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Article type : Original Article: Experimental Allergy and Immunology

Der f 35: an MD-2-like house dust mite allergen that cross-reacts with Der f 2 and Pso o 2

Short title: Der f 35, an MD-2-like house dust mite allergen

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This article has been accepted for publication and undergone full peer review but has not
been through the copyediting, typesetting, pagination and proofreading process, which may
lead to differences between this version and the Version of Record. Please cite this article as
doi: 10.1111/all.13192

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Abstract

Background: *Dermatophagoides farinae* is a source of airborne house dust mite (HDM) allergens. We elucidated IgE-reactive allergens from *D. farinae* by two-dimensional immunoblotting-based allergenome analysis, and identified one new allergen, named Der f 35, that possesses IgE-binding capacity comparable to that of Der f 2. The aim of this study is to clarify the allergenic capacity of new HDM allergen Der f 35.

Methods: We cloned *der f 35* from *D. farinae* mRNA and produced recombinant Der f 35 in *Escherichia coli*. The IgE-binding capacity of Der f 35 and its cross-reactivity with group 2 allergens from *D. farinae* and *Psoroptes ovis* were determined by ELISA and ELISA inhibition assays, respectively.

Results: The deduced amino acid sequence for *der f 35*, which possesses the MD-2-related lipid-recognition domain, showed higher identity with group 2 allergens from *P. ovis* (61.5%) and *Blomia tropicalis* (50.7%) than with Der f 2 (40.8%). Der f 35 showed IgE-binding frequencies of 77.5% (31/40) for the native form upon allergenome analysis and 51.4% (18/35) for recombinant structure by ELISA. Der f 35 showed cross-reactivity with Der f 2 and Pso o 2 in reaction with HDM-allergic patients' IgE by ELISA inhibition assay.

Conclusion: Der f 35 is a candidate major allergen from *D. farinae*, which is more similar to group 2 allergens from sheep scab mite and storage mites. Der f 35 could be responsible for the cross-reactivity among group 2 mite allergens.

Keywords: Allergen, *Dermatophagoides farinae*, Der f 2, Group 2 allergen, House dust mite

Abbreviations

ELISA, enzyme-linked immunosorbent assay; HDM, House dust mite; nDer f 2, native Der f 2; nDer f 35, native Der f 35; nPso o 2, native Pso o 2; rDer f 2, recombinant Der f 2; rDer f 35, recombinant Der f 35; rTF-Der f 35, recombinant TF-fused Der f 35; TF, Trigger factor.

Introduction

House dust mite (HDM) allergens are among the most common airborne allergens and induce severe respiratory disorders, including perennial allergic rhinitis and asthma. Many mite allergens have been identified and characterized from HDM and storage mites (1, 2). In HDM allergens, groups 1, 2, and 23 allergens are considered serodominant (1); group 1 allergens are cysteine proteases, group 2 allergens are MD-2-like lipid-binding proteins, and group 23 allergens are peritrophin-like proteins (3-5). Mite proteases including group 1 and group 3 allergens are considered to play important roles in HDM sensitization by destroying the skin barrier (6-11). Some HDM allergens are important as cross-reactive allergens among mites, foods, and fungi (12-14). Until now, 30 Der f allergens and 19 Der p allergens have been formally registered with the database for systematic allergen nomenclature approved by the World Health Organization and the International Union of Immunological

Societies (WHO/IUIS) Allergen Nomenclature Sub-committee. On the other hand, the results of two-dimensional (2D) Western blotting-based allergenome analysis suggested that many allergens in *D. farinae* extract remain uncharacterized and unregistered ((15) and our unpublished data).

Among the registered HDM allergens, those in groups 1 and 2 are considered the most important not only for diagnosing mite allergy but also as components of vaccines for effective allergen-specific immunotherapy (16, 17). A recent cross-sectional survey showed that group 2 mite allergens were the second most prevalent (18.2%) sensitized allergen out of 112 allergen components among 501 nonselected pupils (12-21 years old) in Austria; the highest prevalence was that of grass allergen, Phl p 1 (26.5%) (18). In another report, high rates of sensitivity to group 2 HDM allergens were observed in patients with severe asthma; and patients with severe persistent asthma were more highly sensitized to HDM allergens, especially to those of group 2 (19). Taken together, these results indicate that group 2 HDM allergens are clinically important due to their high prevalence and strong allergenic capacity.

Group 2 allergens from HDM (*Dermatophagoides farinae* and *D. pteronyssinus*) show cross-reactivity to each other due to their similar three-dimensional (3D) structures (20, 21). Group 2 allergens from storage mites (*Glycyphagus domesticus*, *Lepidoglyphus destructor*, and *Tyrophagus putrescentiae*) also cross-react to each other, but cross-reactivity between the

group 2 allergens from storage mites and HDM is limited (22, 23). Several conflicting reports show positive or negative cross-reactivity of group 2 allergens among HDM, storage mites, and other mite species (24-27).

In this study, we identified a new HDM allergen, Der f 35, by allergenome analysis, and found it has the highest sequence similarity with Pso o 2, a group 2 allergen of sheep scab mite (*P. ovis*). This unique allergen is genealogically more closely related to sheep scab mite allergen and storage mite allergens than to HDM allergens. Its IgE-binding frequency is comparable to that of a major allergen, Der f 2, by 2D Western blotting-based allergenome analysis. Interestingly, Der f 35 shows cross-reactivity with Der f 2 and Pso o 2 in reaction with patients' IgE. These results suggest that Der f 35 is an additional major HDM allergen and could be an important cross-reactive allergen among group 2 mite allergens.

