รายงานวิจัยฉบับสมบูรณ์

โครงการ: การเปลี่ยนแปลงพันธุกรรมของสารก่อภูมิแพ้ของไรฝุ่นในประเทศไทย และผลจากการเปลี่ยนแปลงที่มีต่ออาการภูมิแพ้ของประชากรไทย

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บทคัดย่อ โรคภูมิแพ้ เช่นโรคหอบหืด (Asthma) และ โรคโพรงจมูกอักเสบจากภูมิแพ้ หรือโรคแพ้อากาศ (Allergic rhinitis)จัดเป็นปัญหาสำคัญที่ประเทศไทยกำลังเผชิญในขณะนี้ โดยในช่วงเวลา 10 ปีที่ผ่านมา อัตราความชุกของโรคภูมิแพ้ได้เพิ่มสูงขึ้นประมาณ 3 เท่าในคนไข้ที่เป็นเด็กและผู้ใหญ่ โรคภูมิแพ้เหล่านี้ไม่เพียงแต่จะส่งผลกระทบต่อคุณภาพชีวิต แต่ยังส่งผลกระทบต่อเศรษฐกิจของประเทศไทยอีกด้วย สารก่อภูมิแพ้จากไรฝุ่นจัดเป็นสาเหตุสำคัญที่ก่อให้เกิดโรคภูมิแพ้ จากสารก่อภูมิแพ้จากไรฝุ่นประมาณ 20 ชนิด สารก่อภูมิแพ้กลุ่มที่ 1, 2, และ 3 นับว่าเป็นสาเหตุสำคัญที่ก่อให้เกิดภาวะภูมิแพ้ ในประเทศไทย มีการศึกษาพบว่า ไรฝุ่นบ้านสายพันธุ์ *Dermatophagoides pteronyssinus* (*Dp*) และ *Dermatophagoides farinae* (*Df*) เป็นสายพันธุ์ที่พบมาก แต่ในปัจจุบัน การตรวจหาสาเหตุของโรคภูมิแพ้ ยังจำเป็นต้องใช้ตัวไรฝุ่นสกัดจากทั้งสองสายพันธุ์ ที่นำเข้าจากต่างประเทศ เนื่องจากยังไม่มีการผลิตตัวไรฝุ่นสกัดเพื่อการตรวจสอบในประเทศไทย นอกจากนี้ ยังมีการใช้ตัวไรฝุ่นสกัดจากต่างประเทศในการรักษาอาการโรคภูมิแพ้จากไรฝุ่น (Immunotherapy) ซึ่งอาจจะไม่เหมาะที่จะใช้กับผู้ป่วยในประเทศ เนื่องจาก ปัญหาด้านความหลากหลายทางชีวภาพ (Genetic polymorphisms) ของยีนที่กำหนดการสร้างสารก่อภูมิแพ้ และมีราคาแพง ดังนั้นโครงการวิจัยนี้มีวัตถุประสงค์เพื่อ

1. ศึกษาความหลากหลายทางชีวภาพ (Genetic polymorphisms) ของยีนที่กำหนดการสร้างสารก่อภูมิแพ้ กลุ่มที่ 1, 2, และ 3 จากไรฝุ่น *Dp* และ *Df* ที่พบในประเทศไทย รวมทั้งการผลิตสารก่อภูมิแพ้ (recombinant allergens) เพื่อนำไปพัฒนาเป็นสารเพื่อการตรวจสอบ
2. ศึกษาผลกระทบของความหลากหลายทางชีวภาพของยีนที่กำหนดการสร้างโปรตีนต่อปฏิสัมพันธ์ระหว่างโปรตีนและเซลล์ของภูมิคุ้มกัน IgE และ polyclonal T cells
3. ศึกษาจัดอันดับของสารก่อภูมิแพ้ กลุ่มที่ 1, 2, และ 3 ในการเป็นสาเหตุสำคัญที่ก่อให้เกิดภาวะภูมิแพ้ในผู้ป่วยคนไทย

ผลการทดลองพบว่า มีความหลากหลายทางชีวภาพ (Genetic polymorphisms) ของยีนที่กำหนดการสร้างสารก่อภูมิแพ้ กลุ่มที่ 1, 2, และ 3 จากไรฝุ่น *Dp* และ *Df* ที่พบในประเทศไทย และผลจากความหลากหลายทางชีวภาพของยีน ก็คือการเปลี่ยนแปลงของกรดอะมิโนหลายชนิด ซึ่งมีทั้งที่เป็นการค้นพบใหม่ที่เฉพาะในสารก่อภูมิแพ้ที่พบในประเทศไทย และที่เคยพบแล้วในประเทศอื่นๆ ผลการตรวจสอบสารก่อภูมิแพ้ กลุ่มที่ 1, 2, และ 3 ที่ผลิตได้จาก เซลล์ยีสต์สายพันธุ์ *Pichia pastoris* โดยวิธี SDS-PAGE, Western blot, ELISA, และ mass fingerprint ได้ยืนยันว่าเป็นสารก่อภูมิแพ้ กลุ่มที่ 1, 2, และ 3 จริง ผลการศึกษาผลกระทบของความหลากหลายทางชีวภาพต่อปฏิสัมพันธ์ระหว่างโปรตีนและเซลล์ของภูมิคุ้มกัน IgE พบว่า การเปลี่ยนแปลงของกรดอะมิโนหลายชนิดของสารก่อภูมิแพ้ กลุ่มที่ 1 มีผลไปเพิ่มความรุนแรงต่อปฏิสัมพันธ์ระหว่างโปรตีนและเซลล์ของภูมิคุ้มกัน (IgE) ในขณะที่การเปลี่ยนแปลงของกรดอะมิโนหลายชนิดของ กลุ่มที่ 2 มีผลไปเพิ่ม หรือลด ความรุนแรงต่อปฏิสัมพันธ์ระหว่างโปรตีนและเซลล์ของภูมิคุ้มกัน (IgE) ขึ้นอยู่กับชนิดของกรดอะมิโน นอกจากนี้ การเปลี่ยนแปลงของกรดอะมิโนหลาย

