Characterization of the House Dust Mite Allergen, Der p 23 produced in *Pichia pastoris*.

Mr. Wai Tuck Soh

A Thesis Submitted in Partial Fulfillment of the Requirements
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ลักษณะของสารก่อภูมิแพ้จากไรฝุ่น Der p 23
ที่ผลิตจากยีสต์ *Pichia pastoris*

นายไว ตัก ซอก

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Field of Study	Medical Science
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ไว ตัก ซอก : ลักษณะของสารก่อภูมิแพ้จากไรฝุ่น Der p 23 ที่ผลิตจากยีสต์ *Pichia pastoris* (Characterization of the House Dust Mite Allergen, Der p 23 produced in *Pichia pastoris.*) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: เกียรติ รักษ์รุ่งธรรม, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: อลัน แจ็คเควท, 74 หน้า.

ในปัจจุบันสารก่อภูมิแพ้ไรฝุ่นชนิดที่ 23 เป็นที่รู้จักในนามสารก่อภูมิแพ้ไรฝุ่นที่มีบทบาทในการกระตุ้นการแสดงออกของระดับ IgE ที่สูงมากเมื่อเทียบกับสารก่อภูมิแพ้หลักชนิดที่ 1 และ 2 ในไรฝุ่น การศึกษาลักษณะของสารก่อภูมิแพ้ไรฝุ่นชนิดที่ 23 อย่างละเอียดนี้ มีความจำเป็นต้องผลิตออกมาในรูปแบบของรีคอมบิแน้นท์โปรตีน เนื่องจากปริมาณอันจำกัดของสารก่อภูมิแพ้ในธรรมชาติที่ถูกสกัดโดยใช้บัฟเฟอร์ไม่เพียงพอ ในงานวิจัยครั้งนี้มีจุดประสงค์เพื่อผลิตรีคอมบิแน้นท์โปรตีนชนิดที่ 23 โดยกระบวนการแสดงออกของโปรตีนในยีสต์ *P. pastoris,* เพื่อศึกษาคุณลักษณะเชิงกายภาพพร้อมทั้งคุณลักษณะเชิงกล และวิเคราะห์ปฏิกิริยาความไวต่อการแพ้สารก่อภูมิแพ้ไรฝุ่นชนิดนี้ในกลุ่มตัวอย่างผู้ป่วยไทยเป็นครั้งแรก จึงนำไปสู่การตรวจสอบการกระตุ้นระบบภูมิคุ้มกันที่ติดตัวมาแต่กำเนิด (innate immunity) รีคอมบิแน้นท์โปรตีนที่สมบูรณ์ชนิดที่ 23 นี้สามารถผลิตออกมาได้ในรูปของโปรตีนที่ถูกหลั่งออกมาและละลายน้ำได้ การแสดงออกของโปรตีนที่สูงสุดจะเกิดในระยะ 48 ชั่วโมง ภายใต้การกระตุ้นโดยใช้ 2% ของปริมาณเมทานอล และส่งผลให้เกิดปรากฏการณ์ N-terminal truncation หากกระตุ้นในระยะเวลาที่มากขึ้น รีคอมบิแน้นท์โปรตีนชนิดที่ 23 ได้จัดอยู่ในกลุ่ม mannosylated protein ซึ่งอาจมีการเปลี่ยนแปลงของบริเวณกรดอะมิโน T30-T32 โดยพันธะภายในของกลุ่มไดซัลไฟด์ 2 พันธะนี้ ส่งผลให้โครงสร้างของโปรตีนส่วนใหญ่ไม่มีการม้วนพับ ดังที่แสดงผลใน เทคนิคในการวิเคราะห์ผลการวัดสัดส่วนมวลต่อประจุ (Mass spectrometry) และการศึกษาโครงสร้างทุติยภูมิ (Circular dichorism) โพลีโคนอลแอนติ้บอดีต่อรีคอมบิแน้นท์โปรตีนชนิดที่ 23 สามารถตรวจจับสารก่อภูมิแพ้ธรรมชาติชนิดที่ 23 ได้ในสารสกัดจาก fecal pellets สนับสนุนว่ามีโครงสร้าง B-cell epitopes ที่คล้ายกัน ถึงแม้รีคอมบิแน้นท์โปรตีนชนิดที่ 23 และสารก่อภูมิแพ้ไรฝุ่นชนิดที่ 23 ในธรรมชาตินี้จะคล้ายคลึงกับโปรตีนที่มีสมบัติในการจับกับไคติน แต่ก็ยังไม่สามารถจับกับเม็ดบีทไคตินสังเคราะห์ได้ จากนั้นพบว่ามากกว่าร้อยละ 50 ในกลุ่มผู้ป่วยไทยที่แพ้ไรฝุ่น (n=222) จะมีอาการแพ้จำเพาะต่อสารก่อภูมิแพ้ไรฝุ่นชนิดที่ 23 เมื่อเทียบกับอัตราการแพ้ต่อสารก่อภูมิแพ้ไรฝุ่นหลักชนิดที่ 2 (ร้อยละ 67) ในขณะที่เกิดการแพ้ของสารก่อภูมิแพ้ไรฝุ่นชนิดที่ 23 พบว่ามีการทำงานของกระบวนการดีแกรนูเลชั่นของเซลล์ RBL ที่มีการแสดงออกของ human Fc**Ɛ**RI ร่วมกับ IgE จำเพาะต่อสารก่อภูมิแพ้ธรรมชาติชนิดที่ 23 ทันที อย่างไรก็ตามเซลล์ทางเดินหายใจในมนุษย์ยังสร้างไซโตไคน์ IL-8 เป็นการตอบสนองต่อรีคอมบิแน้นท์โปรตีนชนิดที่ 23 (5-20μg/ml) ผ่านทางกระบวนการส่งสัญญาณโดยตรงต่อ NF-**K**B และ MAPK (MEK, JNK, and p38) การวิจัยครั้งนี้เน้นระดับความสำคัญของการไวต่อการแพ้สารก่อภูมิแพ้ธรรมชาติกลุ่มที่ 23 ในประเทศไทย การไวต่อโปรติเอสของสารก่อภูมิแพ้ธรรมชาติชนิดที่ 23 กับปริมาณการหลั่งที่จำกัดจากสารก่อภูมิแพ้นี้จาก fecal pellets สนับสนุนว่ารีคอมบิแน้นท์โปรตีนชนิดที่ 23 อาจเป็นสารสำคัญที่มาแทนที่มาตาฐานของสารสกัดไรฝุ่นที่ใช้ในการวินิจฉัยอาการไวต่อการแพ้สารก่อภูมิแพ้ธรรมชาติชนิดที่ 23 ในธรรมชาติได้.

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5674663230 : MAJOR MEDICAL SCIENCE
KEYWORDS: HOUSE DUST MITE / DER P 23 / PICHIA PASTORIS / RECOMBINANT ALLERGEN / INNATE IMMUNITY

WAI TUCK SOH: Characterization of the House Dust Mite Allergen, Der p 23 produced in *Pichia pastoris*.. ADVISOR: PROF. DR. KIAT RUXRUNGTHAM, CO-ADVISOR: ASSOC. PROF. DR. ALAIN JACQUET, 74 pp.

Recent identified Der p 23 has been recognized as an important house dust mite (HDM) allergen which displays high IgE reactivity comparable to the major HDM allergens Der p 1 and Der p 2. Due to limited amounts of the natural allergen, the in-depth characterization of Der p 23 requires the production of a recombinant form. Present study aims were to produce a recombinant form of Der p 23 using *P. pastoris* expression system subsequently characterize its physicochemical properties and to analyze for the first time the IgE binding frequency of Der p 23 in a cohort of Thai HDM allergic patients as well as to evaluate the ability to activate innate immunity. The expression of secreted mature rDer p 23 reaches maximum at 48 hrs under 2% methanol induction and resulted in N-terminal truncation with longer induction period. rDer p 23 is characterize as a mannosylated protein (possibly modified at amino acid T^{30}-T^{32}) with two intra-disulfide bonds and adopt mainly unfolded structure. Polyclonal antibodies to rDer p 23 can detect the natural allergen in aqueous fecal pellets extracts suggesting that both forms of Der p 23 share common B-cell epitopes. rDer p 23 as well as the natural corresponding allergen were unable to interact in-vitro with chitin matrices. More than fifty percent of Thai HDM allergic patients (n=222) developed Der p 23-specific IgE which was comparable with the IgE binding frequency of rDer p 2 (67%). Meanwhile, the allergenicity of rDer p 23 was highlighted through degranulation of RBL cells expressing human Fc**ε**RI. Nevertheless, human airway epithelial cells were able to produce IL-8 in response to rDer p 23 (5-20µg/ml) through NF-**K**B and MAPK (MEK, JNK, and p38)-dependent activation signaling pathways. Our findings highlighted important levels of Der p 23 sensitizations in Thailand. The protease sensitivity of Der p 23 as well as the very limited release of this allergen from extracted mite fecal pellets clearly suggested that rDer p 23 may be a valuable material to replace standardized HDM extracts for the diagnosis of Der p 23 sensitivities.

Field of Study: Medical Science
Academic Year: 2014

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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CONTENTS

Page

Page

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

APCs	Antigen Presenting Cells
BMGY	Buffered Glycerol-Complex Medium
BMMY	Buffered Methanol-Complex Medium
$CaCl_2$	Calcium Chloride
CD	Circular Diachroism
cDNA	complementary DNA
CLRs	C-type Lectin Receptors
cm	centimeter
CO_2	Carbon Dioxide
DCs	Dendritic Cells
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
FcεRI	Fc epsilon receptor 1
FP	Fecal pellet
GM-CSF	Granulocyte macrophage colony stimulating factor
HCl	Hydrochloric acid
HDM	House Dust Mite
Hz	Hertz
IgE	Immunoglobulin E
IL	Interleukin
ILC	Innate Lymphoid Cell
KCl	Potassium Chloride
kDa	kilo Dalton
M	Molarity
MB	Mite bodies
m/z	Mass-to-charge ratio
mg	milligram
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulfate
mins	minutes
ml	milliliter
mM	millimolar
NaCl	Sodium Chloride
ng	nanogram
NLRs	NOD-like receptors
PAGE	Polyacrylarmide Gel Electrophoresis
PCR	Polymerase Chain Reaction
RLRs	RIG-I-like receptors

SDS	Sodium Dodecyl Sulfate
T_H2	T-Helper 2
TLRs	Toll-like receptors
TSLP	Thymic Stromal Lymphopoietin
U	Units
µg	microgram
µl	microliter
aa	amino acids

CHAPTER I

INTRODUCTION

House dust mite (HDM) such as *Dermatophagoides pteronyssinus* is well recognized as one of the most common sources of indoor airborne allergens worldwide. Currently, it is considered that HDM allergy affects approximately 20% of the population from industrialized countries (1,2). Allergy is a strong inflammatory response characterized by the ability to generate Immunoglobulin E (IgE) antibody response to environmental allergens (3). HDM sensitizations are strongly associated to allergic asthma but also to atopic rhinitis, dermatitis and conjunctivitis. Up to 85% of allergic asthmatic patients are allergic to HDM allergens (2).

Up to now, more than 20 different HDM allergen groups inducing the production of allergen-specific IgE in humans have been referenced and classified as major, intermediate and minor allergens according to the IgE binding frequencies in multiple cohort studies. Until recently, Der p 1, a cysteine protease, and Der p 2, a LPS-binding protein, were considered as the two most potent HDM allergens. Indeed, not only these allergenic molecules are easily extracted from mite bodies and feces (4), but their IgE reactivities reached 80-90% in HDM allergic patients (5,6). Moreover, these two allergens are able to trigger innate immune signaling pathways which are critical to initiate the allergic response (7,8).

A recent study paid attention to a 8 kDa HDM protein from fecal pellets and designated as Der p 23 (9). Surprisingly, this low abundant allergen in fecal pellets and in house dust was shown to sensitize HDM allergic patients at the levels comparable to those measured for Der p 1 and Der p 2 (>70% of European HDM allergic patients developing Der p 23-specific IgE). The in-depth characterization of natural Der p 23 allergenicity was shown to be challenging because this protein could be poorly extracted from fecal pellets (9). The main reason is due to the

apparent association of natural Der p 23 to the peritrophic membrane of mite feces, a semi-permeable matrix rich in proteins and chitin which can protect the mite midgut from damages by food particle during digestion. In order to circumvent the limited access of natural Der p 23, heterologous expression systems could provide unlimited sources for this allergen production. Although the characterization of Der p 23 was mainly performed with a recombinant form Der p 23 produced in E.coli (9), detailed analysis of Der p 23 amino acid sequence suggested that this protein could contain post-translational modifications through the presence of a N-terminal leader sequence, two regions rich in Threonine residues for O-glycosylations, a putative C-terminal chitin binding domain containing four cysteine involved in two disulfides bonds. Such modifications could not only influence the protein folding but the biological and allergenic properties of Der p 23.

Consequently, in the present study, we reported the production of a recombinant form of Der p 23 using the *Pichia pastoris* expression system. Contrary to *E.coli*, this yeast strain can express and secrete O-glycosylated proteins with appropriate disulfides. Following expression and purification, the prevalence of IgE reactivity to recombinant Der p 23 was, for the first time, examined in a cohort of HDM allergic subjects from Thailand. As chitin could be of importance in the development of the HDM allergic response through notably airway epithelial cell activation (10,11), the chitin-binding activity of Der p 23 was also assayed together with the stimulation of a human airway epithelial cell line by Der p 23.

CHAPTER II
LITERATURE REVIEW

2.1 Allergy and HDM

Allergy can be referred to the unusual tendency of some individuals to develop abnormal reactivity or hypersensitivity reactions against non-infectious environmental substances identified as allergens (12). The clinical manifestations of allergy include allergic asthma, perennial rhinitis, food allergies, and atopic dermatitis, which cause huge economic loss to both individuals and their society. Interesting data from WHO evidenced the increasing trend in allergy prevalence which is currently affecting more than 20% of the populations, mainly from developed countries (13). However, the rising trend of allergy in developing countries particularly in Asia is also evident (14).

Before moving on to HDM allergy, it would be interesting to introduce the subject, House dust mites. They are arachnids, closely related to ticks and spiders, but not to insects (Fig. 2.1). Generally they have a life cycle of 7-10 weeks. They live in dust and can be found in almost every home. House dust not only providing habitat for mites but also contains their foods source, shed human skin scales which colonized by molds, yeasts, and bacteria (15).

HDM is one of the most common sources of airborne allergens. It is believe that HDM allergens could sensitized up to 50% of the allergic population (15,16). Association of HDM with allergy and asthma was first evidenced in the early 1990s (15). It is now well accepted that there is a strong correlation between HDM allergy and allergic asthma as approximately 85% of asthmatics are actually allergic to HDM (2,3,16) (Fig. 2.2). Study also shows that the risk of developing asthma in HDM allergic children is proportional to their anti-HDM IgE titer (17). Asthma disease is affecting over 300 million people particularly among children and contributed to a fatal rate

of 250,000 per year worldwide (2,18). Based on the current rising trend of asthma, it is expected that by 2025 there will be 400 millions of people suffering from asthma worldwide (19).

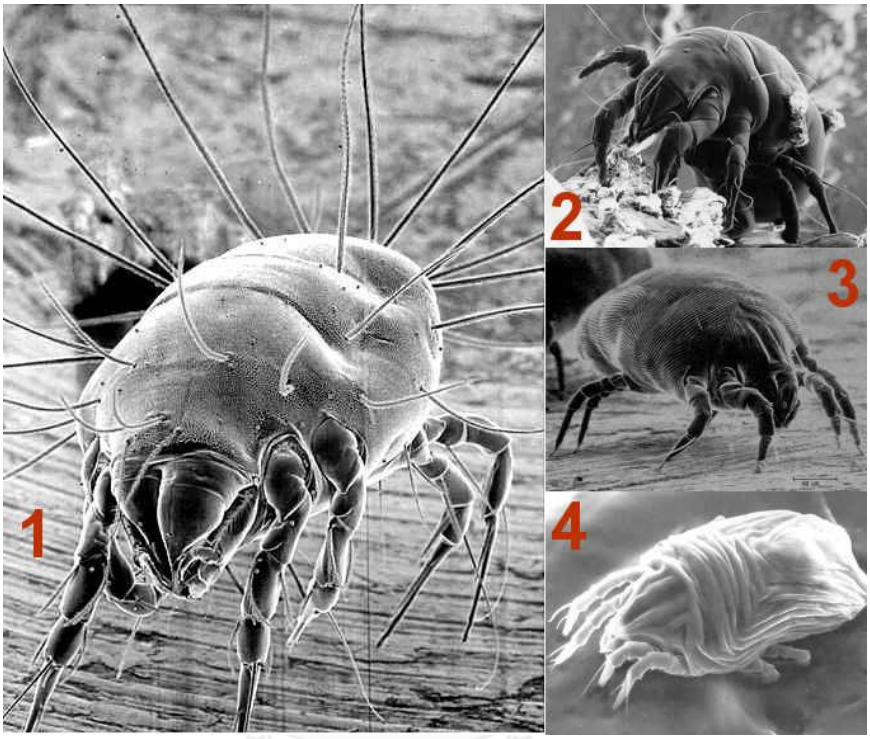

Figure 2. 1 The four most common allergenic dust mite species. 1) *Blomia tropicalis,* 2) *Dermatophagoides pteronysinnus,* 3) *Dermatophagoides farinae,* 4) *Euroglyphus maynei.* Sequence of dust mite species does not correlate to the allergenicity. Picture source (www.allergyaustralia.com.au).

The development of HDM allergy is contributed by mainly three factors: 1) Genetic susceptibility often refers as atopy. Atopy can be defined as the genetic propensity of the immune system to produce IgE antibodies when exposed to an allergen (20,21). 2) Changes of the human indoor living environment such as temperature and humidity has favor the proliferation of house dust mite and mold, which in turn resulted in 3) frequent allergen exposure that could lead to higher risk

of allergy development (22–24). Besides that, hygiene hypothesis that described those individuals who has low exposure to microbial compounds and infection may result in lower immunity, are more susceptible to develop allergy (3).

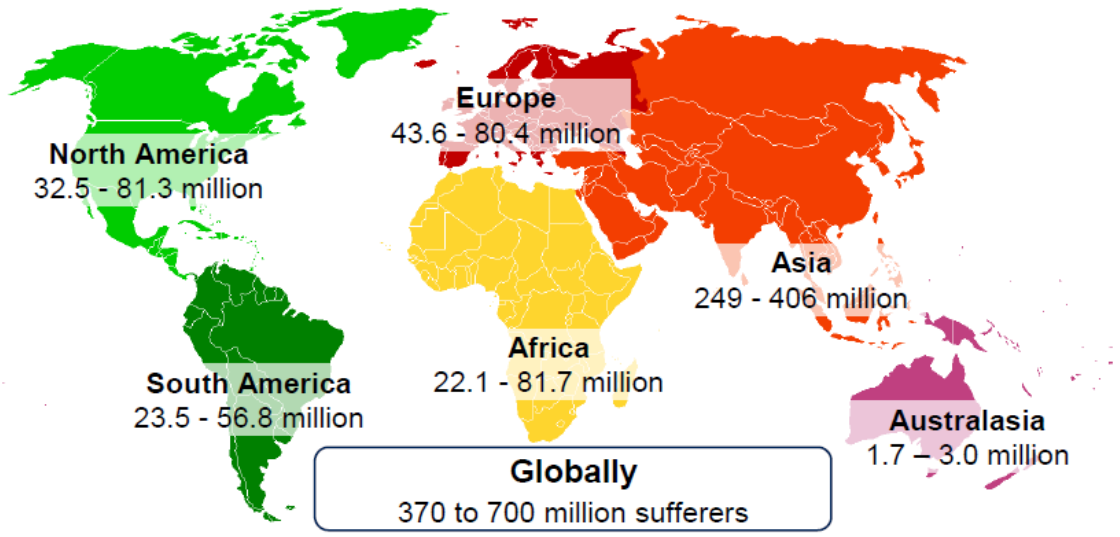

Figure 2. 2 Number of people with HDM induced respiratory allergy worldwide. [Adapted from EAACI 2013 allergy congress].

2.2 Mechanisms of Allergy

Allergic reactions are immunoglobulin-E (IgE) mediated which is classified as type-I hypersensitivity (25). In the context of this study, airway related allergic responses would often use as an example. In general, exposure of a genetic susceptible individual to environmental stimuli such as allergens are more likely to develop a T helper 2 (T_H2) biased immune response which involved the interconnection between innate and adaptive immune pathways. The T_H2 cytokines such as interleukin (IL)-4 and IL-13 release resulted from such immune interactions could then lead to inflammation causes effector organs dysfunction. Repeated exposure to environmental stimuli in long run could resulted in chronic inflammatory disease (3,26). Figure 2.3 give an overall picture on the processes involved in airway

allergic disease development. In the following section, stressed will be given on the interplay between innate and adaptive immune responses.

