

Proteome, Allergenome, and Novel Allergens of House Dust Mite, *Dermatophagoides farinae*

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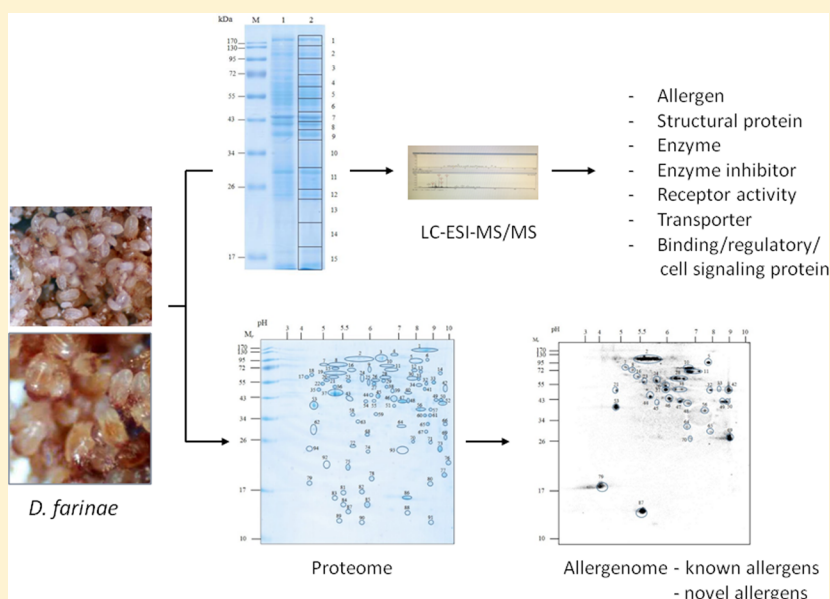
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Supporting Information



Photographs of *D. farinae* mites courtesy of Siriraj Dust Mite Center for Services & Research (SDMC). Copyright 2016.

ABSTRACT: *Dermatophagoides farinae* mite is a predominant source of indoor allergens causing high incidence of allergy worldwide. People with different genetic background respond differently to the mite components, and thus the component-resolved diagnosis (CRD) is preferred to the conventional allergy test based on crude mite extract. In this study, proteome and culprit components in the *D. farinae* whole body extract that sensitized the allergic patients were studied by using SDS-PAGE (1DE) and 2DE-IgE immunoblotting followed by LC-MS/MS and database search for protein identification. From the 1DE, the mite extract revealed 105 proteins that could be classified into seven functionally different groups: allergens, structural components, enzymes, enzyme inhibitor, receptor proteins, transporters, and binding/regulatory/cell signaling proteins. From the 2DE, the mite extract produced 94 spots; 63 were bound by IgE in sera of 20 *D. farinae* allergic patients. One more protein that was not revealed by the 2DE and protein staining reacted with IgE in 2 allergic patients. Proteins in 40 spots could be identified as 35 different types. Three of them reacted to IgE of >50% of the allergic patients, and hence they are major allergens: tropomyosin or Der f 10 (75%), aconitate hydratase (70%), and one uncharacterized protein (55%). Aconitate hydratase is a novel *D. farinae* major allergen unraveled in this study. Several mite minor allergens that have never been previously reported are also identified. The data have clinical applications in the component-resolved diagnosis for tailor-designed allergen-specific immunotherapy.

KEYWORDS: allergen, allergenome, IgE, *Dermatophagoides farinae*, proteome, LC-ESI-MS/MS

