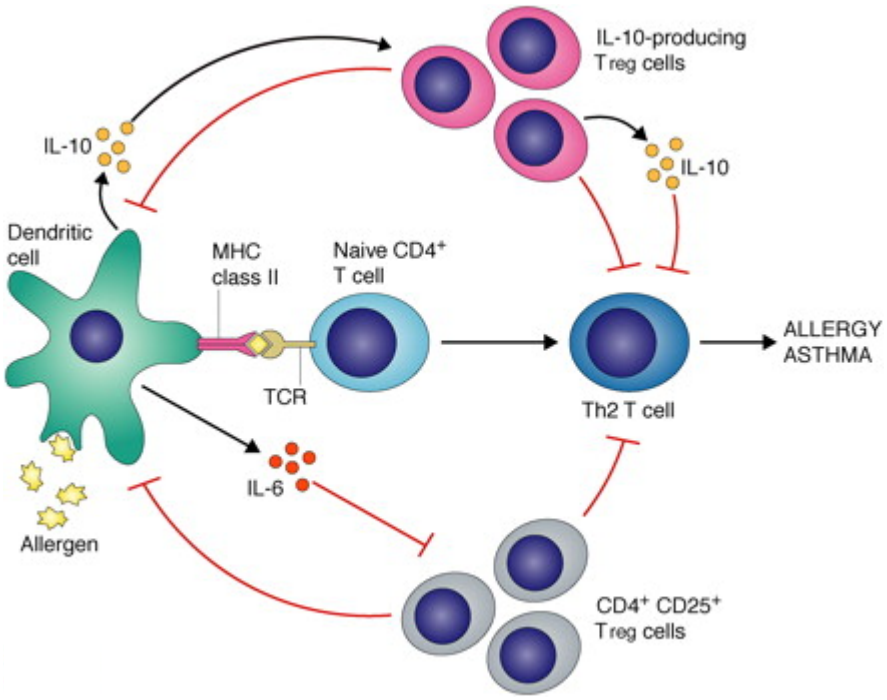

Figure 1.2 Control of allergic airway disease by regulatory T cells. Allergic airway disease is caused by inappropriate Th2-driven immune responses to allergens in the environment. $CD4^+$ $CD25^+$ and IL-10–producing Treg cells can regulate allergic sensitization in vivo through inhibitory effects on Th2 cells or on dendritic cells (DCs) in the lung. (Adapted from Hawrylowicz *et al.*, 2005)

1.3.3 IgE antibody

IgE antibody plays a very important role in the type I hypersensitivity reaction. It is the least abundant antibody class in serum. Sera IgEs from normal ("non-atopic") individuals are about 150ng/ml, much lower than IgGs (about 10mg/ml). IgE levels in sera from atopic individuals can increase up to 10-fold of the normal level. As mentioned previously, cross-linking of allergen to IgE-FcεR1 complex leads to degranulation of mast cells, release of inflammation mediators and induction of inflammation reaction. In addition, allergen cross-linking increases the expression of CD40-ligand (CD40-L), IL-4, and IL-13. CD40-L interacts with B cells and dendritic cells that express CD40 and then activate B cells. B cells induce IgE synthesis under stimulation of IL-4 and IL-13 (Gauchat *et al.* 1993; Pawankar *et al.* 1997). This positive feedback mechanism of IgE synthesis maintains high levels of the local IgE, which enhance the inflammation reactions.

1.4 Allergen

To solve the allergy problem, it is essential to understand allergens, the triggering factors of allergy. Allergens are substances that cause allergic reactions including type I hypersensitivity reaction. Allergens are mostly proteins, but not all proteins are allergens. What makes a protein an allergen in type I hypersensitivity? An allergen exhibits two properties: the induction of IgE response and clinical response upon re-exposure to the same allergen (Akdis, 2006). Allergen should contain B cell epitopes that interact with IgE antibody and form a complex. However, there is no unified theory to explain why some proteins are allergenic while others not. Allergens are commonly derived from

pollen, fungi, mites, endothelial tissues and dander from pets, venom from insects and foods such as egg, milk, fruits, nuts and fish (Kerkhof *et al*., 2003; Burge and Rogers, 2000; Boulet *et al*., 1997; Sporik *et al.*, 1996). Among these sources, mites, endothelial tissues and dander of pets, and fungi are known as indoor allergen sources, while pollen and fungal spore can be found outdoors. Today, more than 2000 allergens including variants and isoforms have been reported from house dust mites, cockroach, weeds, grasses, trees, animal dander, molds, insect venoms, shrimp, soybean, etc.

1.5 House dust mites - important indoor source of allergens

The association between sensitization to dust mite allergens and asthma has been extensively studied since Voorhorst and colleagues (1964) first reported that dust mite *D. pteronyssinus* was a major source of indoor dust that caused allergic reaction. Many studies revealed that dust mite allergy was strongly associated with the allergy asthma in different places of the world such as the United States (Eggleston *et al*., 1998 and Huss *et al.*, 2001), New Zealand (Sears *et al.*, 1989 and Burrows *et al.*, 1995), Ecuador (Valdivieso *et al.*, 1999), Puerto Rico (Montealegre *et al.*, 1997a) and Brazil (Arruda *et al*., 1991). Exposure to more than 2 μg Der p 1 and/or Der f 1 per gram of dust (corresponding to 100 mites per gram of dust) during infancy has been considered to be a risk factor for sensitization to mites and bronchial hyper-reactivity (Lau *et al.*, 1989 and Arruda *et al.,* 1991). Exposure to more than 10 μg Der p 1 per gram of dust (corresponding 500 mites per gram of dust) has been considered a risk factor for both sensitization and asthma development in atopic individuals (Sporik *et al.*, 1990). There is a strong association between increasing exposure to house dust mite and the frequency of sensitization to house dust mite (Warner *et al*., 1996). It was worthwile noting that mite

exposures lower than "threshold" level were also found to be associated with sensitization in some atopic individuals (Warner *et al.,* 1996; Huss *et al.*, 2001). Huss *et al.* reported that exposure to 0.020-2.0 μg/g Group 1 allergen is a risk for sensitization in the subjects with positive family histories of allergy.

1.5.1 Biology of dust mite

Understanding of the biology of dust mite is essential for monitoring and management of dust mite. Mites are very diverse organisms, and their morphological characteristics were described by Colloff *et al.* (1998). Mites belong to the phylum Arthropoda, subphylum Cheliceriformes, class Arachnida and order Acari. Three orders, Opilioacarida, Parasitiformes and Acariformes are usually recognized by acarologists (Arlian *et al.*, 2003). Mites that cause sensitization and allergic reaction belong to order Acariformes including suborder Prostigmata and Astigmata (Table 1.2). Spider mites in Prostigmata were known as allergenic source to induce allergy in orchard workers. Suborder Astigmata contains about 5000 species. Among them, thirteen species have been found in house dust and three of them have been reported to be very common in homes worldwide and are major sources of mite allergens (Arlian *et al*., 2001). These common species are Pyroglyphidae mites: *Dermatophagoides farinae* (*D. farinae*), *Dermatophagoides pteronyssinus* (*D. pteronyssinus*) *and Euroglyphus maynei* (*E. maynei*) which are found mostly in temperate climates (Arlian *et al*., 1992). *Blomia tropicalis* from Glycyphagidae family is prevalent in tropical and subtropical areas worldwide and co-inhabits with *Dermatophagoides spp.* Table 1.3 shows the families and genera of these mite species in the Astigmata.

TABLE 1.2 Abbreviated classification of phylum Arthropoda (Adapted from Arlian *et al.*, 2001)

Phylum Arthropoda

1. Subphylum Uniramia
2. Subphylum Crustacea
3. Subphylum Chelicerata (Cheliceriformes)

Class Arachnida

Order Acari

Suborder Mesostigmata (free-living, predaceous and parasitic mites)

Suborder Prostigmata (chiggers, follicle mites)

Suborder Metastigmata (ticks)

Suborder Astigmata (house dust, storage & scabies mites)

Suborder Oribatids (soil mites)

Table 1.3 Family and genera of allergy-causing mites that belong to the Astigmata (Adapted from Arlian *et al.*, 2003)

Family	Genus	Common species
Acaridae	*Acarus*	*A siro*
	Aleuroglyphus	*A ovatus*
	Rhizoglyphus	—
	Tyrophagus	*T putrescentiae*
Glycyphagidae	*Glycyphagus*	*G domesticus*
	Gohieria	—
Histiostomatidae	*Histiostoma*	—
Hemisarcoptidae	*Hemisarcoptes*	*H cooremani*
Carpoglyphidae	*Carpoglyphus*	—
Pyroglyphidae	*Dermatophagoides*	*D farinae, D pteronyssinus*
	Euroglyphus	*E maynei*
	Malaglyphidae	—
Chortoglyphidae	*Chortoglyphus*	*C arcuatus*
Echimyopodidae	*Blomia*	*B tropicalis*
Aeroglyphidae	*Aeroglyphus*	—
Suidasiidae	*Suidasia*	—
Lardoglyphidae	*Lardoglyphus*	—

There are five stages in the life cycle of a dust mite; from the egg, the larvae stage, then two nymphal stages, and the adult. Laboratory studies showed that the length of life cycle of *D. pteronyssinus* is 122.8 ± 14.5 days at 16°C, 34.0 ± 5.9 days at 23°C, 19.3 ± 2.5 days at 30°C and 15.0 ± 2.0 days at 35°C and 75% relative humidity (Arlian *et al.* 1990). In comparison, few *D. farinae* mites can complete the life cycle at extreme temperatures of 16°C and 35°C, but at 23°C and 30°C, lengths of the life cycle are about the same as *D. pteronyssinus* (Arlian *et al.*, 1996). Relative humidity of the environment also influences the population densities of mites. The densities of *D. farinae* and *D. pteronyssinus* declined when mite cultures were maintained at 21-22 °C and relative humidity of less than or equal to 50% in the laboratory. However, if mite culture was constantly kept at relative humidity over 85%, the densities of these two species in culture also decreased (Arlian *et al.*, 1999). The critical relative humidity (RH) for *Dermatophagoides spp.* growth ranges from 55% to 75% over the temperature from 15°C to 35°C (Arlian *et al.*, 1981a, Arlian *et al.*, 1981b). Hence, mite growth is influenced by both ambient relative humidity and temperature.

The life cycle of mites is strongly associated with its allergenicity. The allergenicity of *Dermatophagoides* mite culture is low at the initial latency period, then reaches the maximum at an exponential growth period, and finally enters a decline stage with a rapid decreasing of living mites (Eraso *et al.,* 1997). The extracts produced from the exponential growth phase of the cultures have six times more allergenic activity than those extracts prepared from latency and death phases when tested by skin prick assay. The specific IgE-binding activities of extracts produced from the exponential growth phase of the cultures are approximately three times higher than those extracts produced

from latency and death phases when tested using RAST method (Eraso *et al.*, 1998). Extracts obtained from *Blomia kulagini* cultures with the highest concentration of live mites (maximum growth phase) also have higher allergenicity (Cardona *et al*., 2004). Thus, extracts produced from maximum growth phase of dust mite provide the best diagnostic results in vivo and in vitro.

1.5.2 Distribution of dust mite

The occurrence of different mites varies in different geographical regions. Surveys of mite fauna have been conducted in many countries and the results showed that *D. pteronyssinus* and *D. farinae* are most common species found in homes in humid regions worldwide (Table 1.4). Initially it was believed that *D. farinae* was the most prevalent mite in the United States, and this species was given the official common name American house dust mite by the Entomological Society of America. *D. pteronyssinus* was given the common name European house dust mite because it was believed to be more prevalent in Europe. However, it is now known that both species are distributed widely in North America and Europe as well as other countries where surveys have been conducted. Interestingly, *D. pteronyssinus* and *D. farinae* normally co-inhabit in most homes worldwide, although one species could be more prevalent in certain homes of a specific geographic area than the other. *D. pteronyssinus* and *B. tropicalis* are found to be the predominant mites in homes of tropical and subtropical regions such as Puerto Rico, Brazil, Taiwan and Singapore. In addition, a study of house dusts from 8 different geographic areas of the United States revealed that the predominant species present varied between homes even within the same geographic area (Arlian *et al.* 1992). These

findings illustrate that a patient may be exposed to more than one species of mites. Hence for clinical diagnosis and immunotherapy, it is necessary to test both *D. pteronyssinus* and *D. farinae* in temperate regions and *D. pteronyssinus* and *B. tropicalis* in tropical and subtropical regions.

Table 1.4. Representative dust mite fauna reported in homes worldwide (Adapted from Arlian *et al.*, 2002)

Location	DF	DP	EM	BT	SM	Study
North America						
United States	X	X	?	?	?	Arlian et al., (1992); Arlian *et al.,* (1982); Chew *et al.*,(1999); Fernandez-Caldas *et al.*, (1990); Hannaway and Roundy (1997); Nadchatram *et al.,* (1981); Nelson et al., (1995); Peterson *et al.*, (1999); Squillace *et al*., (1997)
Puerto Rico	?	X	?	X	?	Montealegre *et al*., (1997b)
Barbados	?	X				Barnes *et al.*, (1997)
Central and South America						
Costa Rica	?	X				Soto-Quiros *et al.,* (1998)
Venezuela (Caracas)	?	X	?	?	?	Hurtado *et al.,* (1987)
Columbia	?	X	?		?	Charlet *et al.,* (1977)
Bogota		X	?	?	?	Charlet *et al.,* (1978)
Peru		?		?		Croce *et al*., (2000)
Brazil	?	X	?	X	?	Binotti *et al.*, (2001)
Chile					?	Franjola *et al.,* (1999)
Europe						
Scotland (Glasgow)		X	?	?	?	Colloff (1987)
England (Birmingham)		X	?		?	Blythe *et al.,* (1974)
Norway	?	X	?		?	Mehl (1998)
Sweden	X	X	?		?	Warner *et al.*, (1999); Munir *et al.*, (1993)
Finland	?	X	?			Stenius *et al.*, (1972)
Estonia	?	X				Julge *et al.,* (1998)
Poland	X	?	?	?	?	Solarz (1998); Racewicz (2001)
Silesia	X	X	?			Solarz (1998)
Denmark	X	X	?		?	Mehl (1998); Stenius *et al.,* (1972)
Germany	X	X				Hirsch *et al.*, (1998)
Switzerland (Basel)	?	X	X		?	Mumcuoglu (1976); Mumcuoglu (1977)
Pavia	X	?				Moscato *et al.*, (2000)
Rome	X	?	?		?	Bigliocchi *et al.,* (1996)
Spain	?	X				Alvarez *et al.*, (1997)
Middle East, Western Asia						
Turkey (Anatolia)	X	X			?	Kalpaklioglu (1997)
Israel	X	X	?		?	Feldman-Muhsam *et al.*, (1985); Mumcoglu *et al.*, (1999)
Uzbekistan		?			?	Nazrullaeva *et al.*, (1999)
Africa						
Egypt	X	X				Morsy *et al*., (1995); Yassin *et al.*, (1997)
Nigeria (Lagos)	?	X		?	?	Somorin *et al.*, (1978)
Eastern Asia						
Japan (Nagoya)	X	X	?		?	Suto *et al*. [117]; Suto *et al.* [118]
Tokyo	?	X			?	Miyamoto and Ouchi [119]
China	?	X	?		?	Chien *et a*l., (1987)
Taiwan	?	X		X		Sun *et al*., (2000); Chang e*t al.*, (1989)
Hong Kong		X				Leung *et al.*, (1998)
Malaysia		X		X		Ho [125]; Mariana *et al.*, (2000)
Singapore	?	X		X	?	Chew *et al*., (1999a); Zhang *et al.*, (1997)
Brunei	?	?		?	?	Woodcock *et al.*, (1980)
Indonesia (Jakarta)	X	?		?	?	Baratawidjaja *et al.*, (1998)
Oceana						
New Zealand		X				Wickens *et al.*, (1997)

DF-*Dermatophagoldes farinae*; DP-*Dermatophagoides pteronyssinus*; EM-*Euroglyphus maynei*; BT-*Blomia tropicalis*; SM-storage mites. X- prevalent or dominant species. ?-species reported to be present.

1.5.3 Mite allergens

Mite allergens are found in mite bodies, secreta and excreta (Tovey *et al.*, 1981). Natural mite allergens can be prepared from aqueous extracts. Since the first allergen was successfully isolated from dust mite *D. pteronyssinus* and named as Der p 1 (Chua *et al.* 1988) using molecular biology technique, more and more mite allergens have been identified from many mite species such as *D. pteronyssinus, D. farinae*, *B. tropicalis, Lepidoglyphus destructor,* etc. As shown in Table 1.5, more than twenty allergens have been identified from dust mites. Some allergens have already been deposited in International Union of Immunologic Societies Subcommittee/ World Health Organization (IUIS/WHO) (http://www.allergen.org/) and Genbank. An allergen should have specific IgE-binding activity in a minimum of 5 patients, or >5% of the individuals tested in atopic population (WHO/IUIS, 1994). According to WHO/IUS Allergen Subcommittee (1994), the allergen name includes the first 3 letters of the genus, the first letter of the species and an Arabic numeral to indicate the chronological order of purification. Allergens from different species but sharing the same biochemical properties are considered to belong to the same group (WHO/IUS, 1994). Some allergens sharing more than 67% identity of amino acid sequences and having similar biological functions are designated as isoallergens. Isoallergen may have multiple forms of closely similar amino acid sequences resulting from polymorphism. These isoforms are called variants and designated by 4 Arabic numerals. The first two numerals 01-99 refer to a particular isoallergen and the two subsequent numerals 01-99 refer to a particular variant of a particular isoallergen.

Table 1.5 IgE-binding frequencies of dust mite allergens and their biological properties.

Allergen groups[a]	Biological function	Molecular weight (kDa)	IgE-binding (%)	References
1	Cysteine protease	25	70-90	Der f 1 (Dilworth *et al.,* 1991), Der p 1 (Chua *et al.,* 1988), Der m 1 (Lind *et al*., 1988), Eur m 1 (Kent *et al.,* 1992), Blo t 1 (Mora *et al.,* 2003).
2	Unknown	14	60-90	Der f 2 (Trudinger *et al.,* 1991), Der p 2 (Chua *et a.,l* 1990), Tyr p 2 (Eriksson *et al.,* 1998), Eur m 2 (Smith *et al.,* 1999a), Gly d 2 (Gafvelin *et al*., 2001), Lep d 2 (Varela *et al*., 1994).
3	Trypsin	28, 30	51-90	Der f 3 (Nishiyama *et al.,* 1995a), Der p 3 (Smith *et al.,* 1994), Eur m 3 (Smith *et al.,* 1999b)[b], Blo t 3 (Cheong *et al.,* 2003).
4	Amylase	57, 60	25-46	Der p 4 (Lake *et al.,* 1991), Der p 4 & Eur m 4 (Mills *et al.,* 1999).
5	Unknown	15	9-70	Der p 5 (Tovey *et al.,* 1989), Blo t 5 (Arruda *et al.,* 1995), Lep d 5 (Eriksson *et al.,* 2001).
6	Chymotrypsin	25	30-40	Der f 6 (Kawamoto *et al.,*1999), Der p 6 (Yasueda *et al.,* 1993).
7	Unknown	22-31	50-62	Der p 7 (Shen *et al*., 1993), Der f 7 (Shen *et al.,* 1995), Lep d 7 (Eriksson *et al.,* 2001).
8	Glutathione-S-transferase	26	40	Der p 8 (O'Neill *et al.,* 1994).
9	Collagenolytic serine protease	30	>90	Der p 9 (King *et al.,*1996).
10	Tropomyosin	33-37	5-80	Der p 10 (Asturias *et al.,* 1998), Der f 10 (Aki *et al.*, 1995), Blo t 10 (Yi *et al.,* 2002), Lep d 10 (Saarne *et al.,* 2003).
11	Paramyosin	92, 98, 110	80	Der f 11 (Tsai *et al.,* 1999), Der p11 (Tategaki *et al*., 2000), Blo t 11 (Ramos *et al.,* 2001).
12	Unknown	14	50	Blo t 12 (Peurta *et al.,* 1996).
13	Fatty acid binding protein	14, 15	10-23	Blo t 13 (Caraballo *et al.,* 1997), Lep d 13 (Eriksson *et al.,* 2001), Aca s 13 (Eriksson *et al.,* 1999).
14	Apolipophorin	177	30[c] 39[d] 70[e]	Der f 14 (Fujikawa *et al.,* 1996), Eur m 14 (Epton *et al.,* 1999), Der p 14 (Epton *et al.,* 2001).

Allergen groups[a]	Biological function	Molecular weight (kDa)	IgE-binding (%)	References
15	98 kDa chitinase	98	?	Der f 15 (McCall *et al.*, 2001). Der p 15 (O'Neil *et al.*, 2006)
16	Gelsolin-like protein/ villin	53	35	Der f 16 (Tategaki *et al*., 2000).
17	EF-hand calcium-binding protein	53	35	Der f 17 (Tategaki *et al*., 2000)
18	60kDa chitinase	60	54	Der f 18 (Weber *et al*., 2003) Der p 18 (O'Neil *et al.*, 2006)
19	anti-microbial peptide	7.2	?	Blo t 19[b]
20	Arginine kinase	40	?	Der p 20[b]
21	Unknown	14	?	Der p 21[b]
Mag 29[f]	Heat shock protein 70 kDa	70	10	Der f (Aki *et al.*, 1994)

Species name of dust mites: Der f (*D. farinae)*, Der p (*D. pteronyssinus*), Eur m (*E. maynei*), Der m (*D. microceras*), Der s (*D. siboney*), Tyr p (*Tyrophagus putrescentiae*), Lep d (*Lepidoglyphus destructor*), Gly d (*Glycyphagus domesticus*), Blo t (*Blomia tropicalis*), and Aca s (*Acarus siro*).

[a] Listed in the WHO/IUIS list of allergens as of December, 2006. http://www.allergen.org/List.htm

[b] Unpublished but sequence data available in WHO/IUIS list of allergen or GenBank.

[c] Data for Mag allergen

[d] Data for recombinant Mag 3 allergen

[e] Data for natural Mag 3 allergen

[f] Not listed in WHO/IUIS list of allergen but published and sequence data available in GenBank.

Dust mite allergen isoforms have been found in Group 1 (Chua *et al.*, 1988; Smith *et al.*, 2001; Piboonpocanum *et al.,* 2006), Group 2 (Schmidt *et al.*, 1995; Chua *et al.*, 1996; Yuuki *et al.*, 1997 Smith *et al.*, 2001; Piboonpocanum *et al.,* 2006) and Group 3 (Smith and Thomas, 1996a; Smith and Thomas, 1996b). These isoforms are the result of the polymorphisms of allelic variations. The effects of polymorphism on the allergenicity (IgE-binding activities) and antigenicities (T-cell response) of dust mite allergens have not been widely investigated. Among the Group 2 allergen isoforms, polymorphic forms of Lep d 2 showed comparable *in vitro* IgE-binding activies to wildtype Lep d 2 (Olsson *et al.*, 1998).

IgE-binding of allergens from taxonomy distant mite species have different patterns. Hales and colleagues (2006) have compared a panel of nine allergens from *D. pteronyssinus* in asthmatic patients in Australia. They reported that Der p 1 and Der p 2 bound 50-65% of the total IgE antibody, Der p 4, 5 and 7 bound about 10% of the IgE antibody individually, and Der p 3, 8, 10 and 20 only had a very low IgE titers. In parallel, IgE-binding pattern of *B. tropicalis* allergens was also studied in asthmatic patients in Taiwan. Their studies demonstrated that the IgE-binding profile of *B. tropicalis* was different from that of *D. pteronyssinus* (Tsai *et al.*, 2003). Blo t 5 was the most important allergenic component in *B. tropicalis* when seven allergens (Blo t 2, Blo t 3, Blo t 5, Blo t 10, Blo t 11, Blo t 12 and Blo t 13) were evaluated.

Mite allergens have different biological functions. The relationship between their biological functions and IgE-binding activities is still not clear. Groups 1, 3, 4, 6, 8, 9, 15 and 18 allergens have enzymatic activity. Interestingly, Groups 1, 3, 6 and 9 allergens are all proteolytic enzymes, but only Group 1 allergens from *D. pteronyssinus* and *D. farinae*

are known to be major allergens. Group 1 allergens belong to the papain-like cysteine protease family. They are mainly found in mite faeces. Der p 1 is the first allergen that was cloned from dust mite using molecular biology tool (Chua *et al.,* 1988). Recombinant Der p 1 had full proteolytic activity and IgE-binding activity only when it was expressed in yeast *Pichia pastoris* and converted into mature form. Its pro-forms exhibited different secondary structures and less allergenicities than the mature forms (Takai *et al*., 2005). These studies indicate that maintenance of the three dimensional structure of mature Der p 1 is important for its IgE-binding. In addition, it has been demonstrated that protease activity of Der p 1 enhances systemic production of IgE and inflammatory cellular infiltration of the lungs in a mouse model (Gough *et al.*, 2003). The increase of allergenicity may be the result of the cleavage of the low-affinity IgE Fc receptor (CD23) from the surface of human B lymphocytes by Der p 1. The author proposed that the loss of cell surface CD23 from IgE-secreting B cells might promote and enhance the IgE immune responses by a feedback mechanism. Since soluble CD23 can promote IgE production, fragments of CD23 released by Der p I may enhance IgE synthesis (Hewitt *et al.*, 1995). Further studies have demonstrated that Der p 1 also cleaves the alpha subunit of the IL-2 receptor (IL-2R or CD25) from the surface of human peripheral blood T cells (Schulz *et al*., 1998 and Ghaemmaghami *et al.*, 1998). Since IL-2R is crucial for the propagation of Th1 cells, the cleavage of IL-2R may bias toward Th2-mediated immune response. Der p 1 eliciting IgE antibody responses can also go through protease-activated receptors (PARs) pathway. PARs can be activated by external protease like Der p 1. Compared with wildtype animals, airway hyperreactivity to inhaled methacholine was diminished by 38% in mice lacking PAR-2 and increased by

52% in mice over-expressing PAR-2 (Schmidlin *et al*., 2002). Hence, biological function of Der p 1 enhances its allergenicity through creating an allergic environment.

Groups 2, 13, 14 and 16 are ligand binding proteins. Among these allergens, Der p 2 allergen was known as a major allergen. Crystallographic and NMR structure studies revealed that Der p 2 allergen contains an ML (MD-2-related lipid-recognition) domain which comprises two anti-parallel beta-pleated sheets and a central hydrophobic cavity. This domain is predicted to be involved in lipid binding. Six cysteine residues in Der p 2 protein form three disulfide bonds which are important for stabilizing the structure (Nishiyama *et al.*, 1993). Mutation of cysteine residues drastically reduces IgE-binding activity (Nishiyama *et al.*, 1995b; Smith and Chapman, 1996), providing evidence that most IgE-binding epitopes of Der p 2 are conformational epitopes. Four studies on conformational IgE epitope mapping of Der p 2 (Smith and Chapman, 1997; Hakkaart *et al.*, 1998; Mueller *et al.*, 2001; Ipsen *et al*., 2004) further identified many other residues on the surface that are important for the IgE-binding.

Groups 10 and 11 allergens, known as tropomyosin and paramyosin respectively, are structure proteins. Between these two allergens, tropomyosin, a highly conserved coiled-coil protein is important because it exists in both invertebrates and vertebrates. Tropomyosin has been identified as a common allergen in the extracts of crustaceans, mollusks, insects such as dust mites, cockroaches, and moths. Dust mite tropomyosins consist of 284 amino acids and share 96% protein sequence identities among Der p 10, Der f 10, Blo t 10 and Lep d 10. The frequencies of Blo t 10 response was 28% in mite allergic patients when tested by ELISA, while Der p 10 had 29% positive response in 77 allergic asthma patients tested by dissociation-enhanced lanthanide fluoroimmunoassay

(DELFIA). Cross-reactivity of tropomyosins was observed due to their high protein sequence homology (Martinez, *et al*., 1997; Asturias, *et al.*, 1999; Ayuso *et al.*, 1999) and may result in the induction of allergic reactions to both foods and inhalants in the same patient.

Groups 5, 7 and 12 are allergens with unknown functions. Among these allergens, Group 5 allergen is the most important one since Blo t 5 has been identified as a major allergen in *B. tropicalis.* In addition to Blo t 5, Der p 5 and truncated Lep d 5 are also identified from *D. pteronyssinus* and *L. destructor* (Lin *et al*., 1994 and Eriksson *et al.*, 2001). Blo t 5 induces IgE in 45-69% of allergic subjects and in more than 90% of asthmatic patients (Arruda *et al.*, 1995; Kuo *et al.*, 2003; Yeoh *et al.*, 2003). And similarly, Der p 5 induces IgE antibody in 50% of allergic subjects (Lin *et al*., 1994). The cross-reactivity between Blo t 5 and Der p 5 is low in UK and Singapore population (Chew *et al.*, 1999b and Simpson *et al.*, 2003). Hence, Group 5 allergens from *D. pteronyssinus* and *B. tropicalis* are species specific. The truncated Lep d 5 was only recognized in 4 out of 45 *L. destructor*-sensitized subjects in Switzerland (Eriksson *et al.*, 2001). Further study of Lep 5 in a larger sample size of allergic patients will be needed to characterize its allergenicity.

1.6 Strategy to identify dust mite allergen

1.6.1 cDNA library screening approach

Classical method for cloning allergens from allergenic sources is based on the screening of the cDNA library using IgE antibodies from atopic individuals. cDNAs are

double-stranded DNA copies of messager RNAs. cDNA fragments can be inserted into an appropriate plasmid, phage, or cosmid vector to construct a cDNA library. A cDNA library represents the entire set of expressed genes in the cell or organism from which the RNA is isolated. By screening the cDNA products of interest using IgE antibodies from patient sera, Der p 1 was first successfully isolated from dust mite *D. pternoyssinus* (Chua *et al.*, 1988). Thereafter, Der p 2, Der p 5 of *D. pteronyssinus* and Blo t 5 of mite *B. tropicalis* were cloned with the same strategy (Chua *et al.*, 1988; Chua *et al.*, 1990; Caraballo *et al.*, 1997). The tittering of cDNA library and the efficiency of cDNA library expression are crucial for this strategy. However, a large proportion of cDNA inserts may not successfully express in this system, as cDNA library is not an ideal system for the expression of recombinant protein. Through screening of cDNA library with patient sera, usually only one allergen can be isolated from the allergenic source. In addition, this screening method also requires a certain amount of patients' sera which are often difficult to be collected. Further more, cDNA clone isolated from cDNA library may not necessarily be a full length clone. For example, Blo t 5 cDNA clone isolated by above strategy in 1996 only contained a truncated cDNA (Caraballo *et al*.). Thus, cDNA library screening by IgE antibody can be effectively applied to isolate allergens from allergenic sources, but there is a need to improve the efficiency of this method.

1.6.2 Phage display approach

To enrich the expressing of the cDNA clones, phage display technology was developed. This technology enables the gene of interest to display on the surface of a phage as a fusion with one of the coat proteins of the virus. By cloning cDNAs into phage,

display library is produced with billions of proteins from an organism displayed on the surface of the viral particle. This method enables rapid isolation of phage populations containing multiply represented clones. The screening efficiency depends on the absolute number of clones displaying the same gene products among the original library and the amount of allergen-specific IgE antibody in serum. Hence, identification of allergens derived from highly abundant mRNAs of the organism is preferable by this method. Tove and his co-workers (2001) were able to clone three new allergens from *Lepidoglyphus destructor* by screening of phage display library. However, phage display based cloning still requires a certain amount of human sera that are usually difficult to be obtained.

The fastest method to clone an allergen is to amplify the open reading frame by reverse transcribed PCR (RT-PCR) from mRNA. However, RT-PCR based cloning requires DNA sequence information, or at least some protein sequences. The sequences of new allergen genes may not be necessarily available.

1.6.3 Expressed Sequence Tag - a useful tool to isolate mite allergen

Expressed Sequence Tag (EST) approach is to obtain mass sequences of the transcriptomes of a particular source of organism, organ or tissue. An EST is a DNA sequence of a randomly picked cDNA clone corresponding to a message RNA (mRNA). mRNAs are transcribed from genes on chromosomes and subsequently translated into proteins. An EST is approximately 500 nucleotides in length with a relatively low quality due to the limitation of current DNA sequencing technology. However, the average cDNA is 2000 nucleotides in length, so an EST is only a tag of the cDNA. This tag is

often sufficient to identify a specific mRNA and its gene. ESTs provide the profile of the expressed genes in the source tissue and demonstrate the relative abundance of these transcripts from the source cDNA library (Adams *et al.,* 1995). EST approach is also a powerful technique for genome investigations as the ESTs reflect the coding regions of genes in genomic sequences. ESTs contain the predicted "functional genome"-transcriptome, which leads to prediction of their protein products, and eventually of their function (Yamamoto and Sasaki, 1997). With the development of DNA sequencing technology, it has been known to be a high throughput method to generate huge amount of DNA sequences from target species at present (Adams *et al.,* 1993; Adams *et al.,* 1995; Ewing *et al.,* 1999; Gross *et al.,* 2001).

In order to obtain the basic DNA information of dust mites, EST approach has been applied in our laboratory to survey the genomic components from important dust mites *D. farinae*, *B. tropicalis, Suidasia medenensis, Tyrophagus putrescentiae* and *Aleuroglyphus ovatus*. Together with bioinformatic analysis, many putative allergen sequences have been identified from these five species including some allergen genes that have already been deposited in WHO/ IUS, or published in international journals, and some putative allergens sharing homology with known allergens from other organisms or species but are new in *D. farinae* / *B. tropicalis*. Although EST approach is proven to be a useful tool for high throughput identifying potential allergen components from dust mites, this method can only identify homologues from known allergens.

With accumulation of dust mite DNA sequences at hand, novel allergenic components which are recognized by SDS-PAGE/Western blotting using patient sera can be further identified by MALDI-TOF Mass Spectrometry (MS). The integration of SDS-

PAGE/western blotting and MS make it possible to identify new allergens from dust mites efficiently.

1.7 Recombinant allergens for research, clinical diagnosis and therapy

With the tremendous progress in molecular biology of allergens over the years, many allergens have been cloned with full length cDNA sequences. This enables us to express recombinant allergens in different expression systems, such as bacteria, yeast, insect cells, mammalian cells and plants with comparable IgE-binding activities to natural allergens. Recombinant allergens can be expressed at high level in vitro and easily purified using established chromatography procedures. High purity of recombinant allergens can be consistently produced without contamination of endotoxin which is crucially important for in vivo study. Hence standardization of recombinant allergen in terms of quantity and quality control can be established. With recombinant allergen at hand, it is possible to achieve a component-resolved diagnosis of dust mite allergy and produce specific IgE-binding profiles for particular patients.

Recombinant allergens also facilitate the study of those allergens which are low-abundant in natural sources. Natural allergen products of dust mite have variable content of specific allergens. Group 1 and Group 2 allergens of *Dermatophagoides spp.* are the only two allergens that may exist in a concentration of 10 μg/ml or above. The concentration of Der p 2 can be 5-10 folds lower than that of Der p 1, while other allergens exist in even much lower concentrations (Thomas *et al.*, 2004). A proteomic analysis also demonstrated that not all allergens are highly expressed in *D. pteronyssinus*.

Only Groups 1, 2, 7, 10, 13 and 20 are found among the most abundant proteins of *D. pteronyssinus* extracts (Batard *et al*., 2006). It is difficult to prepare enough pure natural low-abundant allergens. Hence, studies of these allergens have to heavily depend on recombinant allergens.

In order to reduce the risk of side-effect of allergen-specific immunotherapy, many studies have been conducted to produce hypoallergens aiming to produce modified recombinant allergens with reduced IgE-binding activity but retained T-cell proliferation activities (Takai *et al.,* 1997; Ferreira *et al.*, 1998; van Hage Hamsten *et al.,* 1999; Nopp *et al.,* 2000; Schramm *et al.,* 1999; Kauppinen *et al.,* 1999). Study on the birch pollen Bet v 1 hypoallergen is a good representative. The IgE-binding of Bet v 1 depends on its three-dimensional structure. Separating expression of this allergen into two fragments (1-73 aa and 74-159 aa) resulted in the loss of conformation structure thus showing minimal allergenicity (Vrtala *et al.*, 1997). Trimeric form of Bet v 1 was also expressed in *E. coli* and showed the reduction of IgE-binding activity and histamine release ability with its retained secondary structure (Vrtala *et al.*, 2001).

Cocktail of recombinant allergens could be another choice to be used together with natural allergic products, or used to replace the natural crude extract products. Efficacy of the therapy could be improved by using recombinant cocktail which contains uniform allergen level without contamination of other non-related proteins.

In vitro expression of allergen also opens a door for studying the biological function and three-dimensional structure of the recombinant protein (Ferreira *et al*., 1993 and Chapman *et al*., 2000). Der p 2 produced in *E. coli* has a comparable allergenicity to the purified natural counterpart. *E. coli* expressed Der p 2 was used to determine its three-

dimensional structure through NMR and X-ray crystallography methods (Mueller *et al.*, 1997; Mueller *et al.*, 1998; Derewenda *et al.*, 2002). In contrast, expression of recombinant Der p 1 precursor was established in *P. pastoris* (Jatquet *et al.*, 2002). Additional acid treatment of Der p 1 precursor was needed to trigger the autocatalytic processing of mature Der p 1. The recombinant mature Der p 1 has comparable IgE-binding, histamine release and proteolytic activity to natural Der p 1. The three-dimensional structures of proDer p 1 and mature Der p 1 were solved by X-ray crystallography (Meno *et al.*, 2005 and de Halleux *et al.*, 2006). The structural biology is useful for identifying conformational epitopes of allergen and generation of hypoallergens by genetic engineering.

1.8 Objectives of this study

This study focuses on Group 5 and Group 21 allergens from different dust mite species. Group 5 and Group 21 are homologous proteins in the dust mites. Characterization of these allergens in terms of their IgE reactivities and cross-reactivities leads to a better understanding on the clinical importance of these allergens to mite allergy. Epitope mapping of Blo t 5, the most allergenic molecule of Group 5 and Group 21 allergens will help to explain why this allergen is so important for *B. tropicalis* allergy and the data will provide useful information for generation of hypoallergen vaccine candidates in the future.

The specific aims of this study are:

1. To identify Blo t 21, a Blo t 5 homologue at DNA level and protein expression level using Southern blotting, Western blotting and immuno-localization in *B.*

tropicalis, and to further characterize the Blo t 21 allergen in terms of the IgE - binding activity in vitro and in vivo and cross-reactivity between Blo t 5 and Blo t 21.

2. To study the stability of IgE-binding to Blo t 5 and Blo t 21 by measuring the effect of temperature, pH and chemical denaturant.
3. To identify Der f 21 by Southern blotting and immuno-localization and to characterize its allergenicity by in vitro IgE-binding activity test and cross-reactivity study.
4. To identify Group 5 and Group 21 allergens in *Suidasia medanensis*, *Aleuroglyphus ovatus*, *Tyrophagus putrescentiae*, *Lepidoglyphus destructor* and *Glycyphagus domesticus* and to further compare a panel of eleven Group 5 and Group 21 allergens in local atopic population.
5. To identify specific residues which are important for the IgE-binding of Blo t 5 using site-directed mutagenesis and synthetic peptide mapping.

Chapter 2: Materials and Methods

2.1 Dust mite samples

Mite cultures of *B. tropicalis, D. pteronyssinus, D. farinae, S. medenesis* and *L. destructor* were purchased from the Central Science Laboratory (United Kingdom). Live mites were separated from the feed medium by "heat escape method". This was accomplished by placing the mite cultures on a mesh filter (pore size = 125μm) with a light source nearby. The mites pass through the mesh, away from the medium, and fall onto the receiving pan below when trying to get away from the heat. They were then harvested, weighed, wrapped in aluminum foil before frozen in liquid nitrogen and finally stored at -80^{o}C.

2.2 Cloning of Group 5 and Group 21 allergens and site-directed mutagenesis of Blo t 5

2.2.1 Bacterial strains

XL1-Blue *[N1]Δ(mcrA) 183Δ(mcrCB-hsdSMR-mrr)173 end A1 supE44 thi-1 recA1 gyr 1A96 relA1 lac[F'proAB lacI^{q}Z ΔM15Tn10(Tetr)]*

BL21* (DE3) F- *ompT hsdS*B (rB-mB-) *gal dcm rne131* (DE3)

2.2.2 Identification of Group 5 homologous allergens

ESTs generated from five dust mite species in our laboratory (Angus *et al.*, 2004) were compared with sequences from protein databases available at the National Center

for Biotechnology Information (NCBI) using Translated Basic Local Alignment Search Tool algorithm (BLASTX) (http://www.ncbi.nlm.nih.gov/). Group 5 homologues were identified based on the similarities of their primary protein sequences to Blo t 5, Der p 5 and Lep d 5. Blast results with E-values <0.001 were considered significant identities. cDNA clones homologous to Group 5 allergens were sequenced again for further confirmation of DNA sequences.

2.2.3 Computer-based characterization and analysis

Nucleotide sequences of putative Group 5 allergens were analyzed using DNAMAN version 4.15 (Lynnon BioSoft, Canada), BioEdit sequence alignment editor v7.0.5. (Hall, 1999), ClustalW (Thompson *et al*., 1994), Vector NTI (InforMax) and BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Restriction enzyme maps of nucleotide sequences were generated by webcutter 2.0 (http://www.firstmarket.com/cutter/cut2.html). Amino acid sequences were deduced from cDNA sequences using DNAMAN version 4.15 (Lynnon BioSoft, Canada). Signal peptide cleavage site was analyzed based on the deduced amino acid sequences using SignalP v3.0 (Bendtsen *et al.*, 2004). Secondary structure of the protein was predicted using PredictProtein (http://cubic.bioc.columbia.edu/predictprotein).

2.2.4 Phylogenetic tree generation

Der p 5, Der p 21, Blo t 5 and Lep d 5 protein sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov). Other Group 5 and Group 21 homologous protein sequences from *Blomia tropicalis, Dermatophagoides pteronyssinus, Dermatophagoides farinae, Suidasia medanensis, Glycyphagus domesticus* were obtained from EST

database of our laboratory. All EST sequences used showed homology to known Group 5 allergens in the non-redundant Database of GenBank using BLASTX algorithm (E-value < 0.001). Lep d 5 and Gly d 5 were two Group 5 allergens longer in length. The sequences were separated into two parts: N-terminal and C-terminal regions. The C-terminal regions contain the sequence with similar size of normal Group 5 allergen, while the N-terminal regions contain extra repeat sequence of Group 5. The protein sequences were aligned using ClustalW (Thompson *et al*., 1994). A phylogenetic tree was drawn based on the multiple alignment result using DNAMAN (Lynnon BioSoft, Canada) /BioEdit (Hall, 1999) with neighbor-joining algorithm.

2.2.5 RT-PCR method to isolate Der p 21 allergen

Total RNA was prepared from 250 mg of mites using RNeasy® Midikit (Qiagen GmbH, Germany) according to manufacturer's instructions. The quality of the RNA was examined by electrophoresis separation and spectrophotometer (OD_{260}/OD_{280}). 1μg of *D. pteronyssinus* total RNA was used to generate 5' RACE cDNA using SMART^{TM} RACE cDNA amplification kit (CLONTECH Laboratories, Inc. USA). Specific primers of Der p 21 (listed in Table 2.1) were used to amplify cDNA of Der p 5 and Der p 21. Same sets of primers were used for generation of expression clones.

2.2.6 Cloning of Group 5 and Group 21 allergens in expression vector and site-directed mutagenesis of Blo t 5

Oligonucleotide primers were designed corresponding to the cDNA fragments encoding proteins of Group 5 and Group 21 allergens except the signal peptide. The primers contained suitable restriction enzyme sites to facilitate downstream cloning

(Table 2.1). After the desired PCR products were amplified from cDNA clones, they were further inserted into either pET-15 (Novagen, Germany) or pET-M vectors depending on their restriction enzyme sites. pET15 (Novagen, Germany) and pETM are His-tag fusion vectors for over-expressing recombinant protein in *E.coli*. The expression clones were transformed into *E. coli* strain XL-1 Blue for DNA manipulation and BL21* (DE3) for protein expression (Novagen, Germany). Transformants were picked and employed as templates in PCR mixture to screen the correct clones. PCR was performed using *Taq* DNA polymerase (Fermentas, USA) according to manufacturer's instruction with clone specific primers, i.e. M13 Forward Sequencing Primer and M13 Reverse Sequencing Primer. PCR products were observed using gel electrophoresis. Listed in Table 2.2 are universal primers used for colony screening.

