Allergenicity of native and recombinant major allergen groups 1 and 2 of *Dermatophagoides* mites in mite sensitive Thai patients

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Summary

Background and objective: **Natural allergenic extracts using for diagnosis and immunotherapy may have batch-to-batch variations and contaminations with unrefined allergens or non-allergenic components. Thus, recombinant allergen is believed to overcome these shortcomings. In this study, native and recombinant allergens of group 1 and 2 of *Dermatophagoides* mites were produced and their allergenicities were compared.**

Methods: **Native allergens were prepared by MAb affinity chromatography. All recombinant allergens were produced in *E. coli* expression system. IgE reactivities of these allergens were determined by IgE-ELISA.**

Results: **The native and recombinant Der p 1, Der p 2, Der f 1, Der f 2 had molecular weights of approximately 25, 15, 25 and 15 kDa, respectively. IgE reactivities of nDer p 1, nDer f 1, rDer p 1 and rDer f 1 were 96.67%, 90%, 43.33% and 46.67%, respectively. Allergenicities of nDer p 2, nDer f 2, rDer p 2 and rDer f 2 were 86.67%, 96.43%, 76.67% and 89.29%, respectively. The findings indicated that recombinant group-1 products were minor allergens which revealed no correlation with their native forms. In contrast, recombinant group-2 allergens were major allergens and showed a significant correlation to their native allergens.**

Conclusion: **We successfully produced native and recombinant group-1 and group-2 allergens. According to their allergenicities, recombinant Der p 2 and rDer f 2 have potential to replace native allergen in diagnostic and therapeutic extracts. Moreover, they can employ as a standard reagent to measure the amount of group 2 allergen in the environment by sandwich-ELISA and utilise this as an immunogen for MAb production. *(Asian Pac J Allergy Immunol 2016;34:51-8)***

Keywords: *native allergen, recombinant allergen, Dermatophagoides mites, Allergenicity, IgE reactivities*

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Introduction

Allergy is the IgE-mediated immune response against a normally harmless substance called an allergen.[1] The allergic reaction occurs when the sensitised person is re-exposed to a specific allergen which leads to activate the allergen-specific T helper 2 (T_H2) cells and IgE synthesis.[2] Hence, the level of circulating specific IgE antibodies in the blood is currently used for the diagnosis of allergy as an alternative to skin prick testing.[3]

House dust mites (HDMs), especially *Dermatophagoides pteronyssinus* and *D. farinae*, are the most frequent and potent sources of indoor allergens associated with asthma and other allergic conditions worldwide.[4] Currently, 27 groups of mite allergens have been characterised and established in the IUIS nomenclature database.[5] Among the established allergens, groups 1 and 2 are the major allergens as the IgE-binding activity of both groups has been reported to account for 80-90%.[6-7]

Allergenic extracts, not only commercial products but also in-house preparations used for diagnosis and treatment of allergic diseases, mainly use crude extract from natural allergenic sources. These allergenic products have lots of shortcomings, such as variation in allergen composition and content, contamination with unrefined minor allergens or non-allergenic component, including allergen from other sources. Over 15 years, the initiative has produced recombinant allergens by using genetic engineering to improve extraction performance and to replace crude extraction in diagnosis and immunotherapy. It is believed that recombinant allergens may overcome many problems of conditional crude extracts from natural sources. The advantages of recombinant allergens are the ability to produce on demand in large quantities with a high degree of purity and tremendous advantages in terms of quality control and standardisation. In addition, the recombinant allergen can be used for the immunological response study, especially B cell and T cell epitopes.[8] Therefore, the research into the development of allergy vaccines is now focused on the production of vaccines from recombinant allergens, recombinant hypoallergenic allergen derivatives and allergen-derived T-cell peptides.[9] Many studies have shown that the efficiency of recombinant allergens in the vaccine immunotherapy are the same as conditional crude extracts, but recombinant allergens are safer.[8] Therefore, recombinant allergens could lead to the development of an allergy vaccine in the future.[10] Furthermore, recombinant groups 1 and 2 of *Dermatophagoides* mites should be a better source of allergens. With regard to the genetic variation of mite allergens, the recombinant allergens should be produced upon the different areas. In this study, we made an effort to produce both native and recombinant forms of Der p 1, Der p 2, Der f 1 and Der f 2 as well as to evaluate their IgE reactivities in mite-sensitive patients in Thailand.