ชนิดของ กลุ่มที่ 2 ยังมีผลไปเพิ่มการเพิ่มปริมาณของเซลล์ของภูมิคุ้มกัน polyclonal T cells ผลการศึกษาอันดับของสารก่อภูมิแพ้ ในการก่อให้เกิดภาวะภูมิแพ้ในผู้ป่วยคนไทย พบว่า สารก่อภูมิแพ้ กลุ่มที่ 2 ก่อให้เกิดภาวะภูมิแพ้ในผู้ป่วยคนไทยได้มากที่สุด รองลงมาเป็น สารก่อภูมิแพ้ กลุ่มที่ 1 ผลการศึกษายังพบว่า กลุ่มที่ 2 และ กลุ่มที่ 1 จัดเป็นสาเหตุสำคัญที่ก่อให้เกิดภาวะภูมิแพ้ในผู้ป่วยคนไทย ในทางตรงกันข้าม สารก่อภูมิแพ้ กลุ่มที่ 3 ไม่ได้เป็นสาเหตุสำคัญที่ก่อให้เกิดภาวะภูมิแพ้ในผู้ป่วยคนไทย

ผลงานวิจัยของโครงการนี้แสดงให้เห็นว่า สารก่อภูมิแพ้ กลุ่มที่ 2 ก่อให้เกิดภาวะภูมิแพ้ในผู้ป่วยคนไทยได้มากที่สุด และการเปลี่ยนแปลงของกรดอะมิโนหลายชนิด ซึ่งเป็นผลมาจากความหลากหลายทางชีวภาพของยีนที่กำหนดการสร้างสารก่อภูมิแพ้ สามารถไปเพิ่ม หรือ ลดความรุนแรงต่อปฏิสัมพันธ์ระหว่างโปรตีนและเซลล์ของภูมิคุ้มกัน (IgE) และ สามารถไปเพิ่มปริมาณของเซลล์ของภูมิคุ้มกัน polyclonal T cells อีกด้วย

Abstract In the past decade, the prevalence of respiratory allergic diseases such as asthma and allergic rhinitis among Thai population is increasing dramatically. These allergic diseases have been shown to have a great impact on quality of life as well as economy. House dust mite (HDM) proteins or allergens are the most common allergen causing these allergic diseases in Thai population. Up-to-date, twenty groups of HDM allergens have been identified. However, in Europe and America, group 1, 2, and 3 allergens are shown to be major HDM allergens which >75% of allergic patients have specific IgE against. Recently, genetic polymorphisms, causing missense mutations resulting in changing allergenicity and T-cell responses, of genes coding for group1 and 2 allergens have been reported in many countries. Interestingly, certain mutations have been found to be exclusive to one country while some mutations are common among the same group from many countries. Thus, information regarding local variants would be more useful than information of variant found in other regions. This study aimed to examine genetic polymorphisms of HDM allergen group 1, 2, and 3, as well as examine effects of mutations on allergenicity and T-cell responses. This study, also, determined what might be major allergens for Thai allergic population.

Results of dust sampling around Bangkok showed that *Dermatophagoides farinae* (*Df*) mites were dominant species followed by *Dermatophagoides pteronyssinus* (*Dp*) mites. The genetic polymorphisms analysis showed there were numbers of both nonsense and missense mutations found in 3 allergen groups. There were a number of novel polymorphisms found only in Thai dust mite allergens. Yeast *Pichia pastoris* expressed-recombinant group 1, 2, and 3, of both Der p and Der f proteins were successfully purified and confirmed their identities by SDS-PAGE, western blotting, ELISA, and mass fingerprint. Also, an identity of recombinant group 1, 2, and 3 were confirmed by mass fingerprint. The results of direct binding ELISA and western blotting found that majority of Thai allergic patients had specific IgE to group 2 followed by group 1 allergen whereas a few number of Thai allergic patients had specific IgE to group 3. The results from analysis of missense mutations of group 1 and 2 variants found that all tested group 1 variants were more allergenic than the nature one. However, for group 2, some variants had less allergenic than others. Interestingly, when tested for T-cell responses, three variants had gained ability to stimulate T cell proliferation except one that did not stimulate.

In conclusion, HDM group 2 allergen is the major allergen for Thai allergic patients in Bangkok. Genetic polymorphisms of group 1 and 2 allergens causing missense mutations could alter allergenicity as well as ability to stimulate proliferation of polyclonal T cells

Executive Summary

วัตถุประสงค์ของโครงการ

1. To clone and identify the sequence polymorphisms of genes coding for allergens from house dust mites *Dermatophagoides pteronyssinus* (*Dp*) and *Dermatophagoides farinae* (*Df*). Both species appear to be equally distributed and are the dominant species in dust samples collected from homes in Bangkok.
2. To express and identify which isoforms of major allergens: group 1, 2, and 3, could induce high allergic responses among Thai allergic patients.

