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

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Abstract. Random heptapeptide T7 and random 12mer M13 phage libraries were employed to identify mimotopes binding to monoclonal antibodies (MAb) specific to house dust mite. After selection of bound phage by bio-panning and determination of binding specificity, DNA of selected bound phages was amplified, sequenced and aligned for peptide similarity. Eight mimotopes which were partially matched with Der f 15 allergen were predominant. The amino acid regions, 411-429 and 480-503 of Der f 15 allergen, appeared to be the main epitope clusters. Five mimotopes of MAb B2 and one mimotope of MAb B1 matched with Der p 1 and Der f 2 precursor, respectively.

Keywords: phage display, random peptide library, mimotope, monoclonal antibody, house dust mite

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). Mite allergens are a major cause of allergic diseases such as bronchial asthma, allergic rhinitis and atopic dermatitis (Chapman *et al*, 1983). Like in 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 (Vichyanon, 2002), with 70% of allergic Thai children sensitive to house dust mite (Kongpanichkul *et al*, 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 can produce 19 allergen groups; however, Group I allergens, Der p 1 and Der f 1, are the most important allergens responsible for

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Table 1
Monoclonal antibodies used in this study.

No.	Clone	Specificity[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 panel of crude antigens.
[b]*Dermatophagoides pteronyssinus*; [c]*Dermatophagoides farinae*.

pathogenesis of asthma disease.

Phage display is a powerful tool for selecting peptides or proteins with specific binding properties from a vast number of variants. Its utility lies principally in generating molecular probes against specific targets and for analysis and manipulation of protein-ligand interactions (Smith, 1985; Kay *et al*, 1996). Filamentous M13 bacteriophage random peptide library has been successfully used to identify epitopes specific to monoclonal antibodies (MAbs) of house dust mite (Furmonaviciene *et al*, 1999). Lambda phage (T7) random peptide libraries also have been successfully used for epitope mapping of infections (Tungtrakarnpoung *et al*, 2006; Na-ngam *et al*, 2008).

As the epitopes specific to these MAbs specific to house dust mite, produced at the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand (http://www.li.mahidol.ac.th/thesis/2547/cd364/4336552.pdf) have not been investigated, this study searched for the epitopes or mimotopes reacting with these newly developed MAbs using random heptapeptide T7 and random 12- peptide M13 phage libraries. The identified mimotopes were compared with the sequences of Structural Database of Allergenic Proteins (SDAP) (http://fermi.utmb.edu/SDAP/) and identified the epitopes recognized by MAbs on the Der p 1 and Der f 2 allergens.

MATERIALS AND METHODS

MAbs specific to house dust mite

Six clones of MAbs; 1D4H9E10, 9D2D2G4, 9D4A4, 3C5G7, 7A2D1 and 9C3C7F12 (Table 1) were obtained from Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

Phage display random peptide libraries (PDPL)

Two bacteriophage display libraries were used in this study, random heptapeptide T7 phage and random 12-mer M13 phage library. Random heptapeptide T7 (flanked by cystiene residues) phage library was constructed using T7 select-415 kit from Novagen (Madison, WI) as described previously (Na-ngam *et al*, 2008). The T7 phage library, originally of 3.3×10^7 pfu, was amplified to 4.8×10^{10} /ml before use. The 12-mer M13 phage library was kindly provided by Dr Mondharop Yamabhai, Suranaree University,

Thailand. Each library had a complexity of ~10^9 phages (Kay *et al*, 2001).

