three variants, Der p 1.0113 (D) had the highest specific IgE binding with IC_{50} 0.06 nM. The variant Der p 1.0105 (C) had specific IgE binding with IC_{50} 0.15 nM and Der p1.0114 (B) had IC_{50} 0.21 nM. The results suggested that all point mutations in Der p 1.0113 and 1.0114 somewhat enhance their allergenicity. Interestingly, it also appears that the pro-peptide did not hinder the IgE binding as proposed by others (19, 20).

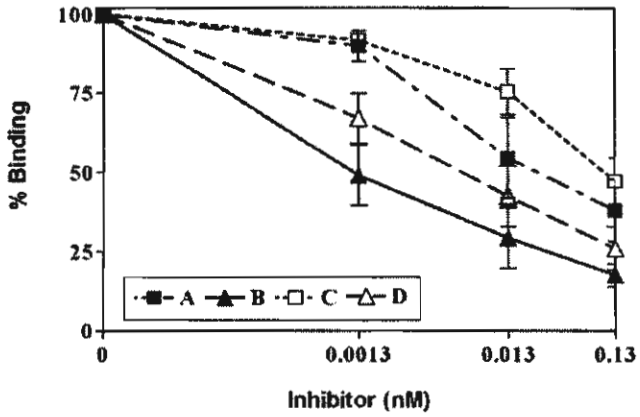

Fig.14. Recombinant Der p 2 variants could inhibit serum specific IgE of allergic donors who had tested to have a high level of specific IgE against Der p 2.

The results from competitive inhibition ELISA showed that two recombinant Der p 2: 2.0103 (B), Der p 2.0110 (D) had higher higher affinity to specific IgE than nature Der p 2 (A).One variant Der p 2.0109 (C) had weaker affinity than all tested variants. The Der p 2.0103 (B), with the 40L, 47S and 114N, had the highest IgE affinity with IC_{50} at 0.001 nM. The Der p 2.0110 (D), with the 47S and 114N, had the IgE affinity similar to the natural Der p 2 (A) with IC_{50} at 0.010 and 0.045 nM, respectively. Interestingly, the rDer p 2.0109 (C), with the 47S, 111L, and 114N, had the weakest IgE affinity with IC_{50} at 0.12 nM. The results suggested that, in addition to 47S and 114N, a point mutation 40L could enhance allergenicity of variant 2.0103 whereas a point mutation 111L could decrease allergenicity of variant 2.0109. Based on these competition results, the variant 2.0109 could be farther developed as a hypoallergenic allergen. The variant 2.0103, on the other hand, could be developed as a reagent for a diagnostic kit.

Effects of genetic polymorphisms of Der p 2 on T-cell proliferation

In addition to altered allergenicity of Der p 1 and Der p 2 variants, it is also unknown whether these point mutations found in each variant might change T-cell responses as well. Stimulation of PBMC proliferation could be used to determine T-cell responses. Thus, together with allergenicity results, proliferation index could be used to identify Der p 2 variants that may have low allergenicity but high stimulation index. Such variants could, further, be developed as a reagent from immunotherapy which requires a high T-cell responses but no allergenicity. This study aimed to examine T-cell responses of Der p 2 variants by stimulation of PBMC proliferation since the study have shown that Der p 2 was a major allergen for Thai allergic donors.

This study examined variants: 2.0101, 2.0103, 2.0104, 2.0109, and 2.0110, as shown below.

AA / Clones	40	47	111	114	Frequency
2.0101	V	T	M	D	0
2.0103	L	S	.	N	2
2.0104	L	S	L	N	5
2.0109	.	S	L	N	3
2.0110	.	S	.	N	2

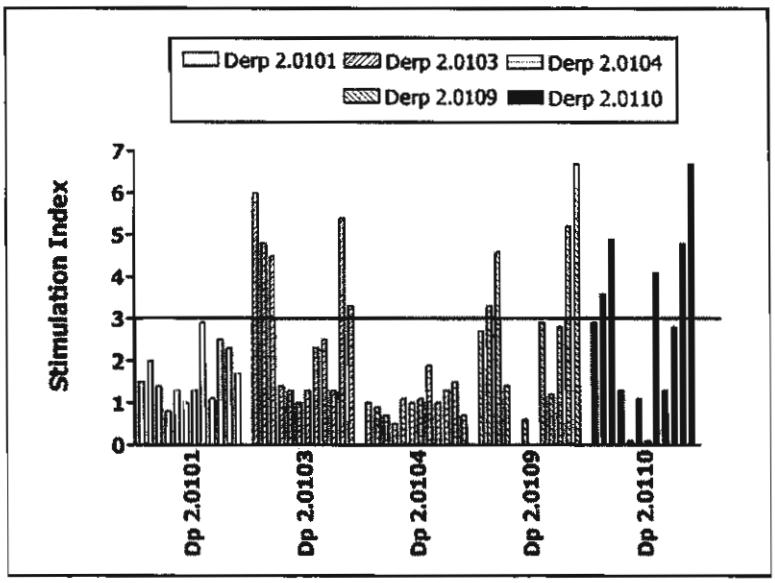

Fig.15. Stimulation index of Der p 2 variants using PBMC cells from 12 Thai allergic donors incubated with 1 μg /ml of Der p 2 variants.

The results of PBMC proliferation showed that variants Der p 2.0103, 2.0109, and 2.0110 could stimulate PBMC to grow as indicated as Stimulation Index of ≥3. Both Der p 2.0101 which is a reference for Der p 2 and 2.0104 which has mutations 40L, 47S, 111L, 114N did not stimulate PBMC proliferation. The results also suggest that 111L mutation of variant Der p 2.0104 altered the effect of three other mutations on stimulation of proliferation.

Furthermore, when examined a relationship between Stimulation Index and Allergenicity of the same allergic donors using Der f 2.0102, the results were shown in the following table (as indicated: T-cell proliferation = Stimulation Index; IgE binding = an absorbance value at OD 450 nm).

	T-cell proliferation	IgE binding
CP	0.70	1.82*
CR	5.40**	0.29
LJ	0.30	1.30*
MA	6.70**	0.72
PR	5.40**	0.24
SR	7.10**	0.43

The results showed that allergic donors, who had a high level of sera specific IgE against Der f 2.0102 as indicated as an absorbance value at OD 450 nm*, their T cells were not stimulated. In contrast, allergic donors, whose T cells were stimulated and proliferated as indicated as a Stimulation Index**, would had a low level of sera specific IgE against Der f 2.0102. These results suggested that allergic donors with a low level of sera specific IgE against Der f 2.0102 may have expansion of polyclonal Th1 cells when stimulated by Der f 2.0102, thus, the Th1 cytokines could suppress IgE production. Further study would need to confirm this observation.