Methods

Serum Collection

The research participants were divided into 2 groups: 10 healthy volunteers in the normal group and 30 allergic patients in the subject group. They attended the Allergy Clinic of Siriraj Hospital. While the subject group in this study must be diagnosed with allergic rhinitis (AR) and reacted with *D. pteronyssinus* and *D. farinae* by the skin prick test, in healthy volunteers without any chronic disease, the skin prick test with HDM and other common allergens showed negative results. In addition, in all of the sera, specific IgE levels to *D. pteronyssinus* and *D. farinae* allergens were measured using ImmonoCAP system (ImmunoCAP® 100E Automate, Sweden) for confirmation of participant sera. This study was approved by the Siriraj Ethical committee (COA No.Si224/210), Faculty of Medicine Siriraj Hospital, Mahidol University.

Preparation of Crude D. pteronyssinus and D. farinae

One gram of 99% purity of fresh whole mite bodies, supplied from the Siriraj House Dust Mite Center for Service and Research, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, was extracted with 0.01M of phosphate-buffered saline (PBS; pH 7.4) using an ultra-sonicator (LABSONIC®P, France) at 30% amplitude with 0.5 cycles for 15 min on ice. After that, the mixture was centrifuged at 12,000 x g for 20 min at 4°C. Then, the supernatant was carefully collected and stored at -80°C until use.

Purification of Native Allergen

The native allergens of Der p 1 (nDer p 1), Der p 2 (nDer p 2), Der f 1 (nDer f 1) and Der f 2 (nDer f 2) were purified from the crude extracts of *D. pteronyssinus* and *D. farinae* by using immune-affinity chromatography. All of the utilised monoclonal antibodies (MAb) were produced from the Laboratory for Research and Technology Development, Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University. CNBr-activated-sepharose 4B (GE Healthcare, Buckinghamshire, UK) were linked to the MAb from individual selected hybridomas specific to each allergen. The MAb affinity resin was blocked with 0.1 M Tris-HCl, pH 8.0 for 2 hours at room temperature to block the remaining active site of the beads. The affinity resin was washed alternately with 0.1 M acetic acid/sodium acetate, pH 4.0 containing 0.5 M NaCl and 0.1 M Tris-HCl, pH 8.0, containing 0.5 M NaCl. After the washing steps, crude extract of HDM (*D. pteronysinus* or *D. farinae*) was added to the column containing MAb affinity resin and rotated for 1 hour at 4°C. The unbound protein was washed and the MAb-bound protein, *i.e.* nDer p 1, nDer p 2, nDer f 1 and nDer f 2, was eluted with 0.1 M glycine-HCl, pH 3.0. The eluted fractions were immediately neutralised with 1 M Tris-HCl, pH 8.0 and kept at -80°C until use.

Production of Recombinant Allergen

Construction of cDNA

Recombinant Der f 1 was previously constructed by the Laboratory for Research and Technology Development. The cloning and production of recombinant Der p 1, Der p 2, and Der f 2 were carried out using the previously described method.[11] Briefly, highly purified house dust mites namely *D. pteronyssinus* and *D. farinae*, were also supplied from the Siriraj House Dust Mite Center for Service and Research. Total RNA was extracted from the approximately 25 mg of mite sample using the Total RNA Mini Kit (Tissue) (Geneaid, Taiwan) according to the manufacturer's instructions. The cDNA, synthesised from total RNA by RevertAid RT Kit (Thermo Scientific, USA) using Anchored Oligo dT Primers (Thermo Scientific), was used as a template to amplify the target gene coding-DNA fragment by PCR using a specific primer. The nucleic acid primers for Der p 1, Der p 2, and Der f 2 were shown in Table 2 with a *Bam*HI and *Xho*I sites at the 5' end of the forward and reverse primers.

Cloning and Expression of Recombinant Allergen

The expected PCR amplicon was purified and cloned into the TA-cloning vector (pTZ57R/T) by using InsTAclone PCR Cloning Kit (Thermo Scientific), and the ligated-product was introduced into *E. coli* strain JM109. The DNA-coding sequence of an insert was confirmed by direct PCR and DNA sequencing. The DNA coding for specific genes was sub-cloned to the expression vector (pET23b+) and introduced into *E. coli* strain BL21 (DE3). The recombinant protein expression was achieved by adding IPTG at the final concentration 0.4. The homogenate was separated as soluble and insoluble fractions. The recombinant protein was purified from the *E. coli* lysate by metal affinity chromatography.

