MIMOTOPE IDENTIFICATION FROM MONOCLONAL ANTIBODIES SPECIFIC TO HOUSE DUST MITE, USING PHAGE DISPLAYED RANDOM PEPTIDE LIBRARIES

NIPAPORN TEWAWONG

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIRMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(TROPICAL MEDICINE)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2006

ISBN

This thesis
Entitled

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was submitted to the Faculty of Graduate Studies, Mahidol University for the degree of Master of Science (Tropical Medicine)
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ACKNOWLEDGEMENTS

The successful of this thesis can be attributed to the extensive support and assistance from my major advisor, Assist. Prof. Pongrama Ramasoota and my co-advisor, Assist. Prof. Thareerat kalambaheti and Lecturer Suwalee Worakhunpiset. I deeply thank them for their valuable advice and guidance in this research.

I wish to thank Assoc. Prof. Pattama Ekpo, Department of Immunology, Faculty of Medicine, Siriraj Hospital, Mahidol University, for kindness in providing monoclonal antibodies sample to use in this research and suggestions for improvement, and who was the chair of the thesis defense. I wish to express my deep vote of thanks to my respected external examiner, Assoc. Prof. Sathaporn Jittapalapong, Department of parasitology, Faculty of Veterinary Medicine, Kasetsart University.

I would like to express my special thank Assist. Prof. Paron dekumyoy, Department of Helminthology, Faculty of Tropical Medicine, Mahidol University, for kindness in examining the research laboratory and providing suggestions for improvement in this research. Best wishes and thanks to co-workers in molecular laboratory, Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University, for their entire time pleasant and taking care during my laboratory performances.

I must express my sincere thanks to DAAD scholarships Granted for Academic Year 2006/2007, SEAMEO-TROPMED, Thailand, for provide financial support.

Finally, I am grateful to my family for their financial support, entirely care and love. I deeply thank my friends, especially Miss Issariya Ieamsuwan and someone special for their kindness suggestions, warmly care and sweetly love. The usefulness of this thesis, I dedicate to my father, my mother and all the teachers who have taught me since my childhood.

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ABSTRACT

Phage display is a powerful technique for identifying peptides or interesting proteins that have desirable binding properties. In this study, random heptapeptide T7 with cysteine flanking library, and random 12mer M13 phage library, were used to identify mimotopes that bind to six monoclonal antibodies (MAbs) specific to the house dust mite, respectively. T7 and M13 phage that bind to MAbs were selected and amplified by biopanning process. The select bound phages were tested for binding specificity against MAbs by indirect ELISA and Western blot. The DNA of the selecting bound phages was amplified and sequenced by PCR and DNA sequencing. Selected bound phages T7/B4 were Western blot positive with MAb specific to the house dust mite and with positive band for molecular weight 42.6 kDa. Altogether, 47 mimotopes were aligned with the similar peptide allergen, using the Structural Database of Allergenic Proteins (SDAP). Eight mimotopes (17.4%) that partially matched Der f 15 allergen predominated followed by Eur m 14 allergen (13.04%) and Per a 3.0202 allergen (10.87%). Various mimotopes matched with Der f 15; Eur m 14 and Per a 3.0202 were aligned. Especially Der f 15 allergen, the amino acid regions 411-429 and 480-503, correlated with overlapping mimotopes and seemed to be the main epitope clusters of the Der f 15 allergen peptide. The alignment of the sequences obtained from T7/B3 (3 mimotopes) and T7/B2 (1 mimotope) revealed a common motif **S**X**TP**XX**T**X**YX**D**. The two sequences (Asn66-Leu67, Ser71-Leu72, Glu74-Phe75), one sequence (Thr41-Pro42, Gln46-Gly48) from MAb B2 and (Asn63, Lys65, Ala67) from MAb B1 were located on the surface protein structures of Der p 1 and Der f 2, respectively. Using BLASTP software, the predominant mimotopes were **LTPCDP**, which matched with dumpy CG33196-PB protein of *D. melanogaster*, found in 4 phages (8.5%), followed by the **CLPYE** match with the LD38710p protein of *D. melanogaster*, found in 3 phages (6.3%). The mimotope **PCCP** matched peptide toxin 4 precursor protein of *M. gigas*, mimotopes **TPC**NN**L**K**KR**, **CPCYPKK**, and **GEMEGL,** matched putative salivary secreted protein of *I. scapularis*, and mimotope **LTPCDP** matched with dumpy CG33196-PB protein of *D. melanogaster*, located extracellular at 66.7, 55.6, 44.4, 43.5 and 26.1% of these mimotopes, respectively. Using phage display technique, mimotope from MAbs specific to the house dust mite could be successfully identified.

KEYWORDS: PHAGE DISPLAY / RANDOM PEPTIDE LIBRARY / MIMOTOPE / MONOCLONAL ANTIBODY / HOUSE DUST MITE

114 pp. ISBN 974-04-7731-3

การศึกษาค้นหา MIMOTOPE จากโมโนโคลนอลแอนติบอดีซึ่งจำเพาะต่อไรฝุ่นโดยอาศัยห้องสมุดเปปไทด์แบบสุ่ม (MIMOTOPE IDENTIFICATION FROM MONOCLONAL ANTIBODIES SPECIFIC TO HOUSE DUST MITE, USING PHAGE DISPLAYED RANDOM PEPTIDE LIBRARIES)

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บทคัดย่อ

เทคโนโลยีการแสดงออกของเปปไทด์บนผิวไวรัสเป็นวิธีการที่มีประโยชน์มาก สามารถนำมาใช้ในการค้นหาเปปไทด์หรือโปรตีนที่เราสนใจโดยอาศัยคุณสมบัติการจับกันอย่างเหมาะสมระหว่างโมเลกุลคู่หนึ่ง ในการศึกษาครั้งนี้มีวัตถุประสงค์เพื่อใช้ห้องสมุดเปปไทด์แบบสุ่ม T7 และ M13 ในการค้นหามิโมโทปจากโมโนโคลนอลแอนติบอดี 6 ตัวอย่างซึ่งจำเพาะต่อไรฝุ่น ห้องสมุดเปปไทด์แบบสุ่ม T7 และ M13 ซึ่งจับกับโมโนโคลนอลแอนติบอดีอย่างจำเพาะจะถูกคัดเลือกและเพิ่มจำนวนโดยวิธี biopanning ฟาจที่ถูกคัดเลือกทั้ง 10 ตัวอย่างจะถูกทดสอบการจับกับโมโนโคลนอลแอนติบอดีที่จำเพาะกับไรฝุ่นโดยวิธี ELISA และ Western blot หลังจากนั้นฟาจที่ได้รับคัดเลือกจะถูกเพิ่มจำนวนดีเอ็นเอโดยวิธี PCR และทำการวิเคราะห์เปปไทด์โดยการหาลำดับเบส ฟาจที่ได้จาก T7/B4 ให้ผลบวกกับโมโนโคลนอลแอนติบอดีที่จำเพาะกับไรฝุ่นด้วยวิธี Western blot ซึ่ง band ที่เกิดมีน้ำหนักโมเลกุล 42.6 kDa จากการหาลำดับเบสของฟาจพบว่ามิโมโทปจากฟาจ M13/B6 ซึ่งมีลำดับกรดอะมิโน AER***WGPW***GVHSW มีความคล้ายคลึงกับเปปไทด์ที่จับกับจานพลาสติก ELISA และเมื่อนำมิโมโทปที่ได้ทั้ง 47 ตัวอย่างไปเปรียบเทียบกับเปปไทด์ของสารก่อภูมิแพ้ในฐานข้อมูลของ SDAP พบว่ามิโมโทปจากฟาจส่วนใหญ่มีลำดับกรดอะมิโนบางส่วนที่เหมือนกับลำดับกรดอะมิโนของสารก่อภูมิแพ้ Der f 15 (17.4%) Eur m 14 (13.04%) และ Per a 3.0202 (10.87%) นอกจากนี้เมื่อพิจารณาการเรียงตัวของลำดับกรดอะมิโนของสารก่อภูมิแพ้ Der f 15 พบมิโมโทปจำนวน 5 ตัวอย่างมีการเรียงตัวของกรดอะมิโนที่ตำแหน่ง 411-429 และ 480-503 และลำดับกรดอะมิโนของมิโมโทปที่ได้จากฟาจ T7/B3 (3 มิโมโทป) และ T7/B2 (1 มิโมโทป) มีการเรียงตัวในรูปแบบเดียวกัน คือ **SXTP**XX**T**X**Y**X**D** มิโมโทปจากฟาจ T7/B2 ที่มีลำดับกรดอะมิโน (Asn66-Leu67, Ser71-Leu72, Glu74-Phe75) (2 มิโมโทป) และThr41-Pro42, Gln46-Gly48 (1 มิโมโทป) และมิโมโทปจากฟาจ T7/B1 ที่มีลำดับกรดอะมิโน Asn63, Lys65, Ala67 (1 มิโมโทป) มีตำแหน่งอยู่บนผิวโครงสร้างโปรตีนของสารก่อภูมิแพ้ Der p 1 และ Der f 2 ตามลำดับ จากการใช้โปรแกรม BLASTP พบว่ามิโมโทป LTPCDP และ CLPYE มีลำดับกรดอะมิโนเหมือนกับโปรตีน CG33196-PB (8.5%) และ LD38710p (6.3%) ของแมลงหวี่ ตามลำดับ และจากโปรแกรม PSORT II ที่ใช้สำหรับทำนายจุดกำเนิดและตำแหน่งของโปรตีนที่ถูกหลั่งออกมาจากเซลล์พบว่ามิโมโทป PCCP ซึ่งมีลำดับกรดอะมิโนเหมือนกับโปรตีนตั้งต้นของพิษแมงมุม มิโมโทป TPCNNLKKR, CPCYPKK และ GEMEGL ซึ่งมีลำดับกรดอะมิโนเหมือนกับโปรตีนของน้ำลายเห็บ และมิโมโทป LTPCDP ซึ่งมีลำดับกรดอะมิโนเหมือนกับโปรตีน CG33196-PB ของแมลงหวี่ โปรตีนเหล่านี้หลั่งมาจากภายนอกเซลล์คิดเป็น 66.7, 55.6, 44.4, 43.5 and 26.1% ตามลำดับ จากการวิจัยครั้งนี้เป็นการแสดงให้เห็นถึงศักยภาพในการประยุกต์ใช้เทคโนโลยีการแสดงออกของเปปไทด์บนผิวไวรัสเพื่อค้นหามิโมโทปจากโมโนโคลนอลแอนติ - บอดีที่จำเพาะต่อไรฝุ่น

114 หน้า ISBN 974-04-7731-3

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LIST OF ABBREVIATIONS

Abbreviation	Term
%	Percent
λ	Lambda
ϕ	Zeta
µg	Microgram(s)
µl	Microlitre(s)
µmol/L	Micromoles per liter
^{0}C	Degree(s) Celcius
aa	Amino acid
Asn	Asparagine
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary Deoxyribonucleic acid
CDR	Complementarity determining region (s)
Df	*Dermatophagoides farinae*
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
Dp	*Dermatophagoides pteronyssinus*
ds	Double strand(ed)
E. coli	*Escherichia coli*
ELISA	Enzyme-Linked Immunosorbent Assay
et al	et alibi (and other)
etc.	et cetera (and so forth)
g	gram

LIST OF ABBREVIATIONS

(continued)

Abbreviation	Term
i.e.	id est (that is)
IFN	Interferon
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
K*d*	Dissociation constant
kDa	Kilo Dalton
kg	Kilogram
MAb	Monoclonal antibody (-ies)
mg	Milligram (s)
mM	Millimolar
mmol/L	Millimole(s) per liter
mRNA	Messenger ribonucleic acid
nm	Nanometer
OD	Optical density
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PFGE	Pulse-Field Gel Electrophoresis
pfu	Plaque forming unit (s)
PPD	*p*-phenylene-diamine dihydrochloride
RF	Replication form
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
Sec	second (s)
Ser	Serine

CHAPTER I
INTRODUCTION

The prevalence and morbidity of persistent asthma are increasing worldwide, particularly in densely populated urban centers in technologically advanced societies. (Brunton and Saphir, 2001). These data have been consistent in showing that the current epidemic is due to increased sensitization to indoor, rather than outdoor, allergens. The majorities are derived from cats, molds, cockroaches, and dust mites. Mite allergens are a major cause of allergic diseases such as bronchial asthma, allergic rhinitis and atopic dermatitis (Chapman *et al*., 1983). Like other parts of the world, prevalence of childhood allergic diseases in Thailand, particularly of asthma and allergic rhinitis, has risen sharply over the past decade (Vichyanond, 2002). House dust mites are the most important source of allergens causing sensitization among allergic Thai children. In Thailand, 58.8 % of the asthma patients less than 13 years old are found positive skin test to house dust mite (Tuchinda *et al*., 1987) and 70% allergic Thai children are sensitive to house dust mite (Kongpanichkul, 1996).

The most common dust mite species around the world include *Dermatophagoides pteronyssinus* (Dp), *Dermatophagoides farinae* (Df), *Euroglyphus maynei* (Em) and *Blomia tropicalis* (Bt) (Milian and Diaz, 2004). House dust mite has been produced 19 allergen groups. However Group I allergen, found in feces of house dust mite, have been the most important group to pathogenesis of asthma disease. Group I allergen is composed of Der p 1, cysteine protease enzyme, which is the major allergen of *Dermatophagoides pteronyssinus*. Der f 1 is the majority allergen of *Dermatophagoides farinae*. From previous study, it was found that group I allergen level over 2-20 μg/g dust have been induced allergic reaction and allergic severity of patient have been related to allergen level in patient's home (NIH, 2002).

A nationwide survey indicated that house dust mites are ubiquitous in Thai homes. Despite the authors' earlier finding that mite allergen levels in Thailand of group-I allergen level was 11 μg/g dust, exceeded the recommended international threshold level to induce asthmatic symptoms (10 μg/g dust), mite allergen levels in homes within the Bangkok area are in the modest range (5 μg/g dust) (Vichyanond, 2002). In 1999, the prevalence of asthma Thai children has been increased 7% and prevalence of rhinitic children has been increased 37% among population in Bangkok (Vichyanond *et al*., 1998). From the review data mite allergy is an enormous public health threat for the Thais and a serious problem for children.

Phage display is powerful tool for selecting peptides or proteins with specific binding properties from vast numbers of variants. Its utility lies principally in generating molecular probes against specific targets and for the analysis and manipulation of protein/ligand interactions. It’s the simple definition, phage display is the expression of peptides, proteins or antibody fragments at the surface of phage particles (Smith, 1985; Winter et al., 1994; Kay and Hoess, 1996). Using phage display, libraries of variant nucleotide sequences with diversities of millions or billions may be converted into populations of displayed variant proteins that can then be conveniently screened for desirable properties. Screening of phage display libraries is usually accomplished by an affinity selection (or bio-panning) process during which phage populations are exposed to targets in order to selectively capture binding phage (Hoogenboom, 1997; Hoogenboom *et al*., 1998; Sparks *et al*., 1996). The genotype of each protein phenotype is carried within phage particles, once proteins of interest have been isolated the sequence encoding them can be readily determined and altered in order to manipulate or refine binding properties. Phage display technique has been shown relationship between phenotype characteristic (expression peptide on surface phage) and genotype characteristic (inserted DNA sequence that encoded peptide on surface phage). Phage display technique was used for specificity selection of many target protein in molecular research such as antibody, receptor, and enzyme and used to study protein reaction.

Monoclonal antibodies (MAbs) specific to house dust mite, have been developed by researcher group at Faculty of Medicine, Siriraj hospital, Bangkok, Thailand (Ekpo *et al.*, unpublished data). There is considerable interest in studying the

molecular interaction between antibody and house dust mite antigen. Since the epitope specific to these MAbs have not been revealed, so in this study, it is aimed to search for the epitopes or “mimotopes” (mimetic sequences of the true epitope), reacting with these newly developed MAbs specific to house dust mite, by using the random heptapeptide T7 and Random 12 peptide M13 phage libraries. The identified mimotope, can be directly used as specific antigen in the form of phage itself (that display mimotope), or can be expressed as a free protein in *Escherachia coli (E. coli)* and purified from supernatant providing a ready supply of the mimotope for further studies.

The specific objectives

1. To apply the random heptapeptide with cysteine flanking T7 phage library and Random 12 peptide M13 phage library for identify mimotope from monoclonal antibodies that specific to house dust mite.

2. To compare bound phages sequences with Structural Database of Allergenic Proteins (SDAP).

3. To identify the epitope recognized by MAbs on the Der p 1 and Der f 2 models using the Brookhaven Protein Databank (PDB) and RasMol V2.5 software.

4. To compare bound phage DNA sequences with GenBank protein sequences and predict the localization of each protein in the cell using BLASTP and PSORT II softwares, respectively.

CHAPTER II
LITERATURE REVIEW

PHAGE DISPLAY TECHNIQUE

Phage display is a powerful technique for identifying peptides or proteins that have desirable binding properties. In this method, a peptide or protein is displayed on the surface of a phage as a fusion to a protein that is normally found in the phage particle. The first phage vectors suitable for surface display were made by Smith and coworkers, using filamentous phage (Smith and Scott, 1993). They also developed simple procedures for selecting phage displaying peptides or proteins that bind to particular targets. Such phage can be selected from large libraries of variants. Both the peptide or protein and its coding sequence are selected at the same time, since the displayed peptide or protein responsible for the binding is encoded in the genome of the bound phage. Phage display has been used to identify peptides that bind to receptors, substrates or inhibitors of enzymes, epitopes, improved antibodies, altered enzymes, and cDNA clones, and new applications are continually arising (O'Neil and Hoess, 1995).

Phage display vectors

Bacteriophages are viruses that infect bacterial cells. Many vectors used in recombinant DNA research are phages that infect the standard recombinant DNA host: the bacterium *E. coli*. The phage display system is divided in to lytic phage system and non-lytic phage system. The lytic phage vector when infect into *E. coli* they replicate and kill host cell for moving outside. A member of this kind of lytic phage vectors is λ phage such as T4 or T7. The non-lytic phage vector do not kill host cell for replication, its usually use filamentous phage for vector. The most widely used phage display vector is based on the use of filamentous phage, a bacteriophage that infects male *E.*

coli. Display systems based on other phage were also developed, including display on phage λ heads, T4 or T7. The key feature of recombinant DNA vectors, including phages, is that they accommodate segments of foreign DNA spieces of human DNA, for instance, or even stretches of chemically synthesized DNA. As vector DNA replicates in its *E. coli* host, the foreign insert replicates along with it as a sort of passenger. An expression vector, including a phage-display vector, has an additional feature compared to vectors in general: the foreign DNA is expressed as a protein (Smith and Patrenko, 1997). Phage display, first introduced by George P. Smith in 1985, is a very effective way for producing large numbers of diverse peptides and proteins and isolating molecules that perform specific functions (Azzazya and Highsmith, 2002). This technique can also be used to study protein-ligand interactions (Cesareni, 1992), receptor and antibody-binding sites, and to improve or modify the affinity of proteins for their binding partners.

Phage display was different from conventional expression systems. However, the foreign gene sequence is spliced into the gene for one of the phage coat proteins, so that the foreign amino acid sequence is genetically fused to the endogenous amino acids of the coat protein to make a hybrid fusion protein. The hybrid coat protein is incorporated into phage particles as they are released from the cell, so that the foreign peptide or protein domain is displayed on the outer surface. Phage display involves the expression of proteins, including antibodies, or peptides on the surface of phage. DNA sequences of interest are inserted into a location in the genome of bacteriophage such that the encoded protein is expressed or displayed on the surface of phage as a fusion product to one of the phage coat proteins. After, the first introduction in 1985, phage display has rapidly become one of the most widely used research tools and it has been applied to almost every discipline of biological research. The advantages of phage display to other techniques are its simplicity and low cost. The convenience and low cost of the phage amplification was a key element to the success of phage display technology over other methods.

Bacteriophage

Bacteriophage can be divided to two kinds, the first is filamentous phage such as M13, f1 and fd, and other is λ phage such as T4 and T7. Bacteriophage T7 is a λ phage, icosahedral shape, dsDNA virus (Dunn and Studier, 1983), and no RNA stage. In this study, T7 phage libraries from Novagen's T7 select® Phage Display System will be used. This system is easy to use and has the capacity to display peptides up to about 50 amino acids in size in high copy number (415 per phage), and peptides or proteins up to about 1200 amino acids in low copy number (0.1–1 per phage) or mid-copy number (5–15 per phage).

Table 1. Phage display vector features.

Vector	Use	Display number	Display limit (amino acids)	Host
T7 select®415-1	Peptides	415	40-50 aa	BL21
T7 select1-1	Peptides or Proteins	≤ 1	900 aa	BLT5403
T7 select1-2	Peptides or Proteins	≤ 1	1200 aa	BLT5403

The T7 select415-1b vector for high-copy number display of peptides has been selected for biopanning with *E. coli* strain BL21. Phage assembly takes place in the *E. coli* cytoplasm and mature phage are released by cell lysis. Bacterial host that use for phageT7 replication is *E. coli* strain BL21. Unlike the filamentous systems, peptides or proteins displayed on the surface of T7 do not need to be capable of secretion through the cell membrane, a necessary step in filamentous phage assembly (Russel, 1991). T7 has additional properties that make it an attractive display vector. It is very easy to grow and replicates more rapidly than either bacteriophage λ or filamentous phage. Plaques form within 3 hours at 37 °C and cultures lyse 1–2 hours after infection, decreasing the time needed to perform the multiple rounds of growth usually required for selection. The T7 phage particle is extremely robust, and is stable to harsh conditions that inactivate other phage. This expands the variety of agents that can be

used in biopanning selection procedures, which require that the phage remain infective. T7 is actually an excellent general cloning vector. Purified DNA is easy to obtain in large amounts, a high-efficiency *in vitro* packaging system is available (Son *et al*., 1988), and the DNA is completely sequenced (39,937 bp), so restriction or DNA sequence analysis of clones is quite straightforward.

T7 structure and assembly

T7 is an icosahedral phage with a capsid shell composed of 415 copies of the T7 capsid protein (gene *10*) arranged as 60 hexamers on the faces of the shell and 11 pentamers at the vertices (Steven *et al*., 1986). Attached at the remaining vertex is the head-tail connector (gene *8*), a short conical tail (genes *11* and *12*) and 6 tail fibers (gene *17*) (shown schematically in Fig. 1). The phage assembly process is similar to that of other double-stranded DNA phages (Cerritelli and Studier, 1996). DNA is packaged into a procapsid shell made up of scaffolding protein (gene *9*), capsid protein, the head-tail connector, and an internal protein structure (genes *13*, *14*, *15*, and*16*). The DNA is packaged from linear concatemers, and as the DNA enters the procapsid shell the scaffolding protein is released and a conformational change occurs in the shell to form the mature particle. Tail and tail fibers attach at the headtail connector vertex. The T7 select® Phage Display System uses the T7 capsid protein to display peptides or proteins on the surface of the phage. The capsid protein is normally made in two forms, 10A (344 aa) and 10B (397 aa). 10B is produced by a translational frameshift at amino acid (aa) 341 of 10A, and makes up about 10% of the capsid protein (Condron *et al*., 1991). However, functional capsids can be composed entirely of either 10A or 10B, or of various ratios of the proteins (Rosenberg and Studier, unpublished observations). This finding provided the initial suggestion that the T7 capsid shell could accommodate variation, and that the region of the capsid protein unique to 10B might be on the surface of the phage and could be used for phage display.