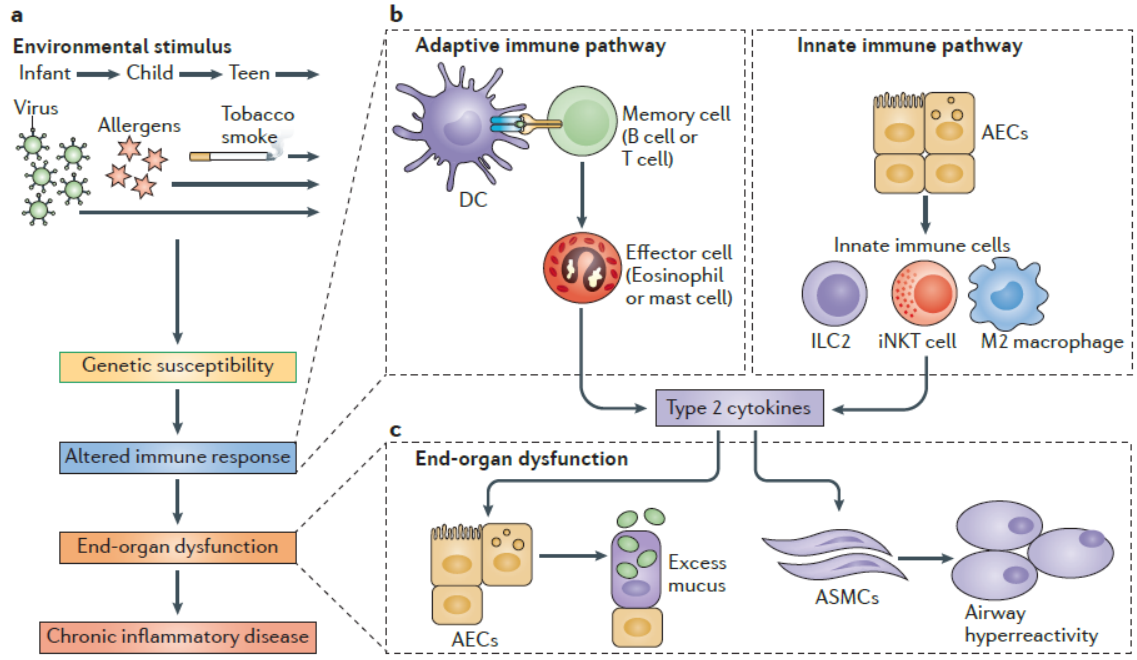

Figure 2. 3 Schematic representation of adaptive and innate immune responses in allergic inflammatory disease development. a) Environmental stimuli could trigger altered immune responses in genetic susceptible individuals and further lead to end-organ dysfunction and chronic inflammatory disease. b) Involvement of adaptive and innate immune pathways in shaping T_H2 polarization. c) T_H2 cytokines such as IL-4, and IL-13 notably that act on end-organ cells resulted in excess mucus production and airway hyperreactivity. [Adapted from (26)]

In the pathogenesis of allergic disease, recent literature pointed out the necessary of innate immune activation through Pattern Recognition Receptors (PRRs) follow by recruitment of innate immune cells and finally trigger the allergic T_H2 response through adaptive immunity (27) (Fig. 2.4). Currently, there are four classes of PRRs have been identified, which include transmembrane proteins Toll-like receptors

(TLRs) and C-type lectin receptors (CLRs), as well as cytoplasmic protein Retinoic acid-inducible gene (RIG)-l-like receptors (RLRs) and NOD-like receptors (NLRs) (28). Besides macrophages and dendritic cells (DCs), these PRRs are also expressed in various nonprofessional immune cells such as epithelial cells, endothelial cells and fibroblasts (27,28).

In allergic asthma, airway epithelial cells are the first contact with aeroallergens particularly HDM allergens. These cells express various PRRs, including TLRs, RLRs, NLRs and to a lesser extent some inducible CLRs (29). Other than serve as first line defense, airway epithelial cells also involved actively in promoting allergic response (27,30). The activation of PRRs by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) will up regulate transcription of genes encoded pro-inflammatory cytokines, type 1 interferons, and chemokines (28). The release of IL-25 and IL-33 by activated epithelial cells are able to stimulate innate lymphoid cells type 2 (ILC-2). This cell is the key sources of T_H2 cytokines such as IL-4, IL-5, IL-9 and IL-13 that participate in subsequent immune responses. Cytokines such as IL-5 and IL-13 released by ILC-2 are particularly important in promoting B cells IgE secretion, eosinophil recruitment and airway remodeling. Furthermore, IL-5 and IL-13 together with Thymic stromal lymphopoietin (TSLP) and IL-4 could drive T_H2 polarization (27). The T_H2 cytokines milieu created by epithelial cells and other innate immune cells provide a suitable environment to trigger T_H2-biased adaptive immunity.

Dendritic cells (DCs) play an important role in bridging the innate to adaptive immunity. Number of cytokines and chemokines released by stimulated airway epithelial cells notably granulocyte macrophage colony-stimulating factor (GM-CSF), IL-1 family members, IL-25, IL-33, CCL2, CCL20 and TSLP, could recruit and activate immature DCs to trigger T_H2-biased airway inflammation (27,31).

The sensitization to allergen occurred when the allergen was captured and presented by antigen presenting cells (APCs) such as macrophages and DCs, to naïve T cell. Together with the help of pro-T_H2 cytokines as mentioned earlier, it is sufficient to drive the differentiation of naïve T cell to T_H2. This will further results in further production of T_H2 cytokines such as IL-4 and IL-13, which could then drive immunoglobulin class switching in B cells to immunoglobulin E (IgE) production. The allergen specific IgE produced would then bind to the high binding affinity IgE receptor (Fc**ε**RI), which is expressed at the membrane surface of mast cells and basophils (12,32–35).

Some epithelial derived chemokines and cytokines could also activate and recruit other innate immune cells such as basophils, mast cells and eosinophils consequently causes allergic inflammation. In addition, IL-8 and GM-CSF produced by epithelial cells play a role in neutrophil recruitment (30). The re-exposure of sensitized allergens will lead to inflammation responses that can be categorized, into early-phase and late-phase. Early-phase reactions could occur within minutes of allergen exposure and conversely late phase reactions would require several hours.

Early phase occur when allergen sensitizes the mast cells and basophils by crosslinking the allergen specific IgE which bound to their membrane surface Fc**ε**RI receptor. Consequently, this trigger a cascade of signaling event that causes degranulation of the cytoplasmic stored granules, which contain biogenic amines (mostly histamine), proteoglycans (such as heparin and chondroitin sulfate), and enzymes (such as tryptases, chymases, and carboxypeptidases). Besides, there are also release of some de novo synthesis of lipid mediators and various cytokines. These biologically active products would then lead to acute signs and symptoms that vary according to the site of reaction. Some typical examples would be erythema of the skin, increase secretion of mucus, vasodilation and contraction of airway smooth muscle (12,32,36).

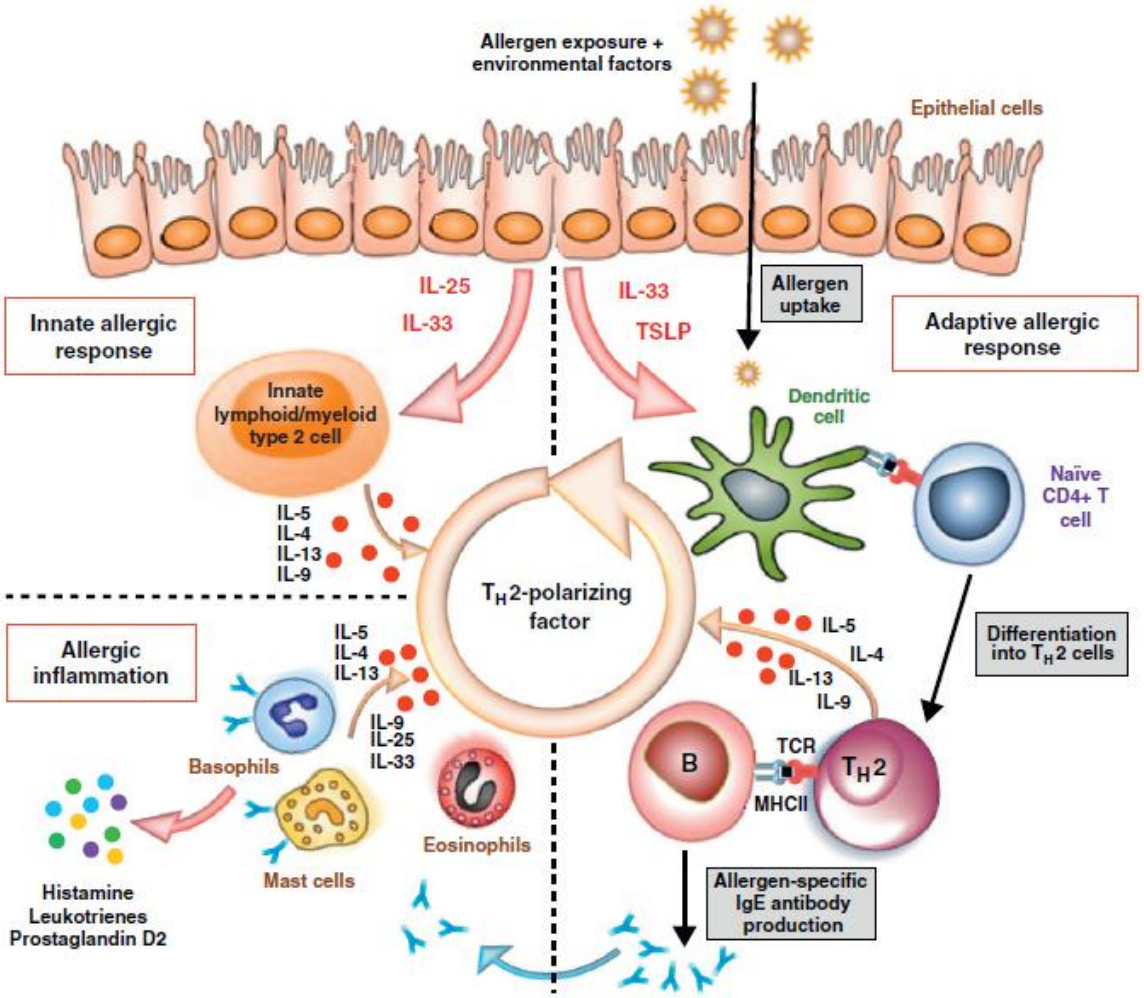

Figure 2. 4 Schematic diagram on the pathogenic processes in allergic disease. Upper strate depicted the exposure of epithelial cells to allergen together with some environmental factors lead to cytokines production. For innate immune response, ILC-2 cells were activated and produce large amount of T_H2 cytokines. For adaptive immune response, allergens are taken up and processed by T_H2 cytokines primed DCs and presented to naïve T cells to drive T_H2 polarization. This further lead to immunoglobulin class switching to IgE in B-cells. [Adapted from (37)]

Late-phase reactions are associated with the slow release of newly synthesized cytokines, chemokines and growth factors, resulted in the accumulation of inflammatory leukocytes such as neutrophils, eosinophils, basophils, and T cells, at the site of allergen exposure. These further complicate the symptoms by releasing more inflammatory cytokines. Late-phase reactions normally develop after 2-6 hours after allergen exposure and frequently peak after 6-9 hours (12,32). It should be noted that late-phase reactions are different from delayed of early phase. Macrophages and T helper 1 (T_H1) cells are dominant in the inflammatory milieu (36).

2.3 HDM Allergy Diagnosis and Immunotherapy

To date, number of HDM extracts based diagnostic tests notably skin prick test, basophil activation assay, and solid phase immunoassay (e.g. ImmunoCAP) are still widely used. Skin prick test is carried out using a lancet to introduce HDM extract into epicutaneous of the patient and evaluate the allergic response based on the wheal size formed on the skin. On the other hand, basophil activation assay is based on histamine released from blood basophil after the addition of HDM extract (3). Nevertheless, ImmunoCAP can be either based on HDM extracts or allergen specific component.

However, major issues with all the HDM extracts based diagnosis is the standardization of the HDM allergen extracts. The extracts contain not only many different important allergens but also proteases that could lead to allergen degradation. The allergen composition is also varies depending on the mite culture conditions, extraction procedures and storage conditions. All these variations have made the HDM extracts standardization impossible. Indeed, analyses on commercially available natural extracts of *D. pteronyssinus* from ten different manufacturers have revealed that some important allergens were not detected in eight of the extracts. This great variability in allergen composition gave false negative diagnosis test results to some patients (38).

Allergen specific immunotherapy has been used for a century long ago by using HDM allergen extracts. It requires administer of very dilute dose of allergen extracts and progressively increase to higher doses until a maintenance dose is reached. This allergen specific immunotherapy typically takes a total period of 3-5 years (3). The administration of crude allergen extracts in an increasing dosage could induce severe life-threatening side effects such as IgE mediated anaphylaxis (32). Not only this therapy is facing the same issue as HDM diagnosis about allergen extracts standardization, concern has also raised on the development of new allergen sensitization during the treatment with HDM allergen extracts (39,40). Some allergens can be poorly extract and/or naturally presence in tiny amount in HDM and resulted in treatment failure. The same apply to HDM extracts based diagnostic that can result in misdiagnosis. Recent identified Der p 23 is apparently difficult to extract using aqueous solution (9).

2.4 House Dust Mite Allergens

Allergens are antigens that are capable to sensitize allergic reactions involving specific IgE and mast cells. The sources of allergens are diverse such as house dust mite products, pollens, fungal spores, drugs, animals and foods (3). In the context of HDM, the main allergenic sources are from the mite feces, which can be easily inhaled into the human respiratory system due to its small size of approximately 10 μm (27,41). More strikingly is that there are well-documented cases where dust mites were found in the sputum of asthmatic patients suggesting that dust mites could also be inhaled into the airway and contributing to asthma development (42,43).

The most commonly found house dust mite species are *Dermatophagoides pteronysinnus* and *Dermatophagoides farinae* (15). Both species are abundant in many European countries as well as in Asia counties. Based on data available for South East Asia countries, *D. pteronyssinus*is dominant in Malaysia and Singapore whereas Thailand has more *D. pteronyssinus* than *D. farinae* (44).

In order to face the rising number of allergens being identified and to avoid confusion with the allergens naming, the allergen nomenclature guidelines were introduced by Allergen Nomenclature Subcommittee of the International Union of Immunological Societies IIUIS) in 1986. Allergens are named using the first three letter of the genus together with the first letter of the species and follow by an Arabic numeral which referring to the order where the allergen group was isolated. For examples, Der p 1 is the first isolated allergen of *Dermatophagoides pteronyssinus.* Similarly, Der f 1 is the allergen from *D. farinae* that belong to the same group as Der p 1, a cysteine protease (3).

Most of the HDM allergens are found in the fecal pellet (3). Currently, there are more than 20 groups of HDM allergens have been identified (Table 1) (27). However, allergenicity of all the HDM allergens was not well characterized (21). Generally, an allergen is consider as major allergen if the frequency of IgE recognition within an allergic population is more than 50% (3). Der p 1 and Der p 2 are well known clinically important major allergens, where their IgE reactivity are more than 70% in most of the HDM allergics cohort studies in European countries includes Germany (45), France (5), Italy (38), Austria and Sweden (6). Some others important HDM allergens include Der p 5, Der p 7, Der p 10, and Der p 21 (6,38). Recently, a newly discovered HDM allergen, Der p 23, displayed a relatively high IgE reactivity of more than 70% in HDM allergic cohort from Austria, Italy and France (9). Although the biological function of Der p 23 is still unknown, bioinformatics analysis on the amino acid sequence has revealed that Der p 23 display homology to chitin binding protein and could be an O-glycosylated protein (9).

Table 2. 1 House dust mite allergens identified in *D.pteronyssinus* and *D.farinae*. [Adapted from(4)]

Allergen groups	Biological function/activity	MW (kDa)	Presence reported in mite species		Sequence homology between the two species	Prevalence of IgE recognition	Location within mites
1	Cysteine protease	24–27	Dp	Df	81%, 24 isoforms for Der p 1 and 10 isoforms for Der f 1	60–80%	Feces
2	Nieman Pick C2 homolog	15	Dp	Df	88%, 15 isoforms for Der p 2 and 17 isoforms for Der f 2	≥70%	Body
3	Trypsin	25–30	Dp	Df	81%	10–15%	Body (Df)
4	α-amylase	56–63	Dp		?	10–25%	Feces
5	Unknown	14	Dp		?	10–40%	Feces
6	Chymotrypsin	25	Dp	Df	75%	41–65%	
7	Unknown	26–31	Dp	Df	86%	20–40%	Feces
8	Glutathione-S-transferase	25–27	Dp	Df	?	10–40%	Feces
9	Collagenolytic serine protease	24–29	Dp		?	?	Feces
10	Tropomyosin	33–37	Dp	Df	98%	5–10%	
11	Paramyosin	98–103	Dp	Df	97%	65–80%	
13	Fatty acid binding protein	15		Df	?	<25%	Feces
14	Apolipophorin	177	Dp	Df	>80%	20–60%	Body
15	Chitinase	98–105	Dp	Df	90%	15–30%	
16	Gelsolin/villin	53–55		Df	?	35–47%	
17	Calcium-binding protein	53		Df	?	35%	
18	Chitin-binding protein	49–60	Dp	Df	88%	30–40%	
20	Arginine kinase	40	Dp		?	5–20%	Body
21	Unknown	14	Dp	Df	?	15–30%	Feces
22	Unknown	67		Df	?	?	Feces
23	Peritrophin-like protein domain	14	Dp		?	30–60%	Feces

Allergen groups 12 and 19 were reported in *Blomia*, but not in *Dermatophagoides* mite species.
Df: *Dermatophagoides farinae*; Dp: *Dermatophagoides pteronyssinus*; MW: Molecular weight.
From [30]/(WHO/International Union of Immunological Societies site providing the official list of registered allergens and their isoforms). Prevalence of IgE recognition was obtained from [4,15,30,35,36] and [PM, Unpublished Data].

The house dust which is the dust mite's living environment, contain many others compounds that could participate in HDM allergy sensitization. Compound such as lipopolysaccharide (LPS) from bacteria; β-glucan from fungi; and chitin from fungi but also mite exoskeleton, are abundant in house dust and are well known PAMPs that can trigger innate immunity (27). Hence, HDM is not only the source of allergens but also a carrier for PAMPs. Experimental evidences in mouse have shown

that low dose LPS induce T_H2 response while conversely, high dose of LPS induced T_H1 responses (46). In HDM allergen, Der p 2 display structural homology with LPS-binding protein that can bind LPS and transport LPS to interact with TLR-4 to trigger innate immune responses and subsequent T_H2 responses. Dimerization of Der p 5 creates a large hydrophobic pocket, which possibly serves as an interacting point with lipid. Other HDM allergens such as Der p 7, 13, 14, and 21 also display sequence homology to lipid-binding proteins. The association of lipid-based PAMPs with these HDM allergens could probably explain their allergenicity (27). A cell wall component of fungi, β-glucan, found in HDM extract has presented to be able to trigger the release of chemokine attractant for immature DCs, CCL20, by airway epithelial cells (47). Chitin, which is a major component in the mite exoskeleton, is also another noteworthy PAMP molecule that can be found in the mite faeces. Study has demonstrated that chitin can stimulate CCL2 and CCL20 chemokine secretion from airway epithelial cells, which could subsequently recruit macrophages and immature DCs to the lung (11). Recent discovered Der p 23 was shown to display chitin-binding domains, which possibly could interact with chitin to trigger innate immune responses (9).

2.5 HDM Sensitization in Thailand

Over the last few decades, there is an apparent increase in allergic diseases in Thailand. One study shows that there is an increasing trend in HDM sensitization particularly in Thai asthmatic children between 2004 and 2009 (48). Data back dated to 1995 revealed that more than 85% of house dust samples collected in Thailand contained house dust mites and the dust mite species identified were mainly *D.pternonysinnus* (49). The most recent prevalence data shows that more than 60% of allergic rhinitis and allergic asthma were sensitized to HDM in Thailand (50).

CHAPTER III
MATERIALS AND METHODS

3.1 Der p 23 Gene Isolation

Mature Der p 23 coding gene sequence (Fig. 3.1) was amplified from total cDNA library of *D. pterronysinnus* (kindly provided by Department of Parasitology of Mahidol University, Bangkok, Thailand) by standard Polymerase Chain Reaction (PCR). The forward and reverse primers (BioDesign, Thailand) (Fig. 3.2A) used were strategically designed to amplify only the mature Der p 23 coding sequence with the incorporation of restriction enzymes digestion site at the 5' and 3' end of the amplicon. In details, 5' end of the amplicon consists of *Xho*I enzyme restriction site with a stretch of yeast secretion leader sequence, whereas, 3' end consists of *Not*I enzyme restriction site only. Generally, the goal of this cloning strategy is to prevent any addition of extra amino acid into the target protein. Figure 3.2 illustrates the cloning strategy.