Purification of Recombinant Allergen

The recombinant proteins were successfully produced in the inclusion bodies (IBs)-insoluble bacterial protein fraction. IBs was then extracted and isolated by repeated washes with washing buffer containing 1% (v/v) Triton X-100. After the IBs isolation process, the purified IBs were solubilised using 10% (w/v) N-Lauroylsarcosine according to Tao et al.[12] The refolded proteins were obtained by mixing with a specific ratio of Triton X-100 and CHAPS. The purification was achieved by metal affinity chromatography using TALON Metal Affinity Resins (Clontech, USA).

SDS-PAGE analysis

SDS-PAGE was carried out as described previously.[13] Briefly, prepared native and recombinant allergens were separated individually in 12% SDS-polyacrylamide gel slabs cast in a Mini-PROTEAN 3 Cell (Bio-Rad, USA). The separated proteins in each gel were stained by using Coomassie Brilliant Blue G-250 (CBB) dye to directly visualise the protein bands

ELISA for IgE-binding Activity Assay

IgE binding activity of all produced native and recombinant allergens was measured by indirect ELISA. Briefly, using native and recombinant Der p 1 as the example, 0.5 μg of purified nDer p 1 and rDer p 1 were coated onto 96-well microtitre plate. The wells were incubated with serum (1:4) at 37°C for 2 hrs. The well was treated with diluted (1:500) mouse-anti-human immunoglobulin E-biotin conjugate (Southern Biotech, USA), and incubated at 37°C for 1 hr. The streptavidin alkaline phosphatase (AP) conjugate (1:2,000; Southern Biotech) was added and incubated at 37°C for 1 hr. For the enzyme reaction, PNPP-phosphatase substrate (Thermo Scientific) was added. The colorimetric reaction was occurred in the dark condition at 25°C for 30 min. The OD of each well was determined at A_{405nm}-A_{630nm}. The postulated cut-off value was mean of OD ELISA values of negative sera + 2 SD. The IgE reactivity was defined as positive when the OD values of the tested sera were higher than the cut-off value.

Statistical analysis

Statistical computer programs (SPSS version 18) were used to analyse the means, standard deviations and cut-offs of the IgE reactivities of native and recombinant allergens. Correlation between IgE reactivities of the produced native and recombinant allergens were analysed by Pearson's correlation test. Differences were considered to be statistically significant when $p < 0.01$

Result

Thirty sera samples were collected from allergic rhinitis patients as the subject group. The mean specific IgE level to *D. pteronyssinus* was 29.34 KAU/L, while to *D. farinae* was 26.62 KAU/L. Moreover, 10 sera samples were collected from healthy volunteers as a control group. The mean

Table 1. Demographic and clinical characteristics of the enrolled subject; 30 mite allergic patients and the 10 healthy volunteers.