กิจกรรมที่ได้ดำเนินการและผล

1) Dust samples have been collected from at least 60 locations around Bangkok. After isolation step, there were only samples from 10 locations that contain dust mites. After further identification using stereomicroscope and pictorial keys, both *Dp* and *Df* mites were isolated and cultured separately.
2) Genes coding 3 major allergens: group 1, 2, and 3 were extracted from *Dp* and *Df* mites, were subcloned and sequenced. Sequencing results showed there were polymorphisms among allergen group 1, 2, and 3, resulting in either change or no change of amino acids. These polymorphisms were also either similarly to the ones previously reported in other countries or novel polymorphisms found only in Thai dust mite allergens.
3) Expressions of recombinant allergens: group 1, 2, and 3, of both Der p and Der f proteins in yeast *Pichia pastoris* were carried out in a small scale production successfully. All recombinant Der p and Der f allergens were secreted in a soluble form in media could be visualized in SDS-PAGE. Only recombinant allergen group 1 and 2 were confirmed by ELISA using commercial monoclonal antibody against Der p 1 and Der p 2, respectively, since both Der p and Der f proteins have a high homology and monoclonal antibodies of each group have been shown to have a cross-reactivity.
4) Also identity of recombinant allergens: group 1, 2, and 3 were confirmed by mass fingerprint.
5) The results of direct binding of serum IgE from Thai allergic patients to all 3 allergen groups showed that 60% and 55% of Thai allergic patients had serum IgE specifically against group 1 and 2 allergen, respectively. Of the same donor group, 52% of Thai allergic patients had higher level of specific IgE to group 2 while only 30% of Thai allergic patients had higher level of specific IgE to group 1. Thus, group 2 allergen is the major HDM allergen for Thais. The results also showed that less than 10% of serum IgE was against Der p 3. Thus, group 3 allergen appears to be only a minor allergen for Thai allergic patients.

ผลงานวิจัยที่ทำของโครงการ

ปัญหาที่ทำการวิจัย และความสำคัญของปัญหา

Allergens from house dust mites are major causes of allergic diseases such as asthma, rhinitis, and atopic dermatitis in many parts of the world [8]. In Thailand, allergens from house dust mites are also found to be a primary cause (57.8%) of allergic diseases among children [1]. Furthermore, a number of allergic reactions among asthmatic patients sensitized to mite extract by skin test were increased in both children (67%) and adult (84.4%) patients [9, 10].

Malainual et al [7] found dust mite *Dermatophagoides pteronyssinus* (*Dp*) was the most common species (66.3%) and *Dermatophagoides farinae* (*Df*) was 24.4% of dust mite species in dust samples collected from Central, North, and Northeastern part of Thailand. In Bangkok, *Dp* is also the most common species found in dust samples collected from homes of allergic patients (Malainual, personal communication). As for allergens of dust mites, a recent study by Vichyanond [1] showed that the amount of group 1 mite allergen was 11 mcg/g dust in samples from the North and Northeastern Thailand, which was exceed both the sensitizing and the symptomatic levels for asthma (2 and 10 mcg/g dust) as recommended in the International Conference on Indoor Allergens and Asthma. This finding confirms that dust mite allergens are likely one of the major causes of allergic diseases. Although, there are 11 different groups of *Dp* allergens listed by WHO/IUIS (www.allergen.org). Due to the limitation of specific antibodies to *Dp* dust mite allergens, these surveys in Thailand reported only sensitivity of allergic patients to only *Dp* allergens group 1 and 2. However, it is unknown whether Thai allergic patients sensitize to other Dp allergen groups. In order to treat dust mite caused allergic diseases effectively, it is necessary to identify *Dp* allergen groups sensitized among Thai allergic patients. To produce 11 recombinant allergen groups to diagnose patients and to develop a diagnostic kit, therefore, is a priority as well as requirement.

Allergens, extracted from cultured dust mites, of *Dp* and *Df* have been identified, cloned, and expressed. Currently, these recombinant allergens have been tested in trials for allergy immunotherapy, the only curative approach for IgE-mediated allergy, because of their homogeneity and purity [11, 12]. However, one of the problems affecting allergy immunotherapy would be sequence diversity of genes encoding allergens because certain changes of amino acids may affect global structure of protein therefore changing epitopes. The sequence diversity is a common feature found in mite allergens of dust mites from Australia, Taiwan, and commercial antigens [13] [14] [15] [16]. Smith et al [16] showed that T cells responded differently to variants of mite allergens, resulting from amino acid substitutions. To overcome this adversity, analysis of polymorphic allergens would be a requirement to identify the most effective stimulant for Thai population, which could, eventually, be used in allergy immunotherapy as well as diagnosis in Thailand.

วัตถุประสงค์

To clone and identify the sequence polymorphisms of genes coding for allergens from house dust mites *Dermatophagoides pteronyssinus* (*Dp*) and *Dermatophagoides farinae* (*Df*). Both species appear to be equally distributed and are the dominant species in dust samples collected from homes in Bangkok.

To express and determine which isoforms of major allergens: group 1, 2, and 3, are major HDM allergens for Thai allergic patients.

To determine effects of genetic polymorphisms of different allergen isoforms on allergenicity and stimulation of T-cell proliferation

ระเบียบวิธีวิจัยโดยย่อ

1. Houst dust samples will be collected from various sources in Bangkok. House dust mites from dust samples will be isolated using a heat lamp. Each mite species will be identified and collected using stereomicroscope and pictorial keys.
2. Total RNA of *Dp* mites will be extracted and be subjected for RT-PCR. cDNA of each allergen group will be ligated into yeast *Pichia pastoris* vector. cDNA sequence will be performed and obtained.
3. Polymorphic allergens of each allergen groups will be expressed and purified using ion-exchange chromatography. Confirmation of each allergen groups via either available specific antibodies or N-terminal protein sequencing.
4. Allergic responses to recombinant proteins from each allergen group will be tested for both specific IgE antibody and T-cell reactivity using sera from allergic patients. Peptide fragments of each allergen group sensitized by Thai patients will also be expressed, purified, and tested for epitopes recognized by either IgE antibodies or T cells from sera of allergic patients.