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■ INTRODUCTION

Allergic diseases that are IgE-mediated (type I) hypersensitivity trigger a wide array of clinical manifestations with different severity from sneezing, stuffy/runny nose, itchy/red/swollen/watery eyes (conjunctivitis), dry and irritating roof of the mouth and throat, urticaria, eczema, nausea, diarrhea, wheezing, and asthma to life-threatening anaphylactic shock.¹ The immunity-gone-wrong entities are posing a global health concern because their prevalence has increased dramatically over the past few decades due to air pollution and global climate change, which enhance flourish and wide distribution of a multitude of allergens. Approximately 30 to 40% of the world population are affected.² The high morbidity rate of the allergic diseases not only demands increased health care services, which causes an economic burden, but also impairs the overall and individual population capacity. A large proportion of the allergic patients are sensitized by allergens that prevailed in the environments, particularly their dwellings and work places, which are arbitrarily called indoor allergens. Among the indoor allergens, Acarid or house dust mites (HDM) of the genus *Dermatophagoides* including *D. pteronyssinus* (Dp) and *D. farinae* (Df) contribute important sources in most geographical regions as 60 to 80% of atopic patients are allergic to them.^{3,4} The allergens may be from the mite bodies or their droppings.⁵

Currently, allergen-specific immunotherapy is the only disease modifying/curative option of allergy.⁶ Mostly, crude extracts that contain not only the allergens to which the patients are allergic but also other unidentified and nonallergenic components are given repeatedly to the patients either parenterally or mucosally over an extended period of time. The aim is to cause a deviation of the patients' immune response from the maladaptive Th2 to the nonpathogenic Th1 or regulatory T cell responses. The protocol received rather low patients' compliance as not only is it time-demanding and prolonged but also the treated patients are at risk to adverse reactions, which may be as serious as life-threatening anaphylaxis. In 1999, a concept of "component-resolved diagnosis (CRD)" was introduced,⁷ and ever since it has received increasing interest in the clinical practice of allergy. On the basis of the CRD, it is advised that a battery of purified allergens should be used for determining IgE reactivity of individual allergic patients instead of the crude extracts to identify the component(s) that the subjects are really allergic to. This would allow a proper design of a tailoring allergen-specific immunotherapy for improving the treatment efficacy.⁸ To do so, it is needed that a comprehensive array of the allergens, both major and minor, from individual allergenic sources should be unraveled. Moreover, it is commonly known that allergic patients of different genetic background have different IgE reactive profiles to a particular causative agent including house dust mites.^{9–11} Therefore, in this study, proteome and allergenome of the whole body (crude) extract of pure cultured *Dermatophagoides farinae* (the predominant species in Thailand) and serum IgE reactivity of individual mite components were determined by using sera of *D. farinae* allergic subjects to pave the way to the more comprehensibility on the mite allergens and a higher effective component-resolved immunotherapy (CRIT)⁸ for the mite allergy. Besides the unique specific IgE reactive profiles of the Thai patients reported herein, we also revealed several novel major and minor allergens of the particular mite species.

■ MATERIALS AND METHODS

Serum Samples

This work received approval from the Ethic Committee of Faculty of Siriraj Hospital, Mahidol University (No. Si224/2010).

Twenty patients (P1–P20) with allergic rhinitis (AR) who seek treatment at the Allergy Clinic of Siriraj Hospital, Mahidol University were enrolled. The patients were positive by skin prick test to the Df extract. Five healthy volunteers without any underlying disease and were skin-prick-test-negative to house dust mite (HDM) extracts and other common allergens in Thailand served as normal controls (N1–N5). Levels of serum IgE specific to Df of all subjects were measured by using ImmunoCAP system (ImmunoCAP 100E Automate, Sweden). Background information on all participants is shown in [Supplementary Table S-1](#).

Mite Rearing

Purified *D. farinae* mites (>99% purity) used in this study was from the Siriraj House Dust Mite Center for Service and Research, Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. The mites were fed with a locally made culture medium (MU Siriraj Mite feed formula), which was a mixture of rat chow and wheat germ. The component analysis of the culture medium was protein 29.4%, fat 6.34%, fiber 2.63%, ash 5.56%, calcium 0.38%, and phosphorus 0.93%. To start a new *D. farinae* culture, we seeded 1 g of the mite stock into individual mite culture flasks containing 5 g of the medium. All culture flasks were kept in specially designed cabinets in the mite-rearing room. Temperature was controlled by air-conditioner, which automatically turned on 12 h per day to keep the ambient at 25–30 °C. Saturated sodium chloride was placed at the bottom of each chamber for keeping the humidity between 75 and 80%. Mites were observed for growth twice weekly. After 4–6 weeks (at which time the insects reached their full development), the mites were harvested. Usually the mite preparation contained not more than 1% contamination of the spent mite medium. The spent medium typically comprised dead mites of all developing stages, skin debris, and the culture medium.