ATG AAA TTC AAC ATA ATC ATC GTT TTT ATT TCG TTG GCC ATT TTG

M K F N I I I V F I S L A I L

GTC CAT TCA TCA TAT GCC GCC AAT GAT AAT GAT GAT GAT CCT ACC

V H S S Y A A N D N D D D P T

ACA ACC GTT CAT CCA ACA ACA ACC GAA CAA CCA GAT GAT AAA TTT

T T V H P T T T E Q P D D K F

GAA TGT CCA AGT AGA TTT GGT TAT TTT GCC GAT CCA AAA GAT CCA

E C P S R F G Y F A D P K D P

CAT AAA TTT TAT ATC TGT TCA AAT TGG GAA GCT GTA CAT AAA GAT

H K F Y I C S N W E A V H K D

TGT CCA GGT AAT ACA CGA TGG AAT GAA GAT GAA GAA ACA TGC ACT

C P G N T R W N E D E E T C T

TAA

Figure 3. 1 DNA gene sequence and deduced amino acid sequence of Der p 23. Bold underlined amino acid sequence represents the natural signal peptide. Primers were designed to amplify mature Der p 23 sequence after the natural signal sequence.

A

Forward primer:
5′ GGCTCGAG**AAAAGAGAGGCTGAAGCT**GCCAATGATAATGATGATGATCCTACCAC 3′
Xho I
Reverse primer:
5′ CCGCGGCCGCTTAAGTGCATGTTTCTTCATCTTC 3′
Not I

B

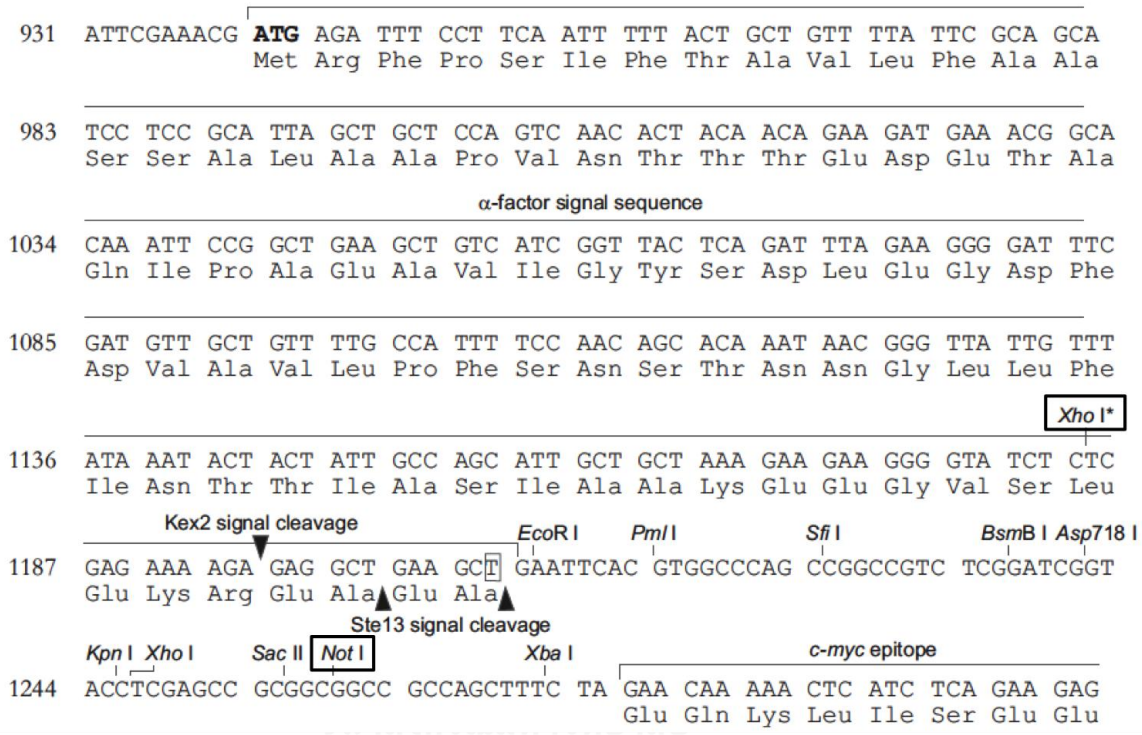

Figure 3. 2 Der p 23 cloning strategy. A) Forward and reverse primers used for Der p 23 PCR amplification. Underlined indicated the restriction enzymes sites. Bolded sequence represent part of α-factor signal sequence. B) Multiple cloning sites for pPICZαA yeast expression vector. Square boxes indicated the restriction enzyme sites used for Der p 23 cloning. [Modified from Invitrogen pPICZα manual]

3.1.1 Standard PCR

PCR reagents used was GoTaq® Flexi PCR reagents (Promega, USA). The PCR was performed in a 50 µl reaction mixture; consist of final concentration of 1X GoTaq buffer, 2 mM magnesium chloride ($MgCl_2$), 0.2 mM deoxynucleotidetriphophates (dNTPs), 0.2 µM of each forward and reverse primer, and 5 U of *Taq* polymerase. Template (HMD total cDNA) was added into PCR reagents mixture prior to the running of PCR. The PCR thermal cycler profile was set to include 5 minutes of pre-denaturation at 95°C, followed by 40 cycles repetition of 30 seconds of denaturation at 95°C, 30 seconds of annealing step at 52°C, and 30 seconds of elongation at 72°C, lastly ended with 5 minutes of final elongation at 72°C and hold at 4°C. The PCR reaction was carried out in Veriti® thermal cycler (Applied Biosystems, Singapore). Amplicon was then checked by agarose gel-electrophoresis and visualized under UV light exposure by using Gel-Doc XR system (Bio-Rad, USA).

3.1.2 Gel extraction of PCR product

The PCR product was extracted from agarose gel by using QIAquick Gel Extraction kit (Qiagen, Italy). According to the manufacturer protocol, excised agarose gel containing DNA fragment was incubated at 50°C for 10 mins in buffer QG (300 µl per 100 mg of gel) of and dissolved the gel. Once the gel was completely dissolved, 1 volume of isopropanol was added. Subsequently, the mixture was transferred to QIAquick column and centrifuged at 13000 rpm for 1 minute. Next, the column was washed with 750µl of buffer PE. Finally, the DNA was eluted with DNase free water into a clean microcentrifuge tube and quantified by Nanodrop® ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, USA) prior to storage at -20°C.

3.1.3 TA cloning

Cloning of gel purified Der p 23 amplicon was performed by using pGEM®-T Easy vector system (Promega, USA). The cloning was performed with an insert to vector ligation ratio of 1:3 in 10µl ligation reaction; consists of 1X rapid ligation buffer, 50 ng of pGEM-T Easy vector, 12 ng of purified Der p 23 DNA insert and 3 U of T4 ligase. The reaction was performed overnight at 16°C.

3.1.4 *E. coli* chemically competent cell preparation

E. coli DH5α competent cell was prepared chemically according to Inoue and colleagues protocol (51). Approximately 10-12 colonies (~2 mm in diameter) were inoculated into 250 ml of SOB medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$) and incubated at 18°C with shaking (200 rpm). Once the culture reaches an optical density of 0.6 at 600 nm, the cells were pre-chilled on ice for 10 mins and pelleted at 3000 rpm for 10 mins at 4°C. Next, the cell pellet was washed with ice-cold transformation buffer (TB: 10 mM Pipes, 15 mM $CaCl_2$, 250 mM KCl, 55 mM $MnCl_2$, pH 6.7) and resuspended in 20 ml of TB containing 7% final concentration of DMSO. The cell suspension was aliquot (150 µl per tube containing approximately $1x10^9$ cells) and frozen immediately under liquid nitrogen and subsequently stored at -80°C.

3.1.4 Bacteria transformation by heat shock

The frozen bacteria competent cells (one tube approximately $1x10^9$ cells) were thawed on ice. Immediately, 10 µl of the overnight ligation was pipetted in and swirled gently while dispensing. Tapped the tube gently to provide mixing. Subsequently, the cells were incubated on ice for 30 mins. Heat shock was carried out at 42°C for 40 seconds and followed by incubation on ice for 2 mins. Next, 0.8 ml of SOC medium were added and incubated at 37°C with 200 rpm agitation for 50 mins. The cells were then pelleted at 13,000 rpm for 5 mins. Supernatant of 500 µl was discarded and the cell pellet was suspended with remaining broth. Then, 200 µl

of cell suspension was spread on LB agar plates containing 100 µg/ml of ampicillin and incubated at 37°C for overnight (16hrs).

3.1.5 Colony PCR

Colony PCR was performed to screen positive clones with Der p 23 specific primers. The overnight grown colonies were picked with sterile toothpicks and swirled into PCR reaction mix. The PCR composition and profile used were the same as described in section 3.1.1 by replacing the DNA template with bacteria colony. The PCR products were then analyzed on a 1.5% agarose gel electrophoresis.

3.1.6 Plasmid extraction

Plasmid pGEMT-Der p 23 extractions were performed using the High-Speed Plasmid Mini Kit (Geneaid, Taiwan) and according to the manufacturer protocol. Briefly, cell pellet of 3 ml bacteria overnight culture was resuspended with 200 µl of PD1 buffer followed by 200 µl of PD2 buffer. Immediately, the tube was gently mixed by inverting for 10 times and incubated at room temperature for at least 2 mins. Next, 300 µl of PD3 buffer was added and mixed gently as before. The mixture was then centrifuged at 10,000 x g for 3 mins. Supernatant containing plasmid was carefully transferred into PD column to trap the plasmid by centrifugation as before for 30 seconds. The column was washed with 600 µl of wash buffer and dried by additional centrifugation for 3 mins. The trapped plasmids were then eluted with 50 µl of DNase free water. Lastly, the purified plasmid was visualized by agarose gel electrophoresis and quantified prior to storage at -20°C. Extracted plasmid was sent for DNA sequencing analysis (U2Bio, Thailand).

3.2 Cloning of Der p 23 into *P.pastoris* KM71

3.2.1 DNA double restriction digestion

pGEMT-Der p 23 purified plasmid (8µg) and pPICZαA expression vector (4 µg) (Invitrogen, USA) were subjected to double restriction enzymes digestion separately in a 50 µl reaction mixture; consist of 35µl of sterile deionized water, 5 µl of 10X restriction enzyme buffer, DNA plasmid, and 20 U of *Xho*I and *Not*I restriction enzymes (NEB, USA) each. The tubes were then incubated at 37°C for 150 mins. Digested products were checked by running 1.5% agarose gel electrophoresis. Der p 23 fragment and linearized pPICZαA vector were gel purified (refer to 3.1.2).

3.2.2 DNA ligation

Gel purified restricted Der p 23 gene was ligated into the expression vector, pPICZαA to generate the pPICZαA-Der p 23 plasmids. DNA ligation was carried out using LigaFast rapid DNA ligation system (Promega, USA) and the ligation steps were performed according to the manufacturer's instructions. The DNA ligation was performed in a 20 µl reaction mixture; consists of 10 µl of 2X rapid ligation buffer, and 6 weiss Units of T4 DNA ligase. Vector to insert molar ratio of 1:3 was used. Ligation was carried out at 16°C for overnight. The ligation was then transformed into DH5α (refer to 3.1.4) with the exception that LB agar containing Zeocin (50 µg/ml) was used. Desired DNA construct selected from colony PCR (refer to section 3.1.5) was sent for DNA sequencing (U2Bio, Thailand).

3.2.3 *P.pastoris,* KM71 strain transformation by electroporation

Purified pPICZαA-Der p 23 plasmids were linearized with *Bgl*II restriction enzyme (NEB, USA) prior to transformation into *P. pastoris* KM71 strain by using Gene PulserXcell™ (Bio-Rad, USA) at 1500 V, 200 Ω in 1 mm electrode cuvette. The transformants were spread on YPDS agar plate (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar) containing 100 µg/mL Zeocin (Invitrogen, USA) and

incubated at 30°C for 2-3 days. Colony PCR (refer to section 3.1.5) was then performed to select the positive clones.

3.3 Expression and Purification of rDer p 23

Positive Colony PCR clone was selected to inoculate an overnight culture in 250 ml BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6, 1.34% yeast nitrogen base, $4x10^{-5}$ % biotin, 1% glycerol) broth at 30°C under shaking condition (200 rpm) for 16 hours. Next day, the culture was pelleted (10 000 rpm, 5 mins) and adjusted to final OD at 600 nm of 5 in BMMY (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6, 1.34% yeast nitrogen base, $4x10^{-5}$ % biotin, 2% methanol) to induce the protein expression at 30°C under shaking condition (200 rpm). Methanol was added every 24 hours to maintain the concentration.

For purification, the culture supernatant was collected by centrifugation (10,000 rpm, 10 mins) and directly diluted 10 times with MiliQ water prior to pH adjustment to pH 3 with HCl. The medium was subsequently, applied onto S Sepharose XL column (5 ml resin) (GE Healthcare Lifesciences, UK) equilibrated with 50mM glycine-HCl pH 3.0 at a flow rate of 20 ml/min using AKTAprime purification system (GE Healthcare Lifesciences, UK). After, the column was washed intensively with equilibrating buffer at the same flow rate until the UV monitor value drop to baseline. Elution was then carried out in a stepwise increase of sodium chloride concentration (0.1-1 M; flow rate, 20 ml/min). The eluted fractions were immediately neutralized with 1 M Tris-HCl, pH 9. Eluted fraction containing rDer p 23 (200 mM NaCl) was concentrated by ultrafiltration with Omega™ 3 kDa cut-off membrane (Pall, USA) followed by gel filtration onto Superdex 75 HR column 10/30 (GE Healthcare Lifesciences, UK) equilibrated with PBS pH 7.3 at a flow rate of 0.5 ml/min. Fractions were then analyzed by SDS-PAGE and coomassie blue staining to evaluate the purity. The fractions containing rDer p 23 were pooled and filtered through 0.22 µm syringe

filter (Millipore, USA). The protein concentration was determined using BCA protein assay kit (Pierce, USA) based on BSA as standard. Subsequently, the protein was aliquoted and stored at -20°C for further analysis.

3.4 Polyclonal anti-rDer p 23 Antibodies Production

Six weeks BALB/c female mice (n=3) were immunized with rDer p 23 formulated with adjuvant (aluminium hydroxide) to raise polyclonal antibodies. Mice were obtained from National Laboratory Animal Center, Mahidol University, Nakornpathom, Thailand. All procedures were carried out according to the regulations of Chulalongkorn University Animal Care and Use Committee (IRB No. 356/2557). Briefly, purified rDer p 23 was incubated with aluminium hydroxide (Sigma, USA) in a protein to adjuvant ratio of 1:100 at room temperature under shaking condition for 30 mins. Next, the mixture was then injected into the peritoneal cavity of the anesthetic mice (10 µg protein each mouse). Three injections were given with every 2 weeks interval and blood was collected 2 weeks post 3rd injection through facial vein. Serum was collected after centrifugation at 300 x g. Pre-immune serum was also collected before the immunization begun as control. Antibodies titers were then evaluated by direct ELISA. All sera were aliquoted and stored at -20°C.

3.4.1 Detection of Der p 23 specific polyclonal antibodies by ELISA

Nunc MaxiSorp® ELISA microplates (Affymetrixe Bioscience, USA) were coated with 500 ng/well of rDer p 23 at 4°C for overnight in 100µl/well of coating buffer (0.1M sodium carbonate, pH 9.5). The plates were then washed with 250 µl/well of PBS-Tween 20, 0.05% (PBS-T) for 5 times using Wellwash™ microplate washer (Thermo Scientific, USA) and blocked with 150 µl/well of PBS-T containing 1% BSA (PBS-T-BSA) for 1 hour at 37°C. Series dilutions of pre-immune and immunized sera were then diluted in PBS-T-BSA and incubated at 37°C for 1 hour (100 µl/well). The plates were then washed again with PBS-T and further incubated with 1/5000 dilution (100 µl/well) of goat anti-mouse antibodies conjugated with horseradish peroxidase

(KPL, USA) at 37°C for 1 hour. Next, the allergen-antibodies complexes were detected with BD OptEIA™ TMB substrate (BD Biosciences, USA), 100µl/well and the reaction was stopped with 50 µl/well of 0.5 M sulfuric acid. Lastly, the wells OD were measured at 450 nm using iMark microplate reader (Bio-Rad, USA).

3.5 Detection of Natural Der p 23 in HDM Faeces Aqueous Extracts

3.5.1 Mite feces extraction

Enriched *D. pteronyssinus* faeces, 36 mg (kindly provided by Dr. Emmanuel Nony, Stallergenes, France) were extracted in 0.2 ml PBS at 4°C for 16 hours under rotating condition. Soluble extract was collected by centrifugation at 13,000 rpm, 5 mins. Natural Der p 23 was then detected into the fecal extracts by Chemiluminescence Immunoblot.

3.5.2 Chemiluminescence Immunoblot

Following 15% SDS-PAGE (1.8 ml of deionized water, 1.25 ml of 1.5 M Tris-HCl, pH 8.8, 0.05 ml of 10% SDS, 1.9 ml of 40% Acrylamide/Bis solution, 0.05 ml of 10% ammonium persulfate, and 10 µl of TEMED) and protein transfer onto nitrocellulose membrane by using Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad, USA). The membrane was then blocked with PBS, 1% (w/v) BSA, 0.05% (v/v) tween 20, at 4°C, for overnight. Next, the membrane was incubated with mouse anti-rDerp23 polyclonal serum (the serum was produced through animal immunization with purified rDer p 23 formulated with Alum adjuvant) at 1/2000 dilution. As control, another set of membrane with the same samples transferred was also incubated with pre-immune serum (collected from the mouse pre-immunized with purified rDer p 23). The membranes were then wash with PBS-Tween 20, 0.05% for 30 mins and followed by the incubation with 1:5000 goat-anti-mouse antibodies conjugated with horseradish peroxidase (KPL, USA). The immunoreactive bands were detected using Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, USA) and following X-ray film exposition (Kodak, USA).

3.6 Alpha Mannosidase Treatment

Purified rDer p 23 was digested with Jack bean α-mannosidase (Sigma-Aldrich, USA) at a 1:20 enzyme/substrate ratio in a pH 4 buffer at 37°C for 16h. The solution was neutralized with 1 M Tris-HCl pH 9 and ultrafiltrated through 30kDa cut-off membrane filter (GE Healthcares, U.K.) to remove mannosidase. The flow through containing de-glycosylated rDer p 23 was subsequently concentrated through 3kDa cut-off membrane (GE Healthcares, U.K.). The filtrate was collected and protein concentration was determined using BCA protein assay kit (Pierce, USA).

3.7 Mass Spectrometry Analyses

3.7.1 NanoESI-MS analysis (direct infusion)

This analysis was performed in collaboration with Dr. Emmanuel Nony of Stallergenes, France. Prior the nanoESI-MS analysis, 20 µg of rDer p 23 was desalted with a C4 ZipTip (Merck Millipore) and was eluted in 50µl of 50% acetonitrile / 0.1% formic acid. Next, the sample was infused at 1 µl/min into an Impact HD mass spectrometer equipped with a Captive Spray source (Bruker Daltonics). Acquisitions were performed in positive mode with end plate offset and capillary voltages set at -500 and 1200 V, respectively. The dry gaz and the dry heater were set at 4.0 L/min and 180°C, respectively. MS spectra were acquired over the m/z range 50-3000 with a scan rate of 1 Hz. MS calibration was performed using the internal lockmass at m/z 1221.9906 (Agilent). Charge state deconvolution was performed using the Maximum Entropy algorithm.

3.7.2 NanoLC-tandem MS (MS/MS)

This analysis was performed in collaboration with Dr. Emmanuel Nony of Stallergenes, France. Collision-induced dissociation experiments were performed over the same m/z window on the most intense precursor ions. nanoLC-tandem MS (MS/MS) analyses were processed with the Compass Data Analysis software (v. 4.02,

Bruker). Mass calibration was performed using the Lock Mass Calibration mode, based on internal calibrants (i.e. 299.2945 and 1221.9906 m/z). MS data were analysed with the PEAKS software v 7 (BSi, Waterloo, Canada). Protein identification was performed using a custom database containing Der p 23 sequence. The mass tolerance in MS and MS/MS were set to 5 ppm and 0.03 Da, respectively.

3.8 Circular Dichroism

This analysis was performed in collaboration with Assoc. Prof. Surapon Piboonpocanum of Institute of Molecular Biosciences, Mahidol University, Thailand. Purified protein was first exchanged in 10 mM NaH_2PO_4 buffer and measured with Jasco J-815 CD spectrometer in 0.1 cm path length quartz cuvette. CD spectra were recorded with 1 nm resolution at speed 50nm/min for 5 cycles. Data were then analyzed with *CDpro*software.

3.9 HDM Allergic Patients' Sera IgE Reactivity to rDer p 23

Sera used were obtained from a collaborative project. The sera consists of 64 sera from healthy individuals and 222 sera from House dust mite allergic patients were collected from four different Hospitals namely, Chulalongkorn Hospital, Phramongkutklao Hospital, Ramathibodi Hospital, and Children Hospital, under the approval of Chulalongkorn Institute Review Board (IRB No 023/55). The sera were aliquoted and stored at -80°C. All sera were subjected to ImmunoCAP™ (Thermo scientific, Sweden) analysis against *D.pteronyssinus.* All the positive sera have an ImmunoCAP specific IgE to *D.pteronysinnus*value over 0.35 kUA/L.