Bio-panning

All six MAbs were used in T7 phage display bio-panning according to the manufacturer's instructions, involving four rounds of bio-panning using microtiter plate coated with 100 µl of MAbs (10 µg ml^{-1}) and unbound sites were blocked with 200 µl of BSA blocking solution [50 mg ml^{-1} in phosphate buffered saline (PBS)]. T7 phage was propagated in *E. coli* strain BL21. Finally, ten single plaques of T7 phage per each MAb were randomly picked and used for further phage amplification and purification (Na-ngam *et al*, 2008). The M13 12-mer library was propagated essentially as previously described (Kay *et al*, 2001), involving three rounds of bio-panning using microtiter plate coated with MAb 9C3C7F12 (200 mg ml^{-1}) and blocked with 150 µl of blocking solution. Finally, eight single plaques of M13 12-mer phage were propagated in F′ *E. coli* strain DH5α.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was performed as a standard protocol to check the binding specificity of ten selected T7 phage clones per each MAb and 8 M13 phage clones. Microtiter wells of ELISA plates were coated with purified phage in carbonate buffer pH 9.6. Phages were allowed to attach to the solid surface of the plates by incubating at 37ºC for 1 hour in a humidified chamber and then at 4ºC overnight. The unbound phages were extensively washed away with the PBS - 0.05% Tween. The unoccupied sites on the wells were blocked with 1% BSA solution at 37ºC in a humidified chamber for 1 hour and washed again before adding MAbs to the wells. Plates were incubated as in the blocking step, washed as described above, and then incubated with rabbit anti-mouse immunoglobulin-horseradish peroxidase conjugate for 1 hour. After washing, 100 µl aliquot of freshly prepared 2,2′-azinobis (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 minutes. The reaction was terminated by adding 1% SDS solution. The optical density (OD) of each well solution was at 405 nm using an ELISA reader (TECAN, Mannedorf, Switzerland). Binding of phage to antibody was considered specific if the OD value is above 0.2. Phages that were ELISA-positive were further prepared their DNA to use in the PCR.

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, washed with PBST and incubated with 3% skim milk in PBST. Each lane of the nitrocellulose membrane was treated with specific monoclonal antibody at room temperature overnight. After washing with PBST, the strips were incubated with rabbit anti-mouse immunoglobulin-horseradish peroxidase conjugate (1/1,000 dilution) at room temperature for 3 hours. The membrane strips were then washed five times with PBST, stained with 20 ml of 2, 6-dichloro-4-nitrophenol in PBS (10 mg/ml) and 5 µg of H_2O_2 until color developed.

PCR and DNA sequencing

Phage DNA was used as the template

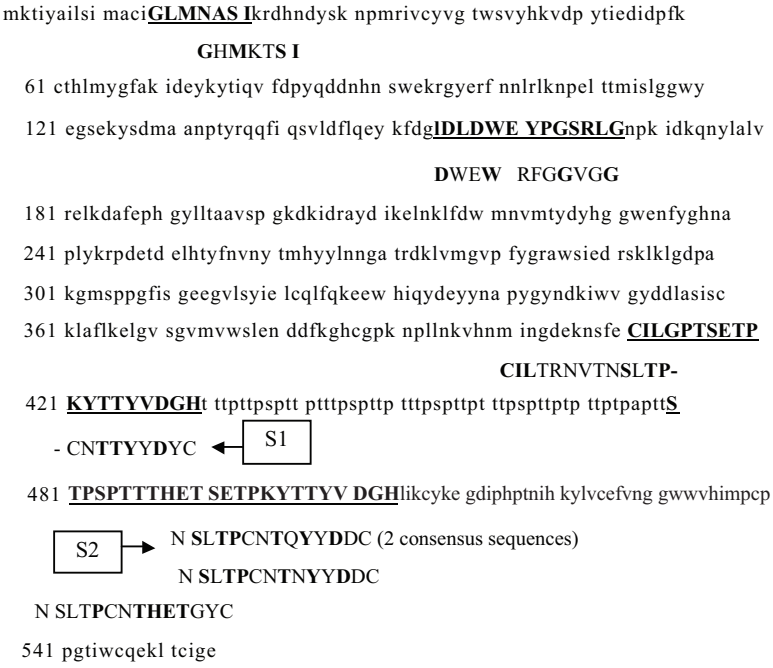

Fig 1–Analysis of the 8 mimotopes appearing on the Der f 15 allergen. The regions that matched are considered as mimotopes and indicated by underlined bold letters. More mimotopes clustered at the regions corresponding to amino acid 411-429 and 480-503. Alignments of the sequences obtained from T7/B3 (S1) and T7/B2 (S3) revealed a common motif of SXTPXXTXYXD.