Methods

Human plasma

Plasma samples were obtained from 35 HDM-allergic patients selected on the basis of their clinical history and a positive CAP-RAST score for HDM (RAST score=3 or higher). Non-allergic plasma samples from 5 healthy volunteers with negative RAST scores were used as negative controls. The study protocol conformed to the standards set by the

Declaration of Helsinki. All experiments were approved by the Institutional Review Board of Takanobashi Central Hospital and were described in detail to all patients before they provided their informed consent and were admitted into the study. The patients' plasma samples for allergenome analysis (N=40) were collected separately for each experiment as a different data set.

Two-dimensional allergenome analysis

Two-dimensional immunoblotting analysis in the allergenome study was carried out using plasma samples from 40 patients allergic to HDM ((14) and unpublished data). Proteins from *D. farinae* extract were loaded onto 2D sodium lauryl sulfate (SDS)-polyacrylamide gel, followed by immunoblotting with HDM-allergic patients' plasma as described previously (14, 28). Protein spots with IgE-binding capacity were characterized by electrospray ionization (ESI)-mass spectrometry (MS) after tryptic digestion, and were identified by peptide mass fingerprinting (Mascot search engine, Matrix Science website) as described previously (28).

Molecular cloning of der f 35 and expression of rDer f 35 in Escherichia coli

Degenerate primers were designed from partial amino acid sequences obtained from *de novo* sequencing of native Der f 35 (nDer f 35) by ESI-MS/MS analysis (YSYNVPAV(L/I)PN(L/I)K and V(L/I)GDNG(L/I)VGC(L/I)K). The full length of cDNA

for *der f 35* was obtained using the degenerate primers and *D. farinae* mRNA as templates by the Gene Amp PCR system 9600 (Perkin-Elmer, Norwalk, CT, USA) using the SMART RACE cDNA Amplification Kit (BD Biosciences, Franklin Lakes, NJ, USA) following the manufacturer's instructions. A mature fragment of *der f 34* cDNA without signal peptide (nucleotide no. 119-499) or *der f 2* cDNA was cloned into pCold TF DNA (Takara, Tokyo, Japan) and expressed as a recombinant trigger factor (TF)-fused protein (rTF-Der f 35 or rTF-Der f 2) with His-tag in Rosetta-gamiTM (DE3) competent cells (Merck Millipore, Billerica, MA, USA). rTF-Der f 35 and rTF-Der f 2 were purified using His-trap HP (GE Healthcare Japan, Tokyo, Japan), and the TF was removed by anion-exchange chromatography after cleavage between it and recombinant Der f 35 (rDer f 35) or recombinant Der f 2 (rDer f 2) by thrombin.

Sequence alignment and 3D structure modeling

Amino acid sequences for the following were aligned using ClustalW: Der f 35 (GenBank ID: LC175222), Der f 2.0102 (D10448) Der p 2.0114 (AM263560), *D. pteronyssinus* cDNA clone LY_YIT_DP1663 (EX1663), Eur m 2.0101 (AF047613), Blo t 2.0101 (AY288141), Lep d 2.0201 (X83875), Gly d 2.0201 (AJ272216), Tyr p 2.0101 (Y12690), Pso o 2 (Q965E2), Sui m 2 (AY497897), Ale o 2 (EU106610), Aca s 2 (EU106609), and Der s 2 (DQ367850). Three-dimensional structure modeling of Der f 2,

Der f 35, and Pso o 2 was performed by SWISS-MODEL (Swiss Institute of Bioinformatics)

using a crystal structure of Der f 2 as a template.

SDS-PAGE and immunoblotting

Proteins for soluble fractions of *E. coli* lysate, rTF-Der f 35 after His-trap affinity chromatography, TF and rDer f 35 after thrombin digestion, and purified rDer f 35 were loaded onto 12.5% SDS-polyacrylamide gel and electrophoresed under reducing conditions. The fractionated proteins were visualized with silver staining following the manufacturer's instructions (2D silver staining kit, Daiichi Kagaku Yakuhin, Tokyo, Japan). The fractionated proteins were blotted onto a PVDF membrane and immunostained with 20-fold-diluted pooled plasma for the HDM-allergic patients, 150-fold-diluted biotin-conjugated anti-human IgE (Vector Laboratories, Burlingame, CA, USA), and 2,000-fold-diluted HRP-streptavidin (Zymed Laboratories, San Francisco, CA, USA) and were visualized with ECL Plus Western blotting detection reagents (GE Healthcare Japan). The area of the PVDF membrane corresponding to the molecular marker was cut after protein transfer and then stained with Coomassie Brilliant Blue to estimate the molecular weights of positive bindings on X-ray film after immunostaining.

Enzyme-linked immunosorbent assay (ELISA) and ELISA inhibition assay

ELISA and ELISA inhibition assay were performed according to a similar method previously described (29). Briefly, 100 ng/well rDer f 35 or rDer f 2 was coated onto a 96-well microtiter plate, which was then blocked with phosphate-buffered saline (PBS) containing 3% skim milk and 1% BSA. A serial dilution of purified human IgE (Athens Research Technology, Athens, GA, USA) was coated onto the plate as a calculator to estimate the positive IgE binding (14). Positive binding equal to the immobilized 1 ng IgE was defined as 1 unit. Then, 10-fold-diluted plasma samples from 35 HDM patients and 5 healthy subjects were added to the plate, followed by incubation with 1,000-fold-diluted biotin-conjugated anti-human IgE (epsilon chain-specific, Biosource International, Camarillo, CA, USA). After incubation with 1,000-fold-diluted alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), AttoPhosTM substrate solution (Promega, Madison, WI, USA) was added to the plate, and fluorescence intensity was measured using a Wallac 1420 ARVOsx Multilabel Counter (Perkin-Elmer, Downers Grove, IL, USA). To analyze cross-reactivity between Der f 35 and Der f 2 or Pso o 2 from *Psoroptes ovis*, rDer f 35 was coated onto a microtiter plate and reacted with 10-fold-diluted plasma from 2 patients whose IgE binds to both rDer f 2 and rDer f 35 were preincubated with serial concentrations of rDer f 35, rDer f 2, nPso o 2, or ribonuclease A as a

negative binding control. Native Pso o 2 was kindly provided by Dr. John H. Pruett (United States Department of Agriculture, Kerrville, TX, USA).