3.10 Rat Basophil Leukemia Assay

Basophil degranulation assay was performed using genetically modified rat basophil leukemia cell line (RBL-SX38) expressing human FcεR1 receptor (52). The cells are maintained and cultured in RPMI1640 supplemented with 10% fetal bovine

serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin at 37°C, 5% CO_2 (Gibco, USA). Cells of $1.5x10^5$/well were seeded in 96-well culture plate and incubated at culture conditions for 3 hours. Cells were then primed for 16 hours with human sera tested positive IgE against rDer p 23 at 1/10 dilution. Cells were then washed with PBS and incubated with RPMI1640 phenol free (Gibco, USA) containing 1 mg/ml BSA for 15 mins before addition of serial rDer p 23 dilutions (0.00001µg/ml-1ug/ml) for 30 mins. Final concentration of 5% (v/v) Triton X-100 was used as maximum released of β-hexosaminidase. The release of β-hexosaminidase was assayed by adding 50 µl of culture supernatant into pre-warm 50 µl of 2.5 mM p-nitrophenyl N-acetyl-β-D-glucosaminide and incubated for 3 hours. Finally, the reaction was stop with 150 µl of 1M Tris-HCl, pH 9 and subsequently read the absorbance at 415 nm by using iMark microplate absorbance reader (Bio-Rad, USA).

3.11 Chitin-binding Assay

Two chitin sources from New England Biolabs (chitin beads) and Sigma (shrimp chitin) were used in this affinity binding assay. The insoluble chitin (50 µl of chitin beads suspension/1 mg of shrimp chitin) was equilibrated in PBS prior to incubation with a protein concentration of 200 µg/ml rDer p 23 or rDer p 5 (selected as a negative control because it contains no chitin binding domain and produced in the same expression system) or wheat germ agglutinin (as a positive control) (Vector Laboratories, USA) or HDM fecal extracts (extracted from 200 mg/ml of fecal pellets) in a volume of 100 µl at room temperature for 60 mins with orbital shaking. Supernatants were collected by centrifugation at 13,000 rpm for 5 mins and the chitin pellets were washed 5 times with PBS. Proteins bound to chitins were then eluted by incubating the chitin matrix in Laemmli buffer at 95°C for 5 mins. Both supernatants and eluted fractions were analysed on 15% SDS-PAGE and coomassie blue staining except HDM fecal extracts were detected with specific rDer p 23 antibodies by immunoblot (refer to 3.5.2).

3.12 Human Bronchial Epithelial Cell Activation Assay

Human bronchial epithelial BEAS-2B cells (ATCC, CRL-9609) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco, USA). Amount of $2x10^5$ cells per well was seeded into 24-well plate and cultured at 37°C, 5% CO_2 for 24 hours. Next day, the cells were then washed with PBS and replaced with serum-free DMEM for another 24 hours. Prior to cell stimulation, the growth medium was replaced with fresh serum-free DMEM and subsequently cells were stimulated with different concentration of rDer p 23 (1-20 µg/ml) for 24 hours at culture conditions. For inhibitory assay, cells were pre-treated with inhibitors (MEK1/2 inhibitor UO126, 20 µM; p38/RK inhibitor SB203580, 1 µM; JNK inhibitor SP600125, 20 µM; IκB inhibitor BAY11-7082, 10 µM) (Invivogen, USA) for 60 mins before exposed to rDer p 23. Poly IC (2 µg/ml) was used as stimulation control. Cell-free supernatants were collected for IL-8 and GM-CSF cytokine measurement with enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, USA) according to the manufacturer's protocol.

3.13 Statistical analysis

Student's t-test was used to compare significant different between treatments in cell stimulation assays. P-values of <0.05 was considered significantly different. Prism 5.0 (GraphPad Software Inc., USA) was used to calculate the significant values.

CHAPTER IV
RESULTS

4.1 Cloning of Der p 23

Using two specific primers derived from the sequence EU414751.1 [GenBank accession number, (9)] a band of approximately 250 bps was successfully amplified from a total *D.pteronyssinus* cDNA preparation (Fig. 4.1A). The amplicon comprised a DNA segment coding for the C-terminus of the yeast α-mating factor leader sequence together with the cDNA encoding the Der p 23 mature form (aa 22-90, 210 bps) (246 bps). The sequence of the PCR product perfectly matched with the mature Der p 23 cDNA sequence from GenBank database (Fig. 4.1B). The PCR product was subsequently cloned into the *P. pastoris* pPICZαA expression vector in order to restore the DNA sequence encoding the yeast α-mating factor leader peptide. This cloning strategy was designed to drive the secretion of authentic mature Der p 23 without any addition of extra N-terminus amino acids. DNA sequencing of the clone confirmed the absence of any codon frame shift in the construction (Fig. 4.1C).

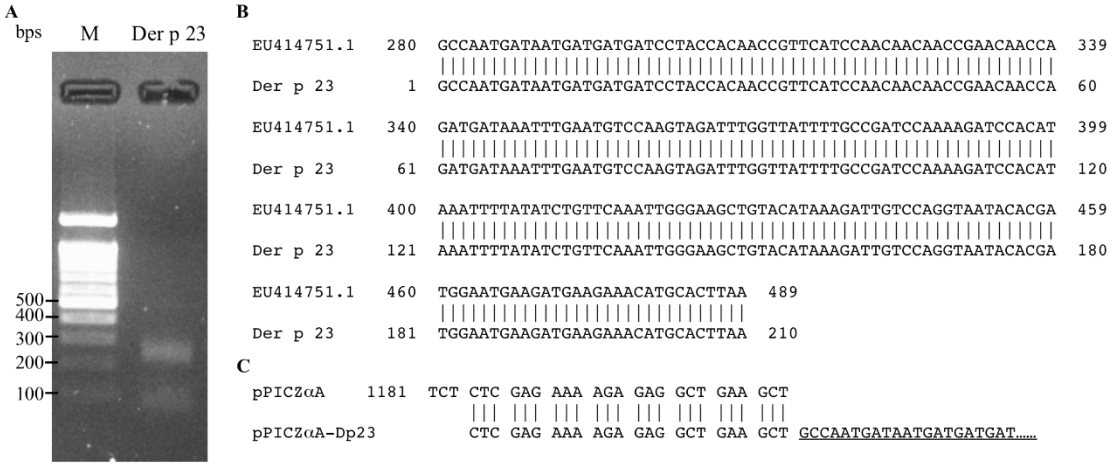

Figure 4. 1 Cloning of Der p 23. A) PCR amplification of cDNA encoding mature Der p 23. B) Pairwise alignment of amplified Der p 23 DNA sequence and theEU414751.1 sequence from GenBank database. C) Partial DNA sequence alignment of segment encoding alpha mating factor C-terminal sequence in empty pPICZαA and pPICZαA-Der p 23 plasmids. Underlined sequence represents the 5' end of mature Der p 23 cDNA sequence.

4.2 Expression and Purification of rDer p 23

Following the transformation of the *P.pastoris* KM71 strain with the recombinant linearized plasmid, zeocin-resistant colonies were screened for the presence of Der p 23 cDNA by colony PCR. PCR-positive colonies were cultured in shake flasks at 30℃ and rDer p 23 expression was triggered by the addition of methanol in the culture medium at a final concentration of 2%. The methanol induction was shown to lead to the secretion of a protein which migrated as a 15 kDa band on SDS-PAGE (Fig 4.2). The protein expression was shown to reach a maximum after 48 hours of induction. For a longer expression period, degradation bands migrating at about 10 kDa were clearly observed (Fig. 4.2A).

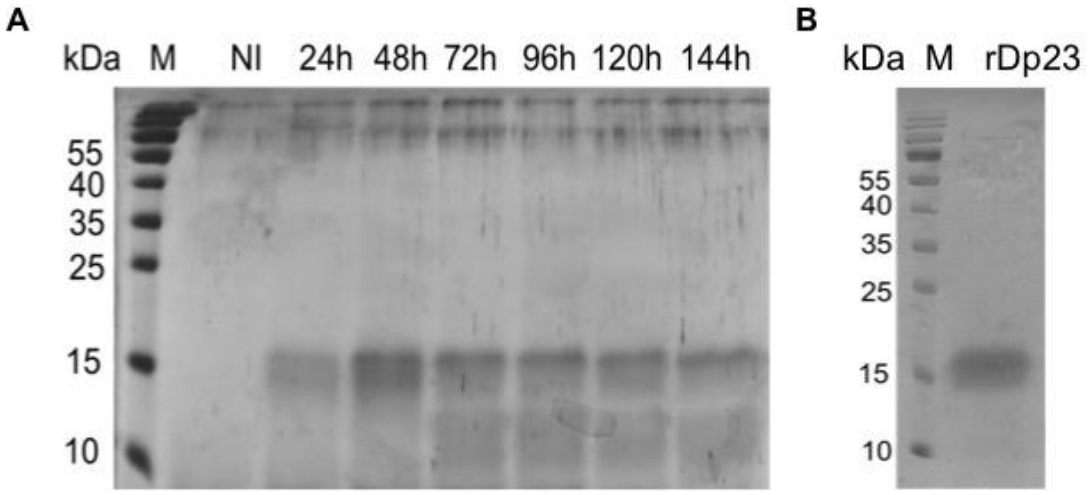

Figure 4. 2 Expression and purification of rDer p 23 from transformed KM71 *P.pastoris.* A) SDS-PAGE analysis of the secretion of expressed rDer p 23 following 2% methanol induction for 24, 48, 72, 96, 120, and 144 hours. NI: non-induced. B) SDS-PAGE profile of purified rDer p 23. Protein detection by coomassie blue staining.

MALDI-TOF MS analyses of tryptic digestion from the SDS-PAGE bands confirmed that both the 15 kDa and 10 kDa bands are indeed rDer p 23 (Fig 4.3). All together the identified peptides covered Der p 23 amino acid sequence from residue 36 to 81 (65% coverage). To analyze rDer p 23 in solution, purified rDer p 23 of 72 hours induction (Fig. 4.4A) was subjected to nanoLC-MS analysis. The liquid chromatography revealed there were two elution peaks and the MS analysis of respective peak showed that peak 1 (associated to 15 kDa band) corresponded to the intact mature rDer p 23 associated to hexose residues (ranging from 8-16 residues) with a mass ranging from 9-10.5 kDa whereas peak 2 (corresponding to 10 kDa band) was two truncated forms of rDer p 23 (form A, 6.4 kDa; form B, 6.2 kDa) which were not associated to hexose (Fig. 4.4B). It must be pointed out that the migration of mature Der p 23 on SDS-PAGE did not correlate with the mature Der p 23 theoretical molecular weight (8 kDa) even with the highest number of hexoses associated (10.5 kDa). Nevertheless, this finding is in agreement with the same SDS-

PAGE gel shift of rDer p 23 produced in *E.coli*. It must also be pointed out that rDer p 23 expressed in *E.coli* is not glycosylated (9).

For the purification of intact mature rDer p 23, large scale recombinant *P.pastoris* cultures were induced for 48h, and the supernatants were used to purify rDer p 23 to homogeneity by a combination of cation exchanger and size exclusion chromatographies (Fig. 4.2B). The SDS-PAGE profile of purified rDer p 23, did not show the presence of both monomeric and dimeric forms as previously reported when rDer p 23 is produced in bacteria (9).

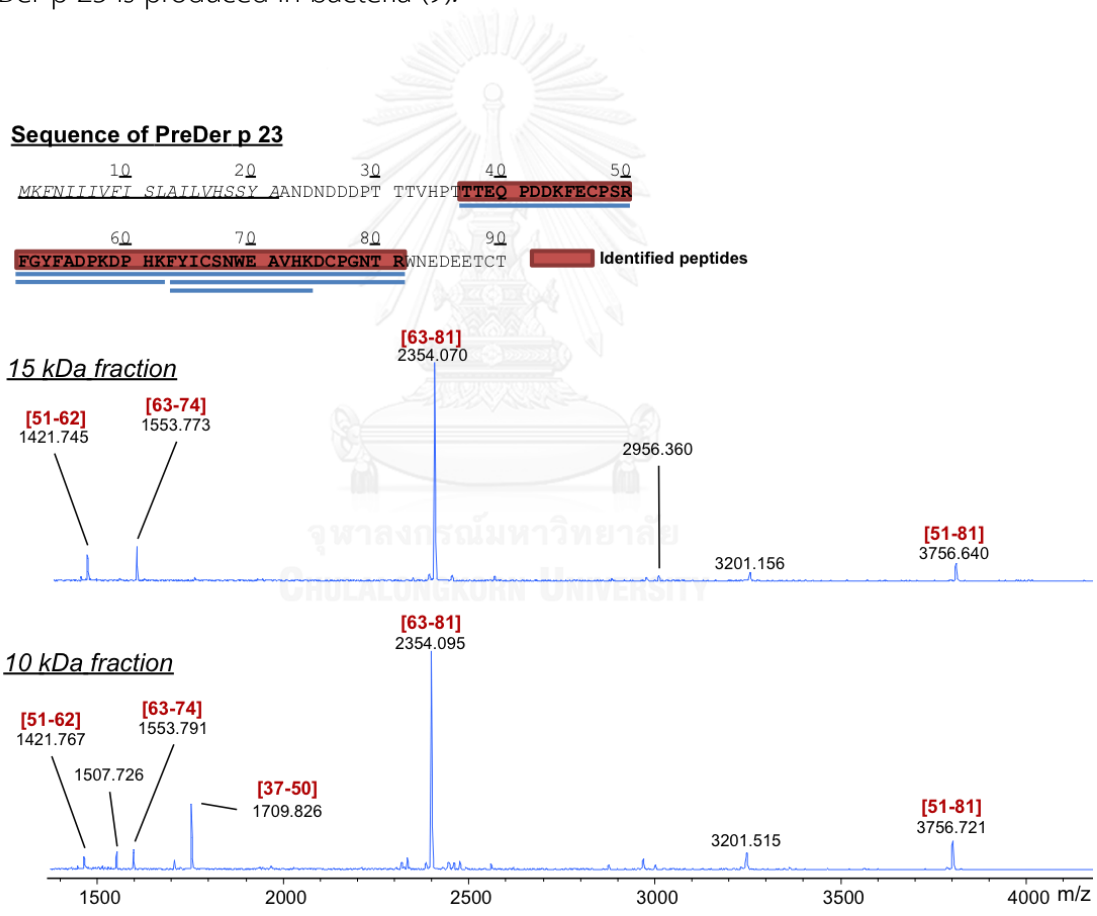

Figure 4. 3 MALDI-TOF MS analysis of trypsin digested rDer p 23 isolated from SDS-PAGE. Underlined sequences are the natural leader sequence (black line) of Der p 23 and the peptides (blue lines) identified. The overall identified peptides of Der p 23 were highlighted in red.

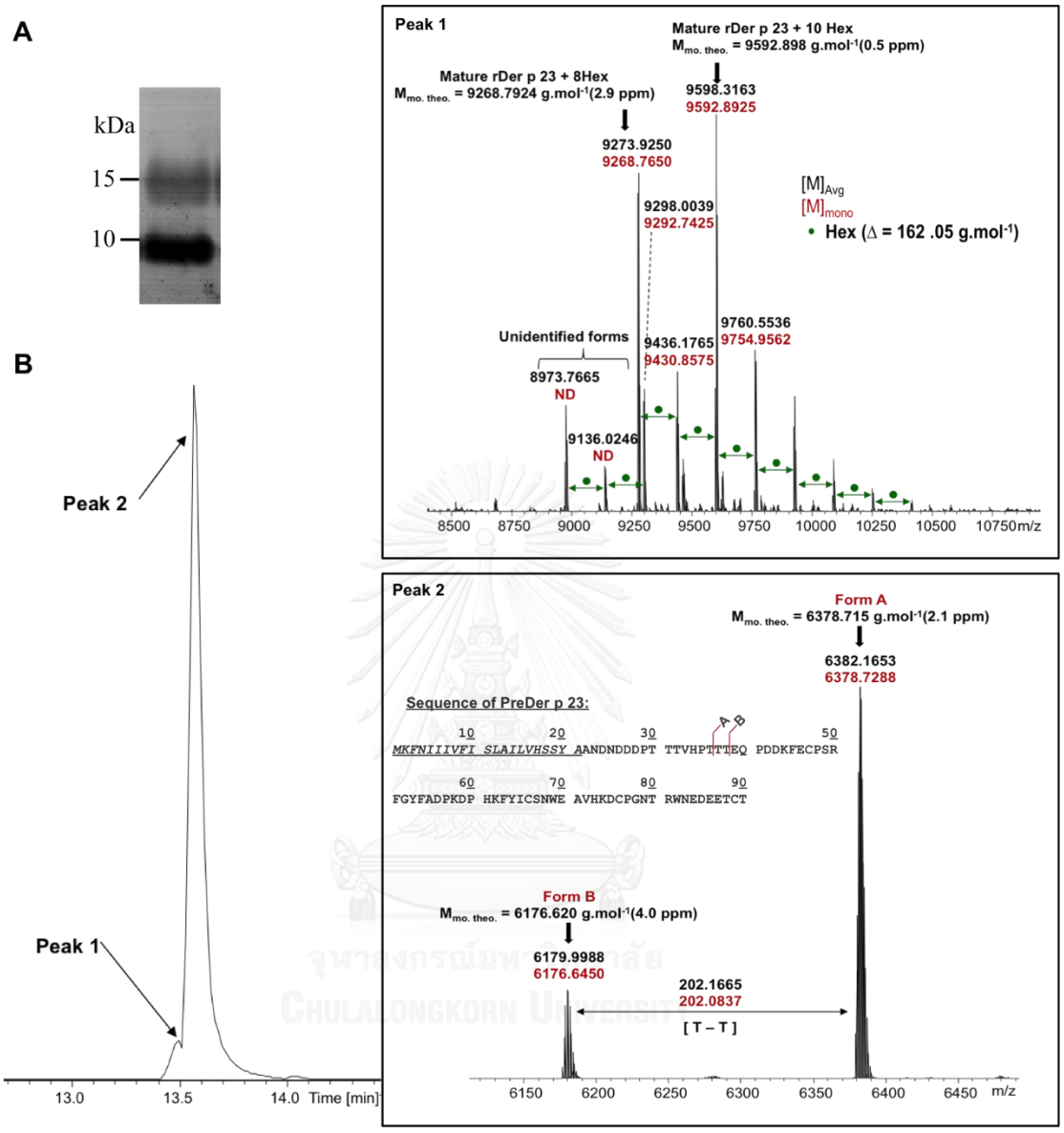

Figure 4. 4 nanoLC-MS analysis of rDer p 23 purified fractions (72 hours induction). A) SDS-PAGE profile of rDer p 23 purified fraction at 72 hours induction. B) Der p 23 subjected to liquid chromatography and MS analysis of respective peak. Peak 1 is the mature form of rDer p 23 associated with hexose (green dot) ranging from 8-16 units whereas peak 2 corresponds to the truncated forms of rDer p 23 (Form A and B) without hexose modification.

4.3 Der p 23 Produced in *P. pastoris* is a O-glycoprotein

Mass spectrometry analysis of purified rDer p 23 (48 hours of induction) preparation demonstrated not only the absence of any detectable host cell (*P.pastoris*) protein contamination but also the presence of N-terminal sequence truncations (Fig. 4.5). Together with a large percentage of intact full-length mature rDer p 23 (aa 22-90, 77%), four truncated forms of rDer p 23 were detected as form A (aa 37-90, 10%), B (aa 39-90, 3%), C (aa 33-90, 5%), and D (aa 36-90, 4%). Form A and B were corresponded to the form A and B identified in previous MS analysis of rDer p 23 (72 hours of induction). It is noteworthy that only the intact form of rDer p 23 was associated with hexose glycans. The amino acid sequence analysis of Der p 23 revealed that there are two stretches of Thr residues (T^{30}-T^{32} and T^{36}-T^{38}) which could be O-glycosylated. MS analysis showed that the truncated forms C and D, containing the T^{36}-T^{38} sequence, were not associated with hexose residues, both the non-hexose associated forms suggesting that the T^{30}-T^{32} site represents the unique O-glycosylation site of rDer p 23 (Fig. 4.5A). This is also further supported by the data that all the peptides identified previously in figure 4.3 were not associated to hexose.

As glycoproteins produced in yeast are commonly O-glycosylated through mannose residues, we treated rDer p 23 with Jack bean α-mannosidase which cleaves α-D-mannose residues. As shown in figure 4.5B, the molecular weight of rDer p 23 treated with the α-mannosidase was reduced as compared to the untreated allergen, confirming that the hexose structures represented O-mannosylation. Altogether, our data indicated that rDer p 23 produced in *P.pastoris* is O-mannosylated at the level of Thr residues 30-32.