Preparation of *D. farinae* Whole Body Extract

One gram of 4-week old laboratory cultured Df was homogenized in 4 mL of 0.01 M of phosphate-buffered saline, pH 7.4 (PBS) by using sonicator (LABSONICP, France) at 30% amplitude with 0.5 cycles for 15 min on ice. The homogenate was centrifuged at 12000g, 4 °C for 20 min; the supernatant was collected and protein concentration in the crude Df extract was determined by Bradford's method, and the concentration was ~9 mg/mL. The preparation was kept in small aliquots at –80 °C until used.

Characterization of *D. farinae* proteome by SDS-PAGE and LC–MS/MS

Ten µg of Df whole body extract was loaded into individual wells in 4% stacking gel. After electrophoresis in 12.5% polyacrylamide gel using Mini-PROTEAN 3 Cell (BioRad), proteins in the gel were stained by Coomassie Brilliant Blue G-250 dye (CBB). The stained gel was cut horizontally into 15 pieces. Gel pieces were destained prior to tryptic digestion. Acetonitrile (50%) in 50 mM NH₄HCO₃ was added to every gel piece for destaining. After the gel pieces were colorless, all solution was removed. To each gel piece was added 10 mM dithiothreitol in 50 mM NH₄HCO₃ and incubated for 15 min at 60 °C for reducing the proteins. After cooling to room temperature, a 55 mM iodoacetamide in 50 mM NH₄HCO₃ was added to alkylate the proteins for 30 min at room temperature in the dark. All solution was removed from the gel pieces, and acetonitrile was added for gel dehydration. Proteomics-grade trypsin purified from porcine pancreas (Sigma-Aldrich, MO) at 0.01 mg/mL concentration was added

to digest proteins, and the gel pieces were incubated at 37 °C overnight. The peptides were extracted by adding acetonitrile to 50% final concentration and subsequently concentrated using concentrator.

Each tryptic-digested fraction was resuspended in 0.1% formic acid containing 2% acetonitrile and analyzed by an Ultimate 3000 nano-LC system (Dionex; Surrey, U.K.) coupled to MicroToF Q II mass spectrometer (Bruker; Bremen, Germany). The separation was done at a flow rate of 300 nL/min under 45 min gradient. The reverse-phase chromatography column was Acclaim PepMap RSLC 75 μm $\times$ 15 cm nanoviper C18, 2 μm particle sizes, 100 Å pore sizes (Thermo Scientific, Waltham, MA). Mobile phase A was 2% (v/v) acetonitrile, 0.1% (v/v) formic acid in HPLC-grade water and mobile phase B was 0.1% (v/v) formic acid in HPLC grade acetonitrile. The gradient started with 10 min 2–10% B, followed by 33 min 10–40% B, ramped rapidly 1 min 40–95% B, and maintained at 95% for 1 min. The eluent was sprayed and ionized in the nanoelectrospray source of the mass spectrometer. Data were acquired using Hystar software.

The survey scan mode covered the mass range of m/z 400–2000. The three most abundant precursors were selected to fragment for 3 s. The collision offset was determined within the collision energy profile, where collision offset values are entered for ions based on their m/z and charge. This value was typically between 25 and 40 V for doubly, triply, and quadruply charged ions with m/z of 400–2000. The MS/MS spectra covered the mass range of m/z 50–1500.