Site-directed mutations of Blo t 5 were generated using Quikchange® Site-directed mutagenesis kit (Statagene) and PCR method with primers containing desired mutation. The target amino acid was mutated to alanine. Primers were listed in Table 2.3. All the recombinant clones were verified by DNA sequence analysis.

Table 2.1 Primers used for generation of expression clones

Primer name	Primer sequence	Restriction enzyme	Amplicon	Vector
pET15-BT51F	ggga**catatg**caagagcacaagccaaagaagg	*NdeI*	Blo t 5 cDNA	pET15
pET15-BT51R	cg**ggatcc**ttattgggtttgaatatccttc	*BamHI*	Blo t 5 cDNA	pET15
pETM-BT52F	gcg**ggatcc**ctaccagtctctaacgataac	*BamHI*	Blo t 21 cDNA	pETM
pETM-BT52R	cgg**gaattc**ttattcggaatcttggactcgc	*EcoRI*	Blo t 21 cDNA	pETM
pET15-Dp5F	ggga**catatg**gaagataaaaaacatgatt	*NdeI*	Der p 5 cDNA	pET15
pET15-Dp5R	cg**ggatcc**ttaaacttcaatctttttaacac	*BamHI*	Der p 5 cDNA	pET15
pET15-DP52F	ggga**catatg**tttattgttggtgacaaaaaaga	*NdeI*	Der p 21 cDNA	pET15
pET15-DP52R	gcg**ggatcc**ttaataatattcatccggatttac	*BamHI*	Der p 21 cDNA	pET15
pET15-DF51F	ggga**catatg**gaaccaaaaaaacatgattatcaa	*NdeI*	Der f 5 cDNA	pET15
pET15-DF51R	cg**ggatcc**tttcaaacttcaatctttttaacacg	*BamHI*	Der f 5 cDNA	pET15
pETM-DF52F	gcg**ggatcc**gaagataaatggcgtaatgcat	*BamHI*	Der f 21 cDNA	pETM
pETM-DF52R	gcc**gaattc**ttaatcatccgattttacagctttaac	*EcoRI*	Der f 21 cDNA	pETM
pET15-LD5F	ccca**catatg**accggtgtcaagactca	*NdeI*	Lep d 5 cDNA	pET15
pET15-LD5R	cg**ggatcc**ttatttggtttcgatggcct	*BamHI*	Lep d 5 cDNA	pET15
pET15-Sm52F	ggga**catatg**gctgacaagaatgactttc	*NdeI*	Sui m 5.01 cDNA	pET15
pET15-Sm52R	cg**ggatcc**ttaggcgtggacattgatag	*BamHI*	Sui m 5.01 cDNA	pET15
pET15-Sm53F	ggga**catatg**gcggacaagaatgactt	*NdeI*	Sui m 5.02 cDNA	pET15
pET15-Sm53R	cg**ggatcc**ttagactttaattgctttg	*BamHI*	Sui m 5.02 cDNA	pET15
pET15-Sm51F	ggga**catatg**gcaccaacccagaacgac	*NdeI*	Sui m 21 cDNA	pET15
pET15-Sm51R	cg**ggatcc**ttacttgttgttggcatca	*BamHI*	Sui m 21 cDNA	pET15

Restriction enzyme sites are indicated in bold.

Table 2.2 List of universal primers used for screening of desired clones

Primer name	**Primer sequence**
M13 Forward Sequencing Primer	GTAAAACGACGGCCAGT
M13 Reverse Sequencing Primer	AGGAAACAGCTATGACCAT
pET upstream primer	GATGCGTCCGGCGTAGA
PETF	GTATGAAAGAAACCGCTGCTGC
PETR	CTAGTTATTGCTCAGCGG

Table 2.3 Primers used for generation of Blo t 5 mutants

Primer name	Primer sequence
N40F	CACTTGTTGATCGAACAGGCAGCCCATGCTATCGAAAAGG
N40R	CCTTTTCGATAGCATGGGCTGCCTGTTCGATCAACAAGTG
BH41AF1	GCTGCTATCGAAAAGGGAGAACATCAA
BH41AR1	GTTTGCCTGTTCGATCAACAAGTG
E44A-1	GGCAAACCATGCTATCGCAAAGGGAGAACATCAAT
E44A-1r	ATTGATGTTCTCCCTTTGCGATAGCATGGTTTGCC
Q49F	CGAAAAGGGAGAACATGCATTGCTTTACTTGCAACACCAACTCG
Q49R	CGAGTTGGTGTTGCAAGTAAAGCAATGCATGTTCTCCCTTTTCG
BN61AF1	GCTGAAAACAAGAGCAAGGAATTGCAAGA
BN61AR1	CAATTCGTCGAGTTGGTGTTGCAAG
BQ69AF1	GCAGAGAAAATCATTCGAGAACTTGA
BQ69AR1	CAATTCCTTGCTCTTGTTTTCATTC
C80F	CGAGAACTTGATGTTGTTGCCGCCATGATCGAAGGAGCC
C80R	GGCTCCTTCGATCATGGCGGCAACAACATCAAGTTCTCG
Q87A-1	CATGATCGAAGGAGCCGCAGGAGCTTTGGAACGTG
Q87A-1r	CACGTTCCAAAGCTCCTGCGGCTCCTTCGATCATG
E91A-1	CCCAAGGAGCTTTGGCACGTGAATTGAAGCG
E91A-1r	CGCTTCAATTCACGTGCCAAAGCTCCTTGGG
BY107AF1	GCAGAAGAGGCTCAAACTCTCAGCAAGAT
BY107AR1	GTTGAATCGTTCCAAAATGTTAAGA
E109A-1	CGATTCAACTACGAAGCGGCTCAAACTCTCAGC
E109A-1r	GCTGAGAGTTTGAGCCGCTTCGTAGTTGAATCG
BQ111AF1	GCAACTCTCAGCAAGATCTTGC
BQ111AR1	AGCCTCTTCGTAGTTGAATCGTTC
BT112AF1	GCTCTCAGCAAGATCTTGCTTAAGGATTTGA
BT112AR1	TTGAGCCTCTTCGTAGTTGAATCGTTC
S114A-1	GAAGAGGCTCAAACTCTCGCCAAGATCTTGCTTAAGGATTT
S114A-1r	AAATCCTTAAGCAAGATCTTGGCGAGAGTTTGAGCCTCTTC
BK115AF1	GCGATCTTGCTTAAGGATTTGAAGGA
BK115AR1	GCTGAGAGTTTGAGCCTCTTCGTAGTT
BT124AF1	GCCGAACAAAAAGTGAAGGATATTCAAA
BT124AR1	TTCCTTCAAATCCTTAAGCAAGATC

2.2.7 DNA sequencing

DNA sequencing reactions were performed according to the manual of ABI Prism^{TM} Dye Terminator cycle sequencing ready reaction kits (Applied Biosystem) which involved a 20 µl mixture containing 2 µl Big Dye v3.1 , 3 µl of 2.5 X Sequencing Buffer (Applied Biosystem,), 300-500 ng plasmid DNA template and 3.2 pmole primer. Cycle sequencing was carried out in PTC-100TM Programmable Thermal Controller (MJ Research, Inc., USA) with the following thermal cycling profile: denaturation at 96 °C for 30 sec, annealing at 50 °C for 15 sec, extension at 60 °C for 4 min and repeated for 30 cycles. After the cycle sequencing reaction, DNA mixtures were purified by ethanol precipitation. 2 µl of 3M sodium acetate (pH4.6), 2 µl of 125 mM EDTA and 50 µl of absolute ethanol were added to the reaction mixture and incubated on ice for 10 minutes, followed by centrifugation for 20 min at 13,000 rpm. The pellet was then washed with 500 µl 70% ethanol and centrifuged at 13,000 rpm for 15 min. The pellet was thoroughly air dried and re-suspended in 12 µl of Hi-Di formamide. The DNA mixture was subjected to Applied Biosystems 3100 fluorescent sequencer (USA) using default parameters.

2.3 Genomic study

2.3.1 Preparation of mite total genomic DNA

Total DNA of the mites was isolated using plant DNA extraction method described by Dixit (1998). The total DNA was purified by repeating the chloroform extraction, followed by ethanol precipitation. Finally, the DNA pellet was washed with 70% ethanol, air-dried and dissolved in 10 mM Tris-HCl buffer (pH 8.0).

2.3.2 Isolation of the genomic organization

Polymerase Chain Reaction (PCR) was used to amplify the genomic organizations of Blo t 5, Blo t 21, Der f 5 and Der f 21 from the total mite genomic DNA. Gene-specific primers were designed according to the 5'- and 3'-end sequences of the open reading frame (ORF). Primer sequences are listed in Table 2.4. The PCR was performed with BD Advantage™ 2 PCR Kits (Clontech, BD Biosciences, USA) using 100 ng of the genomic mite DNA as a template. The amplicons with correct size were cloned into pGEM®-T Easy Vector (Promega, USA) following manufacturer's instruction. After preparation and purification of the plasmid DNA using Qiaprep Miniprep Kit (Qiagen, Germany), the plasmid DNA was sequenced from both 5'- end and the 3'- end with the M13 forward and reverse primers using ABI PRISM Big Dye Terminator v3.1 reagent (Applied Biosystems, USA) by quarter reaction. The products were processed on ABI Prism 3100 Genetic Analyzer/ DNA Sequencer (Applied Biosystems, USA) after cleaned up with ethanol precipitation.

Table 2.4 List of primers used for amplification of genomic DNA fragments

Primer name	**Primer sequence**	**Amplicon**
BT5-1f	ATGAAGTTCGCCATCGTTCTTATTGCC	Blo t 5 genomic DNA
BT5-1r	TTTATTGGGTTTGAATATCCTTC	Blo t 5 genomic DNA
BT5-2f	ATGAAATTTATCATCGCATTGGC	Blo t 21 genomic DNA
BT5-2r	CTATTCGGAAGCTTGGACTCGC	Blo t 21 genomic DNA
DF5-1f	ATGAAATTCATCATTGCTATTGCT	Der f 5 genomic DNA
DF5-1r	TTTCAAACTTCAATCTTTTTAACACG	Der f 5 genomic DNA
DF5-2f	AATGAAATTCATTATTTTCTGTGCC	Der f 21 genomic DNA
DF5-2r	TAATCATCCGATTTTACAGCTTTAAC	Der f 21 genomic DNA

2.3.3 Southern blotting

The total genomic DNA of *B. tropicalis* was completely digested with *Hind III* and *EcoR V*, while total genomic DNA of *D. farinae* was completely digested with *Cla I*, *Hind III* and *EcoR.* These restriction enzyme sites were not present in target genes. The digested genomic DNA was transferred onto a positively-charged nylon membrane (Roche Applied Science, Germany) and then immobilized on the membrane using CL-1000 Utraviolet crosslinker (UVP, USA).

DNA probes were labeled by digoxigenin with PCR probe synthesis kit (Roche Applied Science, Germany) using the plasmid containing Blo t 5, Blo t 21, Der f 5 and Der f 21 gene as a template individually.

The membrane carrying dust mite genomic DNA was hybridized to digoxigenin-labeled DNA probe following the protocol of non-radioactive DIG application manual (Roche, Germany). The detection of the hybridization signal on the DNA blot was performed using immunochemical techniques by testing an alkaline phosphatase conjugate with an anti-digoxigenin antibody. The hybrid bands were exposed to x-ray film after applying the chemiluminescent alkaline phosphatase subtracts CSPD to the membrane.

2.4 Protein expression, purification and CD analysis

2.4.1 Expression and purification of wild type and mutant allergens

Expression clones were transformed into *E. coli* strain BL21*(DE3) (Novagen, Germany). 100 ml cultures were grown to OD_{600} at approximately 0.6 and then induced with 0.5 mM IPTG overnight at 23 °C with continuous shaking at 200 rpm. Cells were

harvested by centrifugation at 2845 g for 15 min at 4 °C and re-suspended in 20 ml of binding buffer (5 mM imidazole, 0.5M NaCl, 20 mM Tris-HCl, pH 7.9). The cells were sonicated on ice for 2 min. Soluble cell lysis was separated from the supernatant by centrifugation at 15,000 x g for 30 min at 4 °C. The over expressed His-tag fusion proteins were purified by Ni-NTA affinity chromatography using the protocol provided by the pET system manual (Novagen, Germany). Briefly, soluble proteins of cell lysis were incubated with charged Ni-NTA resin (Novagen), and the bound proteins were then washed with wash buffer (60 mM imidazole, 0.5 M NaCl and 20 mM Tris-HCl, pH 7.9) and finally eluted with elution buffer (0.5 M imidazole, 0.5 M NaCl and 20 mM Tris-HCl, pH 7.9). The purified proteins were electrophoresed on a 15% SDS-PAGE gel, and the protein bands were visualized with Coomassie blue staining.

2.4.2 Circular Dichroism spectrum

In order to check whether the recombinant protein was folded properly, Circular Dichroism (CD) spectra of recombinant proteins were measured using a Jasco J810 CD spectropolarimeter equipped with PFD-425S Peltier temperature controller. The recombinant proteins were dialyzed in 10 mM sodium phosphate buffer (pH 7.4). 200 µl of samples (0.1mg/ml) were applied in a 1cm quartz cuvette. The spectra were measured at 20 °C and collected from 190-260 nm with a data pitch of 0.1nm. A band width of 1nm was used with a detector response time of 8 seconds and scanning speed of 50nm/min. The spectrum was an average of 8 scans. For thermal denaturation study, the scanning was collected from 10 °C to 90 °C at 1 °C/min with intervals of 10 °C.

2.5 Immunoassays

2.5.1 Patient sera

Sera were obtained from 43 patients with ongoing persistent allergic rhinitis (PAR) and with at least two reporting symptoms (nasal obstruction, rhinorrhea, sneezing and itchy nose) on most days during the past year (Bousquet *et al.*, 2001), who were scheduled to start sublingual immunotherapy. The characteristics of the patients are listed in Table 2.5. Their sensitization to both *B. tropicalis* and *Dermatophagoides spp.*, the almost exclusive pattern of PAR in Singapore, was confirmed by a positive skin prick (SPT) reaction. In addition, all had a positive nasal challenge response to crude extracts (0.06mg/ml) of *B. tropicalis*. The challenge procedure was described previously by Wang *et al.* (2003). Sera from six healthy adult volunteers with no history of any allergic symptoms and a negative SPT to common inhalant allergens were obtained as negative controls.

In addition, sera from 494 consecutive individuals attending outpatient allergy clinics in the hospital over one and a half years for allergic conditions were screened by an immuno-dot blot. A total of 262 were children below the age of 12 years (mean age 7.2 years, 59% males, 78% Chinese, 74% had allergic rhinitis, 37% asthma, 60% atopic dermatitis and 14% had clinical manifestation of all three). The remaining 232 were adults (mean age 31.2 years, 52% males, 80% Chinese, 100% had allergic rhinitis and 35% current or childhood asthma). The sera were collected by three batches. Approval to conduct these studies was obtained from the Institutional Review Boards of the National Healthcare Group, KK Women's and Children's Hospital, and Singapore General Hospital.

Table 2.5 Characteristics of the study patients (n=43)

	Descriptive data Number (%)	**Measurements** Mean (±SD)
Sex		
Male	21 (49%)	
Female	22 (51%)	
Age (year)	31.5 ± 9.81	
Ethnic representations		
Chinese	35	
Malay	3	
Indian	2	
Other	3	
Duration of diseases (year)		
Persistent allergic rhinitis	43 (100%)	
Asthma	9 (21%)	
Childhood asthma	6 (14%)	
Duration of diseases (year)	14.91 ±6.92	
Nasal symptom score*		
Itchy nose		2.00 ± 0.87
Sneezing		2.23 ± 0.72
Rhinorrhea		2.53 ± 0.67
Nasal obstruction		2.51 ± 0.70
Skin Prick test† (Wheal size mm)		
Der p		8.22 ± 4.04
Der f		9.05 ± 3.40
Blo t		8.43 ± 2.74
Blo t (house made‡)		7.43 ± 1.98
IgE test (CAPs) (% Positive)		
Total IgE (>100 IU)		81% (35/43)
(>50 IU)		98% (41/43)
Der p (>0.5 IU/ml, class 3 and above)		93% (40/43)
Der f (>0.5 IU/ml, class 3 and above)		98% (42/43)
Blo t (>0.5 IU/ml, class 3 and above)		95% (41/43)

*: Symptoms are present more than 4 days a week during the past year. These symptoms were recorded using a symptom severity scale: 0 = none: no symptom evident; 1 = mild: symptom clearly present but minimal awareness; 2 = moderate: definite awareness of symptom which is bothersome but tolerable; 3 = severe: symptom is hard to tolerate.

†: Standard crude allergen extracts from Stallergene.

‡: Crude allergen extract made by our own lab

2.5.2 Skin prick test

Skin prick test with the Blo t 5 and Blo t 21 recombinant proteins (0.01μg/ml in PBS with 50% glycerol) was performed using a single prick technique (Solofix, B. Braun, Germany). Negative (PBS with 50% glycerol) and positive controls (histamine 10mg/ml and *B. tropicalis* crude extract (Stallergene))were used for comparison. A wheal response >3mm compared to the negative after 20 minutes was scored as positive.

2.5.3 ELISA for quantification of serum specific IgE

1μg/ml recombinant proteins were coated onto Maxisorp plates (NUNC, Denmark) and incubated overnight at 4°C. The plates were blocked with PBS-0.1% Tween 20 at room temperature for 1 hour, washed with PBS-0.05% Tween 20 and then incubated with 50μl of diluted sera (1:1 ~ 1:20 (v/v) in PBS) at 4°C overnight. Plates were washed, incubated with 100 μl biotinylated anti-human IgE antibodies (1:250 v/v in PBS) at room temperature for 2 hours, and subsequently amplified with avidin-horse radish peroxidase (both from BD Pharmingen, USA) for half an hour at room temperature. Microtitre plates were thoroughly washed with PBS-T between each step. Finally, 100 μl of 3,3',5,5'-tetramethylbenzidine (Sigma) substrate was added and color development was stopped by adding 20 μl of 1M hydrochloric acid after 30 minutes. Color intensity was read using a micro-titer plate reader at 450 nm. Intensities above 2 SD of the mean of negative sera responses were considered positive.

2.5.4 Immuno-dot blot analysis

1 μg of each recombinant protein was dotted onto nitrocellulose membranes together with series diluted IgE standard (National Institute for Biological Standards, United Kingdom) as positive control, 1 μg of BSA as negative protein control and 1 μl of buffer as negative control. Membranes were air dried and blocked with PBS-0.1% Tween-20 and incubated with sera (1:1 v/v in PBS) at 4 ºC overnight, followed by incubation with anti-human IgE antibodies conjugated with alkaline phosphatase (Sigma) (1:1000 v/v in PBS) for 2 hours. The membranes were then developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate (Promega) for 30 minutes and spot intensities were measured using imaging software (Microimage^{TM} v.3.01, Germany). Spot intensities (ranging from 0-255) were normalized by subtracting the local background and intensities >20 (equivalent to 2 standard deviations [SD] above the mean negative sera responses) were considered positive. The inter- and intra-assay concordance was above 90% and 95% respectively, demonstrating good reproducibility of the assay.

2.5.5 Competitive cross-inhibition ELISA

Inhibition ELISA was carried out using the similar protocol described above except that sera were first absorbed with serial 10-fold dilutions of allergens (from 1ng/ml to 100μg/ml) overnight prior to the assay. We also performed end-point inhibition ELISA using 10 sera. Sera were incubated with 0.1mg/ml of specific allergens overnight prior to the assay. The percentage of inhibition was calculated using the formula:

$$\frac{(OD_{450} \text{ without inhibitor} - OD_{450} \text{ with inhibitor})}{(OD_{450} \text{ without inhibitor} - OD_{450} \text{ blank control})} \times 100$$

2.5.6 Effect of temperature, pH and urea on the IgE-binding of Blo t 5 and Blo t 21

For thermal-denaturation study, recombinant Blo t 21 and Blo t 5 were heated at 50°C, 70°C, and 90°C for 10 min. After cooled down to 20°C, proteins were subsequently coated on 96 well plate for ELISA assay as described in section 2.5.3 using seven sera.

The effect of pH was assessed by diluting allergens to 0.1 mg/ml in 200 mM NaOAc (pH4 and pH5), PBS buffer (pH7.4), Cabornate/ biocarbonate buffer (pH9.6), 20 mM HC1 (pH 2.0) and 20 mM NaOH (pH 12.0) individually. After 2 hours at room temperature, the samples were coated on 96 well plates for further ELISA assay as described in section 2.5.3.

To determine the effect of protein denaturants, Blo t 5 and Blo t 21 were treated with 6 M urea in PBS (pH 8.5) for 2 hours and then coated on 96 well plates for ELISA assay as described in section 2.5.3.

2.5.7 Specific IgE-binding to overlapping peptide of Blo t 5

For overlapping peptide mapping, the ELISA procedure described above was slightly modified. Briefly, 100 ug/ml of peptides were coated on Streptavidin coated plate (Pierce). Patient sera used in this study were diluted in 1:1(v/v) with PBS buffer. The secondary antibody was replaced by anti-human IgE antibodies conjugated with alkaline phosphatase (Sigma) (1:250 v/v in PBS). p-Nitropenyl phosphate (PNPP) (Sigma Aldrich, USA) was used as substrate for the assay, and colour reaction was developed for

approximately 30 minutes. Colour intensity was measured using a micro-titer plate reader at 405 nm.

2.5.8 Specific antibody production

Polyclonal antibodies against specific allergens were raised in New Zealand White Rabbits (2.5 to 3 kg). The rabbits were subcutaneously injected with 300µg of recombinant protein diluted in 500 µl of PBS with 500 µl of Freund's complete djuvant (Sigma). Booster shots were given every 3 weeks with the same amount of recombinant protein with incomplete Freund's adjuvant (Sigma) until high titers were obtained. The sera collected were kept at 4^{o}C overnight for complete clotting and then centrifuged at 3000 rpm for 20 minutes. Sera were stored at -20^{o}C.

2.5.9 Western blotting

Whole mite extracts prepared by trichloroacetic acid-acetone method (Damerval *et al.*, 1986) were separated on 15% SDS-PAGE, together with the recombinant proteins in parallel lanes, and transferred onto Hybond nitrocellulose membranes (Amersham Bioscience, USA). Western blotting was performed according to the protocol provided by Amersham Bioscience. Briefly, the membranes were blocked with 0.1% PBST, incubated with rabbit polyclonal antibody (1:2000 v/v in PBST), and then interacted with alkaline phosphatase conjugated goat anti-rabbit secondary antibody (1:2000 v/v in PBST). The specific band was detected by developing with BCIP/NBT (5-bromo-4chloro-3indolyl-phosphate / nitro-blue tetrazolium) colour substrate (Promega, USA).

2.5.10 Immuno-localization

To immuno-localize the allergens on mite sections, live mites were fixed, dehydrated, cleared, and embedded in paraffin and then sectioned into slides of 8μm thick. The sections were de-paraffinized, hydrated, and incubated with the respective antibodies following the western blotting procedure. To remove the non-specific IgG binding, anti-Blo t 21 sera were pre-absorbed with 0.5mg/ml rBlo t 5.

2.5.11 Dust sample collection and processing

Dust samples were collected from kitchens, sofas, carpets and bedrooms from 56 homes in Singapore. Sampling was performed using a modified Kirby Classic III vacuum cleaner (Kirby Co., Cleveland, Ohio, USA) adapted with a chamber that collected dusts onto a filter paper. Each sample was obtained by vacuuming an area of 1 m^2 for 2 mins. All dust samples were stored at -20 ºC until further use. The dust samples were sieved using a 500 μm pore size sieve. 1 ml of PBS was added to every 50 mg of dust sample. Samples were shaked overnight at 4ºC and then centrifuged at 2500 rpm for 20 mins at 4ºC, and the supernatant was stored at -20ºC.

2.5.12 Measurement of allergen in dust samples

100 μl of dust samples were incubated overnight onto 96 well plates (Maxisorp, Nunc) at 4°C. Plates were blocked with 1% bovine serum albumin (BSA) in PBS for half an hour at room temperature and then washed three times with PBS-T (0.05%). The wells were incubated with 100 ul of rabbit sera at 1:5000 dilution in PBS for 1 hour at room temperature. Wells were then washed and incubated with 1:1000 dilution of horseradish

peroxidase conjugated anti-rabbit IgG (BD Pharmingen, USA) in PBS for 1 hour at room temperature. Wells were thoroughly washed before TMB (Sigma) was added. The reaction was stopped using 20 μL of 1M HCl and plates were read at 450 nm. Allergen levels were quantified and denoted as microgram of allergen per gram of fine dust (μg/g).

2.6 Statistical analysis

Statistics were analysed using SPSS 11.5 for Windows (SPSS, USA). Two standard deviations (SD) above negative reactions were used as the cut off points for positive results. Subsequently, 4 SD and 8 SD were used as cut off points for medium and high reactions respectively. All concordances were based on positive and negative reactions. Spearman's Correlation Test was used for all correlation analyses. Cluster Analysis was performed using SAS version 9.1 statistical programme (SAS, USA).

Chapter 3: Identification and Characterization of a Novel Allergen from *Blomia tropicalis*: Blo t 21

3.1 Introduction

Blomia tropicalis is a common house dust mite in tropical regions, usually found together with *Dermatophagoides pteronyssinus* and/or *D. farinae,* and is predominant in the areas of Singapore, Malaysia, Brazil, Colombia, and Puerto Rico (Zhang *et al.*, 1997; Chew *et al.*, 1999; Mariana *et al.*, 2000; Baqueiro *et al.*, 2006; Fernandez-Caldas *et al.*, 1993; Montealegre *et al.*, 1997b; Croce *et al.*, 2000). High prevalence of sensitization to *B. tropicalis* is strongly associated with the presentation of allergic diseases in these countries (Chew *et al.*, 1999c; Puccio *et al.*, 2004; Fernandez-Caldas *et al.*, 1993b).

Many studies have shown that allergens from *B. tropicalis* are species-specific with low to moderate cross-reactivity to other dust mite allergens (Chew *et al.*, 1999b; Stanaland *et al.*, 1994; Morgan, *et al.*, 1996; Johansson *et al.*, 1997). At least 25 IgE-binding protein bands have been identified in *B. tropicalis* by immunoblotting (Stanaland *et al.*, 1994), of which ten allergens (Blo t 1, Blo t 3, Blo t 4, Blo t 5, Blo t 6, Blo t 10, Blo t 11, Blo t 12, Blo t 13 and Blo t 19) have been officially named and deposited into the allergen database (www.allergen.org) (Mora *et al.*, 2003; Flores *et al.*, 2003; Arruda *et al.*, 1997; Yi et al., 2002; Ramos *et al.*, 2001; Puerta *et al.*, 1996; Caraballo *et al.*, 1997). Many other components still remain to be described. Most *B. tropicalis* allergens identified thus far have shown sequence homology to *Dermatophagoides spp.* allergens except Blo t 12 and Blo t 19 which have not yet been found in *Dermatophagoides spp.*.

Blo t 5 is a major allergen of B. tropicalis. A study in Taiwan showed that Blo t 5 was the most prevalent *B. tropicalis* IgE-binding allergen, followed by Blo t 12, Blo t 13 and Blo t 6. Less than 10% of patients responded to Blo t 4 and Blo t 10 (Tsai *et al.*, 2003). Blo t 1 has been reported to be another major allergen (Mora *et al*., 2003; Cheong *et al.*, 2003b). It had low cross-reactivity to Der p 1 or Der f 1 (Cheong *et al.*, 2003b), but experimental results from our laboratory seem to suggest otherwise (Angus *et al.*, 2004).

Our laboratory recently surveyed the transcriptoms of *B. tropicalis* using an Expressed Sequence Tag (EST) strategy. ESTs were obtained from sequences of randomly picked *B. tropicalis* cDNA clones. With this database, we were able to predict many more allergens from *B. tropicalis* through bioinformatic approach. These antigens shared sequence homology to allergens from mites, fungi, pollen, food sources and insects (Ramos *et al*., 2001), including a novel antigen which shared a 64% of sequence similarity to Blo t 5. This novel antigen could be a product of duplication gene of Blo t 5 in *B. tropicalis* genome. Duplication genes sharing a common ancestor in the genome are called paralog genes. In order to verify this antigen, specific IgE antibody response to this antigen was measured in 494 atopic individuals by immuno-dot blot screening and 43 allergic patients with persistent rhinitis by ELISA. Skin prick test was performed in the same group of rhinitis patients to examine whether this antigen can cross-link specific IgE on mast cells and trigger the releasing of proinflammatory mediators such as histamine. ELISA inhibition assays was further performed to investigate the relationship between this antigen and its homologous allergens Blo t 5, Der p 5 and Lep d 5.

3.2 Results

3.2.1 Identification of a novel Blo t 5 homologue (Blo t 21) from *B. tropicalis* EST database

Using the Translated Basic Local Alignment Search Tool algorithm (BLASTX), *Blomia tropicalis* EST database generated from our laboratory (Angus et al., 2004) was compared with sequences from protein databases available at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). Three *B. tropicalis* ESTs were found to have approximately 40-56% amino acid similarity to the Blo t 5 sequence in the Genbank (accession no. AAD10850). These included two full-length clones (accession no. CB282881 and CB283036) and one truncated cDNA which encoded only the C-terminal region (accession no. CB283289). These ESTs can be assembled into a 605-base-pair contig sequence, containing an open reading frame (ORF) of 390 base-pairs (Figure 3.1). Using the 5'- and 3'-end sequence of this contig, specific primers were designed and the full length open reading frame was amplified. The ORF encoding the Blo t 21 protein (accession no. AY800348) has 129 amino acids, including a putative 16-amino acid signal peptide predicted by SignalP (Bendtsen *et al.*, 2004). The presence of a signal peptide suggests that Blo t 21 is a secreted protein. The calculated molecular weight of the mature protein is 13.2 kDa, with a predicted isoelectric point of 5.21, and a predicted secondary structure of the protein made up of mainly alpha-helixes (PredictProtein, http://cubic.bioc.columbia.edu/predictprotein/) (Figure 3.2). The two full-length cDNAs were identical except at position 363 (T>C). Nevertheless, the amino acid coded by the affected codon remained the same (leucine).

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1          CTAGCGGATCCCCCGGGCTGCAGGCTTTTGTAGAACCAATCTCAAACCTTCCAATTAAAC

61         CAACAATGAAATTTATCATCGCATTGGCTGCCCTCATCGCAGTGGCATGTGCCCTACCAG
                 M  K  F  I  I  A  L  A  A  L  I  A  V  A  C  A  L  P

121        TCTCTAACGATAACTTCCGTCACGAGTTTGACCATATGATCGTCAACACCGCTACTCAAC
           V  S  N  D  N  F  R  H  E  F  D  H  M  I  V  N  T  A  T  Q

181        GATTCCATGAGATTGAAAAATTCTTGTTGCATATTACTCATGAAGTTGATGATTTGGAAA
           R  F  H  E  I  E  K  F  L  L  H  I  T  H  E  V  D  D  L  E

241        AAACCGGCAACAAAGATGAGAAGGCACGACTTCTCCGAGAACTTACCGTTTCTGAGGCTT
           K  T  G  N  K  D  E  K  A  R  L  L  R  E  L  T  V  S  E  A

301        TCATCGAAGGATCAAGAGGATATTTCCAACGGGAACTCAAGCGAACTGATTTGGATTTGC
           F  I  E  G  S  R  G  Y  F  Q  R  E  L  K  R  T  D  L  D  L

361        TCGAGAAATTCAACTTTGAAGCTGCATTGGCAACCGGTGATTTGTTGTTGAAGGATCTTA
           L  E  K  F  N  F  E  A  A  L  A  T  G  D  L  L  L  K  D  L

421        AGGCTCTCCAAAAGCGAGTCCAAGATTCCGAATAGAAAGTTCGGTAAAGTCTCGTAGCAG
           K  A  L  Q  K  R  V  Q  D  S  E

481        AAACTTTTGCAAACATCCTCGTTTCTGAAAATAGTGTTCTACATGATGATTTTTTGTCAA

541        TTGATTTGTTTTCAAAAAGATAATAAAATTATCAATTAGATTTGTTTAAAAAAAAAAAAA

601        AAAAAAAAA
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Figure 3.1 Nucleotide and amino acid sequence of Blo t 21. The signal peptide of Blo t 21 is underlined. Start and stop codons of open reading frame are boxed.

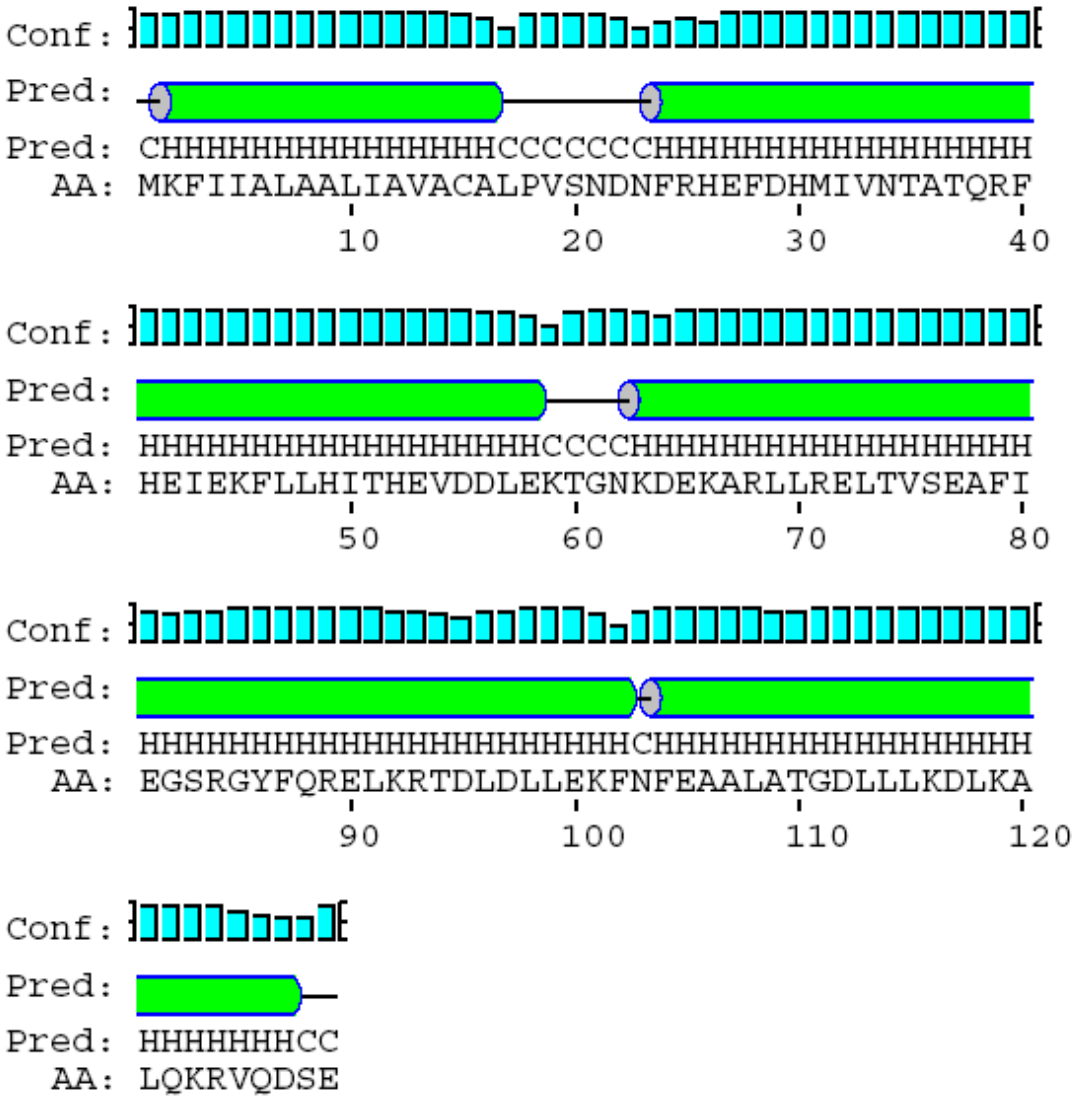


Figure 3. 2 Predicted secondary structure of Blo t 21 by PredictProtein (http://cubic.bioc.columbia.edu/predictprotein/). represents α helix. represents the confidence of prediction. —— represents random coil.

The Blo t 21 sequence, together with its orthologue, Der p 21 (accession no. ABC73706), and other Group 5 allergens from different mite species available in public database were aligned using ClustalW (Thompson *et al*., 1994). Multiple alignments showed that Blo t 21 shared 30%-41% amino acid sequence identity and 45%-59% sequence similarity to Der p 21, Blo t 5, Der p 5, and Lep d 5 throughout the entire length of the protein sequence (Figure 3.3). Pairwise, Blo t 21 shared 41% (52/128) amino acid identity (59% similarity) with Der p 21, and 39% (50/128) amino acid identity (54% similarity) with Blo t 5. In comparison, Der p 21 shared 31% (43/140) amino acid identity (45% similarity) with Der p 5, while Blo t 5 and Der p 5 shared 41% (55/134) amino acid identity (53% similarity). However, no clear conserved domain could be observed within and between the Group 21 and Group 5 protein sequences. Nevertheless, based on the Food and Agriculture Organization – World Health Organization (FAO/WHO) criteria for potential allergenicity (Rome and Agriculture Organization of the United Nations (FAO); 2001), the new gene product shared more than 35% similarity to known allergens over a window of at least 80 amino acids and was considered as potentially allergenic.

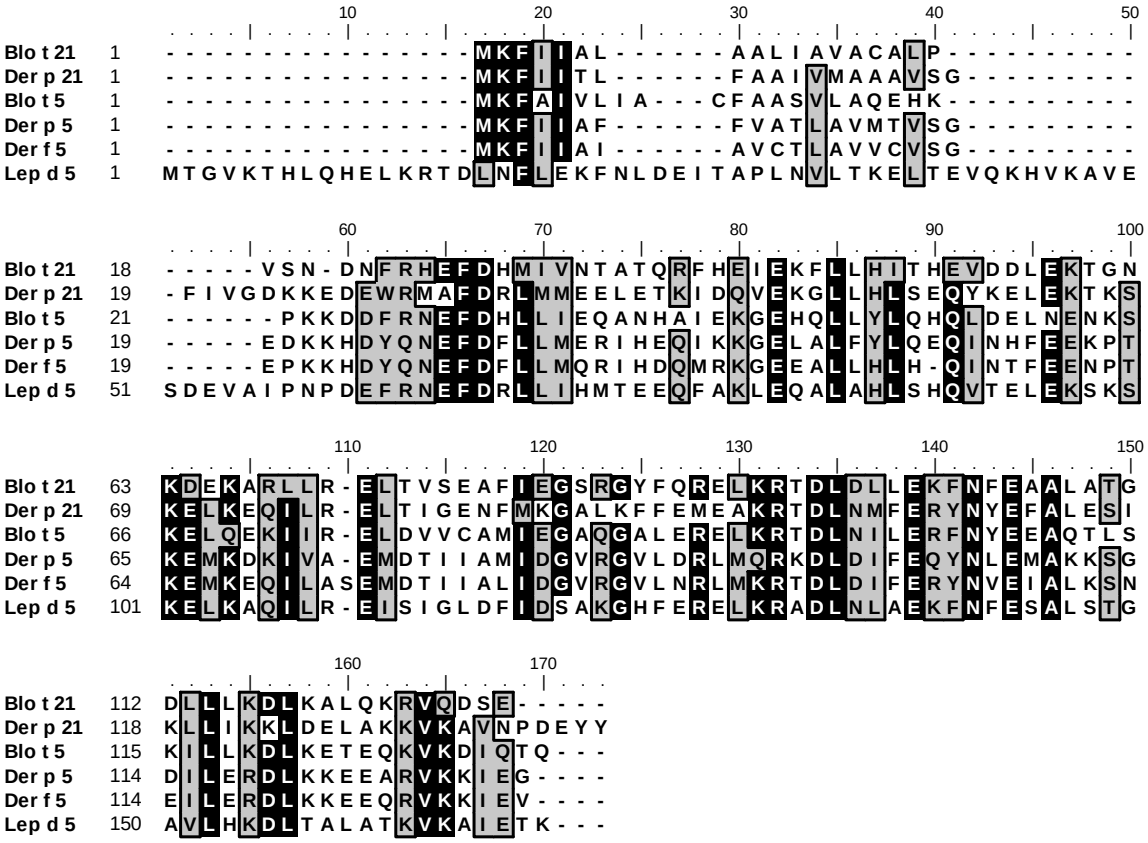


Figure 3.3 Multiple alignments of Blo t 21 protein sequence with Der p 21, Blo t 5, Der p 5, Der f 5 and Lep d 5 protein sequences. Identical and similar sequences are shaded with black and grey respectively.

3.2.2 Genomic organizations encoding Blo t 21 and Blo t 5

Genomic components encoding Blo t 21 and Blo t 5 were isolated by PCR and subsequently cloned into the pGEMT vector. Sequences of ten individual Blo t 21 clones showed that this gene consisted of one intron that interrupted amino acid 18 of the protein. The intron is 56 base pairs long, starting from GT at position 47 and ending with AG at position 102 (Figure 3.4). Four clones (clones 1, 3, 5, 10) were found to be identical to each other, and the others showed nine nucleotide substitutions at eight positions (Table 3.1). One substitution was found in the intron while the remaining seven were in exon coding regions. Four of the exon substitutions affected the amino acids that they coded for. Protein variants occurred twice at V33 to I, once at position T35 to A, once at K59 to E and once at L69 to P. In addition, one nucleotide substitution was observed from one cDNA clone (EST Bt 1304) of Blo t 21 when cDNA and gDNA sequences were aligned. This substitution did not affect the primary protein sequence of Blo t 21.

In contrast, the Blo t 5 genomic DNA sequence revealed a 57 base pairs intron in the 5' end as well (Figure 3.5). From 10 individual clones sequenced, three clones had identical sequences. Eight substitutions were found in the exon region including two substitutions causing protein variants. The variants were at E19>G and K23>E, and both occurred only once in the 10 clones (Table 3.2). The genomic DNA of Blo t 5 and Blo t 21 shared only 50% nucleotide sequence identity (Figure 3.6).