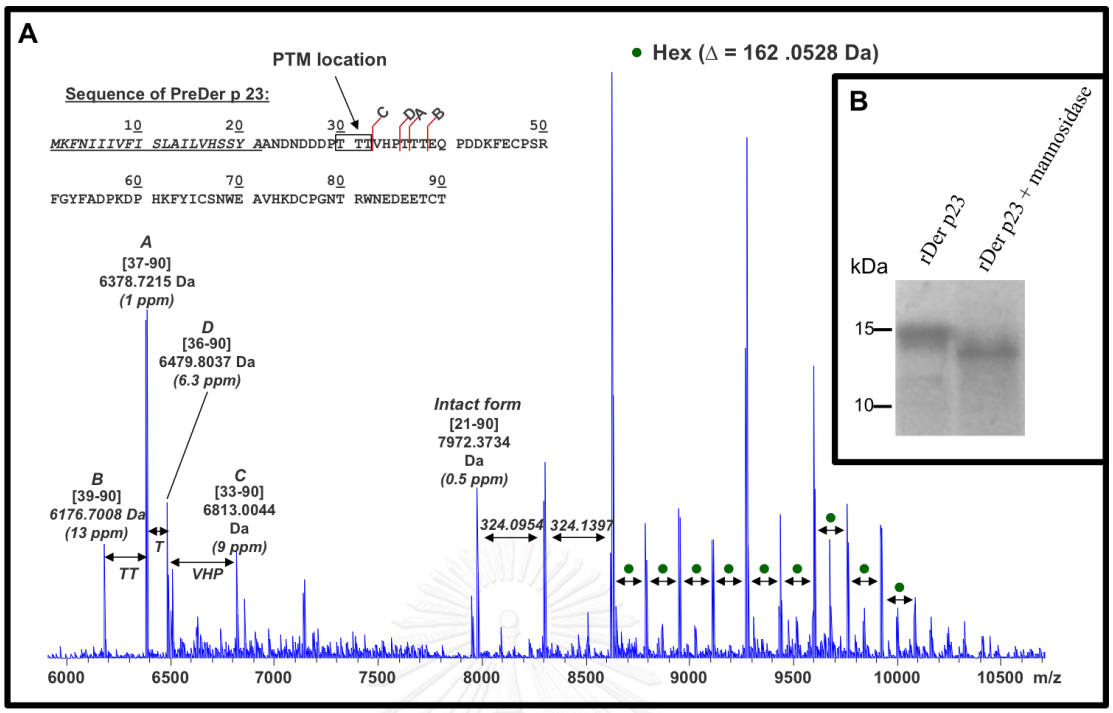

Figure 4. 5 nanoESI-MS analysis and de-glycosylation of rDer p 23. A) Deconvoluated mass spectrum of purified rDer p 23. Full-length amino acid sequence of Der p 23 was shown on the top left corner. The first 21 amino acids (underlined) correspond to the signal peptide whereas the mature full length Der p 23 displays the 22-90 amino acid sequence. Together with an intact form, four truncated rDer p 23 molecules labeled as A (aa 37-90, 10%), B (aa 39-90, 3%), C (aa 33-90, 5%), and D (aa 36-90, 4%) were detected. Hexose residues were represented with green dots. B) Purified rDer p 23 was treated with α-mannosidase overnight at 37°C and analyzed by SDS-PAGE. As control, purified rDer p 23 was also incubated under the same conditions but in the absence of the glycosidase.

4.4 Circular Dichroism (CD) Analysis of Purified rDer p 23

Far-UV CD analyses showed that rDer p 23 is mainly unfolded, with a respective secondary structure composition of 6% helix, 11% beta, 22% turn, and 61% unfolded (Fig. 4.6A). MS results also revealed that rDer p 23 has two intra-disulfide bonds. Changes in the Near-UV CD spectra of rDer p 23 under reducing

conditions (1 or 2mM DTT) confirmed the presence of disulfides (Fig. 4.6B). CD analysis performed from 25°C to 100°C suggested that heating rDer p 23 up to 100°C did not induce more protein unfolding. By contrast, even an increase in β-sheet and α-helix content were observed suggesting that heating induced some conformational changes.

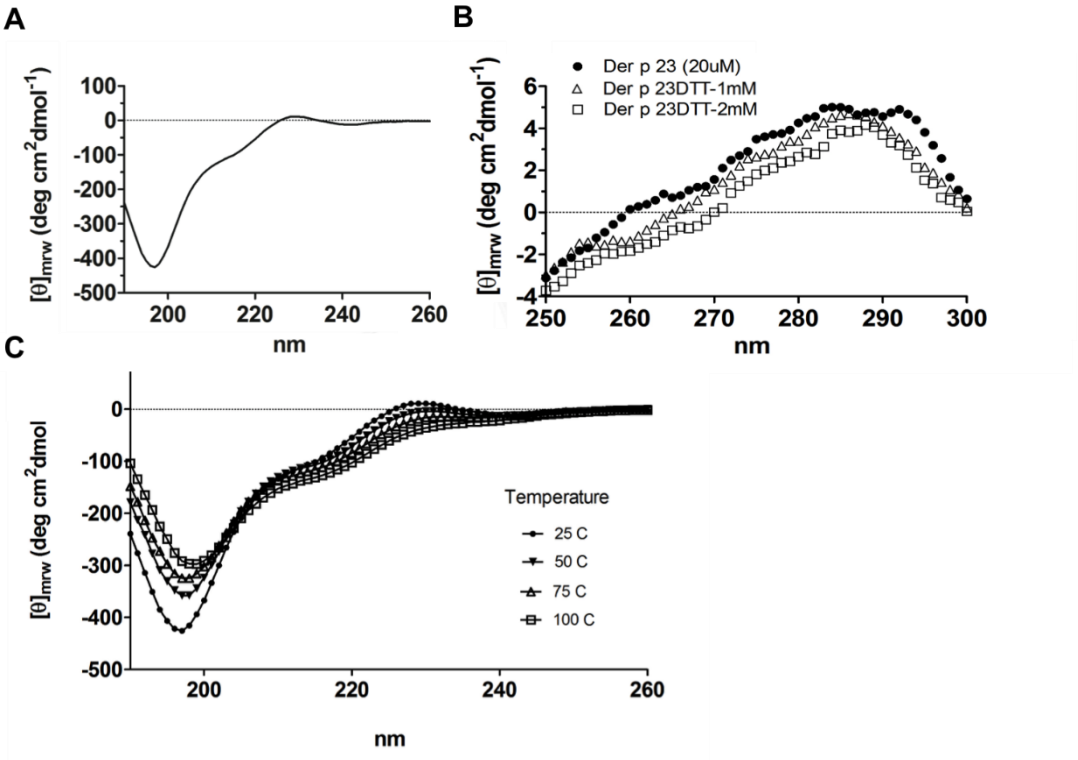

Figure 4. 6 UV-Circular dichroism analysis of purified rDer p 23. A) CD spectra of purified rDer p 23 at 25°C. B) rDer p 23 was treated 1 and 2 mM of DTT. C) CD spectrum of rDer p 23 from 25°C to 100°C. All the analyses were done using J-815 CD spectrophotometer.

Table 4. 1 Secondary structure compositions of rDer p 23 at various temperatures

Temperature, °C	**α**-helix, %	**β**-sheet, %	Turn, %	Disorder, %
25	6	11	22	61
50	5	16	21	58
75	5	16	26	53
100	10	19	19	52

4.5 rDer p 23 Protein Stability

From the protein expression profile to MS and subsequent CD analyses, rDer p 23 displayed largely disorder structure and truncated forms were observed. Consequently, we evaluated the rDer p 23 stability profile at various temperatures in order to optimize the protein storage conditions. Based on SDS-PAGE profile in figure 4.7, we can consider that rDer p 23 integrity is maintained when stored in PBS at temperature -80°C, -20°C (up to 49 days), and 4°C (up to 24 days). Furthermore, we also evaluate the stability of rDer p 23 at higher temperature, 37°C. At such temperature, weak degradation band can be clearly observed at day 7 to 14.

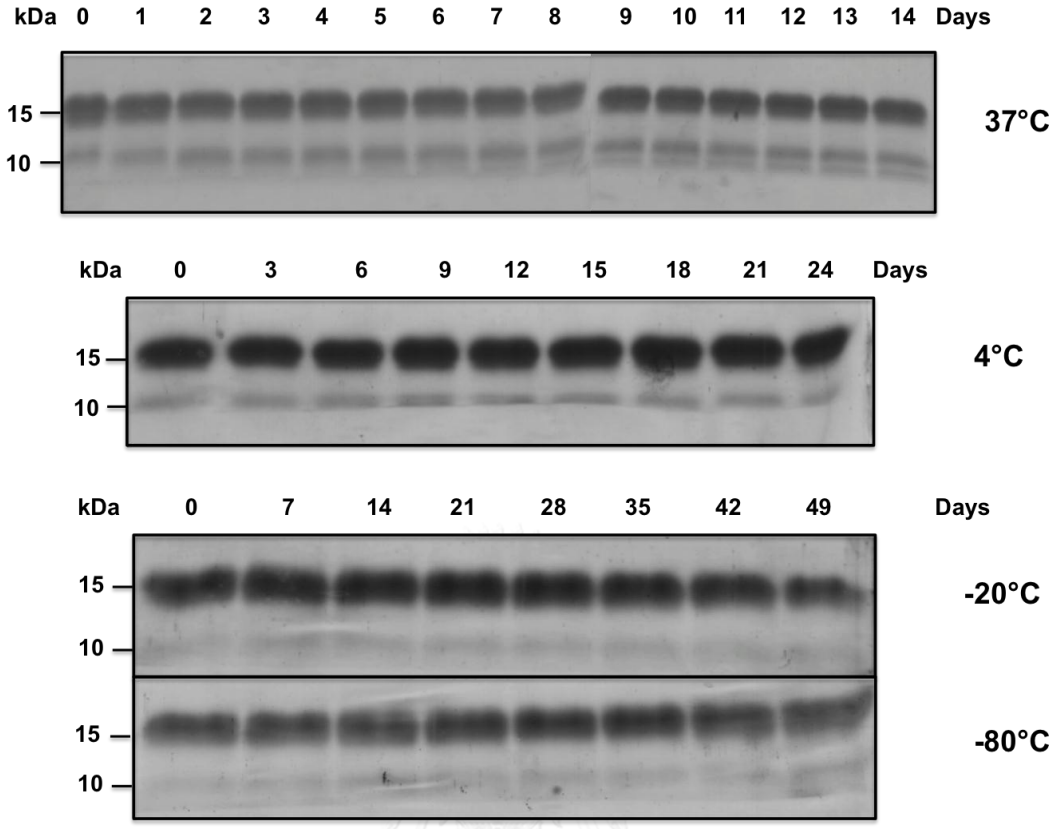

Figure 4. 7 SDS-PAGE profile of rDer p 23 at -80°C, -20°C, 4°C, and 37°C across various time points. Numbers indicated days of incubation rDer p 23 (5 µg/lane) were incubated at indicated temperature for indicated number of days and analyzed by 15% SDS-PAGE and coomassie blue staining.

4.6 Generation of rDer p 23 Polyclonal Antibodies

We generate rDer p 23 polyclonal antibodies in mice as a tool for future experiments and also for the detection of nDer p 23 in the HDM extracts. Figure 4.8 shows the specificity of the mouse polyclonal antibodies generated against rDer p 23. The antibodies raised has a titer of 100,000 and most importantly, it is specific to rDer p 23 as the detection of others recombinant allergens (rDer p 5 and 7) produced using the same expression system as rDer p 23 generated a titer of 100. Moreover, the absence of rDer p 23 detection with the pre-immune control serum was also evidenced.

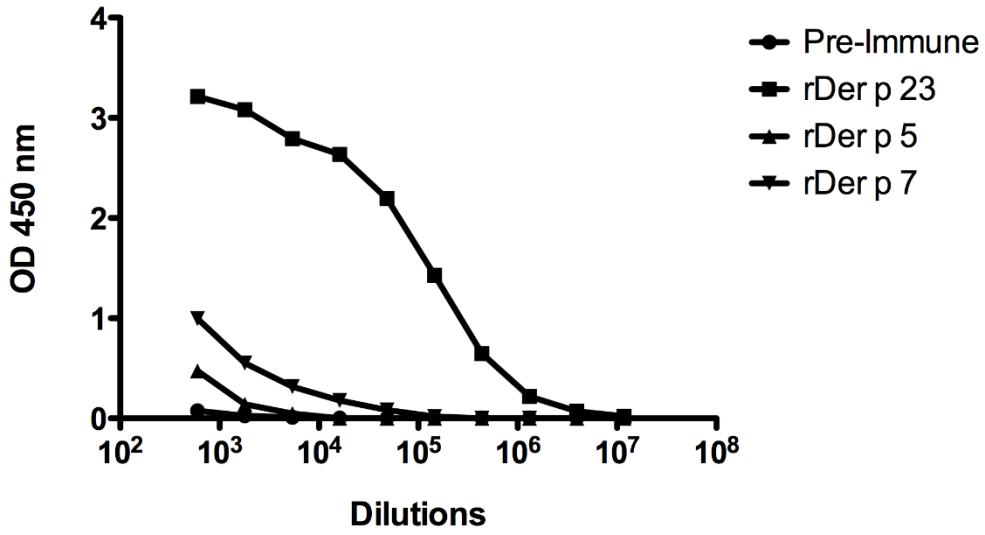

Figure 4. 8 Specificity of ant-rDer p 23 serum. Different recombinant proteins (rDer p 5, rDer p 7 and rDer p 23) were coated onto ELISA plate and detected with series dilutions of pooled mouse polyclonal anti-rDer p 23 antibodies (n=3).

4.7 Detection of Natural Der p 23 in HDM Faeces Extracts

In order to confirm that rDer p 23 produced in *P.pastoris* shared at least some common B-cell epitopes with the corresponding natural allergen, Der p 23-specific polyclonal antibodies were used to detect nDer p 23 into the HDM extracts. Through chemiluminescence-based immunoblot detection, the specific antibodies raised to rDer p 23 but not the preimmune serum were able to detect natural Der p 23 from aqueous extracts of enriched HDM fecal pellets but not in commercially available HDM mite bodies' extracts (Greer, USA) (Fig 4.9). However, it must be pointed out that natural Der p 23 was present in very tiny amounts in the extracts. Our results suggested that recombinant and natural Der p 23 shared at least some common antigenic determinants.

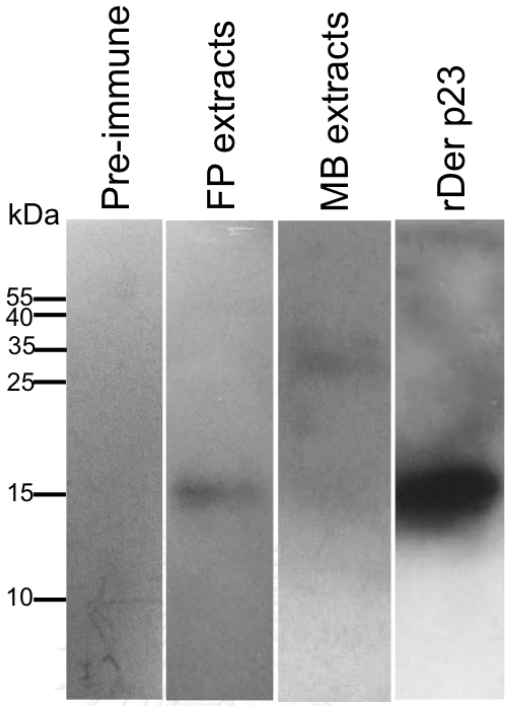

Figure 4. 9 Chemiluminescence detection of Der p 23 in house dust mite *D. pteronyssinus* extracts. Lane pre-immune and FP extracts: loaded with fecal pellet extracts each extracted from 2.5 mg of feces (Provided by Stallergenes, France); Lane MB extracts: was loaded with 100 µg mite bodies extracts (Greer, USA); Lane rDer p 23: 1 µg of rDer p 23. Nitrocellulose-blotted extracts were incubated with mouse polyclonal antibody against rDer p 23 and pre-immune serum of Der p 23 immunized mouse. Bound antibodies were detected with rabbit anti-mouse-HRP and the luminescence signal was captured by radiography film. Mite bodies' extracts incubated with pre-immune has a similar profile as in fecal pellet extracts. MB = mite bodies; FP = fecal pellets.

4.8 IgE Reactivity to rDer p 23

The IgE binding frequency to rDer p 23 was evaluated by direct ELISA in a cohort of Thai HDM allergic patients (n=222, Table 4.2) from four different hospitals in Bangkok. All the selected sera displayed a ImmunoCAP *D.pteronysinnus* specific IgE value higher than 0.35 kU_A/L (Table 4.3). To determine the threshold of positivity,

sixty-four *D.pteronysinnus* ImmunoCAP negative sera were used. Fifty four percent of the HDM-positive sera (i.e. 119 out of 222) showed specific IgE reactivity to rDer p 23 whereas IgE reactivity to rDer p 2 was detected in 67% of the same mite-allergic individuals, confirming that Der p 23 is a major HDM allergen. Figure 4.10 shows the IgE reactivity of Der p 23 and Der p 2 according to specific IgE *D.pteronysinnus* ImmunoCAP grades. There is no patient with grade 1 recognize to neither Der p 2 nor Der p 23. Most of the patients sensitized to Der p 2 and Der p 23 were distributed in grade 3-6. Lastly, we also evaluate the influence of glycan structure of rDer p 23 in IgE binding. By comparing the ELISA OD value obtained from both glycosylated and deglycosylated rDer p 23 (Table 4.4), there is no significant difference between the OD values suggesting that the glycan did not influence the IgE binding.

Table 4. 2 IgE reactivity of Der p 2 and Der p 23 in Thai HDM allergic patients

Sources	Total sera	Der p 2		Der p 23	
		Positive sera	sIgE frequency, %	Positive sera	sIgE frequency, %
Chulalongkorn Hospital	95	68	72	59	62
Phramongkutklao Hospital	14	8	57	7	50
Ramathibodi Hospital	32	18	56	12	38
Children Hospital	81	55	68	41	51
Total	222	149	67	119	54

*The IgE reactivity of Der p 23 is significantly different from Der p 2 ($P<0.05$).

Table 4. 3 HDM patients' Specific IgE to *D.pteronysinnus* ImmunoCAP values

Grade	Unit, kU_A/L	Level	Number
1	0.35-0.7	Weak positive	6
2	0.7-3.5	Positive	33
3	3.5-17.5	Positive	53
4	17.5-52.5	Strong positive	53
5	52.5-100	Strong positive	42
6	>100	Strong positive	35

Note: The grading system employed was refered to diagnostic reports of several diagnostic companies.

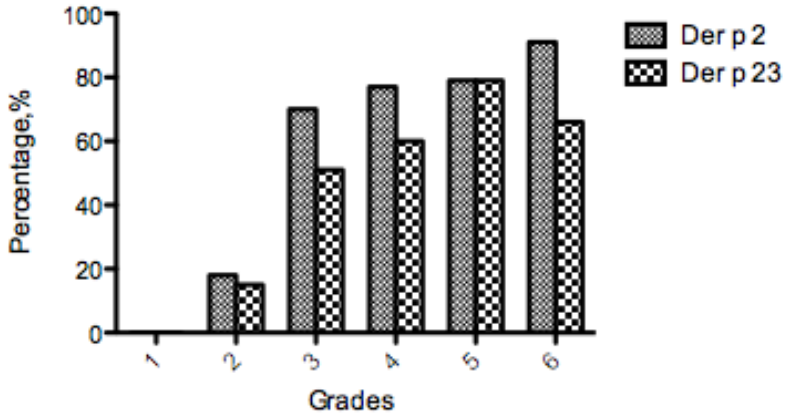

Figure 4. 10 IgE reactivity of Der p 2 and Der p 23 in Thai HDM allergic patients according to specific IgE *D.pteronysinnus* ImmunoCAP grades.

Table 4. 4 Evaluation of specific IgE binding between glycosylated rDer p 23 and deglycosylated rDer p 23 in Thai HDM allergic patients

	CU 017	CU 020	CU 023	CU 027	CU 033	CU 034	CU 036	CU 046	CU 054	CU 066
rDer p 23	0.617	1.087	1.069	0.757	1.117	0.973	0.817	1.119	0.954	0.917
DeDer p 23	0.603	1.068	1.084	0.750	1.043	0.965	0.947	1.195	1.016	0.924

Note: DeDer p 23 is deglycosylated form of rDer p 23. Data are presented as OD values. There is no significant different between rDer p 23 and DeDer p 23 with a P-value of 0.8762.