Conclusion

Df mites were a dominant species, replacing *Dp* mites, found in dust samples collected from at least 60 homes around Bangkok. Analysis of DNA sequence found genetic polymorphisms among allergen group 1, 2, and 3, resulting in either change or no change of amino acids. There were numbers of novel polymorphisms found only in Thai dust mite allergens. Recombinant allergens: group 1, 2, and 3, of both Der p and Der f proteins were expressed successfully. Identity of all recombinant Der p and Der f allergens were confirmed by both western blotting as well as mass fingerprint. Thus, these recombinant allergens could be developed as a reagent for diagnostic kit.

This is the first study to determine what HDM group would be a dominant allergen among Thai HDM allergic donors, although this was a preliminary study, we found that group 2 was the major HDM allergen for Thai allergic sufferers. However, a large population survey in different areas of Thailand is needed to confirm this finding. Group 3 allergen appears to be only a minor allergen for Thai allergic sufferers.

The recombinant allergens could be used in developing as reagents for a diagnostic kit, producing antibodies to use in ELISA or western blot, or further developed as therapeutic agents as some variants have a lesser IgE affinity but a stronger polyclonal T-cell stimulation than others.

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ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

Piboonpocanun S, Malainual N, Jirapongsananuruk O, Vichyanond P, Thomas WR. Genetic polymorphisms of major house dust mite allergens. *Clin Exp Allergy* 2006; 36: 510-516. (ภาคผนวก)

ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับประเทศ

Thanyaratsrisakul S, Ngaotepprutaram T, Jirapongsananuruk O, Nat Malainual N, Piboonpocanun S. Allergenicity Analysis of Thai HDM Der f 2 Variant การศึกษาความสามารถในการก่อให้เกิดภูมิแพ้ของ Der f 2 จากไรฝุ่นที่พบในประเทศไทย. หนังสือรวมผลงานวิชาการหลังการประชุม (Proceedings) ของ การประชุมเสนอผลงานวิจัยระดับบัณฑิตศึกษาแห่งชาติ (Grad-Research) ครั้งที่ 6 ณ จุฬาลงกรณ์มหาวิทยาลัย วันที่ 13-14 ตุลาคม 2549 (ภาคผนวก)

กิจกรรมอื่นๆที่เกี่ยวข้อง

ผลงานอื่นๆ

Genbank submission of polymorphic sequences of Thai group 1 and 2 allergens:

proDerp1 (DQ185508).
proDerf1 (DQ185509).
Derp2.0109 (DQ185510).
Derf2.0109 (DQ185511).

การเชื่อมโยงทางวิชาการกับนักวิชาการอื่นๆ

The project established collaboration with:

Associate Professor Orathai Piboonpocanun M.D. and Professor Pakit Vichyanond M.D., Allergy Unit, Department of Pediatrics, Siriraj Hospital, Faculty of Medicine, Mahidol University, for serum IgE collected from Thai allergic donors (The protocol is approved by the Siriraj Ethics Committee).

Professor Wayne R.Thomas, Centre for Child Health Research, University of Western Australia, Western Australia, Australia, for isoforms of group 1 and 2 allergens that are not found in Thailand. These isoforms (Der p 1.0101 and 2.0101) are used as references in ELISA.

ภาคผนวก

ORIGINAL PAPER

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Genetic polymorphisms of major house dust mite allergens

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Clinical and Experimental Allergy

Summary

Background Polymorphic sequence substitutions in the major mite allergens can markedly affect immunoglobulin E binding and T cell responses, but there are few studies on environmental isolates from *Dermatophagoides pteronyssinus* and none for *D. farinae*.

Objective To determine the sequence variation of the group 1 and 2 allergens from environmental *D. pteronyssinus* and *D. farinae*.

Methods RNA from each species was isolated from homes in Bangkok and the sequence of Der p 1, Der p 2, Der f 1, and Der f 2 determined from cDNA produced by high fidelity polymerase chain reactions.

Results The enlarged data set revealed preferred amino acid substitutions in residues 19, 81, and 215 of Der p 1 as well as sporadic changes. Der p 2 showed frequent variations with clusters of amino acid substitutions, but the canonical Der p 2.0101 was not found in any of 17 sequences. Der f 2 showed variants with clusters of substitutions similar to Der p 2 but in different amino acid positions and without any structural concordance. Der f 1 in contrast to the other allergens had few amino acid sequence substitutions.

Conclusions The sequence information on variants provides data important for the optimal design of allergen formulations and useful for the genetic engineering and structure–function analyses of the major allergens.

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Keywords allergen, Der f 1, Der f 2, Der p 1, Der p 2, house dust mite, polymorphisms
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Introduction

The group 1 and 2 house dust mite (HDM) allergens are products of single genes, but as reported for Der p 1 and Der p 2 they show frequent allelic variation affecting several amino acids. Different variants of Der p 2 have different immunoglobulin E binding activities [1–3] that can differ by 25–50% [2, 3], and the amino acid substitutions can render T cell epitopes active or inactive [4]. The cytokine pattern of T cell responses induced by different variants of rDer p 2 was also found to differ even with a single amino acid substitution [1]. The finding that many patients show up to half the IgE binding to rDer p 2.0101 now extensively used throughout the world compared with a more reactive variant found in the study environment shows the need for optimization and further information. The more reactive variant was representative of half the environmental sample sequenced by Park et al. and essentially corresponded to the more antigenic version of Der p 2 initially described by Hakkaart et al. [5]. for mouse antibodies and then as Der p 2.0104-related variants by Smith et al. [4]. Responses measured to the different variants would depend both on the variants used for testing and the type of variants inducing the sensitization in the study environment, so different results could be expected in different geographical regions.

While both Der p 1 and Der p 2 allergens show a high frequency of variants, the pattern of variation is different. Der p 2 has an evolutionary pattern of allelic variations characterized by amino acid substitutions at positions 40, 47, 110, and 114 that result in two lineages represented in the most diverged variants Der p 2.0101 and Der p 2.0104 [1, 2, 4]. The variations in Der p 1, unlike those found for Der p 2, have to date appeared to consist of sporadic substitutions without a discernable lineage [4].

Several key questions on HDM allergen variants remain to be adequately addressed. The distribution and nature of variants in different geographical environments and how

these relate to the allergens used in extracts and recombinant allergens are unknown. Variants that best represent the allergen in the environment need to be identified, and information on environmental isolates of *D. farinae* similar to that being studied for *D. pteronyssinus* needs to be obtained. The substitutions found in the variants can also provide structure–function information for the engineering of stable recombinant hypoallergens, and this can be particularly useful now that the tertiary structures of the major allergens have been determined [6–13]. This study provides information from a large panel of sequences of the groups 1 and 2 allergens from both *D. pteronyssinus* and *D. farinae* isolated from homes. The different distribution of antigenically different variants is shown and *D. farinae* produces frequent variants with clusters of amino acid substitutions for Der f 2 similar to that revealed for Der p 2. Unlike Der p 1, the Der f 1 had little variation. The extended data found for Der p 1 have elucidated mutational preferences for Der p 1 that modify the previous conclusion of sporadic variation.