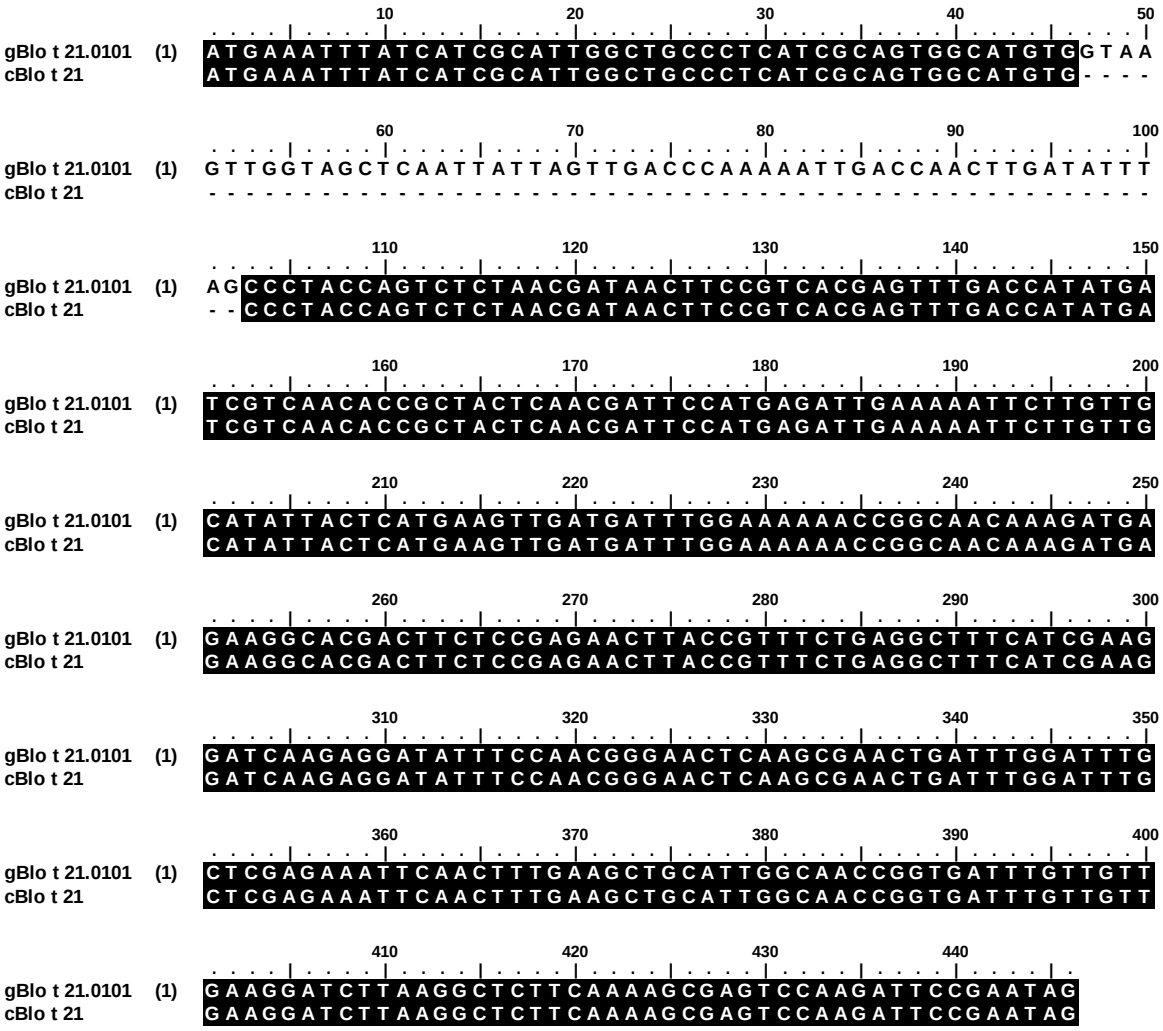


Figure 3.4 Alignment of Blo t 21 cDNA and genomic DNA (gBlo t 21.0101) revealed an intron of 56 base pairs.

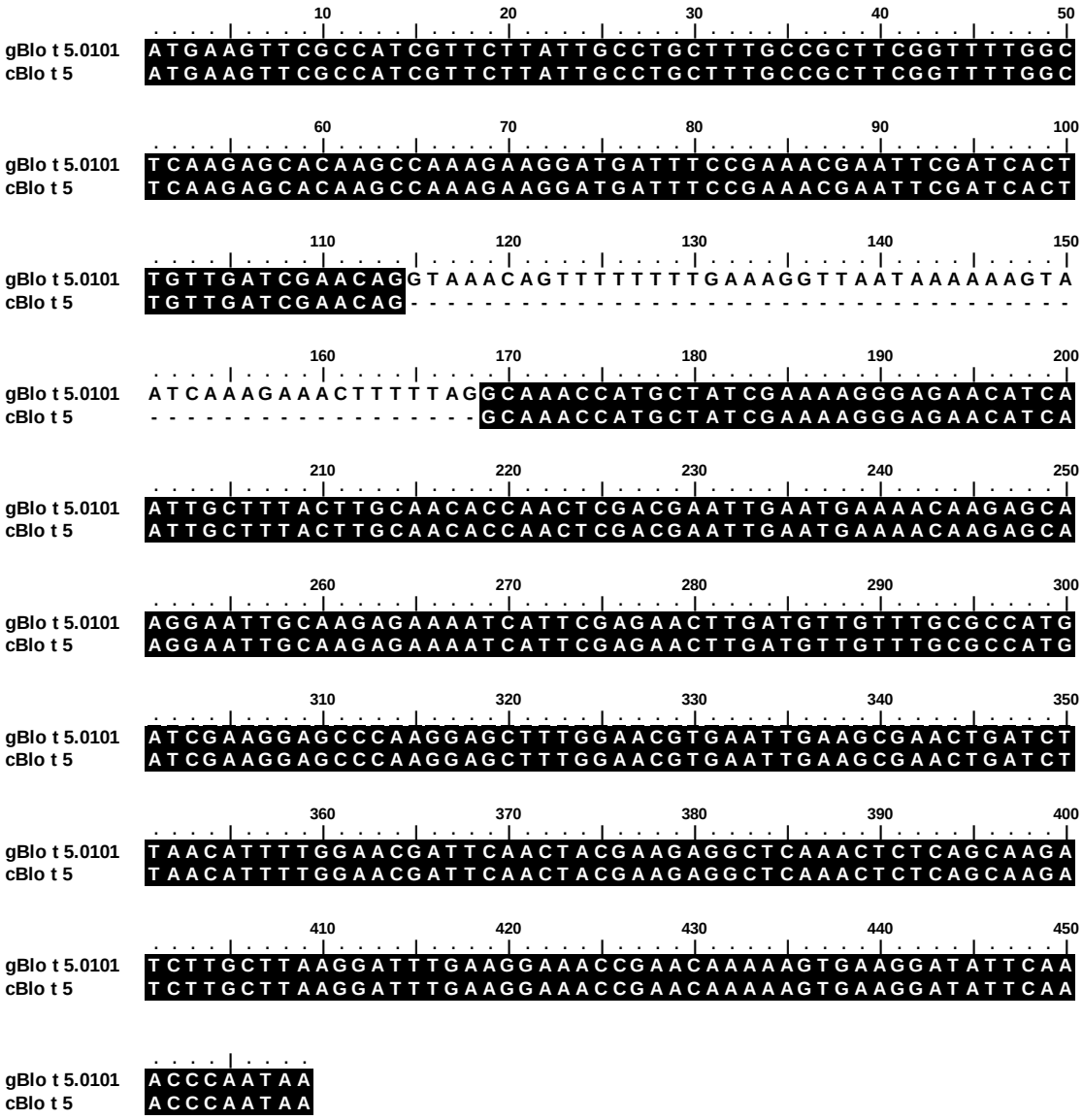


Figure 3.5 Alignment of Blo t 5 cDNA and genomic DNA (gBlo t 5.0101) revealed an intron of 57 base pairs.

Table 3.1 Polymorphisms of genomic components of Blo t 21.

Nucleotide residue Number	AA residue Number	Reference (gBlo t 21.0101*)	Polymorphic (triplet/AA)	Clones contain polymorphism	Frequency
80		a	g (intron)	2	10%
154	33	gct/V	atc/I	7,8	20%
159	35	acc/V	gcc/A	6	10%
167	37	act/T	acc/T	6	10%
231	59	aaa/K	gaa/E	9	10%
262	69	ctt/L	cct/P	3	10%
284	76	tct/S	tcc/S	8	10%
389	111	ggt/G	ggc/G	2	10%
419	121	ctc/L	ctt/L	cDNA (EST 1304)	

* gBlo t 21.0101 refers to the sequence of genomic DNA clones 1, 4, 5, 10.

Table 3.2 Polymorphisms of genomic components of Blo t 5

Nucleotide residue Number	AA residue Number	Reference (gBlo t 5.0101*)	Polymorphic (triplet/AA)	Clones contain polymorphism	Frequency in 10 clones
30	10	tgc/C	tgt/C	10	10%
56	19	gag/E	ggg/G	2	10%
67	23	aag/K	gag/E	11	10%
130	intron	ttt	tttt (i17 insertion)	9,10	20%
130	intron	ttt	ttttt (i17,18 insertion)	11	10%
225	57	ctc/L	ctt/L	9,10	20%
270	72	atc/I	att/I	3	10%
288	78	gtt/V	gta/V	6	10%
321	89	gct/A	gcc/A	4	10%
345	97	act/T	acc/T	7	10%

* gBlo t 5.0101 refers to the sequence of genomic DNA clones 1, 5, 8.

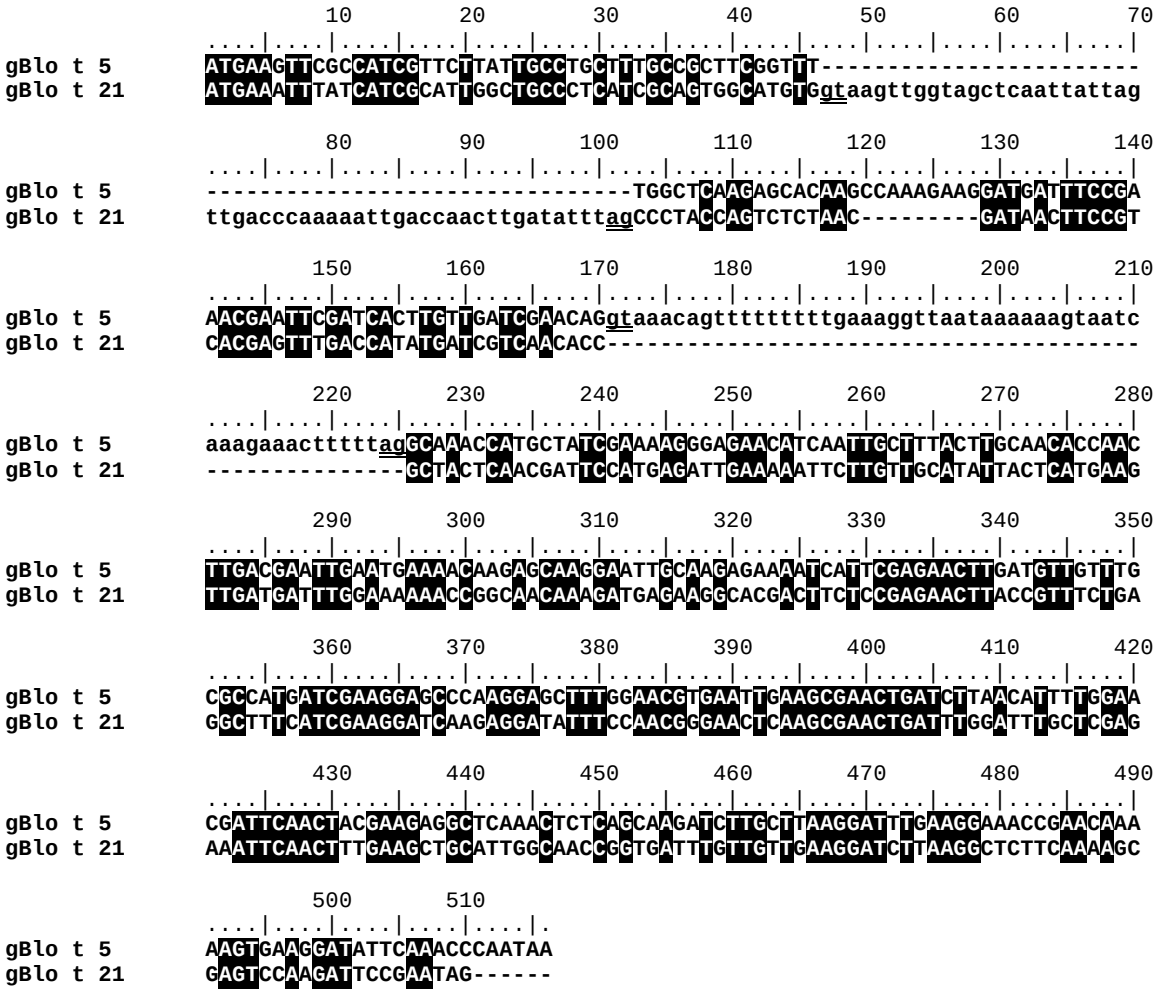


Figure 3.6 Comparison between the genomic organization of Blo t 5 and Blo t 21. The intron borders are underlined. Identical sequences are shaded with black color.

3.2.3 Southern blotting

The Blo t 21 and Blo t 5 genes were further examined in *B. tropicalis* using Southern blotting. Upon hybridization, a single band each for Blo t 21 and Blo t 5 was observed in each lane loaded with enzymes digested *B. tropicalis* genomic DNA (Figure 3.7 A and B). The hybridization patterns for both genes were however different. Hence, Blo t 21 and Blo t 5 were found to be single-copy genes located at different loci of the *B. tropicalis* genome. The nucleotide substitutions in the genomic sequences observed earlier were probably resulted from polymorphisms among different mite individuals rather than different copies of the gene in each mite.

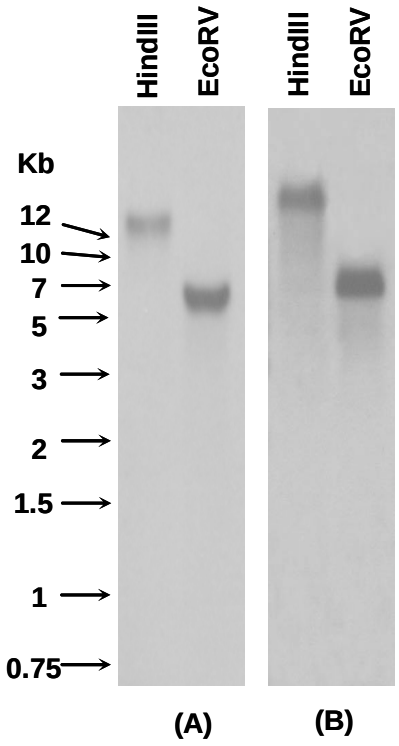


Figure 3.7 Southern blot analysis of *B. tropicalis* using Blo t 5 and Blo t 21 probes. A. Hybridization with a Blo t 5 probe. B. Hybridization with a Blo t 21 probe. In both, genomic DNA was digested with restriction enzymes, *Hind III* and *EcoR V*, respectively.

3.2.4 Secondary structures of Blo t 21 and Blo t 5

Recombinant Blo t 21 and Blo t 5 were expressed in *E. coli*. Figure 3.8A shows the SDS-PAGE of the purified recombinant Blo t 5 and Blo t 21 proteins. Both bands were observed around 14 kDa, corresponding to their respective deduced molecular weights: Blo t 5 (14kDa) and Blo t 21 (13kDa).

To evaluate whether the recombinant proteins were properly folded after purification, far UV circular dichroism (CD) spectra of both rBlo t 5 and rBlo t 21 were measured. The CD profiles of both rBlo t 5 and rBlo t 21 showed that these two proteins were typical α-helix proteins (Figure 3.8B) with two maximum negative peaks at 208 nm and 222 nm and one positive peak at 192 nm. The observed secondary structures were similar to those predicted from the amino acid sequence at PredictProtein server as mentioned in Section 3.2.1. The CD spectra profiles indicated that both recombinant Blo t 5 and Blo t 21 were properly folded in solution, and also shared similar secondary structures.

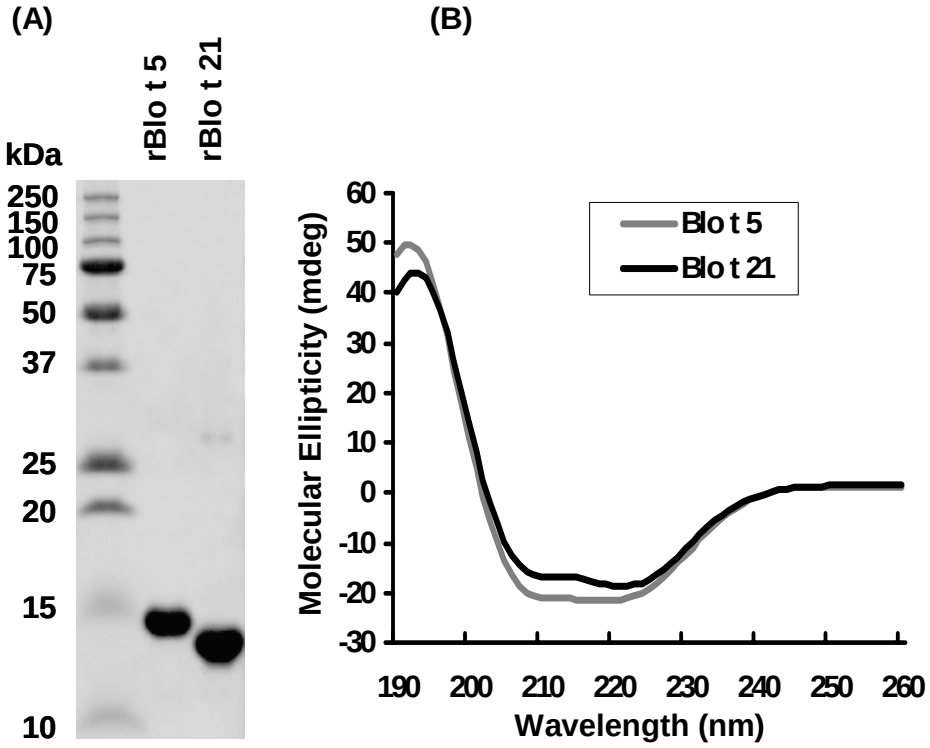


Figure 3.8 Expression of recombinant Blo t 5 and Blo t 21 allergens and their far UV CD spectra. A. Protein profile of recombinant Blo t 5 and Blo t 21 separated on 15% SDS-PAGE gel. B. Far UV CD spectra of rBlo t 5 and rBlo t 21 proteins in solution.

3.2.5 Examination of the natural Blo t 21 and Blo t 5

To evaluate the naturally-occurring Blo t 21 and Blo t 5 in crude mite extract, western blotting was performed using specific polyclonal antibodies. Whole mite proteins were extracted and separated on a 15% SDS gel together with recombinant Blo t 21 and Blo t 5 in parallel lanes (Figure 3.9A). In Figure 3.9B, Blo t 5 polyclonal antibodies recognized rBlo t 5, but not rBlo t 21. A natural Blo t 5 protein band recognized by Blo t 5 antibody was similar to rBlo t 5 (approximately 15 kDa). Two other bands (approximately 20 kDa and 28 kDa) in the mite protein extract were also recognized by Blot 5 antibody. Same Western banding profile of Blo t 5 was also observed by previous study in our laboratory by Mr. Aaron Chen Angus (Data not shown). He had analyzed 20 kDa and 28 kDa bands using Mass spectrometer and results indicated that these mite components were similar to the Group 5 allergen. A non-specific band (at 25 kDa, denoted with an asterisk in the Figure 3.9) was detected in all three Immuno-blotting membranes including the negative control (Figure 3.9D). We also observed that recombinant Blo t 5 existed as a homo-dimer at 30 kDa and homo-trimer at 45 kDa.

In contrast, the Blo t 21 specific polyclonal antibody reacted strongly to rBlo t 21, but showed a weak cross reaction to rBlo t 5 (Figure 3.9C). Consequently, two natural protein bands around 15 kDa were observed in the lane loaded with the crude mite protein extract. The band that ran slightly higher was identified to be natural Blo t 5, while the lower band was identified to be nBlo t 21. The size of nBlo t 21 also matched its predicted molecular weight based on the amino acid sequence. A band that appeared at approximately 20 kDa was found to be similar to Blo t 5. Like rBlo t 5, rBlo t 21 also exists as a homo-dimer at 30 kDa and homo-trimer at 45 kDa.

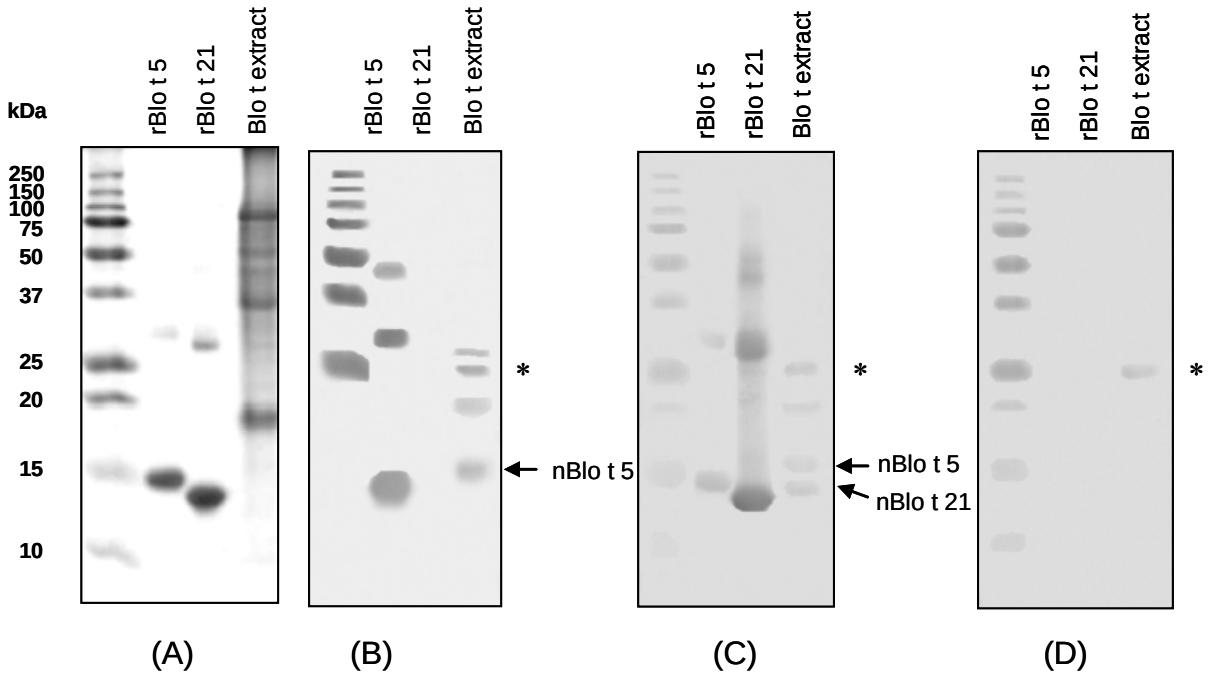


Figure 3.9 Detection of native Blo t 5 and Blo t 21 allergens in the crude extract. (A). SDS-PAGE gel separating profile of rBlo t 5, rBlo t 21 and *B. tropicalis* extract. Immuno-blotting images with (B). anti-Blo t 5 (C). anti-Blo t 21 polyclonal antibodies and (D). PBS buffer (negative control). A non-specific band (*) was detected in all three immuno-blotting membranes.

3.2.6 Prevalence of Blo t 21 and Blo t 5 sensitization in allergic rhinitis patients in Singapore

The allergenicities of Blo t 21 and Blo t 5 were evaluated in forty-three adult patients with ongoing persistent allergic rhinitis who were positive to *B. tropicalis, D. pteronyssinus,* and *D. farinae* and five non-atopic healthy volunteers using direct ELISA method. The responses to these dust mites were verified by skin prick test assay in vivo using extracts prepared from three mites and CAP assay via measuring the IgE antibodies to extract of *B. tropicalis, D. pteronyssinus* and *D. farinae.* The detailed characteristics of these patients were described in Chapter 2 Materials and Methods. ELISA assay results showed that forty out of the forty-three patients (93%) had sera specific IgE response to Blo t 21, while forty-two patients (98%) had specific IgE to Blo t 5. Patients responding to Blo t 21 also specifically responded to Blo t 5 (Figure 3.10).

In order to validate the ELISA system, UniCAP® system was used as a reference assay. *B. tropicalis* extract was applied to test both systems. The ELISA readings versus the UniCAP® ranking values were displayed in Figure 3.11. A good correlation between ELISA absorbance readings and ranking scores of UniCAP was observed. The correlated results obtained from two different assay systems indicated that direct ELISA assay is a reliable semi-quantitative tool to measure allergen specific IgE antibodies when the UniCAP system is used as the bench mark.

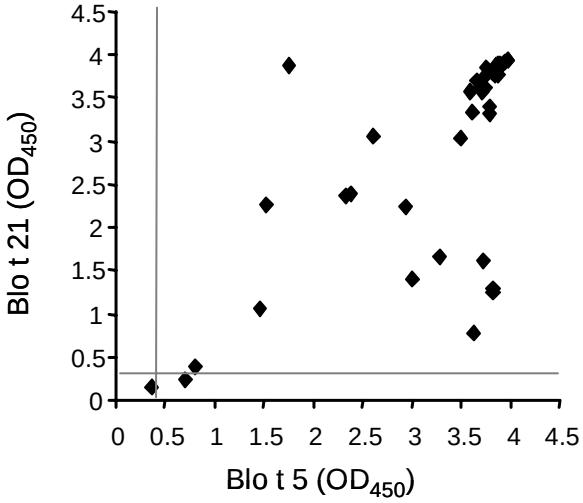


Figure 3.10 Bi-plot comparing the specific IgE levels against Blo t 21 and Blo t 5 assessed by ELISA in sera of 43 allergic rhinitis patients. Grey lines indicate the negative cut- off for the assay.

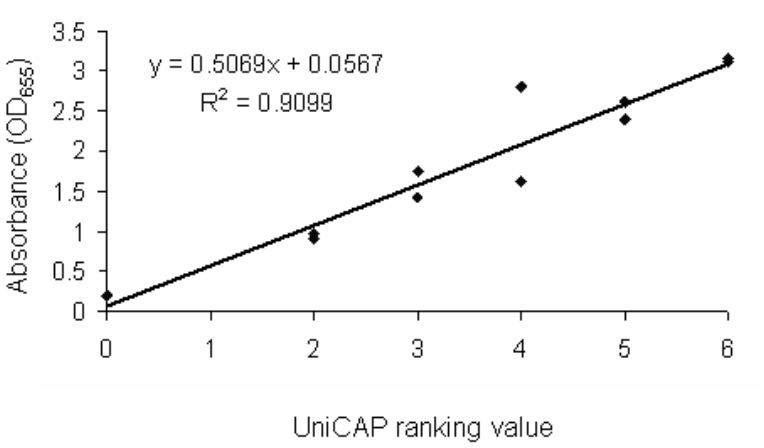


Figure 3.11 Concordance of the ELISA versus UniCAP system.

3.2.7 Prevalence of Blo t 5 and Blo t 21 sensitization in consecutive individuals attending outpatient allergy clinics

The allergenicities of Blo t 21 and Blo t 5 were further evaluated in 181 local atopic individuals attending outpatient allergy clinics over a period of one and a half years using semi-quantitative dot blot immunoarray assay[♣]. Ninty-seven out of 181 (97/181) sera were found responding to *B. tropicalis* extract, and 30 sera responded to Blo t 21 corresponding to 31% (30/97) of *B. tropicalis* positive subjects.

In order to compare Blo t 21 with other allergens of *B. tropicalis*, the IgE responses to a panel of 19 allergens were also included in the immunoarray[♣♣]. Figure 3.12 shows that Blo t 21 (31%) is the third most prevalent IgE response allergen among 19 *B. tropicalis* allergens following Blo t 5 (50%) and Blo t 3 (39%). Blo t 12 and Bt Mag 3 have a mid-range prevalence of 23%, Blo t 2, 6, 7, 11, 13 and Mag 1 have a prevalence of 11% ~19%, and Blo t 1, 4, 8, 9, 10, 15, 20 and Mag 29 have a low prevalence (less than 8%) in this population. Sera with high IgE antibodies to *B. tropicalis* extract tended to respond to many allergens. The specific IgE levels against Blo t 21 and Blo t 5 were compared by intensities of dots detected on membranes (Figure 3.13 A and B), and results revealed that except for one serum, most sera responding to Blo t 21 also responded to Blo t 5.

♣ This work was done by Ms. Tay Angeline.

♣♣ Except Blo t 5 and Blo t 21, the rest 17 *B. tropicalis* allergens were cloned by Dr. Shang Huishen, Kuay Kuee Theng, and Wang Wen Long.

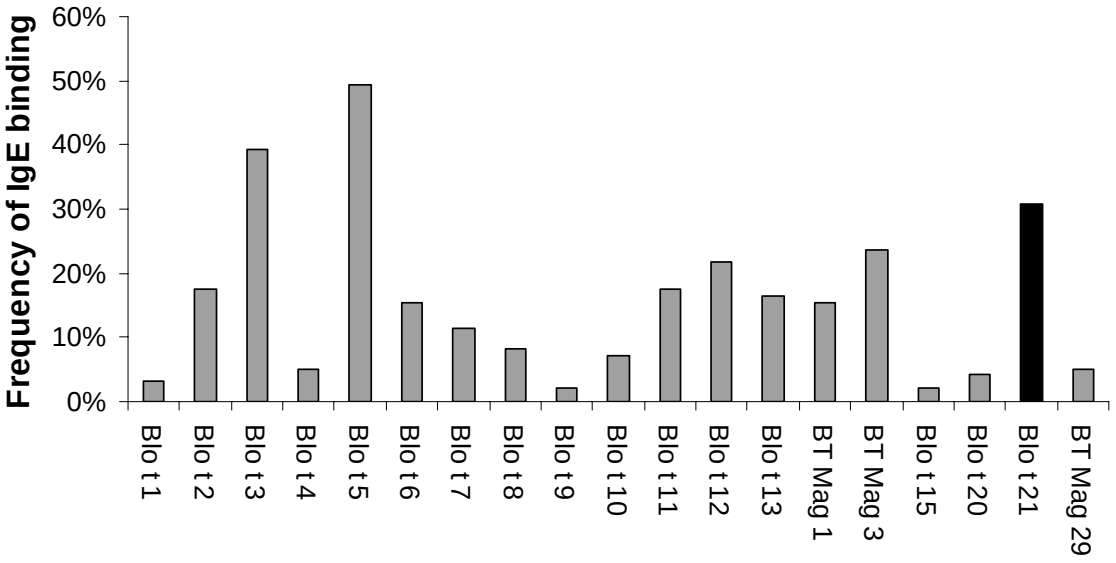


Figure 3.12 IgE-binding frequencies of *B. tropicalis* allergens in the *B. tropicalis* sensitized individuals (n=97).

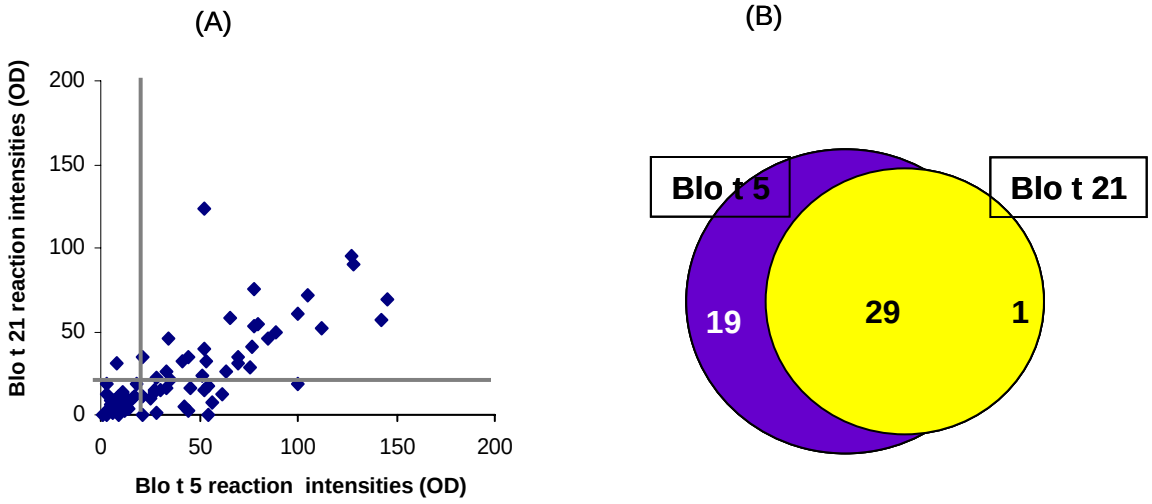


Figure 3.13 Comparison of the specific IgE levels against Blo t 21 and Blo t 5 assessed by Dot blot immunoassay in sera of 97 *B. tropicalis* positive subjects attending outpatient allergy clinic in one and a half years. (A) Bi-plot comparison of IgE-binding of Blo t 5 and Blo t 21 (B) Venn diagram showing IgE-binding to Blo t 5 and Blo t 21.

3.2.8 Skin Prick test of Blo t 21 and Blo t 5 in allergic rhinitis patients

To further confirm the allergenicities of Blo t 21 and Blo t 5 *in vivo*, skin prick test was performed on the same group of allergic rhinitis patients evaluated earlier. Two non-atopic volunteers were also evaluated as controls[♣]. All patients had positive responses to at least one of the allergens, with most of them (n=41) reacting to both allergens (Figure 3.14). The mean wheal diameter was 5.7 mm for Blo t 5 and 6.2 mm for Blo t 21. Healthy subjects did not develop significant wheal and flare reactions (data not shown). The skin prick wheal responses against the two allergens were not correlated.

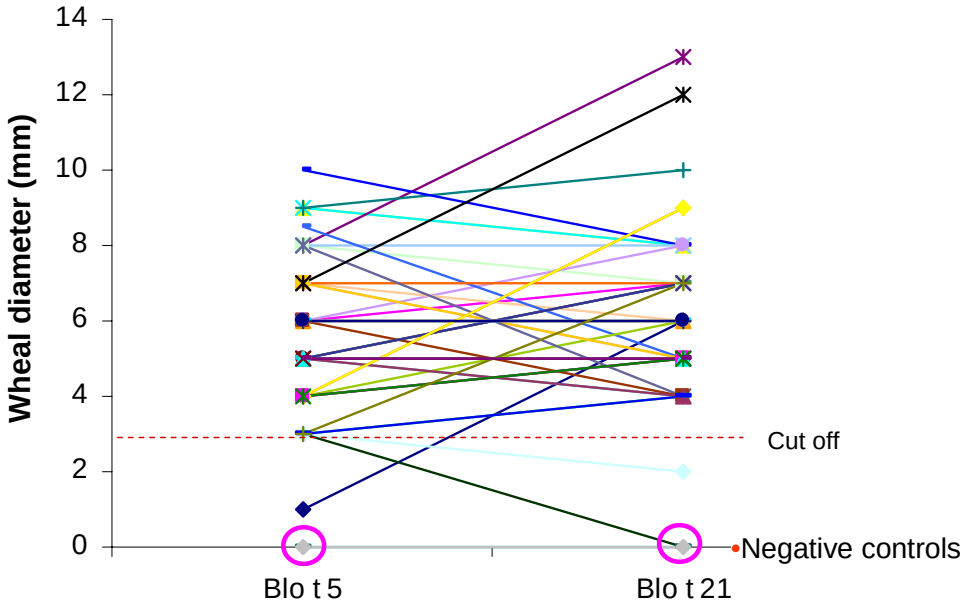


Figure 3.14 Cross comparison between Blo t 5 and Blo t 21 skin prick test responses among 43 allergic rhinitis patients.

♣ This experiment was done by Associate Professor Wang De Yun, Department of Otolaryngology, National University of Singapore.

3.2.9 Dose-response cross-inhibition of IgE-binding to Blo t 21 and Blo t 5

Cross-reactivity between these allergens were evaluated via a dose-response ELISA inhibition study using three sera with high titers of specific IgE to both Blo t 21 and Blo t 5, as well as Lep d 5 and Der p 5. Dose-dependent inhibition up to a maximum of approximately 100% was observed with all homologous antigens in all three sera (Figure 3.15 A and B). IgE-binding to Blo t 21 could be inhibited by the homologous antigen at very low concentrations (0.01 μg/ml) and achieved full inhibition at 100 μg/ml of protein in all three sera (Figure 3.15 A). Heterologous inhibition by Blo t 5, Der p 5 and Lep d 5, however, only occurred after the inhibitor concentration was 10 μg/ml or above in two of the three sera, and could only achieve a maximum of less than 45% inhibition at the concentration of 100 μg/ml of inhibitor. In particular, no cross-reactive specific IgE between Blo t 21 and Group 5 allergens could be detected in serum from patient V1.

In parallel, with Blo t 5 coated on the ELISA plates, partial cross-reactivity between Blo t 5 and Blo t 21, Der p 5 and Lep d 5 was also observed (Figure 3.15 B). When homologous antigen achieved full inhibition of specific IgE-binding to Blo t 5, Blo t 21 reached 55%-71% of the IgE-binding of Blo t 5. Der p 5 and Lep d 5 achieved 18%-75% and 41%-77% inhibition respectively.

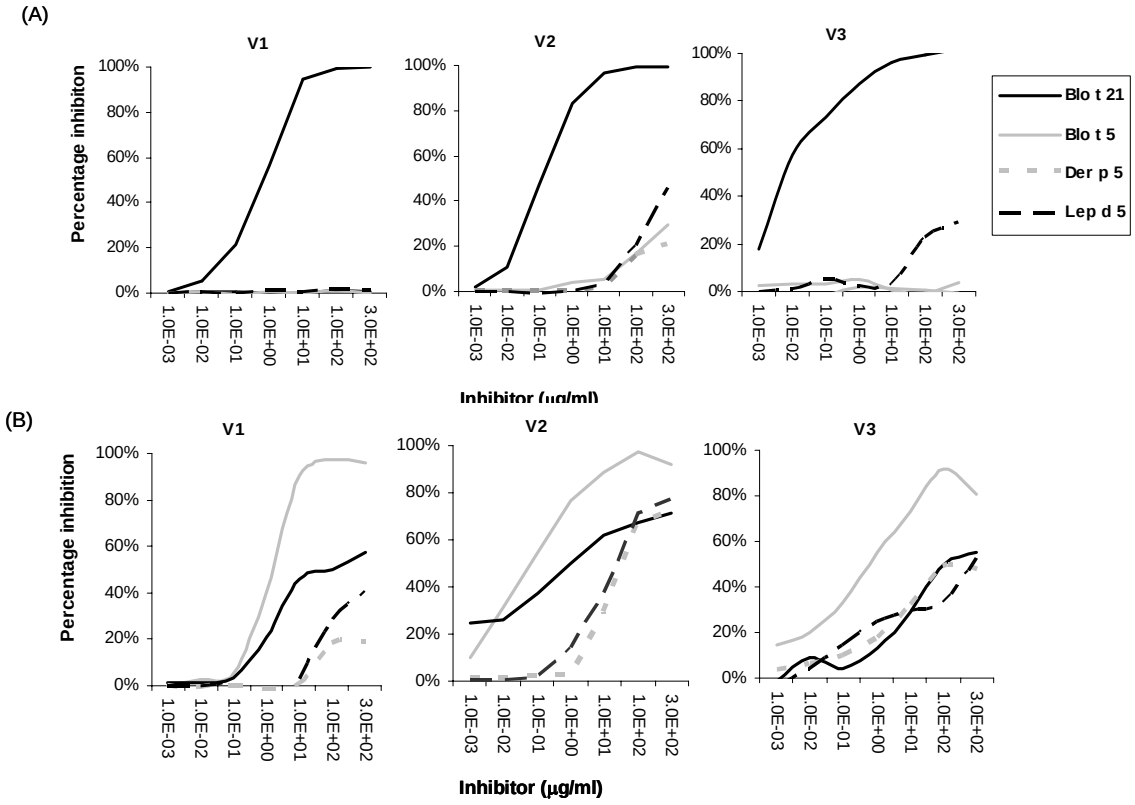


Figure 3.15 Dose-response competitive ELISA assay evaluating the IgE cross-reactivity of Blo t 21 and Blo t 5. Three individual sera (V1, V2 and V3) were pre-absorbed with 10-fold series dilution of inhibitors, and then used to perform allergen specific IgE assay by ELISA. A. Blo t 21 immobilized on solid phase of ELISA plates. B. Blo t 5 immobilized on solid phase of ELISA plate.

3.2.10 Quantitative end point cross-inhibition of Blo t 21 and Blo t 5

To evaluate further, ten additional rhinitis patient sera with specific IgE to both allergens were tested in a quantitative end-point inhibition experiment using only 100 μg/ml of inhibitor. On average, specific IgE-binding to Blo t 21 was partially inhibited by Blo t 5, Der p 5 and Lep d 5 at a mean of 27%, 29% and 28% respectively (Blo t 21 achieved 93% inhibition at 100 μg/ml). In parallel, specific IgE-binding to Blo t 5 was partially inhibited by Blo t 21, Der p 5 and Lep d 5 at a mean of 33%, 51% and 34% respectively (Blo t 5 achieved 85% inhibition at 100 μg/ml) (Table 3.3).

Taken together, cross inhibition studies revealed that Blo t 21 has low cross-reactivity to Blo t 5 and other Group 5 allergens, and vice versa. Specific IgE reactions to Blo t 21 are mainly elicited by its unique IgE epitopes, rather than its shared epitopes to other Group 5 allergens.

Table 3.3 Quantitative end-point cross-inhibition of IgE-binding to Blo t 21 and Blo t 5 in sera of ten atopic individuals.

0.1 mg/ml	A. Inhibition of IgE-binding to Blo t 21				B. Inhibition of IgE-binding to Blo t 5			
Inhibitor	Blo t 21	Blo t 5	Der p 5	Lep d 5	Blo t 5	Blo t 21	Der p 5	Lep d 5
R4	80%	17%	16%	19%	80%	29%	66%	54%
R5	98%	11%	14%	17%	95%	28%	65%	26%
R7	94%	44%	68%	53%	92%	32%	66%	51%
R11	87%	50%	23%	25%	73%	61%	29%	34%
R14	97%	53%	65%	59%	90%	57%	74%	56%
R18	91%	5%	5%	6%	88%	19%	60%	42%
R20	97%	10%	16%	12%	91%	19%	73%	49%
R22	91%	2%	2%	8%	75%	0%	23%	0%
R23	95%	48%	34%	31%	83%	78%	40%	32%
R24	96%	8%	14%	2%	80%	3%	12%	0%
mean	93%	25%	26%	23%	85%	33%	51%	34%

A. rBlo t 21 immobilized on solid phase. B. rBlo t 5 immobilized on solid phase.

3.2.11 Localization of Blo t 21 and Blo t 5 on sections of mite body

Blo t 21 and Blo t 5 were immuno-stained by anti-Blo t 21 and anti-Blo t 5 polyclonal antibodies on paraffin sections of mite bodies. Anti-Blo t 21 antibody bound strongly to mid-gut contents and fecal pellets in the hindgut of the sections (Figure 3.16 A). In parallel, anti-Blo t 5 antibody stained similar positions of the mite sections (Figure 3.16 B). To ensure that the antibodies were not cross reacting, anti-Blo t 21 was pre-absorbed with Blo t 5 prior to immuno-staining. A pre-immune sera control did not stain the mite sections (Figure 3.16 C). Therefore, Blo t 21 and Blo t 5 co-localize in similar positions and may likely pass out of the mite body with feces and exist in the environment as airborne particles.

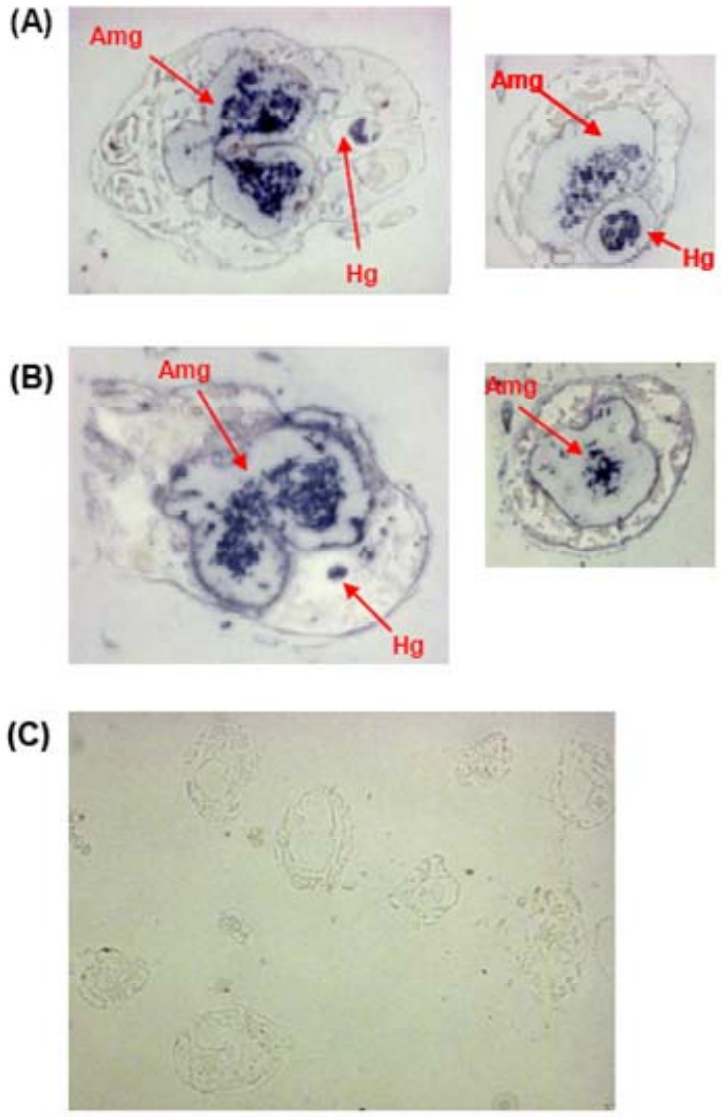


Figure 3.16 Immuno-staining of Blo t 21 and Blo t 5 in paraffin-embedded sections of *B. tropicalis.* (A). Probed with anti-Blo t 21 polyclonal antibody in longitudinal and sagittal mite sections. (B). Probed with anti-Blo t 5 polyclonal antibody. (C). Probed with pre-immune serum. Amg: anterior midgut, Hg: hind gut.