Results

Two-dimensional allergenome analysis of D. farinae body extract

To elucidate *D. farinae* allergens that possess high IgE-binding capacity, we performed 2D allergenome analysis of *D. farinae* mite body extract (14, 28). We found that 113 spots possessed IgE-binding capacity in 40 HDM-allergic patients, and that the IgE-binding frequency of the spots ranged from 2.5% (1/40) to 85.0% (34/40) ((14) and unpublished data). Two spots were identified as a major HDM allergen, Der f 2, with 77.5% (31/40) and 65.0% (26/40) IgE-binding frequency (Fig. 1A and B). A spot located at 14 kDa with pI 5.4 showed IgE-binding frequency of 77.5% (31/40) and an IgE-binding profile similar to those of Der f 2 (Fig. 1A and B). We named this spot originally DFA22 (*D. farinae* allergen no. 22), and renamed Der f 35 after its registration with the WHO/IUIS Allergen Nomenclature Database.

Molecular cloning of a new allergen, der f 35

A cDNA corresponding to *der f 35* was cloned from mRNA of *D. farinae* by a PCR method using degenerate primers constructed from internal amino acid sequences of Der f 35, which were revealed by *de novo* sequencing of tryptic fragments (YSYNVPAV(L/I)PN(L/I)K

and V(L/I)GDNG(L/I)VGC(L/I)K) of nDer f 35. Sequence analysis revealed that the *der f* 35 cDNA consisted of 554 nucleotides containing a 432 bp open reading frame encoding a 143-amino acid polypeptide (Fig. 2). Sequence analysis by InterPro of the European Bioinformatics Institute (EMBL-EBI) revealed that the deduced amino acid sequence of *der f* 35 possesses a putative signal sequence (no. 1-17, indicated by a double line in Fig. 2) and an MD-2-related lipid-recognition domain (ID: IPR003172) (30). The deduced amino acid sequence of *der f* 35 predicts that Der f 35 is a 15.5 kDa protein with pI 5.45 for the full length and 13.7 kDa with pI 5.24 for the mature protein without a signal sequence.

Der f 35 in group 2 mite allergens

Der f 35 showed the highest similarity to group 2 mite allergen Pso o 2 from *Psoroptes ovis* (NCBI GenBank ID: Q965E2) as 61.5% identity and 84.6% similarity. Der f 35 was also similar to group 2 mite allergens such as Blo t 2.0101 from *Blomia tropicalis* (AY288141, 50.7% identity and 80.6% similarity), Ale o 2 from *Aleuroglyphus ovatus* (EU106610, 48.3% and 79.7%), Sui m 2 from *Suidasia medanensis* (AY497897, 48.3% and 77.6%), and Lep d 2.0201 from *Lepidoglyphus destructor* (Q1M2P0, 46.9% and 79.0%). Notably, Der f 35 showed higher identity with, and similarity to, group 2 allergens from sheep scab mite (*P. ovis*), storage mite (*B. tropicalis*, *L. destructor*, *Glycyphagus domesticus*, *Acarus siro*, and *Tyrophagus putrescentiae*) and brown-legged grain mite (*A. ovatus*) than those from HDM (*D.*

farinae, *D. pteronyssinus*, *Suidasia medanensis*, *Euroglyphus maynei*, and *D. siboney*) (Fig.

3A). A genealogical analysis revealed that Der f 35 is distinguishable from group 2 HDM allergens but rather closely related to sheep scab mite, Pso o 2 (Fig. 3B).

The sequence identity between Der f 35 and Der f 22 (DQ643992), an MD-2-like lipid binding domain allergen, is not more significant than those for other group 2 mite allergens (28.8% identity and 65.8% similarity). A cDNA clone named LY_YIT_DP1663 (EX163551) identified from cDNA library of *D. pteronyssinus* showed 86.0% identity with and 97.9% similarity to Der f 35 (Fig. 3A). It suggests that the LY_YIT_DP1663 cDNA is a *D. pteronyssinus* counterpart of Der f 35, and Der f/p 35 is distinct from other MD-2-like allergens, Der f/p 2 and Der f/p 22.

Three-dimensional structure modeling of Der f 35 suggested its beta-strand-rich conformation homologous to those of both Der f 2 and Pso o 2 (Fig. 3). These homologous structures indicate IgE-binding cross-reactivity among Der f 35, Der f 2, and Pso o 2.