The mass spectrometric data was smoothed, centroided, and converted into a mascot generic file (.mgf) using data analysis software version 4.0. The spectra were then searched against the nonredundant NCBI database using MASCOT 2.2 (Matrix Science, USA). The NCBI nr Metazoa database 2014-12-11, which contained 7 457 551 sequence entries, was used to search all MS/MS data. Trypsin was set as enzyme and one possible missed cleavage was allowed at 1. The mass tolerances for precursor and fragment ions were set to 1.2 and 0.6 Da, respectively. The peptide charge was selected as 2+, 3+, and 4+. Oxidation of methionine and carbamidomethylation of cysteines were set as variable modifications. Only peptides with 95% confidence were reported in this study for reducing false-positive identification.

Two-Dimensional Gel Electrophoresis (2DE)

The mite crude extract was cleaned with 2D-cleanup kit (GE Healthcare Bioscience, U.K.) to eliminate detergents and any contaminants. Each IPG strip (pI 3–10; GE Healthcare) was placed into a strip holder (Ettan IPG Phor Electrofocusing System; GE Healthcare) containing 75 μg of *D. farinae* proteins, and 600 μL of PlusOne DryStrip Cover Fluid (GE Healthcare) was overlaid onto each IPG strip to prevent evaporation and urea crystallization. All IPG strips were rehydrated at 20 °C for 12 h. The first dimensional electrophoresis was performed at 300 V for 30 min, 1000 V for 30 min, and 5000 V for 72 min. IPG strips were then subjected to two equilibration steps: first, in 10 mL of SDS-equilibration buffer containing 100 mg dithiothreitol (DTT) at 25 °C for 15 min and second, in 10 mL of the equilibration buffer containing 250 mg iodoacetamide (IAA) for 15 min also. The strips were washed with electrode buffer and placed onto a gel cast in the Hoefer SE 260 system (Amersham Bioscience, U.K.). The electro-focused proteins were separated according to their approximate molecular weights (second dimension) by SDS-PAGE using 4% stacking and 12.5% polyacrylamide gels. Electrophoresis was carried out at 10 mA

per gel during the first 15 min and then 20 mA per gel until the tracking dye reached the lower gel edge. Then, one gel was stained by CBB dye, while the others were subjected further to 2DE-IgE immunoblotting.

2DE IgE-Immunoblotting

The 2DE-separated-Df crude extract was transblotted from the gels onto nitrocellulose membranes (NC), and the empty sites on the NC were blocked by using 5% skim milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T). After washing with the TBS-T, membranes were placed individually in sera of the allergic patients and the normal controls (diluted 1:20 in TBS-T) and kept at 4 °C overnight. Antihuman IgE-horseradish peroxidase (HRP) conjugate (1:2500) (KPL, USA) was used as secondary antibody, and the membranes were kept at 25 °C for 1 h. The IgE-reactive mite components were revealed by placing the NC in a solution of LuminataTM Classico western HRP substrate (Millipore Corporation, USA) at 25 °C for 5 min in darkness. The IgE bound spots were visualized by using ImageQuant LAS 4010 (GE Healthcare). The Df protein spots on the CBB-stained 2DE gel matched with the patient IgE-bound spots, which did not react to the normal sera that were selected for identification. Gel plugs containing the spots of interest were excised and the proteins in the plugs were digested with trypsin before subjecting the peptides to LC–ESI–MS/MS and database search.

RESULTS

Specific IgE Levels in Sera of Allergic Patients and Controls

The age range of the case subjects was 21–59 years, averaged 25.75 years, which was not different statistically from that of the normal controls (averaged 24.6 years). All cases had allergic rhinitis and were allergic to Df by skin prick test. The levels of Df-specific IgE in the patient's sera ranged from 7.06 to >100 kAU/L, whereas those of the controls were negligible (0.01 to 0.02 kAU/L). Hence, all case subjects were regarded as Df-allergic based on both tests.