3.2.12 Concentration of Blo t 21 in the house dusts

Seventy-one dust samples from four niches of 40 homes, namely bedroom floors, living room carpets, living room floors and kitchens floors were assayed for Blo t 21 and Blo t 5 levels. In order to ensure the specificity of the assay, cross-inhibition experiments of Blo t 5 and Blo t 21 antibodies were performed before dust assay. The inhibition study using competitive ELISA method indicated that both Blo t 21 and Blo t 5 antibodies were specific for their own proteins (Figure 3.17).

Wilcoxon-Mann-Whitney rank test showed identical distributions of Blo t 21 and Blo t 5 among different niches where dust samples were collected. Blo t 21 was found in 70 out of 71 dust samples with a geometric mean of 220 ng/g, while Blo t 5 was detected in 67 dust samples with a geometric mean of 163 ng/g. Figure 3.18 shows slightly higher Blo t 21 levels than Blo t 5 in dust samples collected from kitchen floors, bedroom floors and living room carpets. The geometric means of Blo t 21 were higher on kitchen floors and living room carpets than those on bedroom floors and living room floors, while the geometric means of Blo t 5 in four niches were relatively similar. The paired *t* test results showed non-correlation between Blo t 21 and Blo t 5 levels in the dust samples of this study. Bi-plot analysis of the concentration of Blo t 21 and Blo t 5 in house dust samples also indicated the non-correlation of distributions of the two allergens (Figure 3.19).

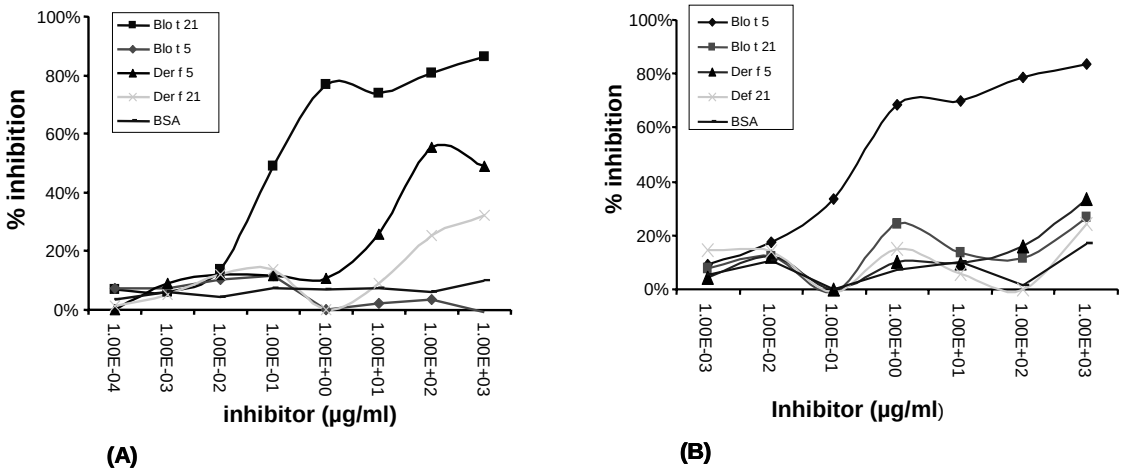


Figure 3.17 Competitive ELISA assay evaluating the IgG specificity of Blo t 21 and Blo t 5. (A) Blo t 21 IgG antibody (B) Blo t 5 antibody.

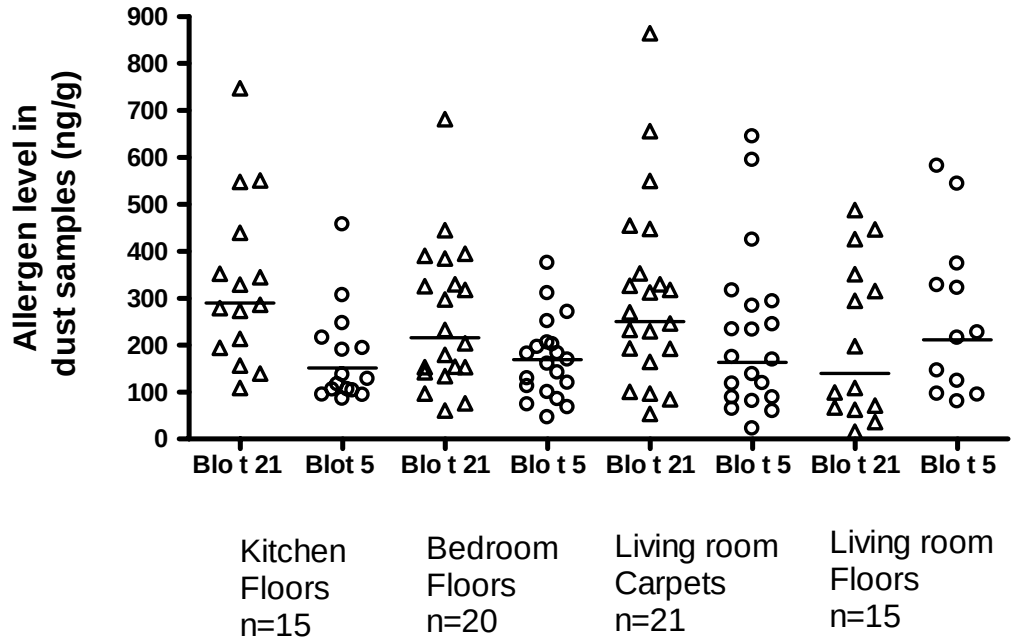


Figure 3.18 Blo t 21 and Blo t 5 levels in 71 house dust samples. The solid lines indicate the geometric means of the dust samples.

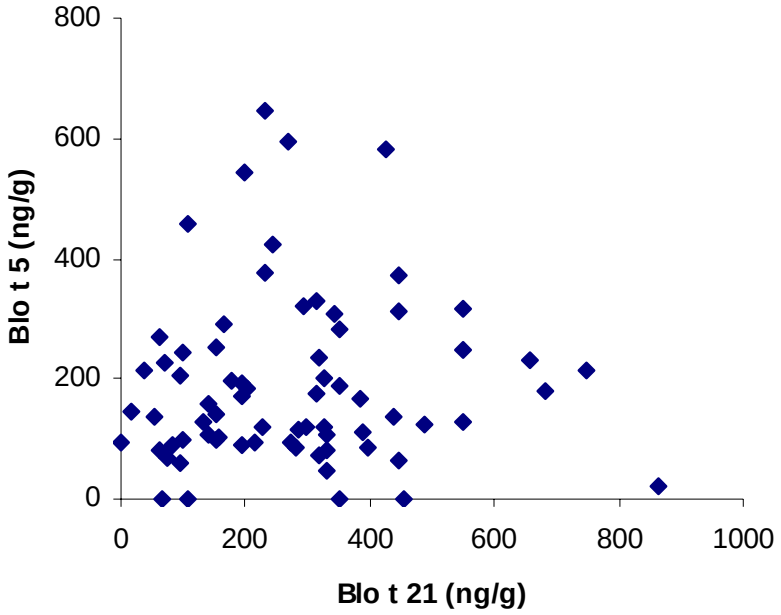


Figure 3.19 Correlation of Blo t 21 and Blo t 5 levels in the house dusts in Singapore.

3.3 Discussion

A novel Blo t 21allergen has been identified from our in-house *B. tropicalis* EST database. It has been discovered in *B. tropicalis* mite at DNA level, transcription level and translation level. Its genomic DNA has been detected by Southern blotting, transcriptional products were obtained from cDNA library by EST approach, and native protein was observed from mite extract by Western blotting.

Blo t 21 is a product of a single-copy gene which contains an intron with 56 base pairs. The open reading frame of its cDNA encodes a protein similar to Group 5 and Group 21 allergens. Additionally, multiple sequence alignment showed that Blo t 21 shared 30-41% amino acid identity with Der p 21 and other known Group 5 allergens. The CD spectra of Blo t 21 and Blo t 5 indicated typical α-helical structures of both proteins, similar to that of Der p 5 (Liaw *et al.*, 2001). These similarities between the primary and secondary structures of Group 21 and Group 5 allergens as well as their co-localization in the dust mite seem to suggest that these two classes of allergens possibly have related functions. The conservation of amino acid sequences in different proteins, originated from the same species is most likely due to a prior gene duplication event within the genome of the organism. Duplicated gene sequences may however change quickly before selective pressure sets in on its new function. Hence, duplicated gene products may have slightly modified structures and functions but show some residual resemblance of each other. These duplicated genes are then called paralogs. Paralogs resulted from gene duplication, are thought to be retained in the organism because of selective benefits. These genes may have overlapping functions in the initial period after

duplication, but this functional redundancy could diminish over the time, as these genes acquire more refined roles (Green *et al.*, 2004).

Many mite proteins with sizes around 15 kDa are allergenic, including the Groups 2, 5, 13 and now Group 21 allergens. Due to the lack of genomic information, identification of new allergens using Immuno-blotting combined with standard molecular biology methods can be difficult and time consuming. Using EST approach (Angus *et al.*, 2004), our laboratory has previously identified putative allergens in the dust mite. Applying the similar approach, a novel Blo t 21 allergen of 13.5 kDa has been identified in this study.

The concurrent IgE-binding responses to Group 21 and Group 5 allergens found in this study were consistently observed in other populations screened in our laboratory including allergic individuals from Italy and South Africa (data not shown) (Potter *et al.*, 2006). We initially reasoned that this might be a result of either co-sensitization to both allergens, or cross-reactions through the presence of common epitopes (Sander *et al.*, 1997; Potter *et al.*, 1991). However, both dose-response inhibition and quantitative inhibition studies showed that Blo t 21 and Blo t 5 are specific low-cross-reactive allergens. They also demonstrated low degree of cross-reactivity to the orthologues between species (Der p 5 and Lep d 5). This is in agreement with studies conducted elsewhere by other researchers (Chew *et al.*, 1999b; Kuo *et al.*, 2003). The scattered patches of identical amino acids across the whole length of Blo t 21, Der p 21 and other Group 5 allergens could be the basis for some cross-reactivities, but the inhibition data strongly support the idea that Group 21 is a distinct group of allergens. IgE antibody

responses to Blo t 21 are mainly elicited by specific epitopes, rather than cross-reactive epitopes on other Group 5 homologous allergens.

The strong co-sensitization profile, not ascribed to antigen cross-reactivity, could then be due to at least two other reasons. Firstly, the Group 5 and Group 21 allergens could both cause sensitizations in susceptible individuals when present in the environment. This is likely due to the wide distribution of Blo t 21 and Blo t 5 in the environment, although the ratio of Blo t 21: Blo t 5 can be different from one room to the other. Secondly, allergen-specific IgE responsiveness towards purified allergens has been shown in some cases to be restricted by certain HLA types and it is possible that immune responses to the two antigens are restricted by similar host factors. Previous study in our laboratory has shown that HLA-DRB1*09 may be a marker of IgE non-responsiveness to Blo t 5 among patients in Singapore (Lee *et al.*, 1998), but not to Der p 5. Whether this is the case for Blo t 21 remains to be evaluated.

Sequence diversity was found in the genomic DNA of both Blo t 21 and Blo t 5. Since Blo t 21 and Blo t 5 present as a single-copy in mite genome, the nucleotide substitutions are most likely resulted from the allele polymorphisms among different mite individuals, and not from multi-gene families of the mite. Using high fidelity Taq polymerase to amplify genomic DNA also ensured that the substitutions were resulted from sequence polymorphisms rather than PCR error. Most substitutions in Blo t 5 genomic DNA were silent changes with only two missense changes of amino acids 19 and 23. These two amino acid substitutions are located closely in the N terminal of the mature protein. In contrast, half of the variations in the Blo t 21 genomic DNA were missense substitutions. Four amino acid substitutions were present at positions 33, 35, 59

and 69. Our observation of protein variants is in agreement with the finding of Blo t 5 isoforms through Two-dimensional Western blotting (Yi *et al.*, 2004). Whether these protein variants stimulate different IgE-binding in atopic patients is to be further investigated. Although polymorphisms of allergens in mites have been reported before, there were no reports for Group 5 and Group 21 polymorphisms prior to this study. It is well known that polymorphisms of Groups 1, 2 and 3 sequences were common in many mite species. Group 1 substitutions were mostly missense changes with low frequency, while the polymorphisms of Group 2 allergens were conserved (Smith *et al.*, 2001). Der p 3 missense substitutions were found in two out of five cDNA clones. All these variants resulted from allele polymorphisms (Smith *et al.*, 1996b).

It has been noticed that charged and polar residues on the surface of protein molecules are important for IgE-binding, particularly for inhalant allergens (Chan *et al.*, 2006). Blo t 21 and Blo t 5 both have more than 40% charged residues and 20% polar residues throughout the entire sequence. Such high percentage of solvent accessible residues could allow IgE antibodies to bind the proteins more readily.

This study clearly showed that sensitization to Blo t 21 is important for *B. tropicalis* allergy, although most patients were co-sensitized to Blo t 5 at the same time. To prevent the *B. tropicalis* allergic diseases, effective allergen avoidance is necessary. Our Immuno-localization result revealed that both Blo t 21 and Blo t 5 were secreted from mite digestive system and mixed with feces in the hind-gut of dust mite, suggesting that Blo t 21 could be passed out with feces and become airborne particles in the environment. Although Blo t 21 and Blo t 5 levels in the environment were below the "threshold" concentration of Der p 1 for sensitization, low levels of exposure to mites

allergen is a risk for inducing sensitization, especially for individuals with positive family histories of allergy (Waner *et al.*, 1996; Wahn *et al.*, 1997; Huss, *et al.*, 2001).

In tropical regions like Singapore, weather conditions are suitable for mite proliferation. Due to relatively constant temperature and humidity, it is difficult to avoid dust mite completely in the environment. At this stage, allergen-specific immunotherapy (SIT) is the only potential specific treatment for IgE-mediated *B. tropicalis* allergy. In current clinical practice, aqueous extracts of dust mite are used for diagnosis and immunotherapy. However, natural allergen extracts consist of complex mixtures of allergenic and non-allergenic components. In addition, endotoxin has been found in the mite extracts from *D. farinae* and *D. pteronyssinus* (Trivedi *et al.*, 2003; Finkelman *et al.*, 2006). In order to improve the quality and consistency of vaccine, multi-component recombinant allergens can be used in diagnosis and treatment to enhance the clinical efficacy. Circular Dichroism Spectroscopy has confirmed that both rBlo t 21 and rBlo t 5 are α-helical proteins. These two recombinant allergens also exhibited IgE-binding activities. Although IgE-binding activities of rBlo t 21 and rBlo t 5 were not compared with natural counterparts in this study, it has been reported that rBlo t 5 expressed in *E.coli* has comparable IgE-binding activity versus natural Blo t 5 (Yi *et al.*, 2004). We believe that rBlo t 21 may also have comparable IgE-binding activity to its natural counterpart like rBlo t 5.

Multiple recombinant allergen vaccine contains only relevant allergens for immunotherapy, and thus could reduce the side-effect caused by contamination of unrelated allergens or other reagents in conventional vaccine. rBlo t 21 and rBlo t 5

produced in our laboratory may facilitate the substitution of natural allergens in specific diagnosis and immunotherapy.

In conclusion, we describe here a novel allergen from *Blomia tropicalis*, Blo t 21, that is able to elicit and bind specific IgE in more than 31% of atopic individuals and 90% of patients with ongoing persistent allergic rhinitis. The allergencity of Blo t 21 is unique, although it shares some primary and secondary structure similarities to Blo t 5 allergen. IgE-binding to this allergen does not cross react to Blo t 5. Blo t 21 is thus an important allergen in *B. tropicalis,* and should be taken into considerstion in the diagnosis and treatment of *B. tropicalis* allergy particularly in tropical regions.

FOOTNOTE

The name Blo t 21 was accepted by the World Health Organization (WHO) / International Union of Immunological Societies (IUIS) Subcommittee of Allergen Nomenclature.

The GenBank accession numbers for the nucleotide sequence encoding for Blo t 21 is AY800348. The GenBank accession numbers for seven Blo t 21 and nine Blo t 5 genomic organizations variants are DQ788677-DQ788683 and DQ788684-DQ788692.

Chapter 4: Effect of Temperature, pH and Chemical Denaturant on Blo t 5 and Blo t 21 IgE-binding

4.1 Introduction

Dermatophagoides pteronyssinus, *Dermatophagoides farinae* and *Blomia tropicalis* dust mites are the three most important allergen sources associated with hypersensitivity reactions in human beings. *B. tropicalis* and *Dermatophagoides spp.* are frequently found in house dusts in tropical and subtropical regions (Baqueiro *et al.*, 2006), while *D. pteronyssinus* and *D. farinae* are domestic mites in temperate regions. Groups 1 and 2 allergens are two most important allergens in *D. pteronyssinus,* as Der p 1 and Der p 2 account for 50-65% of the total IgE antibodies in *D. pteronyssinus* sensitized asthma patients including low and high responders, and the prevalence of Der p 1 and Der p 2 in *D. pteronyssinus* positive patients are about 80% (Hales *et al.*, 2006). Blo t 5 and Blo t 21 are two most important allergens in B. *tropicalis* as mentioned in Chapter 3.

B. tropicalis is the most frequently found dust mite in the rainy season of the tropical regions (Baqueiro *et al.*, 2006; Chew *et al.,* 1999). When dust samples from 459 beds of Brazil residences were surveyed, the percentage of *B. tropicalis* and *D. pteronyssinus* were found to be 71.8% and 39.9%, respectively. When major allergens Der p 1 and Blo t 5 were further quantified by ELISA assay in the same samples, 10 times lower concentration of Blo t 5 (geometric mean=2.8μg/g) was detected compared to that of Der p 1 (geometric mean=30.2 μg/g) (Baqueiro *et al.*, 2006). The lower concentration of Blo t 5 could be resulted from its low expression level in *B. tropicalis* /per mite or the instability of the allergen so that the protein was removed from

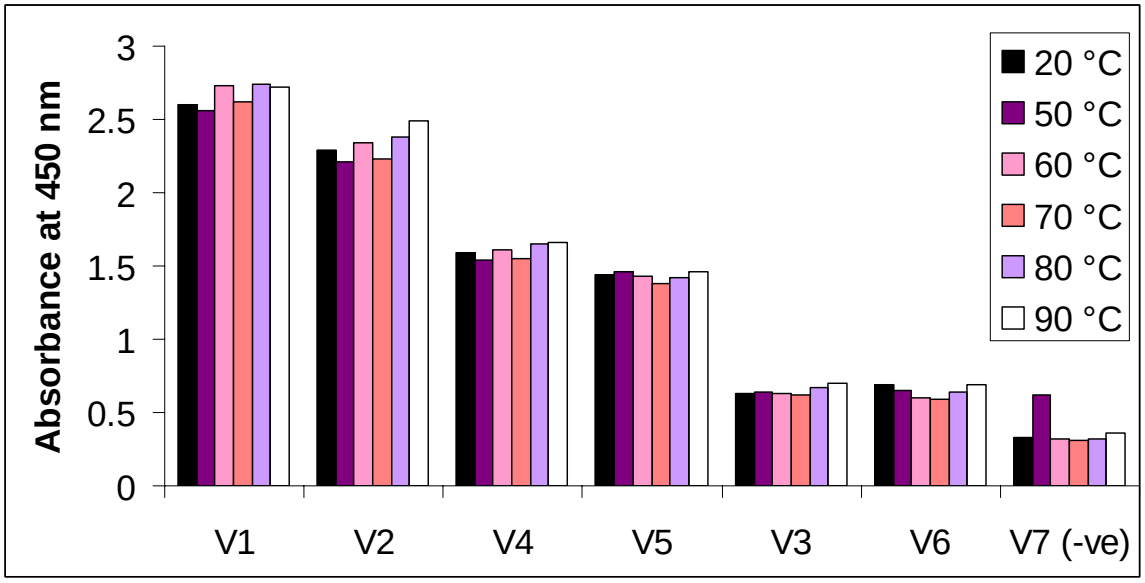
environment quickly. Nevertheless, Blo t 5 at 2.8μg/g in the environment was still above the sensitization threshold of dust mite and thus induced high frequency of sensitization in the atopic subjects. To understand the mechanism behind this, stabilities of IgE-binding activities of Blo t 5 and Blo t 21 were investigated in this chapter.

4.2 Results

4.2.1 Effect of thermal treatment on IgE-binding activities of Blo t 5 and Blo t 21

IgE-binding activities of Blo t 5 and Blo t 21 were retained after thermal treatment. Figure 4.1A showes that there is no significant difference of the IgE-binding activities between treated and non-treated Blo t 5 in sera of seven pre-selected subjects with two high reactors to Blo t 5 (OD_{450} >2.0), two medium reactors to Blo t 5 (OD_{450}=1.0 ~2.0), two low reactors (OD_{450}>0.4) to Blo t 5, and one healthy subject as negative control). Similar results were observed from the IgE-binding activity of heat treated Blo t 21 allergen (Figure 4.1B). Hence, both IgE-binding epitopes of Blo t 5 and Blo t 21 are thermal stable.

The thermal resistance of IgE-binding to Blo t 5 and Blo t 21 could be due to their linear epitopes that are recognized by IgE antibodies. The recognition of IgE antibodies to linear epitopes depends only on their amino acid sequence rather than the tertiary structure of the allergen. Thermal stability of Blo t 5 and Blo t 21 structures could be another reason for the thermal stability of their IgE-binding, so that the conformational epitopes of these allergens are retained after thermal treatment. In order to study the effect of heat treatment on the conformations of Blo t 5 and Blo t 21, the secondary structure changes in the protein were monitored by circular dichroism analysis in the next section.



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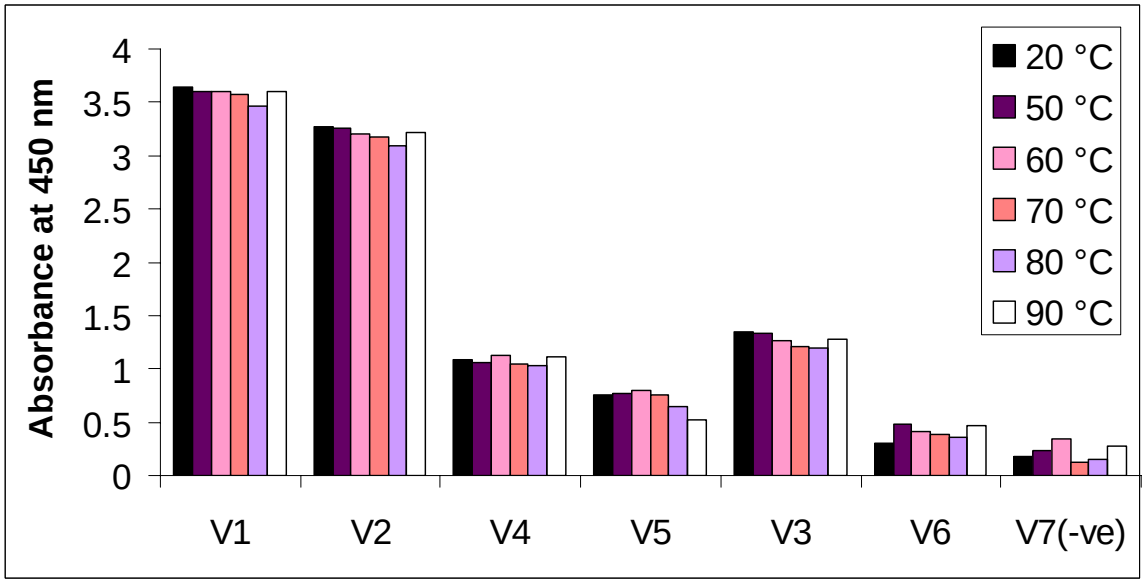


B

Figure 4.1 IgE-binding activities of heat treated Blo t 5 (A) and Blo t 21 (B) in six sera of atopic subjects and one sera of non-atopic healthy subject.

4.2.2 Effect of thermal treatment on Blo t 5 and Blo t 21 folding

The thermal stabilities of Blo t 5 and Blo t 21 were assessed by measuring changes of circular dichroism spectrum with increasing temperature from 10 °C to 90 °C. Firstly, the temperature dependency of the circular dichroism signals at various temperatures were recorded at 222 nm. This wavelength was chosen because Blo t 5 and Blo t 21 have negative peaks at this wavelength and the ellipticity of this wavelength is also important for monitoring the α-helix structure. Hence, any changes in the α-helix structure of Blo t 5 and Blo t 21 allergens can be detected upon thermal treatment. It can be seen from Figure 4.2 that a smooth sigmoid curve of the ellipicity of Blo t 5 was recorded at various temperatures. From 10-40°C, the ellipicity of Blo t 5 slowly increased with increasing temperature. Above 40 °C, an exponential increase of the ellipicity was observed until a plateau was reached at 70 °C. In contrast, the ellipicity of Blo t 21 slightly increased when temperature was changed from 10 to 60 °C, dramatically increased at 70 °C and stopped changing when temperature reached 80 °C. Above 80 °C, Blo t 21 completely lost its α-helix content.

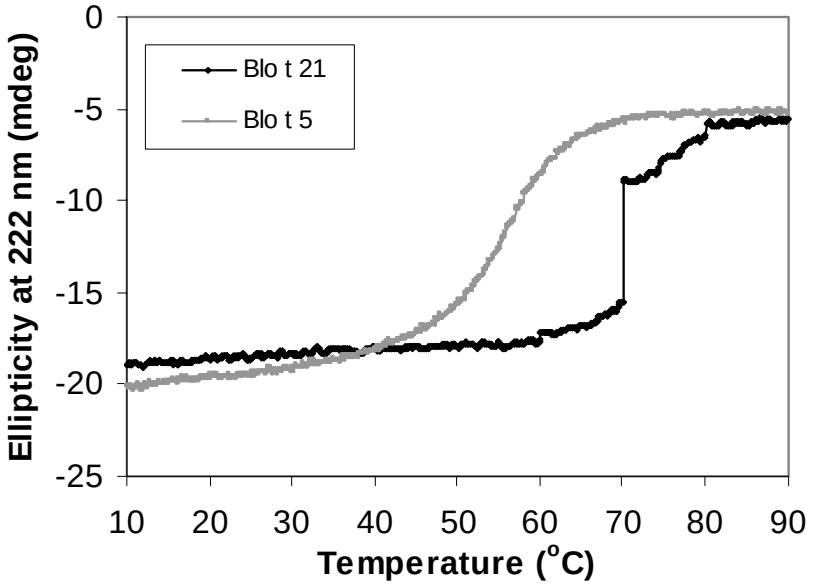


Figure 4.2 Far-UV CD spectrum of Blo t 21 and Blo t 5 at 222 nm recorded at temperatures from 10 °C to 90 °C.

The thermal stability of Blo t 5 and Blo t 21 structures was further examined by recording the circular dichroism spectra at 20, 50, 70 and 90 °C. After heat denatured, the proteins were cooled down to 20 °C, their CD spectra were recorded again and compared to the un-treated protein. Figure 4.3 illustrates that the alternation of the circular dichroism spectra occurred when Blo t 5 and Blo t 21 were denatured by thermal treatment. When temperature increased, the intensity of CD spectra of Blo t 5 and Blo t 21 decreased. The temperature-induced loss of α-helical structure of Blo t 5 was reconfirmed at 70 °C, as the minimal peak was shifted to a shorter wavelength above this temperature. Once temperature was brought back to 20 °C, the CD spectrum of Blo t 5 showed a profile similar to that of the untreated protein. As shown in Figure 4.3, the reduction of the negative peak at 222 nm of the protein cooled down from thermal treatment indicated that Blo t 5 was partially refolded.

The parallel experiments showed that Blo t 21 structure was also very stable as its CD spectrum at 50°C was almost the same as that at 20 °C. However, the intensity of CD spectrum was decreased at 70 °C, indicating the loss of its α-helix content at this temperature. The CD spectrum of Blo t 21 at 90°C lost its minimal peaks at 208 and 222nm, which indicated that Blo t 21 had completely lost its structure at this temperature. However, when the protein was cooled down to 20 °C, part of its α-helix structure was recovered. Its CD profile after denaturing was similar to that at 70 °C. Therefore, Blo t 21 was not completely refolded after heat denaturation.

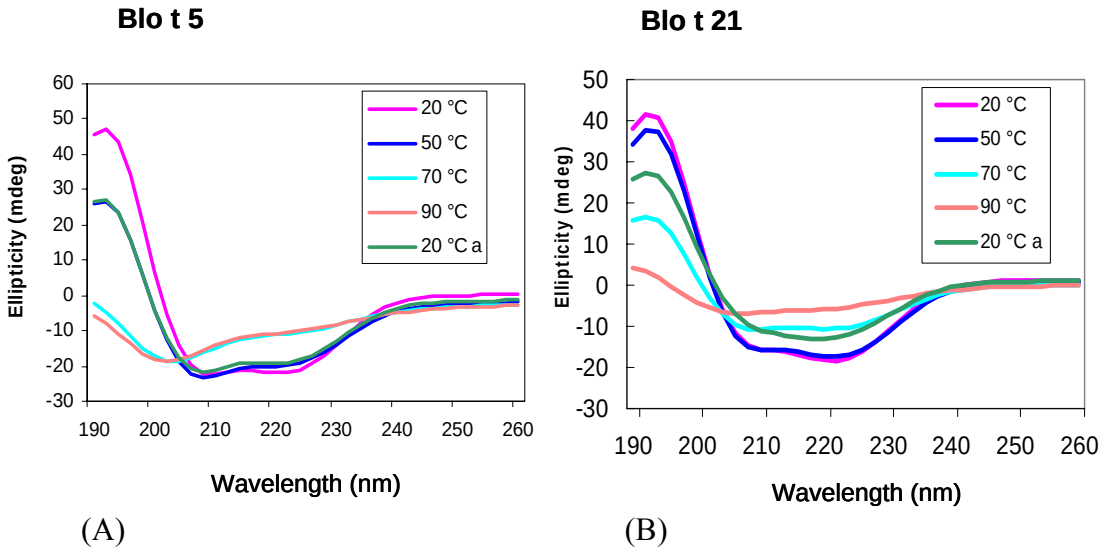


Figure 4.3 CD spectrum of Blo t 5 (A) and Blo t 21 (B) recorded at 20 °C, 50 °C, 70 °C, 90 °C and after cooling down to 20 °C. The spectrum range was 190 to 260 nm.

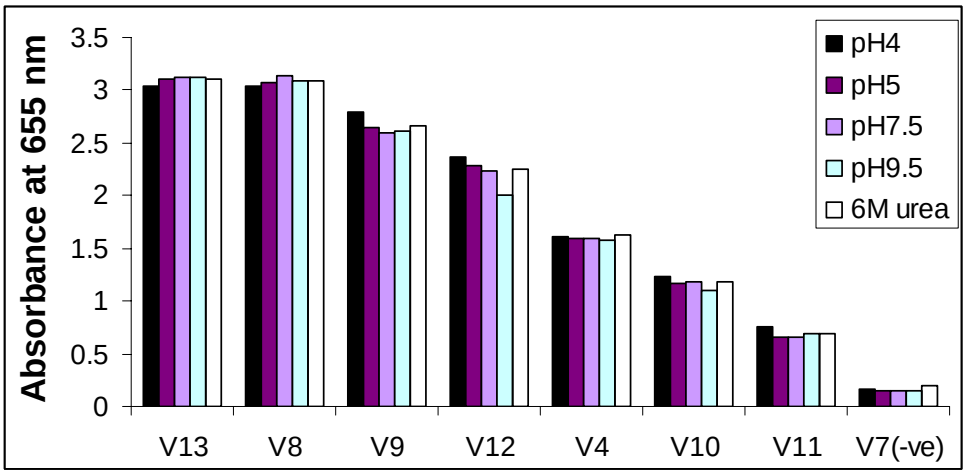
B

4.2.3 Resistance of IgE-binding of Blo t 5 and Blo t 21 to acid, alkaline and urea treatment

The IgE-binding activities of Blo t 5 and Blo t 21 are resistant to acid, alkaline and chemical denaturant urea (6 M). The proteins of Blo t 5 and Blo t 21 were first adjusted to variant pH conditions from pH 4.0 to pH 9.5 for 2 hours before their specific IgE-binding activities were tested. Due to insufficient amount of sera, we were not able to use exactly the same batch of sera for both thermal stability and pH susceptibility studies. In this study, seven sera positive to both Blo t 5 and Blo t 21 were used with a serum from non-atopic healthy volunteer as negative control. Results showed that IgE-binding activites of both Blo t 5 and Blo t 21 were not affected by pH conditions in the range from 4 to 9.5. The ELISA reading of OD_{450} of two proteins showed no significant difference at various pH conditions including neutral condition as shown in Figure 4.4(A) and (B). The extreme pH conditions were then introduced to test the pH susceptibility of Blo t 5 and Blo t 21 allergens. 100 mg/ml of proteins were suspended in 20 mM HCl (pH 2), 20 mM NaOH (pH 12) and phosphorated buffer (pH7.5) for 4 hours and then coated on Maxisop plate with 1 mg/ml concentration in coating buffer. From Figures 4.5 A and B, it can be seen that even after Blo t 5 and Blo t 21 were treated under extreme pH conditions, the ELISA readings were still the same as those proteins under neutral condition. Hence, IgE-binding activities of Blo t 5 and Blo t 21 were stable under a broad range of pH conditions from pH 2 to pH 12.

In addition, the susceptibilities of Blo t 5 and Blo t 21 to 6 M urea were assayed as well. The proteins were suspended in 6 M urea for two hours and then coated on ELISA plate for assay. Results were integrated in Figures 4.4 and 4.5. ELISA reading of

urea-treated and un-treated (in pH7.5 neutralized buffer) Blo t 5 and Blo t 21 showed no significant difference. Blo t 5 and Blo t 21 still retained their IgE-binding activities when the allergens were treated with 6 M urea.



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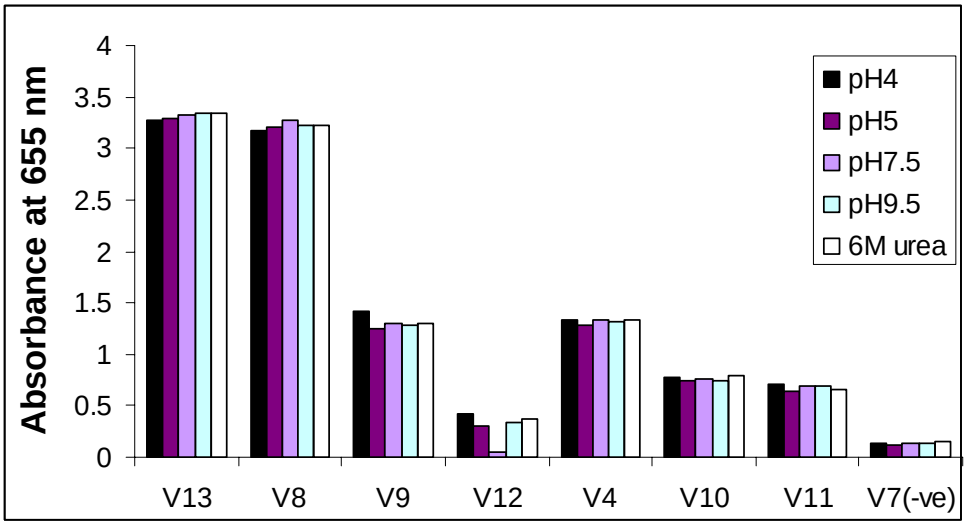
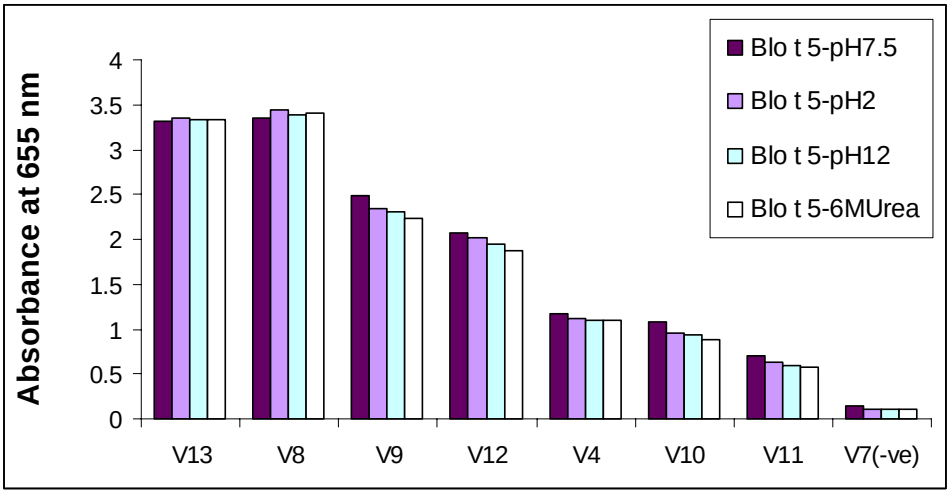
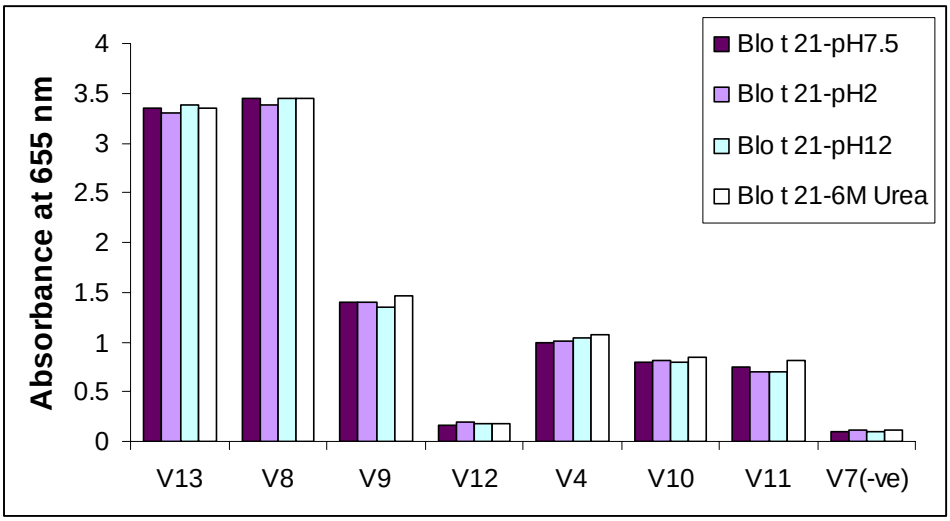


B

Figure 4.4 IgE-binding activities of urea, acid and alkaline treated Blo t 5 (A) and Blo t 21 (B) at different pH ranges from pH 4 to 9.5.



A



B

Figure 4.5 IgE-binding activities of urea, acid and alkaline treated Blo t 5 (A) and Blo t 21 (B) at pH 2, 7.5 and 12.

4.3 Discussion

The IgE-binding activities of both Blo t 5 and Blo t 21 are resistant to thermal treatment. Even after the proteins were treated at 90 °C, their IgE-binding abilities were not affected, despite some loss of the secondary structure contents. This study also showed that Blo t 21 is a thermal-stable protein. It maintains its secondary structure up to 60 °C. After heat denaturing, its secondary structure can be partially refolded. Hence, Blo t 21 major epitopes would present as liner epitopes. Similar to Blo t 21, IgE-binding of Blo t 5 is also thermal stable. In addition, more secondary structures were regained after heat denaturing. In contrast, Group 1 and Group 2 allergens are sensitive to thermal treatment. IgE-binding of Der p 1 and Der f 1 decreased more than 100 folds after proteins underwent treatment at temperatures above 75 °C, and Group 2 lost about 2-fold of IgE-binding activity after the thermal treatment (Lombardero, *et al.*, 1990). Heat denaturation of ProDer p 1 also drastically reduced IgE-binding activity toward human allergic sera using direct ELISA method (Magi *et al.,* 2004). Thus, IgE-binding activities of Blo t 5 and Blo t 21 are more stable against thermal denaturation than those of Der p 1 and Der p 2.

IgE-binding activities of Blo t 5 and Blo t 21 are also resistant to the changes of pH conditions. Even at pH 2 and pH 12, these two allergens still retain their full IgE-binding activities. Group 2 from *Dermatophagoides spp.* is resistant to pH conditions. Same degree of inhibition of mAb and IgE antibody was found among Der p 2 at pH 2, pH 12 and pH 7.4 conditions. In contrast, Der p 1 is sensitive to acid treatment (Lombardero, *et al.*, 1990). At pH 2, the IgE and IgG binding activities to Der p 1 were reduced by 100-1000 folds when compared to proteins at neutral condition (pH 7.4).

Thus, IgE-binding activities of Blo t 5 and Blo t 21 are more resistant to pH changes than Der p 1. Although the secondary structures of Blo t 5 and Blo t 21 between pH 2 and pH 12 were not measured by circular dichroism spectrum in this study, the similar α-helical structure of Der p 5 between pH 2 and pH 11 (Liaw *et al.*, 2001) implies that the secondary structure of Blo t 5 and Blo t 21 may not significantly change as well.

This study has also demonstrated that IgE-binding activity of Blo t 5 and Blot 21 are resistant to 6 M urea treatment. Urea is a strong protein denaturant. 6M urea treated Der p 1 and Der f 1 lost part of their IgE reactivitis. 39% and 45% of cross-inhibitions of urea-treated Der p 1 and Der f 1 to their native proteins were observed when full self-inhibitions were achieved (Lombardero, *et al.*, 1990). Thus, allergenicities of Blo t 5 and Blo t 21 are more resistant to urea-treatment than that of Der p 1.

IgE-binding epitopes of Blo t 5 and Blo t 21 are more stable than Der p 1 and Der p 2. There seems to be no influence of thermal, pH and denaturant treatment on the IgE-binding reactivities of Blo t 5 and Blot 21. Thus, it can be speculated that the IgE-binding epitopes of Blo t 5 and Blo t 21 could be mainly liner epitopes or conformational epitopes located in the structure of the proteins that can be refolded. The core structures of Blo t 5 and Blo t 21 could be regained after heat, extreme pH and denaturant treatment. The stability of IgE-binding epitopes of Blo t 5 and Blo t 21 would be important for the clinical applications of these two allergens, such as dust sample monitoring for allergen avoidance and mite extracted preparation for diagnosis and treatment.

The stability of IgE-binding epitopes of Blo t 5 could also be used to explain why relatively low concentration of Blo t 5 in the environment can induce high frequency of sensitization in the atopic subjects. In contrast, due to its susceptibility to denaturation,

Der p 1 allergen in the environment may not necessarily retain its full IgE-binding activity. Thus, higher amount/concentration of Der p 1 in the environment might be required for inducing sensitization in the atopic individuals.

Chapter 5: Identification and Characterization of Der f 21: a Der f 5 Homologue

5.1 Introduction

House dust mite *Dermatophagoides spp.* was recognized as the most important source of house dust allergens 30 years ago (Voorhorst *et al.,* 1964). Since then, multiple epidemiological studies have demonstrated dose-response relationships between exposure and sensitization to mite allergens. Sensitization to mite allergens is strongly associated with the prevalence of allergic asthma in many countries (Arlian and Platts-Mills, 2001).