4.9 Allergenicity of rDer p 23

To demonstrate that rDer p 23 can trigger basophil degranulation through interactions with FcεRI-bound specific IgE, a mediator release assay was performed. Rat Basophil Leukemia (RBL) SX-38 cells expressing human FcεRI, were passively loaded with sera from HDM-allergic patients positive for rDer p 23-specific IgE and stimulated with different concentrations of the same allergen. Figure 4.11 depicted the typical dose-response bell-shape curve displayed by allergenic activity of rDer p 23 using individual serum from three Der p 23 sensitized HDM-allergic patients. It is generally thought that a cellular response should increase and reach a plateau phase as the stimulant concentration increases. However, FcεRI dose-response will decrease when there is an excessive IgE bridging due to an active turn-off mechanism in the cell (53). rDer p 23 was able to induce basophil degranulation at concentration ranging from 0.0001 to 1 μg/ml with a maximum degranulation at about 0.01 μg/ml except for patients CU082 and A003. No significant degranulation was observed with buffer alone and rDer p 23 alone as well as with non-HDM allergic serum. These data clearly demonstrated that rDer p 23 displays allergenic activity.

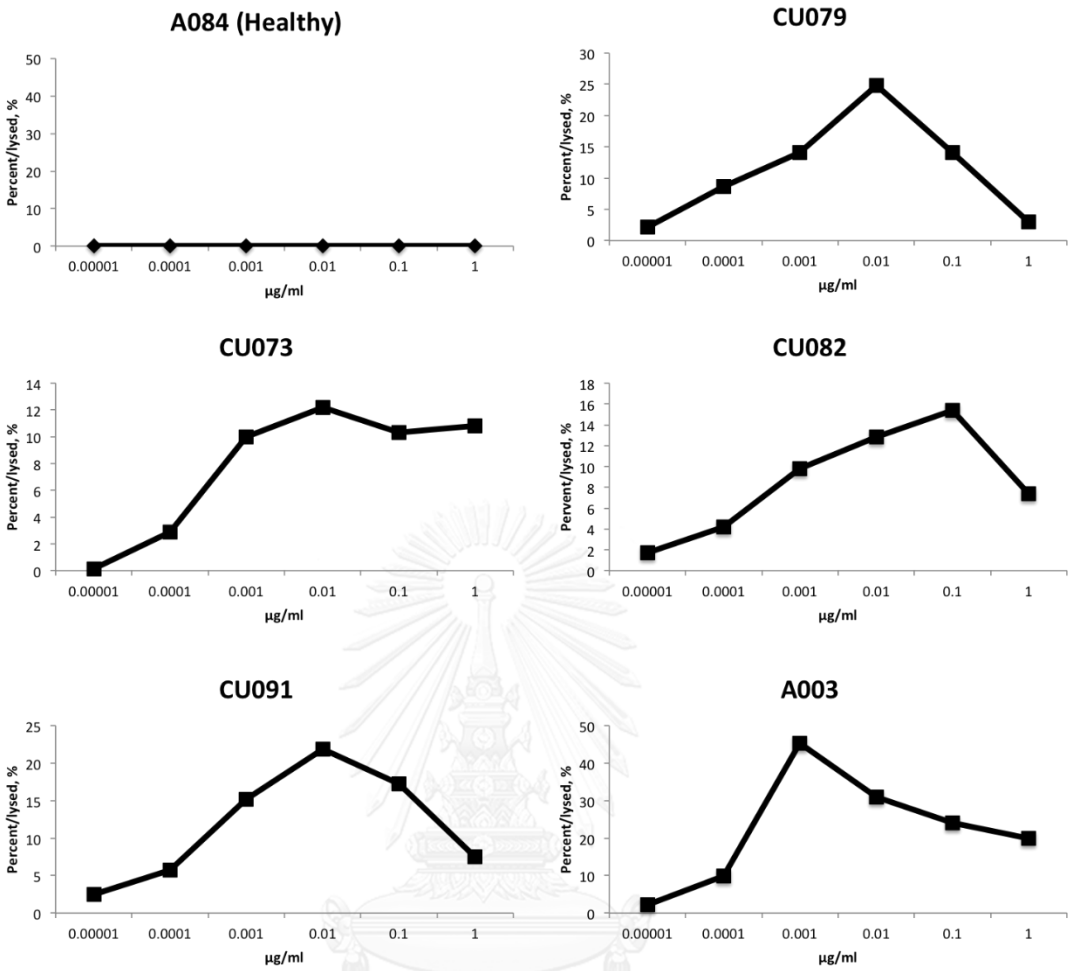

Figure 4. 11 Allergenic activity of rDer p 23 onto IgE-loaded RBL-SX38 cells. Cells were loaded with healthy individual (specific immunoCAP value to *D.pteronysinnus*, 0.04 kUA/L) and HDM allergic patients designated as CU079 (>100 kUA/L), CU073 (>100 kUA/L), CU082 (51.4 kUA/L), CU091 (68.7 kUA/L) and A003 (67.3 kUA/L) sera overnight and stimulated with serial dilution of purified rDer p 23 (0.00001 to 1 µg/ml) for 30 minutes. Degranulation was measured through β-hexasominidases activity. Percentage of degranulation was presented as subtraction of spontaneous released over total lysed with Triton X-100.

4.10 Evaluation of the rDer p 23 Chitin-binding Activity

Amino acid sequence analysis shows that Der p 23 displayed homologies to chitin-binding type 2 and chitin-binding peritrophin-A domains, suggesting that Der p 23 could interact with chitin structures (9). Consequently, we evaluated the chitin-binding activity of rDer p 23 using two different sources of insoluble chitins: chitin beads and shrimp shell chitin. A recombinant form of Der p 5 was used as a negative control because this protein did not display any homologies with chitin-binding proteins. Under our experimental conditions, rDer p 23 as well as nDer p 23 from fecal pellet extracts could not bind to two chitin matrices. By contrast, the positive control wheat germ agglutinin (WGA), a well-known chitin-binding protein, efficiently bound to chitin whereas rDer p 5 cannot interact with the sugar polymers. The absence of binding could not be explained by the presence of residual competing sugars in the purified rDer p 23 preparation as WGA solubilized in the *P.pastoris* induced culture medium can still interact with chitin. Altogether, our data suggested that Der p 23 alone does not displayed any chitin-binding activity (Fig. 4.12).

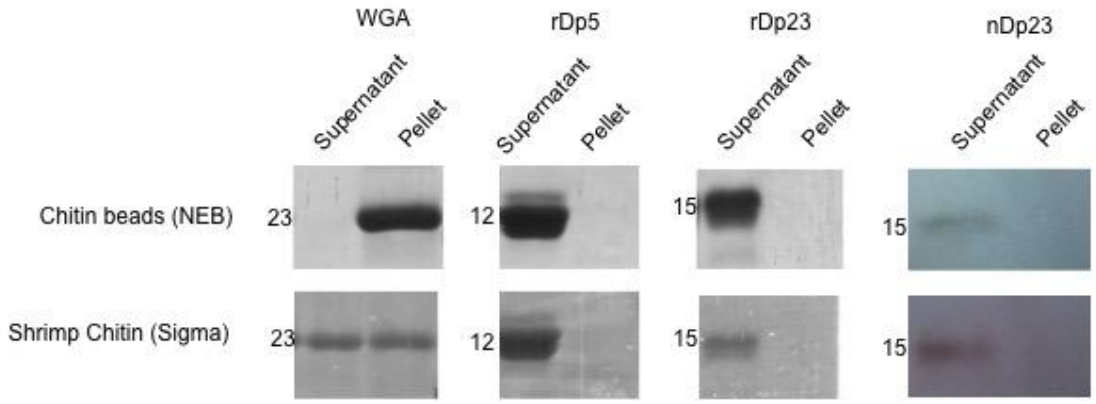

Figure 4. 12 Chitin-binding activity of rDer p23 produced in *P.pastoris*. The binding activity was accessed by incubating the protein with insoluble chitin beads and shrimp chitin. Bound and unbound proteins to chitin beads or shrimp chitin were analysed on SDS-PAGE. The nDer p 23 binding to chitin was analyzed by immunoblot.

4.11 rDer p 23 Induces IL-8 Secretion from Human Airway Epithelial Cells

It is well known that airway epithelium represents the first cell layer exposed to airborne allergens. Recent studies demonstrated that airway epithelial cell activation by allergens in the presence of microbial compounds is a key triggering event in the initiation of the allergic response. Therefore, we investigated the ability of rDer p 23 to stimulate pro-inflammatory cytokine production from human airway epithelial cells (BEAS-2B). As shown in figure 4.13, rDer p 23 triggered the release of IL-8 in BEAS-2B in a concentration (5-20 μg/ml) and time-dependent manner (Fig. 4.13A and B). Polyinosinic:polycytidylic acid (Poly I:C), a well-known toll-like receptor 3 (TLR-3) inducer was used as a positive control. IL-8 secretion did not result from the presence of residual host cell contaminants (proteins, lipids, sugars) in our purified rDer p 23 preparation. Indeed, a methanol-induced culture medium produced with a wild-type KM71 strain and purified using the rDer p 23 purification protocols was unable to activate the IL-8 release under the same experimental conditions (Fig. 4.13C). In addition, we also evaluated the released of GM-CSF as this cytokine is one of the innate pro-Th2 cytokines that can recruit DCs to induce Th2 immunity (2). However, GM-CSF was not detected in any concentration of rDer p 23 tested (1-20 μg/ml) under the same experimental conditions with the exception of Poly I:C (positive control) (data not shown).

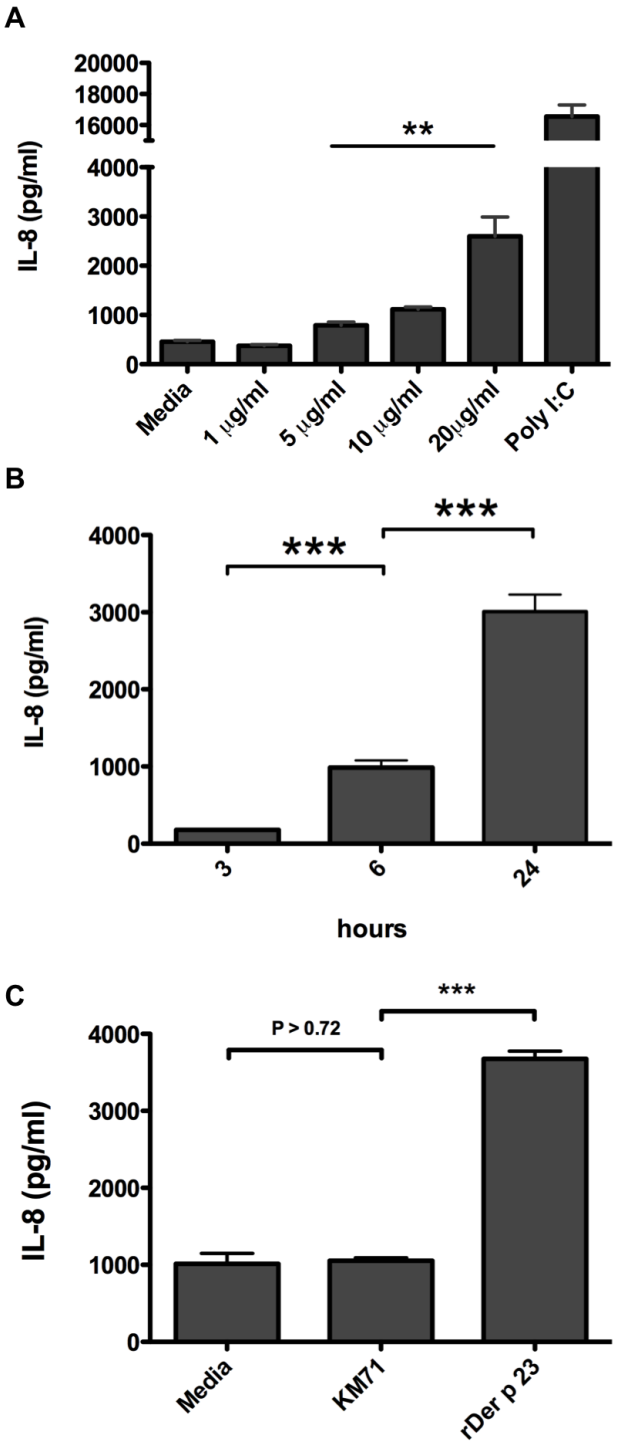

Figure 4. 13 The rDer p 23-induced IL-8 production from BEAS2B cells. A) Cells were stimulated with different concentration of rDer p 23 (1-20 μg/ml) under serum free conditions. Poly I:C (2 μg/ml) was used as a positive control. ** indicated $P<0.01$ versus untreated cells. B) Cells were stimulated with rDer p 23 (20 μg/ml) for

different periods of time. *** indicated P<0.001 versus between time points. C) Cells were stimulated with a methanol-induced culture medium produced with a wild-type KM71 strain and purified using the rDer p 23 purification protocol (corresponding volume to rDer p 23) and rDer p 23 (20 μg/ml). *** indicated P<0.001 compared to non-treated rDer p 23. Secreted IL-8 was quantified by ELISA. All data shown are representative from three experiments and are expressed as the mean ± SD from triplicates.

4.12 Role of MAPK and NF-κB in rDer p 23 Induced IL-8 secretion by Human Airway Epithelial Cells

To further investigate whether rDer p 23-induced IL-8 production is dependent on mitogen-activated protein kinase (MAPK) signaling pathways, BEAS-2B cells were pretreated with the MEK 1/2 inhibitor UO126 (20 μM), JNK inhibitor SP600125 (20 μM), and p38 MAPK inhibitor (1 μM) before any activation with Der p 23. As the inhibitors were solubilized with DMSO, cells were also preincubated with DMSO as negative control. Our results clearly highlighted that the rDer p 23-induced IL-8 secretion was MAPK-dependent (Fig. 4.14A). In addition, we also found that the IL-8 secretion was NF-kB dependent as pretreatment of BEAS-2B cells with the IκB inhibitor BAY11-7082 (10 μM) impaired the cell activation by rDer p 23 (Fig. 4.14B). All these data showed that rDer p 23 could activate in airway epithelial cells both NF-κB and MAPK-dependent innate immune signaling pathways.

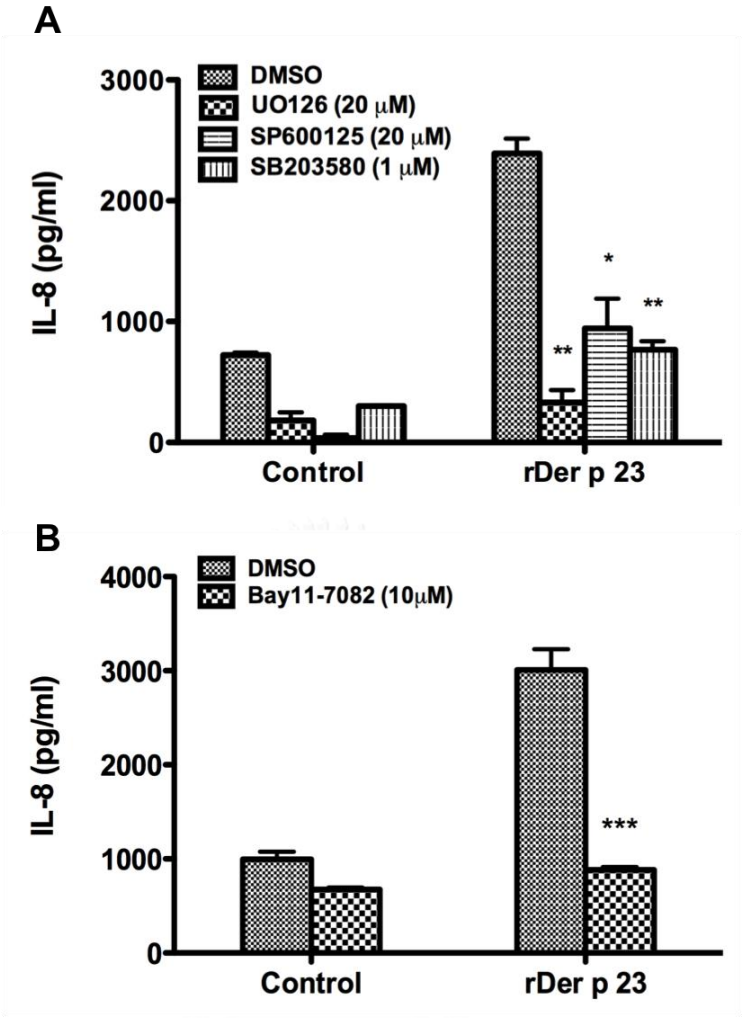

Figure 4. 14 Effect of MAPK and NF-κB inhibitors on rDer p 23-induced IL-8 secretion by BEAS2B. A) Cells were pretreated with indicated concentration of UO126, SP600125, and SB203580 for 60 mins prior to stimulation with 20 μg/ml of rDer p 23 for 24 hours. *P<0.05 and **P<0.01 indicated significant values versus rDer p 23 alone. B) Cells were pretreated with indicated concentration of BAY11-7082 inhibitor for 60 mins prior to stimulation with 20 μg/ml of rDer p 23 for 24 hours. ***P<0.001 indicated significant values versus rDer p 23 alone. Secreted IL-8 was quantified by ELISA. All data shown are representative from three experiments and are expressed as the mean ± SD from triplicates.

CHAPTER V
DISCUSSION

Although eighteen different allergen groups (www.allergome.org) were identified hitherto in the *D.pteronyssinus* mite species, the in-depth characterization of these allergenic proteins remains actually incomplete. With the exception of the proteases Der p 1, Der p 3, Der p 6, Der p 9 together with the LPS-binding protein Der p 2 which were shown to trigger innate immunity (27), the answers to the question: what makes an allergen an allergen remains elusive for most of the allergenic HDM proteins. The main reasons are: 1) it is quite impossible to isolate appropriate amounts of HDM allergens from their natural environment, the house dust (38). 2) With the exception of Der p 1 and Der p 2, the other HDM allergens are present in tiny amount in cultured HDM aqueous extracts from fecal pellets and/or mite bodies (38). 3) Although we can speculate that recombinant forms of HDM allergens are folded as the corresponding natural ones, differences at the level of post-translational modifications, association to ligand(s) (notably for the lipid binding-like proteins) or conformational epitopes could drastically influence their allergenicity (54).

A recent paper successfully identified Der p 23 as a new major *D.pteronyssinus* allergen. The IgE reactivity to a recombinant form of Der p 23 produced in *E.coli* was comparable to those raised to Der p 1 and Der p 2 (>70% in European cohorts of HDM allergic patients) (9). This high IgE-specific frequency was quite challenging as Der p 23 was shown to be mainly an unfolded protein, present in very tiny amount in house dust and its aqueous extraction yield from fecal pellets was very low, probably because Der p 23 could be tightly associated to the peritrophic matrix surrounding the feces. Such semi-permeable matrix, produced in the mite midgut during food digestion to prevent the epithelium from damages by

abrasive food particles and pathogens, is mainly composed of proteins and chitin fibrils (55). According to the capacity of chitin to trigger innate immunity to shape Th2-biased cytokine environment, the potent allergenicity of Der p 23 could consequently result from its association with chitin (11,56). Although the Der p 23 C-terminal region displays homologies with a chitin-binding domain type 2 and peritrophin-A domain, the interactions between the allergen and chitin structures remained to be demonstrated.

In the present study, we selected the *P.pastoris* expression system to produce and characterize a recombinant mature form of Der p 23. Yeast expression system particularly *P.pastoris* can be recognized as one of the most successful systems used in a wide range of recombinant proteins production. The rationale for this selection was based on the presence of a leader peptide in the Der p 23 amino acid sequence for secretion, two threonine stretches (T^{30}-T^{32} and T^{36}-T^{38}) which could represent O-glycosylation sites together with four cysteine residues in the putative C-terminal chitin-binding domain which could be involved in two disulfides. All these three post-translational modifications, which could be critical for the allergenic properties and stability of Der p 23, can be performed in yeast. It must be pointed out that HDM allergens could be naturally glycosylated as glycan structures such as mannosylations were detected in natural Der p 1 and Der p 2 whereas such modifications could not take place when proteins are produced in bacteria (57).

Transformed *P.pastoris* was able to secret soluble full-length mature and hexose-associated rDer p 23. Despite that we did not further characterized the hexose residues which are associated to rDer p 23, we hypothesized that the hexose residues are indeed mannoses since yeast can only mannosylate O-glycosylation sites. (58,59). We further experimentally demonstrated that the hexose residues were removed by mannosidase treatment. Onto SDS-PAGE, the purified rDer p 23 migrated as a 15kDa band whereas the calculated mass by MS analysis ranged from 7.9kDa

(full-length, unglycosylated) to 10.1 kDa (full-length, longest mannose chains). This abnormal SDS-PAGE migration could likely resulted from the proline content in Der p 23 (10%) as proline-rich proteins commonly showed decreased electrophoretic mobility (60). Similar abnormal migration patterns were also observed for non-glycosylated peritrophin-15 protein (14% proline) from *C.bezziana* and *L.cuprina* which displayed a theoretical protein size of 7.9 kDa but migrated as a 15 kDa bands onto SDS-PAGE (55,61). Under our experimental conditions, although Der p 23 was mainly expressed as an intact mature protein, some N-terminal truncations of 11 to 17 amino acids were also observed and particularly, when the Der p 23 expression through methanol induction exceeded more than 48h. Such truncations could result from the host cell proteases released into the medium during the induction or present in the yeast secretory pathway (62). It must be pointed out however that our stability studies clearly indicated that rDer p 23 in PBS displays a conserved SDS-PAGE profile even when the protein was stored at 37°C for at least 6 days, although mild degradation can be observed beyond that period. MS analysis of fecal pellets aqueous extracts evidenced that natural Der p 23 is highly sensitive to proteolysis as the detection of the full-length natural allergen was difficult to achieve (confidential information from Stallergenes). Whether the Der p 23 degradation is mediated by HDM proteases or peptidases from the mite culture medium remains to be identified.