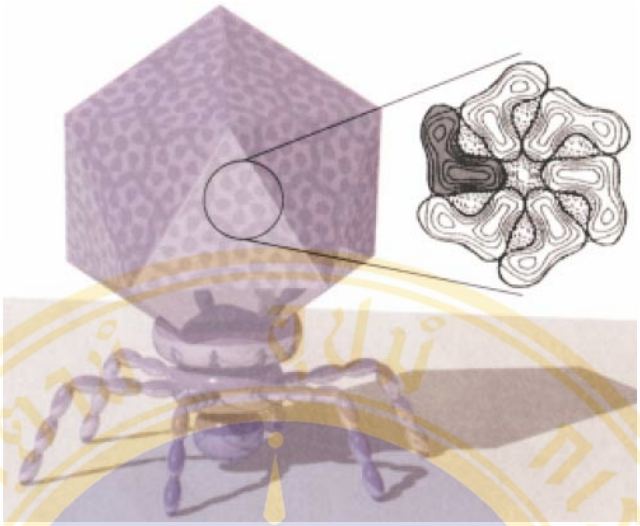

Fig. 1 Structure of the T7 phage particle**.** The capsid shells, head-tail connector, tail, and tail fibers are shown schematically. The diffraction pattern from polyheads showing a hexamer capsid unit has been fit onto the surface of the icosahedral particle (diameter approx. 55 nm). The monomer units are in gray (Adopted from Rosenberg *et al*., 1996)**.**

T7 Select vectors

There are two basic types of T7 Select phage display vectors: the T7 select 415 vector for high-copy number display of peptides, and the T7 select1 vectors for lowcopy number display of peptides or larger proteins (Table 1). In all of the vectors, coding sequences for the peptides or proteins to be displayed are cloned within a series of multiple cloning sites following aa 348 of the 10B protein (Figs. 2 and 3). The natural translational frameshift site within the capsid gene has been removed, so only a single form of capsid protein is made from these vectors. Functional peptides up to 39 amino acids have been displayed from T7 select 415. Expression of the T7 select 415 capsid gene is controlled by the same strong phage promoter (ϕ*10*) and translation initiation site (s*10*) as in wild-type phage (Fig. 3) and the capsid/peptide fusion protein is produced in large amounts during infection.

T7Select415-1b, T7Select1-1b

aa348 aa363

...Met Leu Gly Asp Pro Asn Ser Ser Ser Val Asp Lys Leu Ala Ala Ala Leu Glu

...ATGCTCGGGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGTAACTAGTTAA

*Bam*H I *Eco*R I *Sac*I *Sal* I *Hin*d III *Not*I *Xho* I

T7Select1-2a

aa348 aa368

...Met Leu Gly Gly Ser Asp Ile Glu Phe Glu Leu Arg Arg Gln Ala Cys Gly Arg Thr Arg Val Thr Ser

...ATGCTCGGTGGATCCGATATCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGTAACTAGTTAA

*Bam*H I *Eco*RV *Eco*R I *Sac*I *Sal* I *Hin*d III *Not*I *Xho* I

T7Select1-2b

aa348 aa365

...Met Leu Gly Asp Pro Ile Ser Asn Ser Ser Ser Val Asp Lys Leu Ala Ala Ala Leu Glu

...ATGCTCGGGGATCCGATATCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGTAACTAGTTAA

*Bam*H I *Eco*RV *Eco*R I *Sac*I *Sal* I *Hin*d III *Not*I *Xho* I

T7Select1-2c

aa348 aa366

...Met Leu Gly Ile Arg Tyr Arg Ile Arg Ala Pro Ser Thr Ser Leu Arg Pro His Ser Ser Asn

...ATGCTCGGGATCCGATATCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGTAACTAGTTAA

*Bam*H I *Eco*RV *Eco*R I *Sac*I *Sal* I *Hin*d III *Not*I *Xho* I

Fig. 2 T7 Select vector cloning regions. All vectors contain a multiple cloning site following 10B aa 348. The T7 select1-2a, b, c series contains the cloning sites in all three reading frames and includes a blunt-end site (*Eco*R V).

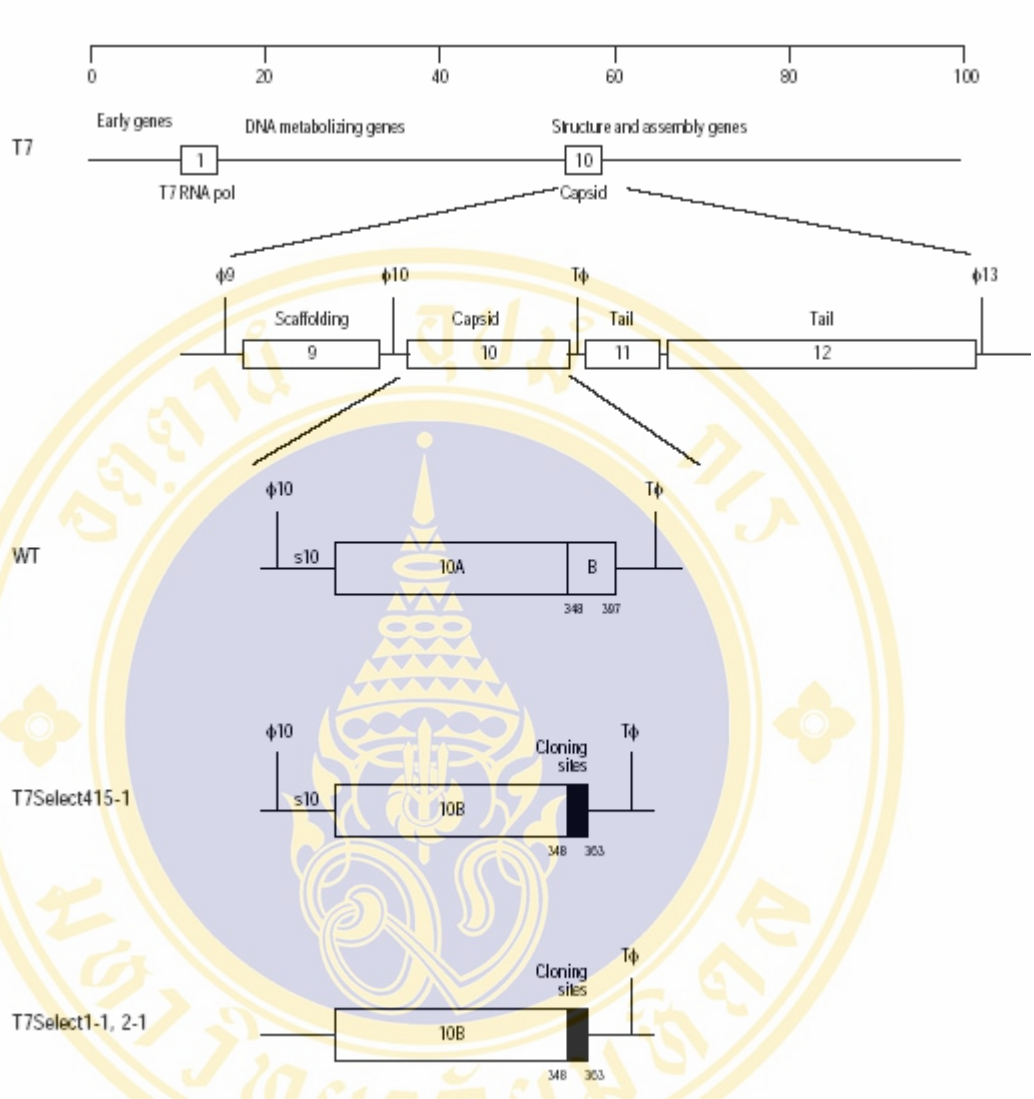

Fig. 3 Phage display vectors and the genetic map of T7. The T7 capsid gene (gene *10*) is located at about position 60 in the T7 genome, within the region of genes coding for proteins involved in the structure and assembly of T7. Capsid protein expression during infection is controlled by a promoter (φ*10*) and terminator (Tφ) for T7 RNA polymerase, and by string translation initiation signals (s*10*). The capsid protein is normally made in two forms, 10A (344 aa) and 10B (397 aa), related by a translational frameshift at 10A aa 341. The T7 select415 and T7 select1 vectors contain a multiple cloning site following aa 348 of a 10B gene that is in a single reading frame, i.e., only the truncated 10B form is made from these vectors. Capsid protein expression from T7 select415 vectors is controlled as in the wild-type phage.

T7 select415 clones usually grow well on normal T7 laboratory hosts, such as *E. coli* BL21. The capsid shell of the phage is composed entirely of the capsid/peptide fusion protein, thereby displaying 415 copies of peptide on the surface of the phage. High copy number display is desirable wherever a strong signal is useful, such as in epitope mapping. It may also be important for obtaining peptides that at best bind only very weakly to their targets. Functional proteins up to slightly more than 1000 amino acids have been displayed from T7 select®1-1 vectors. The T7 select1- 2a, b, c series provides multiple cloning sites in all three reading frames. Peptides or proteins are displayed in low copy number (about 0.1–1 per phage) from these vectors, which makes them suitable for the selection of proteins that bind strongly to their targets. In order to obtain low-copy display, the promoter of the capsid gene was removed and the translation initiation site was altered (Fig. 3). The capsid mRNA is still produced from phage promoters located further upstream of the gene, but production of capsid protein is greatly reduced. T7 select1 phages are grown on a complementing host (BLT5403) that provides large amounts of the 10A capsid protein from a plasmid clone. The 10A gene in the complementing plasmid and the capsid gene in the vectors have been engineered to minimize any recombination between the genes.

The phage T7 library construction was started by synthesizing the random heptapeptide inserted DNA. The inserted DNA was derived from degenerated oligonucleotides, which was synthesized chemically by adding mixtures of nucleotides to growing nucleotide chain. The synthetic oligonucleotides were designed to give a seven-residue long random amino acid sequence flanked by cysteine residues. To limit the occurrence of in-frame stop codons, the degenerated sequence of NNKNNKNNKNNKNNKNNKNNK was used; each N is an equal mixture of A, G, C and T, each K is an equal mixture of G and T. For each NNK, the mixture of 32 nucleotide triplets can be formed, include codons for all 20 natural amino acids and one stop codon (TAG). Each synthesized oligonucleotide was ligated to T7 vector arm. Target peptides were expressed as fusion to the C-terminus of the 10B capsid protein and were displayed on the virion surface, where they were accessible for interaction with other proteins or ligands. The displayed peptide was situated between cysteine residues, and therefore, formation of a disulfide bridge would join the ends of the heptapeptide. The fusion polypeptide is present in 415 copies on each phage

particle. It had an original size of 3.3 x 10^7 pfu/ml but before use it was amplified to a titer of 2.6 x 10^{10} pfu/ml.

Cloning in T7 select 415-1b Vectors Arms and Ligation

T7 select vectors arms are prepared and ligated with target inserts, incubated with an *in vitro* packaging extract, and the phage products used for infection of a suitable host. The T7 select 415-1b vectors as prepared *Eco*RI/*Hind*III arms, ready for directional cloning of appropriately prepared inserts. To provide compatibility with the vector arms and obtain expression in-frame with the 10B protein such that recombinant fusion proteins are displayed, inserts must contain correctly designed termini (Fig.4). The inserts require a 5'-AATT "sticky end" on the top strand (amino-terminal side) and a 5'-AGCT "sticky end" on the bottom strand, either created with oligonucleotides or by *Eco*RI/*Hind*III digestion (Fig.5).

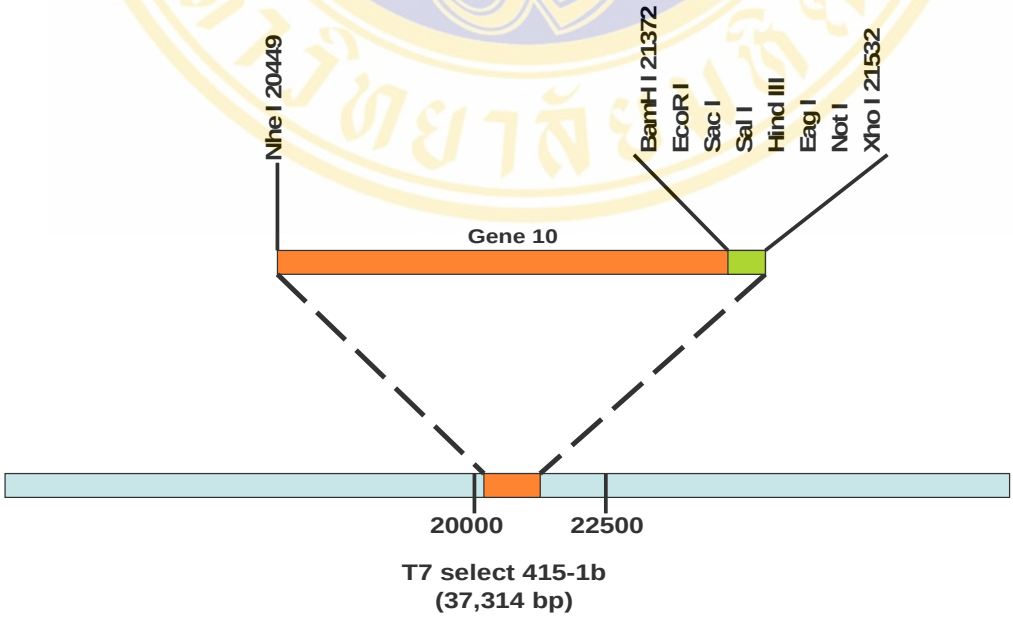

Fig. 4 The location of gene 10B on phage DNA.

gene 10B → insert
left arm...GATCCG AATTXXXX(N)XXXX AGCTT...right arm
left arm...CTAGGCTTAA XXXX(N)XXXXTCGA A...right arm
...AspPro AsnPhe...
AsnLeu...
AsnSer...
AsnTyr...
AsnCys...
AsnTrp...

Reading frame of inserts cloned into *Eco*R I/*Hin*d III T7 select415-1b, T7 select10-3b, and T7 select1-1b vector arms

Fig. 5 The insertion of a 5'-AATT "sticky end" on the top strand (amino-terminal side) and a 5'-AGCT "sticky end" on the bottom strand.

The reading frame requires the AAT (Asn) initial codon, followed by a TXX codon. Use of EcoR I cleavage products places C in the second position, resulting in a TCX codon (Ser). Because the arms are dephosphorylated, inserts should be phosphorylated prior to ligation. For maximal cloning efficiency, it is useful to optimize insert: vector ratios for each insert preparation. The optimal ratio will vary depending on the nature and quality of the insert, but the highest cloning efficiencies are typically obtained with molar ratios between 1:1 and 3:1 (insert: vector). Insert preparations should be free of interfering substances (salts, primers, nucleotides, etc.). Inserts prepared by annealing purified oligonucleotides are suitable for use without further treatment.

The filamentous bacteriophage

The Ff class filamentous phages, including strains M13, f1 and fd, are about 1 mm in length. The phage contains a circular ssDNA genome, which is wrapped inside several thousand copies of the major coat protein pVIII. For a wild type phage genome of 6408 NT, there are 2670 copies of pVIII. At the "head" region of the phage, there are 5 copies each of pIII and pVI, important for host cell binding. At the other end or the "tail" region (Fig. 6), there are 5 copies each of pVII and pIX that are required for

the initiation of phage assembly and for the maintenance of phage stability (Rodi *et al.*, 1998).

As shown figure 6, the life cycle starts by binding of phage, via pIII protein, to the cell receptor for the initiation of infection. Only male *E. coli* strains, i.e., F-plus strains or cells harboring an F episome, are susceptible to infection, although F-minus strains are still capable of producing phage particles once the phage genome is introduced into the cells by other means such as chemical transformation or electroporation (Wilson *et al*, 1998; Rasched *et al*, 1986). Once the single stranded genome (the + strand) is injected into the cell, a complementary (-) strand is synthesized by the host polymerase to form a double stranded phage genome called the replication form (RF). Following this, host mediated protein synthesis results in the production of all ten phage-encoded proteins, including structural proteins (coat proteins pIII, pVI, pVII, pVIII and pIX), proteins for assembly and export (pI and pIV), and proteins for replication (pII, pV and pX). The grouping of gene clusters into two regions regulates levels of expression. Genes for proteins required in large quantities (e.g., pVIII) are preceded by a strong promoter while genes coding for other proteins (e.g., pIII) are controlled by a less efficient promoter.

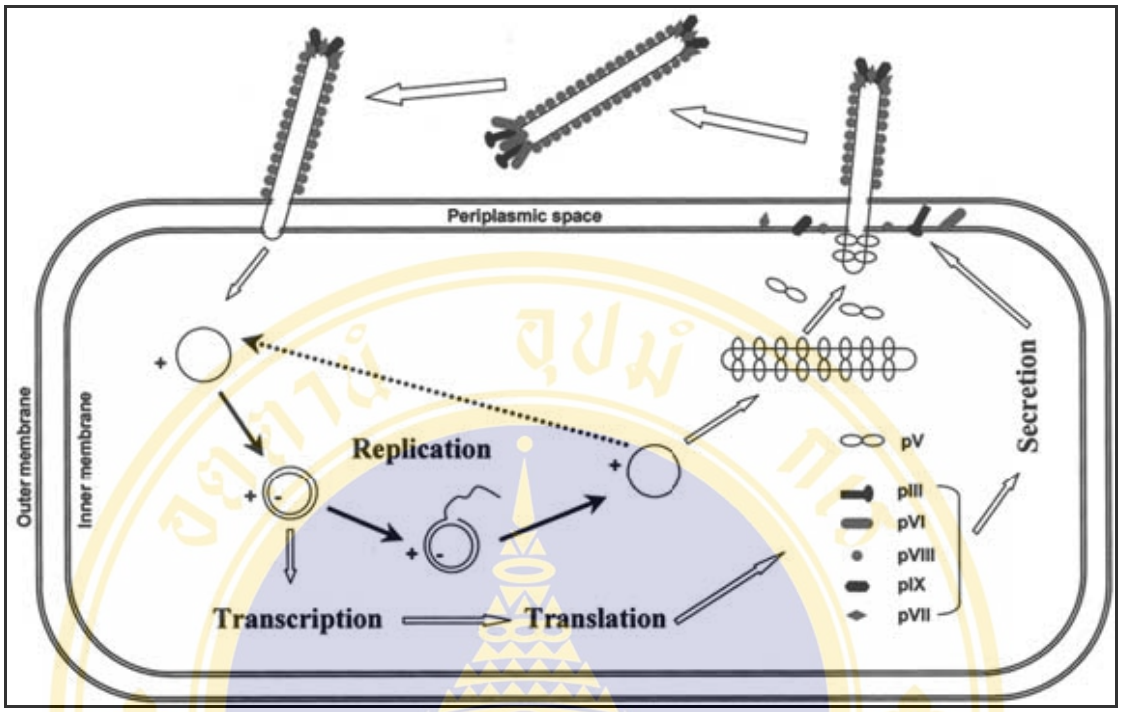

Fig. 6 Life cycle of Ff filamentous phage. The drawing is highly schematic and the scale not proportional. Each of the five different structural proteins is represented in the figure as shown, but not in the actual stoichiometry of the mature virion. The circles represent viral DNA genome, the sense of the single stranded genome is shown (+ or -), and the double stranded replication form (RF) is shown as a double circle (Adopted from Wang *et al*, 2004).

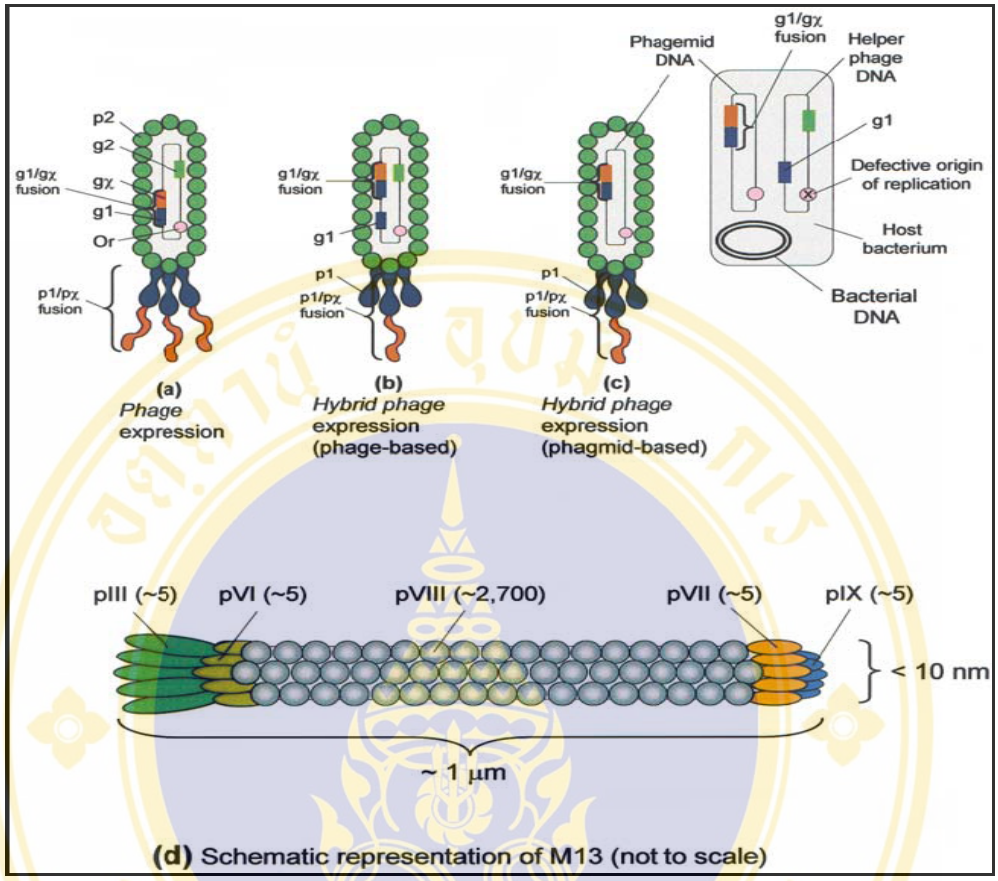

Fig. 7 Schematic representations of M 13 (not to scale) (Adopted from Willats, 2002).

As shown in figure 7**,** Phage display formats (a)–(c) strategies for the expression of proteins at the surface of a simplified hypothetical bacteriophage (see also Figure 1). (a) The simplest format for the expression of a peptide or protein is to fuse the gene (gχ) encoding the foreign protein (pχ) to one of the phage coat protein genes (e.g., g1) (see also Figure 1). This strategy produces phage particles in which all the copies of chosen phage coat protein are fusion proteins (p1/pχ). (b) Hybrid phage may be created by incorporating the gene fusion (g1/gχ) as an additional element in the phage genome. With this arrangement, two versions of the phage coat protein chosen as the fusion partner are encoded - one by the native gene (p1) and one by the fusion gene (p1/pχ). As phage particles are assembled both p1 and p1/pχ are incorporated into the phage coat. (c) Phagemid based systems are also widely used to construct hybrid phage. However, instead of being present on a single genome, the

genes encoding wild type coat protein and fused protein are carried by helper phage and phagemid respectively. Host bacteria contain both phagemid and helper phage DNA and both genomes contribute to the synthesis of hybrid phage particles. (d) M13 bacteriophage are widely used as vehicles for phage display. The pIII coat protein can be used as a fusion partner for a limited number (maximum of five) of proteins while thousands of proteins can be expressed at the phage surface if pVIII is used as a fusion partner. The approximate number of copies of each M13 coat protein is indicated (Willats, 2002).