D. farinae, originally known as American house dust mite, is highly prevalent in the temperate regions worldwide, such as the United States (Arlian *et al*, 1992), Poland (Solarz, 1998), Italy (Bigliocchi *et al.,* 1996), Germany (Kuher *et al*., 1994), France (Dornelas de Andrade *et al*., 1995), United Kingdom (Doull *et al*., 1997), Portugal (Placido *et al*., 1996), Denmark (Mosbech *et al*., 1991), Korea (Ree *et al.*, 1997), Hong Kong (Leung *et al.*, 1997) , Taiwan (Chang and Hsieh, 1989) and Thailand (Malainual *et al.*, 1995). Surveys in the United States demonstrated that most homes contained both *D. farinae* and *D. pteronyssinus* in many locations like Florida (Fernandez-Caldas *et al*, 1990), Virginia (Platts-Mills *et al*. 1987) and California (Mulla *et al.,* 1975), although some homes had predominantly *D. farina*, and some had mostly *D. pteronyssinus.* Up to now, twenty-one groups of dust mite allergens have been identified, 16 groups of allergens have been reported in *D. pteronyssinus*, and 12 groups of allergen have been found in *D. farinae*. IgE responses to a panel of nine *D. pteronyssinus* allergens revealed that Der p 1 and Der p 2 were major allergens of *D. pteronyssinus,* Der p 4, Der p 5, and

Der p 7 were moderate allergens, while Der p 3, 8, 10 and 20 were minor allergens with low IgE prevalence and low IgE titters in allergic asthmatic patients (Hales *et al*. 2006). The quantitative comparison of the IgE-binding among allergens of *D. farinae* has not been studied yet.

Recently, Der p 21 was released from Genbank database as a new putative allergen. Its allergenicity has not been characterized. In our local *D. farinae* EST database, a cDNA was found homologous to Der p 21 and its deduced protein sequence has 66.4% identity to Der p 21. Based on the Food and Agriculture Organization – World Health Organization (FAO/WHO) (2001) criteria for potential allergenicity, we proposed that this antigen might be allergenic. In this study, we aim to characterize Der f 21 in terms of its gene organization, human IgE-binding ability and cross-reactivity to Blo t 21, Blo t 5, Der f 5, and Der p 5.

5.2 Results

5.2.1 Identification of a novel homologous Group 5 allergen-Der f 21 from *D. farinae* EST database

Using local TBLASTN (Basic Local Alignment Search Tool) algorithm, Der f 5 protein sequence was compared with *D. farinae* Expressed Sequence Tags (ESTs) generated from our laboratory (Angus *et al.*, 2004). One *D. farinae* EST was found to have a deduced protein sharing approximately 36% amino acid identity and 55% similarity to Der f 5 sequence (accession no.BAE45865) in the genbank. This EST sequence contains an open reading frame (ORF) of 411 bp encoding Der f 21[♣] with 136 amino acids. It has a putative 17-amino acid signal peptide (predicted by SignalP v3.0 (Bendtsen *et al.*, 2004) (Figure 5.1), suggesting that Der f 21 is a secreted protein. The calculated molecular weight of the mature protein is 14.2 kDa, with a predicted isoelectric point of 4.64, and a predicted majority alpha-helical secondary structure (PredictProtein, http://cubic.bioc.columbia.edu/predictprotein/) (Figure 5.2).

Der f 21 protein sequence, its orthologue – Der p 21 (accession no. ABC73706), Blo t 21 (accession No. AY800348) and their Group 5 counterparts were aligned using ClustalW solfware (Thompson *et al.*, 1994) (Figure 5.3). Pairwise alignments showed that Der f 21 shared 66.4% amino acid sequence identity (77.9% similarity) to Der p 21, 37.5% identity (59.6% similarity) to Blo t 21, but only 28.7% identity (41.2% similarity) to Der f 5, 29.4% identity (44.9% similarity) to Der p 5 and 41.2% identity (53.7% similarity) to Blo t 5. In contrast, Der p 21 shared 41% amino acid identity (59% similarity) to Blo t 21, 30.7% identity (45% similarity) to Der p 5, 32.6% identity

[♣] Accession No. AY800348

```
1          GTGAAACATTAAATCAATCGAATCAAAAACAACGACAAAAAAAATTTTGAAAAATGAAAT
                                                                 M  K

61         TCATTATTTTCTGTGCCATTGTAATGGCTGTTTCTGTTTCCGGTTTCATCGTTGATGTCG
           F  I  I  F  C  A  I  V  M  A  V  S  V  S  G  F  I  V  D  V

121        ATACAGAAGATAAATGGCGTAATGCATTCGATCATATGTTGATGGAAGAATTTGAAGAGA
           D  T  E  D  K  W  R  N  A  F  D  H  M  L  M  E  E  F  E  E

181        AAATGGATCAAATCGAACATGGCCTACTTATGCTCAGTGAACAATATAAAGAATTGGAAA
           K  M  D  Q  I  E  H  G  L  L  M  L  S  E  Q  Y  K  E  L  E

241        AAACCAAAAGCAAAGAATTAAAAGAACAAATTCTTCGTGAATTGACAATCGCAGAAAACT
           K  T  K  S  K  E  L  K  E  Q  I  L  R  E  L  T  I  A  E  N

301        ATTTGCGTGGTGCCTTGAAATTCATGCAACAGGAGGCTAAACGTACCGATTTAAATATGT
           Y  L  R  G  A  L  K  F  M  Q  Q  E  A  K  R  T  D  L  N  M

361        TCGAACGTTATAACTTTGAAACAGCAGTGTCAACTATCGAAATATTGGTTAAAGATTTGG
           F  E  R  Y  N  F  E  T  A  V  S  T  I  E  I  L  V  K  D  L

421        CTGAATTGGCCAAAAAAGTTAAAGCTGTAAAATCGGATGATTAAATACAATCCACAATTT
           A  E  L  A  K  K  V  K  A  V  K  S  D  D

481        AATATAACACAATTTTCATCATCAACATCCCGGAAATAATTATAATATAAAAGACGAAAA

541        ATTTTGAAGAAATAACCG
```

Figure 5.1 cDNA and deduced protein sequence of Der f 21. Start and stop codons of the open reading frame are boxed. The signal peptide of protein sequence is underlined.

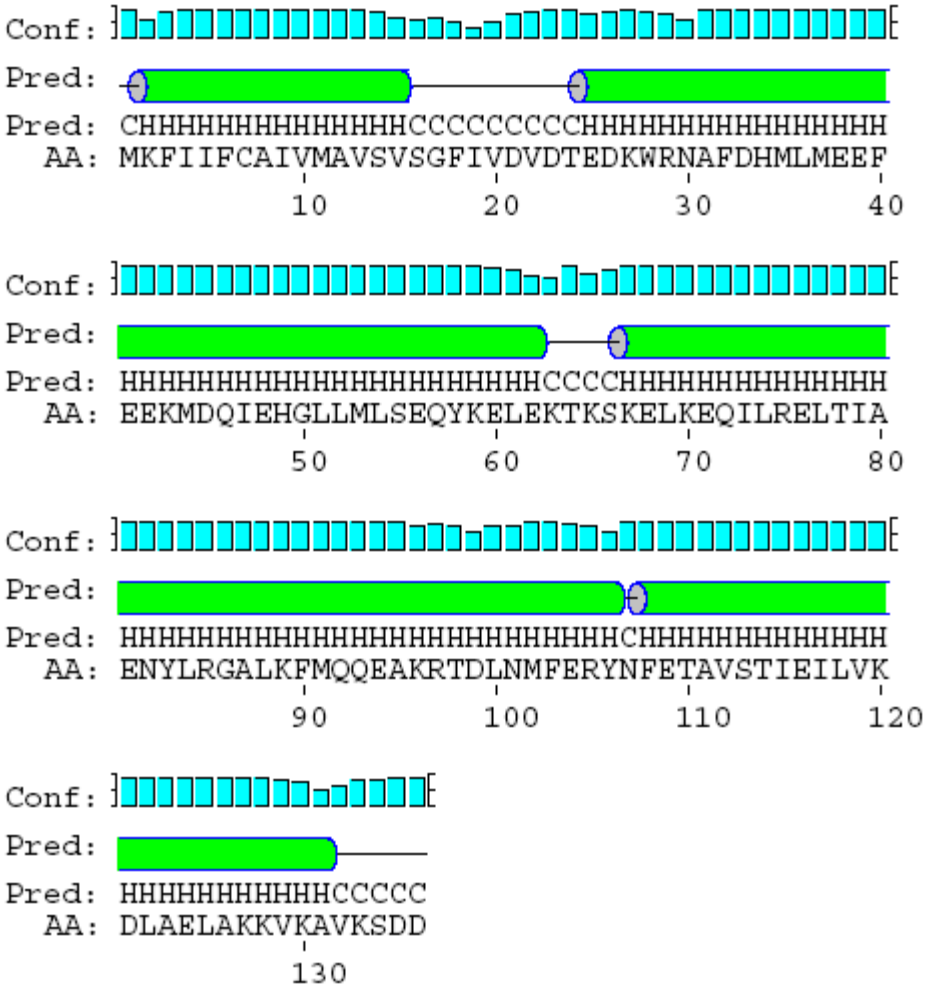


Figure 5.2 Predicted secondary structure of Der f 21 by PredictProtein (http://cubic.bioc.columbia.edu/predictprotein/). represents α helix. represents the confidence of prediction. —— represents random coil. H: helix; C: coil.

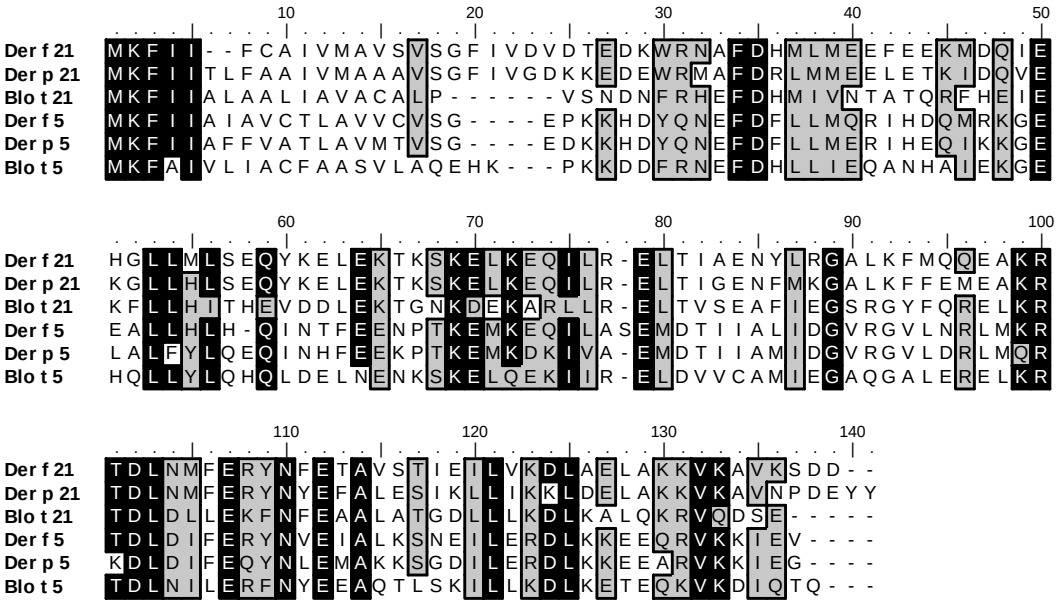


Figure 5.3 Multiple alignments of protein sequences of Der f 21, Der p 21, Blo t 21, Der f 5, Der p 5 and Blo t 5. Identical and similar sequences are shaded in black and gray respectively.

(45.5% similarity) to Der f 5 and 39.3% identity (50.7% similarity) to Blo t 5. From studies on Der f 5, we found that Der f 5 has higher amino acid identity to Der p 5 (75.2% identity) than to Der f 21 (28.7% identity).

Neighbor-Joining (NJ) phylogenetic tree built by DNAMAN (Lynnon BioSoft, Canada) showed that Group 21 allergens and Group 5 allergens clustered in two major clades (Figure 5.4). Group 21 and Group 5 allergens are from the same Pyroglyphidae family cluster in the same sub-clade. The Phylogenetic tree clearly indicated that Group 21 and Group 5 allergens of *Dermatophagoides pteronyssinus*, *D. farinae* and *Blomia tropicalis* were originated from duplicated allergen before speciation. However, no clear conserved domain could be observed within and between the Group 21 and Group 5 protein sequences.

5.2.2 Genomic organization of the gene encoding Der f 21 and Der f 5

Genomic organizations of Der f 21 and Der f 5 were amplified by PCR method and cloned into the pGEMT vector and subsequently sequenced. Sequences of ten individual clones showed that Der f 21 has an intron that interrupts amino acid 17 of the protein. The introns of 6 individual clones were 58 base pairs long, while the other four had an extra T insertion at intron position of 26. Among ten individual clones, clones 2, 3 and 4 were identical. There were 10 nucleotide variants in the entire genomic sequence, including two variants in the intron and the remaining eight in the exon regions. Six exon substitutions affected the amino acids that they were coding for. Protein variants occurred once at N30 to G, once at E41 to G, four times at M44 to I, once at L61 to S, once at L69

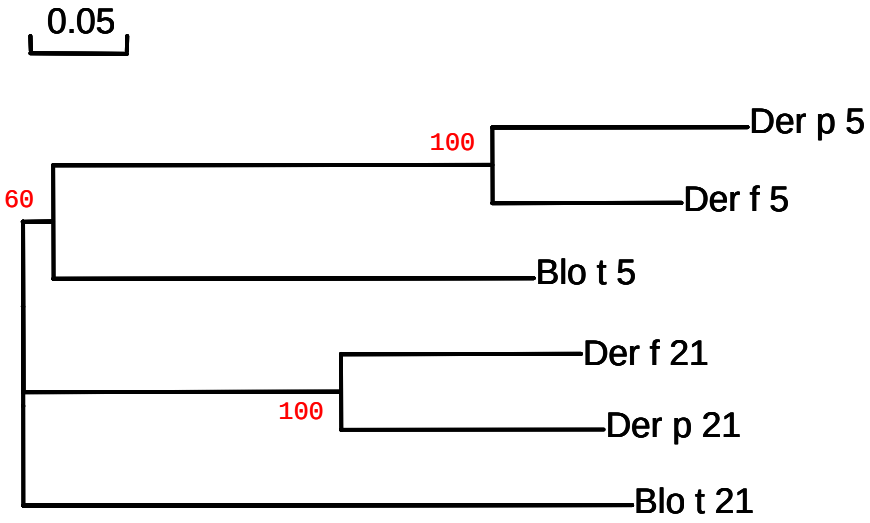


Figure 5.4 Phylogenetic tree of Group 5 and Group 21 allergens in three important mite species, *Dermatophagoides pteronyssinus*, *D. farinae* and *Blomia tropicalis.*

to P and once at S113 to P. In addition, protein sequences deduced from 10 genomic DNA clones of Der f 21 were different from that deduced from cDNA at position 34 (R>H) (Table 5.1).

In contrast, out of ten Der f 5 genomic DNA clones, seven had a 65 base pairs intron and three had an extra insertion of T in the intron. Intron disrupted amino acid 14 of the protein. Among sequences obtained from 10 clones, five from clones 1, 2, 8, 9, 10 were identical. In total, six substitutions were found in the exon region and four substitutions resulted in protein variants. The variants were at K22>E, R43>K, K68>E and T77>A, and all of them occurred only once in the 10 clones. Der f 5 protein sequence deduced from cDNA was also slightly different from that deduced from genomic DNA. There were two substitutions of E40>D and G73>A and one deletion of Q53. The insertion of additional three nucleotide acids in the cDNA sequence resulted in the replacement of G72 with A and S (Table 5.2). Figure 5.5 compares the genomic DNA sequences of Der f 21 and Der f 5. It can be seen that both Der f 21 and Der f 5 have one intron and their intron-exon splice borders are in agreement with the GT-AT rule (Mount, 1982). However, the position of the intron insertion shifted.

Table 5.1 Polymorphisms of the genomic component of Der f 21.

Nucleotide residue Position	AA residual number	Reference (gDer f 21.0101)	Polymorphic (triplet/AA)		Clone number	Frequency in 10 clones
g75	--	ttt	tttt (i26)	Intron	2,3,4,8	40%
g130	24	act/T	aca/T	Exon	1,6,7,9	40%
g146-147	30	aat/N	ggt/G	Exon	10	10%
g164	36	ttg/L	ctg/L	Exon	10	10%
g181	41	gaa/E	gga/G	Exon	1	10%
g190	44	atg/M	att/I	Exon	1,6,7,9	40%
g240	61	ttg/L	tcg/S	Exon	6	10%
g395	113	tca/S	cca/P	Exon	8	10%
c101	34	cgt/R	cat/H	cDNA	cDNA	10%

gDer f 21.0101 refers to the sequence of genomic DNA clones 2, 3, 4. g represents genomic DNA, and c represents cDNA. The polymophic amino acid is in red.

Table 5.2 Polymorphisms of the genomic component of Der f 5.

Nucleotide residue Number	AA residual number	Reference (gDer f 5. 0101)	Polymorphic (triplet/AA)		Clone number	Frequency in 10 clones
g49	--	ttt	tttt (i9)	Intron	4, 5, 7	30%
g54	--	cgt	cgc (i15)	Intron	11	10%
g129	22	aaa/K	gaa/E	Exon	3	10%
g193	43	aga/R	aaa/K	Exon	5	10%
g206	47	gag/E	gaa/E	Exon	4, 5	20%
g267	68	aaa/K	gaa/E	Exon	6	10%
g275	70	cag/Q	caa/Q	Exon	5, 7	20%
g294	77	act/T	gct/A	Exon	5	10%
c120	40	gaa/E	gac/D	Exon	cDNA	10%
c141	47	gag/E	gaa/E	Exon	cDNA	10%
c159	53	caacat/QH	cat/H	Exon	cDNA	10%
c215	72	ggt/G	gcaagt/AS	Exon	cDNA	10%

gDer f 5.0101 refers to the sequence of genomic DNA clones 1, 2, 8, 9, 10. g represents genomic DNA, and c represents cDNA. The polymophic amino acid is in red.

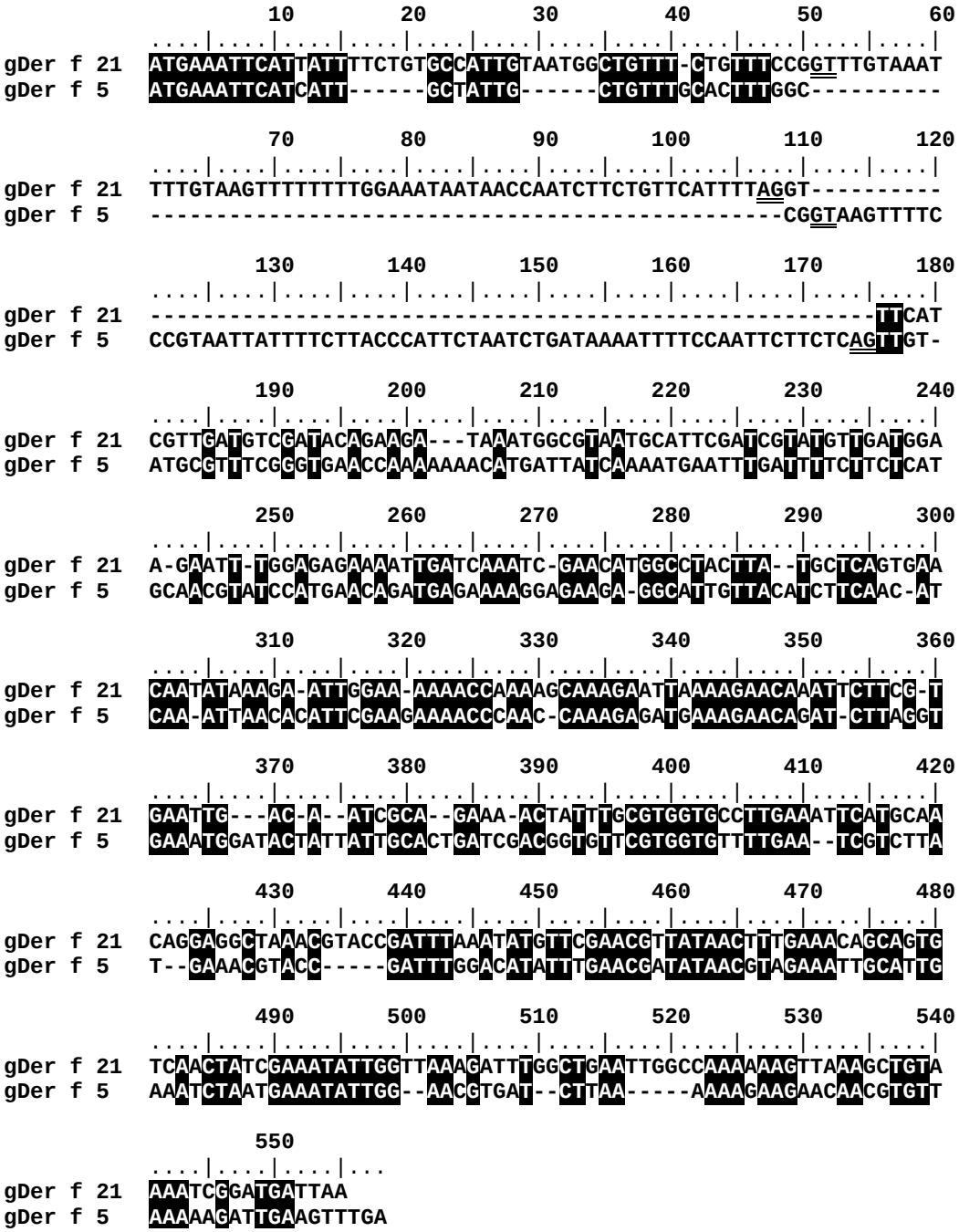


Figure 5.5 Pairwise alignment of genomic DNAs of Der f 21 and Der f 5. Identical sequences are shaded in black. The GT-AG intron borders were underlined.

5.2.3 Southern blotting of Der f 21 and Der f 5

Der f 21 and Der f 5 genes were further examined in *D. farinae* using Southern blotting. Upon hybridization, different banding patterns for Der f 21 and Der f 5 were observed and a single hybrid band for both genes was detected in each lane loaded with different enzyme digested *D. farinae* genomic DNA (Figure 5.6). Hence, Der f 21 and Der f 5 were located at different loci of the *D. farinae* genome with a single-copy. The nucleotide substitutions in the genomic sequences observed earlier were likely due to polymorphisms among different mite individuals rather than different copies of the gene in each mite.

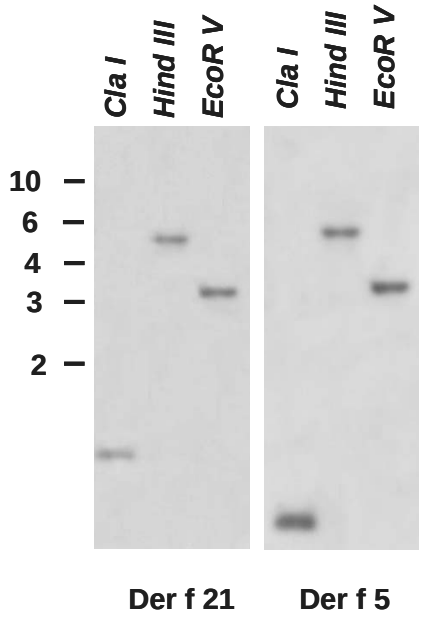


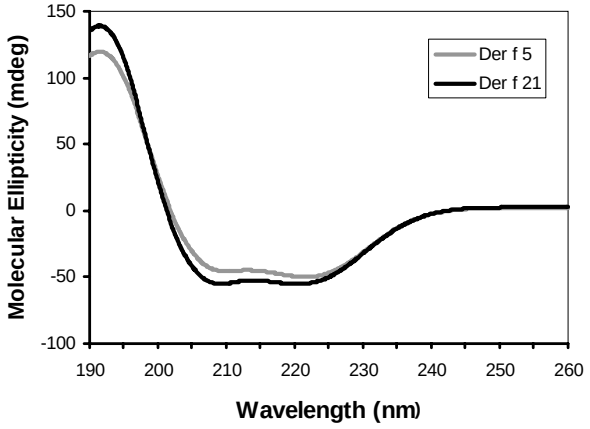
Figure 5.6 Southern blot analysis of Der f 21 and Der f 5 genes in mite genome. Genomic DNA was digested with restriction enzymes, *Hind III* and *EcoR V*, respectively.

5.2.4 Secondary structures of Der f 21 and Der f 5

Recombinant Der f 21 and Der f 5 were expressed and purified from *E. coli*. SDS-PAGE electrophoresis bands of the purified recombinant Der f 21 and Der f 5 proteins were about 14 kDa (Figure 5.7A), corresponding to their respective deduced molecular weights: Der f 21 (13 kDa) and Der f 5 (13.5 kDa).

To evaluate whether the recombinant proteins were properly folded after purification, far UV circular dichroism (CD) spectra of both rDer f 21 and rDer f 5 were measured. The CD profiles of both rDer f 21 and rDer f 5 revealed a typical α-helix structure of protein (Figure 5.7B) with two maximum negative peaks at 208 and 222 nm and one positive peak at 192 nm. The observed secondary structures were similar to those predicted from the amino acid sequences at PredictProtein server, as well as the Der p 5 secondary structure published previously by Liaw *et al.* (2001). The CD spectra profiles indicated that both recombinant Der f 5 and Der f 21 properly folded in solution, and also shared similar secondary structures.

(A)



(B)

Figure 5.7 Expression of recombinant Der f 21 and Der f 5 allergens and their far UV CD spectra. **(**A) Protein profile of recombinant Der f 5 and Der f 21 separated on 15% SDS-PAGE gel. (B) Far UV CD spectra of rDer f 5 and rDer f 21 proteins in phosphate buffer (pH7.4).

5.2.5 IgE-binding of Der f 21 and Der f 5 in consecutive atopic individuals in Singapore

Specific IgE antibodies to Der f 21, Der f 5, together with a panel of 9 allergens from *D. farinae,* were measured using semi-quantitative immuno-dot blot assay[♣]. Among 181 consecutive atopic individuals attending outpatient allergy clinics in a period of one year and a half, 74 were positive to *D. farinae* extract. Fourteen individuals (19% of *D. farinae* positive individuals) responded to Der f 21, and the same number of individuals responded to Der f 5. Twelve out of 14 sera responding to Der f 21 also responded to Der f 5. In general, specific IgE antibodies toward Der f 21 were lower than those of Der f 5 (Figure 5.8).

In a panel of 12 recombinant allergens of *D. farinae* tested among 74 atopic individuals positive to *D. farinae*, Der f 21 and Der f 5 were the second most prevalent allergens following Der f 2 in this population (Figure 5.9), suggesting that Der f 21 is an important allergenic component of *D. farinae*. Comparison of the IgE-binding capacities of Der f 2 and Der f 21 revealed that 64% (9/14) of Der f 21 positive sera strongly responded to Der f 2 (Figure 5.10). These sera had higher Der f 2 titer than Der f 21. However, 34% of sera only reacted to Der f 21, but not to Der f 2.

[♣] Semi-quantitative immuno-dot blot assay was performed by Tay Angeline, and ten *D. farinae* allergens included in this screening were cloned by Dr. Ong Siew Then and Dr. Shang Huishen.

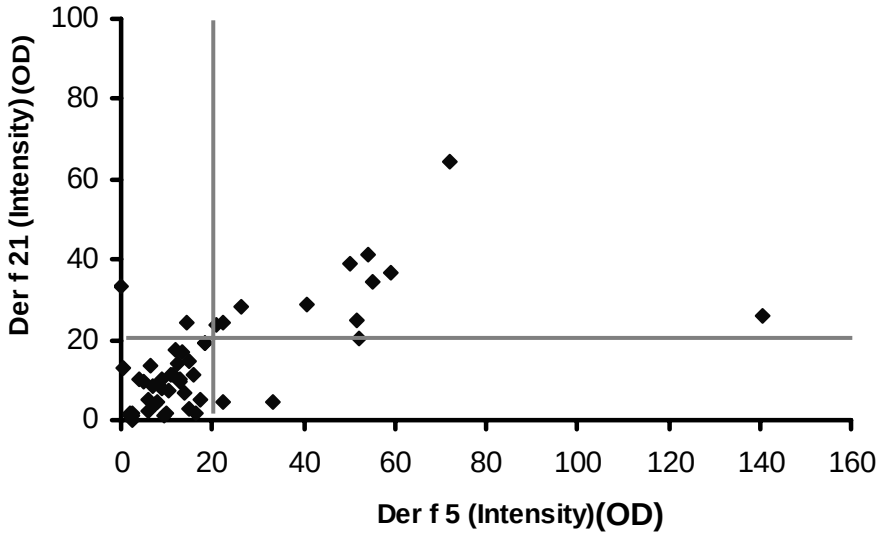


Figure 5.8 Comparison of the specific IgE levels against Der f 21 and Der f 5 assessed by dot blot immunoassay in sera of 74 *D. farinae* positive subjects attending outpatient allergy clinics over one and a half years. Grey lines indicate negative cut-off for the assay.

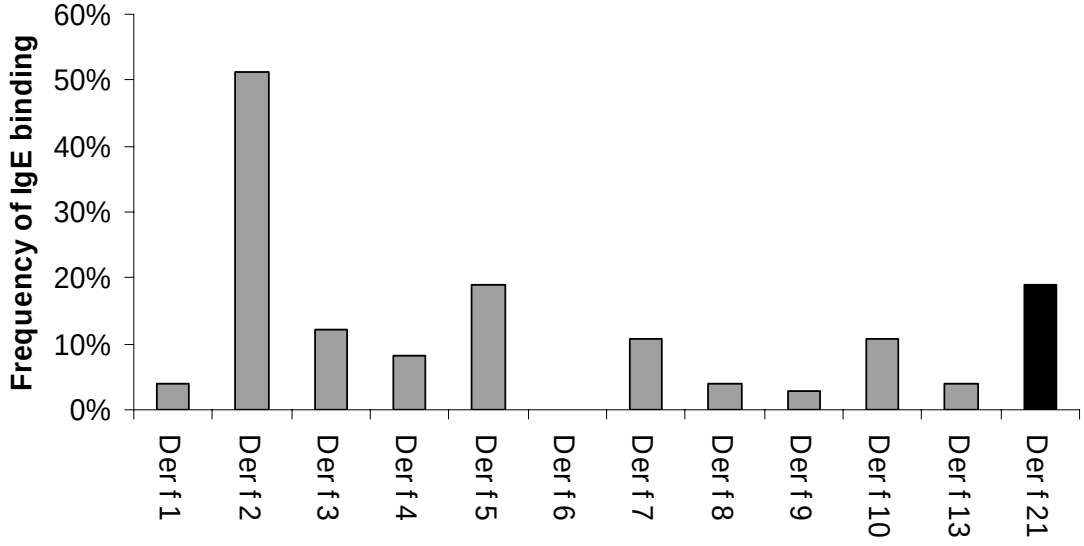


Figure 5.9 Frequencies of IgE-binding to a panel of 12 recombinant allergens of *D. farinae* in 74 mite positive subjects.

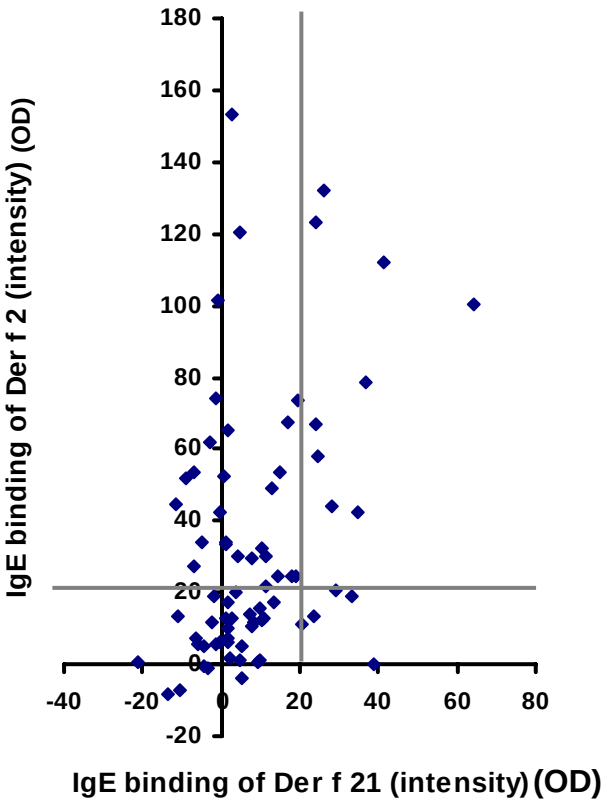


Figure 5.10 Comparison of the specific IgE levels against Der f 21 and Der f 2 assessed by dot blot immunoassay in sera of 74 *D. farinae* positive subjects attending outpatient allergy clinics over one and a half years. Grey lines indicate negative cut-off for the assay.

5.2.6 IgE-binding of Der f 21 in patients with onging persistent allergic rhinitis

The allergenicities of Der f 21 and Der f 5 were further evaluated using direct ELISA in thirty adult patients with ongoing persistent allergic rhinitis (R1-R31) who were positive to *B. tropicalis, D. pteronyssinus* and *D. farinae* and two non-atopic healthy volunteers. The characteristics of patients were described in Chapter 2 Material and Methods. Twenty-five out of thirty patients (83%) had specific IgE response to Der f 21, while twenty-eight patients (93%) had specific IgE to Der f 5 (Figure 5.11). Patients responding to Der f 21 also specifically responded to Der f 5. Compared with the results obtained in previous section, allergenicities of Der f 5 and Der f 21 were found more prevalent in patients with severe allergic rhinitis disease than in consecutive atopic individuals.

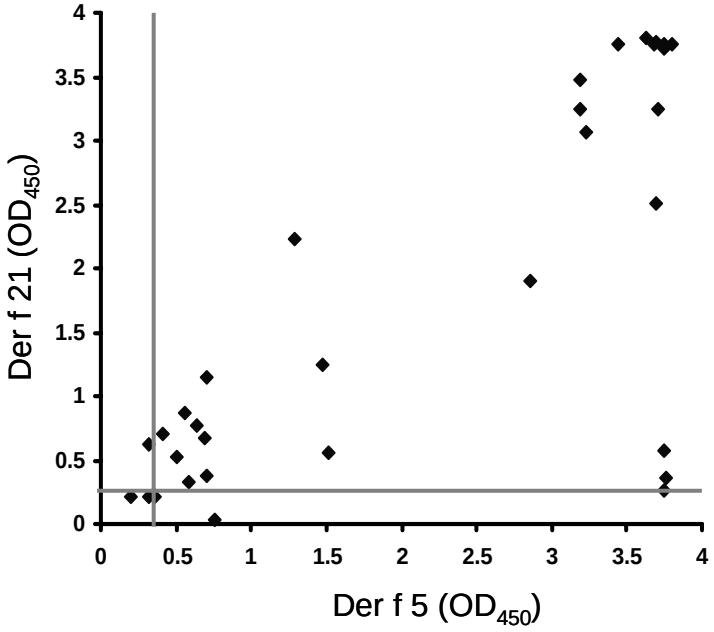


Figure 5.11 Bi-plot comparison of specific IgE levels against Der f 21 and Der f 5 assessed by ELISA in sera of 30 patients with ongoing persistent allergic rhinitis. Grey lines indicate the negative cut- off for the assay.

5.2.7 Dose-response cross-inhibitions of Der f 21 and Der f 5

The dose-response inhibitions of Der f 21 and Der f 5 to each other, as well as to Der p 5, Blo t 5 and Blo t 21, were further evaluated using ELISA in three sera positive to both Der f 21 and Der f 5. These sera were selected from the immunoarray screening. It can be seen from Figure 5.12 that heterogeneous inhibition curves of Der f 21 were much lower than the self-inhibition curves in two of three patients tested individually. Der f 21 self-inhibition occurred at a very low concentration (0.01 μg/ml) of inhibitor and achieved full self-inhibition at 100 μg/ml. In contrast, heterogeneous inhibition occurred after the inhibitor concentration reached 10 μg/ml, and could only achieve 34%-68% for Blo t 21, 14%-41% for Der f 5, 29%-52% for Der p 5, and 15%-38% cross-reactive with 100 μg/ml of inhibitor. Similar observation was obtained in these two sera when Der f 5 was immobilized on the solid surface (Figure 5.12 B).

In addition, high degree of cross-reactions of Der f 5, Der f 21, Blo t 5, Blo t 21 and Der p 5 were observed in one patient. As shown in the inhibition curve measured from the sera of patient V3, both Der f 21 and Der f 5 were completely inhibited by Blo t 21 and Blo t 5, and partially inhibited by Der p 5. The heterogeneous inhibitions of Blo t 5 and Blo t 21 were even stronger than self-inhibitions of Der f 21 and Der f 5. Hence this patient was predominantly sensitized by Blo t 21 and Blo t 5, and then cross-reactive to Der f 21 and Der f 5. Knowing that *B. tropicalis* is a local predominant mite, it is not surprising to see this phenomenon.

Partial inhibitions between Der f 5 and Der p 5 were observed in all three sera, suggesting that even though Der f 5 is closer to Der p 5 than to Blo t 5 and Group 21 allergens, specific epitopes still exist between these two molecules.

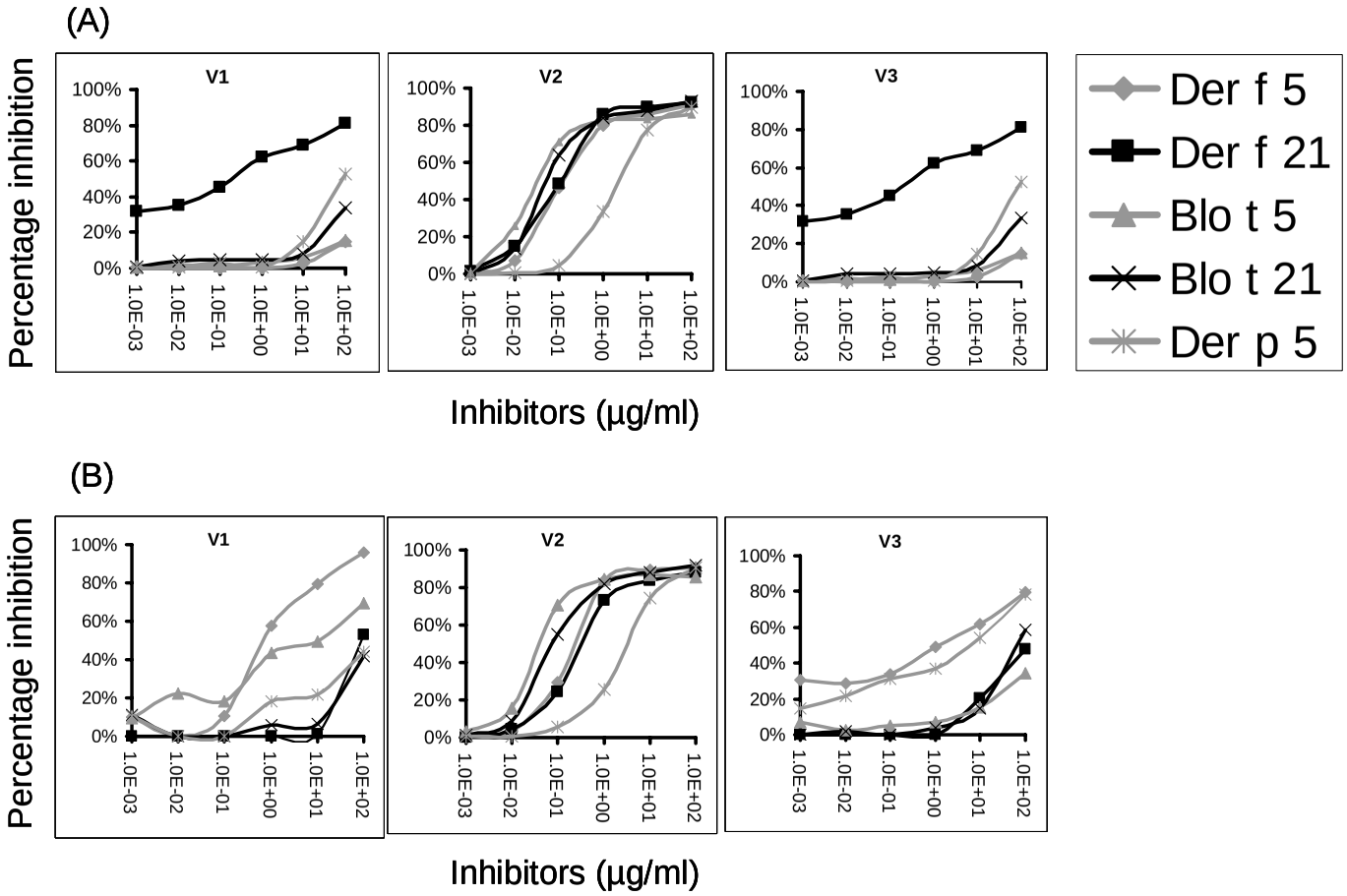
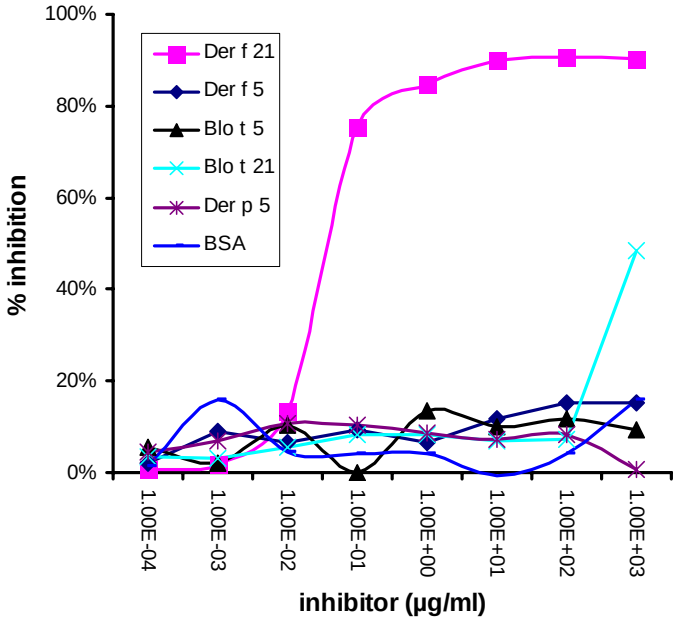


Figure 5.12 Dose-response competitive ELISA assay of Der f 21 and Der f 5 using three atopic patients sera. (A) Der f 21 immobilized on solid phase of ELISA plate. (B) Der f 5 immobilized on solid phase of ELISA plate.

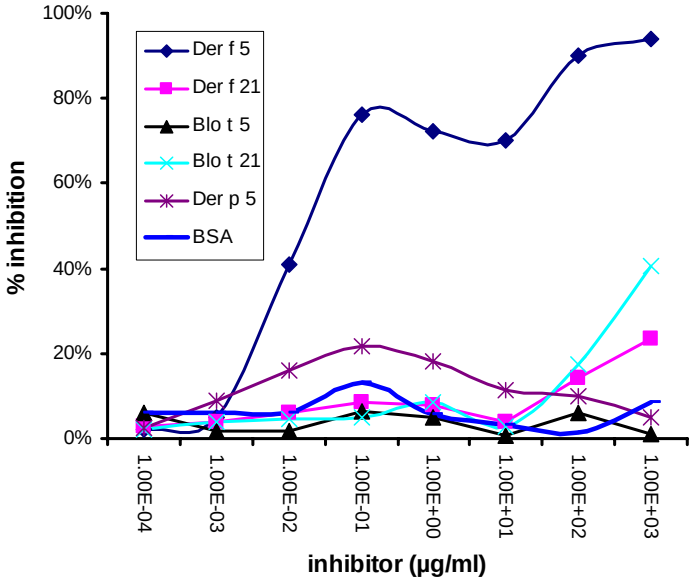
5.2.8 Localization of Der f 21 on sections of mite body

To ensure that reaction to antibody was specific, cross-reaction of anti-Der f 21 and anti-Der f 5 polyclonal antibodies was tested before anti-Der f 21 antibody was used for localization. Inhibition study indicated that anti-Der f 21 polyclonal antibody was unique to Der f 21 protein. It did not cross react to Blo t 21, Der f 5, Der p 5 and Blo t 5. BSA, a non-specific inhibitor, was used as negative control of the experiment. Similarly, anti-Der f 5 polyclonal antibody was also found specific to its own protein, Der f 5 (Figure 5.13).