ผลงานวิจัยที่ได้รับ

There were only dust samples from 16 out of 80 locations that contain dust mites after isolation step. Further identification using stereomicroscope and pictorial keys showed *Df* mites. Further identification using stereomicroscope and pictorial keys showed mites from 2 of 16 locations were *Dp* mites. We, also, found mixed dust mite species: *Dp*, *D. farinae* (*Df*), and storage mites *Blomia tropicalis* (*Bt*) from another 2 locations. Interestingly, we found only dust mite *Df* mites in 12 of 16 locations. In some locations, dust samples contained more than one dust mite species. Multiple rounds of sub-culturing of dust mites were performed until one species was obtained. From these samplings, *Df* mites appeared as a predominant species found in Bangkok.

Genetic polymorhisms analysis of HDM major allergens

Sequencing of cDNA encoding 3 major allergens: group 1, 2, and 3, amplified from total RNA extracted from purified *Dp* and *Df* mites, were performed. The results showed there were polymorphisms resulting in either sense or nonsense mutations as shown as follow.

A. Der f 1. There were only 4 of 19 clones that had missense mutations. Moreover, these mutations were sporadic. The dominant Der f 1 variant found in Bangkok was proDer f 1.0101. (proDer f 1 genbank accession number DQ185509)

AA / Clone	1	72	113	152	162	Frequency (of 19)
Ref. 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

B. Der p1. Polymorphisms that cause a point mutation were found in both prepro and mature-form. The dominant Der p 1 variant found in Bangkok was proDer p 1.0105 which has a point mutation H50Y. The mutations in all Der p 1 clones appear to be sporadic as shown in the following table: (proDer p1 BK clone genbank accession number DQ185508)

AA / Clone	19	50	52	81	108	113	124	125	132	160	170	215	Frequency (of 28)
Ref. 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

C. Der f 2. Polymorphisms were found in all 15 BK clones. The NINV substitution pattern appeared as the dominant mutations found in 9 of 15 Der f 2 BK clones. Other frequent substitutions found were at I63 (11 of 15), F75 (5/15), V76 (9 of 15), I88 (7 of 15), and G125 (4 of 15). (Der f 2.0109 genbank accession number DQ185511)

AA / Clone	21	57	58	59	63	75	76	88	111	125	128	Frequency (of 15)
Ref. 2.0101	C	S	L	D	I	F	V	I	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	.	.	.	.	1

D. Der p2. Polymorphisms of Der p 2 were found to be pattern. 70% of the clones found to have mutations with different combinations in these amino acids: V40L, T47S, M111L, and D114N. The dominant Der p 2 Bk clone was Der f 2.0104 as shown in the following table: (Derp2.0109 genbank accession number DQ185510).

AA / Clone	40	47	49	76	111	114	Frequency (of 17)
Ref. 2.0101	V	T	K	M	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
2.0111	L	S	.	V	.	N	1
2.0112	.	.	.	V	I	N	3
2.0113	.	.	N	V	.	N	1

E. Der f 3. Polymorphisms of Der f 3 were found sporadic except L15W, A136P or S, and V219I, which were the dominant mutations for Der f 3 BK clones as shown in the following table:

Amino acid	Reference	Mutation	Occurrence (Of 36)
14	I	T	7
15	L	W	36
35	Q	K	1
46	Q	L	1
72	Q	P	1
83	T	S	1
95	V	A	1
98	I	T	1
136	A	P	12
136	A	S	7
159	S	N	1
159	S	G	1
165	E	G	1
180	D	G	1
219	V	I	13
258	S	P	2

F. Der p3. Polymorphisms of Der p 3 were found in the mature region of protein (aa12 to aa243). These polymorphisms appear to be pattern. The F83Y, Y91F, Q92K, S104T, and N109D, are found in >80% of Der p 3 clones as shown in the following table:

Amino acid	Reference	Mutation	Occurrence (Of 20)
83	F	Y	16
84	A	S	2
91	Y	F	14
92	Q	K	14
104	S	T	16
109	N	D	18
114	K	R	2
115	V	A	2
171	N	D	2

Yeast *Pichia pastoris* cells were transformed with plasmid pPIC$_Z$ containing cDNA encoding HDM allergen group 1, 2, or 3 of both Dp and Df species. Yeast transformants were selected for integration of HDM allergen-coded cDNA before be induced to express recombinant Der p and Der f allergens under 0.5%-3% methanol varied by each recombinant protein. All recombinant proteins were filtered and purified through a gel filtration (superdex G-75) column. All purified recombinant proteins were in PBS and kept at -20 °C until used in following experiments: western blotting, ELISA, mass fingerprint, and polyclonal T-cell proliferation.

SDS-PAGE electrophoresis analysis of recombinant HDM allergens

Secreted recombinant HDM allergens in culture media could be visualized in SDS-PAGE as shown in the following figures.

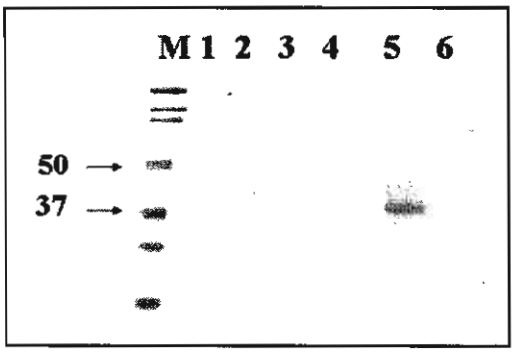

Fig.1. Reducing 12% SDS-PAGE gel of recombinant Der p1 and Der f 1: lane M: Broad-range MW marker, lane 1: media from a culture of *Pichia* transformed with pPICz vector, lane 2-4: different variants of recombinant proDer p 1, lane 5: recombinant proDer f 1, lane 6: 1 μg of BSA.