Phage display library

Phage display library is a heterogeneous mixture of such phage clones, each carrying a different foreign DNA insert and therefore displaying a different peptide on its surface (Smith and Petrenko, 1997). Each peptide in the library can replicate, when the phage attaches and infects a fresh bacterial host cell, it multiplies to produce a huge crop of identical progeny phages displaying the same peptide. The mutation of the phage's DNA at the peptide coding sequence, the mutated DNA is passed on to the phage's progeny and can affect the structure of the peptide. Because of phage display peptide accessibility to solvent, a displayed peptide often behaves essentially as it would if it were not attached to the virion surface. All many techniques that apply to compounds free in solution can be applied more or less unaltered to peptides tethered to a phage. The affinity purification, in which an immobilized receptor is used to specifically capture ligands from a complex mixture of compounds, can equally be used to capture phage displaying receptor-binding peptides from a large phage library displaying many different peptide structures. Infecting them into fresh cells and culturing the cells to yield a large crop of progeny phages, which can serve as the input for another round of affinity purification, amplify the captured phages.

The most common type of phage-display constructs is a random peptide library. In constructing random peptide libraries, the peptides are encoded by synthetic oligonucleotides, with each random residue encoded by a degenerate codon. In the degenerate sequence is a mixture of 32 triplets that include codons for all 20 natural amino acids. For example, if constructed 4-residue peptides, the entire 12-base

sequence is an equimolar mixture of over a million (32^4) different molecular species collectively encoding all 160,000 (20^4) possible 4-residue peptides. The major advantage of this type of library is its universal nature. The random peptide libraries can be used for mapping of epitopes recognized by different antibodies, for isolating mimotopes of proteins or carbohydrates (Wang and Yu, 2004). Other phage library is a genomic library, its construct by inserts the fragments of total chromosomal DNA. Thus, all coding sequences in the organism's genetic complement (i.e., genome) are potentially represented among the displayed peptides. Similarly, in cDNA libraries the inserts are DNA copies of mRNAs extracted from some tissue or cell population; again, a huge diversity of coding sequences is potentially represented in a sufficiently large cDNA library. In the remaining constructs, the phages display all or part of a specific peptide or protein domain.

The ability of phage library associated with affinity and avidity. The meaning of affinity is the strength of the binding between a single binding site of a molecule (e.g., an antibody) and a ligand (e.g., an antigen). The affinity of molecule X for a ligand Y is represented by the dissociation constant (K_d), which is the concentration of Y that is required to occupy the combining sites of half the X molecules present in a solution. A smaller K_d indicates a stronger or higher affinity interaction, and a lower concentration of ligand is needed to occupy the sites. Avidity means the overall strength of interaction between two molecules, such as an antibody and antigen. Avidity depends on both the affinity and the valency of interaction. Therefore, the avidity of a pentameric IgM antibody, with 10 antigen binding sites, for a multivalent antigen may be much greater than the avidity of a dimeric IgG molecule for the same antigen. Avidity can be used to describe the strength of cell-cell interaction, which is mediated by many binding interaction between cell surface molecules. Affinity and avidity of phage library have affected to the result of selection and biopanninig or affinity selection. The copy number of phage has affected to the level of affinity and avidity. Wang and Yu (2004) have been described the relation of affinity and avidity with copy numbers of phage. For this relation, the affinity of a specific ligand-selector interaction plays an important role in the overall success of any panning experiment. In the most random peptide libraries, the apparent affinity or the avidity is determined by two factors. First, the absolute affinity of the ligand-selector interaction is crucial

difficult to measure experimentally. Second, the copy number of the peptides displayed per virion. Depending on the display format used, this number can vary from one to several hundred. For most applications, it is desirable to have a ligand with true high affinity, rather than one with apparently high avidity. One way to achieve this is to reduce the copy number by using the phagemid display format "3+3". It should be pointed out that it is now possible to modulate the displayed peptide copy number without having to use a different display format. In the "3+3" display system, the peptide copy number can be adjusted simply by using different helper phage.

The filamentous phage is a mostly used for phage libraries (Smith, 1985). The filamentous phages such as phage Ff class including strains M13, f1 and fd. The filamentous phage contains a circular ssDNA genome, which is wrapped inside several thousand copies of the coat protein. The successful of the filamentous phages for epitope mapping of infectious diseases has been demonstrated such as Dengue hemorrhagic fever (Kesakarn *et al.*, 1999), *Neisseria meningitidis* (Grothaus *et al.*, 2000), *Escherichia coli* (Tayapiwatana *et al.*, 2003). The random peptide libraries that constructed in filamentous phage based on coat protein pIII and pVIII. For example, a combinatorial library of random peptide 12-mers fused to minor coat proteins (pIII) of M13 phage. The display peptide 12-mers is expressed at the N-terminus of pIII. A short spacer and then the wild type pIII sequence follow the peptide. The peptide library consists of ~2.7×10^9 electroporated sequence, amplified once to yield ~55 copies of each sequence in 10 µl of the supplied phage. Because of phage M13 is not a lytic phage so plaques are due to diminished cell growth rather than cell lysis and are turbid rather than clear. The M13 coat protein pIII mediates infectivity by binding to the F-pilus of the recipient bacterium. Display of foreign peptide as N-terminus fusion to pIII appears to attenuate infectivity of the library phage relative to wild type M13.

The λ phage is other type of phages that used for construct the random peptide libraries. The λ phage such as T4 or T7, their libraries has been used in many studies. The successful of the λ phage (T7) libraries for epitope mapping of infectious diseases such as *Bordetella pertussis* (Wilson *et al.*, 1998), Polyomavirus (Houshmand *et al.*, 1999), Leptospirosis (Tungtrakarnpoung *et al*, 2006), Hepatitis E virus (Gu *et al.*, 2004), *Plasmodium falciparum* (Casey *et al.*, 2004), their result showed the successful

of the application of λ phage (T7) random libraries. The λ phage is a lytic phage and has difference plaques formation from filamentous phage. Therefore, plaques of λ phage are due to diminished cell lysis than cell growth and are clear rather than turbid.

Selection

Smith has described selection in 1985 (Smith, 1985). Selection is important in phage display for the success of phage library usage. In the meaning of selection, selection consists of culling an initial population of phage-borne peptides to give a subpopulation with increased fitness according to some user-defined criterion. Mostly, the input to the first round of selection is a very large initial library and the selected subpopulation is a tiny fraction of the initial population, fitter clones being overrepresented. This population can be amplified by infecting fresh bacterial host cells, so that millions of copies in the amplified stock represent each individual phage in the subpopulation. The amplified population can then be subjected to further rounds of selection (perhaps accompanied by mutagenesis) to obtain an ever-fitter subset of the starting peptides.

There are two pivotal parameters of selection (Smith and Petrenko, 1997), which can often be manipulated to some extent in order to enhance the efficacy of selection. First, stringency is the degree to which peptides with higher fitness are favored over peptides with lower fitness. Second, yield is the fraction of particles with a given fitness that survive selection. The goal of selection is usually to isolate peptides with high fitness, but this does not mean that stringency should be increased without bound, since increasing stringency usually entails decreased yield. High yield of the fittest clones is importance in the very first round of selection, whose input consists of all clones in a very large initial library. The yield can then safely be decreased in favor of high stringency. These occurring can demonstrated if each clone in the library including the very fittest clones and the yield for the fittest clones is not greater than 1%, such clones have a good chance of being lost and of course can never be recovered. Those clones that do survive the first round of selection are amplified and are thus represented by millions of phages each in subsequent rounds. However, there is a limit to stringency, because in practice selection techniques are imperfect

and there is an unavoidable background yield of all phages regardless of their fitness. If stringency is set too high, the yield of a specifically selected phage will fall far below the background of a nonspecifically isolated phage, and all power of discrimination in favor of high fitness is lost.

Affinity Selection

The most popular selection methods include affinity selection, (also called biopanning) that the most common selection pressure imposed on phage displayed peptide populations is affinity for a target receptor. Affinity selection is ordinarily accomplished by minor modifications of standard affinity purification techniques (Smith and Petrenko, 1997). The experiment that good demonstrated to biopanning, Smith's first selection experiment (Smith, 1985) was performed by coating a polystyrene dish with a polyclonal antibody against the EcoRI endonuclease, and then selectively binding phage that displayed a fragment of the protein via pIII from a pool containing a large excess of phage bearing no insert. Phage expressing functional insert were retained on the plate during extensive washing. They were eluted in acidic conditions (pH 2.2) that denatured the immobilized antibody (allowing release of the phage) but did not affect the integrity of the phage. After neutralization, the eluates were used to infect *E. coli* cells, which amplified the enriched pool of antibody-binding phage. The eluted phages are still infective and are propagated simply by infecting fresh bacterial host cells, yielding an amplified eluate that can serve as input to another round of affinity selection. Phage clones from the final eluate (typically after 2-3 rounds of selection) are propagated and characterized individually. The amino acid sequences of the peptides responsible for binding the target receptor are determined simply by ascertaining the corresponding coding sequence in the viral DNA.

Parmley and Smith (1988) presented an improved selection procedure, which they called biopanning. This procedure entailed biotinylating the selecting molecule, mixing it with phage displaying a targeted peptide or protein fragment, and then capturing the complexes of phage and biotinylated screening molecule on a plate coated with streptavidin (which binds biotin with a 10–14 to 10–15 K_d). Panning

entailed capture of a biotinylated screening molecule; hence the name biopanning. Phage library screening entailed several rounds of selection, with each round followed by a phage-amplification step. Consecutive, multiple rounds of selection without amplification were found to be less effective, because background binding by phage increased significantly after the buffered-acid elution step. In general, low densities of the target receptor favor high stringency and by monovalent display of the foreign peptide; high stringency is almost invariably accompanied by relatively low yield.

Because selected phages are in the end cloned and characterized one by one, it is feasible to use a complex mixture of receptors, rather than a single receptor, to capture phage. For instance, total human serum immunoglobulin, comprising hundreds or thousands of individual antibody specificities, can be used to select a diversity of peptides, each recognized by one of the specificities. This is the basis of epitope discovery, a strategy for identifying diagnostic peptides and synthetic vaccine components. The progress of affinity selection through succeeding rounds is ordinarily reflected in increasing affinity of individual phage clones or of entire eluate populations for the target receptor. The affinity of individual clones or entire eluate populations can be assessed quantitatively by standard enzyme-linked immunosorbent assay (ELISA). Alternatively; a few hundred individual clones from an eluate can be sampled on “plaque lifts” and tested qualitatively for ability to bind the receptor.

Recently, Wang and Yu given the opinion about affinity selection has bias of phage growth competition found within any biological system, i.e., certain phage clones grow much better than others (Wang and Yu, 2004). In an ideal system, the enrichment of specific binders would be solely determined by the affinity of the ligand-selector interaction and the subsequent infection of and amplification in *E. coli* would only increase the total number of each binder selected without changing the ratio of different clones represented. However, it is well documented that growth competition poses a serious challenge in the overall success of phage display technology. In each round of panning, affinity selection is followed by growth competition of the selected phage clones. It is possible that good affinity selected binders are poor growers and will be overgrown by poor binders or non-specific binders which have much better growth characteristics. To solve this problem, several modifications have been reported to minimize this growth competition effect. These

include direct screening of a large number of first round binders using the colony or plaque lift method, reducing rounds of amplification and carrying out statistical analysis on a large number of sequences, replacing liquid medium with solid media (on plate) for amplification and growing individual clones separately and then pooling them together for subsequent panning. All of these methods are labor intensive and tedious to operate. So far there is really no easy alternative to circumvent this obstacle.

HOUSE DUST MITE

In most temperate humid areas of the world, house dust mites are a major source of multiple allergens in house dust. Mite allergens sensitize and induce perennial rhinitis, asthma, or atopic dermatitis in a large portion of patients with allergic disease (Arlian, 2001). The most important house dust mites are *Dermatophagoides pteronyssinus (D. pteronyssinus)* and in drier areas *Dermatophagoides farinae (D. farinae)* (Fig. 8). In subtropical and tropical regions the glycyphagid mite *Blomia tropicalis* is a major source of allergen, which co-exists with *D. pteronyssinus* (Thomas, 2004).

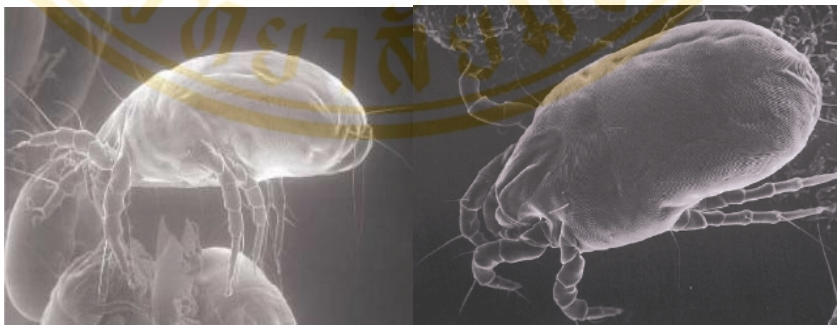

Fig. 8 The important house dust mite are *D. pteronyssinus* (European House Dust Mite) and in drier areas *D. farinae* (North American House Dust Mite)

Classification and relationship to other Arthropods

The phylum Arthropoda is comprised of 3 subphyla of living organisms: Chelicerata, Uniramia, and Crustacea. House dust mites are arthropods belonging to the subphylum Chelicerata, class Arachnida, order Acari, and suborder Astigmata . Other suborders of mites include Mesostigmata, Metastigmata (ticks), Prostigmata, and Oribatida. The chelicerae for the house dust mite are pincer-like and consist of both movable and fixed digits. For comparison, other chelicerates have either stylet-like chelicerae for piercing (eg, spider mites and chiggers) or sickle-like chelicerae for cutting an incision in the host skin (eg, ticks).

Habitat of House dust mite

The dust mite thrives in the modern environment of fully-carpeted, double-glazed, draft-proof homes, and is comfortable at 25 degrees Celsius and 75% relative humidity. The mites are particularly common in carpets and bedding. The mite generally lives on shed human skin cells, which are pre-digested by the fungus *Aspergillus repens*. An average person sheds about 1.5 grams of skin a day (approximately 3-4.5 kg per year), which is enough to feed roughly a million dust mites. Further, dust mites in bedding derive moisture from human breathing, perspiration, and saliva. Many mite species live in bird's nests, in barns, among stored grain, straw, etc. House dust mites are cosmopolitan in distribution with much of the research previously done in Europe. One of the major limiting factors in mite survival and population development is the availability of water for sorption. Highest mite densities occur in the humid summer months and lowest in drier winter periods. Dust mite populations are highest in humid regions and lowest in areas of high altitude and/or dry climates. Due to the large quantity of skin scales sloughed off daily by humans, mites have an abundant food supply. Dust mite antigen levels are measured in bed dust, floor dust, and room air samples. Detection in room air was best during cleaning and bed-making activities.

Distribution of House dust mite

House dust mites belong to the suborder Astigmata and family Pyroglyphidae. However, mites belonging to other families are also present in house dust, and the term domestic mites include both mites from the Pyroglyphidae family or dust mites and mites from other families (Hart, 1998). Astigmatid mites are unusual in that they lack organized respiratory systems and associated external openings for ventilation. They are aerobic and apparently exchange O_2 and CO_2 through their general body surface. They feed principally on skin scales and other organic detritus that collects in homes. The family Pyroglyphidae contains about 16 genera and 46 species (Wharton, 1976; O'Connor, 1982). A phylogenetic perspective, the evolutionary ecology, and the cladistic relationships of families and species of astigmatic mites, can be found in several reports (Norton *et al*., 1992; O'Connor, 1982).

Thirteen species have been found in house dust, 3 of which are very common in homes worldwide and are the major source of mite allergen. The most common of these species are *Dermatophagoides farinae, Dermatophagoides pteronyssinus,* and *Euroglyphus maynei,* which are found in temperate climates. In tropical climates, the storage mite *Blomia tropicalis* (Family Echymyopodidae) can be a prevalent mite in dwellings, along with other Pyroglyphid mites. In addition, other astigmatid mites (storage mites) can be found in homes and are a potent source of allergens. Most notable are species in the families Glycyphagidae *(Glycyphagus domesticus* and *Lepidoglyphus destructor),* Acaridae *(Tyrophagus putrescentiae* and *Acarus siro),* and Chortoglyphidae *(Chortoglyphus ancutatus)*. Predaceous mites (eg, Cheyletus) and parasitic mites of plants (Tetranychidae [spider mites] and Tarsonemidae) can also be present in homes. The significance of these as sources of indoor allergens is yet to be determined.

Prevalence of House dust mites

Worldwide and in the United States, the most prevalent pyroglyphid mites found in homes are *D. farinae* and *D. pteronyssinus. E. maynei* may also be prevalent in some temperate geographic areas, and at times, their density may even exceed that

of *D. farinae* and *D. pteronyssinus. Blomia tropicalis* is an important source of allergen in homes in the subtropics and tropics. In a US study of homes located in 8 different geographic areas (Arlian *et al*., 1992), most houses were coinhabited by *D. farinae* and *D. pteronyssinus.* However, one species usually predominated in each home and constituted greater than 70% of the total mite population. Only a few homes in each geographic area of this study had populations containing one species only. An important discovery was that within the same geographic area, the predominant species present varied between homes. These findings illustrate the need to test for both species when a patient undergoes skin testing and when using immunotherapy.

Like other parts of the world, prevalence of childhood allergic diseases in Thailand, particularly of asthma and allergic rhinitis, has risen sharply over the past decade (Vichyanond, 2002). House dust mites are the most important source of allergens causing sensitization among allergic Thai children. A nationwide survey indicated that house dust mites are ubiquitous in Thai homes. In Thailand, 58.8 % of the asthma patients under 13 years old are positive skin test to house dust mite (Tuchinda *et al.*, 1987) and 70% allergic Thai children are sensitive to house dust mite (Kongpanichkul *et al.*, 1996).

Mechanism of allergy

The type 1 hypersensitivity reaction. IgE produced by B cells is captured at the cell surface by Fc epsilon receptor I (FceRI) present on mast cells and eosinophils. Cross linking of this receptor during subsequent encounter with antigen stimulates release of a variety of toxic products that together elicit atopic disease. The central role of the Th2 cell is evident: stimulated by antigen, these cells produce the IL-4 and IL-13 required for IgE synthesis and the IL-5 required for eosinophil growth and differentiation.

Source of Mite Allergens

Much has been learned about dust mite allergens in the last 20 years. It is clear that mite bodies and mite feces are the sources of many allergens (Arlian *et al.*, 1987;

Tovey *et al.*, 1981). The allergens associated with mite fecal matter are enzymes that originate from the mite's digestive tract. Possible sources of other allergens include enzymes associated with the molting process that occurs as mites change from one life stage to the next. Some allergens may be components of mite saliva that is left in the environment on food substrates where mites feed. Secretions from the supracoxal glands that are involved in the active uptake of water likely contain proteins, as well as sodium and potassium chloride (Wharton and Furumizo, 1977). After death, soluble protein in body fluids may be released as the body disintegrates. Some proteins from all of these sources could be allergenic.

Characteristic of Mite allergens

Mite allergens are divided into specific groups 19 denominated allergens on the basis of their biochemical composition, sequence homology, and molecular weight (Thomas *et al*., 2002). The majorities have enzymatic activity; studies have shown that mite extracts contain a variety of biochemically active enzymes, including trypsin, chymotrypsin, carboxypeptidase A and B, glucoamylase and lysozyme. Marked differences in the relative concentrations of some of these enzymes, particularly trypsin and carboxypeptidase A, occur in different mite extracts (Stewart *et al*., 1992; Stewart *et al*., 1994). Several of these enzymes are physicochemically similar to corresponding enzymes from vertebrate and invertebrate sources. The designation for a characterized allergen is the first 3 letters of the genus, the first letter of the species name, and a number designating the order in which the allergen was isolated or the number for other already characterized allergens it matches in homology and molecular weight. The characteristics of group 1 and 2 allergens are shown in Table 2.

Table 2. Characteristics of group 1 and 2 allergens and percentage of patient reactivity to them

Allergen group	Specific allergens characterized	Molecular weight (kd)	Frequency of reactivity (%)	Homology
Group 1	Der f 1, Der p 1, Eur m 1, Der m 1	25	>90	Cysteine protease, homology similar to enzymes papain, actinidin, cathepsin H and B, bromelain, frein
Group 2	Der f 2, Der p 2, Eur m 2, Tyr p 2, Lep d 2	14	>90	Homology with primate Epididymus protein

Group 1 allergens are glycoproteins with cysteine protease activity similar to that of some plant and mammalian enzymes (Platts-Mills *et al,* 1997; Stewart, 1995). They originate from cells lining the intestinal tract of the mite (Tovey and Baldo, 1990; Thomas *et al.,* 1991). Der p 1 can cleave the CD23 IgE receptor on the membrane of human B cells and the CD25 subunit of the T-cell IL-2 receptor, which enhances its allergenicity (Shakib *et al.*, 1998; Schulz *et al.*, 1998). Der f 1 and Der p 1 have sequence homology of 80% and have cross-reactive epitopes. However, they also have species-specific epitopes (Dilworth *et al.*, 1991). There is some evidence that the group 1 allergens may be released as preproproteins that are activated by gluthathione found in respiratory secretions when deposited on mucus membranes.

Group 2 allergens are 14-kd nonglycosylated proteins that have high sequence homology (Heymann *et al.*, 1989; Lind, 1985). Der f 2 and Der p 2 shows 88% sequence homology. Group 2 allergens seem to be associated with secretion from the male mite reproductive tract (Thomas and Smith, 1998) although Lep 2 is also associated with the gut and other areas of the mite's anatomy (Varela *et al*, 1994). The mAb 1D8 that recognizes Der p 2 but not Der f 2 also binds to Eur m 2 (Morgan et *al.*, 1997). This indicates that Der p 2 and Eur m 2 share an epitope not present on the Der f 2 allergen.

Allergy test

Since avoidance of the provoking allergen/s is the best therapy it is vital to identify the offending allergens accurately as soon as possible. In many cases a careful and systemic history combined with clinical examination may provide useful clues to the nature of the potential allergens. Avoidance based on guesswork may cause more harm to the child or adult in question. Allergy tests should be performed quickly to accurately identify the suspected allergen triggering the symptoms.

There are two types of allergy test available:

(1) Skin Prick Test, and

(2) Allergy Blood Test, based on 'Cap RAST' performed on a blood sample.

Skin Prick Test: In this test small drops of allergens in variable concentration is placed on the forearm and the dermis punctured with a special lancet. The development of a raised area (called wheal) similar to a mosquito bite appears within minutes and the area surrounding the wheal becomes reddened (flare). The response is compared to negative and positive controls run at the same time. A positive reaction for the allergen is confirmed when the swelling reaches a certain size which is bigger than the negative control. This test has many limitations. Skin testing is dangerous in highly sensitive patients and there have been fatalities recorded in the past. Patients must stop taking anti-histamines for at least 7 to 28 days (depending on drug) because the drug suppresses the skin reaction causing the development of a false negative result. The skin test is not useful for babies and the elderly. In general skin tests are not useful indicators for food allergies because of the frequent false positive reactions. Skin testing is being rapidly replaced with allergy blood testing.