Der f 21 immuno-staining was performed using anti-Der f 21 rabbit sera on paraffin sections of mite bodies. The Der f 21 antibody bound strongly to mid-gut contents of the mite sections (Figure 5.14), similar to our previous localization study of Blo t 21. Pre-immune rabbit sera showed no staining on mite body sections.



(A)



(B)

Figure 5.13 Competitive ELISA assay evaluating the IgG specificities of Der f 21 and Der f 5. (A) Der f 21 IgG antibody (B) Der f 5 antibody.

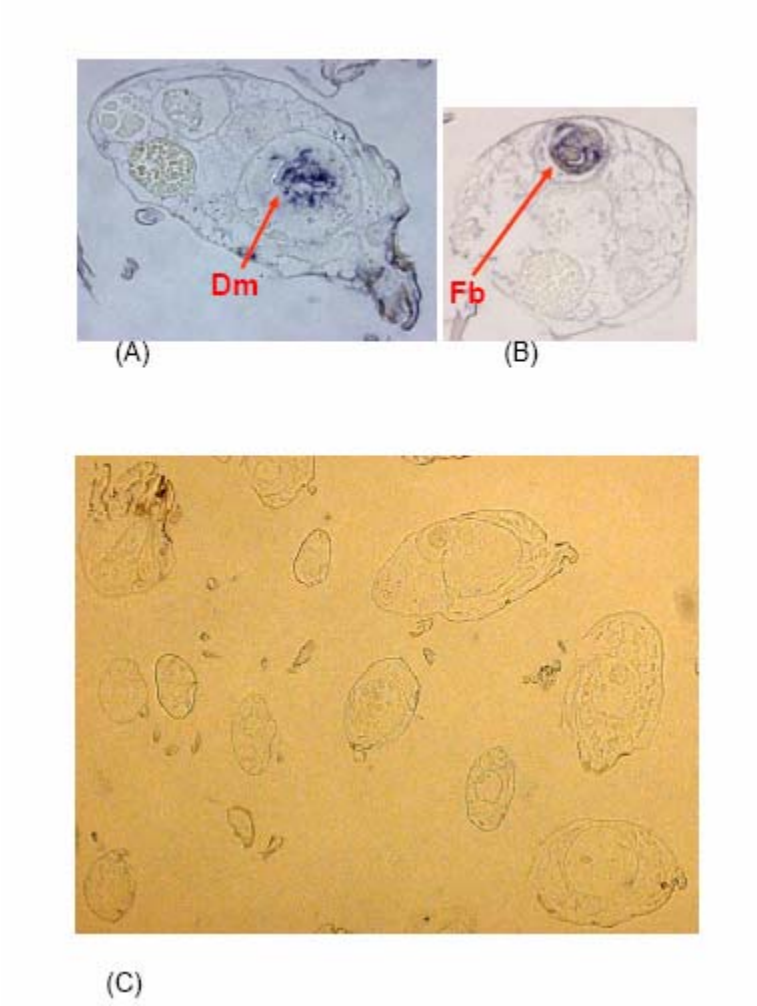


Figure 5.14 Immuno-staining of Der f 21 in paraffin-embedded sections of *D. farinae* probed with anti-Der f 21 polyclonal antibody in longitudinal (A) and sagittal mite sections (B). Section probed with pre-immune sera (C).

5.2.9 Der f 21 and Der f 5 in house dust samples

Seventy-seven dust samples from four niches of 40 homes, namely bedroom floors, living room carpets, living room floors and kitchen floors were assayed for Der f 21 and Der f 5 levels. Wilcoxon-Mann-Whitney rank test showed identical distributions of Der f 21 and Der f 5 among different niches where dust samples were collected. Der f 5 was detected in 50 out of 77 dust samples with concentrations ranging from 39 to 470 ng/g and the geometric mean was 74 ng/g. In parallel, Der p 21 was detected in 66 dust samples ranging from 10 to 350 ng/g with a geometric mean of 36 ng/g. Both allergens were present in the indoor dusts with low concentration in all four niches. Der f 21 and Der f 5 concentrations on living room carpets and bedroom floors were slightly higher than those on kitchen floors and living room floors. The overall concentrations of Der f 21 were lower than those of Der f 5 in all four niches where dust samples were collected.

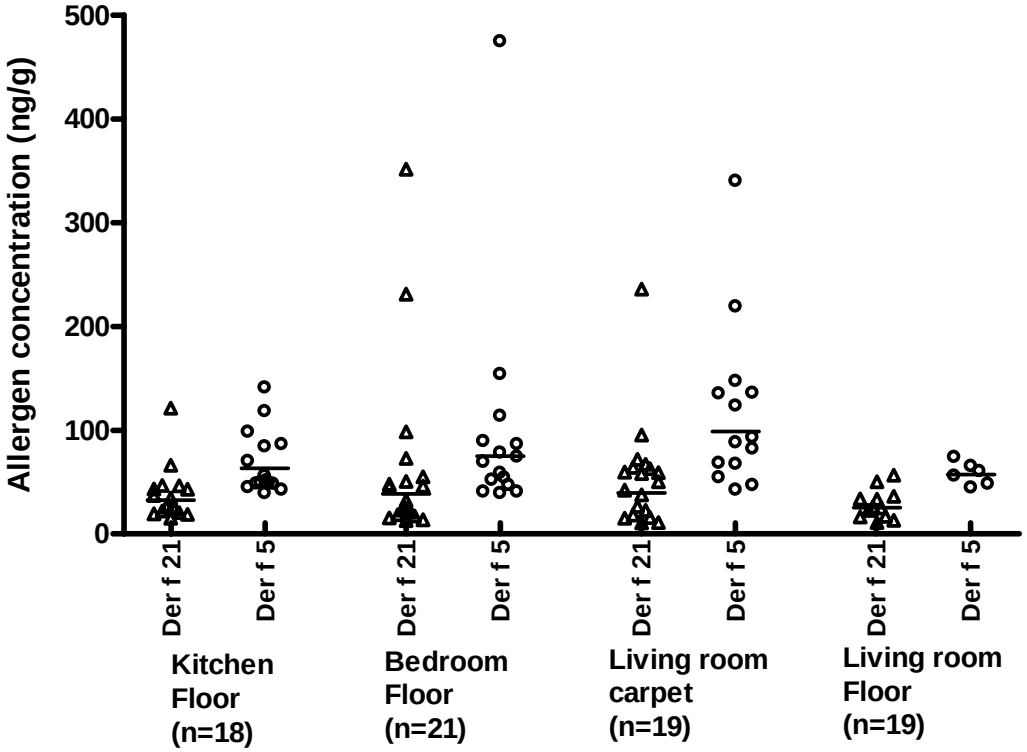


Figure 5.15 Der f 21 and Der f 5 levels in 76 house dust samples. The solid lines indicated the geometric mean of dust samples.

5.3 Discussion

In this study, a novel allergen, Der f 21, has been identified from our in-house *D. farinae* EST database using local TBLASTN searching against Der f 5. It is a product of a single-copy gene with an intron of 58 base pairs. The cDNA of Der f 21 encodes a protein similar to Der p 21, Blo t 21, as well as their Group 5 counterparts. Multiple sequence alignment showed that Der f 21 protein sequence is more related to Der p 21 (66.4% identity) than Der f 5 (28.7% identity). In parallel, Der f 5 was also found to share higher percentage identity to Der p 5 (75.2%) than Blo t 5 and other Group 21 allergens. According to sequences diversities of Group 21 and Group 5, phylogenetic tree was built using Neighbor-Joining (NJ) method (Figure 5.4). This phylogenetic tree shows that Group 21 and Group 5 allergens of three major dust mite species clustered in two major clades, indicating that Group 21 and Group 5 allergens may occur due to a prior event of gene duplication from the same ancestor and the duplication occurred before speciation. Since Der f 21 and Der f 5 had introns at different positions, it can be speculated that their introns may insert into genes after gene duplication.

It is of great interest to know whether Der f 21 is allergenic. IgE response to Der f 21 and Der f 5 was thus investigated in 181 consecutive atopic individuals attending outpatient allergy clinics over one year and a half and 30 severe rhinitis patients who were positive to *D. pteronyssinus*, *D. farinae* and *B. tropicalis* and needed to undergo further immunotherapy. Frequency of IgE-binding to Der f 21 is the same as Der f 5 allergens in atopic individuals. Sera responding to Der f 21 mostly responded to Der f 5 as well. Lower IgE level towards Der f 21 than Der f 5 has been observed in atopic individuals and rhinitis patients.

IgE-binding of Der f 21 could be resulted from co-sensitization or cross-reaction through common epitopes of Der f 21 and Der f 5. Dose-response inhibition study was performed to further investigate the reason for IgE-binding to Der f 21. Inhibition study revealed that in two out of three sera, Der f 21 was a low cross-reactive allergen against Der f 5, Blo t 21, Der p 5 and Blo t 5. One subject was found predominantly sensitized to Blo t 5 and Blo t 21 and cross-reactive to Der f 5 and Der f 21. Hence, different patients had different immuno-responses to Der f 21. Allergic response of Der f 21 could be either due to sensitization to specific epitopes of Der f 21, or cross-reactivity to Blo t 5 and Blo t 21 through the common epitopes existing in the conserved amino acid sequences between molecules of Der f 21, Der p 21, Blo t 21 and Blo t 5.

Immuno-localization experiment in this study revealed that Der f 21 localizes in the digestive system of dust mite like Der p 1 and Der p 2. Similar to Der p 1 and Der p 2, Der f 21 may also get out of the mite body and become airborne particles in the environment. The environmental levels of Der f 21 and Der f 5 were examined in 77 samples from 4 niches of 40 homes. Although Der f 21 and Der f 5 levels observed in this study were below the commonly suggested risk level for sensitization (2 μg/g of dust), they were similar to Der p 5 levels measured in houst dusts in Taipei, Taiwan (Li et al., 1996), but much lower than the level of Der p 1 or Der f 1 in the dust samples of Singapore by other studies (Li *et al*., 1994; Sporik et al., 1990). It is interesting to note that exposure to low levels of mite allergens would lead to sensitization especially for individuals with positive family histories of allergy (Waner *et al.*,; 1996 Wahn *et al.*, 1997; Huss, *et al.*, 2006).

This study revealed that Der f 21 alone was able to elicit IgE response in many susceptible individuals. Hence, it is important to include this allergen for allergy diagnosis and allergen-specific immunotherapy (SIT). Up to now, *D. farinae* extract is used for SIT in clinical practice. However, it has been reported that mite extract is unstable and may vary from batch to batch. In addition, endotoxin has ever been found in *D. farinae* extract (Trivedi *et al*., 2003). To overcome these problems, standardized formulation of mixed allergens to mimic natural mite extract could be used for better immunotherapy. Recombinant Der f 21 used in this study has retained its secondary structure and IgE-binding capacity. It can be used as a good candidate to form formulation allergens for immunotherapy.

In conclusion, a novel allergen Der f 21 was identified and characterized in *D. farinae* in this study. It is possible to elicit specific IgEs and bind to these IgEs. The cross-linked IgEs cause allergic response in many atopic subjects who were sensitized to *D. farinae*. Although Der f 21 shares some primary and secondary structure similarities to Der f 5, IgE antibodies binding to this allergen do not cross-react to Der f 5 in some patients. Der f 21 is thus an important allergen of *D. farinae* and should be considered in the diagnosis and treatment of *D. farinae* allergy.

Chapter 6: Identification and Characterization of Group 5 and Group 21 in *S. medanensis* and Cross Comparison of a Panel of 11 Group 5 and Group 21 Allergens

6.1 Introduction

Wide spectrums of mite species were identified from house dusts (Chew *et al.*, 1999a) in Singapore. *B. tropicalis* is a predominant mite species in this region, but high counts (>100 mites/g) of other less commonly occuring dust mites such as *Tyrophagus putrescentiae*, *Austroglycyphagus malaysiensis* were also found in dust samples. High temperatures (24-31 °C) and high relative humidity (64-96%) throughout the year could be the reason for the growth and proliferation of such a wide spectrum of dust mites.

Exposure to more than 100 mites/g of dust mites is considered as an independent risk factor for sensitization. Sensitization to dust mite is associated with development of allergic diseases. Skin prick test and sera IgE antibodies measurement in allergic asthma and allergic rhinitis patients in Singapore demonstrated high prevalence of sensitization to *B. tropicalis* (96.2%), *D. pteronyssinus* (93.4%), *D. farinae* (92.3%), *A. malaysiensis* (78.2%), *S. brasiliensis* (71.6%) and *T. putrescentiae* (71.3%). High degrees of sensitization to less commonly occuring dust mites may be caused by exposure to high counts of mites in house dusts or cross-reactivity of predominant mites like *B. tropicalis*.

B. tropicalis and *Dermatophagoides spp.* have been extensively studied. Blo t 5 and Der p 5 have been reported as major allergens that cause allergic diseases, because they can bind IgE antibodies in mite sensitized subjects with high frequencies and intensities (Lin *et al.,* 1994; Arruda *et al.,* 1997). Low cross-reactivity between Blo t 5 and

Der p 5 has been reported in several studies (Chew *et al.*, 1999b; Kuo *et al.*, 2003). Thus, it can be inferred that Blo t 5 and Der p 5 are able to specifically induce allergy by their specific epitopes.

In 2006, a cDNA of Group 5 homologous gene, Der p 21 was deposited in the public database (Genbank, http://www.ncbi.nlm.nih.gov/). Its allergenicity has yet to be characterized. From *B. tropicalis* and *D. farinae* EST database available in our laboratory, Blo t 21 and Der f 21 have been identified as well. Characterization of Blo t 21 and Der p 21 allergens in Chapter 3 and Chapter 5 demonstrated that Blo t 21 and Der f 21 are also specific for sensitization and development of allergic diseases. It is of interest to find out whether Group 5 and Group 21 in less commonly occurring mite species are also allergenic, since these mites are also present in the environment. The EST projects conducted in our laboratory provide a large amount of DNA sequences of eight mite species, including some storage mites existing in house dusts worldwide. Such information opened a door for further identification of Group 5 and Group 21 from storage mites such as *Suidasia medanensis*, *Tyrophagus putrescentiae* and *Aleuroglyphus ovatus*.

6.2 Results

6.2.1 Group 5 and Group 21 allergens in *Suidasia medanensis*

6.2.1.1 Identification of Group 5 and Group 21 in *S. medanensis*

From the expressed sequence tagging database of *S. medanensis* prepared in the laboratory[♣], twenty-five ESTs homologous to Group 5 dust mite allergens were identified by local BLASTX searching. Group 5 homologous ESTs were among the top ten most abundant ESTs in *S. medanensis.* Multiple alignments of these ESTs using DNAMAN (Lynnon BioSoft, Canada) and subsequent generation of an unrooted phylogenetic tree based on the diversities between these sequences using a bootstrap value of 1000 with the same software provided a clear picture of the relationship between these EST sequences (Figure 6.1). These 25 ESTs were clustered into three separated clades, suggesting that there could be three different "sub-groups" of Group 5 homologoues in *S. medanensis*. The presence of multiple copies of cDNA sequences in each clade offers additional positive support to the above assumption.

[♣] *S. medanensis* cDNA library construction and ESTs generation were conducted in my previous studies and not described in this thesis. The sequencing of cDNAs was performed together with Kavita Reginald.

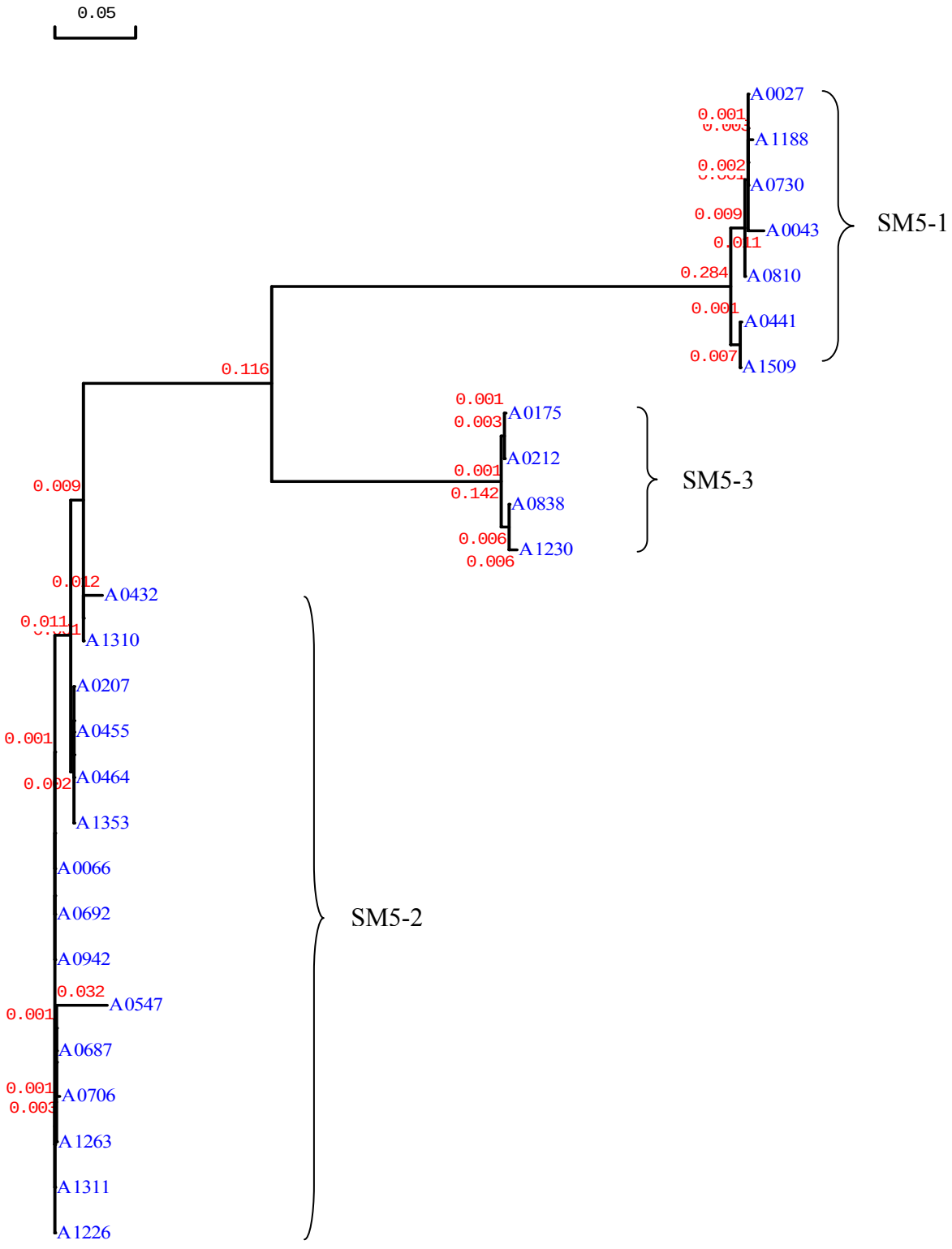


Figure 6.1 Unrooted phylogenetic tree of 25 *S. medanensis* cDNAs encoded Group 5 and its homologues drawn using a bootstrap value of 1000 (DNAMAN).

cDNAs from representative ESTs, the most frequently occurring polymorphism in each clade were given their temporary names as SM5-1, SM5-2 and SM5-3 according to the sequential of cloning, before officially named by the WHO/IUIS nomenclature committee. The open reading frames of SM5-1, SM5-2 and SM5-3 cDNAs encode proteins in the lengths of 134, 130 and 128 amino acids (Appendix 1-III). Each protein contains a 16 amino acids signal peptide in the N-terminal of the protein predicted by SignalP v3.0 (Bendtsen *et al.*, 2004), suggesting that these proteins may be secreted proteins. Since *S. medanensis* is taxonomically closer to *B. tropicalis* than *Dermatophagoides spp.*, protein sequences of SM5-1, SM5-2, and SM5-3 were compared with Blo t 5 and Blo t 21 using ClustalW (Thompson *et al*., 1994) integrated in BioEdit (Hall, 1999) (Figure 6.2A). The unrooted phylogenetic tree was built subsequently using Neighour-Joining algorithm in BioEdit (Figure 6.2 B). These five proteins fall into 2 major clades with Blo t 5, SM5-2 and SM5-3 clustering in one, and Sm5-1 and Blo t 21 in the other. In pair-wise comparison, SM5-2 has 76.9% identity to SM5-3, 48.9% to Blo t 5, but 39.6 % to SM5-1. According to the guideline of WHO/ IUIS nomenclature committee, SM5-2 and SM5-3 are renamed as Sui m 5.01 and Sui m 5.02, and SM5-1 is renamed as Sui m 21.

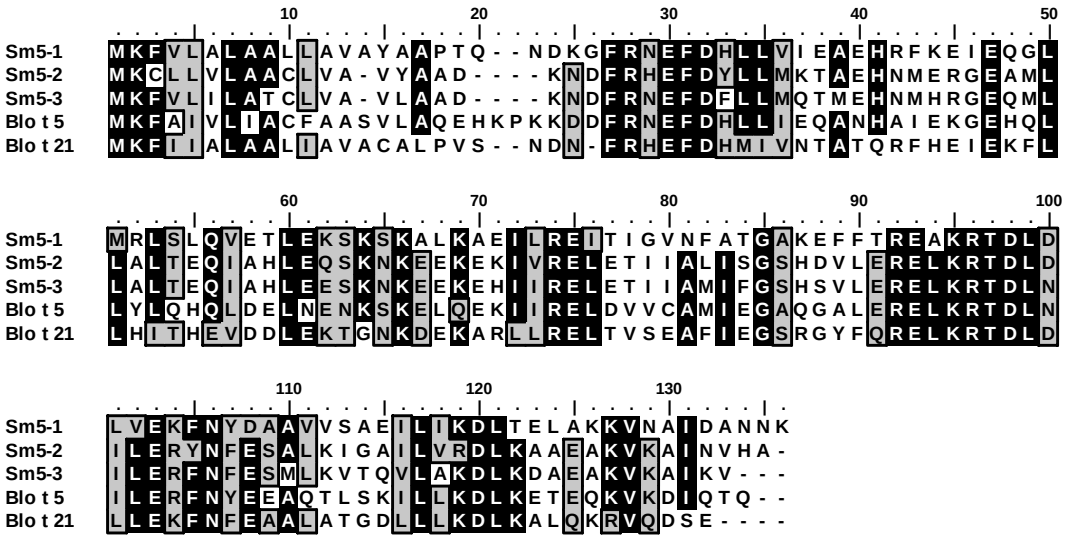


Figure 6.2A Multiple alignments of SM5-1, SM5-2, SM5-3 with Blo t 5 and Blo t 21. Identical and similar sequences are shaded with black and grey respectively.

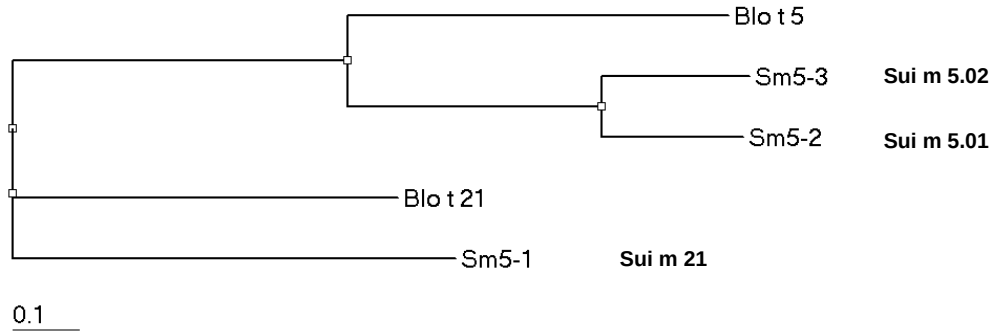


Figure 6.2B Phylogenetic tree of Group 5 homologues in *S. medanensis*, Blo t 5 and Blo t 21 drawn by Neighbor-Joining method.

6.2.1.2 Expression of Sui m 5.01, Sui m 5.02 and Sui m 21 in *E.coli*

In order to study these putative allergens, cDNAs of Sui m 5.01, Sui m 5.02 and Sui m 21 encoding secreted part of proteins were cloned into pET15 vector to produce six histidine tagged N-terminal fusion proteins. Group 5 homologues proteins were over-expressed as soluble proteins in *E. coli* BL21* (DE3) cells. The proteins purified by a Ni-NTA column were about 13 kDa when analyzed on a 15% SDS-PAGE gel stained with Coomassie blue (Figure 6.3).

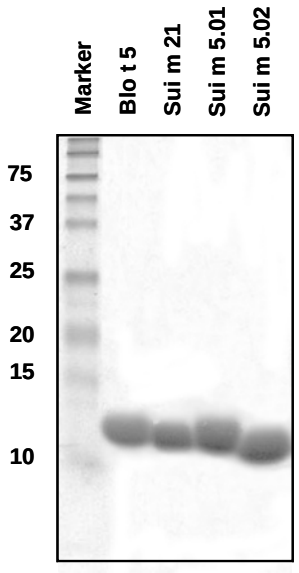


Figure 6.3 SDS-PAGE profile of purified Blo t 5, Sui m 5.01, Sui m 5.02 and Sui m 21 proteins.

6.2.1.3 IgE-binding activities of Sui m 5.01, Sui m 5.02 and Sui m 21

Specific IgE antibodies against Sui m 5.01, Sui m 5.02, Sui m 21 and other [♣]eight allergens of *S. medanensis* were measured by semi-quantitative immuno-dot blot assay using recombinant proteins[♣♣]. These eight allergens are Sui m 1 to Sui m 3, Sui m 7 to Sui m 10 and Sui m 13. Among total 181 consecutive atopic individuals attending outpatient allergy clinics over one year, 82 subjects were positive to *S. medanensis* extract, and 75 out of 82 subjects responded to both *S. medanensis* and *B. tropicalis*. 28% (23/82) of *S. medanensis* positive subjects responded to Sui m 5.01, 19% (16/82) to Sui m 5.02 and 16% (13/82) to Sui m 21. Sui m 5.01 was thus the most common allergen in the total of 11 allergens, and Sui 5.02 and Sui 21 were the third and fourth most common allergens in the panel (Figure 6.4A).

When IgE-binding intensities of an individual allergen in all tested sera are summed together, this integral value could represent the specificity of IgE to that particular allergen source in the population. According to the information obtained from *B. tropicalis* and *D. pteronyssinus,* major allergens were normally among Group 1 and Group 13. The sum of the IgE activities towards Groups 1, 2, 3, 5, 7, 8, 9 10, 13 and 21 allergens in *S. medanensis* may contribute to the majority of total *S. medanensis* IgE activities. In this study, IgE antibodies of Sui m 5.01, Sui m 5.02 and Sui m 21 accounted for 20%, 16% and 10% of the total IgE levels of *S. medanensis* (Figure 6.4B). IgE antibodies against these three allergens contributed to 46% of the total sera IgE, suggesting the importance of Group 5 and Group 21 in *S. medanensis* allergy. A strong

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[♣] Group 1 to Group 10 allergens of *S. medanensis* were cloned in pET32 in my previous study. This part of work was not included in this thesis. Sui m 6 and Sui m 13 were constructed by Kavita Reginald.

[♣♣] Immuno-dot blot assay was done by Ms Tan Angeline.

correlation between IgE antibodies of Sui m 5.01 and Sui m 5.02 was observed, while low correlation between Sui m 5.01 and Sui m 21 IgE antibodies was found by scatter plot analysis (Figure 6.4 C and D).

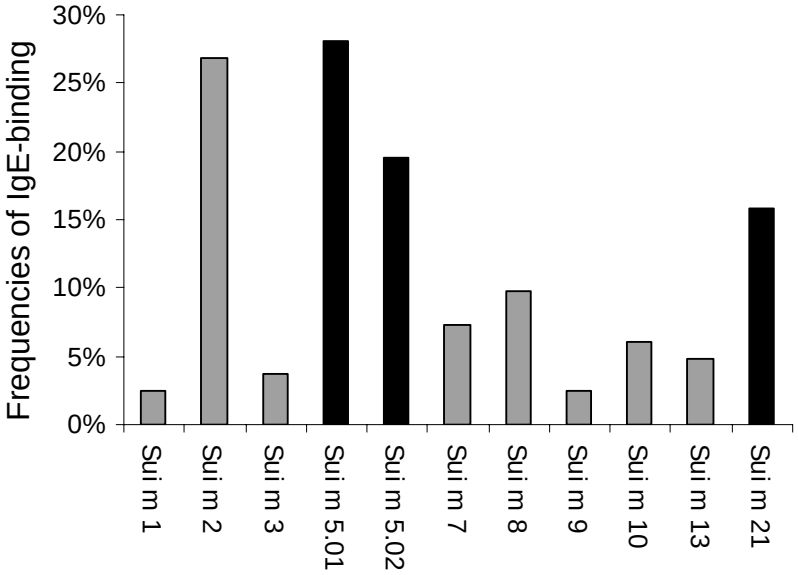


Figure 6.4A Frequencies of IgE-binding to a panel of 11 allergens of *S. medanensis* in 82 sensitized subjects.

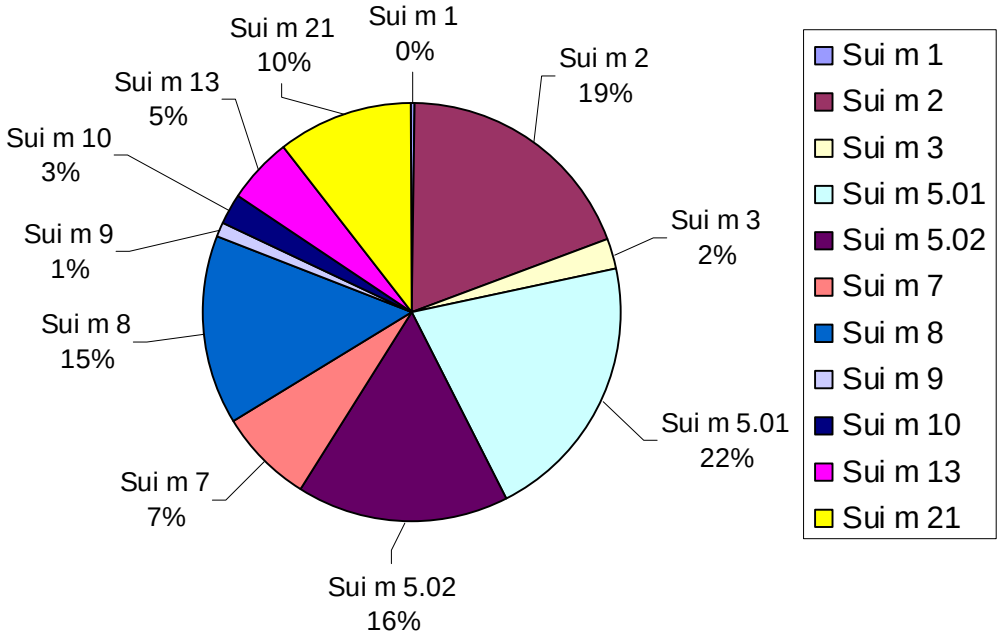
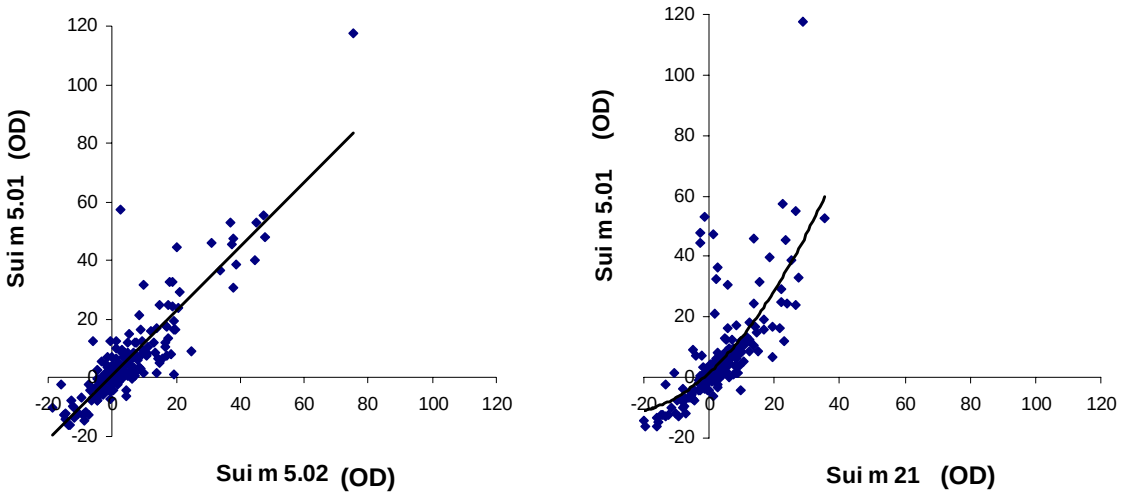


Figure 6.4B Percentage of specific IgE activities of Groups 1, 2, 3, 5, 7, 8, 9 10, 13 and 21 allergen in total IgE of *S. medanensis*.



(C) (D)

Figure 6.4 C and D Correlation of specific IgE antibodies between Sui m 5.01 and 5.02 (C), and between Sui m 5.01 and Sui m 21 (D).

6.2.2 Identification of Ale o 5

From the expressed sequence tag (EST) database of *Aleuroglyphus ovatus* available in the laboratory (Angus *et al.*, 2004), sequences showing homology to Blo t 5 and Blo t 21 were identified using local BLASTX. Four cDNA sequences were fished out from EST database when searched against either Blo t 5 or Blo t 21. All four cDNA clones had similar DNA sequences. The BLAST score of Blo t 5 searching was higher than that of Blo t 21 searching, indicating that these sequences were closer to Group 5. Hence, putative allergen encoded by these cDNAs was named Ale o 5 (accession number AAS75832), in accordance with the allergen nomenclature guidelines (WHO/IUS Allergen Nomenclature Subcommittee World Health Organization, 1994). The open reading frame of Ale o 5 encodes 135 amino acid residues (Appendix IV). Ale o 5 contains a signal peptide with 16 amino acids predicted by SignalP v3.0 (Bendtsen *et al.*, 2004).

6.2.3 Expression of recombinant Ale o 5, Gly d 5, Lep d 5 and Der p 5 in *E. coli*

The cDNA encoding for the mature protein of Ale o 5 was amplified using PCR method and cloned into pET15 vector. The primers were designed according to the cDNA sequences of Der p 5 and Lep d 5 available in Genbank database. Der p 5 and Lep d 5 cDNAs were then amplified by RT-PCR and subsequently inserted into pET15 vector. Gly d 5[♣] was expressed as thioredoxin fusion protein. Thioredoxin tag helped increase the expression and solubility of Gly d 5.

The recombinant Ale o 5, Der p 5, Lep d 5 and Gly d 5 were over-expressed in *E. coli* BL21* (DE3) strain and produced as His-tag fusion protein. Ale o 5, Der p 5, Lep d 5 and Gly d 5 purified by a Ni-NTA column were about 14 kDa, 14 kDa, 20 kDa and 42 kDa when analyzed on a 15% SDS-PAGE gel stained with Coomassie blue (Figure 6.5).

[♣] Gly d 5 expression clone was provided by Mr. Aaron Chen Angus.

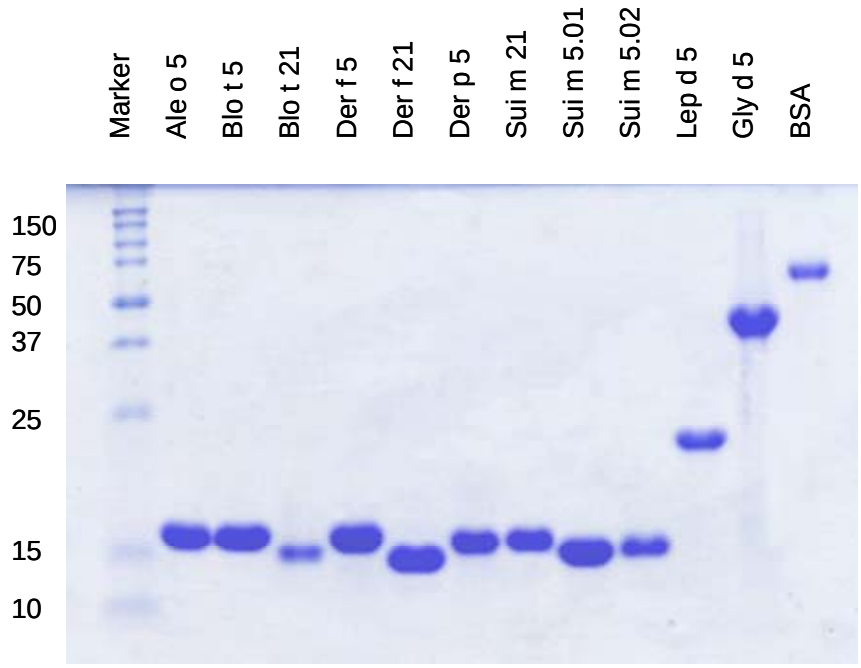


Figure 6.5 SDS-PAGE profile of a panel of Group 5 and Group 21 proteins including Ale o 5, Der p 5, Lep d 5 and Gly d 5.

6.2.4 IgE-binding profiles of 11 Group 5 and Group 21 allergens

Specific IgE activites to 11 Group 5 and Group 21 from *B. tropicalis*, *D. farinae*, *D. pteronyssinus*, *Lepidoglyphus destructor, G. domesticus* and *S. medanensis* were studied in 118 local mite positive sera using semi-quantitative immunoarray assays. These sera were recruited from consecutive atopic subjects attending similar outpatient allergy clinics in one year and a half. Dust mite crude extracts and thioredoxin-tag protein were dotted on membrane as positive and negative protein controls. Sera from atopic individuals did not bind to thioredoxin-tag protein produced by pET32a.

In this batch of sera, 97 responded to *B. tropicalis*, 74 to *D. farinae*, 80 to *D. pteronyssinus,* 58 to *Aleuroglyphus ovatus,* 82 to *S. medanensis,* 63 to *L. destructor* and 82 to *G. domesticus.* Majority of *D. farinae* (68/74) and *D. pteronyssinus* (73/80) positive sera were positive to *B. tropicalis*. Thus *B. tropicalis* allergy was the most common mite allergy tested in this atopic Singaporean population.

Among mite positive subjects, 41% (48/118) had specific IgE antibodies to Blo t 5, followed by Ale o 5 (27%, 32/118), Blo t 21 (25%, 30/118) and Sui m 5.01 (19%, 22/118). Frequencies of IgE-binding to Der f 5, Der p 5, Gly d 5, Lep d 5, Sui m 5.02, Der f 21, and Sui m 21 were below 14 % (Figure 6.6). Scatter plot analysis of IgE antibodies to Blo t 5 and other Group 5 allergens demonstrated the predominance of IgE antibodies towards Blo t 5 in mite positive sera tested in this batch (Figure 6.7A). Similar predominance of Blo t 21 was found against Der f 21 and Sui m 21 (Figure 6.7B).

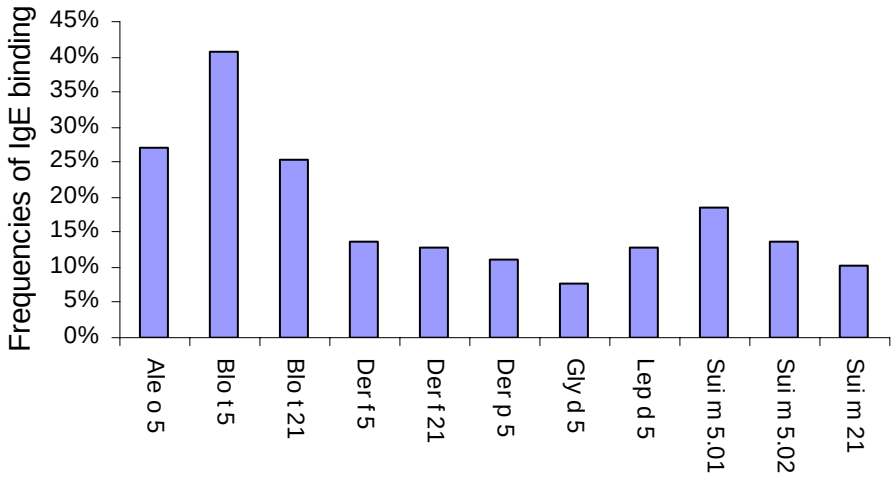


Figure 6.6 Frequencies of IgE-binding toward a panel of 11 Group 5 and Group 21 allergens from seven mite species existing in local environment tested in 118 local mite positive sera visiting outpatient clinic.

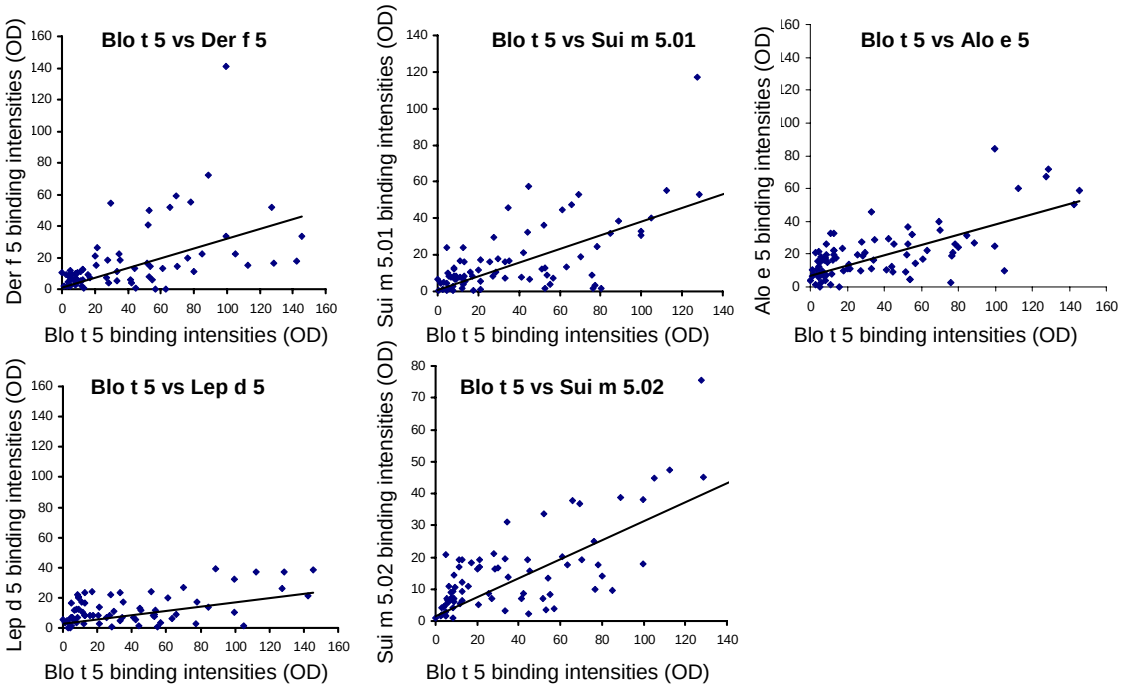


Figure 6.7(A) Bi-plot assay of IgE activities of Blo t 5 to Der f 5, Lep d 5, Ale o 5, Sui m 5.01 and Sui m 5.02.