Patient/ Control No.	Sex	Age (years)	Diagnosis	Skin prick test positive to crude extract of	Specific IgE to Df (KAU/L)	Specific IgE to Dp (KAU/L)
P1	F	50	AR	Dp, Df, CR	6.94	9.76
P2	M	21	AR	Dp, Df, cat, pollen ,CR, kapok	23.8	14.1
P3	F	51	AR	Dp, Df, CR	6.43	11.9
P4	M	23	AR	Dp ,Df, dog, pollen, housefly, kapok	10.8	11
P5	F	38	AR	Dp, Df, cat, dog, pollen, MQ, CR	13.0	16.3
P6	F	21	AR	Dp, Df, cat, dog, pollen, CR	21.6	78.5
P7	M	56	AR	Dp ,Df, CR	5.28	1.8
P8	F	29	AR	Dp ,Df, CR	21.6	17.5
P9	F	23	AR	Dp, Df, cat, dog, pollen, MQ, CR	10.3	6.22
P10	F	35	AR	Dp, Df, cat, dog , pollen, CR	15.8	11.3
P11	M	23	AR	Dp, Df, CR	8.2	6.97
P12	F	15	AR	Dp, Df, cat, dog, pollen, MQ, CR	>100	>100
P13	F	71	AR	Dp, Df, cat, dog, pollen, MQ, CR, housefly, kapok	3.28	6.37
P14	F	30	AR	Dp, Df, cat , pollen, CR	7.5	6.36
P15	F	28	AR	Dp, Df, cat, dog, pollen, CR	13.4	13
P16	F	19	AR	Dp, Df, cat, dog, pollen, MQ, CR	94.6	96.2
P17	F	19	AR	Dp, Df, cat, dog, pollen, MQ, CR	67.3	69.5
P18	F	19	AR	Dp, Df, cat, dog, pollen, MQ, CR	61.4	85.1
P19	F	20	AR	Dp, Df, cat, dog, pollen, MQ, CR, housefly, kapok	2.2	5.78
P20	M	34	AR	Dp, Df, cat, dog, pollen, MQ, CR, housefly, kapok	7.4	12.9
P21	F	34	AR	Dp, Df, cat, dog, pollen, MQ, CR	39.0	52.5
P22	F	22	AR	Dp, Df, cat, dog, pollen, MQ, CR	43.8	15.3
P23	F	59	AR	Dp, Df, cat, dog, pollen, CR	7.06	9.61
P24	M	21	AR	Dp, Df, cat, dog, pollen, MQ, CR, housefly, kapok	6.72	7.52
P25	F	33	AR	Dp, Df, cat, dog, pollen, CR	15.2	16.4
P26	F	21	AR	Dp, Df, cat, dog, pollen, MQ, CR	74.1	>100
P27	M	21	AR	Dp, Df, cat, dog, pollen, MQ, CR, housefly, kapok	17.2	20
P28	F	22	AR	Dp, Df, cat, dog, pollen, CR	10.8	18.7
P29	M	22	AR	Dp, Df, cat, dog, pollen, MQ, CR, housefly, kapok	4.48	4.81
P30	F	23	AR	Dp ,Df, pollen, CR	79.5	54.7
P1c	F	23	-	-	0.04	0.03
P2c	M	23	-	-	0.02	0.02
P3c	F	26	-	-	0.01	0.01
P4c	F	36	-	-	0.06	0.09
P5c	M	22	-	-	0.02	0.04
P6c	M	26	-	-	0.02	0.02
P7c	F	22	-	-	0.01	0.02
P8c	F	22	-	-	0.03	0.04
P9c	M	22	-	-	0.02	0.01
P10c	F	26	-	-	0.06	0.05

Abbreviation: M, Male; F, Female; AR, Allergic rhinitis; P, Patient; C, Control; Skin prick test to crude allergenic extracts was determined positive for a mean diameter of wheal size larger than 3 mm than negative control (saline diluent); Dp, *Dermatophagoides pteronyssinus*; Df, *Dermatophagoides farinae*; MQ, Mosquito; CR, Cockroach.

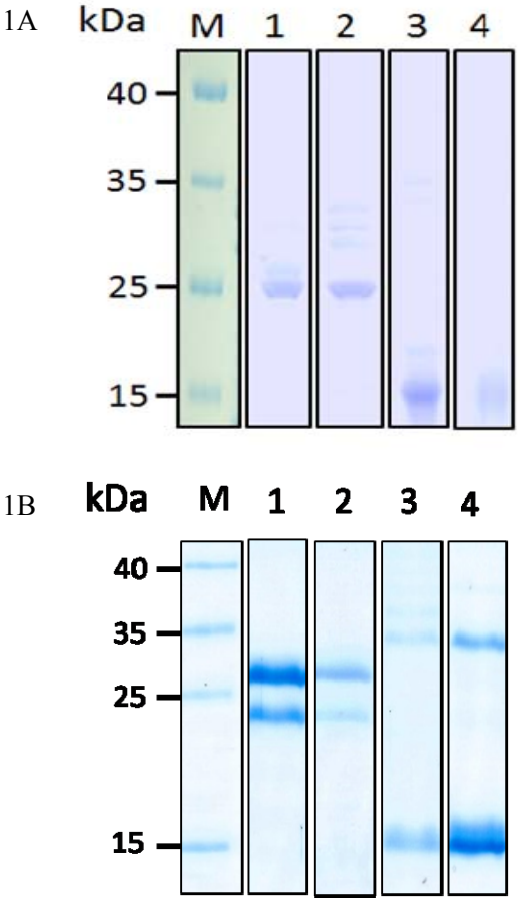

Figure 1. SDS-PAGE patterns of the native allergens (A) and recombinant allergens (B) of *Dermatophagoides* mites