Allergy Blood Tests: There are many types of cheap allergy blood testing methods available but most of them have poor reproducibility. Moreover, these tests normally report the results with fancy colorful charts, which appear impressive but provide little information to the professionals. These tests often give false positive or

false negative results that may cause more harm than good. In general, hospitals worldwide do not use these types of allergy testing methods but use the cap RAST technique as the only method of choice.

The Cap RAST method uses a state-of-the-art technique that is accepted world wide as the in-vitro Gold Standard. Moreover, the IgE level estimated by the equipment is standardized against the World Health Organization immunology standards for IgE maintained by them. The Cap RAST reports the results in both quantitative and qualitative terms for professional assessment. Therefore, the allergy blood test using the cap RAST is the method of choice because it is sensitive, accurate, safer, convenient and unaffected by medication taken by the patient. The allergy blood test can be performed in babies and elderly and in persons with severe eczema in whom it is difficult to do the skin test.

The choice of the allergens selected for test depends on the clinical history. Usually the doctor may select about 12 to 15 allergens based on his experience but in some cases he may select less allergen for testing. Skill and experience is required to interpret the result and in most cases the test results often fit the clinical observation and confirm the diagnosis. Appropriate avoidance measures should be immediately implemented. The benefits of the avoidance measures usually become apparent within the first few days.

Treatment

The three (3) basic treatments for dust mite allergy are;

1. Dust mite avoidance
2. Medicines prescribed by your doctor
3. Immunotherapy (allergy shots).

As one might expect, reduction of the dust mite allergen or avoidance works best to relieve symptoms.

THE APPLICATIONS OF RANDOM PEPTIDE PHAGE DISPLAYED LIBRARY IN ALLERGY RESEARCH

Random peptide libraries may potentially cover the sequence or structural diversity of many allergens. They are self renewing, inexpensive and information can be generated within days. Peptide epitopes can also be offered in constrained or unconstrained forms so that mimotopes of discontinous epitopes can be identified (Luzzago *et al*., 1993; Felici *et al*., 1993). Once the mimotope is identified, it can be expressed as a free protein in *E. coli* and purified from supernatant providing a ready supply of the mimotope for further studies. Phage display thus offers an additional approach to the investigation of IgE epitopes and also provides a way of generating a pure source of epitopes. In the following section several studies where phage display was used to identify IgE binding epitopes in allergens will be discussed.

Phage display has also been used to identify IgE epitopes on Der p 1, a cysteine protease and important allergen of dust mite (Furmonaviciene *et al.,* 1999). The study utilized the mouse monoclonal 2C7, an antibody that binds to the same epitope on Der p 1 as that recognized by a large proportion of allergic human serum IgE (McElveen *et al*., 1998). The MAb 2C7 was used to select phage present in an unconstrained 15mer peptide library, expressed on p8. Several clones were isolated, each having the consensus sequence DXXXR or KXXGR. The consensus sequences corresponded well to the region of leu147-gln160 present in the crystal structure of Der p 1 (Topham *et al*., 1994) as part of a β pleated sheet. Similar structures were then found on the related molecules papain, chymopapain and actinidin tha.t are also cysteine proteases. Two phage clones having the peptide sequences of TPLCDYAAARVGACG and NCLSSDEPLHIRWCQ inhibited the binding of purified Der p 1 to the mAb 2C7 in an ELISA. As both clones contain cysteine residues within their sequence, it was thought that this assisted in Der p1 binding. Other phage clones with internal cysteines could not inhibit binding of Der p 1 to 2C7.

The identification of IgE binding epitopes using random peptide libraries has clearly provided insight into the structural motifs or regions of allergens, which are important in allergic patients. In addition, these mimotopes may have therapeutic

potential since they can only bind to one of the antigen binding sites on IgE. The mimotopes could therefore interfere between the binding of IgE and native allergens by binding but not crosslinking cell bound IgE. As the cell bound IgE would not be crosslinked, degranulation of mast cells or basophils would not occur. Ongoing work is required to determine the full clinical potential of this approach.

CHAPTER III
MATERIALS AND METHODS

Preparation and purification of specific monoclonal antibodies to House dust mite

Six clone of MAbs; 1D4H9E10, 9D2D2G4, 9D4A4, 3C5G7, 7A2D1 and 9C3C7F12 (Table 3) were obtained from researcher group at Faculty of Medicine, Siriraj hospital, Bangkok, Thailand. Theses monoclonal antibodies were selected for use in panning experiments with random heptapeptide T7 phage and bacteriophage M13 display libraries, in order to determine the mimotopes. Four kinds of antigens preparing from *D. pteronyssinus* and *D. farinae* mites (secretory antigens, phenol extract antigens, sonicated antigens and spent mite medium) were immunized mices for monoclonal antibodies production. Secretory antigens preparation by live mite bodies were suspended in 0.15 M PBS pH 7.2 and rotated for overnight at 4°C. The supernatant was pelleted by centrifugation at 500 x g for 20 minutes at 4 °C and stored at –20°C in small aliquots. Phenol extracted antigens preparation by live mite bodies were collected, dehydrated by lyophilization for 24 hours, defatted by diethyl ether and dried at room temperature. Ten grams of the dried mite were extracted in PBS with 0.4 % phenol in 0.5% NaCl for 120 hours with continuous stirring by using electric stirrer. Centrifuge (1,000 x g) at 4 °C for 20 minutes and the supernatant was collected, dialyzed against PBS by using dialysis bag with molecular weigh cut off 3,500. Sonicated antigens preparation by live mite bodies suspended in 0.15 M phosphate buffer saline (PBS) pH 7.2 were immediately frozen in liquid nitrogen (–196 °C) and then sonicated for 20 minutes with sonicator heat system ultrasonic in the condition of 50% output power, 50% duty cycle and level 4.5 of output control. The supernatant was collected after centrifugation at 500 x g for 20 minutes at 4°C and stored at –20°C in small aliquots. Each hybridoma cells were cultured in a serum free

medium to late log phase. The spent culture media (with monoclonal antibodies) were dialyzed and checked for antibody titers by an indirect ELISA. The MAbs were tested for specificity by indirect ELISA using crude panel antigens (more detailed about specificity of MAbs in Appendix C). The monoclonal antibodies were coated on the microtiter plate (respectively) for further panning experiment.

Table 3. Monoclonal antibodies used in this study

No.	Clone	Specific [a]	Isotype
1	1D4H9E10 (B1)	Dp [b]	IgG1 κ
2	9D2D2G4 (B2)	Dp + Df [c]	IgG1 κ
3	9D4A4 (B3)	Dp + Df	IgG2a κ
4	3C5G7 (B4)	Dp + Df	IgG1 κ
5	7A2D1 (B5)	Df	IgG1 κ
6	9C3C7F12 (B6)	Broadly	IgM κ

[a]: specificity of the MAbs tested by indirect ELISA using crude panel antigens

[b]: *Dermatophagoiedes pteronyssinus*

[c]: *Dermatophagoiedes farinae*

Bacteriophage T7 Peptide Library

Random heptapeptide (flanked by cystiene residues) phage display library was previously constructed by using the T7 select-415 kit from Novagen (Wisconsin, USA). The T7 bacteriophage has icosahedral shape. The library construction was started by synthesizing the random heptapeptide inserted DNA. The inserted DNA was derived from degenerate oligonucleotides, which was synthesized chemically by adding mixtures of nucleotides to growing nucleotide chain. The synthetic oligonucleotides was designed to give a seven-residue long random amino acid sequence flanked by cysteine residues. To limit the occurrence of in-frame stop codons, the degenerate sequence of NNKNNKNNKNNKNNKNNKNNK was used;

each N is an equal mixture of A, G, C and T, each K is an equal mixture of G and T. For each NNK, the mixture of 32 nucleotide triplets can be formed, include codons for all 20 natural amino acids and one stop codon (TAG). Each synthesized oligonucleotide was ligated to T7 vector arm. Target peptides were expressed as fusion to the C-terminus of the 10B capsid protein and were displayed on the virion surface, where they are accessible for interaction with other proteins or ligands. The displayed peptide was situated between cysteine residues, and therefore, formation of a disulfide bridge would join the ends of the heptapeptide. The fusion polypeptide is presented in 415 copies on each phage particle. It had an original size of 3.3×10^7 pfu but before use it amplifies to a titer of 2.6×10^{10} pfu per milliliter. The library has been successfully used to map epitopes of antibodies against *Leptospira spp.* (Tungtrakarnpoung *et al*., 2006).

Random 12 peptide M13 phage library

Phage libraries: M13 bacteriophage libraries displaying X_{12} peptides, where X is any amino acid encoded by NNK codons and C is cysteine. Each library has a complexity of ~10^9 members (Kay *et al*., 2001).

Bacterial Strains

E. coli BL21 was obtained from Dr.Gunnar Froman, Uppsala University, Sweden and was generally used as the host cell for phage manipulation and amplification.

E. coli TG1 (K12 Δ (lac-proAB) Sup E thi D5/F' tra D36 pro A^+ $lacI^q$ lacZΔM15) was obtained from Dr.Montarop Yamabhai, Suranaree University of Technology, Thailand and was used for propagation of phage.

Helper phage KM13

KM13 used as a helper phage for supplying the wild-type and other proteins necessary for M13 phage assembly and function (Viera and Messing, 1987) was obtained from Dr.Montarop Yamabhai, Suranaree University of Technology, Thailand.

Measurement of Monoclonal antibodies concentration

The conventional of each MAbs were measured, using Coomassie Plus Protein Assay Reagent Kit (PIERCE). The Pierce Coomassie Plus Protein Assay Reagent is a modification of the well-known Bradford, Coomassie, dye-binding, colorimetric method for total protein quantification (Bradford, 1976).

The Coomassie – protein reaction scheme

Protein + Coomassie G-250 in an acidic medium $\longrightarrow$ Protein-Dye complex

(blue color, measured at 595 nm.)

The Micro Protocol: Microwell Plate Version (Working Range = 1 to 25 μg/ml). The 150 μl of each standard (BSA 20, 10, 5, 2.5, 1.25, 0.625 25 μg/ml) and nine MAbs were added in to the appropriate microwell. The 150 μl of diluent were added to the blank wells. The 150 μl of Coomassie Plus Protein Assay Reagent were added to each well, the plate was mixed for 30 seconds. The absorbance was measured at or near 595 nm on ELISA reader. The standard curve was prepared by plotting the average blank corrected 595 nm reading for each BSA standard versus its concentration in μg/ml. Using the standard curve, the protein concentration was estimated for each MAbs.

Screening phage mimotopes by random heptapeptide with cystiene flanking T7 phage library

Bio-panning

All six MAbs were used in T7 phage display panning experiments to characterize their binding epitopes. Purified MAbs were diluted in PBS to 10 μg/ml and 100 μl portions were adsorbed to the wells of microtiter plate for 2 hours at 25 °C. The coated wells were blocked by incubation for 18 hours at 4 °C with 200 μl BSA containing 50 mg/ml in PBS. Adsorptions of virus particles to each particle were performed by incubating the amplified phage library, or sub library, for 15-40 min at 25 °C under agitation. Unbound phages were washed off; bound phages were released by incubation in 1% SDS and used to infect *E. coli* BL21 cells, to produce a sub-library for the next panning round. Four repetitive panning rounds were done depending on the efficiency of selection. Finally, ten single plaques of T7 phage per each MAb, were randomly picked, and use for further phage amplification and purification.

Phage purification

Each single picked plaque were amplified in the *E. coli* strain BL21 until the host cells lysis. For precipitation, 5 ml of 5 M NaCl were added to the 50 ml culture, centrifuge at 7,000 rpm, for 10 min at 4 ^{o}C. Then phage in the supernatant were extracted, by adding 1/6 volume of 50% polyethylene glycol (PEG) 8000, vortexed vigorously. To precipitate the phage, the PEG mixture were placed on ice for 30 min, and then centrifuged at 7,000 rpm for 10 min, the supernatant were decanted. Then the precipitate was resuspended with 1.2 ml of 1 M NaCl, 10 mM Tris-Cl, pH 8.0 and 1 mM EDTA. Then this purified phage can be further used in ELISA experiment.

ELISA (Enzyme Linked Immunosorbent Assay)

ELISA was performed as a standard protocol, to check the binding specificity of ten selected phage clones per each MAb (respectively). Microtiter well of ELISA plates, were coated with purified phage (from previous method) in carbonate buffer pH 9.6. Then phages were allowed to attach to the solid surface of the plates, by

incubating at 37 ^{0}C for 1 hour, in humid box and then at 4 ^{0}C, overnight. Then the unbound phages were extensively washed away with the PBS-Tween. The unoccupied sites on the wells were blocked with 1% BSA at 37 ^{0}C, in a humidified chamber for 1 hr and washed again. After washing, MAb were added to appropriate wells. The plates were incubated, as done for the blocking step, and then they will be washed as mentioned above. Incubated with the rabbit anti-mouse immunoglobulin-horseradish peroxidase conjugate for 1 hour. The excess conjugate was washed away. Then freshly prepared 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate solution containing 0.05% H_2O_2 was added to each well, and the plates were kept at room temperature, for 30 min. The reaction was stopped by adding 1% SDS solution. The optical density (OD) of the content in each well was determined compared with the blank at 405 nm, using an ELISA reader. Binding of phage to antibody consider specific if the absorbance value at 405 nm was above 0.2. Phages that shown positive results with ELISA were further prepare for its DNA to use in PCR experiment.

Western blot analysis

The lack of suitable monoclonal antibodies to T7 phage meant that the phage could not be detected by ELISA. Alternatively, Western blot analysis was used to check the binding specificity of ten selected phage clones per each MAb. The purified phages were separated by 10% SDS-PAGE and blotted onto nitrocellulose membranes (NC), washed with PBST and blocked with 3% skim milk in PBST. Each lane of the NC membrane was cut and reacted with specific monoclonal antibody at room temperature for overnight with constant shaking. After washing with PBST, the strips were incubated with rabbit anti-mouse immunoglobulin-horseradish peroxidase conjugate (1/1000 dilution) at room temperature for 3 hours. The membrane strips were then washed five times with PBST, stained strips with 2, 6-dichloro-4-nitrophenol in PBS 20 ml. and 5 µl H_2O_2 until color developed.

PCR and DNA sequencing

The phage DNA was used as the template for PCR and sequencing experiments. For analysis of peptide sequences of bound phage, a segment of the 10B capsid protein of T7phage DNA was amplified, according to the manufacturer's instruction (Novagen 2000) using the T7 select up (5'-AGC TGT CGT ATT CCA

GTC A-3') and down (5'-ACC CCT CAA GAC CCG TTT A-3') as primers. A total PCR reaction mixture (50 µl) was consisted of the following reagents:

5 µl	T7 selected up primer (5 pmol/µl)
5 µl	T7 selected Down primer (5 pmol/µl)
5 µl	10 x buffer
10 µl	$MgCl_2$ (25 mM)
2 µl	*Taq* DNA polymerase (1U/µl)
1 µl	dNTP (25 mM)
12 µl	H_2O
10 µl	phage DNA

The reaction mixture was placed in the thermal cycler, using the following program; one cycle at 94 ^{O}C for 2 min, 35 cycles of (94 ^{O}C for 20 sec, 50 ^{O}C for 20 sec and 72 ^{O}C for 45 sec) and final cycle complete extension at 72 ^{O}C for 4 min. PCR products were purified by commercial kit (QIA quick PCR purification kit). Then purified PCR products were sent together with T7 select up primers, for the automate DNA sequencing procedure.

Screening phage mimotopes by random 12peptide M13 phage library

Immobilization of Targets onto ELISA-Ready Microtiter Plates

Add 1–10 µg of 9C3C7F12 (B6) monoclonal antibody in 100 µl 100 mM $NaHCO_3$ (pH 8.5) onto microtiter wells of three ELISA-ready plates. (Plate 1 will be used in the first- round panning, while plates 2 and 3 were used for the second and third rounds, respectively.) Use a control target protein, such as streptavidin or a GST fusion protein to the Src SH3 domain. Leave a space between different targets to prevent cross-contamination. Seal the wells with tape to avoid evaporation, and incubate the plates at room temperature for 1 h. Use either strips of Scotch Tape to cover individual wells, or microtiter plate-sized tape or plastic wrap to cover to the entire plate. Add 150 µl blocking solution to each well to block nonspecific binding. Seal the wells with tape and incubate the first plate at room temperature for 1 h. Incubate the second and third plates overnight at 4^{o}C.

Affinity Purification of Binding Phage: First-Round

Wash the wells three times with PBS–0.1% Tween 20, which can be introduced into the wells by pipetting or with a plastic squeeze bottle. The wash solution can be flicked into the sink after each wash. Remove residue liquid by slapping the dish against a clean Kimwipe. Do not let the wells dry out completely. Add 25 µl of a combinatorial peptide library (concentrated to ~10^{14} plaque-forming units (PFU)/ml, with a complexity of 10^9) in 125 µl PBS–0.1% Tween20 to each well. To ensure successful isolation of a particular peptide, we use 2500 copies of each phage in the first round of panning. Seal the wells and incubate plate at room temperature for 2 hours. Remove nonbinding phage by washing the wells five times as described on above. Elute bound phage by adding 50 µl of 50 mM (DMSO) or dimethylformamide (DMF) HCl (pH 2.0) to each well and incubate at room temperature for 15 min. Neutralize the solution by transferring eluted phage to a new well containing 50 µl neutralization solution.

Amplification of Recovered Binding Phage

Dilute 30 µl of an overnight culture of F' *Escherichia coli*, such as DH5αF, in 3 ml sterile 2xYT. Add 290 µl of the eluted phage and incubate the culture at 37°C, with vigorous agitation, for 8 hours. To minimize proteolytic degradation of displayed peptides, do not incubate longer than 16 hours.

Affinity Purification of Binding Phage: Second and Third- Round Panning

Second Round

Collect the amplified phage by spinning out cells at 4°C, 4000*g* for 10 min and transfer the phage supernatant to a new tube (4-ml Falcon). Wash the wells of the second plate three times with PBS–0.1% Tween 20 by flicking the wash solution into the sink. Remove residue liquid by slapping the dish against a clean Kimwipe. Add 150–200 µl amplified phage (~10^{10} PFU) from the first-round panning. Seal the wells and incubate the plate at room temperature for 2 hours. Wash the wells five times as described on above. Elute bound phage by adding 50 µl of 50 mM glycine–HCl (pH 2.0) to each well and incubating the plate at room temperature for 15 min. Neutralize the solution by transferring eluted phage to a new well containing 50 ml neutralization solution. Dilute 30 µl of an overnight culture of F' *E. coli*, the eluted phage and

incubate the culture at 37°C, with vigorous agitation, for 8 hours. To minimize proteolytic degradation of displayed peptides, do not incubate longer than 16 hours.

Third Round

Collect the amplified phage by spinning out cells at 37°C, 4000*g* for 10 min and transfer the phage supernatant to a new tube (4-ml Falcon). Wash the wells of the second plate three times with PBS–0.1% Tween 20 by flicking the wash solution into the sink. Remove residue liquid by slapping the dish against a clean Kimwipe. Add 150–200 ml amplified phage (~10^{10} PFU) from the second round of panning to each well. Seal the wells and incubate the plate at room such as unrelated bacterial fusion partner proteins, and temperature for 2 hours. Wash the wells five times as described on above. Elute bound phage by adding 50 ml of 50 mM glycine–HCl (pH 2.0) to each well and incubate the plate at room temperature for 15 min. Neutralize the solution by transferring eluted phage to a microcentrifuge tube containing 50 µl neutralization solution. This represents the output population of binding phage from three rounds of affinity purification.

Isolation of Affinity-Purified Phage Clones

Perform a 10-fold serial dilution of each recovered phage on a microtiter plate (U-bottom). Add 180 ml PBS into the wells (rows A–E) with an eight-channel pipettor. The number of the columns should equal the number of targets. Add 20 µl of each recovered phage to be tittered in row A. Mix by pipetting up and down. Transfer 20 µl of the phage from row A to row B. Mix and continue the dilution series through row G. (Before starting, melt the top agar in a microwave and keep it at 55°C). Add 10 µl of diluted phage from rows A–E with 200 µl DH5αF' overnight culture into a 4-ml sterile Falcon tube. Premix 3 ml of molten 0.8% top agar with 30 ml 2% IPTG and 30 ml 2% X-Gal; keep at 55°C. Add the 3 ml of agar mixture to each tube containing diluted phage, invert several times, and pour onto a 2xYT Petri plate that has been prewarmed at 37°C. Allow the plates to then sit undisturbed for a few minutes (~5 min), until the top agar hardens. Incubate the plates inverted at 37°C overnight, then keep in 4°C until ready to pick up the isolated phage clones. Phage are viable as plaques for at least 1 month if the plates are kept at 4°C.

Propagation of Individual Phage Clones

Dilute an overnight culture of DH5αF' 1:100 into sterile 2xYT. For each isolated plaque to be propagated, add 3 ml of the mixture into 15-ml tubes. Pick (touch and twist) and inoculate the blue isolated plaques into each 15-ml tube with sterile long wooden toothpicks. Incubate the tubes at 37°C, with vigorous agitation, for 8–10 hours.

Confirmation of Binding Activity of Affinity-Purified Phage Clones by ELISA

For each phage clone to be tested, add 1 μg of target protein and 1 μg of a negative control protein, such as unrelated bacterial fusion partner proteins, and BSA in 100 μl of 100 mM $NaHCO_3$ (pH 8.5) into adjacent microtiter wells. Seal the wells with tape to avoid evaporation, and incubate the plate at room temperature for 1 hour. Add 150 μl blocking solution to each well to block nonspecific binding. Seal the wells with tape and incubate overnight at 4°C. Pellet the bacterial cells in the culture tubes by centrifugation at 4°C, 4000*g* for 10 min. Transfer the phage supernatant into a new tube. Keep the bacterial pellet for preparation of replicative form DNA. Wash the wells three times with PBS–0.1% Tween 20 by flicking the wash solution into the sink. Remove residue liquid by slapping the dish against a clean Kimwipe. Do not let the wells dry out completely. Add 100 μl of each recovered phage into a separate pair (target/negative control) of wells and incubate for 2 hours at room temperature. Keep the rest of the supernatant as a phage stock in 4°C (for a long-term storage, add glycerol to a final concentration of 20% and keep at -70°C). Wash the wells five times as described above. Dilute horseradish peroxidase-conjugated antiphage antibody 1:5000 in PBS–Tween 20. Add 100 μl of the diluted conjugate to each well. Seal the wells and incubate the plate at room temperature for 1 hour. Wash the wells five times as described above.

Add 100 μl ABTS reagent containing 0.05% H_2O_2 to each well. Incubate the plate at room temperature until the color reaction develops (10–30 min) Quantify the reaction by measuring the absorbance at 405 nm with a microtiter plate reader. Positive signals gave optical density (OD) of sample (phage bind with MAb) three times higher than that of the control (BSA).

Preparation of Plasmid from Positive Phage Clone for DNA Sequencing

Use the cell pellet of *bona fide* binding phage to prepare double stranded DNA for automated fluorescence DNA sequencing. Elute DNA with 50 µl sterile H_2O. Measure DNA concentration by reading the OD value at 260 nm (1 OD = 50 µg/ml). Add 0.7 µg of DNA, 10 pmol primer (1 µl of 10 mol/µl gene III downstream primer) (5'-GCC CTC ATA GTT AGC GTA ACG-3'), and sterile H_2O to bring the total volume to 20µl into a 0.65 ml microcentrifuge tube. Label each tube with the sender's name, submission date, and sample number. Submit to a sequencing facility for fluorescent dideoxynucleotide sequencing.