Materials and methods

Amplification of group 1 and group 2 allergen cDNA

Dust samples were collected by a vacuum cleaner with a filter apparatus from homes in Bangkok, Thailand. Total RNA was extracted from >100 mites using QIAGEN® RNA extraction kit (QIAGEN®, Hilden, Germany). RT-PCR with a high fidelity DNA polymerase (DyNAzyme™ EXT DNA Polymerase, Finnzyme, Espoo, Finland) was performed to amplify the groups 1 and 2 coding region using a pair of these following primers:

Dp1F: 5′ GTCCTCTCGAGAAAAGAGAGGCTCGTCCATCATCGATCAAAAC 3′
Dp1R: 5′ GTGGTGCGGCCGCTTAGAGAATGACAACATATGGATATTC 3′
Df1F: 5′ GTCCTCTCGAGAAAAGAGAGGCTCGTCCAGCTTCAATCAAAATC3′
Df1R: 5′ GTGGTGCGGCCGCGTTCACATGATTACAACATATGGATATTG3′
Dp2F: 5′ CGCATATGGAATTCGATCAAGTCGATGTCAAAGATTG 3′
Dp2R: 5′ CAGGTCGACATCGCGGATTTTAGCATGAGTAG 3′
Df2F: 5′ CGCATATGGAATTCGATCAAGTCGATGTTAAAGATTGTG 3′
Df2R: 5′ CAGGTCGACATCACGGATTTTACCATGGGTAG 3′

The cDNAs of groups 1 and 2 were purified and restriction endonucleases digested before being inserted into the pPICZ$_{\alpha A}$ expression vector (Invitrogen, Carlsbad, CA, USA). Extracted DNA of positive clones was sequenced at Macrogen (Korea). DNA sequences were analysed using program BioEdit (www.mbio.ncsu.edu/BioEdit/). The selected variants were submitted to Genbank database, the accession numbers of which are DQ185508 for Der p 1, DQ185509 for Der f 1, DQ185510 for Der p 2.0109, and, DQ185511 for Der f 2.0109, respectively.

The numbering of the allergen residues of each allergen reported in this paper is consistent with previous reports of allergen variants. For Der p 1 and Der f 1 variants, position 1 corresponds with position 99 of the prepropolypeptides of the group 1 allergens in the Swiss database (Der p 1 (P08176) and Der f 1 (P16311)). For Der p 2 and Der f 2 variants, position 1 corresponds with position 18 of the prepolypeptides of the group 2 allergens in the Swiss database (Der p 2 (P49278) and Der f 2 (Q00855)).

Results

Der p 1 variants

Twenty-eight Der p 1 cDNA (Bangkok, BK) clones were sequenced and analysed (Table 1). There were amino acid substitutions in 12 of the amino acid positions compared with Der p 1.0101. Interestingly six of these positions (19, 50, 81, 124, 125, and 215) were among the 12 positions previously reported to be polymorphic, which, given Der p 1 has 221 residues, is not random (χ^2, $P < 0.001$). Of the 12 polymorphic positions in the BK mites, two were substituted at high frequency. The substitution H50Y was found in all 28 clones and A124V was found in seven of 28 BK sequences. Three other positions (19, 81, and 215) showed multiple substitutions in the BK clones. The substitutions for M19 were conservative, being either I (0113 and 0118) or L (0116), while the substitutions for E81 and E215 were non-conservative. The E81K was found in Der p 1.0103 [4] while the E81A and G were new to the BK variants (Der p 1.0114 and 0119). The E215G substitutions in clones 1.0113, 1.0121, and 1.0123 were unique to the BK mites, but substitutions of Q and K had previously been reported for this position in Der p 1.0104, 1.0111, and 1.0114 [4]. All the positions with a single mutation also had dissimilar amino acid substitutions (N52S, A108T, I113T, N125S, A132T, Q160R, and H170Y).

The Der p 1.0102 and 1.0105 were predominant variants as found in five and 12 of the 28 BK clones, respectively. All other BK clones showed unique substitutions or a unique combination of substitution and have been denominated as the new variants Der p 1.0113 to Der p 1.0123.

Der f 1 variants

Nineteen Der f 1 cDNA BK clones were analysed (Table 2). Only four clones had polymorphisms constituting a significantly lower frequency than Der p 1 ($P < 0.05$, χ^2),

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Table 1. Polymorphisms of environmental Der p 1 Bangkok variants

	AA											
Clone	19	50	52	81	108	113	124	125	132	160	170	215	Frequency (of 28)
1.0101	M	H	N	E	A	I	A	N	A	Q	H	E	0
1.0102	.	Y	.	.	.	.	V	.	.	.	.	.	5
1.0105	.	Y	.	.	.	.	.	.	.	.	.	.	12
1.0113	I	Y	.	.	.	.	.	S	.	.	.	G	1
1.0114	.	Y	.	A	.	.	.	.	.	.	.	.	1
1.0115	.	Y	.	.	T	.	.	.	.	.	.	.	1
1.0116	L	Y	.	.	.	.	.	.	.	.	Y	.	1
1.0117	.	Y	.	.	.	.	.	.	T	.	.	.	1
1.0118	I	Y	.	.	.	.	.	.	.	.	.	.	1
1.0119	.	Y	.	G	.	.	V	.	.	.	.	.	1
1.0120	.	Y	.	.	.	.	.	.	.	R	.	.	1
1.0121	.	Y	S	.	.	.	V	.	.	.	.	G	1
1.0122	.	Y	.	.	.	T	.	.	.	.	.	.	1
1.0123	.	Y	.	.	.	.	.	.	.	.	.	G	1

New variants found by this study: clones 1.0113–1.0123.

Table 2. Polymorphisms of environmental Der f 1 Bangkok variants

	AA					
Clone	1	72	113	152	162	Frequency (of 19)
1.0101	T	C	G	D	H	15
1.0102	.	.	.	.	R	1
1.0103	.	.	.	G	.	1
1.0104	I	R	.	.	.	1
1.0105	.	.	D	.	.	1

New variants found by this study: clones 1.0102–1.0105.

although like Der p 1 each substitution was to a dissimilar residue (Table 3). Although the results shown suggest that the *D. farinae* examined could be from a genetically restricted pool, the nucleotide sequences (DQ185509) show frequent changes, and, as will reported below, the Der f 2 amino acid sequences from the same mites were highly polymorphic.