The secondary structure analysis by circular dichroism confirmed that, similarly to the recombinant form produced in bacteria, our Der p 23 preparation was mainly unfolded, despite the presence of disulfides, suggesting that Der p 23 likely displays more linear than conformational antibody-binding epitopes on the allergen surface (63). This is in accordance with the capacity of purified specific polyclonal antibodies to detect rDer p 23 under native (ELISA) or denaturing (Immuno-blot after SDS-PAGE) conditions. Heating to rDer p 23 did not further unfold the protein secondary structure. In contrast, a slight increase in α-helix and β-sheet contents could be measured. We considered that the increase in beta sheet structures did not correspond to protein aggregation, as commonly observed. Indeed, similar native gel

filtration profile could be measured between native and heat-treated rDer p 23 (data not shown).

As mentioned earlier, Der p 23 has high IgE reactivities of 74 % (n=347) in European populations of HDM allergic patients, measured using a recombinant form produced in E.coli (9). Using sera from a cohort of Thai HDM-allergic patients, we confirmed that Der p 23 is an important allergen as the frequency of Der p 23-specific IgE reached more that 50% (54%, n=222). It is noteworthy that this IgE reactivity was lower than that measured for the typical major allergen Der p 2 (67%) in the same population. To our knowledge, this is the first time that the IgE binding frequencies of Der p 2 and Der p 23 was determined in HDM allergic patients from Thailand. RBL degranulation assays obviously evidenced the potent allergenic activity of Der p 23. The lower prevalence of Der p 23 sensitivity in Thai HDM allergic patients could not be explained by the presence of O-mannosylations at the N-terminal domain of our recombinant allergen preparation which could mask some IgE binding epitopes. Indeed, it was recently demonstrated that major IgE epitopes of Der p 23 are present at the C-terminal part corresponding to the putative chitin-binding domain (63). Moreover, different countries and regions could have variation in sensitivity profile. For instance, Der p 1 and Der p 2 are generally accepted to have high IgE binding frequencies (>70%) in countries such as France, Italy, and Austria. However, countries like Belgium and New Zealand have much lower IgE frequency (<50%) (www.allergome.org). Interestingly, our cohort study showed that, through the IgE reactivities to Der p 23 or Der p 2, up to 80% of HDM-allergic patients can be diagnosed.

The poorly extractable amount of nDer p 23 has made the characterization difficult or almost impossible to convince our findings. It is also challenging the commonly accepted concept that major allergens are easily extracted and usually present in high amount in the extracts. For instance, two major HDM allergens Der p

1 and Der p 2 are abundant in HDM extracts (38). Perhaps, one of the reasons could be the presence of many enzymes such as chitinases, lysozymes, human defensins, surfactant proteins and etc. into the bronco-alveolar lavage fluid (64,65) that could help in releasing Der p 23 from the fecal pellets more efficiently or slowly to provide long exposure resulted in innate immunity activation.

To date, it is increasingly accepted that allergenicity of HDM allergens, can result from their interactions with microbial compounds which are able to activate innate immune signaling pathways, leading to the induction of T_H2-biased allergen-specific adaptive responses (21). In that context, and according to the presence of putative chitin-binding domain at the Der p 23 C-terminus, its allergenicity could be related to its chitin-binding reactivity. Using two different sources of chitin, our results suggested that both recombinant and natural Der p 23 were not capable to bind to this sugar polymer. This absence of chitin binding activities could be related to the presence of only four cysteine residues at the C-terminus. Commonly, typical Peritrophin-A and chitin binding 2 domains contains at least 6 conserved cysteines. Moreover, chitin binding activity has been commonly demonstrated in proteins containing at least 2 of these domains (66,67). Figure 5.1 shows a multiple protein sequence alignment of Der p 23 to the chitin-binding domains (CBM_14 and ChtBD2) from the database and two other proteins (AgChi-1 and CB15) which their chitin-binding activities has been demonstrated.

```
AgChi-1    STTTEGNPGTTRPPSGDGPCAGGRYGFVPHPT-NCARYYICLTADTY-YE 498
CB15       ACVLACDPDGNNQP----QCSSNNVNVPVRNFWDPTHYWLCKSASGVAES 60
CBM_14     -------------------CEGRPDGLYPDPG-DCSKYYQCSNGKA--VV 28
ChtBD2     -----------------NECPGRGDGLYPHPT-DCSKYYQCSNGRP--IV 30
Der p 23   DPTTTVHPTTTEQPDDKFECPSR-FGYFADPK-DPHKFYICSNWEA--VH 52
                            * .   .       :  ::: * .

AgChi-1    FTCPPGTLFDPALHICN----WADQVKCPNE 525
CB15       VPCPVAEGFDPAKGACVPFDQWKWTEPCPKA 91
CBM_14     FTCPAGLVFDPALGTCD-------------- 45
ChtBD2     GSCPAGLVFNPATQTC--------------- 46
Der p 23   KDCPGNTRWNEDEETCT-------------- 69
             **    ::     *
```

Figure 5. 1 Multiple protein sequence alignment of Der p 23 to chitin-binding domains. rDer p 23 protein sequence was aligned against chitin-binding domain of type-1 peritrophic protein (AgChi-1), *C.bezziana* peritrophin-15 (CB15), Chitin-binding domain type 2 (CBM_14), and chitin-binding peritrophin-A domain (ChtBD2). Below protein sequence is a key indicating conserved sequence (*), conservative (:), and semi-conservative (.). Important cysteines residues were highlighted in yellow.

An important example from the HDM Blomia tropicalis allergen, Blo t 12 which contained five cysteine residues could display chitin-binding activity to chitin (the same chitin sources as we used). To our knowledge, the interaction between chitin and proteins harboring a domain with only four cysteines was not demonstrated up to now. Consequently, the localization of Der p 23 into the peritrophic matrix (composed of chitin and proteins) of the mite could result from interactions with other molecular partners besides chitin or from the existence of oligomeric forms of the allergen exposing appropriate four cysteine-based chitin-binding domains to interact with chitin. This lead to our hypothesis that perhaps Der p 23 could interacts with partner protein, notably chitinases such as Der p 15 and Der p 18 allergens which probably form a multi chitin-binding domains to interact with chitin substrates. Numbers of studies have shown that chitin-binding protein

plays a synergetic role in chitinase activities (68,69). Nevertheless, we cannot exclude that the Der p 23 chitin binding activities is particularly dependent on the chitin sources, the chitin materials used in our study and the chitin from the peritrophic matrix could adopt different structures (70). Some chitin-binding protein can only interact with certain chitin conformations (55). It must be also pointed out that the recently identified *D.farinae* allergen Der f 23 was shown to contain a putative chitin binding domain with five cysteine residues, four out the five cysteine residues are conserved in Der p 23 (Fig. 5.2) (71). However, the chitin binding properties of Der f 23 remains to be elucidated.

```
Der_p_23        MKFNIIIVFISLAILVHSSYAANDNDDDPTTTVHP-TTTEQPDDKFECPS 49
Der_f_23        MKFNITIAFVSLAILIHSSYADIDHDDDPTTMIDVQTTTVQPSDEFECPT 50
                ***** *.*:*****:*****  *:****** :.  *** **.*:****:

Der_p_23        RFGYFADPKDPHKFYICSNWEAVHKDCPGNTRWNEDEETCT 90
Der_f_23        RFGYFADPKDPCKFYICSNWEAIHKSCPGNTRWNEKELTCT 91
                ********** **********:**.*********.* ***
```

Figure 5. 2 Protein sequence alignment of Der p 23 and Der f 23. Below protein sequence is a key indicating conserved sequence (*), conservative (:), and semi-conservative (.). Important cysteines residues were highlighted in yellow.

Besides, the role of glycosylation in the recognition and uptake of allergens by the innate immune system through C-type lectin receptors (CLRs) was shown also to be important for the initiation of the allergic response (72). In the present study, we evidenced a O-glycosylation site formed by a stretch of Threonine residues in Der p 23 ($T^{30-}T^{32}$). Although the presence of O-glycans remains to be demonstrated in natural Der p 23, O-glycosylation of natural HDM allergens can be expected as previously shown for the HDM chitinase Der f 15 (73). Moreover, western blot detection of natural Der p 23 from mite extracts indicated that the natural protein migrated onto SDS-PAGE at the same molecular weight as rDer p 23, suggesting that

natural Der p 23 could be also glycosylated. However, we can expect that the glycosylation pattern of rDer p 23 is not fully shared by natural Der p 23 as cells from HDM (arachnid cells) could perform more complex N- and O-glycosylations than the typical yeast hypermannosylations (74). Taken together, our results showed that Der p 23 is a O-glycoprotein and its glycan structures could influence its uptake by dendritic cells (DCs) as well as the T_H2 polarization through different DC activation levels.

In term of innate immunity activation, pro-inflammatory cytokines such as TSLP, IL-33, IL-25, IL-1β and GM-CSF produced by innate immune signaling play a critical role in the initiation of the allergic responses at the level of airway epithelium which is the first cell layer in contact with HDM allergens (airborne allergens) (27). In order to determine whether Der p 23 is able to activate airway epithelial cells (BEAS-2B), we evaluated the IL-8 production following the cell stimulation. IL-8 was selected: 1) because IL-8 is a typical pro-inflammatory cytokine which can be easily produced and detected by ELISA notably. 2) It also plays an important role in lung inflammatory diseases such as asthma. One study has shown that asthmatic HDM allergic patients have significantly increase in IL-8 found in bronco-alveolar lavage fluid after allergen challenge as compared to non-asthmatic HDM patients (75). Our data clearly showed that rDer p 23 is able to trigger the IL-8 release by airway epithelial cells in a concentration- and time-dependent manner. It must be pointed out that similar IL-8 production was triggered by Der p 2- or Der p 21-treated airway epithelial cells (8,76). It is important to stress that cells activated by rDer p 23 is not due to host cell proteins or LPS contaminations. It is well described that under serum-free condition, lipopolysaccharide (LPS) is not able to stimulate IL-8 release by BEAS2B cells up to 10µg/ml (77). In addition, our group has also previously confirmed the complete absence of cell activation by LPS under serum-free conditions (unpublished results). It is worth to remind that MS analysis show no detectable host proteins in the purified rDer p 23. Together with the control (same purified fraction

from methanol-induced *P.pastoris*), we considered that the IL-8 response observed is due to rDer p 23.

Commonly, cytokines productions following airway epithelium stimulation are mediated by MAPK signaling pathways as well as by the activation of transcription factors such as NF-κB. Normally, IL-8 production is modulated by nuclear factor (NF)-κB and activating protein (AP)-1. NF-κB is essential for IL-8 production whereas AP-1 is used as an enhancer (78). Though the used of the IκB inhibitor BAY11-7082, we demonstrated that IL-8 production in response to rDer p 23 is NF-κB dependent. AP-1 is activated by mitogen-activated protein kinase (MAPK). IL-8 gene expression can be modulated by three different MAPK pathways which are extracellular-regulated protein kinase (ERK), cJun-N-terminal protein kinase (JNK), and p38 MAPK cascades (78). By using specific inhibitors for each pathway, we identified that all these three pathways are involved in IL-8 production in response to rDer p 23 in airway epithelial cells. All the data showed that rDer p 23 stimulates NF-κB and MAPK dependent IL-8 production in BEAS-2B. Although similar results were observed with rDer p 2 (8). It was impossible, due to very limited amount, to confirm our results through the cell activation by natural Der p 23.

Although our results clearly suggest that Der p 23 can trigger innate signaling pathways, the receptor(s) activated by Der p 23 remain(s) to be identified. C-type lectin (CLR) receptors could represent the more plausible candidates, as highly purified rDer p 23 alone, devoid of microbial contaminations, is capable to trigger IL-8 through its O-glycan structures. CLR expression in BEAS-2B cells, however, was not clearly demonstrated, with the exception of one study which evidenced the up-regulation of Dectin-1 in cells activated by β-glucans. It would be also interesting to evaluate the Der p 23-induced innate immune activation in normal airway epithelial cells as the expression of innate receptors could be different compared with the BEAS-2B cell line.

CHAPTER VI
CONCLUSION

Newly identified major HDM allergen, Der p 23 with an IgE frequency of more than 70% in European cohort study was mainly characterized as a recombinant form produced in bacteria expression system (9). The *P.pastoris* yeast expression system employed during this work could address some limitations from the bacteria expression system to optimize the secretion of Der p 23, its glycosylation, and the formation of disulfide bonds. The rDer p 23 IgE screening using 222 Thai HDM allergic patients represented the first evaluation of Der p 23 sensitization in Thailand. According to an IgE binding frequency of 54% which was considerably high indicating that Der p 23 was also an important allergen in Thai population. Despite being an important allergen, natural Der p 23 is poorly extracted from the HDM fecal extracts. In addition, this minimally structured allergen displayed no chitin-binding activity under our experimental conditions although exhibited sequence homology with chitin-binding domain. All of these have led to new research questions on how to explain this poorly extracted allergen that has no chitin-binding activity could have high IgE reactivity. Even though we could demonstrate that mannosylated rDer p 23 trigger AP-1 and NF-κB dependent cytokine production in airway epithelial cells, but which receptor(s) and pathway(s) are involved need to be addressed further.

The evaluation of Der p 23 release from the HDM fecal pellets using HDM allergic patients' bronco-alveolar lavage fluid can be interesting. Further evaluation on the synergistic effect of Der p 23 with HDM chitinases Der p 15 and Der p 18 could possibly explain the chitin binding activity of Der p 23. Finally the elucidation of the Der p 23-induced innate immune signaling pathways using primary cells would greatly shed new light on the allergenic determinant(s) of Der p 23.

Overall, our findings confirmed that Der p 23 displays potent allergenic activity, despite the fact that this allergen is poorly extractable from the mite fecal pellets. Consequently, recombinant forms of Der p 23 must be included instead of HDM allergen extracts for the diagnosis of Der p 23 sensitization as well as in the design of specific immunotherapeutic treatments.

REFERENCES

1. Zock J-P, Heinrich J, Jarvis D, Verlato G, Norbäck D, Plana E, et al. Distribution and determinants of house dust mite allergens in Europe: the European Community Respiratory Health Survey II. J Allergy Clin Immunol. 2006;118(3):682–90.

2. Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. Trends Immunol. Elsevier Ltd; 2011;32(9):402–11.

3. Holgate S, Church M, Broide D, Martinez F. Allergy. 4th ed. Elsevier Ltd.; 2011. 432 p.

4. Moingeon P. Progress in the development of specific immunotherapies for house dust mite allergies. Expert Rev Vaccines. Informa UK, Ltd.; 2014;13(12):1463–73.

5. Bronnert M, Mancini J, Birnbaum J, Agabriel C, Liabeuf V, Porri F, et al. Component-resolved diagnosis with commercially available D. pteronyssinus Der p 1, Der p 2 and Der p 10: relevant markers for house dust mite allergy. Clin Exp Allergy. 2012;42(9):1406–15.

6. Weghofer M, Thomas WR, Kronqvist M, Mari A, Purohit A, Pauli G, et al. Variability of IgE reactivity profiles among European mite allergic patients. Eur J Clin Invest. 2008;38(12):959–65.

7. Post S, Nawijn MC, Jonker MR, Kliphuis N, van den Berge M, van Oosterhout a JM, et al. House dust mite-induced calcium signaling instigates epithelial barrier dysfunction and CCL20 production. Allergy. 2013.

8. Osterlund C, Grönlund H, Polovic N, Sundström S, Gafvelin G, Bucht A. The non-proteolytic house dust mite allergen Der p 2 induce NF-kappaB and MAPK dependent activation of bronchial epithelial cells. Clin Exp Allergy. 2009;39(8):1199–208.

9. Weghofer M, Grote M, Resch Y, Casset A, Kneidinger M, Kopec J, et al. Identification of Der p 23, a peritrophin-like protein, as a new major Dermatophagoides pteronyssinus allergen associated with the peritrophic matrix of mite fecal pellets. J Immunol. 2013;190(7):3059–67.

10. Koller B, Müller-Wiefel AS, Rupec R, Korting HC, Ruzicka T. Chitin modulates innate immune responses of keratinocytes. PLoS One. 2011;6(2):e16594.

11. Roy RM, Wüthrich M, Klein BS. Chitin elicits CCL2 from airway epithelial cells and induces CCR2-dependent innate allergic inflammation in the lung. J Immunol. 2012;189(5):2545–52.

12. Galli SJ, Tsai M, Piliponsky AM. The development of allergic inflammation. Nature. 2008;454(7203):445–54.

13. Pawankar R, Canonica G. WAO white book on allergy. WI World Allergy Organization 2011.

14. Prescott SL, Pawankar R, Allen KJ, Campbell DE, Sinn JK, Fiocchi A, et al. A global survey of changing patterns of food allergy burden in children. World Allergy Organ J. 2013;6(1):21.

15. Colloff MJ. Dust Mites. Dordrecht: Springer Netherlands; 2009.

16. Jacquet A. The role of the house dust mite-induced innate immunity in development of allergic response. Int Arch Allergy Immunol. 2011;155(2):95–105.

17. Vrtala S, Huber H, Thomas WR. Recombinant house dust mite allergens. Methods. Elsevier Inc.; 2013;66(1):67–74.

18. Bousquet J, Khaltaev N. Global surveillance, prevention and control of chronic respiratory diseases: a comprehensive approach. Haden A, Desloovere P, editors. Switzerland: World Health Organization; 2007.

19. Pawankar R, Canonica GW, Holgate ST, Lockey RF. Allergic diseases and asthma: a major global health concern. Curr Opin Allergy Clin Immunol. 2012;12(1):39–41.

20. Arshad SH. Does exposure to indoor allergens contribute to the development of asthma and allergy? Curr Allergy Asthma Rep. 2010;10(1):49–55.

21. Jacquet A. The role of innate immunity activation in house dust mite allergy. Trends Mol Med. 2011;17(10):604–11.

22. Grundy J, Matthews S, Bateman B, Dean T, Arshad SH. Rising prevalence of allergy to peanut in children: Data from 2 sequential cohorts. J Allergy Clin Immunol. 2002;110(5):784–9.

23. Punekar YS, Sheikh A. Establishing the incidence and prevalence of clinician-diagnosed allergic conditions in children and adolescents using routinely collected data from general practices. Clin Exp Allergy. 2009;39(8):1209–16.

24. Platts-Mills TA, Vervloet D, Thomas WR, Aalberse RC, Chapman MD. Indoor allergens and asthma: report of the Third International Workshop. J Allergy Clin Immunol. 1997;100(6 Pt 1):S2–24.

25. Abbas AK, Lichtman AH. Basic Immunology: Functions and Disorders of the Immune System. 3rd ed. Saunders Elsevier; 2010. 312 p.

26. Holtzman MJ, Byers DE, Alexander-Brett J, Wang X. The role of airway epithelial cells and innate immune cells in chronic respiratory disease. Nat Rev Immunol. Nature Publishing Group; 2014;14(10):686–98.

27. Jacquet A. Innate immune responses in house dust mite allergy. ISRN Allergy. 2013;2013:735031.

28. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. Elsevier Inc.; 2010;140(6):805–20.

29. Lee H, Yuk J, Shin D, Jo E. Dectin-1 is Inducible and Plays an Essential Role for Mycobacteria-Induced Innate Immune Responses in Airway Epithelial Cells. 2009;795–805.

30. Lambrecht BN, Hammad H. The airway epithelium in asthma. Nat Med. Nature Publishing Group; 2012;18(5):684–92.

31. Willart M, Hammad H. Lung dendritic cell-epithelial cell crosstalk in Th2 responses to allergens. Curr Opin Immunol. Elsevier Ltd; 2011;23(6):772–7.

32. Larché M, Akdis C a, Valenta R. Immunological mechanisms of allergen-specific immunotherapy. Nat Rev Immunol. 2006;6(10):761–71.

33. Akdis C a, Akdis M. Mechanisms of allergen-specific immunotherapy. J Allergy Clin Immunol. 2011;127(1):18–27; quiz 28–9.

34. Jacquet A. New Insights into the Molecular Basis of the House Dust Mite-Induced Allergic Response. Open Allergy J. 2009;2(1):38–44.

35. Lambrecht BN, Hammad H. The role of dendritic and epithelial cells as master regulators of allergic airway inflammation. Lancet. Elsevier Ltd; 2010;376(9743):835–43.

36. Wambre E, James EA, Kwok WW. Characterization of CD4+ T cell subsets in allergy. Curr Opin Immunol. 2012 24(6):700–6.