Comparison of bound phage sequences with Structural Database of Allergenic Proteins (SDAP).

The obtained sequences were compared with the matched sequences from SDAP (http://fermi.utmb.edu/SDAP/index.html)

In this study, all of the finding phage mimotopes were compared with previously reported "target-unrelated peptides" (TUP) from published paper of Menendez and Scott (2005) [TUP means phage that selected from phage-displayed random peptides libraries (PDPLs), may react with constant antibody regions or other components of the screening system, such as the beads, plates, or the capturing molecule (streptavidin, protein A, etc.).

Locating the epitope recognized by MAbs on the Der p 1 and Der f 2 models

To study locating the epitope recognized by MAbs on the Der p 1 and Der f 2 models using the Brookhaven Protein Databank (PDB) and RasMol V2.5 software.

Comparison of bound phage sequences with GenBank sequences

The obtained sequences were compared with the matched sequences from GenBank, using BLASTP software (http://www.ncbi.nlm.nih.gov/BLAST/), and were compared with vectors sequences from gene bank using VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html).

Prediction of protein sequence with PSORT II Prediction

After comparison phage sequence with BLAST software, the protein sequence that matched with our phage mimotope were checked for its localization in the cell and the surface protein activity by using PSORT II program (http://psort.ims.u-tokyo.ac.jp/). PSORT II is a computer program for the prediction of protein localization sites in cells. It receives the information of an amino acid sequence and its source origin.

CHAPTER IV
RESULTS

Measurement of Monoclonal antibodies concentration

The concentration of six MAbs specific to house dust mite were measured using Coomassie Plus Protein Assay Reagent Kit (PIERCE). The standard curve was prepared by plotting the average blank corrected of optical density (OD) at 595 nm, reading for each BSA standard (20, 10, 5, 2.5, 1.25, 0.625 µg/ml) (the curve not shown). This standard curve showed correlation coefficient 0.994. The MAbs were diluted with distilled water (dilution 1:100) in Coomassie reagent kit and measured using ELISA reader at OD 595 nm. Using the standard curve, six monoclonal antibodies concentration were estimated in Table 4.

Selection of phage mimotopes by biopanning

A phage library displaying random heptapeptides on the capsid fusion peptides of T7 phage was used for selecting phage that interact with 10 µg/ml MAbs specific to house dust mite immobilized on the microtiter plate. For each round of panning, the total amount of phages added (input) and recovered (output) was determined (Table5). Increments in the titer of the phages through successive rounds of selection (Table 5) suggest that there was an enrichment of the phages that reacted with MAbs specific to house dust mite.

Table 4. Concentration of MAbs specific to house dust mite measured using Coomassie Plus Protein Assay Reagent Kit.

Monoclonal antibodies	Mean (OD)	Standard Deviation	%CV	Concentration (μg/ml)	Concentration (μg/ml) (x100)
B1	0.2770	0.006	2.30e+000	13.707	1370.7
B2	0.4915	0.021	4.32e+000	25.252	2525.2
B3	0.5115	0.042	8.29e+000	26.329	2632.9
B4	0.5190	0.011	2.04e+000	26.732	2673.2
B5	0.4420	0.037	8.48e+000	22.588	2258.8
B6	0.4890	0.002	4.34e-001	25.118	2511.8

Table 5. Input and output of the phages from the four/five rounds of biopanning (from random heptapeptide with cystiene flanking T7 phage library)

Round of biopannaing	Phage titer of input (PFU/ml.)	Phage titer of output (PFU/ml.)
1D4H9E10 (B1)		
First	9.5×10^{16}	3×10^{11}
Second	9.5×10^{16}	6×10^{10}
Third	9.5×10^{16}	1×10^{11}
Fourth	9.5×10^{16}	1.2×10^{13}
Fifth	9.5×10^{16}	7×10^{11}
9D2D2G4 (B2)		
First	1×10^{15}	9×10^{7}
Second	2×10^{12}	2×10^{8}
Third	4×10^{12}	2×10^{9}
Fourth	3×10^{14}	4×10^{10}
9D4A4 (B3)		
First	1×10^{15}	1.7×10^{8}
Second	4×10^{12}	1.3×10^{8}
Third	2×10^{16}	1.3×10^{8}
Fourth	2×10^{14}	2.0×10^{9}
3C5G7 (B4)		
First	1×10^{15}	2×10^{8}
Second	6×10^{12}	2×10^{8}
Third	6×10^{14}	5×10^{8}
Fourth	1×10^{14}	1×10^{9}
7A2D1 (B5)		
First	2×10^{13}	3×10^{7}
Second	2×10^{12}	2×10^{8}
Third	6×10^{12}	4×10^{8}
Fourth	1×10^{14}	1.2×10^{9}
9C3C7F12 (B6)		
First	9.5×10^{16}	2×10^{11}
Second	9.5×10^{16}	3×10^{10}
Third	9.5×10^{16}	1×10^{11}
Fourth	9.5×10^{16}	2×10^{12}
Fifth	9.5×10^{16}	2×10^{13}

Confirmation of the binding specificity of bound phages using ELISA/Western blot

Random heptapeptide with cystiene flanking T7 phage library

After selection of bound phages with each MAb by biopanning for four/five rounds, ten bound phages were randomly selected for test the binding specificity with each house dust mite MAbs respectively using indirect ELISA. The selected bound phages of T7/B1 and T7/B6 with ELISA positive for house dust mite MAbs (Table 6) were further amplified and checked the sequence of its inserted DNA using PCR and DNA sequencing. In table 6 showed that the selected bound phages of T7/B6.4 and T7/B6.5 had high titer positive at 1:320 and all selected bound phages of T7/B1 had titer positive at 1:20. On the other hand, the selected bound phages of T7/B2, T7/B3, T7/B4 and T7/B5 were found to have ELISA negative with house dust mite MAbs (data not shown). The selected bound phages of T7/B2, T7/B3, T7/B4 and T7/B5 could not be detected by ELISA. Alternatively, those were screened on Western blot. The 10% SDS-PAGE and Western blot analysis showed that selected bound phages of T7/B2, T7/B3 and T7/B5 were negative by the test. Only the selected bound phages of B4 was found to have Western blot positive with MAb specific to house dust mite (lanes 3-12, Fig. 9). Figure 9 showed positive band with molecular weight of about 42.6 kDa (lanes 3-12) and wild type T7 phage library was negative by the test. However, all selected bound phages with negative result were also amplified and checked for the inserted DNA sequence using PCR and DNA sequencing.

Table 6. Absorbance of selected bound phages of B1 and B6 MAbs from random heptapeptide with cystiene flanking T7 phage library.

Phage clone	Absorbance of phage clones					Positive titer
	1:20	1:40	1:80	1:160	1:320	
B1.3	0.296	0.173	0.098	0.007	-0.046	1:20
B1.4	0.253	0.137	0.031	0.021	-0.067	1:20
B1.5	0.271	0.146	0.049	0.022	-0.060	1:20
B1.6	0.291	0.165	0.033	0.020	-0.059	1:20
B1.7	0.346	0.199	0.057	0.019	-0.017	1:40
B1.8	0.357	0.191	0.081	0.009	-0.029	1:20
B6.3	0.920	0.737	0.475	0.265	0.134	1:160
B6.4	1.175	0.884	0.563	0.408	0.199	1:320
B6.5	1.053	0.789	0.597	0.343	0.208	1:320
B6.6	1.115	0.861	0.560	0.350	0.180	1:80
B6.7	0.926	0.589	0.370	0.163	0.036	1:80
B6.8	1.068	0.759	0.520	0.237	0.092	1:160

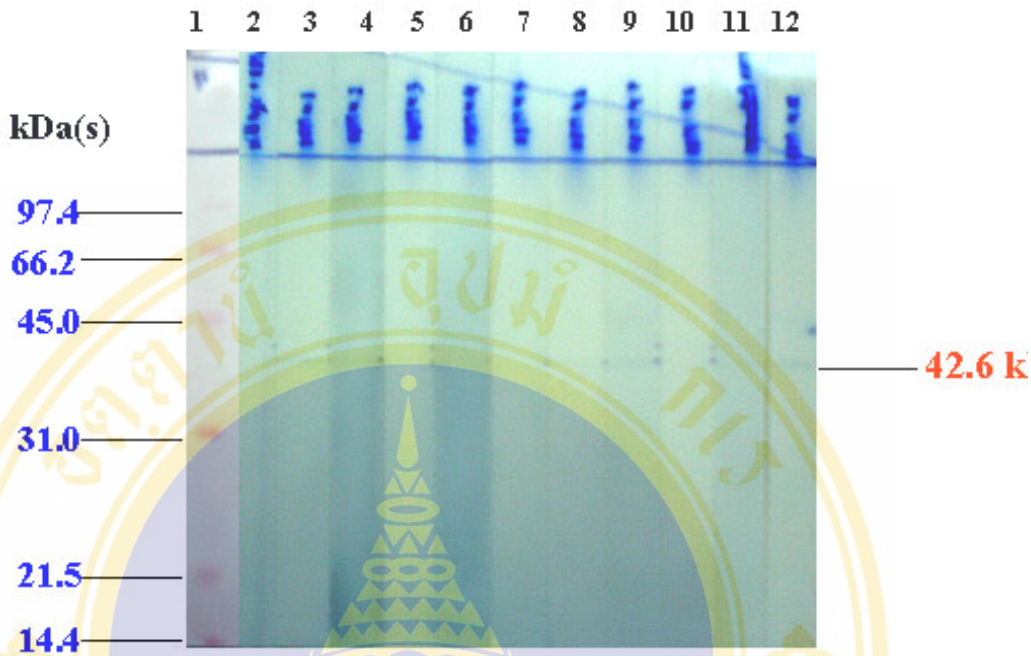

Fig. 9 Western blot (10% SDS-PAGE) patterns showing reactivity of selected bound phages of B4 against house dust mite MAb.

Lane 1 = Molecular weight marker

Lane 2 = Western blot pattern of wild type T7 phage library (control)

Lane 3-12 = Western blot patterns of selected bound phages B4

Random 12peptide M13 phage library.

After selection of bound phages with B6 MAb by biopanning for three rounds, eight bound phages were randomly selected for test the binding specificity with house dust mite MAb clone B6 using indirect ELISA. Table 7 showed optical density value of eight selected bound phages. Bound phages that shown positive result of binding specificity was the one that gave OD of sample (phage bind with MAb) three times higher than that of the control (BSA). Six from eight selected bound phages (75%) gave positive ELISA result (Table 7). The sequences of the selected bound phages were determined by DNA sequencing of the insert random DNA in the pIII gene.

Table 7. Absorbance of selected bound phages of B6 MAb from random 12peptide M13 phage library.

Phage clone	Sample (OD)	BSA control (OD)	Sample (OD)/ BSA control (OD)	Interpretation of results [a]
A	0.759	0.124	6.12	Positive
B	0.764	0.235	3.25	Positive
C	0.767	0.071	10.80	Positive
D	0.701	0.15	4.67	Positive
E	0.752	0.464	1.62	negative
F	0.272	0.101	2.69	negative
G	0.707	0.159	4.45	Positive
H	0.723	0.122	5.93	Positive

[a] positive result were consider from OD of sample should be three times higher than that of the BSA control.

PCR and DNA sequencing

Random heptapeptide with cystiene flanking T7 phage library

The DNA encoded for random hetapeptide was amplified using PCR and further sequenced. Figure 10 showed that the PCR product of selected bound phages were located at 133 bps. The PCR products of selected bound phages were purified using commercial kit (QIA quick PCR purification kit). The nucleotide sequences of purified PCR products were determined by using T7 select up primers for DNA sequencing.

Random 12 peptide M13 phage library

The nucleotide sequences of phage DNA were determined by using the -96 sequencing down primer for the DNA sequencing reaction.

Fig. 10 PCR product analysis of the selected phage clones. Each PCR product was electrophoresis on 1.5% agarose gel, stained with gel star and visualized under the dark reader. Lane M; 100 bps DNA ladder plus; lane 2.2, 2.3, 2.4, 2.5, 3.1, 3.3, 3.4 and 3.5; amplified DNA of selected phage clones were positive band at 133 bps; lane 3.2 amplified DNA of selected bound phage was disappeared.

Comparison of bound phage sequences with Structural Database of Allergenic Proteins (SDAP).

Peptide-displaying phage types were assigned according to the selection procedure with each monoclonal antibody: T7/B1, T7/B2, T7/B3, T7/B4, T7/B5, T7/B6 and M13/B6 (Table 8). All mimotopes were compared with previously reported "target-unrelated peptides" (TUP) (Menendez and Scott, 2005) and also compared with vector sequences in GenBank using Vecscreen software. All mimotopes were not similar to vector sequences, only one phage M13/B6 with sequence AER***WGPW***GVHSW was similar with that of TUP.

All 47 mimotope sequences were aligned with the peptide similarity of allergens using SDAP. All mimotopes were partially matched with allergen sequences and their possible functional roles were listed (Table 9). Table 9 showed that 8 mimotopes (17.4%) were matched with Der f 15 allergen (GenBank Accession number AAD52672), 6 mimotopes (13.04%) were matched with Eur m 14 allergen (GenBank Accession number AAF14270), 5 mimotopes (10.87%) were matched with Per a 3.0202 and Lep d 7 allergen (GenBank Accession number AAB62731 and Q9U1G2), 3 mimotopes (6.52%) were matched with Der f 6, Der p 1 and Der f 7 allergen (GenBank Accession number P49276, P08176 and Q26456) and 2 mimotopes (4.35%) were matched with Der p 1 and Der p 4 allergen (GenBank Accession number AAA28296 and AAD38942). Interestingly, mimotope **AE**RW**G**PW**G**VHSW that similar with TUP was matched with Lep d 2 allergen (GenBank Accession number P80384).

Table 10 showed the summary of mimotopes from each MAbs that matched with allergen protein sequences, the majority of mimotope sequences from T7/B1, T7/B2, T7/B3 and T7/B5 were matched with Der f 6 allergen (33.33%), Der p 1 allergen (55.55%), Der f 15 allergen (62.5%) and Eur m 14 allergen (42.87%), respectively. Most of mimotope sequences from T7/B4 were matched with Der f 7 allergen (33.33%) and Lep d 7 allergen (44.44%).

The partially matched mimotopes may contain more than one matching site along the amino acid sequences of the allergen. Various mimotope sequences were aligned with Der f 15, Eur m 14 and Per a 3.0202 and some mimotopes were appeared

at more than one location along the sequences (Figs. 11, 12 and 13). As shown in figure 11, the regions: amino acid 411-429 and 480-503, correlated with overlapping mimotopes and seemed to be the main epitope clusters of the Der f 15 allergen peptide. The duplicate sequences N**S**L**TP**CN**T**Q**Y**Y**D**DC appeared to be partial match with amino acid 490-503. Alignments of the sequences obtained from T7/B3 (3 mimotopes) and T7/B2 (1 mimotope) revealed a common motif of SXTPXXTXYXD (Fig. 11).

Table 8. Deduced amino acid and sequences of capsid fusion peptides of T7 and M13 phage that respectively bind to 6 monoclonal antibodies of House dust mite.

Libraries/antibody	Display peptides
T7/B1 (6)	CYPKKNRAC (2), CSNKKSARC (1), CNNLKKRAC (1), CKPKRPGNC (1), CTSKKKVNC (1)
T7/B2 (9)	CSIQGGSNC (2), CSLTEFGSC (2), CNTNYYDDC (1), CSLTDTSNC (1), CSLTYYYSC (1), CNNPLNSDC (1), CSLQGAANC (1)
T7/B3 (8)	CNTQYYDDC (2), CNTHETGYC (1), CNTTYYDYC (1), CCLIRTIYC (1), CRKNSKGSC (1), CCP* (1), CILTRNVTC (1)
T7/B4 (9)	CDP* (4), CLPYEHGDC (3), CMIKLTDYC (1), CSPFEHGDC (1)
T7/B5 (7)	CRTSLRTC (1), CGLARALG* (1), CQSLSVVP* (1), CIIC* (1), CGLIWNNFC (1), CGHMKTSIC (1), CPLASLSPC (1)
T7/B6 (2)	CTTNSKRKC (1), CPNPISNLC (1)
M13/B6 (6)	QPLRVEEMPGLE (1), AER***WGPW***GVHSW (1)[a], NGRGVEDLVAWH (1), WGEMEGLWWQGK (1), DWEWRFGGVGVG (1), DWDWTDPRGNAN (1)

* Mean stop codon

[a] Phage sequence similar to plastic binder (polystyrene microtiter plate) (Menendez *et al*, 2005)

Table 9. Comparison of phages peptide sequence with Allergen sequences from Structural Database of Allergenic Proteins (SDAP).

Allergen	Accession No. (NCBI/SWISS-PROT)	Protein name	Peptide mimotope	Frequency
1.Der f 15	AAD52672	98kDa HDM allergen of *D. farinae*.	N**SL**T**P**CN**T**N**Y**Y**D**DC NSLT**P**CN**THET**GYC N**SL**T**P**CN**T**Q**Y**Y**D**DC N**SL**T**P**CN**TTY**Y**D**YC **CIL**TRNV**T**C **G**H**M**KT**SI** **D**WE**W**RFG**G**VGV**G**	1 1 2 1 1 1 1
2.Eur m 14	AAF14270	High molecular weight allergen M-177 precursor of *E. maynei*.	**NSL**T**P**CTSKK**K**V**N**C NSLTPC**CP*** **G**L**A**RA**LG*** N**SL**TPCQS**LS**V**V**P* **GLI**WN**N**F **CP**NP**I**SN**L**C	1 1 1 1 1 1
3. Per a 3.0202	AAB62731	Allergen of *P. Americana*.	**K**P**K**RP**G**N **NSL**T**P**CS**L**TYYYSC CLI**R**T**IY** **RKN**SKG**S** **NSL**TPCS**PF**EHGDC	1 1 1 1 1
4. Lep d 7	Q9U1G2	Mite allergen Lep d 7 Precursor of *L. destructor*.	NS**L**T**P**C**DP* Q**PL**R**VEE**MPG**L**E	4 1
5. Der p 1	P08176	Major mite fecal allergen Der p 1 Precursor.	**N**S**L**TPC**SL**T**EF**GSC **C**SLQ**G**A**A**NC	2 1
6. Der f 6	P49276	Mite allergen Der f 6 Precursor *D. farinae*.	N**SL**TPC**Y**PKKN**R**AC **N**SLT**P**CI**IC***	2 1
7. Der f 7	Q26456	Mite allergen Der f 7 Precursor of *D. farinae*.	**L**P**Y**EH**GD**	3
8. Der p 1	AAA28296	Major house dust allergen of *D.pteronyssinus*	NSL**TP**CSI**QGG**SNC	2
9. Der p 4	AAD38942	Alpha-amylase of *D. pteronyssinus*	N**N**PL**NS**D **D**W**T**D**P**RGNA**N**	1 1
10. Der p 3	P39675	Mite allergen Der p 3 Precursor.	**SL**TDT**S**N	1
11. Der p 8	P46419	Glutathione S-transferase of *D.pteronyssinus*	NS**L**T**P**CP**L**A**SL**SP**C**	1

Table 9. Comparison of phages peptide sequence with Allergen sequences from Structural Database of Allergenic Proteins (SDAP). *(Continued)*

Allergen	Accession No. (NCBI/SWISS-PROT)	Protein name	Peptide mimotope	Frequency
12. Der f 2	Q00855	Mite group 2 allergen Der f 2 Precursor.	CS**N**K**K**S**A**RC	1
13. Der f mag	BAA03064	Mag protein of *D. farinae*	W**G**EME**G**LWWQ**GK**	1
14. Blo t 1	AAK58415	Cysteine protease precursor of *B. tropicalis*.	NSLTPC**N**N**L**KK**RA**C	1
15. Per a 1.0103	AAB8240	Cr-PII protein of *P.Americana*	TTN**SKRK**	1
16. Per a 3	Q25641	Allergen Cr-PI Precursor of *P. Americana*	**N**SLT**P**C**R**T**S**L**RT**C	1
17. Per a 3.0201	AAB09632	Allergen of *P. Americana*	NG**R**GV**EDLVA**WH	1
18. Eur m 3	O97370	Mite allergen Eur m 3 Precursor of *E. maynei*	CM**IKL**TDYC	1
19. Lep d 2	P80384	Mite group 2 allergen Lep d 2 [Precursor]	**AE**RW**G**PW**G**VHSW	1

* Mean stop codon

Bold letter mean the display peptide of bound phage that match with part of Allergen protein sequence from Structural Database of Allergenic Proteins (SDAP).

Table 10. Summary mimotopes from each MAbs that matched with Allergen sequences from SDAP Structural Database of Allergenic Proteins.

Libraries/ MAbs	Peptide	allergen	Protein name	Frequency (%)
T7/B1 (6)	N**SL**TPC**Y**PKKN**R**AC	Der f 6	Mite allergen Der f 6 Precursor *D. farinae*.	33.33
	CS**N**K**KS**A**R**C	Der f 2	Mite group 2 allergen Der f 2 Precursor.	16.67
	NSLTPC**NN**L**KK**A**R**C	Blo t 1	Cysteine protease precursor of *B. tropicalis*.	16.67
	KP**K**RP**G**N	Per a 3.0202	Allergen of *P. Americana*.	16.67
	NSLT**P**CTSKK**K**V**N**C	Eur m 14	High molecular weight allergen M-177 precursor of *E. maynei*.	16.67
T7/B2 (9)	**NSL**TPC**SL**T**EF**GSC	Der p 1	Major mite fecal allergen Der p 1 Precursor.	22.22
	NSL**TP**CSI**QGG**SNC	Der p 1	Major house dust allergen of *D.pteronyssinus*	22.22
	CSLQ**G**A**A**NC	Der p 1	Major mite fecal allergen Der p 1	11.11
	SLTDT**S**N	Der p 3	Mite allergen Der p 3 Precursor.	11.11
	NN**P**L**NS**D	Der p 4	Alpha-amylase of *D.pteronyssinus*	11.11
	N**SLTP**CN**T**N**Y**Y**D**DC	Der f 15	98kDa HDM allergen of *D. farinae*.	11.11
	NSLT**P**CS**L**TYYYSC	Per a 3.0202	Allergen of *P. Americana.*	11.11
T7/B3 (8)	NSLT**P**CN**THE**T**G**YC	Der f 15	98kDa HDM allergen of *D. farinae*	12.5
	N**SLTP**CN**T**Q**Y**Y**D**DC	Der f 15	98kDa HDM allergen of *D. farinae*	25
	N**SLTP**CN**TTY**Y**D**YC	Der f 15	98kDa HDM allergen of *D. farinae*	12.5
	CILTRNV**T**C	Der f 15	98kDa HDM allergen of *D. farinae*	12.5
	CLI**R**T**IY**	Per a 3.0202	Allergen of *P. Americana*.	12.5
	RKNSKG**S**	Per a 3.0202	Allergen of *P. Americana*	12.5
	NSLTPC**CP***	Eur m 14	High molecular weight allergen M-177 precursor of *E. maynei.*	12.5
T7/B4 (9)	**L**P**Y**EH**GD**	Der f 7	Mite allergen Der f 7 Precursor of *D. farinae*.	33.33
	NS**L**T**P**C**DP***	Lep d 7	Mite allergen Lep d 7 Precursor of *L. destructor*.	44.44
	NSLTPCS**PF**EHGDC	Per a 3.0202	Allergen of *P. Americana*	11.11
	CM**IKL**TDYC	Eur m 3	Mite allergen Eur m 3 Precursor of *E. maynei*	11.11

Table 10. Summary mimotopes from each MAbs that matched with Allergen sequences from SDAP Structural Database of Allergenic Proteins. ***(Continued)***

Libraries/ MAbs	Peptide	allergen	Protein name	Frequency (%)
T7/B5 (7)	**N**SLT**P**C**R**TSL**RT**C	Per a 3	Allergen Cr-PI Precursor of *P. Americana*	14.29
	GL**A**RA**LG***	Eur m 14	High molecular weight allergen M-177 precursor *of E. maynei*	14.29
	N**SL**TPCQS**LS**V**V**P*	Eur m 14	High molecular weight allergen M-177 precursor *of E. maynei*	14.29
	GLIWNN**F**	Eur m 14	High molecular weight allergen M-177 precursor *E. maynei*	14.29
	NSLT**P**CI**IC***	Der f 6	Mite allergen Der f 6 Precursor of *D. farinae*	14.29
	GH**M**KT**SI**	Der f 15	98kDa HDM allergen of *D. farinae*	14.29
	NS**LT**P**C**P**LASL**SP**C**	Der p 8	Glutathione S-transferase of *D.pteronyssinus*	14.29
T7/B6 (2)	TTN**SKRK**	Per a 1.0103	Cr-PII protein of *P. Americana*	50
	CPNP**I**SN**L**C	Eur m 14	high molecular weight allergen M-177 precursor of *E.maynei*	50
M13/B6 (6)	Q**PL**R**VEE**MPG**L**E	Lep d 7	Mite allergen Lep d 7 Precursor of *L. destructor*	16.67
	AERW**G**PW**G**VHSW	Lep d 2	Mite group 2 allergen Lep d 2 [Precursor]	16.67
	NG**R**GV**EDL**V**A**WH	Per a 3.0201	allergen of *P. Americana*	16.67
	W**G**EME**G**LWWQ**GK**	Der f mag	Mag protein of *D. farinae*	16.67
	DWE**W**RFG**G**VGV**G**	Der f 15	98kDa HDM allergen of *D. farinae*	16.67
	DWTD**P**RGNA**N**	Der p 4	Alpha-amylase of *D. pteronyssinus*	16.67

* Mean stop codon

Bold letter mean the display peptide of bound phage that match with part of Allergen protein sequence from SDAP Structural Database of Allergenic Proteins.