Der p 2 variants

Amino acid substitutions were found in six positions of the 17 Der p 2 BK clones (Table 4). The four frequent substitutions reported in other studies predominated (V40L (8/17), T47S (13/17), M111L (8/17) or I (3/17), and D114N (17/17), but the proportions were different. In particular, Der p 2.0101 was absent. A further frequent substitution M76 V has not been reported (Table 4).

Most of the 17 BK clones (13/17) were constituted by five variants (13/17) with Der p 2.0104-type substitutions

Table 3. Summary of all reported Der p 1 polymorphisms from different regions

	Smith ($n = 20$)*	BK variants ($n = 28$)	Occurrence (of 48)
19M	1L	2I, 1L	4
21T	1P	–	1
44E	1D	–	1
50H	18Y	28Y	46
52N	–	1S	1
81E	1K	1A, 1G	3
108A	–	1T	1
113I	–	1T	1
124A	9V	7V	16
125N	1S	1S	2
129E	1K	–	1
132A	–	1T	1
136E	2T	–	2
138I	1M	–	1
160Q	–	1R	1
161R	1H	–	1
170H	–	1Y	1
215E	2K, 1Q	3G	6

*The results from Smith et al. (2001) showed sequences from cDNA of CSL, Australia, Perth, Australia, and from genomic DNA of UK and Sydney, Australia.

characterized by LSLN at positions 40, 47, 111, and 114, with the most frequent variant in fact being Der p 2.0104.

Der f 2 variants

In contrast to Der f 1 the Der f 2 sequences showed 11 variants from 15 clones with 11 amino acid substitutions

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Table 4. Summary of polymorphisms of environmental Der p 2 Bangkok variants and all reported variants from different regions

Clone	40	47	49	76	98	111	114	116	127	Country
2.0101(6)	V	T	K	M	A	M	D	V	I	a, c
2.0102 (1)	.	S	.	.	.	.	N	.	L	a
*2.0103 (3)	L	S	.	.	.	.	N	.	.	a, b, d
*2.0104 (7)	L	S	.	.	.	L	N	.	.	c, d
2.0105 (2)	L	S	.	.	.	.	.	.	.	b
2.0106 (1)	.	.	.	.	T	.	.	.	.	c
2.0107 (2)	.	S	.	.	.	.	.	.	.	c
2.0108 (1)	.	S	.	.	.	.	.	A	.	c
*2.0109 (3)	.	S	.	.	.	L	N	.	.	d
*2.0110 (2)	.	S	.	.	.	.	N	.	.	d
*2.0111 (1)	L	S	.	V	.	.	N	.	.	d
*2.0112 (3)	.	.	.	V	.	I	N	.	.	d
*2.0113 (1)	.	.	N	V	.	.	N	.	.	d
Occurrence (of 33)	13	22	1	3	1	15	21	1	1	

*Environmental Der p 2 Bangkok variants. New variants found by this study: 2.0109–2.0113. Country of dust mite cDNA clones: a, CSL, Australia; b, (genomic), Sydney, Australia; c, Perth, Australia; d, Bangkok, Thailand. Occurrence, number of clones from the total of 33 clones (combined with the results from Smith et al 2001). Number in parentheses, number of clones with an indicated variant.

Table 5. Summary of polymorphisms of environmental Der f 2 Bangkok variants and all reported variants from different regions

Clone	11	21	36	52	57	58	59	63	75	76	88	100	108	111	125	128	Country
2.0101(1)	N	C	T	I	S	L	D	I	F	V	I	K	V	I	G	R	a
*2.0102(2)	.	.	.	.	.	.	.	.	.	M	.	.	.	.	.	.	a,e
*2.0103(2)	.	.	.	.	.	.	.	.	.	M	A	.	.	V	A	.	a,e
2.0104(1)	.	.	.	T	.	.	.	.	.	M	A	.	.	V	A	.	b
2.0105(1)	.	.	.	.	.	.	.	.	.	M	A	E	.	V	.	.	c
2.0106(1)	H	.	N	.	N	I	.	V	Y	I	.	.	.	V	.	.	d
2.0107(1)	.	.	.	.	N	I	N	.	.	.	.	.	.	.	.	.	d
2.0108(1)	.	.	.	.	N	I	.	.	.	.	.	.	.	V	.	.	d
*2.0109(1)	.	R	.		N	I	N	V	.	.	.	.	.	.	A	.	e
*2.0110(1)	.	.	.	.	N	I	N	A	Y	I	A	.	.	.	A	.	e
*2.0111(1)	.	.	.	.	N	I	N	V	Y	I	A	.	.	.	A	.	e
*2.0112(1)	.	.	.	.	.	.	.	.	.	M	A	.	.	V	.	.	e
*2.0113(1)	.	.	.	.	.	.	.	T	.	M	A	.	.	.	.	.	e
*2.0114(3)	.	.	.	.	N	I	N	V	.	.	.	.	.	.	.	.	e
*2.0115(1)	.	.	.	.	N	I	N	V	.	.	.	.	.	.	.	C	e
*2.0116(2)	.	.	.	.	N	I	N	V	Y	I	A	.	.	.	.	.	e
*2.0117(1)	.	.	.	.	N	I	N	V	Y	I	.	.	.	.	.	.	e

*Environmental Der f 2 Bangkok variants. New variants found by this study: 2.0109–2.0117. Country of dust mite cDNA clones: a, Japan; b, CSL, Australia; c, Korea; d, Germany; e, Bangkok, Thailand. Number in parentheses, number of clones with an indicated variant.

(Table 5). Like Der p 2, substitutions at certain positions occurred in clusters. Accordingly, 10 of the clones had substitutions of S57N, L58I, and D59N and either I63 V or I63A. The substitutions F75Y (5/15), V76I (5/15) were also associated with this cluster, although they were not as frequent. The other substitutions including the frequent V76M (4/15) did not associate closely with the cluster.