All recombinant Der p 1 and Der f 1 variants appeared in a smear-broad band migrating from 37 to 50 kDa. This is much higher than the reported molecular weight, which is 25 kDa, of mature-form nature Der p 1 or Der f 1. This discrepancy could be due to different glycosylated by *Pichia* yeast and a lack of maturation processing to cleave the pro-peptide off from the mature-form protein (18). Maturation occurs naturally in enzyme cysteine protease family where the pro-peptide is cleaved allowing the enzyme to be fully function. Contrary to recombinant Der p 1, recombinant Der f 1 appears to have a partial maturation processing since there was a protein band mobilized at 25 kDa as well.

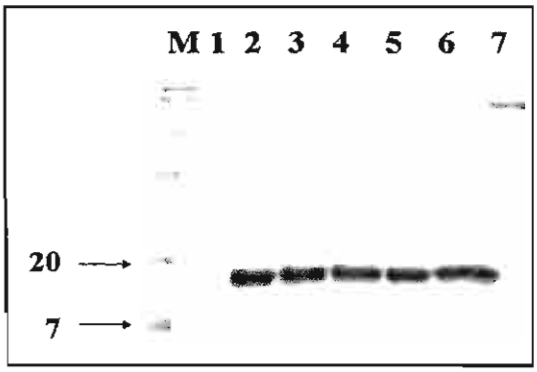

Fig.2. Reducing 18% SDS-PAGE gel of recombinant Der p2 and Der f 2: lane M: Broad-range MW marker, lane 1: media from a culture of *Pichia* transformed with pPICz vector, lane 2-4: different recombinant Der p 2 variants, lane 5-6: different recombinant Der f 2 variants, lane 7: 1 μg of BSA.

All recombinant Der p 2 and Der f 2 variants appeared in a sharp band migrating at 15 kDa which is the reported molecular weight of nature Der p 2 or Der f 2.

Fig.3. Reducing 12% SDS-PAGE gel of recombinant Der p3: lane M: Broad-range MW marker, lane 1: media from a culture of *Pichia* transformed with pPICz vector, lane 2: recombinant proDer p 1, lane 3: recombinant proDer p 3.

Recombinant Der p 3 appeared as two smear-small bands. As indicated as *A, a band migrated from 37 to 50 kDa. This is much higher than the reported molecular weight 25 kDa of mature-form nature Der p 3. Also, as indicated as *B, another band migrated around 25 kDa corresponding to mature-form protein. Similar to recombinant Der p 1, this discrepancy could also due to different glycosylated by *Pichia* yeast and an incompletion of maturation processing to cleave the pro-peptide off the mature sequence of Der p 3 which is an enzyme serine protease.

Western blot analysis of recombinant HDM allergens

In addition to SDS-PAGE analysis, to confirm their identity, different recombinant variants of both group 1 and 2 were transferred onto a nitrocellulose membrane and incubated with monoclonal antibodies against either Der p1 or 2. Because of high homology between proteins of two mite species, the monoclonal antibody against Der p 1 has cross-reactivity to Der f 1, while that against Der p 2 also has cross-reactivity to Der f 2, respectively. However, for Der p 3, we incubated specific serum IgE against Der p 3 to confirm identity of recombinant Der p 3 since there is not commercial antibody against Der p 3.

Results from western blot analysis of recombinant allergens: group 1, 2, and 3, were shown in the following figures.

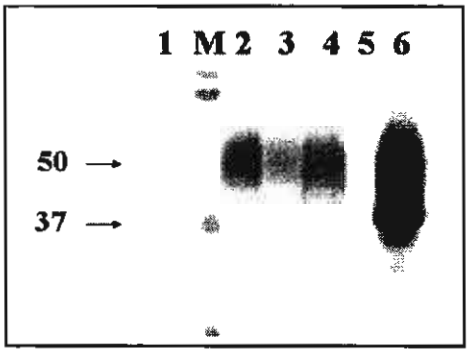

Fig.4. Western blot analysis of recombinant Der p1 and Der f 1 using monoclonal antibody against Der p1, lane 1: media from a culture of *Pichia* transformed with pPICz vector, lane M: Broad-range MW marker, lane 2-4: different recombinant proDer p 1 variants, lane 6: recombinant proDer f 1.

This result confirmed that these proteins were Der p 1 and Der f 1. The results also suggested that the pro-peptide did not hinder the binding of monoclonal antibody to these recombinant group 1 proteins (19, 20).

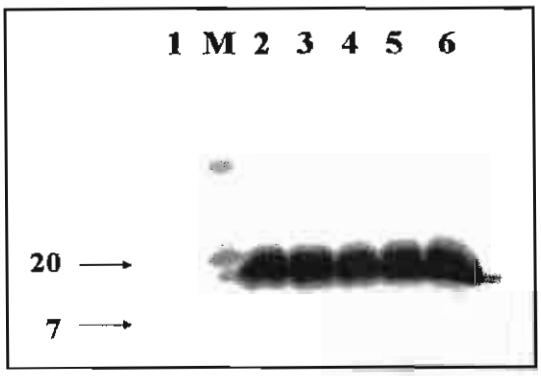

Fig.5. Western blot analysis of recombinant Der p 2 and Der f 2 using monoclonal antibody against Der p 2, lane 1: media from a culture of *Pichia* transformed with pPICz vector, lane M: Broad-range MW marker, lane 2-4: different recombinant Der p 2 variants, lane 5, 6: recombinant Der f 2 variant.