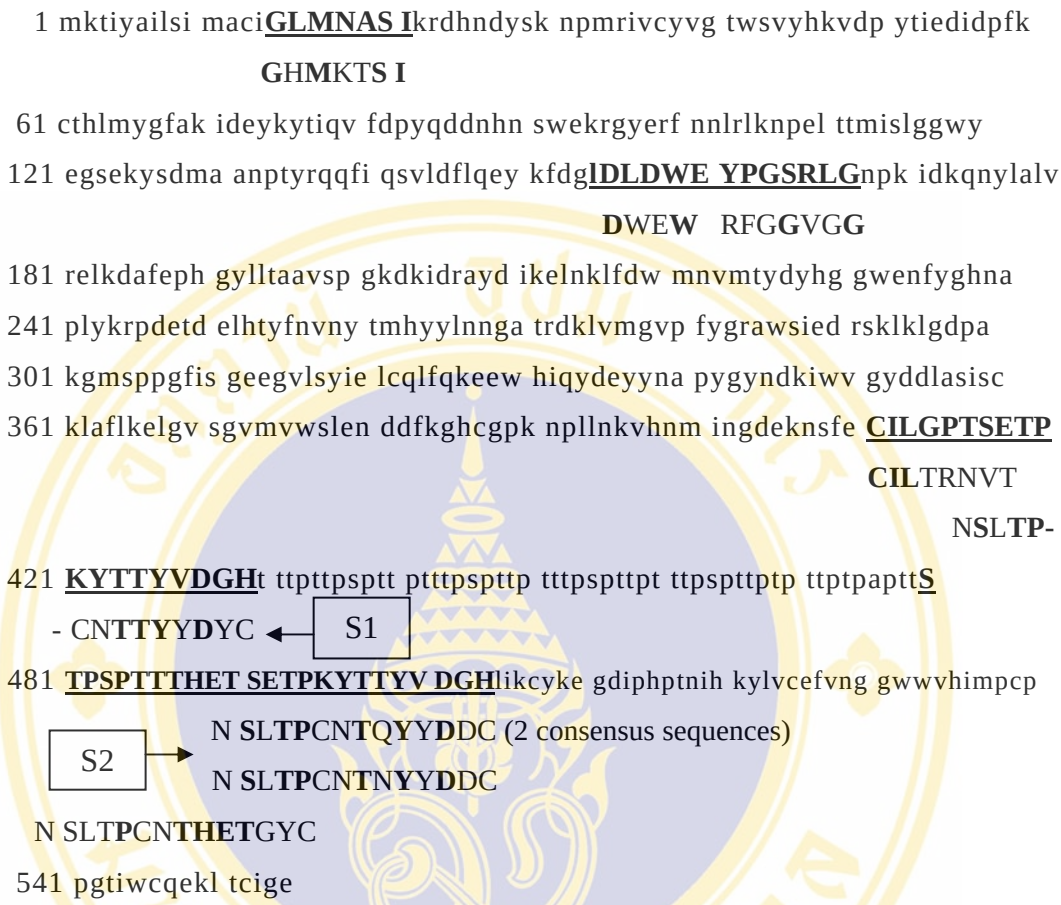

Fig. 11 Analysis of the 8 mimotopes which appeared on the Der f 15 allergen. The regions that matched were considered as mimotopes and indicated by the underlined bold letters. More mimotopes clustered at the regions corresponding to amino acid 411-429 and 480-503. Alignments of the sequences obtained from T7/B3 (S1) and T7/B2 (S3) revealed a common motif were SXTPXXTXYXD.

1 mrvialllta cllglgqaqh ctva**CPKSIP QLI**npkaqst yvysldaktv ltprdsqkvt

CPNP**I**S N**L**C

61 ikadaevaiv ssceavlrlq nvaidgvpng aelaaelaak sfafgyfngr ilgvcpandd

121 qdwslnvkka ivsalqvqfd enkdkvee**TD FSGTCP**teyr kirsdddntv vmekrkdlnl

NSLTPC**CP***

181 cddrrvdlrq tpdqalgqlk eiirhymhpm dsdlmcrmtl kdkvvsevdc eerhvlvhrs

241 hkpihlsyvk mmlkqskd**GV AADLG**qtdse pkrpylsfdh khknptetdv vqvlkklcte

GL **A**RA**LG***

301 itepqasiet sftfhklvdk lrylsaeeta tvdesvktai cpahakrire lfldasafaa

361 sdgsirtlvk ahenqelsvt rstalftvaa ikaapnketv nvllpviase ktirpmllgf

421 svlvrrycek sadcasnsgv kdardaylar lavakdpser mtivralenl nvntedvdnm

481 inamdeiiks tdaepslraa avnalpsdas hmdrykslvm desmpneari aafhkmmqng

541 gmthikdlfa vkgdcmknyv ltyvdnmqks nndlrrrtva advelpekpk remgitrnia

601 reygpytfey dviypethen vtrsingrli rakndklkel vqiqitqngf drelnnamsl

661 lekksfqsvm qfvrdtlkml aqirknaddn hhmkvsvkvn gknvyytdvf qdlkklkell

721 vkraekiine kkvdrsiggv lldsklalpt itglplvykf gdnfvlrydg efsgekgdrh

781 irlnggvvag fygrvkllvk dqkmgyeydg klaytplvdm diqkkehsll lrfntkdvdq

841 htvfrfkqsl rekratgeek dyenevtpes rsdrcfsffl mnycrkashi k**GLILPNV**y

GLIWN**N**F

901 yvmkpekevt alelllkset edktrrymae ltavgspsnk qaraqlevtk geqyrvtlkl

961 pehefnteft insdknnlkm hmdfpnvlqa dltgsfehdk ennvrknrln lqykfanddq

1021 phtveyenef sfnlkrsske knsgldyrak yvsshfpiln hklnvqfkyr pfkvnelnle

1081 gefgrefqhk fqlmrnsqme veevrpfkmh gnsdiklman dldidldlks efkyesnkgt

1141 pielqykvsg kdrskraael gaedvegvid yknngspids kmhahlkakg nhyeydselk

1201 qtqpqqyegk itmskndkki finhksemtk ptntfhlktd advsysdsem kkhyqmefkk

1261 endiytlrst verdgqlfye nyltihkggk lnlnyrrndr killdld**NAL SPREGTMKLN**

NSL T**P**CTSKK **K**V**N**

1321 **I**kdreynfal krdplryrdi tvegnenayv khgklh**LSLM DPSTLSLVT**k adgkidmtvd

N**SL**T PCQS**LS**V**V**P*

Fig. 12 Analysis of the 6 mimotopes which appeared on the Eur m 14 allergen. The regions that matched were considered as mimotopes and indicated by the underlined bold letters.

1 lnafn**MYFRY IY**ptwfnttl ygktfdrrge qfyytyhqiy aryflerls**N SLPDVKPFQY**
CLI **R**T **IY** **N SL**TPCS **PF**EH
61 **SKP**lktgynp hlryqngeem parpsnmypt nidlfyvsdi knyesrveka idfdafdehr
GDC
121 tpyslyhdqh gmdylgqmie gtsnspyqyf ygsifhfyrl lvghvvdpyh k**NGLAPSALE**
NS **L**T**P**CS **L**T
181 **HHQTA**lrdpa fyqlwkridh ivqkyknrlp rytydelsfp gvkienvdvg klytyfehfe
YYYSC
241 hslgnamylg kledvlkani rarhyrlnhk pftynievss dkaqdvyvri flgpkydslg
301 hecelderrh yfvemdrfvh kveagktvie **RKSHDSS**iis dshdsyrnlf kkvsdalegk
RKNSKG**S**
361 dqyyidnshk ycgypenlll p**KGKKGGQ**tf tfyvivtpyv kqdehdlesy hykaftycgv
KP **K**R P**G**N
421 ghgrkypddk plgfpfdrki hdydfytpnm yfkdvvifhk kydevhnetn

Fig. 13 Analysis of the 5 mimotopes which appeared on the Per a 3.0202 allergen. The regions that matched were considered as mimotopes and indicated by the underlined bold letters.

Locating the epitope recognized by MAbs on the Der p 1 and Der f 2 models

A three-dimensional model of Der p 1 (Meno *et al*., 2005) and Der f 2 (Johannessen *et al*., 2005) has previously been published. The protein 3D structure with PDB ID 1XKG (Der p 1) and 1XWV (Der f 2) were downloaded from the Brookhaven Protein Databank (Bernstein *et al*., 1977). We then carried out molecular modeling of the Der p 1 and Der f 2 sequences using RasMol V2.5 software (Kraulis, 1991).

The result from part 5 found that the five mimotopes [**NS**L**L**TPC**SL**T**EF**GSC (2), **CS**LQ**G**A**A**NC (1) and NSL**TP**CSI**QGG**SNC (1)] of MAb B2 were matched with major dust allergen of *D. pteronyssinus* (Der p 1). Only one mimotope (CS**N**K**K**S**A**RC) was matched with mite group 2 allergen Der f 2 Precursor. These mimotopes were aligned and displayed on the molecular structure of Der p 1 and Der f 2 models using Rasmol V2.5 (figs. 14 and 15). As figure 14 shown, the sequences (Asn66-Leu67, Ser71-Leu72, Glu74-Phe75) and (Thr41-Pro42, Gln46-Gly48) were located on the surface three dimensional model of Der p 1, but sequence (Cys102-Ser103, Gly106, Ala108) were located inside the protein structure of Der p 1. Only one sequence (Asn63, Lys65, Ala67) was located on surface of the protein structure of Der f 2 (fig. 15).

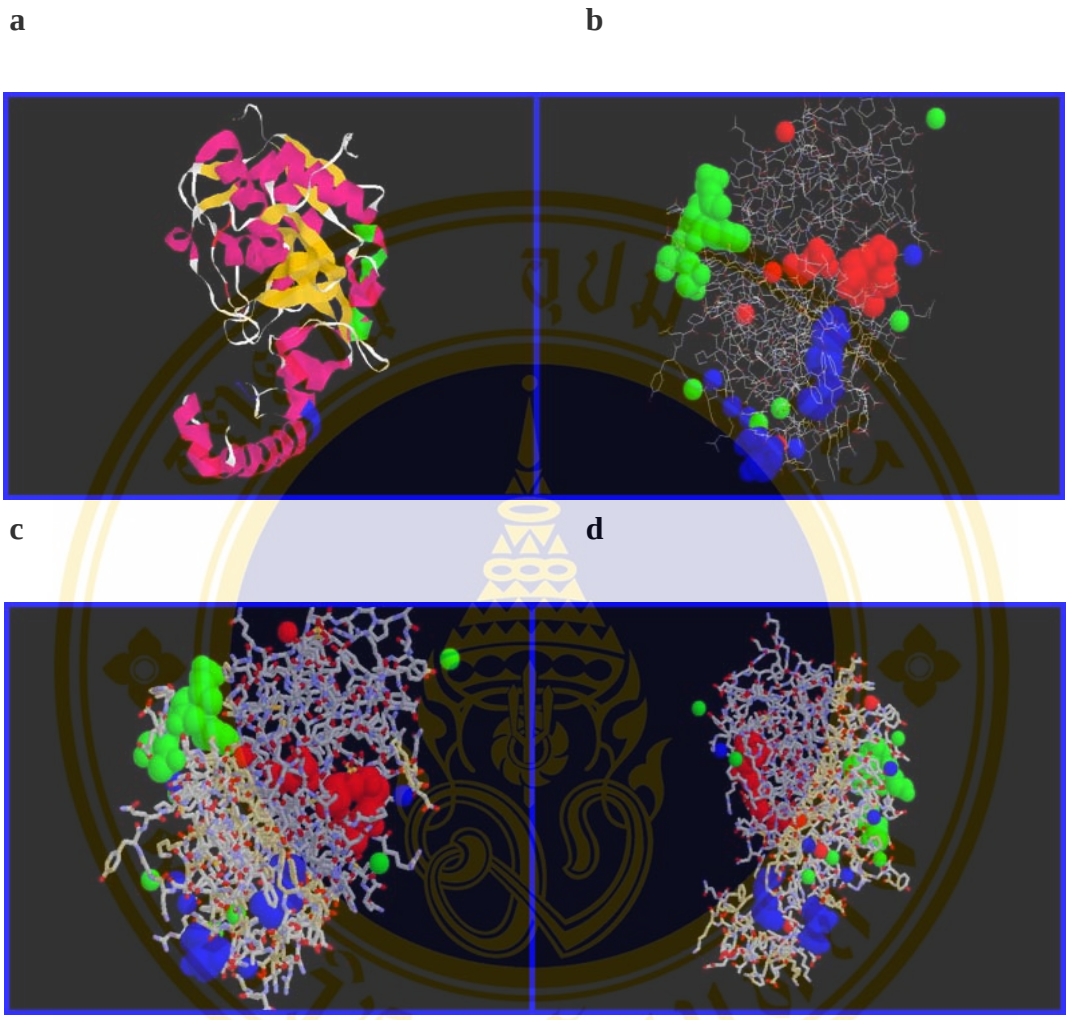

Fig. 14 Three-dimensional model of Der p 1 showing the three epitopes recognized by MAb B2 (a, b, c and d). The epitopes (Asn66-Leu67, Ser71-Leu72, Glu74-Phe75), (Cys102-Ser103, Gly106, Ala108) and (Thr41-Pro42, Gln46-Gly48) colored in green, red and blue respectively.

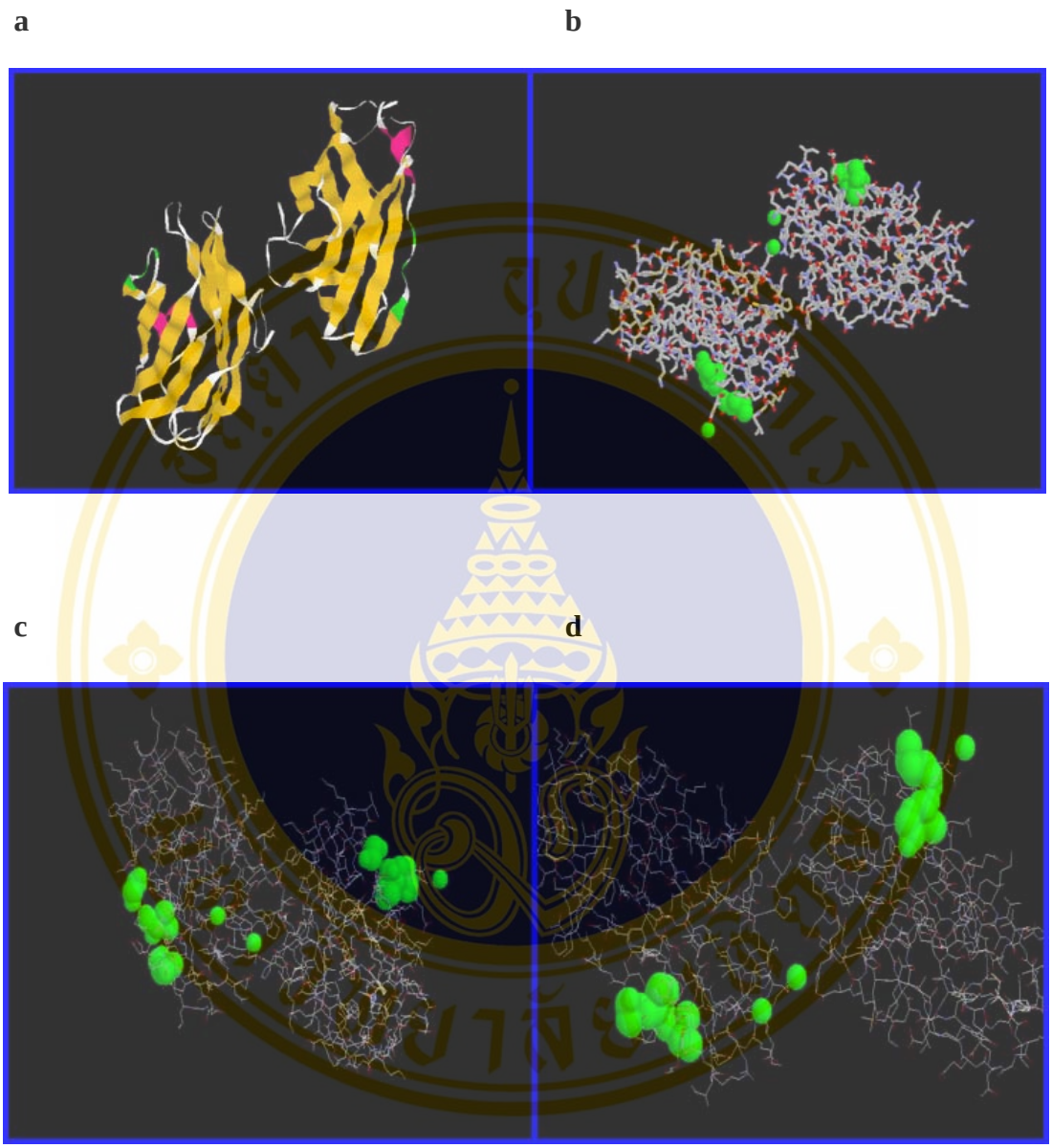

Fig. 15 Three-dimensional model of Der f 2 showing the epitope (Asn63, Lys65, Ala67) recognized by MAb B1 colored in green (a, b, c and d).

Comparison of bound phage sequences with GenBank sequences

Moreover, all 47 mimotope sequences were compared with arthropod protein sequence from GenBank database using BLASTP. Eventhough, Table 11 shown the predominant mimotopes were mimotope with sequence **LTPCDP** that matched with dumpy CG33196-PB protein of *Drosophila melanogaster*, found in 4 phages (8.5%), followed by **CLPYE** match with LD38710p protein of *Drosophila melanogaster*, found in 3 phages (6.3%), 2 phages (4.26%) each mimotopes **PCYPKK**, **SLTEFG**, **SIQGGSN** and **NTQYYD**, 1 phage (2.13 %) each of mimotopes **TPC**SN**KK**, **TPC**NN**L**K**KR**, **PKRPGNC**, **TSKKKVN**, **TNYYDD**C, **SLTDT**SN, **TYYYSC**, **NNPLNS**, **LQGAANC**, **CNTHE**, **NTTYYD**, **CCLIR**, **RKNSKG**, **PCCP**, **LTRNVT**, **IKLTDY**, **CPLA**P**LSP**, **RTSLRT**, **LARALG, SLSVVP**, **SLTPCII**, **LIWNNF**, **TPCGHMK**, **EHGDC**, **TNSKRK**, **NPISNL**, QPL**RVEE**M**PGLE**, **NGR**GVE**DLVA**, W**GEMEGL**, **FGGVGVG**, D**WDWT**D**P**.

Table 11. Comparison of phages peptide sequence with Arthropod sequences from GenBank.