for PCR and sequencing experiments. For analysis of peptide sequences of bound phage, a segment of the 10B capsid protein of T7 phage DNA was amplified, according to the manufacturer's instruction (Novagen, Madison, WI, 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') primers. The total PCR reaction mixture (50 µl) consisted of the following reagents: 5 µl each of T7 Selected Up and Down primers (5 pmol μl^{-1}), 5 µl of 10x buffer, 10 µl of $MgCl_2$ (25 mM), 2 µl of Taq DNA polymerase (1 U μl^{-1}), 1 µl of dNTP (25 mM), 12 µl H_2O, and 10 µl of phage DNA. The thermal cycling conditions were as follows: 94°C for 2 minutes, 35 cycles of 94°C for 20 seconds, 50°C for 20 seconds and 72°C for 45 seconds; and a final heating at 72°C for 4 minutes. PCR amplicons were purified using a commercial kit (QIA Quick PCR Purification Kit). Purified amplicons were sequenced using BigDye Terminator v3.1 cycle sequencing kit (ABI Applied Biosystem, Bedford, MA). For 12-mer M13 phage library, cell pellet of *bona fide* binding phage was used to prepare double stranded DNA for automated fluorescence DNA sequencing by eluting DNA with 50 µl of sterile H_2O and measuring DNA concentration at 260 nm (1 OD = 50 µg ml^{-1}). Then, 0.7 µg of DNA and 10 pmol primer (1 µl of 10 mol μl^{-1} gene III downstream primer) (5'-GCC CTC ATA'GTT AGC GTA ACG-3') were added, followed by sterile H_2O to bring the total volume to 20 µl before being subjected to sequencing.

Table 2
Deduced amino acid and sequences of capsid fusion peptides of T7 and M13 phages that bind to six monoclonal antibodies of house dust mite.

Library/antibody (numbers)	Display peptide (numbers)
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] *WGPW* matched with Target Unrelated Peptide (TUP).

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

The sequences obtained were compared with the matched sequences from SDAP (http://fermi.utmb.edu/SDAP/index.html). In addition, all of the found phage mimotopes were compared with those previously reported "target-unrelated peptides" (TUP) of Menendez and Scott (2005).

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

In order to study the location of the epitopes recognized by MAbs on Der p 1 and Der f 2 models, the 3D protein structures of PDB ID1XKG (Der p 1) and 1XWV (Der f 2) were downloaded from PDB database and analyzed using RasMol V2.5 software.

RESULTS

Phage sequence selected by MAb specific to house dust mite

After the bio-panning process, binding specificity of selectively bound phages was identified using ELISA and western blot analysis. The bound phages of T7/B1, T7/B6 and M13/B6 showed ELISA positivity for house dust mite MAbs, while only the bound phage of T7/B4 showed positive result with B4 MAb and positive western blot band of 42 kDa with bound phages of T7/B2, T7/B3, T7/B4 and T7/B5 (Fig 1).

Sequence analysis and molecular modeling

All selected phage clones, both positive and negative with ELISA and western blot analysis, were checked for the inserted DNA sequences. Peptide-displaying phage types were assigned

Table 3
Summary of mimotopes from MAbs that matched allergen sequences from SDAP.