Lane M: Pre-stained standard protein ladder

Lane 1: nDer p 1(A) and rDer p 1(B) allergens

Lane 2: nDer f 1(A) and rDer f 1 (B)

Lane 3: nDer p 2 (A) and rDer p 2 (B)

Lane 4: nDer f 2 (A) and rDer f 2 (B)

Numbers at the left are molecular masses of proteins in kDa

specific IgE level to *D. pteronyssinus* was 0.033 KAU/L, while to *D. farinae* it was 0.029 KAU/L.

The protein patterns of individual native forms of group 1 and 2 allergens of *Dermatophagoides* mites revealed by SDS-PAGE and CBB staining are shown in Figure 1 A, whereas all recombinant products are shown in Figure 1 B. The molecular weight of native and recombinant allergens of Der p 1, Der p 2, Der f 1 and Der f 2 were approximately 25, 15, 26 and 15, respectively. All produced allergens were confirmed to be dust mite allergens by amino acid sequencing (data not shown).

The comparison of IgE-reactivity from native and recombinant allergens of Der p 1, Der p 2, Der f 1, and Der f 2 is shown in Table 3. The calculated cut-off values of native allergens of Der p 1, Der p 2, Der f 1 and Der f 2 were 0.119, 0.094, 0.097 and 0.028, respectively, whereas those of individual recombinant allergens were 0.047 0.087, 0.061 and 0.042, respectively. In the group 1 allergens, the IgE binding activity of nDer p 1 was 96.67%, while that of rDer p 1 was only 43.33%; this is significantly lower than for the native form. In accordance with the result of Der p 1, nDer f 1 showed a more significant difference of IgE binding activity (90%) than that of rDer f 1 which was only 46.67%.

For group 2 allergens derived from *Dermatophagoides,* mites were also tested and the results are shown in Table 3. IgE reactivities of nDer p 2 and rDer p 2 were 86.67% and 76.67%, respectively, which were not significantly different. Similar outcomes were also observed in the Der f 2 allergen. IgE reactivity for nDer f 2 was 96.43%, while that for rDer f 2 was 89.29%. Thus, both forms of prepared Der f 2 allergen were considered major allergens.

When analysing the correlation between native and recombinant products of each allergen, group 2 allergens, nDer f 2 versus rDer f 2, showed a strong positive correlation: $r = 0.745$; $p < 0.01$ (Figure 2 D). Similarly, IgE reactivities of nDer p 2 positively correlated with that of rDer p 2 $r = 0.545$; $p < 0.01$ (Figure 2 B). For group 1 allergens, native and recombinant products of Der p 1 and Der f 1 exhibited no correlation.

Discussion

In this study, native allergens were purified by MAb affinity chromatography. All recombinant allergens had been produced in *E. coli;* thus, this can generate plenty of proteins which are in the soluble part that is easily separated from bacterial lysate by centrifugation. The results from SDS-PAGE (Figure 1) and amino acid sequencing confirmed that the produced proteins are dust mite allergens, which is in accordance with previous reports.[11, 14]

The comparison of allergenicity between native and recombinant allergens was performed using the IgE-ELISA technique, measuring the reaction of IgE antibodies in the sera. The IgE reactivity of group 1 allergens (Table 2) and their correlation (Figure 2 A and C) revealed that nDer p 1 and nDer f 1 were major allergens, while rDer p 1 and rDer f 1 were the minor allergens. This implied that the recombinant allergens produced had allergenicity