Consensus sequence from four phages T7/B4 4/47 (8.5%)
NS**LTPCDP***
LTPCDP
Part of amino acid sequences from dumpy CG33196-PB protein [*Drosophila melanogaster*] (GenBank accession number NP_787974)

Consensus sequence from three phages T7/B4 3/47 (6.3%)
NSLTPC**CLPYE**HGDC
CLPYE
Part of amino acid sequences from LD38710p protein [*Drosophila melanogaster*] (GenBank accession number AAM27521)

Consensus sequence from two phages T7/B1 2/47 (4.26%)
NSLTP**CPCYPKK**
LTDW**CPCYPKK**
Part of amino acid sequences from putative salivary protein [*Ixodes scapularis*] (GenBank accession number AAY66672)

Consensus sequence from two phages T7/B2 2/47 (4.26%)
NSLTPC**SLTEFG**SC
SLTEFG
Part of amino acid sequences from gag-like protein [*Drosophila melanogaster*] (GenBank accession number CAB99191)

Consensus sequence from two phages T7/B2 2/47 (4.26%)
NSLTPC**SIQGGSN**C
SIQGGSN
Part of amino acid sequences from CG9626-PA protein [*Drosophila melanogaster*] (GenBank accession number NP_649781)

Consensus sequence from two phages T7/B3 2/47 (4.26%)
NSLTPC**NTQYYD**DC
NTQYYD
Part of amino acid sequences from CG8671-PA, isoform A protein [*Drosophila melanogaster*] (GenBank accession number NP_724324)

Sequence of one phage T7/B1
NSL**TPC**S**NKK**
TPCG**NKK**
Part of amino acid sequences from truncated secreted metalloprotease [*Ixodes scapularis*] (GenBank accession number AAM93652)

Table 11. Comparison of phages peptide sequence with Arthropod sequences from GenBank. ***(Continued)***

Sequence of one phage T7/B1
NSL**TPC**NN**L**K**KR**AC
TPCGNG**KKR**
Part of amino acid sequences from putative secreted salivary protein [*Ixodes scapularis*] (GenBank accession number AAY66587)

Sequence of one phage T7/B1
NSLTPC**PKRPGNC**
PKRPGNC
Part of amino acid sequences from ENSANGP00000016875 protein [*Anopheles gambiae* str. PEST] (GenBank accession number EAA01110)

Sequence of one phage T7/B1
NSLTPC**TSKKKVN**
TSKKKVN
Part of amino acid sequences from leucine-rich transmembrane protein [*Aedes aegypti*] (GenBank accession number EAT34302)

Sequence of one phage T7/B2
NSLTPCN**TNYYDD**C
TNYYDD
Part of amino acid sequences from flightin CG7445-PA protein [*Drosophila melanogaster*] (GenBank accession number EAA00837)

Sequence of one phage T7/B2
NSLTPC**SLTDT**SNC
SLTDTTN
Part of amino acid sequences from Myosin heavy chain-like CG31045-PB, isoform B protein [*Drosophila melanogaster*] (GenBank accession number NP_732109)

Sequence of one phage T7/B2
NSLTPCSL**TYYYSC**
TYYYSC
Part of amino acid sequences from IP15830p protein [*Drosophila melanogaster*] (GenBank accession number ABE73228)

Sequence of one phage T7/B2
NSLTPC**NNPLNS**DC
NNPLNS
Part of amino acid sequences from cytochrome oxidase subunit II protein [*Ephippiger ephippiger*] (GenBank accession number AAZ67720)

Table 11. Comparison of phages peptide sequence with Arthropod sequences from GenBank. ***(Continued)***

Sequence of one phage T7/B2
NSLTPCS**LQGAANC**
LQGAANC
Part of amino acid sequences from PREDICTED: similar to ENSANGP00000021020 protein [*Apis mellifera*] (GenBank accession number XP_394028)

Sequence of one phage T7/B3
NSLTP**CNTHE**TGYC
CNTHE
Part of amino acid sequences from GA13308-PA protein [*Drosophila pseudoobscura*] (GenBank accession number EAL27271)

Sequence of one phage T7/B3
NSLTPC**NTTYYD**YC
NITYYDY
Part of amino acid sequences from GA16045-PA protein [*Drosophila pseudoobscura*] (GenBank accession number EAL27331)

Sequence of one phage T7/B3
NSLTP**CCLIR**TIYC
CCLIR
Part of amino acid sequences from ENSANGP00000017193 protein [*Anopheles gambiae* str. PEST] (GenBank accession number EAA03889)

Sequence of one phage T7/B3
NSLTPC**RKNSKG**SC
RKNSKG
Part of amino acid sequences from ENSANGP00000010757 protein [*Anopheles gambiae* str. PEST] (GenBank accession number EAA07743)

Sequence of one phage T7/B3
NSLT**PCCP***
PCCP
Part of amino acid sequences from peptide toxin 4 precursor protein [*Macrothele gigas*] (GenBank accession number BAD13405)

Sequence of one phage T7/B3
NSLTPCI**LTRNVT**C
LTRNVT
Part of amino acid sequences from RNA-binding protein S1 protein [*Drosophila melanogaster*] (GenBank accession number CAD30680)

Table 11. Comparison of phages peptide sequence with Arthropod sequences from GenBank. ***(Continued)***

Sequence of one phage T7/B4
NSLTPCM**IKLTDY**C
IKLTDY
Part of amino acid sequences from RE60936p protien [*Drosophila melanogaster*] (GenBank accession number AAM48431)

Sequence of one phage T7/B4
NSLTPCSPF**EHGDC**
EHGDC
Part of amino acid sequences from ENSANGP00000009614 protein [*Anopheles gambiae* str. PEST] (GenBank accession number EAA00837)

Sequence of one phage T7/B5
NSLTPC**RTSLRT**C
RTSLRT
Part of amino acid sequences from GM08204p protein [*Drosophila melanogaster*] (GenBank accession number AAO25045)

Sequence of one phage T7/B5
NSLTPCG**LARALG***
LARALG
Part of amino acid sequences from RH08828p protein [*Drosophila melanogaster*] (GenBank accession number AAQ22428)

Sequence of one phage T7/B5
NSLTPCQ**SLSVVP***
SLSVVP
Part of amino acid sequences from SMC6 protein [*Anopheles gambiae*] (GenBank accession number CAD59408)

Sequence of one phage T7/B5
N**SLTPCII**C*
SLTPCII
Part of amino acid sequences from PREDICTED: similar to CG8844-PA, isoform A protein [*Tribolium castaneum*] (GenBank accession number AAO25045)

Sequence of one phage T7/B5
NSLTPCG**LIWNNF**C
LIWNNF
Part of amino acid sequences from RE10888p protein [*Drosophila melanogaster*] (GenBank accession number AAM51993)

Table 11. Comparison of phages peptide sequence with Arthropod sequences from GenBank. ***(Continued)***

Sequence of one phage T7/B5
NSL**TPCGHMK**TSIC
TPCGHMK
Part of amino acid sequences from GA16932-PA protein [*Drosophila pseudoobscura*] (GenBank accession number EAL29972)

Sequence of one phage T7/B5
NSLTP**CPLA**S**LSP**C
CPLAP**LSP**
Part of amino acid sequences from PREDICTED: similar to CG12541-PA protein [*Tribolium castaneum*] (GenBank accession number XP_975223)

Sequence of one phage T7/B6
NSLTPCT**TNSKRK**C
TNSKRK
Part of amino acid sequences from bin3, bicoid-interacting 3 protein [*Tribolium castaneum*] (GenBank accession number XP_969281)

Sequence of one phage T7/B6
NSLTPCP**NPISNL**C
NPISNL
Part of amino acid sequences from CG4281-PA protein [*Apis mellifera*] (GenBank accession number XP_001121068)

Sequence of one phage M13/B6
QPL**RVEE**M**PGLE**
M**RVEE** L**PGLE**
Part of amino acid sequences from l (3)70Da protein [*Drosophila melanogaster*] (GenBank accession number CAB51031)

Sequence of one phage M13/B6
NGRGVE**DLVA**WH
NGRSED**DLVA**
Part of amino acid sequences from putative metalloprotease protein [*Ixodes scapularis*] (GenBank accession number AAO85918)

Sequence of one phage M13/B6
W**GEMEGL**WWQGK
GEMEGL
Part of amino acid sequences from putative salivary secreted protein [*Ixodes scapularis*] (GenBank accession number AAY66675)

Table 11. Comparison of phages peptide sequence with Arthropod sequences from GenBank. ***(Continued)***

Sequence of one phage M13/B6 DWEWR**FGGVGVG** **FGGVGVG** Part of amino acid sequences from GA11391-PA protein [*Drosophila pseudoobscura*] (GenBank accession number EAL31485)
Sequence of one phage M13/B6 D**WDWT**D**P**RGNAN **WDWTEP** Part of amino acid sequences from ENSANGP00000010900 protein [*Anopheles gambiae* str. PEST] (GenBank accession number XP_310176)
Bold letter mean the display peptide of bound phage that match with part of Arthropod protein sequence from GenBank

Prediction of the localization in the cell for mimotope matched using PSORT II software

After comparison phage sequences using BLASTP software, using PSORTII software to analyze and predict the protein localization in the cell (Table 12). In the table 12, mimotope **PCCP** that matched with peptide toxin 4 precursor protein of *Macrothele gigas* (GenBank accession number BAD13405), mimotope **TPC**NNL**KKR** that matched with putative secreted salivary protein of *Ixodes scapularis* (GenBank accession number AAY66587), mimotope **CPCYPKK** that matched with putative salivary protein of *Ixodes scapularis* (GenBank accession number AAY66672), mimotope **GEMEGL** that matched with putative salivary secreted protein *Ixodes scapularis* (GenBank accession number AAY66675) and mimotope **LTPCDP** that matched with dumpy CG33196-PB protein of *Drosophila melanogaster* (GenBank accession number NP_787974), were found that to locate at extracellular at 66.7%, 55.6%, 44.4%, 43.5% and 26.1% those matched proteins, respectively.

Table 12. Information and origin of protein sequences with match phage mimotope by using PSORT II program.

Peptide	Ortholog protein	GenBank Accession No.	% predict origin of protein sequence
1. NS**LTPCDP***	dumpy CG33196-PB protein of *Drosophila melanogaster*	NP_787974	26.1 %: extracellular, including cell wall
2. **CLPYE**HGDC	LD38710p protein of *Drosophila melanogaster*	AAM27521	47.8 %: cytoplasmic
3. **CPCYPKK**	putative salivary protein of *Ixodes scapularis*	AAY66672	44.4 %: extracellular, including cell wall
4. **SLTEFG**SC	gag-like protein of *Drosophila melanogaster*	CAB99191	73.9 %: nuclear
5. **SIQGGSN**C	CG9626-PA protein of *Drosophila melanogaster*	NP_649781	69.6 %: nuclear
6. **NTQYYD**DC	CG8671-PA, isoform A protein of *Drosophila melanogaster*	NP_724324	69.6 %: nuclear
7. NSL**TPC**S**NKK**	truncated secreted metalloprotease of *Ixodes scapularis*	AAM93652	47.8 %: nuclear
8. **TPC**NN**LKKR**A	putative secreted salivary protein of *Ixodes scapularis*	AAY66587	55.6 %: extracellular, including cell wall
9. C**PKRPGNC**	ENSANGP00000016875 protein of *Anopheles gambiae* str. PEST	EAA01110	78.3 %: nuclear
10. C**TSKKKVN**C	leucine-rich transmembrane protein of *Aedes aegypti*	EAT34302	34.8 %: cytoplasmic
11. CN**TNYYDD**C	flightin CG7445-PA protein of *Drosophila melanogaster*	EAA00837	52.2 %: nuclear
12. C**SLTDTSN**C	Myosin heavy chain-like CG31045-PB, isoform B protein *of Drosophila melanogaster*	NP_732109	26.1 %: plasma membrane
13. CSL**TYYYSC**	IP15830p protein of *Drosophila melanogaster*	ABE73228	47.8 %: nuclear
14. C**NNPLNS**DC	cytochrome oxidase subunit II protein of *Ephippiger ephippiger*	AAZ67720	60.9 %: cytoplasmic
15. CS**LQGAANC**	similar to ENSANGP00000021020 protein of *Apis mellifera*	XP_394028	47.8 %: nuclear
16. **CNTHE**TGYC	GA13308-PA protein of *Drosophila pseudoobscura*	EAL27271	39.1 %: plasma membrane
17. CN**TTYYD**YC	GA16045-PA protein of *Drosophila pseudoobscura*	EAL27331	47.8 %: endoplasmic reticulum
18. **CCLIR**TIYC	ENSANGP00000017193 protein of *Anopheles gambiae* str. PEST	EAA03889	69.6 %: cytoplasmic
19. C**RKNSKG**SC	ENSANGP00000010757 protein of *Anopheles gambiae* str. PEST	EAA07743	39.1 %: nuclear
20. NSLT**PCCP***	peptide toxin 4 precursor protein of *Macrothele gigas*	BAD13405	66.7 %: extracellular, including cell wall
21. CI**LTRNVT**C	RNA-binding protein S1 protein of *Drosophila melanogaster*	CAD30680	82.6 %: nuclear
22. CM**IKLTDY**C	RE60936p protien of *Drosophila melanogaster*	AAM48431	47.8 %: cytoplasmic
23. CSPF**EHGDC**	ENSANGP00000009614 protein of *Anopheles gambiae* str. PEST	EAA00837	39.1 %: plasma membrane
24. C**RTSLRT**C	GM08204p protein of *Drosophila melanogaster*	AAO25045	21.7 %: nuclear

Table 12. Information and origin of protein sequences with match phage mimotope by using PSORT II program. ***(Continued)***

Peptide	Ortholog protein	GenBank Accession No.	% predict origin of protein sequence
25. CQ**SLSVVP***	SMC6 protein of *Anopheles gambiae*	CAD59408	17.4 %: vesicles of secretory system
26. CG**LARALG***	RH08828p protein of *Drosophila melanogaster*	AAQ22428	60.9 %: nuclear
27. N**SLTPCII**C*	similar to CG8844-PA, isoform A protein of *Tribolium castaneum*	AAO25045	44.4 %: endoplasmic reticulum
28. CG**LIWNNF**C	RE10888p protein of *Drosophila melanogaster*	AAM51993	43.5 %: cytoplasmic
29. **TPCGHMK**TSIC	GA16932-PA protein of *Drosophila pseudoobscura*	EAL29972	47.8 %: endoplasmic reticulum
30. **CPLASLSP**C	similar to CG12541-PA protein of *T. castaneum*	XP_975223	65.2 %: nuclear
31. CT**TNSKRK**C	bin3, bicoid-interacting 3 protein of *T. castaneum*	XP_969281	87.0 %: nuclear
32. CP**NPISNL**C	CG4281-PA protein of *Apis mellifera*	XP_001121068	69.6 %: nuclear
33. QPL**RVEEMPGLE**	l (3)70Da protein of *Drosophila melanogaster*	CAB51031	47.8 %: cytoplasmic
34. **NGR**GVE**DLVA**WH	putative metalloprotease protein of *Ixodes scapularis*	AAO85918	44.4 %: endoplasmic reticulum
35.W**GEMEGL**WWQGK	putative salivary secreted protein *Ixodes scapularis*	AAY66675	43.5 %: extracellular
36. DWEWR**FGGVGVG**	GA11391-PA protein of *Drosophila pseudoobscura*	EAL31485	73.9 %: nuclear
37. D**WDWT**D**P**RGNAN	ENSANGP00000010900 protein of *A. gambiae* str. PEST	XP_310176	39.1 %: cytoplasmic

* Mean stop codon

Bold letter mean the display peptide of bound phage that match with part of Arthropod protein sequence from GenBank

CHAPTER V
DISCUSSION

Phage display is powerful tool for selecting peptides or proteins with specific binding properties from vast numbers of variants. Its utility lies principally in generating molecular probes against specific targets and for the analysis and manipulation of protein/ligand interactions. Antibody-antigen interactions can be investigated using libraries of random amino acids, generated by cloning random oligonucleotides into the gene coding for p3 (Wright *et al*., 1995) or gene 10B of T7 capsid protein (Steven and Trus, 1986). The random sequences can range from 6-43 amino acids (Cwirla *et al*., 1990; McConnell *et al*., 1996) and can be displayed in one of two formats. First, each random peptide can be displayed as a linear molecule upon the phage protein (Cwirla *et al*., 1990; Scott and Smith, 1990; Devlin *et al*., 1990) that will assume a number of possible conformations. Thus the same peptide sequence can theoretically be presented as different conformations within the library. The second format is to conformationally constrain the peptide sequence using conserved cysteine residues encoded on either side of the pepide cloning site (Mc Connell *et al*., 1994; Felici *et al*., 1993). The cysteine residues in many cases likely form a disulfide bond presenting the peptide as a loop, allowing for a more natural conformation to be assumed. Internal cysteine residues can also be designed within the peptide sequence (Furmonaviciene *et al*., 1999) allowing the formation of internal constraints.

Filamentous M13 bacteriophage random peptide library have been successfully used to identify epitopes specific to MAb (Furmonaviciene *et al*., 1999) and patient's sera (Felici *et al*., 1993). λ phage (T7) random peptide libraries also have been successfully used for epitope mapping of infectious diseases such as *Bordetella pertussis* (Wilson *et al.*, 1998), Polyomavirus (Houshmand *et al.*, 1999), Leptospirosis (Tungtrakarnpoung *et al*, 2006), Hepatitis E virus (Gu *et al.*, 2004), *Plasmodium falciparum* (Casey *et al.*, 2004).

The aim of this study was to search for the epitopes or “mimotopes” reacting with newly developed MAbs specific to house dust mite, by using T7 random heptapeptide with cysteine flanking, and random 12 mer M13 phage display libraries, respectively.

Screening of phage mimotopes against MAbs specific to House dust mite

The biopanning result revealed that increments in the titer of the phages through successive rounds of selection (table 5) suggested that the enrichment of phages with high affinity binding where increasing the numbers of affinity selection process. For T7 phage selection with some MAb like B1, in each round of biopanning, the titer of phage recovered were decreased. Which can be influence from, stringency of the washes, the number of rounds of panning, and the concentration of the target molecule (if initial binding is done in solution) or the density of the immobilized target molecule (if panning involves direct binding of phage to immobilized target), all of this prior mention conditions can be affect the diversity and affinity of clones selected. In general, the diversity of phage clones decreases after multiple rounds of purification, and with higher-stringency selection conditions (i.e. with a low concentration or density of target molecule) (Irving *et al*., 2001).

To increase the target-specific binders, during biopanning step, the concentration of target coated protein should be reduced two times in each next round of panning (Hogenboom, 2005).

Ten randomly selected bound phages per each MAb were tested for the binding specificity with each house dust mite MAbs using indirect ELISA. The selected bound phages of T7/B1, T7/B6 and M13/B6 gave positive ELISA result, especially selected bound phages of T7/B6.4 and T7/B6.5 had high titer positive at 1:320. But the selected bound phages of T7/B2, T7/B3, T7/B4 and T7/B5 gave negative indirect ELISA result. Our result corroborate with that of Furmonaviciene et al (1999) in that their selected bound phage with MAb specific to Der P1, could not be detected by ELISA, therefore, selected bound phages were further screened using Western blot analysis. In this thesis study, phages T7/B2, T7/B3, T7/B4 and T7/B5 were further screened using Western blot analysis, only the selected bound phages of T7/B4 (25%) were found to have positive result with B4 MAb, and positive band with molecular weight of 42.6

kDa (figure 9). This result also in concordance with that of Furmonaviciene et al (1999), in that only 20% of their selected bound phage were shown positive result with Western blot analysis.

Comparison of bound phages sequences with Structure Database Allergen Protein (SDAP)

The screening of phage-displayed random peptides libraries (PDPLs) typically contains a small population of phage clones expressing peptides that bind selectively to the antigen-combining site of a particular antibody. Phages from this group are ‘‘target-specific binders’’, which can be further divided into scarce high-affinity binders and the much more abundant low-affinity binders. PDPLs also contain a population of ‘‘target-unrelated peptides’’ (TUP), which may react with constant antibody regions or other components of the screening system, such as the beads, plates, or the capturing molecule (streptavidin, protein A, etc.).

In this study, all of the found mimotopes were compared with previously reported TUP (Menendez and Scott, 2005; Adey *et al*., 1995) and also compared with vector sequences in GenBank using Vecscreen software. All mimotopes were not similar to vector sequences, but one mimotope from phage M13/B6 with sequence AER***WGPW***GVHSW, was found to be similar with TUP sequence. Recovery of TUP in PDPL screenings can often be minimized by careful experimental design. Retention of solid-phase binders may be diminished by immobilization of the target at high density, as suggested by Adey, *et al* (Adey *et al*., 1995) however; use of high density of target may also lower the stringency of the selection. Preadsorption of the input phage to the solid phase coated with the capturing reagent (protein A or streptavidin) helps to reduce plastic and capturing reagent binders and should be an integral part of any screening procedure (Menendez et al., 2001; Messmer and Thaler, 2001). In cases in which streptavidin is used as the anchoring reagent, a short pulse with biotin just before the washes may be introduced to help displace streptavidin binders.

All together 47 phages mimotope were aligned with the allergenic peptide from the Structural Database of Allergenic Proteins (SDAP). SDAP is a web server

that integrates a database of allergenic proteins with various computational tools that can assist structural biology studies related to allergens. SDAP is an important tool in the investigation of the cross-reactivity between known allergens, in testing the FAO/WHO allergenicity rules for new proteins, and in predicting the IgE-binding potential of genetically modified food proteins. Using this internet service through a browser, it is possible to retrieve information related to an allergen from the most common protein sequence and structure databases (SwissProt, PIR, NCBI, PDB), to find sequence and structural neighbors for an allergen, and to search for the presence of an epitope from other collection of allergens.

In this study, from the total 47 selected phages mimotope, 8 mimotopes (17.4%) that partially matched with Der f 15 allergen was predominant, followed by Eur m 14 allergen (13.04%) and Per a 3.0202 (10.87%) (Table 9). Der f 15 is the 98 kDa HDM allergen of *D. farinae* (GenBank Accession number AAD52672). Der f 15 allergen were characterized in group 15 mite allergens that are homologous to insect chitinases. In *D. farinae*, they have been shown to be located in the gut, suggesting that they have a function in digestion rather than in moulting. The Group 15 allergens are potentially very significant because they are the major allergens recognised by dogs and cats, and because they are highly glycosylated, consisting of almost 50% carbohydrate (Thomas *et al*., 2002). Five mimotopes of T7/B3, one each of mimotope from T7/B2, T7/B5 and T7/B6 were partially matched with Der f 15 allergen. There is considerable about specificity of MAbs against crude panel antigen using indirect ELISA, MAb B5 was specific with *D. farinae* antigen and MAbs of B2, B3, B6 were specific with *D. farinae* and *D. pteronyssinus* (Table 3 in chapter III). This prior mentioned results showed that the mimotopes that matched with Der f 15 allergen were correlated well with MAbs specific to *D. farinae* antigen.

The majority of mimotope sequences from T7/B2 was partially matched with Der p 1 allergen. The major Group 1 mite allergens Der p 1 and Der f 1 were first isolated as cysteine proteases, some study reported that natural Der p 1 exhibits mixed cysteine and serine protease activity (Takai *et al*., 2005). Der p 1 and Der p 2 have been shown to be potent inducers of nitric oxide release from alveolar macrophages (Peake *et al*., 2003). Group 7 mite allergens comprised of Der p 7, Der f 7 and Lep d 7.

In this study, most of mimotope sequences from T7/B4 partially matched with Der f 7 allergen and Lep d 7 allergen. The proliferative and cytokine response to the group 7 allergens for *D. pteronyssinus* and *D. farinae* indicates that there is a high degree of T-cell cross-reactivity between the whole purified allergens from each species (Johansson *et al*., 1997). Lep d 7 has been shown to bind with sera from *L. destructor*-sensitised subjects but usually at low titres (Eriksson *et al*., 2001). The majority of mimotope sequences from T7/B5 were partially matched with Eur m 14 allergen. Eur m 14 is the member in group 14 mite allergens that have sequence homology to a vitellogenin or apolipophorin-like protein. These molecules are for lipid transport or lipid storage, which may explain their instability in aqueous extracts (Thomas *et al*., 2002).

Two mimotope sequences from T7/B1 were partially matched with Der f 6 allergen. Der f 6 allergen is the 279 amino acid polypeptide which conserves a primary structure characteristic for chymotrypsin-like serine proteases found in mammals (Kawamoto *et al*., 1999). There is considerable about specificity of MAbs against crude panel antigen using indirect ELISA, MAb B1 was specific with *D. pteronyssinus* (Table 3 in chapter III). This result showed that the mimotopes that were not matched with Der p 1 allergen were differenced with MAbs specific to *D. pteronyssinus* antigen. The only one mimotope sequence from T7/B1 partially matched with Blo t 1 allergen. Blo t 1, a Group 1 mite allergen, a cysteine protease, a major allergen, and a homologue of Der p 1 (Cheong *et al*., 2003; Mora *et al*., 2003; Fonseca and Diaz, 2003). Recombinant Blo t 1 has been shown to have 90% frequency of reactivity with IgE in sera from asthmatic children and a 65% frequency in sera from asthmatic adults, indicating that represents a major allergen. Cross-reactivity between the Group 1 mite allergens of *B. tropicalis* (Blo t 1) and *D. pteronyssinus* (Der p 1) was demonstrated to be low (Cheong *et al*., 2003; Mora *et al*., 2003).