Discussion

The immunological consequence of the sequence variation in HDM allergens has been shown by IgE binding, T cell response to peptides, and the induction of cytokines. Results from studies of other allergens have shown that sequence changes to allergen need not be extensive. The crystallisable recombinant Bet v I allergen binds IgE very poorly compared with its highly allergenic Bet v Ia counterpart, but only differs in nine residues [7, 14]. The study of variants also provides information for the modification of allergens by genetic engineering. Modified allergens that retain a stable and hence consistent structure would provide an advantage in quality control. The use of modified allergens could also provide therapeutic advantages, for example by a reducing antibody-

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Table 4. Summary of polymorphisms of environmental Der p 2 Bangkok variants and all reported variants from different regions

Clone	40	47	49	76	98	111	114	116	127	Country
2.0101(6)	V	T	K	M	A	M	D	V	I	a, c
2.0102 (1)	.	S	.	.	.	.	N	.	L	a
*2.0103 (3)	L	S	.	.	.	.	N	.	.	a, b, d
*2.0104 (7)	L	S	.	.	.	L	N	.	.	c, d
2.0105 (2)	L	S	.	.	.	.	.	.	.	b
2.0106 (1)	.	.	.	.	T	.	.	.	.	c
2.0107 (2)	.	S	.	.	.	.	.	.	.	c
2.0108 (1)	.	S	.	.	.	.	.	A	.	c
*2.0109 (3)	.	S	.	.	.	L	N	.	.	d
*2.0110 (2)	.	S	.	.	.	.	N	.	.	d
*2.0111 (1)	L	S	.	V	.	.	N	.	.	d
*2.0112 (3)	.	.	.	V	.	I	N	.	.	d
*2.0113 (1)	.	.	N	V	.	.	N	.	.	d
Occurrence (of 33)	13	22	1	3	1	15	21	1	1	

*Environmental Der p 2 Bangkok variants. New variants found by this study: 2.0109–2.0113. Country of dust mite cDNA clones: a, CSL, Australia; b, (genomic), Sydney, Australia; c, Perth, Australia; d, Bangkok, Thailand. Occurrence, number of clones from the total of 33 clones (combined with the results from Smith et al 2001). Number in parentheses, number of clones with an indicated variant.

Table 5. Summary of polymorphisms of environmental Der f 2 Bangkok variants and all reported variants from different regions

Clone	11	21	36	52	57	58	59	63	75	76	88	100	108	111	125	128	Country
2.0101(1)	N	C	T	I	S	L	D	I	F	V	I	K	V	I	G	R	a
*2.0102(2)	.	.	.	.	.	.	.	.	.	M	.	.	.	.	.	.	a,e
*2.0103(2)	.	.	.	.	.	.	.	.	.	M	A	.	.	V	A	.	a,e
2.0104(1)	.	.	.	T	.	.	.	.	.	M	A	.	.	V	A	.	b
2.0105(1)	.	.	.	.	.	.	.	.	.	M	A	E	.	V	.	.	c
2.0106(1)	H	.	N	.	N	I	.	V	Y	I	.	.	.	V	.	.	d
2.0107(1)	.	.	.	.	N	I	N	.	.	.	.	.	.	.	.	.	d
2.0108(1)	.	.	.	.	N	I	.	.	.	.	.	.	.	V	.	.	d
*2.0109(1)	.	R	.		N	I	N	V	.	.	.	.	.	.	A	.	e
*2.0110(1)	.	.	.	.	N	I	N	A	Y	I	A	.	.	.	A	.	e
*2.0111(1)	.	.	.	.	N	I	N	V	Y	I	A	.	.	.	A	.	e
*2.0112(1)	.	.	.	.	.	.	.	.	.	M	A	.	.	V	.	.	e
*2.0113(1)	.	.	.	.	.	.	.	T	.	M	A	.	.	.	.	.	e
*2.0114(3)	.	.	.	.	N	I	N	V	.	.	.	.	.	.	.	.	e
*2.0115(1)	.	.	.	.	N	I	N	V	.	.	.	.	.	.	.	C	e
*2.0116(2)	.	.	.	.	N	I	N	V	Y	I	A	.	.	.	.	.	e
*2.0117(1)	.	.	.	.	N	I	N	V	Y	I	.	.	.	.	.	.	e

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facilitated presentation [15], but clinically useful medicaments must be produced in reproducible and verifiable structures [16]. The natural polymorphisms can provide information on the residues that can be substituted while maintaining a stable structure. In a broader sense knowledge of the natural variation of allergens would be key information for the mutational analysis of allergen structure.

There are now a total of 23 variants reported for Der p 1 and substitutions in 20 amino positions worldwide (Table 3). The key results for Der p 1 are that the most representative variants can be identified as Der p 1.0102 and Der p 1.0105. The combined results from the present and previous reports show that 25/48 variants could be represented by these sequences (Table 3). They only differ in the frequent V124A polymorphism, and as this substitution is important in the T cell responses of man [4] and mouse [17] both variants should be used.

Except for the substitutions at 124 the pattern of Der p 1 substitution has been described as frequent but sporadic [4]. The combined data now available show that substitutions are found in preferred positions in the molecule. Specifically the residues M19, E81, and E215 are identified as mutational hot spots (Table 3). Substitutions of more than one residue were found at each of these positions, and for positions 81 and 215 amino acids from different physicochemical groups were substituted. Unlike residue 124, which is buried and only has substantive bonding to its sequentially neighbouring residues, the residues 19, 81, and 215 have some surface exposure and bind to residues besides their sequential neighbours.

The substitutions N52S and H170Y were only found once, but might also affect structure or function of natural Der p 1. The substitution N52S would ablate the canonical N-glycosylation site. The glycosylation structure of natural Der p 1 is not known, but Meno et al. [9]. present evidence for other N-glycosylation sites for recombinant Der p 1 produced in *Pichia pastoris*. H170Y may have an effect on a function of S' subsite, which is predicted as a binding region for a large hydrophobic side chain.

The only polymorphic residue with a very high degree of surface exposure found for Der p 1 was the Y50H, and the original 50H variant has only been found in two clones constructed mites grown on a commercial culture (Der p 1.0101, CSL Ltd., Melbourne, Australia, Table 1). The four sequences reported from a commercial culture (Der p 1.0101–4) had the variants for 81, 124, and 215, but they lacked variants at the frequent 19 position, and substitutions at positions 81 were not to the same residues found in the BK mites. Clearly the sample was small, so the commercial mites should be reanalysed in the light of this knowledge.

Because human B cell epitopes of Der p 1 are conformational epitopes, any changes that alter the secondary and tertiary structures because of charge or size of side chain would also affect the structure of the B cell interaction site [18]. Thus, it is possible that Der p 1 BK variants with multiple dissimilar substitutions on the same molecule, such as 1.0113, 1.0120, and 1.0121, might have an altered structure of the B cell binding sites. Testing these variants for specific IgE binding might help identify potential hypoallergens found in the environment. Alternatively potential hypoallergens could be constructed by multiple substitutions of the known polymorphic residues with a significant degree of surface exposure positions (e.g. 19, 50, 52, 81, 125, 161, and 215).