IgE-binding capacity of Der f 35 in HDM-allergic patients

To elucidate the IgE-binding capacity of Der f 35, we produced rDer f 35 using the pCold TF DNA expression system in Rosetta-gamiTM *Escherichia coli*. Recombinant TF-Der f 35 was purified by nickel affinity chromatography, and the fused TF was removed by anion exchange chromatography after thrombin digestion (Fig. 5A). The rTF-Der f 35 and

purified rDer f 35 showed IgE-binding capacity upon Western blotting analysis using pooled plasma from HDM-allergic patients (Fig. 5B). In 2D allergenome analysis, nDer f 35 showed positive IgE binding in 31 of 40 patients (77.5%), which is comparable to that of nDer f 2 (31 or 26 of 40; 77.5% or 65.0%) (Fig. 1B). Among the 31 patients who showed positive binding to nDer f 35, 27 patients (77%) also showed positive binding to nDer f 2. We further confirmed the IgE-binding capacity of Der f 35 by ELISA using plasma from 35 HDM-allergic patients. We defined IgE binding as positive if a patient's specific IgE titer exceeded a threshold indicated by the broken line in Fig. 5C (8.8 or 6.1 unit/ml for rDer f 35 or rDer f 2, respectively). The IgE-binding frequency of rDer f 35 was 51.4% (18/35) and that of rDer f 2 was 94.2% (33/35) (Fig. 5C). The IgE-binding titers of rDer f 35 were positively correlated with those of rDer f 2 as $p < 0.05$ ($R_s = 0.38$) in all HDM-allergic patients ($N = 35$) determined by Spearman's correlation test. Notably, in patients who reacted to both rDer f 35 and rDer f 2 ($N = 18$), the IgE-binding titers of rDer f 35 correlated strongly with those of rDer f 2 as $p < 0.001$ ($R_s = 0.82$). This result suggests that Der f 35 shares its IgE-binding epitopes with Der f 2.

Consequently, we analyzed the cross-reactivity of Der f 35 with Der f 2 and Pso o 2, because Der f 35 showed the most sequence similarity to, and the high homologous structure with, Pso o 2 and Der f 2 (Fig. 3A and Fig. 4). We analyzed whether binding between rDer f 35 and IgE from 2 patients who were positive to both rDer f 35 and rDer f 2 (indicated as

black symbols in Fig. 5C) was inhibited by nPso o 2, rDer f 2, or ribonuclease A as a negative control (Fig. 5D and E). One patient showed positive cross-reactivity among rDer f 35, nPso o 2, and rDer f 2 (Fig. 5D), but the other patient showed limited positive cross-reactivity between rDer f 35 and nPso o 2 and negative cross-reactivity between rDer f 35 and rDer f 2 (Fig. 5E). These data indicate that Der f 35 shares part of its IgE-binding epitope with Der f 2 and Pso o 2 in some patients.

Discussion

We identified a new HDM allergen, Der f 35, from *D. farinae*, that shows a similar primary sequence to group 2 allergens from sheep scab mite and storage mites. Native Der f 35 showed 77.5% (31/40) IgE-binding frequency in HDM-allergic patients, comparable to nDer f 2 (77.5% and 65.0%), upon allergenome analysis (Fig. 1B). On the other hand, rDer f 35 showed 51.4% (18/35) IgE-binding frequency, which was less than those of nDer f 35 (77.5%) and rDer f 2 (94.2%, 33/35), by ELISA analysis (Fig. 5C). This slightly poor IgE-binding capacity of rDer f 35 may be attributable to the difference in post-translational modification or partial alteration of the tertiary structure of recombinant protein produced in *E. coli*, albeit fused TF may help its proper folding of rDer f 35 (31). Isolation of the native protein from *D. farinae* extract will be necessary to accurately estimate the IgE-binding frequency of nDer f 35 by ELISA.

Der f 35 showed the highest sequence similarity to Pso o 2 and higher similarity to group 2 allergens from storage mites (Blo t 2, Lep d 2, Gly d 2, Tyr p 2, and Aca s 2) and brown-legged grain mite (Ale o 2) than those from HDM (Der f 2, Der p 2, Eur m 2, and Der s 2) (Fig. 3A). A genealogical analysis showed that Der f 35 does not belong to HDM or to the storage mite group 2 family, but genealogically is closely related to a sheep scab mite group 2 allergen (Fig. 3D). These results indicate that Der f 35 is an MD-2-like lipid binding domain protein that differs genealogically from other well-known group 2 mite allergens.

IgE titers specific to rDer f 35 showed significant correlation with those to rDer f 2, especially in patients who were double positive to rDer f 35 and rDer f 2 (Fig. 5C). Furthermore, rDer f 35 showed positive cross-reactivity with rDer f 2 in 1 of 2 patients and with nPso o 2 in 2 of 2 patients (Fig. 5D and E). IgE-binding epitopes of Der f 2 are considered conformational rather than sequential (32). In fact, the identity and similarity of amino acid sequences for putative IgE-binding epitopes (residues 1-24, 25-29, and 121-123) of Der f 2 (32) are partially conserved in Der f 35: 31% identical and 72% similar in residues 1-24, 62% identical and 92% similar in residues 25-29, and 0% identical and 33% similar in residues 121-123; and two lysine residues (K18 and K77) of Der f 2, which are important for IgE-binding (33), are not conserved in Der f 35. Conversely, beta-strand-rich conformations are conserved among Der f 35, Der f 2, and Pso o 2 in their 3D structure models (Fig. 4).

The evidence that six cysteine residues responsible for three intermolecular disulfide bonds (Cys8-Cys119, Cys21-Cys27, and Cys73-Cys78), which are important for the 3D structure of Der f 2 (34), are conserved in Der f 35 and Pso o 2 (shown in boldface in Fig. 3A) supports the homology of the Der f 35, Der f 2, and Pso o 2 structures. These results suggest that this homology contributes to the cross-reactivity among the allergens. To confirm this hypothesis, analysis of the 3D or tertiary structures of the allergens by circular dichroism spectrum or nuclear magnetic resonance will be necessary.