The partially matched mimotopes may contain more than one matching site along the amino acid sequences of the allergen. Various mimotopes that matched with Der f 15, Eur m 14 and Per a 3.0202 were aligned, with each matched protein (respectively) and some mimotopes were appeared at more than one location along the sequences. As shown in figure 11, the region of amino acid 411-429 and 480-503,

correlated with overlapping mimotopes and seemed to be the main epitope clusters of the Der f15 allergen peptide. Interestingly, the alignment of the sequences obtained from T7/B3 (3 mimotopes) and T7/B2 (1 mimotope) revealed a common motif were **S**X**TP**XX**T**X**Y**X**D**. This result showed that MAbs clone B2 and B3 bind to the same epitope at Der f 15 allergen.

In general, a library of constrained peptides will represent far fewer three-dimensional shapes than a library of unconstrained (but otherwise comparable) peptides. The constrained peptide whose accessible conformations happen to overlap extensively with active conformations may possess far higher activity than any unconstrained peptide (Smith GP *et al*, 1997). The most common constraint on displayed peptides is a disulfide bond between two flanking cystine residues of the random peptide sequence. The disulfide bond has been required for the ability of the displayed peptide to bind with target receptor. In this study, T7 random heptapeptide with cysteiene flanking and 12peptide M 13 libraries, were used. The result showed that mimotopes from T7 random heptapeptide with cysteiene flanking (constrained displayed peptide) yeilded more consensus sequences and matched with *Dermatophagoides spp*. allergen than those of 12peptide M 13 library (unconstrained displayed peptide).

Locating the epitope recognized by MAbs on the Der p 1 and Der f 2 models

Rasmol V2.5 program is a molecular graphic software intended for the visualization of proteins, nucleic acids and small molecules. The program is aimed at display, teaching and generation of publication quality images (Kraulis, 1991). From previous studies on structure of house dust mite allergen, Kåre Meno and colleagues provided a detailed description of the crystal structure of the major house dust mite allergen Der p 1 (Meno *et al*., 2005), Johannessan and colleagues were study structure of the house dust mite allergen Der f 2 using X ray Crystallization (Johannessen *et al*., 2005). We could download protein 3D structure with PDB IDs 1XKG (Der p 1) and 1XWV (Der f 2) from the Brookhaven Protein Databank. Then Rasmol V 2.5 program was used for locating mimotopes that display on the Der p 1 and Der f 2 models. The

result showed that mimotopes T7/B1 and T7/B2 were partially matched with major dust allergen of *D. pteronyssinus* (Der p 1 allergen). The sequences (Asn66-Leu67, Ser71-Leu72, Glu74-Phe75) and (Thr41-Pro42, Gln46-Gly48) from mimotope of phage T7/B2 were located on the surface of the protein structure of Der p 1. One sequence (Asn63, Lys65, Ala67) from mimotope of phage T7/B1 were located on the surface of the protein structure of Der f 2. In general, antigenicity properties is depend upon several factors; such as allowing a substance to combine specifically with antibodies or T cell receptor, complexity of antigen, locating outside the antigen molecule (easily interact with paratope of antibody) etc. From these prior mentioned, four sequences from phages mimotopes located on the surface of protein structure of Der p 1 and Der f 2 may have antigenic or allergen properties. For further study, the Dermatophagoides allergenic proteins (that matched with phage mimotopes); Der p 1 [from residues (Asn66-Phe75) and (Thr41-Gly48)] and Der f 2 [from residue (Asn63-Ala67)] should be further test for its antigenicity, allergen and specific epitope (that interact with antibody specific to *Dermatophagoides* spp.*)* properties.

Our finding of phage mimotopes that partially matched with Der P1 also corroborated with that of Furmonaviciene, *et al* (1999), in that their finding phage mimotope also located on Der P1 but at different position at Leu 147-Gln160.

Comparison of bound phage sequences with GenBank sequences

When all 47 mimotope sequences were compared with arthropod protein sequence from GenBank database using BLASTP, all mimotopes were not found to match with protein sequences of house dust mite (Table 11). Since, these mimotopes were partially matched with allergen sequences using SDAP (table 9). It can be explain reasons why all mimotopes were not found matched with house dust mite sequences in SDAP and GenBank that, first, the GenBank is the biggest genome database of organism so that shortly mimotope sequences could have more chance to match with others arthropod proteins with full complete genome such as *Drosophila melanogaster* or *Ixodes scapularis* (that have more than 10,000 proteins in database), than match with house dust mite proteins database (that have only 90 proteins in database). Second, the house dust mite MAbs that used in this study were not specific

only *D. farinae* or *D. pteronyssinus* antigen (table 3 in chapter III), such as MAb B6 were positive with others antigen like Cockroach antigen (*Periplaneta Americana* and *Blatella germanica*), house fly antigen etc.

Prediction of protein sequence using PSORT II software

PSORTII software was used for analysis and prediction the protein localization in the cell that matched with phage mimotope sequences. The result showed that mimotope **PCCP** matched with peptide toxin 4 precursor protein of *Macrothele gigas*; mimotopes **TPC**NNL**KKR**, **CPCYPKK** and **GEMEGL** were matched with putative salivary secreted protein of *Ixodes scapularis* at extracellular. The peptide toxin 4 precursor protein is neurotoxin in spider which has function on blocking voltage-gated sodium channels. The putative salivary secreted protein of *Ixodes scapularis* contains antiinflammatory and immunomodulatory compounds that prevent immune reactions from disrupting the feeding process (Wikel and Alarcon-Chaidez, 2001). In this study, phage mimotope that matched with peptide toxin 4 precursor and putative salivary secreted protein may be only structural mimic but different functional mimic. The matched protein may not relate with secretion of house dust mite or pathogenesis of allergy mechanism in human or animals. Since mite bodies and mite feces are the main sources of many allergens (Arlian *et al*., 1987; Tovey *et al*., 1981). The allergens associated with mite fecal matter are enzymes that originate from the mite's digestive tract.

The successful identification for the epitopes or mimotopes, reacting with MAbs or patient' s sera, using the random peptide library relies upon several factors; such as quality of MAbs, process during selection and screening (biopanning), type of random peptide library, good laboratory experiences etc. Thus far, the random peptide library has been applied to search for the epitopes of MAb and patient's sera (Tungtrakarnpoung *et al*., 2006; Furmonaviciene *et al*., 1999). It is generally much cheaper and easier than alternative epitope mapping methods that require chemical synthesis of short peptide segments of the ligand's amino acid sequence (Geysen *et al*., 1987). It would be interesting to apply technology to search and identify specific

epitopes reacting with newly developed MAbs specific to house dust mite. Then we can utilized the information from SDAP, SWISS-PROT and PDB database including RasMol and PSORT II software to identify epitope location in the structure of protein sequences and predict antigenic of mimotopes from phages. The results of which will open avenues for development of study the molecular interaction between antibody and house dust mite antigen including epitope mapping of allergen in allergy field.

CHAPTER VI
CONCLUSIONS

The most important findings of this study can be summarized as follows;

Random peptide phage display libraries

- The selected bound phages of B1 and B6 with ELISA positive for house dust mite MAbs, especially selected bound phages T7/B6.4 and T7/B6.5 gave positive high titer with MAb against house dust mite.
- Selected bound phages of T7/B4 were found to have Western blot positive with MAb specific to house dust mite and positive band with molecular weight at 42.6 kDa.

Comparison of selected bound phages using SDAP and Rasmol V2.5 software

- From the total 47 selected phages mimotope, 8 mimotopes (17.4%) that partially matched with Der f15 allergen was predominant, followed by Eur m 14 allergen (13.04%) and Per a 3.0202 (10.87%).
- The majority of mimotope sequences from T7/B1, T7/B2, T7/B3 and T7/B5 were matched with Der f 6 allergen (33.33%), Der p 1 allergen (55.55%), Der f 15 allergen (62.5%) and Eur m 14 allergen (42.87%), respectively. The most of mimotope sequences from T7/B4 were matched with Der f 7 allergen (33.33%) and Lep d 7 allergen (44.44%).
- Only one mimotope **AE**RW**G**PW**G**VHSW that was matched with Lep d 2 allergen.
- Various mimotope matched with Der f 15, Eur m 14 and Per a 3.0202 were aligned and some mimotopes were appeared at more than one location along the sequences. Especially Der f 15 allergen, the regions: amino acid 411-429

and 480-503, correlated with overlapping mimotopes and seemed to be the main epitope clusters of the Der f 15 allergen peptide.

- The alignment of the sequences obtained from T7/B3 (3 mimotopes) and T7/B2 (1 mimotope) revealed a common motif of **S**X**TP**XX**T**XYX**D**.
- Two mimotope sequences (Asn66-Leu67, Ser71-Leu72, Glu74-Phe75), and one mimotope sequence (Thr41-Pro42, Gln46-Gly48) from phage T7/B2 were located on the surface of the protein structure of Der p 1. Mimotope sequence (Asn63, Lys65, Ala67) from phage T7/B1 was located on the surface of Der f 2.

Comparison of selected bound phages using BLASTP and PSORTII software

- Using BLASTP software; The predominant mimotopes were **LTPCDP** that matched with dumpy CG33196-PB protein of *Drosophila melanogaster*, found in 4 phages (8.5%), followed by **CLPYE** matched with LD38710p protein of *Drosophila melanogaster,* found in 3 phages (6.3%).
- Using PSORTII software; The mimotope **PCCP** that matched with peptide toxin 4 precursor protein of *Macrothele gigas*, mimotope **TPC**NNL**KKR**, **CPCYPKK**, and **GEMEGL** matched with putative salivary secreted protein *Ixodes scapularis* and mimotope **LTPCDP** matched with dumpy CG33196-PB protein of *Drosophila melanogaster* were found that to locate at extracellular including cell wall 66.7%, 55.6%, 44.4%, 43.5% and 26.1% of that mimotopes, respectively.

Future recommendation;

- To increase the target-specific binder phage from biopanning experiment in the future, the concentration of target coated protein should be reduced two times in each next round of panning.
- For random peptide library screening, preadsorption of the input phage to the solid phase coated with the capturing reagent (protein A or streptavidin) helps to reduce plastic and capturing reagent binders and should be an integral part

of any screening procedure. In cases in which streptavidin is used as the anchoring reagent, a short pulse with biotin just before the washes may be introduced to help displace streptavidin binders.

- The Dermatophagoides allergenic proteins (that matched with phage mimotopes); Der p 1 [from residues (Asn66-Phe75) and (Thr41-Gly48)] and Der f 2 [from residue (Asn63-Ala67)] should be further test for its antigenicity, allergen and specific epitope (that interact with antibody specific to *Dermatophagoides* spp.*)* properties.

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APPENDIX

APPENDIX

Appendix A:

0.05 M Carbonate buffer, pH 9.6 (coating buffer)

Na_2CO_3	0.159	g
$NaHCO_3$	0.293	g
DW	100.0	ml, keep at 4°C

Phosphate buffered saline-Tween (PBS-T)

Nacl	8.9	g
Na_2HPO_4	1.28	g
$NaH_2\ PO_4.2H_2O$	0.156	g
Tween-20	0.50	ml
DW	1,000.0	ml, keep at 4°C

Phasphate buffered saline (PBS), pH 7.4, 0.01 M

Na_2HPO_4	1.42	g
$NaH_2\ PO_4.2H_2O$	1.56	g
Nacl	0.58	g
DW	1,000.0	ml, keep at 4°C

ABTS (2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)

(Substrate pH 4.5)		
Citrate buffer	2	ml
ABTS	0.0006	g

Citrate buffer

$Na_3C_6H5O_7$. 2H2O	2.94 g
Citric acid	2.1 g
DW	100 ml

Stopping reaction reagent, 5 N H_2SO_4

H_2SO_4	28.0 ml
DW	172.0 ml

LB top agarose

LB broth, powder	25.0 g
Agarose	6.0 g
DW	1,000.0 ml, autoclave at 121°C, 15 min

1% Sodium dodecyl sulfate (SDS)

SDS	1.0 g
DW	100.0 ml

Tris-EDTA buffer (TE buffer)

10 mM Tris-HCl, pH 8.0 + 1 mM EDTA

50% PEG 8000

PEG 8000	100.0 g
DW	100.0 ml

Stir overnight and autoclave at 121°C, 15 lbs. pressure, 15 min

1% Bovine serum albumin (BSA)

BSA	1.0 g
Sterilized PBS	100.0 ml

Horse-radish peroxidase (HRP)-conjugate

Dilution peroxidase-conjugate rabbit anti-mouse immunoglobulins, ratio 1: 1,000 in 1% BSA-PBST

Phage extraction buffer (mixing in ratio 1:1:1)

20 mM Tris-Hcl : 100 mM Nacl: 6 mM MgSO4, pH 8.0

PCR reaction mixture, 50 µL/PCR tube

T7 selection up primer, 10.0 pmol/µL	2.5 µl
T7 selection down primer, 10.0 pmol/µL	2.5 µl
Mg free 10 x thermal buffer for DyNAzyme DNA	5.0 µl
$MgCl_2$, 50 mM	5.0 µl
Taq DNA polymerase, 5.0 u/µl	0.4 µl
dNTP, 25 mM	1.0 µl
UDW	23.6 µl
DNA sample	10.0 µl

Stock Tris-borate EDTA buffer 10 x (TBE 10 x)

Tris base	121.2 g
Boric acid	61.8 g
$Na_2EDTA.2H_2O$	100.0 ml
DW	1,000.0 ml

1.5% Agarose

Agarose	1.5 g
0.5 x TBE buffer	100.0 ml

Pre-reaction mixture for 20 samples

Premix Dye	80.0	µl
5 x Sequencing buffer	40.0	µl
T7 selection up primer, 10.0 pmol/µL	20.0	µl
UDW	160.0	µl

Divide into 15 µl/PCR tube, 20 tubes

Add 5 µl PCR product into each tube

Acrylamide: Bis-acrylamide solution

Acrylamide	30.0	g
Bis-acrylamide	0.8	g
DW to	100.0	ml

The solution should be stored in a dark bottle at 4^{o}C.

0.5 M Tris-HCl buffer, pH 6.8

Tris (Hydroxymethyl aminomethane)	6.05	g
DW	50.00	ml

The pH will be adjusted to 6.8 with 1 N HC1 then DW will be added until the volume will be reached 100 ml. The buffer will be kept at 4^{o}C.

1.5 M Tris-HCl buffer, pH 8.8

Tris	18.15	g
DW	50.00	ml

The pH will be adjusted to 8.8 with 1 N HC1 then DW will be added to be 100 ml. The buffer should be stored at 4^{o}C.

10% Sodium dodecyl sulfate (SDS)

SDS	10.0	g
DW	100.0	ml

The solution will be kept at room temperature (RT).

10% Ammonium persulfate

Ammonium peroxodisulfate	1.0	g
DW	10.0	ml

The solution will be freshly prepared or made a stock solution and stored at 4°C.

N, N, N', N'-Tetra-methylethylenediamine (TEMED)

The solution is commercially prepared by Bio-Rad Laboratories and stored at 4°C.

Sample buffer or denaturing buffer (3X)

0.5 M Tris-HCl, pH 6.8	4.7	ml
SDS	1.7	g
2-Mercaptoethanol	3.8	ml
Glycerol	7.5	ml
0.5% Bromphenol blue	0.8	ml

The final volume will be adjusted to 25 ml with DW. The buffer will be stored in small plastic tubes at 44°C. Working sample buffer (1.5X) will be prepared by diluting the 3X sample buffer with an equal volume of DW.

Electrode buffer or running buffer or tris-glycine buffer, pH 8.3

Tris	6.06	g
Glycine	28.80	g
SDS	2.00	g
DW	2,000.00	ml

The buffer will be kept at 4°C.

10% gels (40 ml)

DW	16.1	ml
1.5M Tris-HCl,pH8.8	10	ml
Acrylamide: Bis-acrylamide	13.34	ml
10% SDS	400	µl
10% Ammonium persulfate	133.34	µl
TEMED	26.66	µl

The solutions will be gently mixed.

5% stacking gel (5 ml)

DW	2.90	ml
0.5 M Tris-HCl, pH 6.8	1.25	ml
Acrylamide: Bis-acrylamide	800.00	µl
10% SDS	50.00	µl
10% Ammonium persulfate	25.00	µl
TEMED	4.00	µl

The solution will be gently mixed and poured on the top of the 10% separate gel.

Coomassie brilliant blue stain

Coomassie brilliant blue R-250	5.0	g
Absolute methanol	400.0	ml
Glacial acetic acid	50.0	ml
Glycerol	25.0	ml
DW	500.0	ml

The staining solution will be kept at RT.

Destaining solution

Absolute methanol	500.0 ml
Glacial acetic acid	100.0 ml
Glycerol	50.0 ml
DW	1,400.0 ml

The solution will be stored at RT.

Transfer or blotting buffer, pH 8.3

Tris	6.06 g
Glycine	28.80 g
Absolute methanol	400.00 ml
DW	1,600.00 ml

The buffer will be kept at 4^{o}C.

Phosphate-buffered saline (PBS), pH 7.4 (10X)

$NaH_2P0_4 \cdot H_2O$	4.14 g
$Na_2HP0_4 \cdot 2H_2O$	30.24 g
NaCl	170.00 g
DW	2,000.00 ml

Working PBS solution (IX) will be prepared by diluting 100 ml of 10 X PBS solutions with 900 ml of DW.

Washing buffer: 0.05% Tween 20 in PBS (PBS-T)

Tween 20	0.5 ml
PBS, pH 7.4 (IX) to	1,000.0 ml

Blocking solution

Skim milk	2.0 g
20% NaN_3	0.2 ml
PBS, pH7.4 (lX) to	100.0 ml

Diluent of enzyme-conjugated

PBS-T	12.0	ml

Substrate solution

2, 6-Dichlorophenol-indophenol	0.02	g
PBS, pH7.4 (lX)	10.00	ml
30%H_2O_2	10.00	µl

The solution will be freshly prepared before being used.

Appendix B:

Table 13. The genetic codes

FIRST POSITION (5' END)	SECOND POSITOIN				THIRD POSITION (3' END)
	U	C	A	G	
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	Stop	Stop	A
	Leu	Ser	Stop	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

Note: Given the position of the bases in a codon, it is possible to find the corresponding amino acid. For example, the codon (5') AUG (3') on mRNA specifies methionine, whereas CAU specifies histidine. UAA, UAG, and UGA are termination signals. AUG is part of the initiation signal, and it codes for internal methionines as well. (From L. Stryer, Biochemistry, 3d ed., W. H. Freeman, 1988)

Source: Watson *et al*, 1992

Table 14. Codes of amino acids

Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic acid	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamic acid	Glu	E	Serine	Ser	S
Glutamine	Gln	Q	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

Source: Watson *et al*, 1992; Panbankaed and Udomwarapane, 1993

Appendix C: The MAbs were tested for specificity by indirect ELISA using crude panel antigens.

Table 15. Specificity of the MAbs tested by indirect ELISA using crude panel antigens.

Clone numbers	Clone names	OD405													
		Dp [1]	Df [1]	Bt [2]	Ld [2]	Tp [2]	CR-Pa [1]	CR-Bg [1]	housefly [1]	mosquito [1]	ant [1]	bermuda [1] grass	para [1] grass	careless [1] weed	typhaceae [1]
1	ID4H9	**5+**	0	0	0	0	0	0	0	0	0	0	0	0	0
2	7D2A1	0+	**3+**	0	0	0	0	0	0	0	0	0	0	0	0
3	9D2D2	**3+**	**4+**	0	0	0	1+	1+	1+	1+	0	0	0	1+	0
4	9C3C7	**5+**	**5+**	0	0	0	1+	1+	1+	0	0	0	0	1+	0
5	9D4A4	**3+**	**4+**	0	0	0	1+	1+	0	0	0	0	0	1+	0
6	9D3B5	**4+**	**5+**	0	0	0	0	0	0	0	0	0	0	0	0
7	3C5G7	**1+**	**2+**	0	0	0	0	0	0	0	0	0	0	0	0
8	8B4A1	**1+**	**1+**	0	0	0	0	0	0	0	0	0	0	0	0
9	5D5C12	**4+**	**3+**	**3+**	**3+**	**1+**	**5+**	**5+**	**5+**	**5+**	**2+**	**2+**	**3+**	**5+**	**2+**

0 = OD 0.000 - 0.200, 1+ = OD 0.201-0.400, 2+ = OD 0.401-0.600, 3+ = OD 0.601-0.800, 4+ = 0.801-1.000, 5+ = > 1.000

Dp = *Dermatophagoides pteronyssinus*, Df = *Dermatophagoides farinae*, Bt = *Blomia tropicalis*, Ld = *Lepidoglyphus destructer*,

Tp = *Tyrophagus putrescentiae*, CR-Pa = Cockroach- *Periplaneta americana*, CR-Bg = Cockroach- *Blatella germanica*

[1] phenol extracted antigen, [2] sonicated antigen

Table 16. Specificity of the MAbs tested by 4 kinds of antigens preparing from Dp and Df mites

Clone number	Clone name	Isotype	OD405								nDer p 1
			Dp				Df				
			sonicate	secretory	SMM	phenol extract	sonicate	secretory	SMM	phenol extract	
1	1D4H9	IgGκ	0.648	0.176	0.084	1.048	0.175	0.123	0.118	0.146	0.499
2	7A2D1	IgG1κ	0.063	0.057	0.040	0.117	0.108	0.092	0.085	0.626	0.260
3	9D2D2	IgG1κ	0.606	0.559	0.123	0.810	0.587	0.601	0.403	1.241	0.903
4	9C3C7	IgMκ	0.750	0.743	0.271	1.273	1.251	1.069	0.738	2.120	1.044
5	9D4A4	IgG2aκ	0.463	0.198	0.101	0.602	0.385	0.228	0.142	0.880	0.464
6	9D3B5	IgG1κ	0.576	0.089	0.039	0.718	0.210	0.150	0.137	1.086	0.330
7	3C5G7	IgG1κ	0.180	0.122	0.082	0.345	0.158	0.131	0.096	0.472	0.427
8	8B4A1	IgMκ	0.401	0.818	0.230	0.325	0.752	2.123	1.532	0.368	0.738
9	5D5C12	IgMκ	0.788	0.719	0.169	0.999	0.799	0.593	0.825	0.509	1.239

Table 17. The specificity of the MAbs tested by indirect ELISA using 4 kinds of antigen prepared from Dp and Df mites

Clone numbers	Clone names	Isotypes	OD405								
			Dp				Df				nDer p 1
			sonicate	secretory	SMM	phenol* extract	sonicate	secretory	SMM	phenol* extract	
1	1D4H9	IgG1 κ	3+	0	0	5+	0	0	0	0	2+
2	7A2D1	IgG1 κ	0	0	0	0	0	0	0	3+	0
3	9D2D2	IgG1 κ	3+	2+	0	4+	2+	3+	2+	5+	4+
4	9C3C7	IgM κ	3+	3+	1+	5+	5+	5+	3+	5+	5+
5	9D4A4	IgG2a κ	2+	0	0	3+	1+	1+	0	4+	2+
6	9D3B5	IgG1 κ	2+	0	0	3+	2+	0	0	4+	1+
7	3C5G7	IgG1 κ	0	0	0	1+	0	0	0	2+	2+
8	8B4A1	IgM κ	2+	4+	1+	1+	3+	5+	5+	1+	3+
9	5D5C12	IgM κ	3+	3+	0	4+	3+	2+	4+	2+	5+

0 = OD 0.000 - 0.200, 1+ = OD 0.201-0.400, 2+ = OD 0.401-0.600, 3+ = OD 0.601-0.800, 4+ = 0.801-1.000, 5+ = > 1.000

* phenol extracted antigens from Dp and Df mites were used as in Table 1

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