The data for *D. farinae* are the first compilations of variant sequences from this important species. As well as providing the knowledge to define the allergens, two general points emerged. The first was that Der f 1 had a very low degree of polymorphism. It was not because of a lack of genetic diversity in the mites because there were frequent polymorphisms in the Der f 2 sequences and there were frequent silent mutations in Der f 1 (accession number DQ185509). The lack of polymorphisms in Der f 1 provides evidence against the possibility that the degree of sensitisation to major mite allergens is enhanced by the sequence variation but there is also no clear data on the prevalence of sensitisation to *D. farinae*. The *D. farinae* usually coexists with *D. pteronyssinus* and the assay used to measure IgE binding to Der f 1 would not distinguish *D. farinae* sensitisation with cross reactivity [19]. None of the substitutions have a high degree of surface exposure, but two of the Der f 1 BK variants have the substitutions D155G and H162R that are at the pro-region binding loop [9]. These conceivably could affect the folding process.

The standout result found for the BK Der p 2 sequences was the lack of the canonical Der p 2.0101, which is the prevalent sequence, found in Perth (Table 4) and also in the CSL Ltd. commercial mites. Smith et al. [4]. found that the Der p 2.0101 variant (characterized by VTMD at positions 40, 47, 111, and 114) constituted over half the sequences, whereas Der p 2.0104-like substitutions in these positions (LSLN) were sporadic (Table 4). The absence of Der p 2.0101 in the BK clones, except for a few similar sequences with VT in positions 40 and 47 (Table 4), was offset by substitutions in 11 clones that were similar to the highly divergent Der p 2.0104 (2.0104, 2.0109, and 2.0112) (Table 4). The CSL mites (sample of three reported) showed the LSLN-type sequences were represented in a commercial culture (Table 4). The amino acid positions 40, 47, 111, and 114 where the substitutions differentiate between the 0101 and 0104 variants occur in close proximity in or near a loop structure. Residues 40 and 47 are poorly accessible, but residues 111 and 114 are highly accessible perhaps accounting for their effect on binding of monoclonal antibody and specific IgE. None of the residues substituted in the Der p 2 BK variants are associated with the lining or entrance of the lipid cavity.

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One inference from the BK data is that people in Bangkok would be poorly exposed to Der p 2.0101 and would not respond to it optimally in extracts or to the widely used rDer p 2.0101. The Der p 2.0104-like sequences were also the most abundant reported from a sample in Korea (Table 4), and for many of the sera tested the IgE binding to this variant was almost twice that to Der p 2.0101. All Der p 2 BK variants were very similar to Der p 2 .0104 or had the D114N, which has been found to be responsible for the ability to bind the 1D8 and 4G7 monoclonal antibodies [20]. It could therefore be proposed that this variant is more immunogenic and Der p 2 could be a more potent allergen in Bangkok relative to other HDM allergens (Table 4). Furthermore, variants with the substitution T47S, which have been shown to have a higher IgE binding (with or without 114D) [1], could potentially contribute to this. As suggested by van Ree [3], Der p 2.0104 could be a better diagnostic reagent for mite sensitivity, but given the frequent occurrence of the Der p 2.0101 in at least some regions it is probable that a mixture of both variants would be optimal for immunotherapy.

In contrast to Der f 1, the Der f 2 revealed the same degree of sequence variation as Der p 2 and with a similar focus on 2 clusters of residues. Although variants of Der f 2 have been known for some time, the available sequences have not been compiled. Table 5, therefore, presents the denominated variants in the IUIS isoallergen list as well as the Bangkok sequences. There is a high degree of coordinated sequence variation especially for residues of positions 57–59 and 63, and to a lesser degree for 75 and 76. This constitutes a cluster pattern reminiscent of Der p 2, but the substitutions are quite different and from completely different regions on the 3-D structure of the molecule. The nature of these substitutions, thus, appears to result from the pattern of inheritance rather than from structure–function reasons. Polymorphism was found in position 111, but it was not frequent and the V111I substitution was conservative.

The Bangkok variants have a high frequency of the NIN substitutions in residues 57–59 and 63. The NIN-type variants had been recorded in the sequences from Germany, but their high prevalence was not appreciated because of the paucity of data (Table 5). Most other reported variants from other regions have SLD in these positions. The residues 57–59 and 63 are found at the end of a β sheet, which is a part of the opening of the lipid binding cavity (I58, I63) and in residues that line the cavity (L58, F75, I88), based on the X-ray crystallographic data of the group 2 allergens [8, 13]. Besides the functional effect, the NIN-type variants might have the altered antigenic surface molecules as well. The changes in Der f 2 BK variants are mostly conservative, except I63T found in BK2.0113, and to residues found in the homologous sequences from other mite species. The substitutions at positions 63, 75, 76, and 111 have some surface exposure, but they are conserved while G125A is not exposed. The present data indicate that a mixture of Der f 2.0101 and Der f 2.0110 could provide a good representation of the divergent sequences, although the environmental data are from Bangkok.

Taken together the results show the predominant variants in different regions can be different. Although the difference may be a few substitutions, these changes could alter the immune responses. Determining allergenicity of the predominant variants might generate new information for developing more effective and safer materials for immunotherapy as well as developing specific diagnostics for local patients.

Acknowledgements

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Allergenicity Analysis of Thai HDM Der f 2 Variant

การศึกษาความสามารถในการก่อให้เกิดภูมิแพ้ของ Der f 2 จากไรฝุ่นที่พบในประเทศไทย

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Abstract

House dust mite *Dermatophagoides farinae* group 2 (Der f 2) is one of the major allergens stimulating allergic responses in large portion of allergic donors. To determine allergenicity of Thai isoform of Der f 2, the clone of Thai Der f 2.0102 cDNA encoding allergen Der f 2.0102 was selected and expressed in yeast *Pichia pastoris* under methanol induction. The recombinant Der f 2.0102 was secreted in yeast media and confirmed by specific monoclonal antibody and mass fingerprint. After purification by a size-exclusion column, rDer f 2.0102 was analyzed for its allergenicity among Thai allergic donors by direct binding ELISA and T-cell proliferation assay. The results showed that 80% of the donors had specific IgE against rDer f 2.0102. However, rDer f 2.0102 was recognized by specific T-cell in only 20% of allergic Thai donors.

Introduction

The prevalence of asthma and allergic rhinitis in Thailand have increased sharply like the rest of the world, it were found to be 3 and 2 folds increased, respectively, within 10 years from 1989 to 1999 [1]. A study in 1997 reported that about 70-80% of allergic Thai patients have allergic responses to house dust mite extracts in skin prick test [2].