Although nPso o 2 showed positive cross-reactivity with rDer f 35, rDer f 2 showed limited cross-reactivity with rDer f 35 (Fig. 5D and E). We speculate Der f 35 possesses different sequential epitopes from Der f 2 in some patients, because the primary sequence of Der f 35 is more identical with that of Pso o 2 (50.7%) than Der f 2 (40.8%). Another possibility is some conformational epitopes of rDer f 35 are slightly altered from those of nDer f 35, because the IgE-binding frequency of rDer f 35 (51.4%) was slightly lower than that of nDer f 35 (77.5%) (Fig. 1B and 5C). Further investigations are needed to clarify the cross-reactivity between Der f 35 and Der f 2 by using deletion mutants and native allergens.

In summary, our results suggest that Der f 35 is an additional major HDM allergen that cross-reacts with another major HDM allergen, Der f 2. It will be important to elucidate the clinical significance of Der f 35 in the cross-sensitization of group 2 allergens for the precise diagnosis of HDM, sheep scab mite, and storage mite allergy.

Acknowledgments: We thank Dr. John H. Pruett (United States Department of Agriculture, Kerrville, TX, USA) for kindly providing us with nPso o 2 protein. This work was supported by a Grant-in-Aid for Scientific Research (B), JSPS KAKENHI Grant Number 14360209. The nucleotide sequence reported in this paper has been submitted to DDBJ/GenBank/EBI Data Bank under accession number LC175222. The sequence of Der f 35 has been accepted in the database for systematic allergen nomenclature approved by the World Health Organization and the International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee as the newly registered *D. farinae* allergen, Der f 35.

Conflicts of Interest Statement: The authors declare that they have no conflicts of interest regarding the contents of this article.

Author Contributions: TI and AM performed experiments and analyzed experimental data; TH helped to obtain plasma from HDM patients and healthy volunteers, as well as the clinical data for them; TF, TA, KO, and SK analyzed experimental data: TF, TA, and SK wrote the paper and revised the manuscript; TA, KO ,and SK conceived, arranged, and supervised the study; TF and SK agreed to be accountable for all aspects of the work and to ensure that questions related to the accuracy or integrity of any part of the work are appropriately

investigated and resolved; SK gave final approval of the version of the manuscript to be published. All authors read and approved the manuscript for publication.

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Figure Legends

Figure 1 Allergenome analysis of *Dermatophagoides farinae*. (A) *D. farinae* body extract was fractionated by 2D electrophoresis and visualized by silver staining. After immunoblotting with 40 HDM-allergic patients, the IgE-binding frequency was calculated for each spot, and identities were characterized by ESI-MS for spots that reacted with more than one patient's IgE. Arrows show the spots corresponding to Der f 35 and Der f 2 (a and b). (B) The IgE-binding profiles of spots for nDer f 35 and nDer f 2. Y-axis shows individual patients (N=40) and solid column shows positive reaction for each allergen spot with each patient. IgE-binding frequency for each allergen spot appears at the bottom of each column.

Figure 2 A nucleotide sequence of *der f 35* and its deduced amino acid sequence. A putative signal sequence (no. 1-17) is indicated by a double line.

Figure 3 Similarity analysis and genealogical analysis of Der f 35 in group 2 mite allergens.

(A) Amino acid sequence of Der f 35 is compared to those of mite group 2 isoallergens: Pso o 2 from *Psoroptes ovis* (NCBI accession no. Q965E2); Blo t 2.1010 from *Blomia tropicalis* (AY288141); Sui m 2 from *Suidasia medanensis* (AY497897); Ale o 2 from *Aleuroglyphus ovatus* (EU106610); Lep d 2.0201 from *Lepidoglyphus destructor* (X83875); Gly d 2.0201 from *Glycyphagus domesticus* (AJ272216); Try p 2.0101 from *Tyrophagus putrescentiae* (Y12690); Aca s 2 from *Acarus siro* (EU106609); Der f 2.0102 from *D. farinae* (D10448);

Der p 2.0114 and cDNA clone LY_YIT_DP1663 from *Dermatophagoides pteronyssinus* (AM263560 and EX163551); Eur m 2.0101 from *Euroglyphus maynei* (AF047613); and Der s 2 from *D. siboney* (DQ367850). Six cysteine residues (Cys8, 21, 27, 73, 78, and 119) conserved in Der f 35, which are responsible for three intermolecular disulfide bonds for Der f 2, are shown in boldface. (B) A genealogical tree for Der f 35 and mite group 2 allergen isoforms registered with both the allergen nomenclature database of the WHO/IUIS Allergen Nomenclature Sub-committee and the NCBI GenBank database, and to those of mite group 2 allergens deposited only in the NCBI GenBank database: Pso o 2 from *P. ovis* (NCBI accession no. Q965E2); Blo t 2.1010, 2.0102, and 2.0103 from *B. tropicalis* (AY288141, AY288140, and AY288139); Sui m 2 from *S. medanensis* (AY497897); Ale o 2 from *A. ovatus* (EU106610); Lep d 2.0101, 2.0102, 2.0201 and 2.0202 from *L. destructor* (X81399, AJ487972, X83875, and AJ487973); Gly d 2.0101 and 2.0201 from *G. domesticus* (AJ249864 and AJ272216); Try p 2.0101 from *T. putrescentiae* (Y12690); Aca s 2 from *A. siro* (EU106609); Der p 2.0101, 2.0110, 2.0114, and 2.0115 from *D. pteronyssinus* (AF276239, FM177223, AM263560, and FM177223); Der f 2.0101, 2.0102, 2.0103, 2.0105, 2.0106, 2.0107, 2.0108, 2.0109, 2.0112, and 2.0116 from *D. farinae* (D10447, D10448, D10449, AY066008, AJ862836, AJ862837, AJ862838, DQ185511, AY283288, and EF205129); Eur m 2.0101 and 2.0102 from *E. maynei* (AF047613 and AF047614); and Der s 2 from *D. siboney* (DQ367850).