37. Abbas AK, Lichtman AHH, Pillai S. Cellular and Molecular Immunology [Internet]. 7th Editio. Saunders; 2011. 560 p.

38. Casset A, Mari A, Purohit A, Resch Y, Weghofer M, Ferrara R, et al. Varying allergen composition and content affects the in vivo allergenic activity of commercial Dermatophagoides pteronyssinus extracts. Int Arch Allergy Immunol. Karger Publishers; 2012;159(3):253–62.

39. Moverare R, Elfman L, Vesterinen E, Metso T, Haahtela T. Development of new IgE specificities to allergenic components in birch pollen extract during specific immunotherapy studied with immunoblotting and Pharmacia CAP Systemtm. Allergy 2002;57(5):423–30.

40. Ball T, Sperr WR, Valent P, Lidholm J, Spitzauer S, Ebner C, et al. Induction of antibody responses to new B cell epitopes indicates vaccination character of allergen immunotherapy. Eur J Immunol. 1999;29(6):2026–36.

41. Tovey ER, Chapman MD, Platts-Mills TA. Mite faeces are a major source of house dust allergens. Nature. 1981;289(5798):592–3.

42. Wilson HTH. Tropical Eosinophilia in E. Africa. BMJ. 1947;1(4509):801–4.

43. Soysa E, Jayawardena MDS. Pulmonary Acariasis and Asthma. BMJ. 1945;1(4383):1–6.

44. Thomas WR. Review article Geography of house dust mite allergens. Asian Pacific J Allergy Immunol. 2010;28:211–24.

45. Becker S, Kramer MF, Havel M, Welz C, Markmann S, Gröger M. IgE reactivity profiles among house dust mite allergic patients in Bavaria. Eur Arch Otorhinolaryngol. 2013;270(12):3177–82.

46. Eisenbarth SC, Piggott D a., Huleatt JW, Visintin I, Herrick C a., Bottomly K. Lipopolysaccharide-enhanced, Toll-like Receptor 4-dependent T Helper Cell Type 2 Responses to Inhaled Antigen. J Exp Med. 2002;196(12):1645–51.

47. Nathan AT, Peterson E a, Chakir J, Wills-Karp M. Innate immune responses of airway epithelium to house dust mite are mediated through beta-glucan-dependent pathways. J Allergy Clin Immunol. Elsevier Ltd; 2009;123(3):612–8.

48. Yuenyongviwat A, Koonrangsesomboon D, Sangsupawanich P. Recent 5-year trends of asthma severity and allergen sensitization among children in southern Thailand. Asian Pac J Allergy Immunol. 2013;31(3):242–6.

49. MALAINUAL N, VICHYANOND P, PHAN-URAI P. House dust mite fauna in Thailand. Clin Exp Allergy. 1995;25(6):554–60.

50. Bunjean K, Sukkasem K, Noppacroh N, Yamkaew N, Janthayanont D, Theerapancharern W, et al. Prevalence of allergic rhinitis and types of sensitized allergen in adult at Wat Intaram community, Hua Raeu, Phra Nakhon Si Ayutthaya District, Phra Nakhon Si Ayutthaya Province, Thailand. J Med Assoc Thai. 2012;95 Suppl 5:S63–8.

51. Inoue H, Nojima H, Okayama H. High efficiency transformation of Escherichia coli with plasmids. Gene. 1990;96:23–8.

52. Wiegand TW, Williams PB, Dreskin SC, Jouvin MH, Kinet JP, Tasset D. High-affinity oligonucleotide ligands to human IgE inhibit binding to Fc epsilon receptor I. J Immunol. 1996;157(1):221–30.

53. Huber M. Activation/Inhibition of mast cells by supra-optimal antigen concentrations. J Cell Commun Signal. 2013;11(1):7.

54. Bordas-Le Floch V, Bussières L, Airouche S, Lautrette A, Bouley J, Berjont N, et al. Expression and characterization of natural-like recombinant Der p 2 for sublingual immunotherapy. Int Arch Allergy Immunol. 2012;158(2):157–67.

55. Tellam RL, Wijffels G, Willadsen P. Peritrophic matrix proteins. Insect Biochem Mol Biol. 1999;29(2):87–101.

56. Reese T a, Liang H-E, Tager AM, Luster AD, Van Rooijen N, Voehringer D, et al. Chitin induces accumulation in tissue of innate immune cells associated with allergy. Nature. 2007;447(7140):92–6.

57. Al-Ghouleh A, Johal R, Sharquie IK, Emara M, Harrington H, Shakib F, et al. The glycosylation pattern of common allergens: the recognition and uptake of Der p 1 by epithelial and dendritic cells is carbohydrate dependent. PLoS One 2012;7(3):e33929.

58. Strahl-bolsinger S, Gentzsch M, Tanner W. Protein O -mannosylation. 1999;1426:297–307.

59. Tanner W, Lehle L. Protein glycosylation in yeast. Biochim Biophys Acta - Rev Biomembr. 1987;906(1):81–99.

60. Hames BD. Gel Electrophoresis of Proteins: A Practical Approach. OUP Oxford; 1998. 372 p.

61. Wijffels G, Eisemann C, Riding G, Pearson R, Jones a, Willadsen P, et al. A novel family of chitin-binding proteins from insect type 2 peritrophic matrix. cDNA sequences, chitin binding activity, and cellular localization. J Biol Chem. 2001;276(18):15527–36.

62. Salamin K, Sriranganadane D, Léchenne B, Jousson O, Monod M. Secretion of an endogenous subtilisin by Pichia pastoris strains GS115 and KM71. Appl Environ Microbiol. 2010;76(13):4269–76.

63. Banerjee S, Weber M, Blatt K, Swoboda I, Focke-Tejkl M, Valent P, et al. Conversion of Der p 23, a new major house dust mite allergen, into a hypoallergenic vaccine. J Immunol. 2014;192(10):4867–75.

64. Whitsett J a, Alenghat T. IMMUNOLOGY OF THE LUNG Respiratory epithelial cells orchestrate pulmonary innate immunity. Nature Publishing Group; 2015;16(1):27–35.

65. Li Y, Wang Y, Liu X. The role of airway epithelial cells in response to mycobacteria infection. Clin Dev Immunol. 2012;2012.

66. Shen Z. A Type I Peritrophic Matrix Protein from the Malaria Vector Anopheles gambiae Binds to Chitin. J Biol Chem. 1998;273(28):17665–70.

67. Wijffels G, Eisemann C, Riding G, Pearson R, Jones A, Willadsen P, et al. A novel family of chitin-binding proteins from insect type 2 peritrophic matrix. cDNA sequences, chitin binding activity, and cellular localization. J Biol Chem. 2001;276(18):15527–36.

68. Tran HT, Barnich N, Mizoguchi E. Potential role of chitinases and chitin-binding proteins in host-microbial interactions during the development of intestinal inflammation. Histol Histopathol. 2011;26(11):1453–64.

69. Gutiérrez-Román MI, Dunn MF, Tinoco-Valencia R, Holguín-Meléndez F, Huerta-Palacios G, Guillén-Navarro K. Potentiation of the synergistic activities of chitinases ChiA, ChiB and ChiC from Serratia marcescens CFFSUR-B2 by chitobiase (Chb) and chitin binding protein (CBP). World J Microbiol Biotechnol. 2014;30(1):33–42.

70. Hajji S, Younes I, Ghorbel-Bellaaj O, Hajji R, Rinaudo M, Nasri M, et al. Structural differences between chitin and chitosan extracted from three different marine sources. Int J Biol Macromol. 2014;65:298–306.

71. Chan T-F, Ji K-M, Yim AK-Y, Liu X-Y, Zhou J-W, Li R-Q, et al. The draft genome, transcriptome, and microbiome of Dermatophagoides farinae reveal a broad spectrum of dust mite allergens. J Allergy Clin Immunol. 2015;135(2):539–48.

72. Salazar F, Sewell HF, Shakib F, Ghaemmaghami AM. The role of lectins in allergic sensitization and allergic disease. J Allergy Clin Immunol. 2013;132(1):27–36.

73. McCall C, Hunter S, Stedman K, Weber E, Hillier A, Bozic C, et al. Characterization and cloning of a major high molecular weight house dust mite allergen (Der f 15) for dogs. Vet Immunol Immunopathol. 2001;78(3-4):231–47.

74. Altmann F, Staudacher E, Wilson IBH, März L. Insect cells as hosts for the expression of recombinant glycoproteins. Glycoconj J. 1999;16(2):109–23.

75. Lopuhaa CE, Out TA, Jansen HM, Aalberse RC, van der Zee JS. Allergen-induced bronchial inflammation in house dust mite-allergic patients with or without asthma. Clin Exp Allergy. 2002;32(12):1720–7.

76. Pulsawat P, Theeraapisakkun M, Nony E, Le Mignon M, Jain K, Buaklin A, et al. Characterization of the house dust mite allergen Der p 21 produced in Pichia pastoris. Protein Expr Purif. Elsevier Inc.; 2014;101:8–13.

77. Schulz C, Farkas L, Wolf K, Krätzel K, Eissner G, Pfeifer M. Differences in LPS-induced activation of bronchial epithelial cells (BEAS-2B) and type II-like pneumocytes (A-549). Scand J Immunol. 2002;56(3):294–302.

78. Hoffmann E, Dittrich-breiholz O, Holtmann H, Kracht M. Multiple control of interleukin-8 gene expression. 2002:72.

APPENDIX

Published Abstract in *Allergy* (69 Suppl. 99) 457. No1251.

1249
Influences of environmental triggers and lifestyle on the development of allergic sensitisations

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Background: The number of patients suffering from allergic diseases has been increasing in the last decades, especially in industrialized countries. However, the underlying reasons for this development still remain unclear. The aim of this study is to investigate the influences of environmental triggers and lifestyle on the development of an allergic sensitisation.
Methods: The study was conducted within a randomized cohort of 450 Austrian pupils aged 13–19 years. Schools in different geographical regions were involved, enrolling subjects from rural, urban and alpine areas. Living conditions were surveyed with a detailed questionnaire. Demographic data, self-reported health status including allergies, and other lifestyle conditions (diet, sports, alcohol consumption and smoking) were requested from all participants. In addition, blood samples were collected from each subject and analyzed for IgE sensitisation using the ImmunoCAP ISAC system.
Results: The influence of the geographical region is evaluated regarding general health condition and the development of allergies. Clinically confirmed allergies were declared by 23% of the subjects. Fourty-two percent stated to suffer from any allergy including self-reported adverse reactions. IgE reactivity to 112 different purified allergens is categorized according to allergen sources and number of sensitisations and statistically correlated with data obtained from surveyed living conditions.
Conclusion: The study investigated both, allergic and non-allergic subjects of young age. Therefore, the comparison allowed the determination of positive and negative influence parameters for the development of an allergic sensitisation.

The study was funded by Sparkling Science, a program of the Federal Ministry of Science and Research, Vienna, Austria.

1250
Characterisation of mite allergens for house dust mite allergy therapy – specific IgE antibody reactivities to group 1 and group 2 allergens

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Introduction: The aim of this study was to investigate whether concurrent IgE reactivities to group 1 and group 2 allergens of *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* are based on cross reactivity or arises from independent sensitisation.
Methods: In total, 381 serum samples were tested for specific IgE to extracts of *D. pteronyssinus*, *D. farinae*, recombinant group 1 allergens rDer p 1.0105 S54G and rDer f 1.0107 N53W, and group 2 allergens rDer p 2.0101, rDer p 2.0114, rDer f 2.0106, rDer f 2 0108 and rDer f 2.0103. The IgE antibody levels were measured using extract-coated paper disks, purchased from Omega Diagnostics (Reinbek, Germany). Paper disks coated with Der p 1, Der f 1, Der p 2 or Der f 2 isoallergens were prepared at Allergopharma.
Results: Of these 381 sera, 318 sera had specific IgE to any of the test antigens, and 304 sera had IgE to any of the two group 1 and five group 2 isoallergens tested. Of these 304 sera, 232 (76.3%) had IgE to at least one group 1 allergen, and the majority (n = 182, 78.4%) reacted to both Der p 1 and Der f 1. Exclusive reactivity to Der p 1 and Der f 1 was observed in 36 (15.5%) and 14 (6.0%) sera, respectively. All these sera with exclusive IgE reactivity to either Der p 1 or Der f 1 had low specific IgE (EAST class $\leq$3) to these allergens. Of the 304 sera with IgE to any of the two group 1 and five group 2 allergens tested, 293 (96.4%) had IgE to any of the two Der p 2 and the three Der f 2 isoforms. Of those 293 sera 290 (99.0%) reacted to any Der p 2 isoform and 292 (99.7%) reacted to any Der f 2 isoform. 284 (96.6%) of these 293 sera had similar IgE levels (=identical EAST classes) to all five group 2 isoforms of both mite species.
Conclusion: The observed similarities in IgE reactivity patterns of group 1 and group 2 isoallergens irrespective of species indicate allergen cross-reactivity rather than independent, species-specific sensitisation.

1251
Proteomic analysis of the house dust mite allergen Der p 23 produced in *Pichia pastoris*

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Background: Recombinant forms of house dust mite (HDM) allergens are very useful compounds not only for the component-resolved diagnosis of HDM allergy, the development of new immunotherapeutic treatment but also for the characterisation of their allergenicity. The objective of this study is the production and characterisation of the recently identified HDM allergen Der p 23 using the *Pichia pastoris* expression system.
Method: Total mRNA from *D. pteronyssinus* were extracted and retrotranscripted to amplify the cDNA encoding mature Der p 23 by PCR. The amplicon was cloned into the expression vector pPICZαA. *P. pastoris* KM71 strain was transformed with the recombinant plasmid and rDer p 23 expression was subsequently induced by methanol. The recombinant allergen was purified to homogeneity by cation-exchange and gel filtration chromatographies. Proteomic analysis of rDer p 23 was performed using MALDI-TOF MS and nanoLC-MS.
Results: Mature rDer p 23 was successfully expressed under a secreted form following induction with 2% methanol at 30°C. Optimisation of the induction time evidenced that the highest expression level was reached after 48 h whereas protein truncated forms were clearly detected for longer induction period. MS analysis of purified intact rDer p 23 showed the presence of two disulfide bridges as well as several hexose residues ranging from 8 to 16 units. By contrast, truncations of rDer p 23 occured at the N-terminus residues T_{16} and E_{18}, leading to the loss of putative O-glycosylations sites (T_9–T_{11} and T_{15}–T_{17}). The absence of sugar residues in truncated rDer p 23 suggesting that intact rDer p 23 is O-glycosylated.
Conclusion: rDer p 23 produced in *P. pastoris* could be appropriate for future characterisation of its allergenicity but also for HDM allergy diagnosis as well as future recombinant allergen-based specific immunotherapy.

Poster Presented in EAACI Congress 2014, Copenhagen, Denmark.

#TPS54: Proteomic analysis of the house dust mite allergen Der p 23 produced in *P. pastoris*

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STALLERGENES

Background
Recombinant forms of house dust mite (HDM) allergens are very useful compounds not only for the component-resolved diagnosis of HDM allergy, the development of new immunotherapeutic treatment but also for the characterization of their allergenicity. The objective of this study is the production and characterization of the recently identified HDM allergen Der p 23 using the *Pichia pastoris* expression system.

Methods
Total mRNA from *D.pteronyssinus* were extracted and retrotranscripted to amplify the cDNA encoding mature Der p 23 by PCR. The amplicon was cloned into the expression vector pPICZαA. *P.pastoris* KM71 strain was transformed with the recombinant plasmid and rDer p 23 expression was subsequently induced by methanol. The recombinant allergen was purified to homogeneity by cation-exchange and gel filtration chromatographies. Proteomic analysis of rDer p 23 was performed using MALDI-TOF MS and nanoLC-MS.

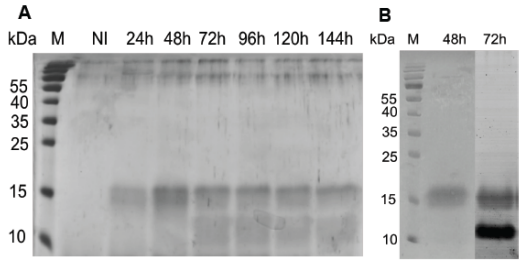

Figure 1. rDer p 23 expression and purification from transformed *P. pastoris* KM71
Panel A. Der p23 expression profile after 24, 48, 72, 96, 120, and 144h methanol induction . NI: non induced . The secretion of expressed rDer p 23 was monitored by SDS-PAGE and coomassie blue staining. Panel B: Purified rDer p 23 from 48h and 72h induction (after a combination of ion exchange and gel filtration chromatographies).

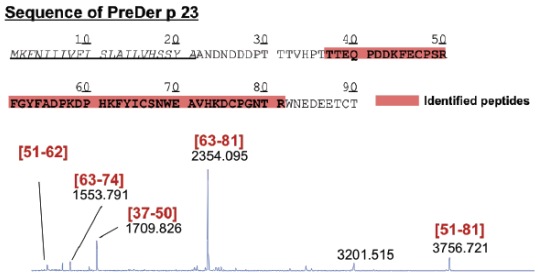

Figure 2. MALDI-TOF MS analysis of rDer p 23 tryptic digest.
Underlined sequence is the natural leader sequence of Der p 23. The identified peptides were highlighted in red.

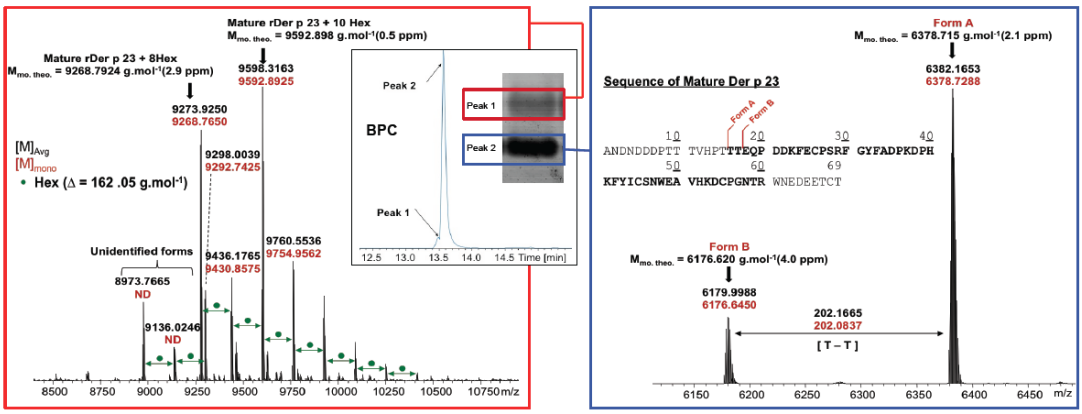

Figure 3. nanoLC-MS analysis of rDer p 23.
Base peak chromatogram (BPC) [400-1200] of purified rDer p 23 (72h) shows 2 peaks at 13.5 mins (Peak 1) and 13.6 mins (Peak 2). Deconvoluated mass spectra of peak 1 (Red box) shows that mature form of rDer p 23 was identified with several hexose ranging from 8-16 units while peak 2 (Blue box) shows two truncated forms of rDer p 23 (Form A and B) with no hexose detected. Purified rDer p 23 (48h) has similar profile as peak 1 (data not shown).

- rDer p 23 was expressed as secreted form the yeast *P.pastoris.*
- The highest expression level was reached after 48h whereas protein truncated forms were clearly detected for longer induction period.
- MS analysis of purified mature rDer p 23 showed the presence of two disulfide bridges as well as several hexose residues ranging from 8-16 units. By contrast, truncations of rDer p 23 occured at the N-terminus residues T_{16} and E_{18}, leading to the loss of putative O-glycosylations sites (T_9-T_{11} and T_{15}-T_{17}). The absence of hexose residues in truncated rDer p 23 suggesting that rDer p 23 is O-glycosylated.

Conclusion
rDer p 23 produced in *P.pastoris* could be appropriate for future characterization of its allergenicity but also for HDM allergy diagnosis as well as future recombinant allergen-based specific immunotherapy.

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In relation to this presentation, I declare that there are no conflicts of interest.

VITA

The author, Mr. Soh Wai Tuck is a Malaysian born in April 22nd, 1989 in Kuala Lumpur, the capital city of Malaysia. Since he was a kid, he is passionate about science and ambitious to become a great scientist. After completing his high school in 2006, he took a diploma course in Biotechnology in Technology Park Malaysia College, Kuala Lumpur. There he met his best friend Mr. Saranpal Singh, who shares a strong interest and passion in science. Upon completed his diploma in Biotechnology with distinction, together with his best friend, they continued their Bachelor studies in Biotechnology in UCSI University in Kuala Lumpur. There he met his inamorata, Ms. Chiam Nyet Cheng. In 2012, he obtained his Bachelor degree with first class honour and was immediately offered a research assistant position in the same University. In 2013, he was awarded a scholarship from Chulalongkorn University to pursue his Master in Medical Sciences in Bangkok, Thailand under the supervision of his mentor Assoc. Prof. Alain Jacquet and Prof. Kiat Ruxrungtham. In 2015, he completed his two years study in the field of molecular allergology and produced this thesis.