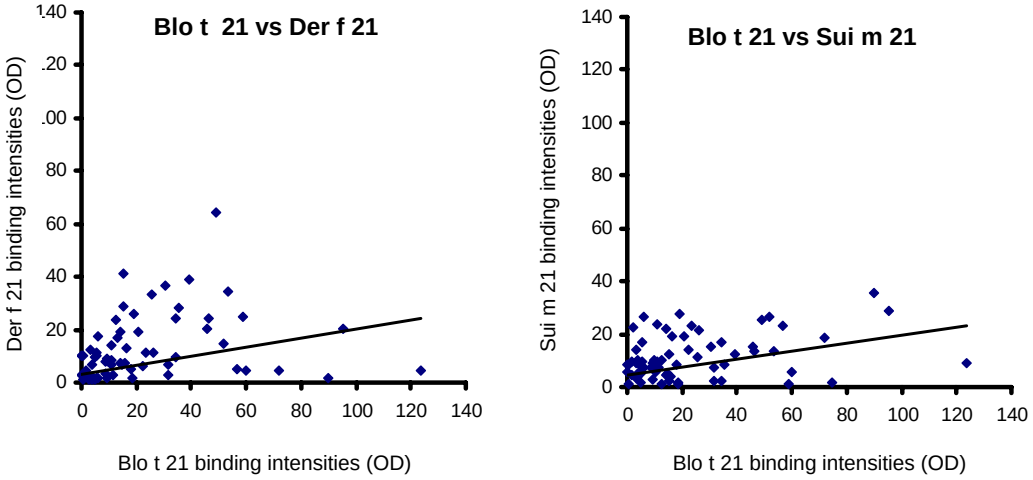


Figure 6.7(B) Bi-plot assay of IgE activities of Blo t 21 to Der f 21 and Sui m 21.

6.2.5 Cross-reactivity of Group 5 and Group 21 allergens

Cross-inhibitions of Group 5 and Group 21 allergens between different species are very important for clinical diagnosis and immunotherapy. With a large panel of Groups 5 and 21 allergens at hand, it is possible for us to study the specificities of these allergens. Due to limited amount of sera collected, cross-inhibition was only performed by ELISA end-point competition using seven Group 5 and Group 21 allergens from important mite species as inhibitors at the concentration of 100 μg/ml.

6.2.5.1 Cross-reactivity of Blo t 5 and Blo t 21

Three sera (V1-V3) screened by previous studies showing positive to Blo t 5, Blo t 21 and with sufficient amount were selected for the inhibition study of Blo t 5 and Blot 21. When Blo t 5 was immobilized on the solid phase, complete homozygous inhibition of Blo t 5 was achieved in all three patients tested at the concentrations of 100 μg/ml. Heterozygous inhibitors were only able to partially inhibit IgE-binding activities to Blo t 5 (49%-67% for Blo t 21; 19%-77% for Der f 5; 52%-54% for Der f 21; 18%-67% for Der p 5; 28%-71% for Lep d 5; and 2%-37% for Ale o 5) (Figure 6.8), indicating low to moderate cross-reactivity between these allergens.

Same sera were used to conduct competition study for Blo t 21. Results showed that Alo e 5 was not able to inhibit the IgE-binding acticities to Blo t 21 in all three sera (< 2% inhibition). Blo t 5 and Der p 5 were not able to inhibit Blo t 21 in two sera and only had low inhibition to Blo t 21 in one sera (16% inhibition). Lep d 5 demonstrated low inhibition in all three sera (17%-23% inhibition). Der f 5 and Der f 21 showed low to moderate inhibition to Blo t 21 (18%-69%) (Figure 6.9).

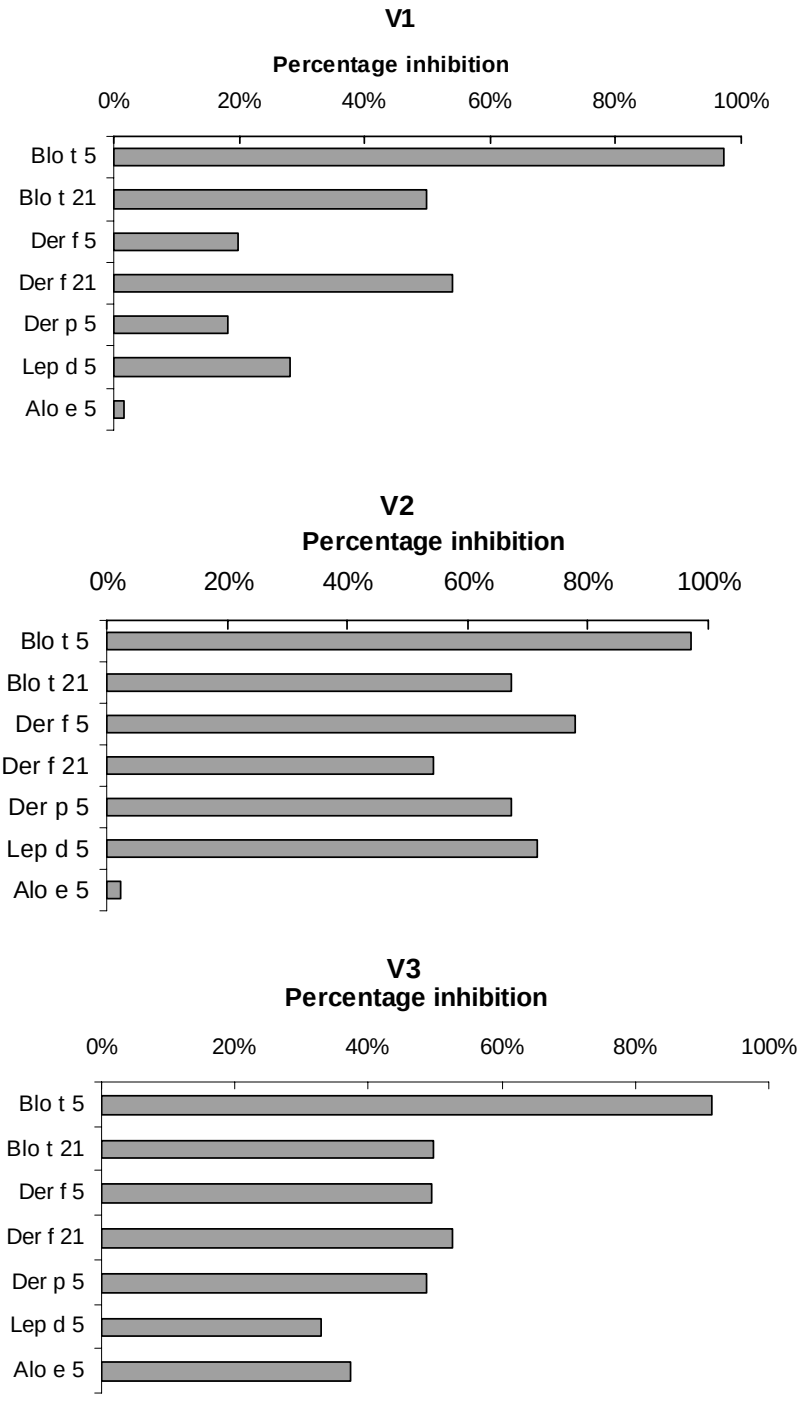


Figure 6.8 Inhibition of Blo t 5 by Group 5 and Group 21 inhibitors from *B. tropicalis*, *D. farinae*, *D. pteronyssinus*, *L. destructor* and *A. ovatus* using three sera.

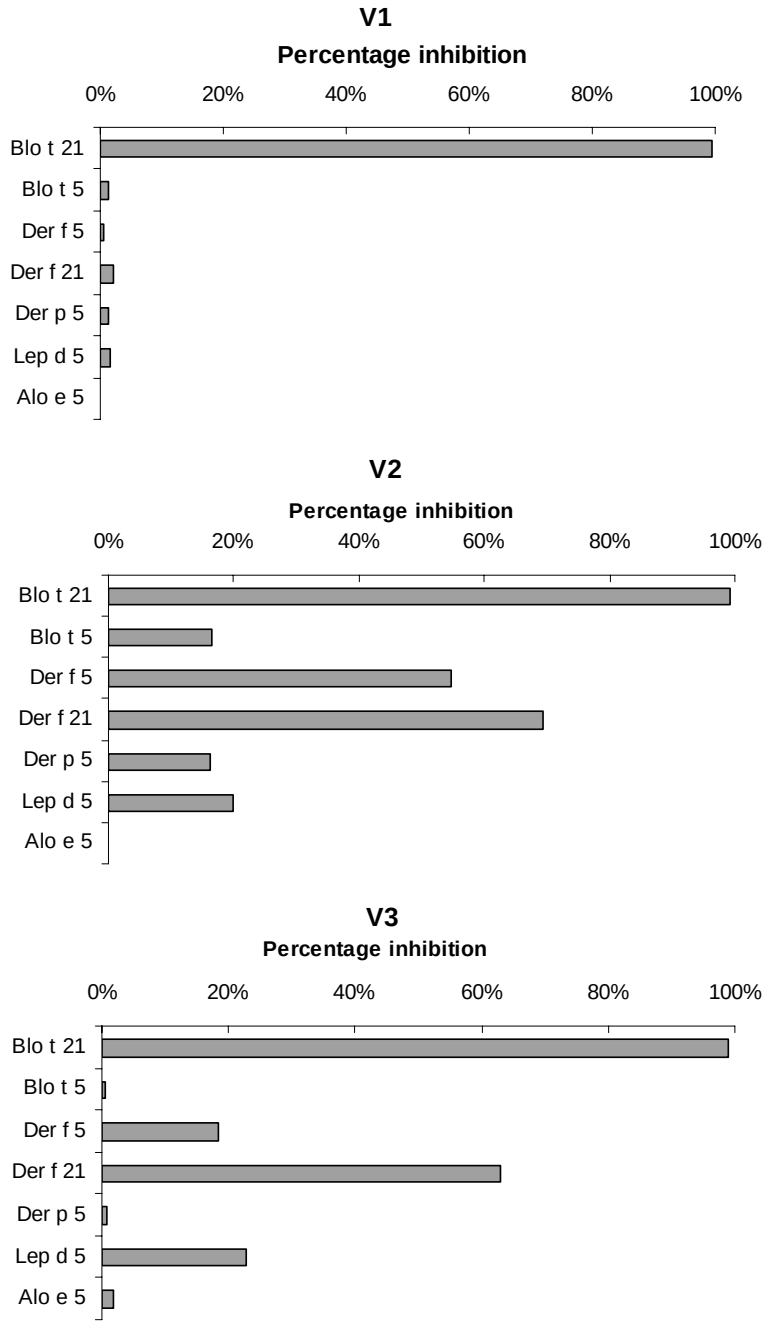


Figure 6.9 Inhibition of Blo t 21 by Group 5 and Group 21 inhibitors from *B. tropicalis*, *D. farinae*, *D. pteronyssinus*, *L. destructor* and *A. ovatus* using three sera.

6.2.5.2 Cross-reactivity of Der f 5 and Der f 21

Cross-inhibition of Der f 5 and Der f 21 was evaluated as well using three sera. In this study, serum V3 was replaced by serum V4 due to its low specific IgE titer to Der f 5 and Der f 21. Sera V1, V2 and V4 showed high IgE activities to Der f 5, Der f 21, Blo t 5 and Blo t 21. High homozygous inhibitions (>79.6%) of Der f 5 were observed in three sera. Ale o 5 was not able to fully inhibit Der f 5 in all three sera. Der f 21, Blo t 5, Blo t 21 and Lep d 5 were able to partially abolish IgE-binding to Der f 5 at 100 μg/ml in sera V1 and V4 (41%-58% inhibition), and fully inhibit the IgE of serum V2. Der p 5 was able to partially inhibit Der f 5 in serum V1 (44% inhibition), and fully inhibit in sera V2 and V4 (>80% inhibition) (Figure 6.10).

When Der f 21 was immobilized on solid surface of ELISA plate, Ale o 5 was not able to completely inhibit IgE-binding to Der f 21 in all three sera. Der f 5, Der p 5, Blo t 5, Blo t 21 and Lep d 5 partially inhibited Der f 21 at low to moderate level (11%-70%) in sera V1 and V4 and completely inhibited Der f 21 in serum V2 (Figure 6.11).

In subject V2, Blo t 5 and Blo t 21 were partially cross-inhibited by Der f 5 and Der f 21. In contrast, Der f 5 and Der f 21 were completely inhibited by Blo t 5 and Blo t 21, suggesting that this subject was predominantly sensitized by Blo t 5 and Blo t 21, and cross-reactive to Der f 5 and Der f 21.

Thus, IgE activities to Der f 5 and Der f 21 in our local atopic population are most probably caused by two reasons: firstly primary sensitization to *D. farinae* and secondly cross-reaction to *B. tropicalis*. Der f 5 and Der f 21 IgE were partially inhibited by Ale o 5, a Group 5 allergen isolated from non-commonly occurring mite *A. ovatus*.

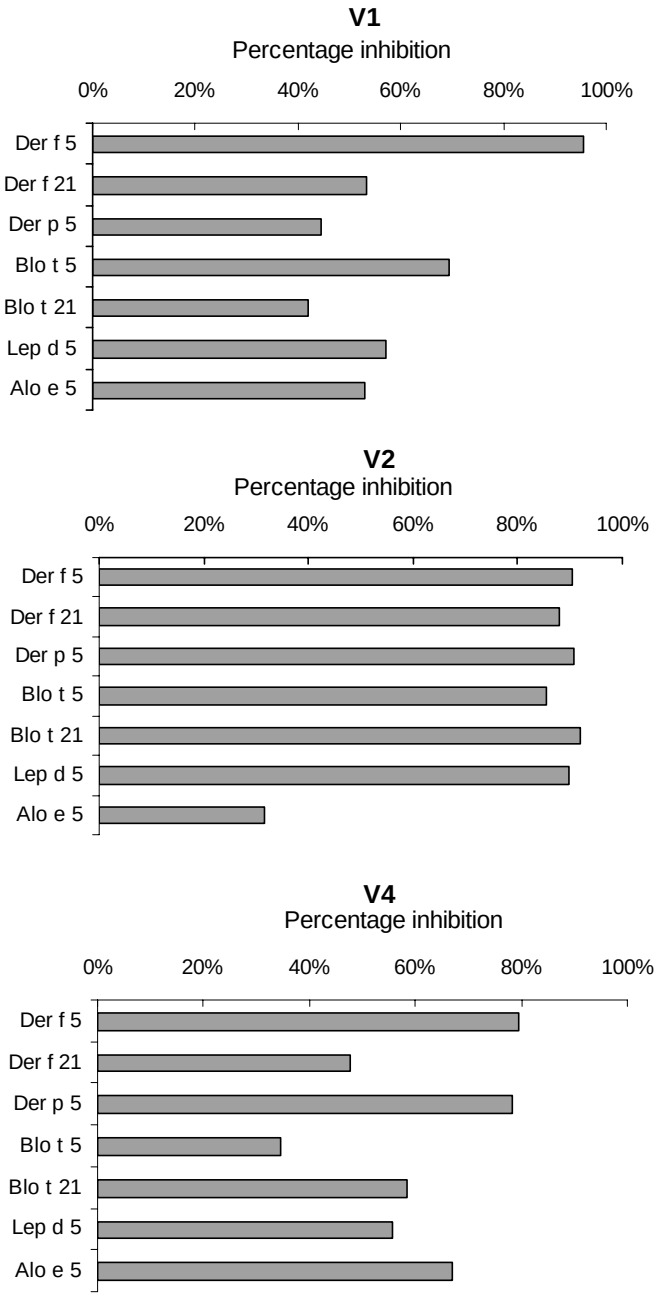


Figure 6.10 Inhibition of Der f 5 by Group 5 and Group 21 inhibitors from *B. tropicalis*, *D. farinae*, *D. pteronyssinus*, *L. destructor* and *A. ovatus* using three sera.

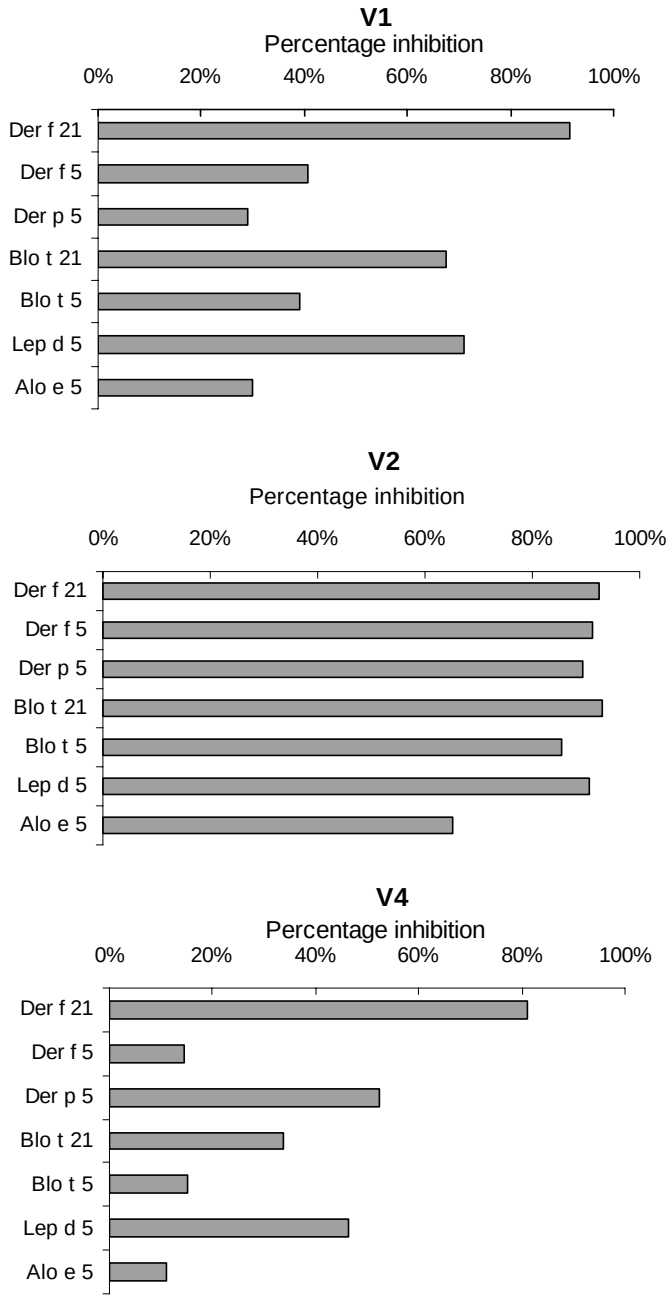


Figure 6.11 Inhibition of Der f 21 by Group 5 and Group 21 inhibitors from *B. tropicalis*, *D. farinae*, *D. pteronyssinus*, *L. destructor* and *A. ovatus* using three sera.

6.2.6 Cross-reactivity of Der p 5 and Lep d 5

Cross-inhibition of Der p 5 and Lep d 5 was evaluated in serum V1, the only serum that had high IgE titer to Der p 5 and Lep d 5 with enough amounts to perform competitive ELISA experiment. Serial dilutions of inhibitors ($1x10^{-3}$ – $1x10^{2}$ µg/mL) were pre-incubated with serum before they were reacted with either Der p 5 or Lep d 5 on the solid surface of ELISA plate.

When complete homologous inhibition of Der p 5 was achieved at the concentration of 300 µg/ml, Blo t 5, Der f 21, and Ale o 5 were able to fully inhibit IgE-binding to Der p 5. Hence, Der p 5 showed high cross-reactivity to Der f 21, Blo t 5, Lep d 5 and Alo e 5. It also displayed a moderate cross-reactivity to Der f 5 and Blo t 21 (50% inhibition) and a high cross-reactivity to Lep d 5 (Figure 6.12A).

When Lep d 5 was immobilized on the solid surface, full (>80% inhibition) inhibition was achieved by its own protein at the concentration of 100 µg/ml. Der f 21 was able to completely inhibit IgE-binding of Lep d 5. Both Blo t 21 and Der f 5 were able to inhibit IgE-binding to Lep d 5 at high levels (> 75 % inhibition). Blo t 5 and Ale o 5 were able to inhibit IgE-binding to Lep d 5 at moderate levels, while Der p 5 was only able to inhibit Lep d 5 at a low level (Figure 6.12B). Hence, subject V1 was predominantly sensitized by Group 5 and Group 21 from both *B. tropicalis* and *D. farinae* and partially cross-reactive to Der p 5, Lep d 5 and Ale o 5. Since only one serum was used in the inhibition experiment, further studies using sera from more subjects would be needed to provide a better picture of cross-reactivity of Group 5 and Group 21 isolated from different species.

It is noteworthy that the majority of individuals in this screening showed higher IgE titers to Blo t 5 than to other Group 5 and Group 21 allergens (Figure 6.6), suggesting that individuals in this group might develop more specific IgE antibodies to epitopes of Blo t 5, Blo t 21, Der f 5 and Der f 21 than those of Group 5 and Group 21 allergens in less commonly occurring dust mites.

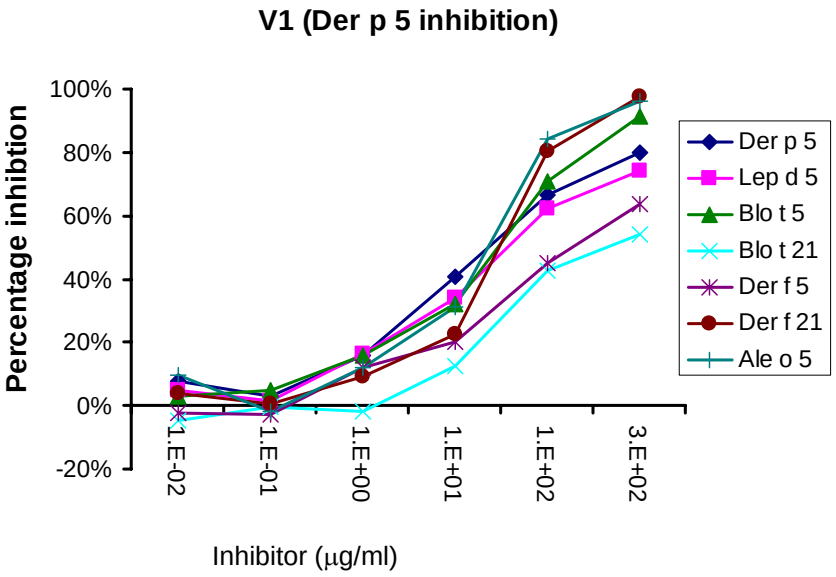


Figure 6.12(A) Inhibition of Der p 5 by Group 5 and Group 21 inhibitors from *B. tropicalis*, *D. farinae*, *D. pteronyssinus*, *L. destructor* and *A. ovatus*.

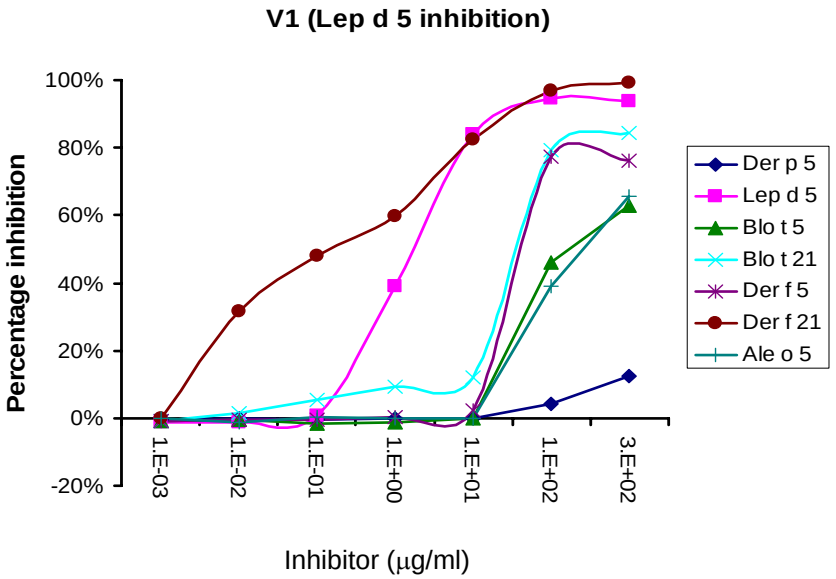


Figure 6.12(B) Inhibition of Lep d 5 by Group 5 and Group 21 inhibitors from *B. tropicalis*, *D. farinae*, *D. pteronyssinus*, *L. destructor* and *A. ovatus*.

6.3 Discussion

Group 5 and Group 21 allergens are universal in dust mites. They were found in different mite species from different families. Together with published Group 5 and Group 21 allergens and those identified from Chapter 3 and Chapter 5, a total of 13 Group 5 and Group 21 allergens were identified from seven mite species. Highly abundant Group 5 and Group 21 homologous ESTs have been found in *S. medanensis*, indicating high expression of Group 5 and Group 21 homologues in this species. Two Group 5 isoforms were first reported from this species. It seemed that Group 5 isoforms were not just restricted in one species, they were also observed in *T. putrescentiae* when *T. putrescentiae* ESTs were surveyed. It can be speculated that more Group 5 isoforms may be identified in other species of mites. However, Group 5 and Group 21 genes are only found in dust mites up to the present. No homologous genes can be found in other organisms using homologous based searching in public database.

Group 5 and Group 21 allergens isolated from *B. tropicalis*, *D. pteronyssinus*, *D. farinae*, *A. ovatus* and *S. medanensis* have similar protein sizes, and their proteins all contain a signal peptide, while Group 5 allergens isolated from Glycyphagidae family (Lep d 5 and Gly d 5) have extra duplication in the N-terminal of the protein. Part of duplicated DNA was lost in Lep d 5 and this resulted in the loss of signal peptide. Multiple alignments of protein sequences of Group 5 and Group 21 allergens and the drawing of an unrooted Neighbor-Joining phylogenetic tree based on the diversities of these sequences indicated two major clusters: Group 5 and Group 21 (Figure 6.13). In this phylogenetic tree, Group 5 and Group 21 allergens of *T. putrescentiae* were added, but an outgroup gene was not included, because Group 5 and Group 21 were only found

in mites so far, and it is impossible to find a homologous gene in distant species other than mites. This phylogenetic tree clearly demonstrated that Group 5 and Group 21 were evolved separately in the species. The duplication event was most likely taking place before speciation. The phylogenetic tree of Group 5 and Group 21 based on sequence diversity was also in a good agreement with the phylogenetic tree of dust mite generated from the observation of morphology. It can be seen from the phylogenetic tree that Group 5 and Group 21 from *D. pteronyssinus* and *D. farinae* always fall into the same clades. *D. pteronyssinus* and *D. farinae* belong to Pyroglyphidae family. Gly d 5 and Lep d 5 whose gene duplication occured within the gene seemed to cluster as outgroups of normal Group 5 and Group 21 allergens. Lep d 5 and Gly d 5 clustered into the same clades. Interestingly, N-terminal parts of two allergens clustered in one clade and C-terminal parts in another, suggesting that gene duplication most probably happened before speciation. Gly d 5 and Lep d 5 clustering together also reflected the taxonomy relationship between *L. destructor and G. domesticus,* which belong to the same Glycyphagidae family.

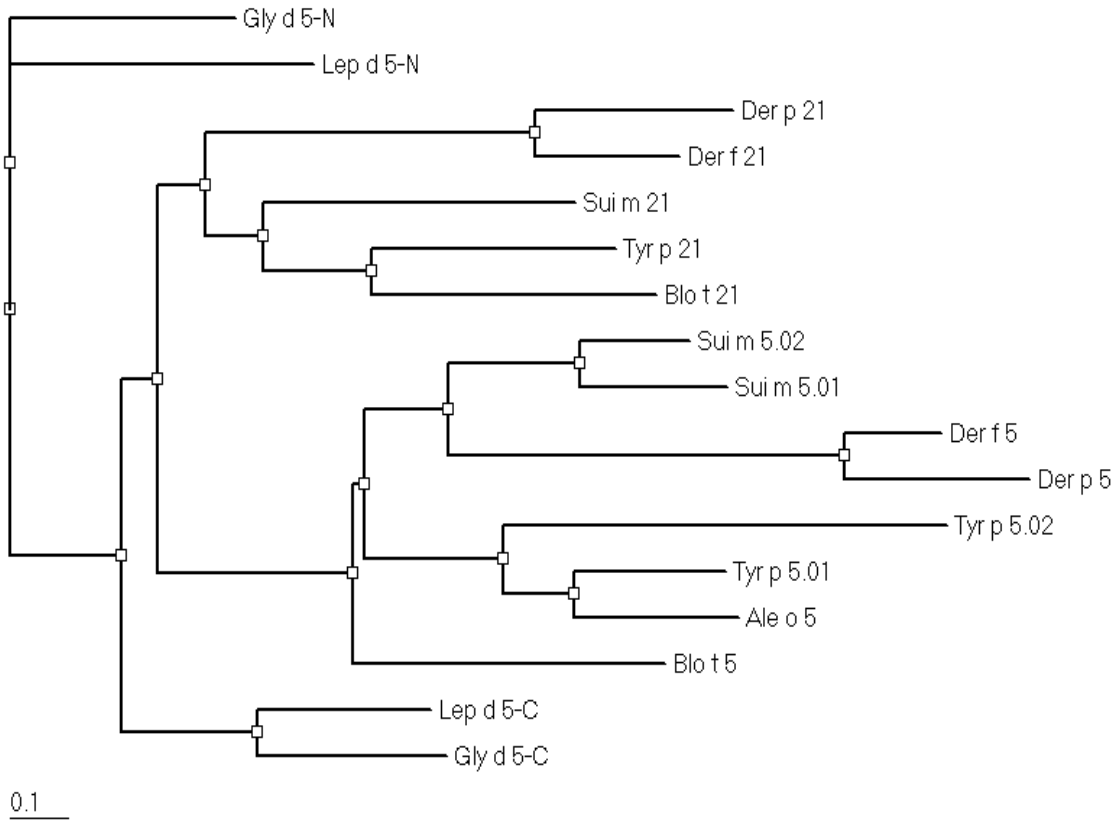


Figure 6.13 Neighbor-Joining phylogenetic tree of Group 5 and Group 21 allergens from eight mite species.

Gene duplication plays an important role in evolution. In the initial period of duplication, the duplicated gene has overlapping function as the original one. Later on, random mutations occurred in the duplicated gene during evolution. Due to the presence of two copies of the same gene, mutation only in one copy often has no effect on the organism. This leads to an accumulation of large amount of mutations in the second copy. The mutations could lead to either gain of a new function or loss of any function. The function-losing mutations (pseudogene) will be deleted in the genome later on. Group 5 and Group 21 are most likely paralogs in mite species, which may exist after gene duplication. However, the biological functions of these two groups of allergens are still under investigation.

Despite the sequence diversity, both Group 5 and Group 21 allergens from predominant mite *B. tropicalis* and *D. farinae* are able to induce species specific allergy in mite positive atopic individuals in Singapore. Group 5 and Group 21 from other less commonly occurring mites also showed some degree of IgE-binding. When 11 allergens (Groups 1~3, Groups 5 ~ 10, Group 13) of *S. medanensis* were studied in 82 *S. medanensis* positive subjects attending outpatient clinic, Sui m 5.01, 5.02 and 21 were found contributing about half of the total IgE of 11 allergens, suggesting that Group 5 and Group 21 were important allergens in *S. medanensis*. However, when Sui m 5.01, 5.02 and 21 were studied together with a panel of Group 5 and Group 21 in the screening, frequencies and intensities of Sui m 5.01 and Sui m 5.02 were lower than that of Blo t 5, and lower frequency and intensity of Sui m 21 were found compared to Blo t 21. Ale o 5, Der f 5, Der p 5, Lep d 5 and Gly d 5 in the same screening also showed lower IgE-binding frequencies and intensities than Blo t 5, and Der f 21 and Sui m 21 showed lower

IgE-binding frequencies and intensities than Blo t 21. These results reflected the predominant sensitization of *B. tropicalis* in local atopic subjects. This hypothesis was supported by the result of cross-inhibition studies. Cross-inhibition studies revealed that atopic individuals were predominantly sensitized by Group 5 and Group 21 from both *B. tropicalis* and *D. farinae* species and partially cross-reactive to Der p 5, Lep d 5 and Ale o 5.

In addition, positive responses to Blo t 5 and Blo t 21, together with other *Blomia tropicalis* major allergens, may be indicative of more severe atopic disease or concomitant clinical manifestation of all three atopic diseases in the tropics (Kidon *et al.*, 2007). Studies in our laboratory revealed that significant increases in the number of sensitizations to discrete mite allergenic groups (including the Group 21 allergens) in atopic children correlated well with an increasingly complex allergic phenotype (manifestation of all three atopic diseases) (Kidon *et al.*, 2007). Whether this can be used as a predictive risk factor for future development of asthma and allergic rhinitis in young children presenting first with atopic dermatitis requires further prospective cohort studies.

Chapter 7: B Cell IgE-binding Epitope Mapping of Blo t 5

7.1 Introduction

House dust mites have been known to play an important role in the pathogenesis of asthma and allergic diseases. In Singapore, one in five school children has doctor diagnosed asthma and 140,000 individuals suffer from current asthma (Goh *et al.*, 1996). More than 100 individuals die of this disease annually and the annual economic cost on asthma management has been estimated over US$33.93 million (Chew *et al.*, 1999d). A major risk factor for asthma in Singapore is dust mite allergy (Chew *et al.*, 1996), and dust mite allergy was mainly caused by sensitization of *Blomia tropicalis* and *Dermatophagoides spp.* (Chew *et al.*, 1996). Current clinical strategies for mite allergy management include allergen avoidance by patients, pharmacotherapy and allergen-specific immunotherapy. Among these, only allergen-specific immunotherapy provides a curative treatment. It is based on the repeated administration of increasing doses of mite crude extracts to allergic patients to obtain allergen-specific non-responsiveness. However, with the administration of crude extracts, patients may develop new IgE activity to previously non-IgE-binding allergen components in the crude extracts after treatment. In addition, patients may encounter the risk of anaphylaxis that can be fatal. Intensive study on allergens is necessary toward a better understanding of the mechanism of dust mite allergy and safe immunotherapy.

It has been demonstrated in Chapter 3 that Blot 5 and its homologous allergen Blo t 21 are important allergenic components of *B. tropicalis*. Both of them are able to induce specific IgE response in 34%-50% of allergic subjects and in more than 90% of allergic

rhinitis patients in Singapore. In the study with a panel of 11 Group 5 and Group 21 allergens in Singaporean atopic subjects in Chapter 6, Blo t 5 was found to be the most important allergen in Group 5 and their homologous allergens (Group 21). Further investigation of the IgE-binding area of Blo t 5 will provide useful information on the pathogenesis of Group 5 and Group 5 homologous allergens.

There are many methods to identify B cell epitopes of an allergen. Phage display technique is able to define the mimotopes that mimic natural epitopes, but they do not correspond to the linear sequence of the cognate allergen. X-ray crystallography and Nuclear Magnetic Resonance Spectroscopy (NMR) are able to solve the structure of IgE antibody-allergen complexes, so as to define the antibody binding site of the allergen. Naturally occurring variants which have less allergenic activity reflect the IgE-binding site of wildtype allergen. However, not all natural occurring variants have reduced IgE reactivity. Site-directed mutagenesis is commonly used to determine IgE epitopes of allergens. It can be used to selectively modify target amino acid. The substitutes affecting the allergen-IgE interaction can be measured by immuno-protein array or ELISA assay. Such site-directed mutagenesis approaches have been successfully applied to epitopes mapping of Der p 2 (Mueller, 2001), Bet v 1 (Spangfort, 2003), and Der f 13 (Chan *et al.*, 2006). Synthetic peptides approach is another powerful method for identification of linear B cell epitopes. This method has been deployed to identify epitopes of tropomyosin (Ayuso *et al.,* 2002), Cry j 1 (Takagi *et al.,* 2005) and Ole e 1 (Gonzalez *et al.*, 2006). However, this method was not able to determine the IgE epitopes of Der p 1 and Der p 2 due to lack of continuous epitopes (Chua *et al.*, 1991; van 't Hof *et al.*, 1991).

In this study, specific IgE epitopes of Blo t 5 were determined by site directed mutagenesis of charged and polar amino acids. These mutants were then assayed for IgE-binding activities by immuno-dot blot method.

7.2 Results

7.2.1 Identification of Blo t 5 specific epitopes using site-directed mutagenesis

7.2.1.1 Selection of residues for site-directed mutagenesis

From previous studies in Chapter 3 and Chapter 6, it has been revealed that Blo t 5 is the most important allergen among Group 5 and their homologous allergens (Group 21). The allergenicity of this allergen is very specific compared to other Group 5 and Group 21 allergens, because low to moderate levels of cross-inhibitions of Blo t 5 were achieved by Der f 5, Der p 5, Lep d 5, Blo t 21 and Der f 21 inhibitors. Hence, specific epitopes may play important roles in Blo t 5 allergy. The residues which may affect Blo t 5 specific epitopes were selected based on the alignment of amino acid sequences of Blo t 5, Der f 5, Der p 5, Blo t 21 and Der f 21. The amino acid residues most different among Blo t 5 and other Group 5 and Group 21 were firstly selected. To further refine this selection, residues with similar side chain properties (D-E, K-R, and S-T) were excluded. Hydrophobic residues were not included in the selection either, because these residues are normally not involved in antibody interactions (Davies *et al.*, 1996). Based on these considerations, a total of 16 residues were selected for site-directed mutagenesis to

determine the IgE-binding epitopes, including five charged residues (Glu, Lys and His) and ten polar residues (Gln, Asn, Ser, Thr and Tyr) (Figure 7.1). All these selected residues were mutated into alanine.

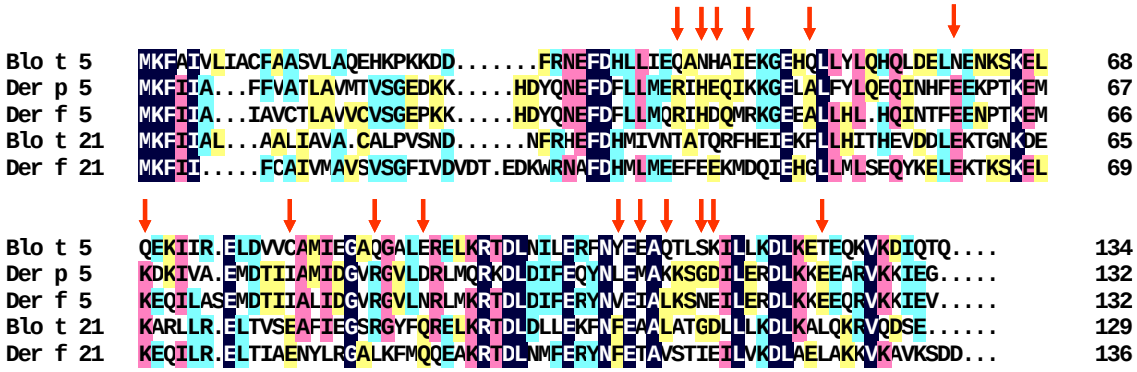


Figure 7.1 Sequence alignment of Blo t 5 with Der p 5, Der f 5, Blo t 21 and Der f 21. 100%, 75%, 50% and 33% identical residues are shaded in black, red, light blue and yellow. Arrows indicate the residues that have been selected for mutagenesis.

7.2.1.2 Expression of Blo t 5 mutations

Selected amino acid residues were mutated into alanine by PCR method using DNA of Blo t 5 expression clone (pET15-BT5) as a template. The mutation clones were confirmed by DNA sequencing. The mutants were transformed in *E. coli* BL21 (DE3) strain for expression. *E. coli* strains carrying mutant DNA were cultured until OD_{600} 0.8 and induced by IPTG. The expressed protein was purified by Nickel-NTA resin and separated by SDS-PAGE electrophoresis. Figure 7.2 shows electrophoresis bands of the purified mutant proteins.

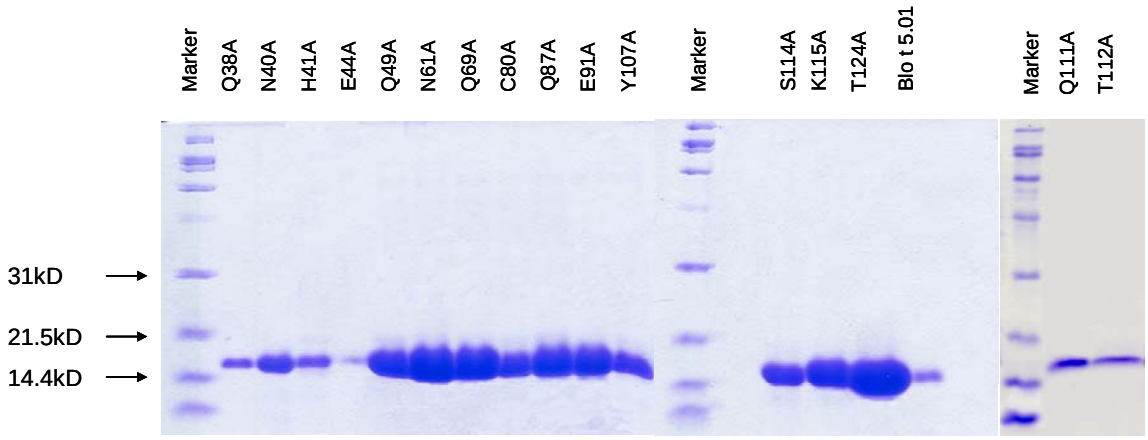


Figure 7.2 Electrophoresis profile of Blo t 5 mutants on SDS-PAGE gel.

7.2.1.3 Influence of amino acid substitutions on IgE-binding

IgE-binding capacities of Blo t 5 mutants were measured by immuno-dot blot assay using 10 Blo t 5 positive sera and 6 Blo t 5 negative sera from local consecutive patients. The IgE-binding activities of mutants in immuno-dot blot were calculated for each sera using wild-type Blo t 5 as a reference (100% IgE-binding activity). Negative serum showed no reactions towards Blo t 5 wild-type and mutants (Data not shown). Major IgE-binding residues were identified when more than 50% of sera tested showed significant reduction of IgE-binding.

Different IgE-binding patterns of Blo t 5 mutants were obtained when different sera were tested (Figure 7.3). It seemed that IgE-binding residues of Blo t 5 were distributed throughout its whole molecule (Figure 7.4). Epitopes at the N-terminal were observed mainly at positions N40-E44, N61 and Q81, while C-terminal epitopes were observed mainly at positions E91 and T124. Sera 3576, 3578 and 3626 responded to the epitopes at the N-terminal of Blo t 5 molecule, while sera 3640 and 3641 mainly responded to the epitopes at the C-terminal. In addition, sera 3604 and 3688 responded to the epitopes at both N-terminal and C-terminal.