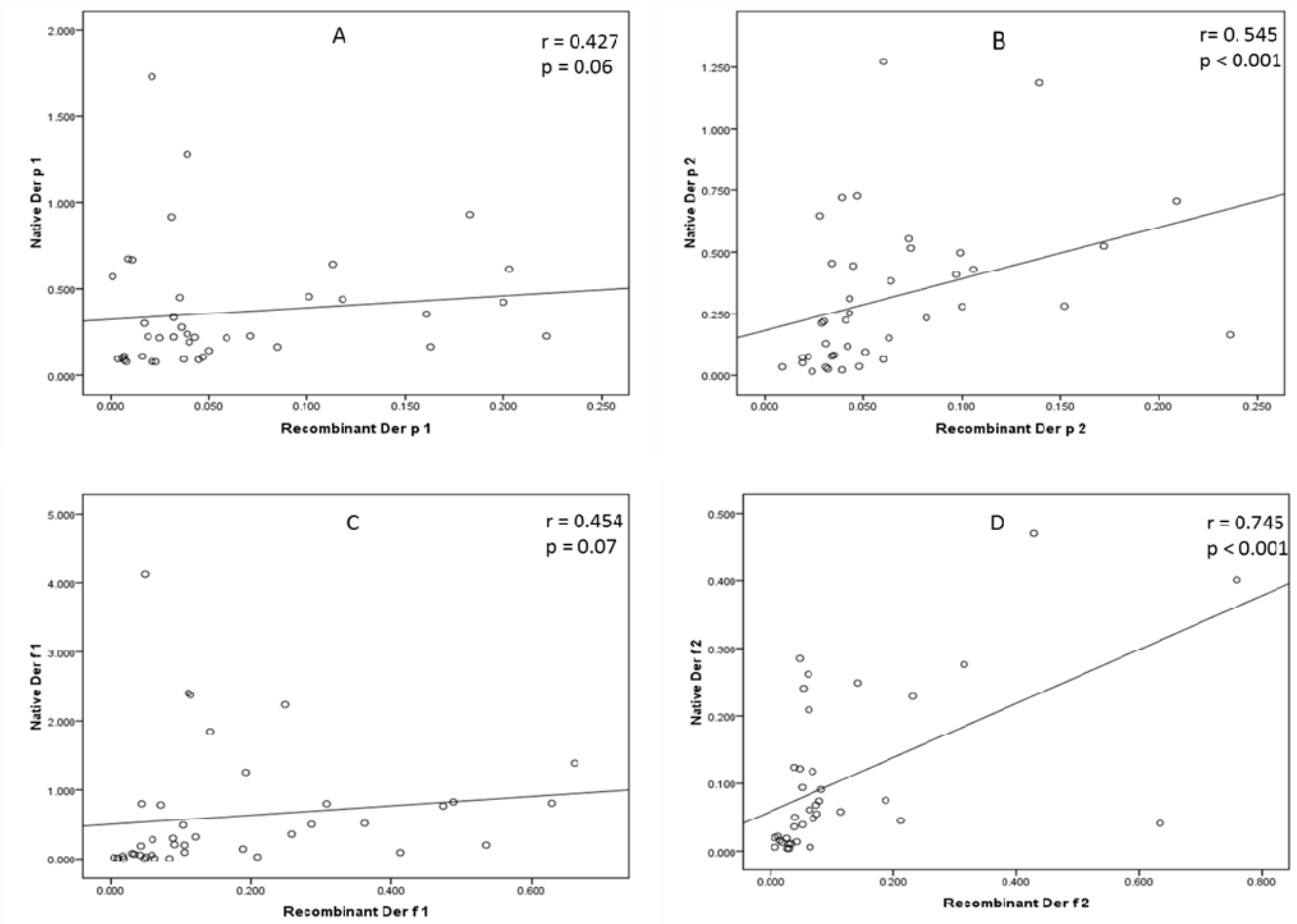

Figure 2. Correlation between IgE binding activities of native allergens and recombinant products: A, nDer p1 and rDer p 1; B, nDer p 2 and rDer p 2; C, nDer f 1 and rDer f 1; D, nDer f 2 and rDer f 2.
Pearson's correlation coefficients (r) and statistical significance at the 0.01 level are also indicated in the upper right corner.

and allergenic potency lower than native allergen, which might be related to the IgE binding epitopes of recombinant allergens. Therefore, the recombinant allergen might not be used to replace native allergens in clinical practice. However, these recombinant allergens may be used as a standard reagent in sandwich ELISA to measure the amount of group 1 allergen in house dust, but their allergenicity and allergenic potency had to be increased to correlate with native forms. The concentration of recombinant allergen should be increased in order to increase the chance of the specific IgE antibody binding to a specific epitope on recombinant allergen.