Library/ MAbs (numbers)	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*	33.3
	CS**N**K**KS**A**R**C	Der f 2	Mite group 2 allergen Der f 2 precursor	16.7
	NSLTPC**NN**L**K**K**RA**C	Blo t 1	Cysteine protease precursor of *B. tropicalis*	16.7
	KP**K**RP**G**N	Pera 3.0202	Allergen of *P. Americana*	16.7
	NSL**T**PCTSKK**K**V**N**C	Eur m 14	High molecular weight allergen M-177 precursor of *E. maynei*	16.7
T7/B2 (9)	**NSL**TPC**SL**T**EF**GSC	Der p 1	Major mite fecal allergen Der p 1 precursor	22.2
	NSL**TP**CSI**QGG**SNC	Der p 1	Major house dust allergen of *D. pteronyssinus*	22.2
	CSLQ**G**A**A**NC	Der p 1	Major mite fecal allergen Der p 1	11.1
	SLTDT**S**N	Der p 3	Mite allergen Der p 3 Precursor	11.1
	N**N**PL**NS**D	Der p 4	Alpha-amylase of *D. pteronyssinus*	11.1
	N**SLTP**CN**T**N**YY**D**D**C	Der f 15	98 kDa HDM allergen of *D. farinae*	11.1
	NSLT**P**C**SL**TYYYSC	Per a 3.02	Allergen of *P. americana*	11.1
T7/B3 (8)	NSLT**P**CN**THET**GYC	Der f 15	98 kDa HDM allergen of *D. farinae*	12.5
	N**SLTP**CN**TQYYD**DC	Der f 15	98 kDa HDM allergen of *D. farinae*	25.0
	N**SLTP**CN**TTYYD**YC	Der f 15	98 kDa HDM allergen of *D. farinae*	12.5
	CILTRNV**T**C	Der f 15	98 kDa HDM allergen of *D. farinae*	12.5
	CLI**RTIY**	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)	**LPYEHGD**	Der f 7	Mite allergen Der f 7 precursor of *D. farinae*	33.3
	NS**LTPCDP***	Lep d 7	Mite allergen Lep d 7 precursor of *L. destructor*	44.4
	NSLTPCS**PF**EHGDC	Per a 3.0202	Allergen of *P. americana*	11.1
	CM**IKL**TDYC	Eur m 3	Mite allergen Eur m 3 precursor of *E. maynei*	11.1
T7/B5 (7)	**N**SLT**PCRTSLRT**C	Per a 3	Allergen Cr-PI precursor of *P. americana*	14.3
	GLARALG*	Eur m 14	High molecular weight allergen M-177 precursor *of E. maynei*	14.3
	N**SL**TPCQS**LS**V**VP***	Eur m 14	High molecular weight allergen M-177 precursor *of E. maynei*	14.3
	GLIWN**N**F	Eur m 14	High molecular weight allergen M-177 precursor *E. maynei*	14.3

Table 3 (Continued).

Library/ MAbs (numbers)	Peptide	Allergen	Protein name	Frequency (%)
	NSLT**P**CI**IC***	Der f 6	Mite allergen Der f 6 precursor of *D. farinae*	14.3
	GH**M**KT**SI**	Der f 15	98 kDa HDM allergen of *D. farinae*	14.3
	NS**L**T**P**CP**L**A**SL**SP**C**	Der p 8	Glutathione S-transferase of *D.pteronyssinus*	14.3
T7/B6 (2)	TTN**SKRK**	Per a 1.0103	Cr-PII protein of *P. americana*	50.0
	CPNP**I**SN**L**C	Eur m 14	high molecular weight allergen M-177 precursor of *E. maynei*	50.0
M13/B6 (6)	Q**PL**R**VEE**MPG**L**E	Lep d 7	Mite allergen Lep d 7 Precursor of *L. destructor*	16.7
	AERW**G**PW**G**VHSW	Lep d 2	Mite group 2 allergen Lep d 2 [Precursor]	16.7
	NG**R**GV**EDLVA**WH	Per a 3.0201	allergen of *P. americana*	16.7
	W**G**EME**G**LWWQ**GK**	Der f mag	Mag protein of *D. farinae*	16.7
	DWE**W**RFG**G**VGV**G**	Der f 15	98 kDa HDM allergen of *D. farinae*	16.7
	DW**TDP**RGNA**N**	Der p 4	Alpha-amylase of *D. pteronyssinus*	16.7

* Stop codon.
Bold letter means the display peptide of bound phage that could match with part of allergen protein sequence from SDAP.