This result confirmed that these proteins were Der p 2 and Der f 2. As mentioned before, the monoclonal antibody to Der p 2 could cross-react to the Der f 2 as well.

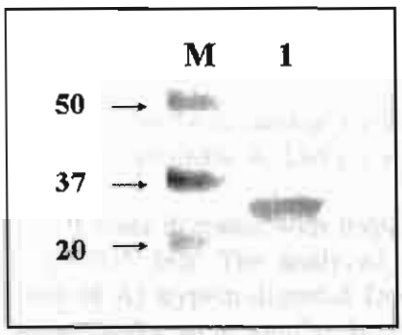

Fig.6. Western blot analysis of recombinant proDer p 3 using specific serum IgE of HDM allergic donors, lane M: Broad-range MW marker, lane 1: recombinant Der p 3.

This analysis result showed recombinant proDer p 3 had an auto-processing when stored at 4 °C and appeared at 25 kDa as a mature Der p 3. The result also confirmed that this protein was Der p 3. However, it appears that Der p 3 may not be one of the dominant allergens as previously thought since we found only specific serum IgE against Der p 3 from 1 of 10 allergic donors.

Mass fingerprint analysis of recombinant HDM allergens

In addition to western blotting, identity of all recombinant allergens: group 1, 2, and 3, were also confirmed by mass fingerprint using MALDI-TOF MS. The results of predicted mass of each allergen group were shown in the following figures.

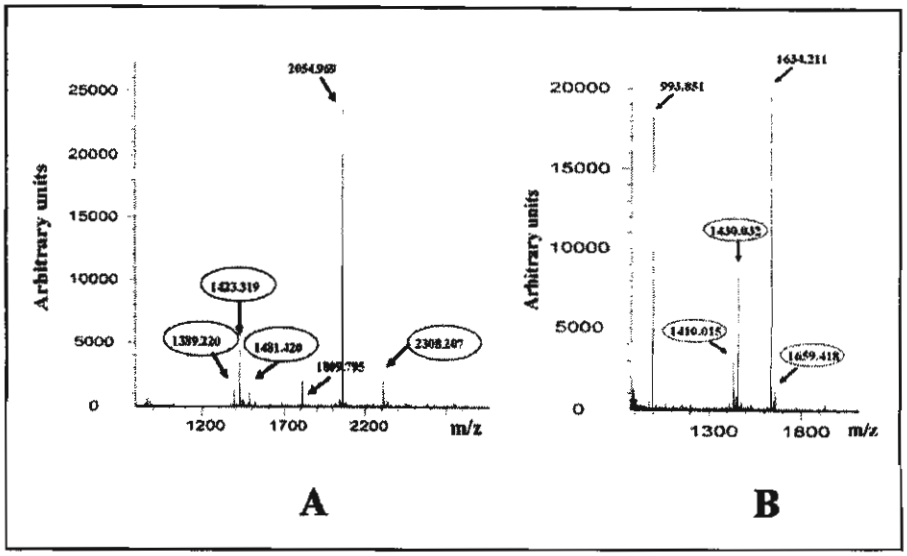

Fig.7. Results of Mass fingerprint analysis showed mass to charge ratio (m/z) of trypsin-digested peptides of recombinant A) Der p 1 and B) Der f 1.

The recombinant Der p 1 and Der f 1 were digested with trypsin before the digested peptides were analyzed by MALDI-TOF MS. The analyzed prominent m/z peaks corresponded to the predicted mass of A) trypsin-digested Der p 1 and B) trypsin-digested Der f 1. The circled mass peaks were similar to the predicted mass of prosequence of A) trypsin-digested Der p 1 and B) trypsin-digested Der f 1.

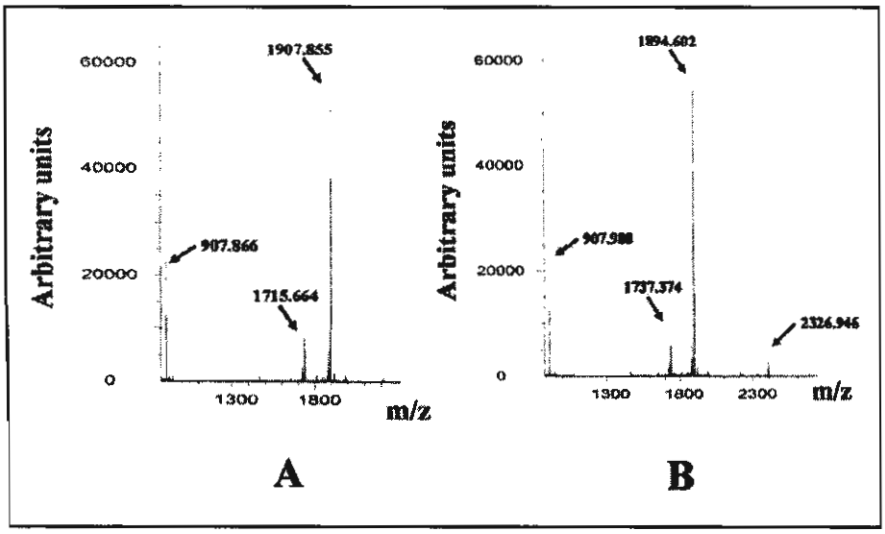

Fig.8. Results of Mass fingerprint analysis showed mass to charge ratio (m/z) of trypsin-digested peptides of recombinant A) Der p 2 and B) Der f 2.

The recombinant Der p 2 and Der f 2 were digested with trypsin before digested peptides were analyzed by MALDI-TOF MS. From the mass fingerprint analysis, the prominent mass peaks corresponded to the predicted mass of A) trypsin-digested Der p 2 and B) trypsin-digested Der f 2.