Currently, allergic treatment using whole mite extracts has several drawbacks [3]. Whole mite extract is an intricate mixture of both known and unknown allergens, and the quantity and quality of allergens are not uniform. Because of these mentioned factors, the use of whole mite extract always includes a risk of anaphylaxis. Moreover, dust mites that were used for preparation of whole mite extract is not from Thai house dust mites, it may result in unsuccessful treatment due to genetic polymorphisms. The partially purified mite extract fraction can reduce adverse effects, but its composition has not been defined and difficult to gain a uniform composition. Ideally, recombinant mite allergens might be the best tool to treat allergic patients because engineered hypoallergenic recombinant allergens could be expressed and be used with individual allergic patient that has an immune response to. Thus, expression and analysis of single recombinant allergen is a prerequisite for specific allergic treatment.

Thai house dust mite survey revealed that house dust mites were ubiquitously found all over Thailand. It was shown that 88% of house dust samples contained house dust mites [4]. There are two predominant species of Thai HDM which are

Dermatophagoides pteronyssinus (Dp) and *Dermatophagoides farinae (Df)*. Currently, there are 20 groups of HDM allergens based on their basis immunological and physicochemical properties. HDM major allergens could be identified by high IgE reactivity (>80% of allergic patients). Group 2 mite allergens, considered as one of the major HDM allergens, are 14 kDa proteins with unknown function. However, number of studies showed group 2 allergens might contain a large lipid-binding pocket that may involve in cholesterol transport [5, 6, 7]. Recently, 11 variants of Der f 2 were found in Thailand (Table 1). Interestingly, Der f 2.0101, the first reported sequence found in Australia and Japan, was not found in Thailand [8]. This study was aimed to determine allergenicity as well as T-cell recognition of Thai isoform (Der f 2.0102) using serum IgE as well as PBMC of Thai allergic donors.

Methods: Der f 2.0102 cDNA were cloned and expressed in yeast *P. pastoris* under methanol induction. The secreted recombinant Der f 2.0102 was analyzed on 15% SDS-PAGE gel electrophoresis. After that, the rDer f 2 was digested with trypsin and analyzed by mass peptide fingerprint. Moreover, direct binding ELISA using mAb against mite group 2 allergens (7A1) [Indoor Biotechnologies, UK.] was performed to confirm expressed recombinant protein. The rDer f 2.0102 was purified through a size-exclusion Superdex 75 column and eluted with PBS pH 7.4.

CD spectra of the rDer f 2.0102 dissolved in PBS at a final concentration of 300 ng/ml was measured in 0.2 mm path length cuvette at a step resolution of 1 nm with a scan speed of 50 nm/min from 190 to 250 nm. The five scans were single-averaged.

IgE reactivity or allergenicity of recombinant Der f 2.0102 was determined by direct binding ELISA using serum IgE of Thai mite-allergic patients. In brief, a 96-well plate was pre-coated with rDer f 2.0102 was incubated with serum IgE for 1 h. Then, incubated the plate with HRP conjugated anti-human IgE antibody for 1 h. Bound HRP anti-human antibodies were measured absorbance at OD.450 nm.

Ability of Der f 2.0102 to stimulate T-cell proliferation was determined by PBMC proliferation assay. PBMC purified from donor serum were incubated with rDer f 2.0102 for 4 days before addition of ^{3}H-thymidine. After 18 h, ^{3}H-thymidine incorporation was counted.

Table 1. Der f 2 isoforms found in Thai environment (modified from S. Piboonpocanun et al.).

aâ / clone	21	57	58	59	63	75	76	88	108	111	125	128	Frequency (of 14)
2.0101	C	S	L	D	I	F	V	I	V	I	G	R	0
2.0102	.	.	.	.	.	.	M	.	.	.	.	.	1
2.0103	.	.	.	.	.	.	M	A	.	V	A	.	1
2.0109	R	N	I	N	V	.	.	.	.	.	A		1
2.0110	.	N	I	N	A	Y	I	A	.	.	A	.	1
2.0111	.	N	I	N	V	Y	I	A	.	.	A	.	1
2.0112	.	.	.	.	.	.	M	A	.	V	.	.	1
2.0113	.	.	.	.	T	.	M	A	.	.	.	.	1
2.0114	.	N	I	N	V	.	.	.	.	.	.	.	3
2.0115	.	N	I	N	V	.	.	.	.	.	.	C	1
2.0116	.	N	I	N	V	Y	I	A	.	.	.	.	2
2.0117	.	N	I	N	V	Y	I	A	A	.	.	.	1

Results: Expression of rDer f 2.0102 in *P. pastoris* was induced by 3 % methanol for 3 days. Supernatants containing secreted rDer f 2.0102 were analyzed on 15% SDS-PAGE (Fig. 1). The rDer f 2.0102, MW size about 15 kDa, was successfully expressed in secreted form.

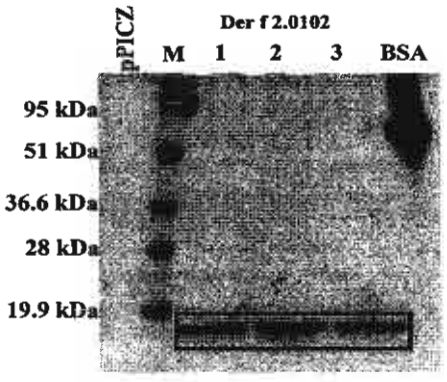

Figure 1. SDS-PAGE of secreted rDer f 2.0102 from 3 different clones (in square). 25 µl of the supernatants were analyzed on 15%SDS-PAGE. The supernatant from yeast containing empty plasmid was used as a negative control (pPICZ). M: prestained broad range marker. BSA: BSA 1 ug.

ELISA using mAb against mite group 2 allergens (mAb 7A1) confirmed secreted rDer f 2.0102 (data not shown). Moreover, the mass fingerprint also confirmed an identity of secreted rDer f 2.0102 (Fig. 2). Mass to charge ratio of rDer f 2.0102 matched to the calculating mass to charge ratio of trypsin digested Der f 2.

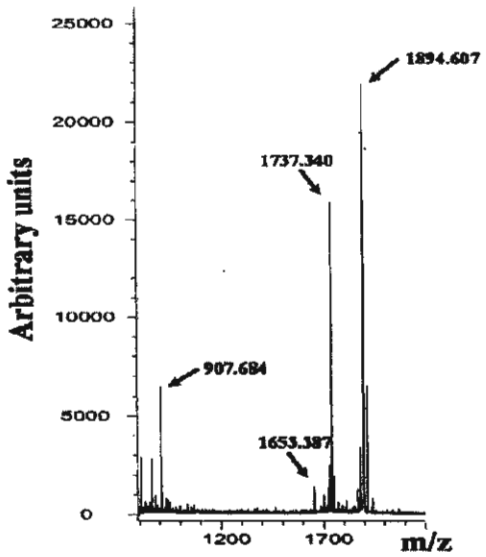

Figure 2. Mass fingerprint of rDer f 2.0102. Trypsin digested rDer f 2.0102 was analyzed by MALDI-TOF MS. The numbers show mass to charge ratio of digested rDer f 2.0102 corresponding to that of calculated Der f 2.