according to the selection procedure with each MAb (Table 2). All mimotopes were compared with previously reported in Target Unrelated Peptide (TUP) (Menendez and Scott, 2005) and also compared with vector sequences in GenBank using Vecscreen software. The results showed that 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 for peptide similarity of allergens using SDAP. All mimotopes partially matched with allergen sequences whose possible functional roles are listed in Table 3. The majority of mimotope sequences from T7/B1, T7/B2, T7/B3 and T7/B5 were matched with Der f 6 allergen (33.3%), Der p 1 allergen (55.5%), Der f 15 allergen (62.5%) and Eur m 14 allergen (43%). Most of the mimotope sequences from T7/B4 matched Der f 7 allergen (33.3%) and Lep d 7 allergen (44.4%). Five mimotopes of T7/B3, one each mimotope from T7/B2, T7/B5 and T7/B6 were partially matched with Der f 15 allergen.

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 3D protein structures PDB ID 1XKG (Der p 1) and 1XWV (Der f 2) were downloaded from the Brookhaven Protein Databank (Bernstein *et al*, 1977). Five mimotopes (**NSL**TPC**SL**T**EF**GSC (2), **CS**-LQ**G**A**A**NC (1) and NSL**TP**CSI**QGG**SNC (1)) of MAb B2 matched with the major dust allergen of *D. pteronyssinus* (Der p 1). Only one mimotope (CS**N**K**KSA**RC) matched with mite group 2 allergen

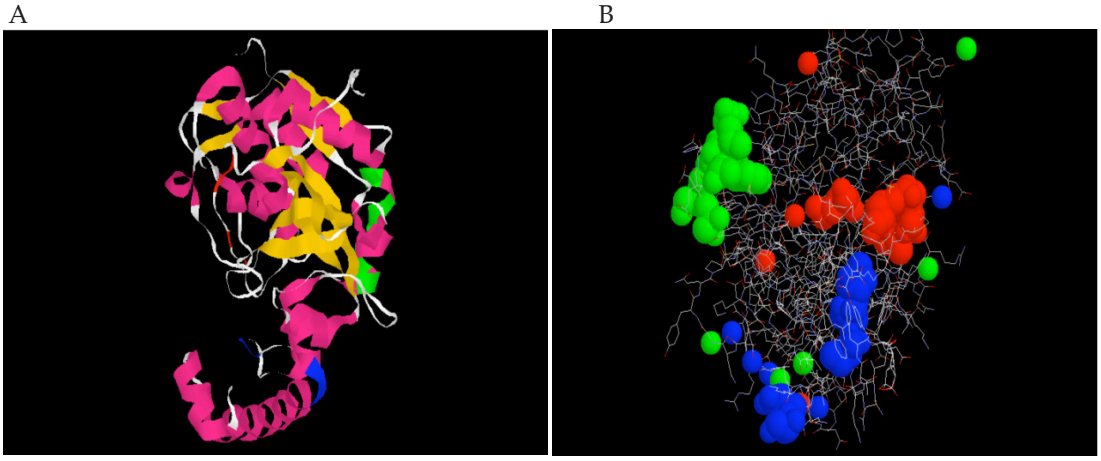

Fig 2–Three-dimensional model of Der p 1 showing the three epitopes recognized by MAb B2. The epitopes Asn66-Leu67, Ser71-Leu72 and Glu74-Phe75; Cys102-Ser103 and Gly106, Ala108; and Thr41-Pro42, and Gln46-Gly48 are colored in green, red and blue, respectively.