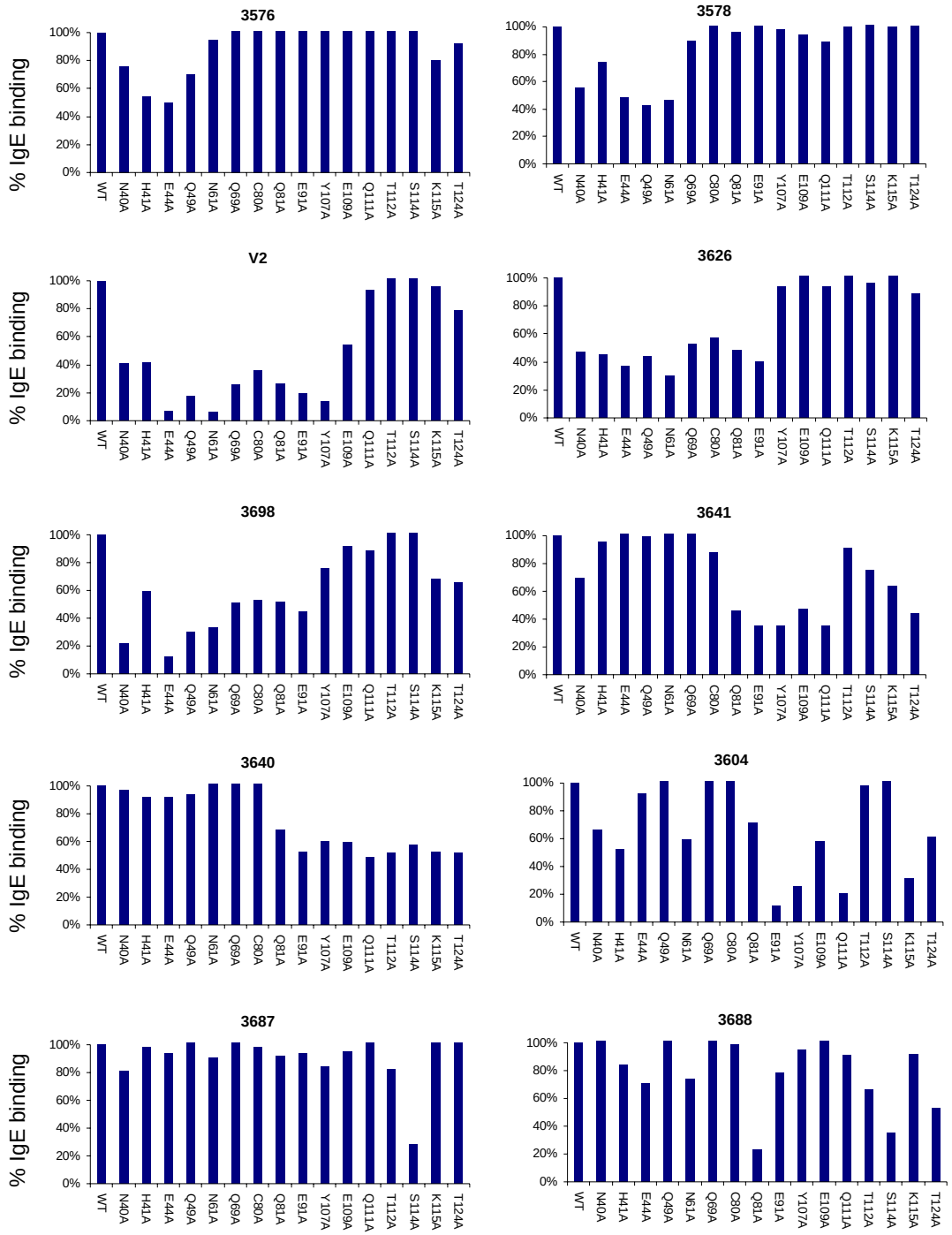


Figure 7.3 IgE-binding profiles of Blo t 5 mutants from ten Blo t 5 positive sera.

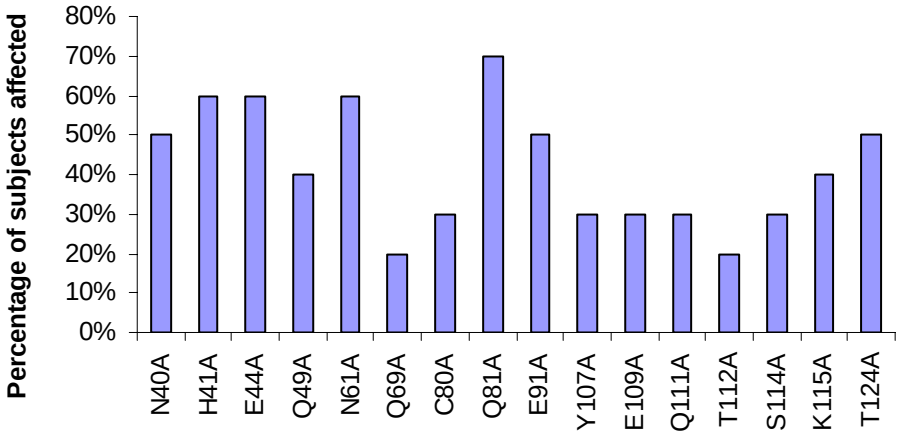


Figure 7.4 Percentage of subjects whose sera showed reduced IgE-binding activity to mutants of Blo t 5.

7.2.2 Identification of Blo t 5 IgE-binding regions using systematic synthetic peptide

The IgE-binding regions on Blo t 5 were determined by peptide mapping approach. This method has been used to systematically identify linear epitopes of antigens in many studies (Shreffler *et al.*, 2005, Ayuso *et al.,* 2002, Takagi *et al.,* 2005). Thirteen overlapping peptides were initially designed to span entire Blo t 5 molecule. Each peptide was 16 amino acids long with an offset of 8 residues. According to site-directed mutagenesis based mapping result, certain regions like N40 to N61 may contain predominant epitope of Blo t 5. To further increase resolution of the IgE-binding epitope mapping, eight extra peptides were added into the assay. Thus, certain regions of Blo t 5 were spanned by overlapping peptides with four residues offset. In total, 21 peptides covering Blo t 5 primary protein sequence were designed as Biotin-SGSG-Peptide-Amide and purchased from commercial company (GL Biochem(Shanghai) Ltd) (Figure 7.5) and 20 peptides were obtained except peptide 10. Blo t 5 molecule was still covered by the remained 20 peptides.

Synthesized peptides were coated on strep-avidin-coated plate and tested with 10 Blo t 5 positive sera and one negative serum from healthy people using ELISA assay. As displayed in Figure 7.6, nine out of ten Blo t 5 positive sera responded to at least two peptides, except that one serum did not respond to any peptides. Negative serum showed no response to any peptide with baseline absorbance reading observed. In general, each serum had its own specific IgE responding profile to peptides. Fifteen out of twenty peptides interacted differently with specific IgE antibodies in patient sera tested in this study (Figure 7.7). These peptides were distributed in the entire Blo t 5 molecule

including N-terminal, Center and C-terminal regions. Peptides 14 and 15 were recognized by eight out of ten sera, followed by Peptide 16 by six sera, Peptides 1 and 11 by three sera. The rest peptides were recognized by only one serum. Interestingly, five Peptide 16 positive sera were able to react strongly to Peptides 14 and 15 as well. This finding led to identification of a common epitope broader region DLNILERF (98-105).

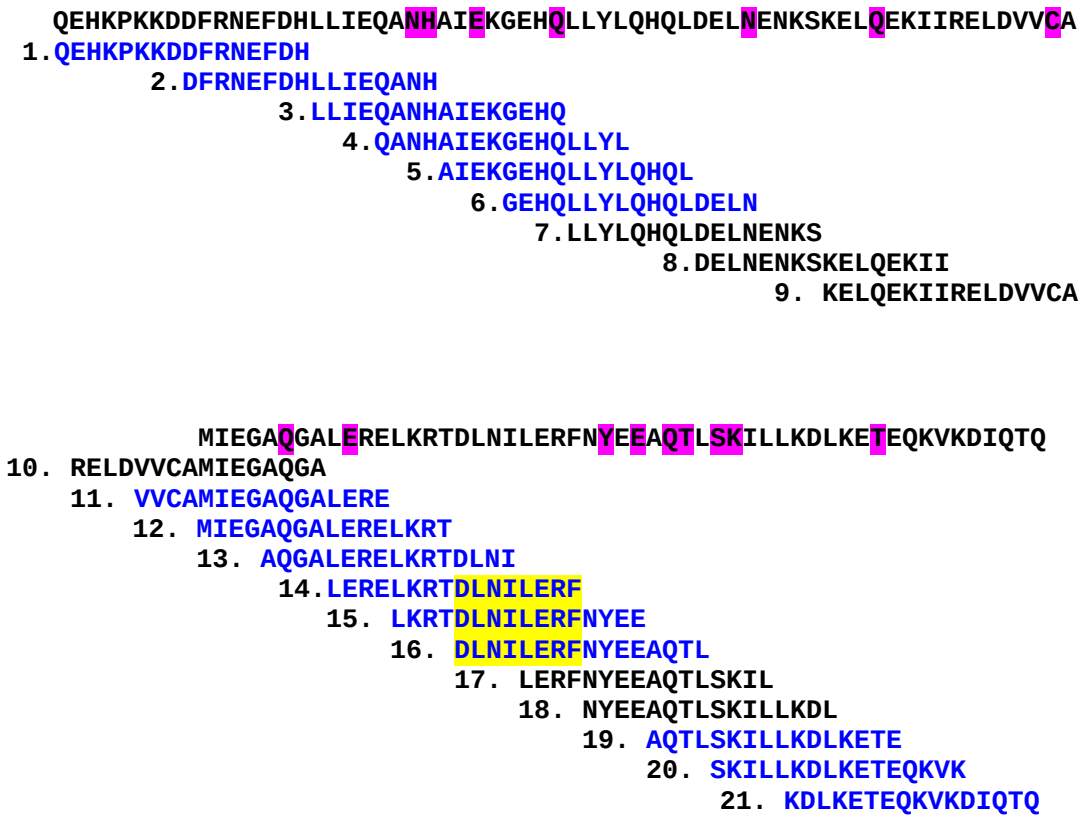


Figure 7.5 Overlapping peptides covering Blo t 5 molecule. Residues substituted by alanine in site-directed mapping study are marked in violet. Peptides showing response to IgE antibody of patients were in blue. The predominant IgE-binding region is shaded in yellow.

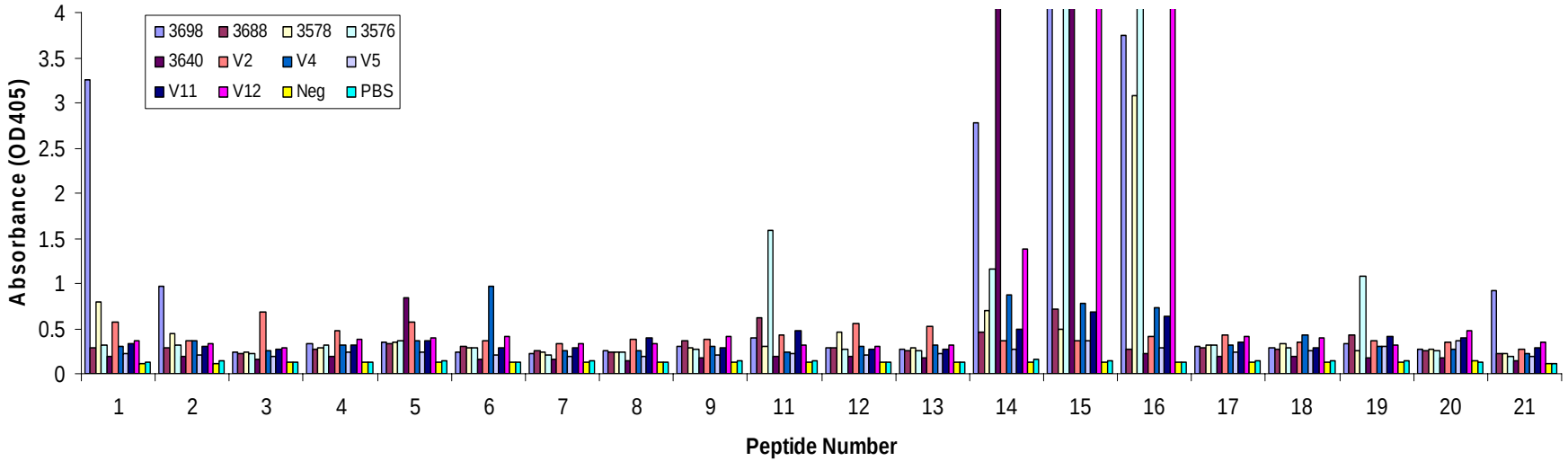


Figure 7.6 IgE-binding activities of 20 overlapping peptides using 10 Blo t 5 positive sera.

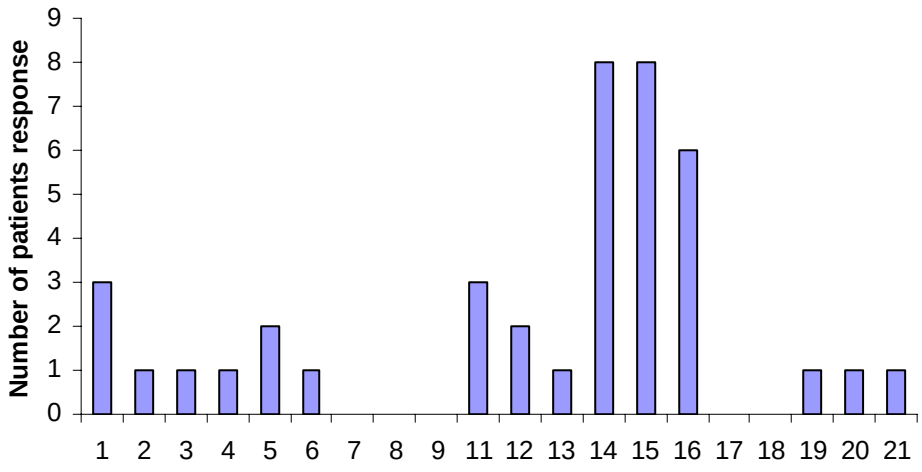


Figure 7.7 Number of sera reacting to the overlapping peptides of Blo t 5 (total sera number=10).

7.2.3 Overlapping peptide mapping rabbit IgG binding regions of Blo t 5

The immunogenicity of the overlapping peptides of Blo t 5 was investigated using polyclonal rabbit antiserum raised against purified Blo t 5 protein by ELISA method. The results were shown in Figure 7.8. A large proportion of peptides of Blo t 5 were immunogenic in rabbit with 60% (12/20) peptides having strong immune response (ELISA reading >2.5) and 35% (7/20) peptides having low immune response. Peptide 11 was not immunogenic in rabbit. Many epitopes were scattered in the entire Blo t 5 molecule, since peptides responding to rabbit polyclonal IgG almost covered the sequences of the whole molecule. For example, sequence between amino acids 82 and 113 (MIEGAQGALERELKRTDLNILERFNYEEAQT) may contain many epitopes, because overlapping peptides (Peptide 12-Peptide 16) covering this area all showed high IgG responses.

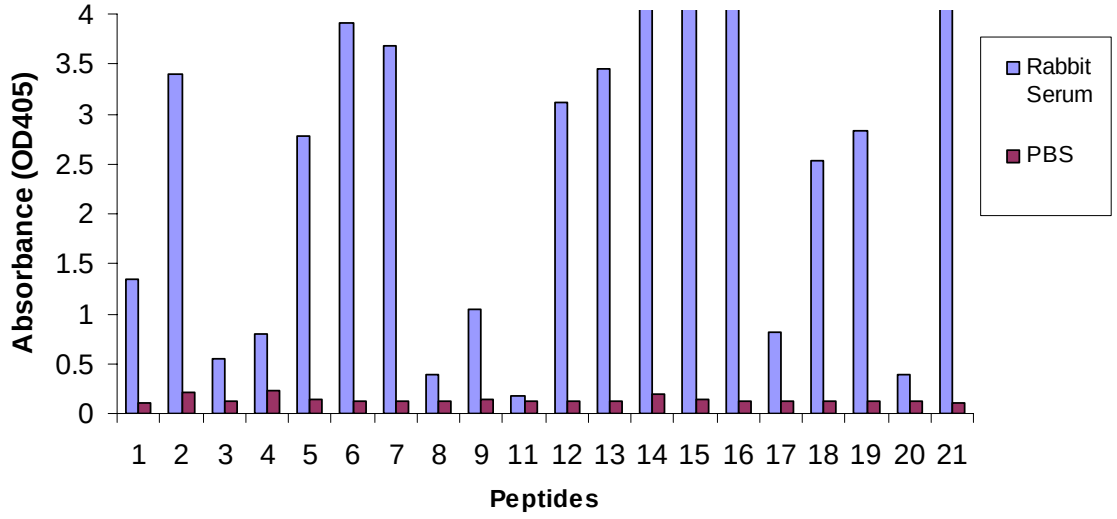


Figure 7.8 Immuno-response of rabbit polyclonal antiserum to overlapping peptides of Blo t 5.

7.3 Discussion

Studies of the interaction areas of Blo t 5 with its IgE and IgG specific antibodies can help define the molecular basis of its allergy. The ideal method to identify the interaction area of epitope is direct scanning of the solvent accessible amino acid on the molecule surface area. However, due to lack of information of the three-dimensional structure of Blo t 5, structure-based epitope analysis can not be applied to this molecule. In this study, two methods have been used to investigate the interaction of Blo t 5 with IgE and IgG specific antibodies, site-directed mutagenesis and overlapping peptide mapping methods.

Site-directed mutagenesis is a useful strategy to identify residues critical for IgE-binding. Epitopes on Der p 2 (Mueller, 2001), Der f 13 (Chan *et al.*, 2006), Bet v 1 (Spangfort, 2003) and Lol p 5 (Swoboda *et al.*, 1995) have been identified by replacing target amino acid with alanine. It is also a powerful tool to disrupt the overall conformational structure of target protein by replacing cysteine, proline or other residues. Substitution of cysteine residues on Der p 2 resulted in destruction of disulfate bonds, which led to conformational change of the structure (Smith *et al.*, 1996). Conformational change of Der p 2 indirectly disrupts the conformational epitopes and causes reduction of IgE-binding activity. Substitutions on Blo t 5 might either directly affect IgE epitopes of the molecule, or disrupt the structure of molecule which indirectly disrupted conformational IgE epitopes. Although detailed structure of Blo t 5 has not been released yet, analysis of primary amino acid sequence has revealed the three coiled-coil structure of the protein by computer programme Matcher (Fischetti *et al.,* 1993). Coiled-coil structure contains haptad repeats $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$. Positions "a" and "d" are usually apolar

residues which form the hydrophobic core, while polar residues are in other positions forming the solvent-exposed part. Single substitution of coiled-coil protein on solvent-exposed region may not significantly affect its coiled-coil structure. It has been reported that twelve substitutions on dominant epitopes of Pen a 1, a major shrimp allergen with coiled-coil structure, did not significantly change the secondary structure (Reese *et al.,* 2005). CD spectroscopy study revealed that these substitutions still maintained the α-helical profiles (Reese *et al.,* 2005). Residues selected for mutagenesis in this study are also located at the solvent-exposed region. We supposed that replacement of these residues with alanine may not significantly affect the coiled-coil structure. Thus, any changes in the allergenic potency of Blo t 5 mutants could be attributed to mutations at the critical amino acid positions of IgE-binding. CD spectrum assay of the mutant proteins needs to be further examined to monitor the conformational change in future studies.

This study revealed that single mutation of Blo t 5 is able to reduce the IgE-binding acticity. Different sera display different IgE-binding profiles to the mutants. Some sera showed reduced IgE reactivity to the substitutions located on N-terminal of the molecule, while some sera showed the reduction mainly on C-terminal regions and some sera on both N and C-terminal regions. Predominant epitopes are located most probably at N40-E44, N61, Q81, E91 and T124, because more than 50% sera tested had reduced IgE-binding activities to substitutions of these residues.

Overlapping peptides mapping is another systematic approach to study the antigen–antibody interactions. Identification of a number of B epitopes from different allergens has been achieved by this method, such as Blo t 11, Pen a 1 and Ole e 1 (Ramos *et al.*, 2003, Ayuso *et al.*, 2002, Gonzalez *et al.*, 2006). Pen a 1 is a typical α-helical

coiled-coil protein, which may have the similar structure to Blo t 5. Ayosu *et al.* (2002) reported five major linear epitopes scattered in Pen a 1. It is possible that Blo t 5 also contains a number of linear epitopes. In order to find all linear epitopes on Blo t 5 molecule, the ideal strategy is to test the overlapping peptides with an offset of one residue. However, this approach is not cost effective. In this study, a set of 16 amino acids overlapping peptide was initially designed with an overlap of 8 amino acids. To increase the resolution of mapping, the overlap between peptides was increased to 12 amino acids in the regions that may contain potential epitopes based on the study using side-directed mutagenesis. The peptides were biotinylated at N-terminal to avoid the non-physiological charge. They were also amidated at C-terminal to mimic the amide form of an internal peptide sequence. Ser-Gly-Ser-Gly was recommended as a spacer inserting between the N-terminal Biotin and Blo t 5 sequence to eliminate the steric inhibition of binding (Carter *et al.,* 2004). When peptides were tested for IgE-binding activitis with ten Blo t 5 positive sera, it was found that most sera responded to at least two peptides, four out of ten sera responded to more than six peptides. These peptides were scattered along Blo t 5 molecule at region Q18 - N61 and V78-Q134. Peptides 7, 8, 9, 17 and 18 did not interact with any IgE antibodies from all sera tested. Thus, region L50-A81 and L102-L121 of the molecule may not contain any entire linear epitope. From reaction patterns of five Blo t 5 sera, a predominant IgE-binding region DLNILERF (98-105) was identified. When looking into this protein sequence in other Group 5 and Group 21 protein sequences at hand, we found that this sequence was conserved throughout all Group 5 and Group 21 proteins. This conserved sequence can be summarized as **DL**(N_{10}/D_6)(I_8/L_5/F_2/M_1)(L_9/F_3/V_3/A_1)**E**(K_8/R_6/Q_2)(F_{11}/Y_4/I_1). The subscript numbers

indicate the occurrence of that particular amino acid. Sui m 5.02 and Tyr p 5.01 have sequences identical to this epitope broader region, suggesting that this common epitope might cause cross-reactive response throughout all Group 5 and Group 21 allergens. Other IgE-binding peptides were distributed along the molecule, including those located on the "less-conserved areas" of the molecule, i.e. Peptides 3, 4, 11, 19, 20. Epitopes on these peptides may induce specific IgE response of Blo t 5.

Rabbit IgG antibodies were able to bind almost all overlapping peptides of Blo t 5 with different activities except for Peptide 11. It seems that most sequences of allergen are immunogenic for rabbit. This result was in accordance with other studies on polyclonal IgG epitopes mapping such as Hev b 1 (Chen *et al.*, 1996) and Chi t 1.01 (van Kampen *et al.*, 2001).

Results of epitope mapping could be affected by the strategies applied and sera selected for the study. Site-directed mutagenesis method enabled us to narrow down the regions which were specific for Blo t 5. These regions might contain linear epitopes and/or conformational epitopes. Systematic overlapping peptide approach helps identify regions which may contain linear epitopes. Regions narrowed down by two methods may not be necessarily identical. That is in agreement with the studies of Chi t 1.01 epitopes (van Kampen *et al.*, 2001). Indeed, the parallel studies using two methods provided more information for Blo t 5 and sera antibody interaction. It appears that there are many epitopes on Blo t 5 molecule, including linear epitopes and conformational epitopes. These epitopes seem to be scattered throughout most of the Blo t 5 protein sequence. We found that different subjects recognized different epitopes of Blo t 5. That could be the reason why Blo t 5 is a major allergen of dust mite and why it is so specific for mite

allergy. Further investigations, particularly on the generation of hypoallergen with preserved T cell activity but abolished IgE-binding activity may help produce a better vaccine for allergen-specific immunotherapy.

Chapter 8: Conclusions and Future Direction

8.1 Conclusions

Sensitization to dust mites is one of the major causes of airway allergic diseases worldwide. *D. pteronyssinus* and *D. farinae* are predominant mites in the temperate regions, while *Blomia tropicalis* is predominant in the tropics. Up to now, more than twenty groups of mite allergens have been characterized, but only Group 1 and Group 2 allergens have been extensively studied. This study focused on Group 5 and their homologous allergens, Group 21 allergens, aiming toward a more comprehensive understanding of mite allergy.

cDNA sequences homologous to Group 5 allergens have been identified from EST database for seven dust mite species using local Translated Basic Local Alignment Search Tool algorithm. These seven dust mite species included *B. tropicalis*, *D. farinae*, *S. medanensis*, *A. ovatus, L. destructor, G. domesticus* and *T. putrescentiae*. At least two clusters of Group 5 homologous sequences have been found in six species out of the above seven. Sequences clustering together with Blo t 5 and Der p 5 were named as Group 5, and the others were named as Group 21, according to the World Health Organization (WHO)/ International Union of Immunological Societies (IUIS) Subcommittee of Allergen Nomenclature.

In order to understand Group 5 and their homologous allergens, Group 21 allergens, we initially focused on two new Group 21 allergens in *B. tropicalis* and *D. farinae*, and then *S. medanensis,* a less commonly occurring dust mite. Eleven Group 5 and Group 21 allergens from six mite species were compared in local atopic population when all these allergens were expressed in *E.coli* system.

A cDNA showing homology to Blo t 5 from the *B. tropicalis* cDNA library was isolated and characterized. Amino acid sequence deduced from this cDNA had 39% identity to Blo t 5. However, this cDNA was transcripted from a single-copy gene, which was different from Blo t 5. Genomic organization studies revealed that both Blo t 21 and Blo t 5 had an intron insertion in different positions, implying the separation evolution of these genes within species. Allergen encoded by this cDNA was named Blo t 21 by the World Health Organization (WHO) / International Union of Immunological Societies (IUIS) Subcommittee of Allergen Nomenclature.

Blo t 21 is a clinically important major allergen of *B. tropicalis*. Ninety-three percent (40/43) of positive responses to Blo t 21 were obtained by ELISA assay and 95% (41/43) by skin prick test (SPT) from 43 adult patients with ongoing persistent allergic rhinitis. In addition, Blo t 21, following Blo t 5 and Blo t 3, was found to be the third most prevalent IgE response allergen (31%) after studies in a panel of 19 *B. tropicalis* allergens with 97 *B. tropicalis* positive individuals who attended outpatient allergy clinics over a period of one and a half years. The majority (>75%) of sensitized individuals were co-sensitized to both Blo t 5 and Blo t 21, as low to moderate degrees of cross-reactivity to these two allergens were observed in above two populations. Co-sensitization of Blo t 5 and Blo t 21 in the same population could be resulted from the low protein sequence identity between these two allergens due to lack of conserved domains, or co-exposure to both allergens in the environment.

Immuno-staining studies indicated that both Blo t 21 and Blo t 5 localize to mid- and hind-gut contents of *B. tropicalis* as well as fecal particles, suggesting that Blo t 21 and Blo t 5 could pass out with feces and become airborne particles in the environment.

The distributions of Blo t 21 and Blo t 5 were then examined in 77 house dust samples. Results showed identical distributions of Blo t 21 and Blo t 5 with low concentrations (<2.0 μg/g). Identical distributions in the environment lead to co-exposure of these allergens in the population.

IgE-binding of Blo t 21 and Blo t 5 are stable. Thermal treatment does not affect their IgE-binding even at 90 °C. In this study, Blo t 5 and Blo t 21 lost their secondary structures at 90 °C. However, their secondary structures could be partially refolded back after they were cooled down to 20 °C. When treated under extreme alkalic and acidic conditions, the IgE-binding of Blo t 5 and Blo t 21 were not affected either. Their IgE-binding activities were even retained after treatment with 6 M urea, a strong protein denaturant. IgE-binding of Blo t 21 and Blo t 5 seem more stable than those of Der p 1 and Der p 2, in terms of thermal stability, pH and chemical denaturation resistance. Hence, the IgE-binding epitopes of these molecules could be linear epitopes whose primary amino acid sequences are recognized by the specific IgE antibodies regardless of their structures. The epitopes might be located at core structures of these proteins and these core structures could be regained after denaturation. The high stability of IgE-binding of Blo t 5 and Blo t 21 explains why these two allergens are so important for *B. tropicalis* allergy.

Der f 21 is another Group 5 homologous allergen studied in this thesis. It is also a product of a single-copy gene with its amino acid sequence sharing 66.4% identity to Der p 21 and 28.7% to Der f 5. The allergenicity of Der f 21 was evaluated in thirty adult patients with ongoing persistent allergic rhinitis, and 83% (25/30) of them had specific IgE response. Patients responding to Der f 21 also had specific response to Der f 5. When

Der f 21 was compared in a panel of 12 recombinant allergens of *D. farinae* in 74 atopic individuals positive to *D. farinae*, Der f 21 and Der f 5 were the second most prevalent allergens following Der f 2 in this population, suggesting the importance of Der f 21 in *D. farinae*.

Der f 21 also localizes to mid- and hind-gut contents of *D. farinae* as well as fecal particles like Blo t 5 and Blo t 21. Consistent immuno-staining results confirmed that Group 21 and Group 5 were overlappingly expressed at the same positions. Both Der f 5 and Der f 21 can be detected by ELISA assay in the environment at low concentrations.

Group 5 and Group 21 allergens of *S. medanensis* were studied as examples representing non-common dust mites. Two Group 5 isoforms and one Group 21 allergen have been identified from this species. They were highly transcribed in *S. medanensis*, as multiple copies of cDNA clones were obtained from the cDNA library. When Group 5 and Group 21 were studied in a panel of 11 recombinant allergens of *S. medanensis* in 82 atopic subjects responding to both *S. medanensis* and *B. tropicalis*, Sui m 5.01, Sui m 5.02 and Sui m 21 were found to be the first (28%), third (19%) and fourth (16%) most prevalent allergens, respectively. Sui m 5.01, Sui m 5.02 and Sui m 21 together contributed 46% of the total sera IgEs of 11 allergens. These data implied the importance of Group 5 and Group 21 allergens in *S. medanensis.* Thus, Group 5 and Group 21 allergens were important in both predominant and less commonly occurring dust mite species.

It seems that Group 5 homologous genes are common in dust mite. When EST database from six mite species were analyzed, Group 5 homologous sequences kept popping out. However, no Group 5 homologous sequences from other organisms have

been reported. Analysis of 15 Group 5 homologous sequences from eight mite species revealed that these sequences were able to be grouped in two major clusters. Sequences closer to Blo t 5 and Der p 5 were named Group 5, while sequences closer to Der p 21, a Group 5 homologous allergen, were named Group 21. Phylogenetic tree generated from these 15 sequences clearly showed that Group 5 and Group 21 evolved separately in the species. Hence, gene duplication was most likely taking place before speciation.

In order to compare Group 5 and Group 21 allergens from different species, 11 Group 5 and Group 21 from *A. ovatus*, *B. tropicalis*, *D. farinae*, *D. pteronyssinus*, *Lepidoglyphus destructor*, *G. domesticus* and *S. medanensis* were evaluated in 118 mite positive sera from local atopic subjects using semi-quantitative immunoarray assays. Among these sera, 97 were positive to *B. tropicalis*, 74 to *D. farinae*, 80 to *D. pteronyssinus,* 58 to *Aleuroglyphus ovatus,* 82 to *S. medanensis,* 63 to *L. destructor* and 82 to *G. domesticus.* Most *D. farinae* (68/74) and *D. pteronyssinus* (73/80) positive sera were also positive to *B. tropicalis*. Interestingly, all these tested Group 5 and Group 21 allergens were allergenic. In addition, 41 percent of sera were positive to Blo t 5, followed by Ale o 5 (27% positive) and Blo t 21 (25% positive). The rest of Group 5 and Group 21 allergens showed low frequencies of IgE-binding (8%-19%) in these sera. This result implies that Blo t 5 is the most important allergen in this panel and reflects the predominant sensitization to *B. tropicalis* in the local population.

In order to get a deeper insight into Group 5 and Group 21 allergens, Blo t 5, the most important allergen in Group 5 and Group 21 panel, was further studied to identify the IgE-binding regions of this molecule. Site-directed mutagenesis and systematic overlapping peptide approaches were applied to obtain more information on the epitopes.

Results from both strategies showed that IgE-binding epitopes were scattered throughout the entire molecule, including N-terminal, Center and C-terminal of the protein. Majority of patients responded to multiple epitopes of Blo t 5 and different patients reacted to different epitopes. Site-directed mutagenesis method located the possible predominant epitopes at N40-E44, N61, Q81, E91 and T124, as more than 50% of sera tested had reduced IgE-binding activities to substitutions of these residues. On the other hand, systematic overlapping method spotted a predominant IgE-binding region at the position 98-105 (DLNILERF) of the molecule, as 5 out of 10 sera responded to this epitope. This IgE-binding region was found to be conserved throughout all Group 5 and Group 21 proteins that have been identified thus far. It can be speculated that reaction to this epitope might induce cross-reaction among different Group 5 and Group 21 allergens.

In summary, this thesis has identified and characterized novel Group 5 homologous allergens from predominant dust mites, *B. tropicalis* and *D. farinae*, and non-commonly occurring mite *S. medanensis*. All these allergens have been found to be important in mite species, no matter whether they are predominant mites or non-common mites. When a panel of 11 Group 5 and Group 21 allergens in six mite species were compared in local mite positive sera, Blo t 5 was found to be the most important allergen among them. In addition, IgE antibody-interaction regions of Blo t 5 were narrowed down on the molecule. These results provide useful information for diagnosis, management and immunotherapy of Group 5 and Group 21 allergy.

8.2 Future direction

Allergic immune reactions require a pre-sensitization of the host and re-exposure of the similar antigens. The reactions are complex and influenced by genetic susceptibility and allergen exposure. In Singapore, there are four major races in the society, i.e. Chinese, Malay, Indian and Eurasian. It would be very interesting to study allergic immune responses in subjects of a particular race, so as to understand the influences of genetic background on the allergic immune reactions. Group 5 and Group 21 allergens characterized in this thesis can be used as representative allergens for these studies. The immune response profiles from different races can be compared to one another to get better insight into Group 5 and Group 21 allergy.

In this thesis, Group 5 and Group 21 induced allergic immune responses have been studied in atopic Singaporean individuals. Atopic Singaporean individuals are sensitized to *B. tropicalis* and/or *D. farinae* because of the predominance of *B. tropicalis and D. farinae* in the local environment. Since Blo t 5 is a major allergen in *B. tropicalis*, it is not surprising to find the predominance of Blo t 5 in a panel of 11 Group 5 and Group 21 allergens from seven dust mite species. However, in temperate areas such as Switzerland, the United Kingdoms and China, D. *pteronyssinus* and/or *D. farinae* are the predominant mite species. Further studies on allergic immune responses of Group 5 and Group 21 with respect to the populations from these temperate areas will be required to achieve a more comprehensive understanding on the mite allergy.

Specific allergen immunotherapy (SIT) represents the only curative treatment of inhalant and insect venom allergies, and it has been used for almost 100 years for

desensitizing. However, extensive use of allergen specific immunotherapy is limited due to problems related to safety of vaccines, adjuvants, treatment schedules and side effects. In order to produce safer vaccines for immunotherapy, several strategies have been applied to reduce the allergenicity while maintaining the immunogenicity of the vaccines. These strategies include site-directed mutagenesis to remove IgE epitopes of the molecule, non-IgE-binding fragments of the entire molecule, unfolded recombinant allergens, and soluble synthetic peptide. IgE-binding epitope studies in this thesis revealed that there are multiple IgE-binding epitopes existing on Blo t 5, including several linear epitopes. Atopic individuals tend to respond to multiple epitopes of Blo t 5. Thus, single mutant or double mutants may not necessarily be able to remove the majority of IgE-binding activity of Blo t 5 for certain atopic individuals. Systematic overlapping peptides mapping of Blo t 5 revealed that Peptides 7, 8, 9, 17 and 18 do not bind to any specific IgE antibodies from all ten sera tested. However, Peptides 8 and 17 were able to bind rabbit IgG at medium levels (OD=0.8 ~1.0) and Peptide 18 at a high level (OD=2.5). Hence, Peptides 8, 17 and 18 could be used as potential vaccines for immunotherapy.

Peptide vaccines have been evaluated in immunotherapy studies. Peptides of phospholipase A2, a major allergen of honeybee venom, were used to treat five highly allergic patients and successfully induced allergen-specific T-cell anergy to bee venom (Carlallido *et al.*, 1993). Peptides from cat allergen Fel d 1 were examined in many studies. Peptide vaccine treatment changed immune responses, such as generation of allergen-specific T regulatory cells (Treg) and reduction in specific IgE levels. In addtition, peptide vaccine treatment decreased the asthmatic reaction and nasal problems to cat dander exposure (Oldfield *et al*., 2001; Alexander *et al.*, 2005).

To examine the potency of Peptides 8, 17 and 18 for immunotherapy, it is necessary to establish a mouse model of Blo t 5 allergy. Peptides can be then introduced into Blo t 5 sensitized mice for desensitization. The effects of allergen-specific immunotherapy can be examined by testing the following parameters. (1) Decrease in specific IgE levels at late stages of SIT. (2) Increase in specific IgG_4 levels. (3) Increase in IL-10 and TGF-β levels. (4). Generation of Treg cells. (5) Decrease in allergen-induced proliferation of T cells. (6) Decrease in mediator release, e.g. Histamine levels. Based on the results from animal model, clinical trial could be started by introducing of peptide vaccine to Blo t 5 allergic subjects.

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Appendix I Nucleotide and amino acid sequence of SM5-1. The signal peptide was underlined. Start and stop codons of open reading frame were boxed.

```
1          GGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCGGCACGAGGCACGCG

61         TAAAACAGACCCATTCCTCAAAAATGAAGTTCGTCCTTGCTCTCGCTGCCCTCCTGGCTG
                                 M  K  F  V  L  A  L  A  A  L  L  A

121        TGGCTTATGCTGCACCAACCCAGAACGACAAGGGATTCCGAAACGAGTTTGACCACCTTT
           V  A  Y  A  A  P  T  Q  N  D  K  G  F  R  N  E  F  D  H  L

181        TGGTGATCGAAGCCGAGCACCGATTCAAGGAGATCGAACAGGGTCTGATGAGGTTGAGCC
           L  V  I  E  A  E  H  R  F  K  E  I  E  Q  G  L  M  R  L  S

241        TCCAGGTGGAGACCCTCGAGAAGAGCAAATCGAAGGCGTTGAAGGCGGAGATTCTGCGTG
           L  Q  V  E  T  L  E  K  S  K  S  K  A  L  K  A  E  I  L  R

301        AGATCACCATCGGCGTGAACTTTGCCACCGGCGCCAAGGAGTTCTTCACCCGTGAGGCCA
           E  I  T  I  G  V  N  F  A  T  G  A  K  E  F  F  T  R  E  A

361        AGCGAACCGATCTCGATCTGGTCGAGAAGTTCAACTACGATGCCGCCGTTGTGTCCGCCG
           K  R  T  D  L  D  L  V  E  K  F  N  Y  D  A  A  V  V  S  A

421        AAATTCTCATCAAGGATCTCACCGAGCTTGCCAAGAAGGTCAACGCCATTGATGCCAACA
           E  I  L  I  K  D  L  T  E  L  A  K  K  V  N  A  I  D  A  N

481        ACAAGTAGATGATGATGAGAATGAACTAAAATTGATGAGCAAAGTTCTCTAACTTTGTTA
           N  K

541        TTCAATGAATAAAATAAAGTAAATAAATAATAATTTTCTGTAAAGAAAACAAAACTAAAA

601        AAAAAAAAAAAAAA
```

Appendix II Nucleotide and amino acid sequence of SM5-2. The signal peptide was underlined. Start and stop codons of open reading frame were boxed.

```
1          GGGCTGCAGGAATTCGGCACGAGGCTCCTTCCCACTCCCCAAAAAACCCAAACCAAAAAA

61         TGAAGTGCCTTCTGGTTCTCGCTGCCTGCCTCGTCGCCGTCTATGCGGCTGACAAGAATG
           M  K  C  L  L  V  L  A  A  C  L  V  A  V  Y  A  A  D  K  N

121        ACTTTCGTCACGAGTTCGACTACTTGTTGATGAAGACCGCTGAGCACAACATGGAACGTG
           D  F  R  H  E  F  D  Y  L  L  M  K  T  A  E  H  N  M  E  R

181        GTGAGGCAATGCTTCTGGCTTTGACCGAGCAGATTGCTCATCTCGAACAGTCGAAGAACA
           G  E  A  M  L  L  A  L  T  E  Q  I  A  H  L  E  Q  S  K  N

241        AGGAGGAGAAGGAGAAGATTGTCCGAGAGCTCGAGACCATCATCGCTCTCATCTCTGGAT
           K  E  E  K  E  K  I  V  R  E  L  E  T  I  I  A  L  I  S  G

301        CGCACGATGTCTTGGAACGTGAACTCAAGCGTACTGACTTGGACATTCTCGAGCGTTACA
           S  H  D  V  L  E  R  E  L  K  R  T  D  L  D  I  L  E  R  Y

361        ACTTTGAGTCTGCCCTGAAGATCGGCGCCATCTTGGTTCGTGACCTCAAGGCCGCCGAGG
           N  F  E  S  A  L  K  I  G  A  I  L  V  R  D  L  K  A  A  E

421        CTAAGGTGAAGGCTATCAATGTCCACGCCTAGAAAGCCCACCTTGTCACCTCAGTGTTCT
           A  K  V  K  A  I  N  V  H  A

481        GTGTGTTTGTCTAAATTATAATCATAAAA
```

Appendix III Nucleotide and amino acid sequence of SM5-3. The signal peptide was underlined. Start and stop codons of open reading frame were boxed.

```
1          CCCCCGGGCTGCAGGAATTCGGCACGAGGATTTCAGGTCGCTGAAAATCGCACAATCCAG

61         ATTCAAAAGAAAAATAATTTCAAAATTCTAAAATGAAGTTTGTTTTAATTCTTGCAACTT
                                           M  K  F  V  L  I  L  A  T

121        GTTTGGTTGCCGTGCTTGCCGCGGACAAGAATGACTTTCGCAACGAGTTTGACTTTCTGC
           C  L  V  A  V  L  A  A  D  K  N  D  F  R  N  E  F  D  F  L

181        TGATGCAAACCATGGAACACAACATGCACCGTGGAGAGCAGATGCTTCTGGCCCTAACCG
           L  M  Q  T  M  E  H  N  M  H  R  G  E  Q  M  L  L  A  L  T

241        AACAGATCGCCCACCTTGAAGAGTCGAAGAACAAGGAGGAGAAGGAGCACATCATTCGTG
           E  Q  I  A  H  L  E  E  S  K  N  K  E  E  K  E  H  I  I  R

301        AATTGGAAACCATCATCGCAATGATCTTCGGATCACACTCGGTTCTCGAACGTGAACTCA
           E  L  E  T  I  I  A  M  I  F  G  S  H  S  V  L  E  R  E  L

361        AGCGTACCGATTTGAACATTCTCGAGAGGTTCAACTTTGAGAGCATGCTTAAGGTTACCC
           K  R  T  D  L  N  I  L  E  R  F  N  F  E  S  M  L  K  V  T

421        AGGTTTTGGCCAAAGACCTCAAGGATGCAGAGGCTAAGGTCAAAGCAATTAAAGTCTAAA
           Q  V  L  A  K  D  L  K  D  A  E  A  K  V  K  A  I  K  V

481        CTTGTTTGTTTGTTACATACACCAAAA
```

Appendix IV cDNA and protein sequences of Alo e 5. Signal peptide was underlined, and the start and stop codons were boxed.

```
1          GATCCTTTTGTGTTTCCTCTCNGAAGACGCCAAAGTCCCACCCAACCTCAACCATGAAGT
                                                              M  K

61         TTGCCATTCTCGCTCTTGCCTTCCTCGCTACGGCTTTGGCTGCCGATGTCCCCAAGGTCC
           F  A  I  L  A  L  A  F  L  A  T  A  L  A  A  D  V  P  K  V

121        CACCAAAGGGTGACTTCCGCAATGAATTTGACCATCTCCTAGTCGCCCAGCTGCAGGCTG
           P  P  K  G  D  F  R  N  E  F  D  H  L  L  V  A  Q  L  Q  A

181        GTATGGCACGTGGTGAGCAGCATCTACTTCGTCTGACGGCGGAGATTGCTCATCTGGAGC
           G  M  A  R  G  E  Q  H  L  L  R  L  T  A  E  I  A  H  L  E

241        AGACCAAGACAAAGGCTGAACAGGAGCGAATTGTCAATGAGATCAATGTGACGGTGGCCT
           Q  T  K  T  K  A  E  Q  E  R  I  V  N  E  I  N  V  T  V  A

301        TTATTGAGGGTGCCCAGGGTGTCATCAGCCGTGAGCTTGAACGAAAGGATCTGAACATTC
           F  I  E  G  A  Q  G  V  I  S  R  E  L  E  R  K  D  L  N  I

361        TGGAGAAGTTCAACTTTGAAGAGGTTCAGGCTATCTCGAAGATTCTGATTAAGGATCTTA
           L  E  K  F  N  F  E  E  V  Q  A  I  S  K  I  L  I  K  D  L

421        AGGAGGCGGAAGCTAAGGTGAAGGCGGTCAAGACTCACTAAACTGTGGCTGCGGTTCCCG
           K  E  A  E  A  K  V  K  A  V  K  T  H

481        GCTAAATTTATGGAAATTTTTGTATAATTTCTTTTTTTTGAATTTTAAATAAAATATCTT


541        GATTCCCANGAAANACCGTCAAAAAAAAAAAAA
```