Figure 4 Predicted 3D structure of (A) Der f 35, (B) Der f 2, and (C) Pso o 2. The 3D structure models were constructed by SWISS-MODEL (Swiss Institute of Bioinformatics) using the structure of Der f 2 as a template.

Figure 5 Purification of rDer f 35 and its allergenic activity. (A) Recombinant TF-fused Der f 35 was produced in Rosetta-gamiTM *Escherichia coli* and the soluble fraction was collected (Sol.). rTF-fused Der f 35 was purified from the soluble fraction by nickel-affinity column (Ni), and TF was removed by thrombin digestion (Dig.). Purified rDer f 35 without TF was prepared by removing TF by anion exchange chromatography (Pur.). MK shows the molecular weight marker. (B) The proteins shown in A were immunostained with pooled plasma from HDM-allergic patients. Molecular weights for positive bands were estimated based on images of molecular weight markers where the areas of PVDF membranes were cut after protein transfer, and then were stained with Coomassie Brilliant Blue. (C) IgE-binding reactivity of rDer f 35 and rDer f 2 in 35 HDM-allergic patients and 4 healthy volunteers. The Y-axis shows IgE titer specific to rDer f 2, and the X-axis shows IgE-titer specific to rDer f 35. Dashed lines show threshold values for positive binding for each allergen (6.1 or 8.8 unit/ml for rDer f 2 or rDer f 35, respectively). Specific IgE titers for healthy volunteers (RAST=0, N=4) and for HDM-allergic patients with RAST=3 (N=4), RAST=4 (N=10), RAST=5 (N=13), or RAST=6 (N=8) are shown as asterisks, circles, triangles, diamonds or

squares, respectively. The patients' IgE values corresponding to solid black symbols were used for ELISA inhibition assay. (D, E) Cross-reactivity of rDer f 35 with nPso o 2 and rDer f 2 in 2 patients who showed positive IgE binding to both rDer f 35 and rDer f 2. (D) One patient, whose RAST score=5, is shown as a black diamond in C and (E) the other patient, whose RAST score=4, is shown as a black triangle in C, were used for the ELISA inhibition assay. The binding between rDer f 35 and a patient's serum IgE was inhibited by serial dilution of rDer f 35 as a positive control, or by nPso o 2, rDer f 2, or Ribonuclease A as a negative control. The value for 0% inhibition was determined without an inhibitor, and 100% inhibition was set as the saturated value of rDer f 35 as a positive control.

Figure 1

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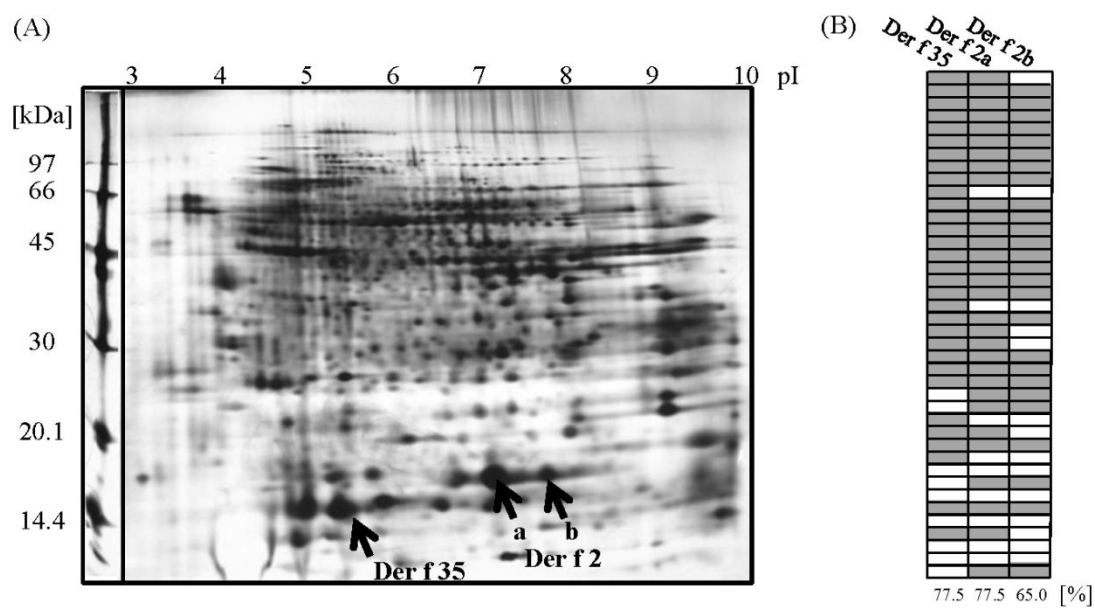