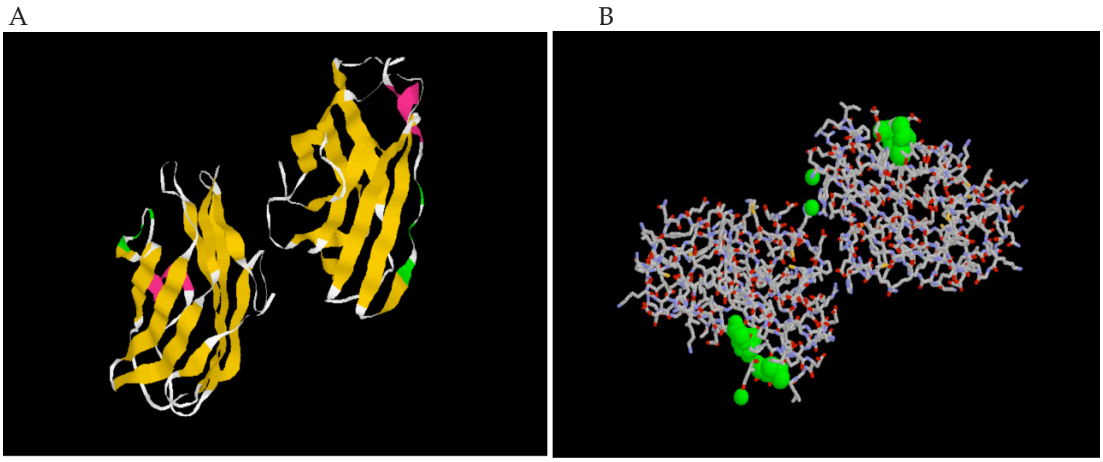

Fig 3–Three-dimensional model of Der f 2 showing the epitopes Asn63, Lys65, Ala67 recognized by MAb B1 colored in green.

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 2 and 3). The sequences Asn66-Leu67, Ser71-Leu72, and Glu74-Phe75, and Thr41-Pro42 and Gln46-Gly48 from mimotope of phage T7/B2 were located on the surface of the Der p 1. One sequence (Asn63, Lys65, Ala67) from mimotope of phage T7/B1 was located on the surface of the protein structure of Der f 2.

DISCUSSION

Our results showed 25% of the bound phages with ELISA negative but positive by western blot analysis, in agreement with the study of Furmonaviciene *et al* (1999), in that 20% of their selected bound phages with ELISA negative for MAb specific to Der P1 are positive by western blot analysis. There was one report concerning the specificity of MAbs against house dust mite antigen using ELISA (Yong *et al*,

1999). In our study, MAb B5 is specific with *D. farinae* antigen and MAbs of B2, B3, while B6 was specific with *D. farinae* and *D. pteronyssinus*. Our results showed that the mimotopes that were matched with Der f 15 allergen correlated well with MAbs specific to *D. farinae* antigen.

Menendez *et al* (2005) recommended that the recovery of TUP in PDPL screenings can often be minimized by careful experimental design. Pre-adsorption of the input phage to the solid phase coated with the capturing reagent (protein A or streptavidin) helped to reduce plastic and capturing reagent binders and should be an integral part of any screening procedure.

The partially matched mimotopes may contain more than one matching site along the amino acid sequences of the allergen. In this study, various mimotope sequences were aligned with Der f 15, Eur m 14 and Per a 3.0202, and some mimotopes appeared at more than one location along the sequences. As shown in Fig 1, 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**T-P**CN**TQ**Y**YD**DC appeared to partially 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 SXTPXX-TXYXD, indicating that MAbs clones B2 and B3 could bind to the same epitope at Der f 15 allergen.

In general, antigenicity properties depend upon several factors, such as allowing a substance to combine specifically with antibodies or T cell receptor, complexity of antigen, and location outside the antigen molecule (easily interacting with paratope of antibody). Our studies revealed four sequences from phages mimotopes located on the surface of Der p 1 and Der f 2 and these may have antigenic or allergen properties. The dermatophagoides allergenic proteins (that matched with phage mimotopes), Der p 1 (from residues Asn66-Phe75 and Thr41-Gly48) and Der f 2 (from residues Asn63-Ala67) should be tested for their antigenicity, allergen and specific epitope properties. Our finding of phage mimotopes that partially matched with Der p 1 are in agreement with that of Furmonaviciene *et al* (1999), but their mimotope was located on Der P1 but at a different position, Leu 147-Gln160.

In summary, the results of this work will open avenues for studies on the molecular interaction between antibody and house dust mite antigen including epitope mapping of allergen in allergy field. The dermatophagoides allergenic proteins (that were 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 tested for their antigenicity, allergen and specific epitope properties. These epitopes may be used in the future for vaccine development and immunotherapy in mite allergic patients.

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