For group 2 allergens, not only nDer p 2 and nDer f 2, but also rDer p 2 and rDer f 2 were major allergens (Table 2). Moreover, both recombinant allergens had a positive correlation to their native forms (Figure 2 B and 2 D). Therefore, the recombinant group 2 allergen had an allergenicity and allergenic potency similar to that of native forms. This indicates that the IgE binding epitope of recombinant and native allergens are likely to be similar. Therefore, the recombinant group 2 allergens might have the potential to replace native allergens in therapeutic allergen vaccines or diagnostic reagents.

In this study, the recombinant allergens were produced in inclusion bodies of *E. coli*, which present IgE-reactivity in the minority of patients (group 1 allergen) might due to prokaryotic bacterial does not have post-translational modification such as glycosylation for active form protein.[15] In addition, the protein aggregates and incorrect folding of the inclusion body in bacterial cell may lead to expression of inactive from allergen or the lack of some IgE-binding epitope. Therefore, the produced recombinant allergens from inclusion body *E. coli* were needed solubilisation and

Table 2. The specific primers for DNA synthesis

Primers	The nucleic acid sequence
F-Der p 1 mat-*Bam*HI	5'- CGGGATCCGACTAACGCCTGCAGTATC -3'
R-Der p 1-*Xho*l	5'- TGCTCGAGAATGACAACATATGGA-3'
F-Der p 2 mat-*Bam*HI	5'- CGGGATCCGGATCAAGTCGATGTCAAAG-3'
R-Der p 2-*Xho*l	5'- TGCTCGAGATCGCGGATTTTAGCATG-3'
F-Der f 2 mat-*Bam*H	5'- CGGGATCCGGATCAAGTCGATGTTAAAG-3'
R-Der f 2-*Xho*l	5'- TGCTCGAGATCACGGATTTTACCATGG-3'

Table 3 Percentage of IgE binding activities of Thai mite sensitive patients to produced native and recombinant Der p 1, Der p 2, Der f 1 and Der f 2,

	IgE reactivity (%)	
	Native allergen	Recombinant allergen
Der p 1	96.67[a]	43.33
Der p 2	86.67	76.67
Der f 1	90.0[a]	46.67
Der f 2	96.43	89.29

[a] Entries with superscripts are significantly different of reactivity in the same row at $p < 0.05$ by Chi's square analysis

refolding processes to recover the native-like secondary structure that results in active form allergens.[16]

Many studies have attempted to increase the IgE-reactivity of recombinant allergens to levels equivalent to native allergens; the eukaryotic expression system was the alternative choice. Besides, the insect cell system[16] and mammalian cell system[17] were also used as expression systems for recombinant allergens. However, the yield of recombinant allergens from insect cells and mammalian cells was low compared to that of yeast systems.[8] Even the eukaryotic expression system has almost the same IgE-reactivity as the native allergen but the enzymatic activity of cysteine protease (Der p 1) was still ineffective.[18] Our findings for low allergenicities of rDer p 1 and rDer f 1 were relevant to previous studies because it had been documented that group 1 mite allergens exhibited strictly conformational IgE binding epitopes, resulting in low IgE-binding activity.[19] Additionally, the recombinant production of mature Der p 1 is unsuccessful in terms of correct folding with constitution of aggregation.[20] However, even having low or no IgE binding activity, the recombinant hypoallergenic allergens still preserve T-cell epitopes to induce T-cell responses.[21] Hence, this disadvantage is beneficial for new approaches to develop a safe allergen-specific vaccine as the risk of anaphylaxis might be reduced. Moreover, aggregated forms of recombinant Der p 1 were demonstrated to be promising vaccine candidates against mite allergy.[22]

In contrast, native and recombinant Der p 2 have been characterised as major allergens, in a result that was similar to a previous report by Bordas-Le Floch who described the comparison of allergens between the production from *P. pastoris* as protein secretion and the production from inclusion bodies of *E. coli*.[23] The results showed that both native and recombinant Der p 2 were expressed from *P. pastoris* and *E. coli* displayed comparable allergenicity, despite native and recombinant Der p 2 having different secondary structures. Nevertheless, it is necessary to observe the secondary structure of native and recombinant allergens by using circular dichroism (CD) analysis.

In conclusion, native and recombinant allergens of Der p 1, Der p 2, Der f 1, Der f 2 were successfully produced. Regarding their allergenicities, all native forms of group 1 and group 2 are major HDM allergens in Thailand. Recombinant Der f 2, as well as rDer p 2, have the potential to replace native allergens in diagnostic and therapeutic extracts.

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