Figure 2

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10 20 30 40 50 60
 ACGCGGGGATCCAATTGTTCTTCTTCCTTTTCTTCTTTGCCAAAATAACGACCGAAAAAC
 70 80 90 100 110 120
 AATCAAAATGATCAAATTTTGTGCATCTTTGCTCTCACTTTTGCTGTGCTTCAGCTGG
M I K F L C I F A L T F A V A S A G
 130 140 150 160 170 180
 CAAAATGAAATTCGTCGACTGCGGACACAAAGAAGTAATTTCACTCGATGTCTCTGGTTG
 K M K F V D C G H K E V I S L D V S G C
 190 200 210 220 230 240
 CGAAGGCGATTATTGTGTTCTTCATAAAGGCAAAACCATCGATTGGATATGAAATGTAA
 E G D Y C V L H K G K T I D L D M K C K
 250 260 270 280 290 300
 ATCAAACCAAGATTGAGAACATCTTAAATTAATCATCTCTGCCGATGTCAATGGAATCGA
 S N Q D S E H L K L I I S A D V N G I E
 310 320 330 340 350 360
 AATCGAAGTTCCCGGATTGATCAAGATGGTTGCCACTATGTCCAATGTCCAATTCACAA
 I E V P G F D Q D G C H Y V Q C P I H K
 370 380 390 400 410 420
 AGGTCAAGATTATGATATCAAATACAGTTACAATGTTCCAGCAGTTCTGCCAAACATCAA
 G Q D Y D I K Y S Y N V P A V L P N I K
 430 440 450 460 470 480
 AGGCACCCCTACAGCTAAAGTCATCGGTGATAATGGTTTAGTTGGCTGTTTGAAACTTAA
 G T L T A K V I G D N G L V G C L K L N
 490 500 510 520 530 540
 TGGTGAAATCGCCGATTAAATATTTCTGTAATTAAATTTTTATTGGTCATTTCATAAAA
 G E I A D *
 550
 GTGATTATTGATG

Figure 3

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A

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Der f 35 : -MIKFLCIFALTFAVASAGMKFVDCGHKEVISLDVSG-CEG-DYCVLHKGKTIDLDMKCKSNQDSEHLKLIISADVNGIEI
Pso o 2 : -.M.T.VVL.I.L..V...V...Q...KG..E..E.E.-.S.-...I...KL..AISVT...AN...D.V..I..VQ.
Blo t 2 : MFKFICLALLPFWSR.A..DV..T..A.G..T...L..-S.-.H.II...SFT.KTFFIA...K.EIK...IM.N..V
Sui m 2 : ---MKFI.L.MFV...A..E...Q...G..KK.L..D-S.-...II...KLSMEADFVA...PTAVIK...K...V.L
Ale o 2 : --MLKFAVL..FV..VA..DF..S...G..TK..L.D-S.-.SH..I...EFKMSADFVA..S..KVEIK.I.K...L..
Lep d 2 : --MMKFIALFALV.....T.K...G..TE..I..-S.-.T..I.R.QKMT..A.FAA...TNKVTIKVL.K.A.TT.
Gly d 2 : -----K...KG..TE..ITD-S.-.F..I.R..PLT.EA.FAA...TTKATIKVL.K.A.TP.
Tyr p 2 : ---MKFL.LFALV...A..QV..T...K..IA.VA.D.-...L..I..S.PVHVIAEFTA...TCKIEVKVTGQL..L.V
Aca s 2 : --MLQNVCLP.FV....TPIPYT...NH.LSK..ANLH.A.-.L.TI...ELK..AQFTV...TA.V.IQLT.SMG...
Der f 2 : -.SKILCLS.LV.AVV.DQVDVK..ANN.IKKVM.D.-.H.S.P.II.R..PFT.EALFDA..NTKTA.IE.K.SLD.L..
Der p 2 : -.MYKILCLS.LV.AVA.DQVDVK..ANH.IKKVL.P.-.H.SEP.II.R..PFQ.EALFEA..NTKNA.IE.K.SID.L.V
DP1663 : -.M.....I...N..K.....-.....N...V.....V.....
Eur m 2 : --MYKILCLS.LV.AVA.DQVDIK..ANH.IKKVM.P.-.K.SEP..I.R.TAFQ.EAVFDA..N.NAA.IE.K.TID.V..
Der s 2 : -.SKILCLS.LV.AVV.DQVDVK..ANN.IKKVM.D.-.H.S.P.II.R..PFT.EALFDA..NTKTA.IE.K.NID.L.V
      .      **.: *: :.      * * . * :*: . :      **: : : : : :

Der f 35 : EVPGFDQDGCCHYVQCPIHKGQDYDIKYSYNVPAVLP-NIKGTLTAKVIGDNLVGCCLKLNGEAD-
Pso o 2 : ....V.H.....K...K...HF.V..T.SI..I..-TT.AKII..I...K..G..IVI...Q.- 50.7%/80.6%
Blo t 2 : P...V.K...KHTT..LK...K.ELD..LII.TI..-L.TVT..SLV..H.V.A.G.V.T.VV.- 49.7%/80.4%
Sui m 2 : Q...IETN...HMK..LV...S.QF..DLVI.QI..-V.ADV..SLT.AH..LA.GTVH..VQN- 48.3%/77.6%
Ale o 2 : P...VESN..NHMK..VT...K.TFN.GISI.KL..-ALV...L..H.VLA..IVD...KN- 48.3%/79.7%
Lep d 2 : Q...LET...KVLK...K..EAL.FN.GMTI..IT.-K..ADV..ELV..H.VMA.GTIH.QVE- 46.9%/79.0%
Gly d 2 : Q...LET...KF.K...K..DPI.F..TTT...I..-KV.AEV..ELV..H.VLA.GRFGQVE- 39.9%/68.5%
Tyr p 2 : PI..IET...KVLK..LK..TK.TMN..V...S.V.-TVVKLLAT.EH.VLA.GAV.TDVKP- 42.7%/78.3%
Aca s 2 : P...VGT...K.IK..LV..EKKTFP.T.L..KI..-SVT.DVK.VFT..H.PL...FH.K.V.- 43.8%/72.9%
Der f 2 : D...I.TNA..FMK..LV...Q...TW...KIA.KSENVVV.V.L...VLA.AIATHGKIRD 40.8%/77.6%
Der p 2 : D...I.PNA...K..LV...Q...TW...KIA.KSENVVV.V..L...VLA.AIATHAKIRD 40.8%/79.6%
DP1663 : .....I...V...Q..V...TI.S...-AN...T..Q..L...Q..... 86.0%/97.9%
Eur m 2 : D...I.NNL..FMK..LV...E...TW...RIA.KSENVVV.V.LL...VLA.AIATHAKIRD 40.4%/78.1%
Der s 2 : D...I.TNA..FIK..LV...Q..A..TW...KIA.KSENVVV.V.L...VLA.AIATHAKIRD 38.8%/77.6%
      :*: : *: :*: **      * :*: * . .      * :* . *