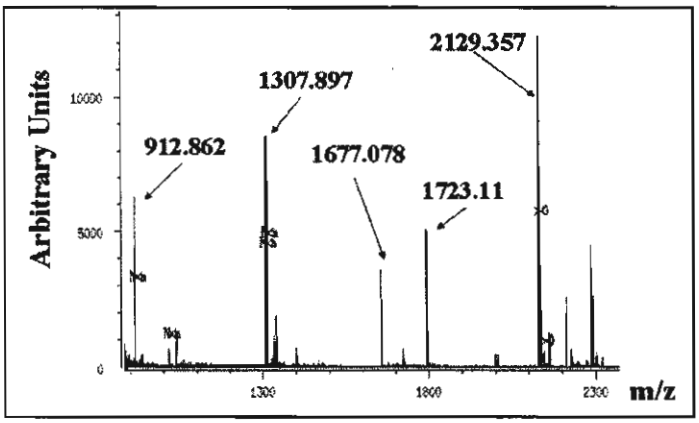

Fig.9. Results of Mass fingerprint analysis showed mass to charge ratio (m/z) of digested peptides of recombinant Der p 3.

The recombinant Der p 3 was digested with trypsin before digested peptides were analyzed by MALDI-TOF MS. The analyzed prominent mass peaks corresponded to the predicted mass of trypsin-digested Der p 3.

Analysis of serum specific IgE against Der p 1 or Der p 2 in Thai allergic populations

Sera of individual donor from 33 Thai whose had positive skin-prick test for whole dust mite extract was used in two-site ELISA for Der p 1 and Der p 2. The results from ELISA are shown in the following figures.

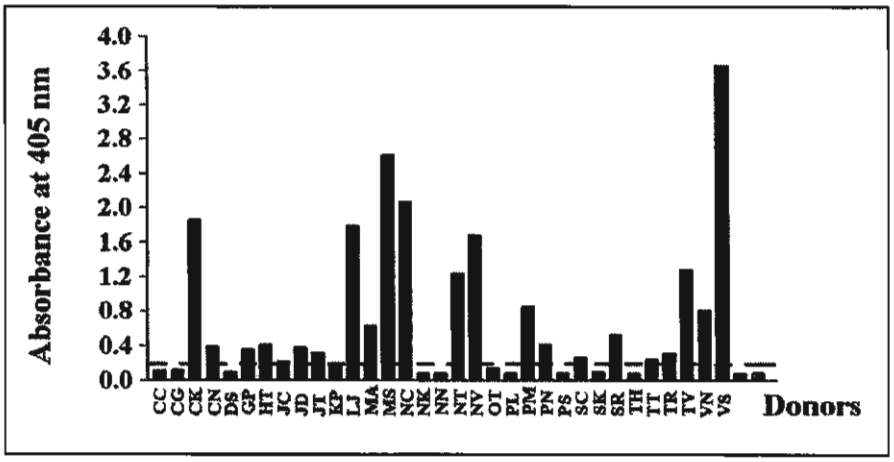

Fig.10. Results of two-site ELISA for serum specific IgE against Der p 1 in 33 Thai allergic donors.

Results of two-site ELISA for serum specific IgE against Der p 1 showed that 18 of 33 allergic donors had serum specific IgE against Der p 1. However, of 18, only 10 donors had a high level of specific IgE against Der p 1 with absorbance value > 0.8 at 405 nm while another 8 donors had a low level of specific IgE with absorbance value ~0.4-0.6 at 405 nm. The cut-off value for positive absorbance value is 0.3 which is 3 O.D. of value from negative control.

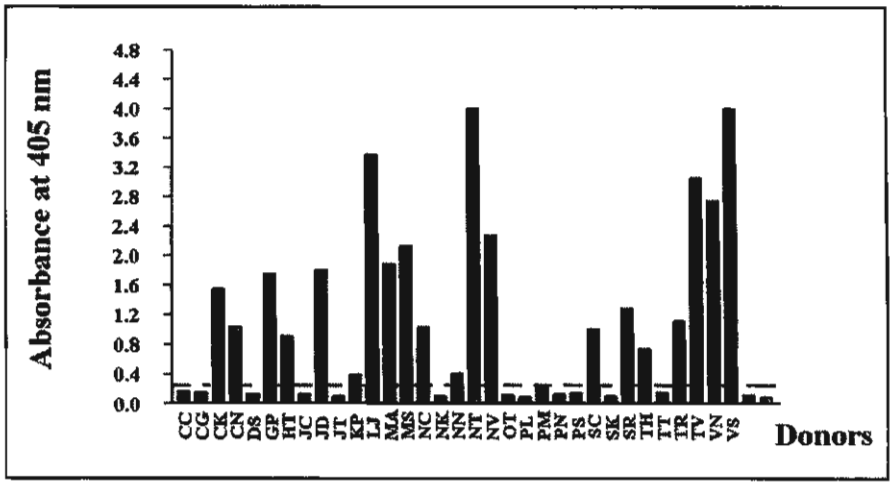

Fig.11. Results of two-site ELISA for serum specific IgE against Der p 2 in 33 Thai allergic donors who were also tested for serum specific IgE against Der p 1.

Results of two-site ELISA for serum specific IgE against Der p 2 using the same 33 donors testing for specific IgE against Der p 1 showed that 20 of 33 (60%) allergic donors had serum specific IgE against Der p 2. However, unlike the ELISA results of Der p 1, of 20, 17 donors had a high level of specific IgE against Der p 2 with absorbance value > 0.8 at 405 nm while only 3 donors had a low level of specific IgE with absorbance value ~0.4-0.7 at 405 nm. The cut-off value for positive absorbance value is 0.3 which is 3 O.D. of value from negative control.