The rDer f 2.0102 was purified by a size exclusion Superdex 75 column and stored in PBS pH 7.4 at -30°C until use. The conformational of recombinant protein

was confirmed by CD spectral analysis. The CD spectrum pattern of purified protein indicated a β-strand structure (Fig. 3).

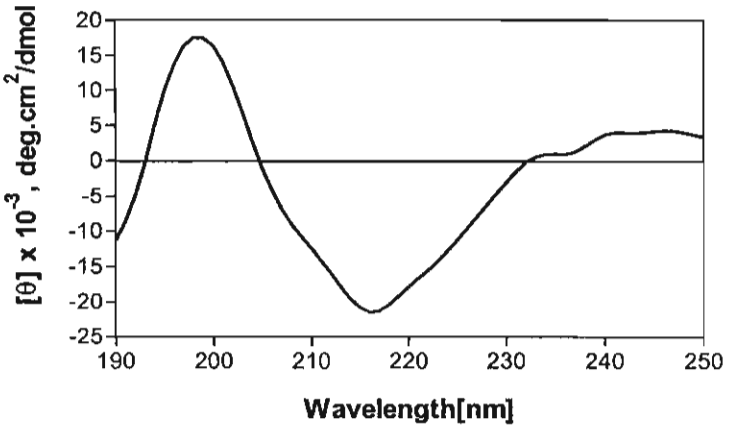

Figure 3. CD spectrum of rDer f 2.0102. The rDer f 2.0102 in PBS was subjected to CD spectral analysis. CD spectrum was recorded from 190-250 nm using JASCO J-715 spectropolarimeter.

Results of direct binding ELISA using sera from 28 HDM allergic donors with positive SPT to crude mite extract showed 23 donors (82%) had specific IgE against rDer f 2.0102 (Fig. 4). Furthermore, 14 of 23 donors showed high level of specific IgE (>3x of a cut-off value). However, results from T-cell proliferation using PBMC from 18 HDM allergic donors (Fig. 5), only 4 donors (22%) had a high T-cell stimulation index (>SI of 3) suggesting this Der f 2 variant may be very allergenic.

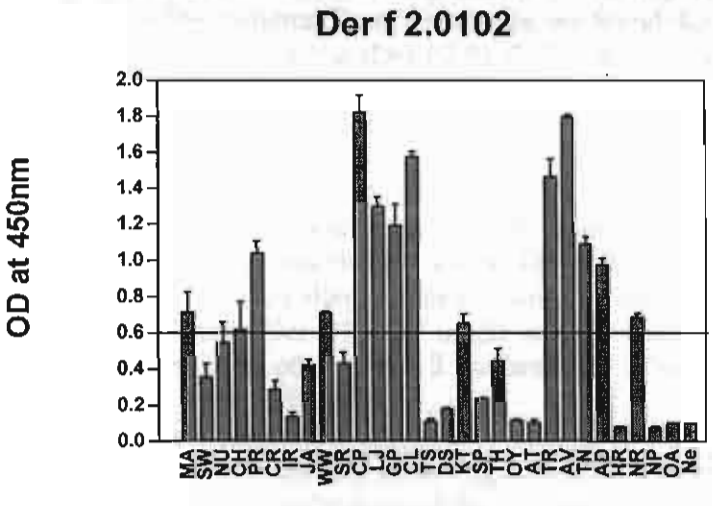

Figure 4. Direct binding ELISA. IgE-binding activity of 28 HDM allergic donors was determined by direct binding ELISA with rDer f 2.0102. OA is non atopic donor and Ne is the negative control without recombinant allergen. The black line shows the 3x above a cut-off value.

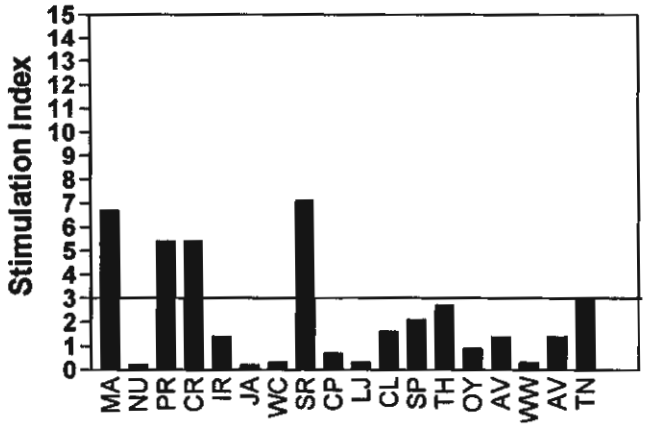

Figure 5. The stimulation index profile of PBMC proliferation assay. The black line shows the Stimulation Index of 3 as a cut-off value.

Discussion: Thai house dust mite Der f 2 variant (rDer f 2.0102) was successfully expressed and purified. Both Mass finger print and ELISA analysis confirmed the purified protein was indeed rDer f 2. The rDer f 2.0102 has a molecular weight of 15 kDa as the same as MW of nature Der f 2. This indicates that the yeast expressed rDer f 2.0102 did not alter its physical properties. In addition, the conformational structure confirmed by CD spectral analysis indicated the rDer f 2.0102 formed the Ig-like or β-structure as the same structure as reported by others [5, 9].

Although Vichyanond et al. [1] reported that 50% of allergic patients were sensitized to HDM extracts, however, to date, there is still no report identifying a major HDM allergen among Thai patients. From our results, we found that 80% of Thai HDM allergic donors had specific IgE to the rDer f 2.0102. When compared to another major HDM allergen (rDer p 1, results not shown), it appears that a number of Thai HDM allergic donors sensitized to Der f 2 were 1.7 fold higher than that of those sensitized to Der p 1.

This study also determined whether rDer f 2.0102 could stimulate PBMC proliferation. If the rDer f 2.0102 stimulates T-cell to proliferate well, it could be useful in developing a reagent for immunotherapy where Th1-cell stimulation is required. As results shown, only 20% of Thai allergic donors showed T-cell Stimulation Index (SI) above a cut-off value. Thus, rDer f 2.0102 might only be useful for developing as a diagnostic agent. Determining other Der f 2 isoforms for a high T-cell proliferation index is underway.

In summary, the allergenicity analysis of Thai rDer f 2 variant expressed by yeast system showed this protein has potent IgE binding reactivity, but low T-cell stimulation efficiency. These results suggested that Der f 2.0102 was very allergenic and it could be developed as a diagnostic agent but not as a therapeutic agent.

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