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B

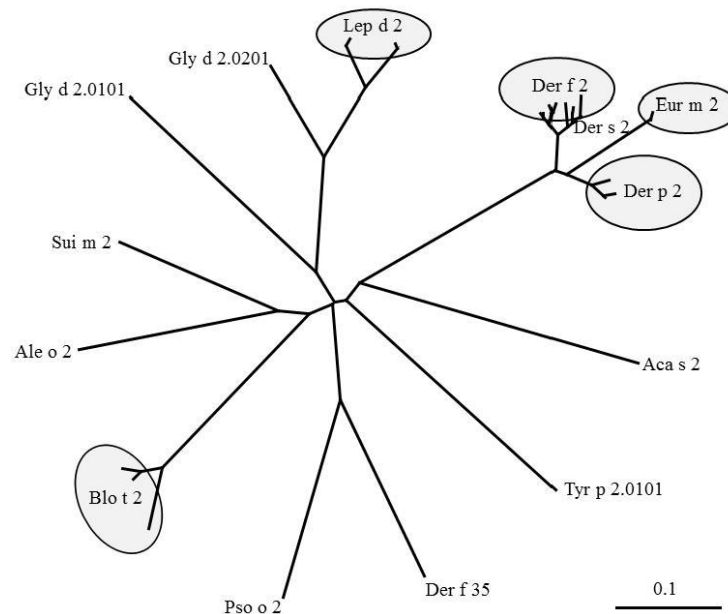


Figure 4

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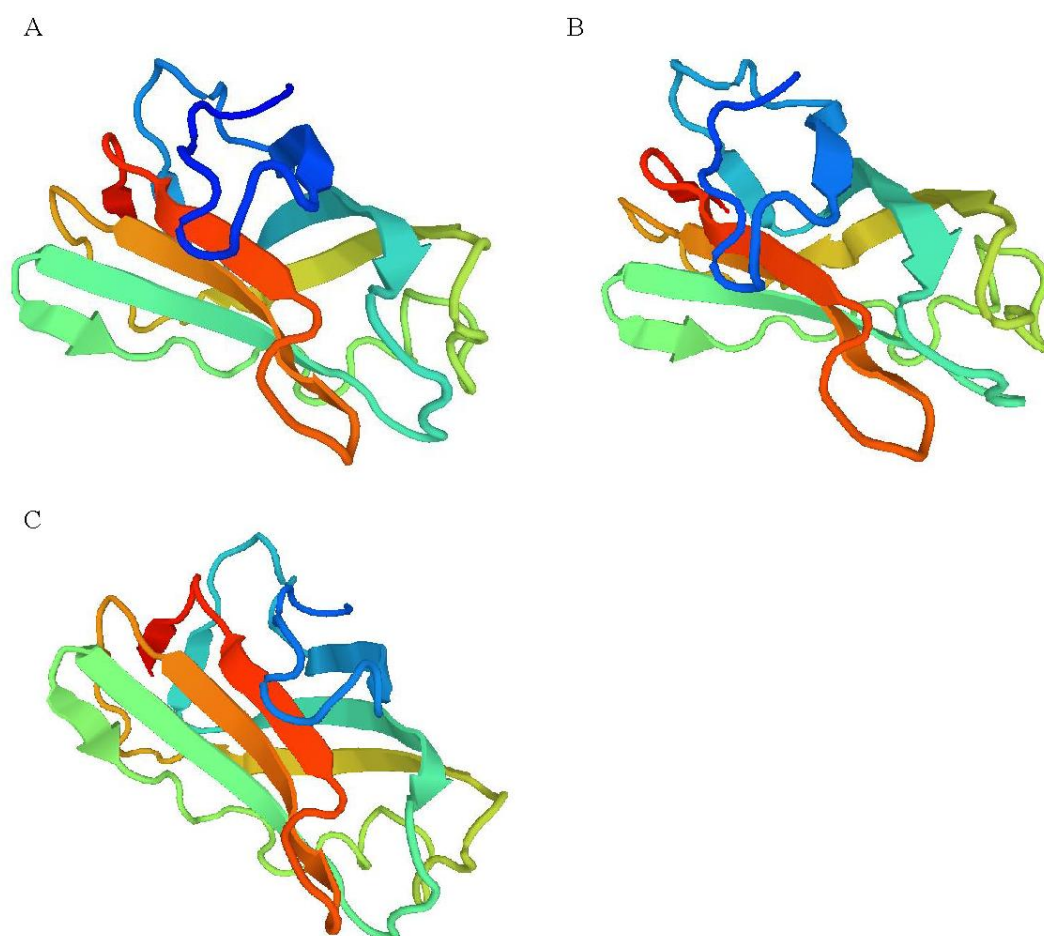


Figure 5

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