Further analysis of the ELISA results, only 8 allergic donors had a high level of serum specific IgE against both Der p 1 and Der p 2. Of these 8 donors, 5 donors had a higher level of serum specific IgE against Der p 2 than that against Der p 1.

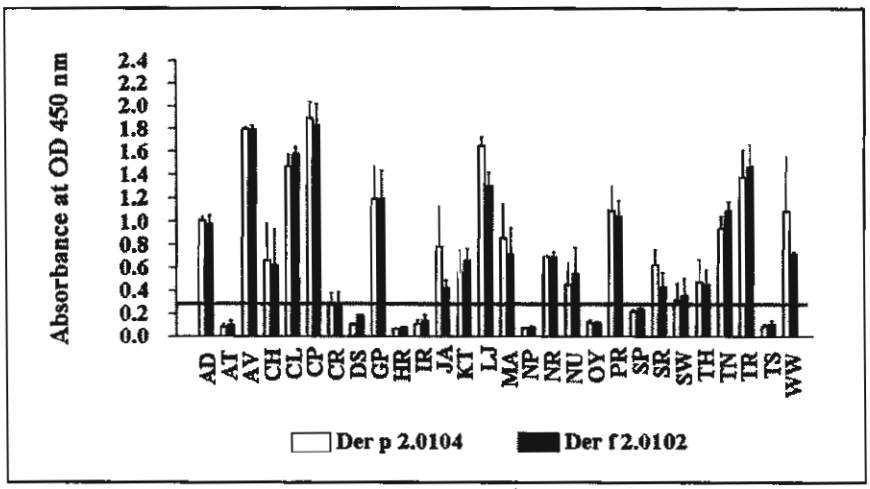

Fig.12. Results of two-site ELISA for serum specific IgE against Der p 2 and Der f 2 in 28 Thai allergic donors some of whom were different from the tests reported in Fig. 10 and 11.

Results of two-site ELISA for Der p 2.0104 and Der f 2.0102 using serum IgE of the same donors showed that 20 of 28 (71%) allergic donors had serum specific IgE against both Der p 2 and Der f 2. Of 20, 17 donors had a similar level of specific IgE against Der p 2 and Der f 2 suggesting that specific IgE may cross-react to both allergens. The cut-off value for positive absorbance value is 0.3 which is 3 O.D. of value from negative control.

For analysis of Thai allergic population against Der p 3, two-site ELISA for Der p 3 could not be performed because, currently, there is no antibody available, and a preliminary test by direct binding ELISA found that Der p 3 did not bind to the ELISA plate. This study had performed western blotting using 10 individual sera of the same allergic donors. The results of western blotting (not show here) found that only one of 10 donors had sera specific IgE against Der p 3. However, based on this study, it appears that Der p 3 might not be a major HDM allergen. This finding is similar to the recent finding by other (21).

Effects of genetic polymorphisms of Der p 1 and Der p 2 on IgE binding activity

There are numbers of Der p 1 and Der p 2 variants which have different mutations found in houses in Bangkok. However, it is unknown whether these point mutations found in each variant might change affinity by serum specific IgE or allergenicity. In one environment, as one might expect, these variants could be found together in house dust. Thus, a ratio of each variant in a gram of house dust could be an important factor for HDM allergenicity. This study aimed to examine allergenicity or IgE binding of these variants by competitive inhibition ELISA.

For Der p 1, this study examined variants: 1.0105, 1.0113, and 1.0114, as shown below.

AA / Clone	19	81	125	215	Frequency
Ref. Der p 1.0101	Met	Glu	Asn	Glu	**0**
Der p 1.0105	Met	Glu	Asn	Glu	**12**
Der p 1.0113	**Ile**	Glu	**Ser**	**Gly**	**1**
Der p 1.0114	Met	**Ala**	Asn	Glu	**1**

As for Der p 2, this study examined variants: 2.0103, 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**
2.0109	**.**	**S**	**L**	**N**	**3**
2.0110	**.**	**S**	**.**	**N**	**2**

The results of competitive inhibition ELISA are shown in the following figures.

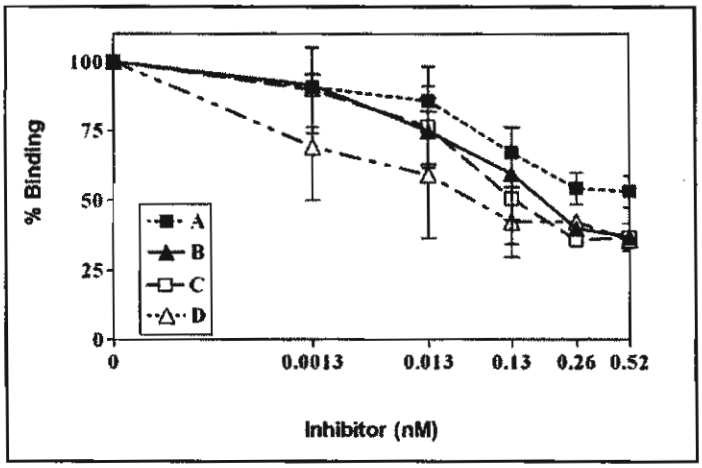

Fig.13. All recombinant proDer p 1 variants could inhibit serum specific IgE of allergic donors who had tested to have a high level of specific IgE against Der p 1.

The results from competitive inhibition ELISA showed that all tested recombinant proDer p 1 variants had higher affinity to specific IgE than nature Der p 1 (A). Of the