Proteome of the *D. farinae* Extract

SDS-PAGE-separated pattern of the Df crude extract under a reducing condition after staining with CBB is shown in Figure 1. The molecular masses of the proteins ranged from ~10 to >170 kDa. The results of the NCBI nr database search of the in-gel trypsin digested Df proteins contained in the 15 1DE gel pieces after LC–ESI–MS/MS are shown in the Supplementary Table S-2. A total of 105 proteins were identified. Among all of the gel pieces, the gel piece no. 1 contained as many as 24 proteins, while the gel piece no. 12 did not contain any protein. Several of the 105 were unknown proteins. The known proteins could be classified into seven functionally different groups (Table 1) including the previously known mite and other allergens (listed in group 1 of Table 1), structural/cytoskeletal proteins, enzymes, enzyme inhibitor, receptor proteins, transporters, and binding/regulatory/cell signaling proteins. Many proteins in the high-molecular-weight gel slices might degrade during sample collection process. Therefore, some high-molecular-weight proteins appeared at much lower molecular weight than the expected gel migration.

Allergenome of the *D. farinae*

A total of 94 isolated protein spots of pI 3–10 and molecular size range of 26–170 kDa were revealed by the 2DE (Figure 2). An example of the 2DE-IgE immunoblotting results is shown in Figure 3. Among the 94 protein spots, 63 were recognized by IgE in sera of the 20 Df allergic patients, while the control sera did not

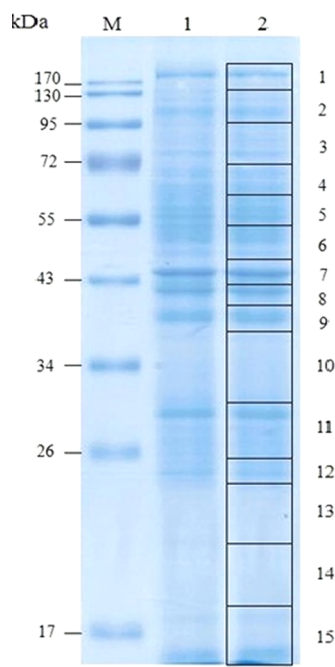


Figure 1. SDS-PAGE-separated pattern of *D. farinae* crude extract stained with CBB dye. Lanes (from left to right): M, protein molecular weight standard; 1 and 2, SDS-PAGE-separated components of *D. farinae* crude extract. Numbers at the left are protein molecular masses in kilodaltons. Numbers at the right are gel pieces containing the *D. farinae* proteins that were subjected to protein identification by LC–ESI–MS/MS and database search.

react to any of the spots (data not shown). There was one more spot (no. 95 in Figure 3) that was not seen in the CBB-stained 2DE gel but was recognized by IgE in serum samples of two Df allergic subjects. (This protein was not identified.) Percent allergenicity of the proteins in the 63 spots is shown in Table 2. Of the 64 spots, proteins in 24 spots were unknown protein function, while those in the remaining 40 spots could be designated to 35 different protein functions. Three of them were bound by IgE in sera of >50% of the allergic patients; therefore, they are regarded as major Df allergens. These are tropomyosin or Der f 10 (75%), aconitate hydratase (70%), and one unknown protein (55%). The aconitate hydratase has not been previously reported as the mite allergen, so this protein is a novel allergen. Five proteins, that is, apolipoprotein or Der f 14, arginine kinase or Der f 20, chitin-binding protein or Der f 18, heat shock protein-70 or Der f 28, and triosephosphate isomerase or Der f 25, bound to IgE of 50% of this set of DF allergic patients. The remaining proteins were minor allergens as they reacted to IgE of <50% of the allergic patients. Among the minor allergens, 17 functionally different proteins are novel Df allergens indicated by this study including deoxyribonuclease-2-beta, alpha-enolase, fructose biphosphate aldolase, glyceraldehydes 3-phosphate dehydrogenase, phosphoenolpyruvate carboxylase, NADH dehydrogenase, cytochrome-*b*, aldehyde dehydrogenase or Alt a 10, actins, heat shock protein-60, glutamate dehydrogenase, ATP synthetase, citrate synthase, phosphoglycerate kinase, serine hydroxymethyl transferase, transketolase-like protein, and hexoseaminidase.

DISCUSSION

In the present study, whole body extract of the laboratory cultured *D. farinae* was characterized by 1DE and 2DE. Mite-rearing

Table 1. Classification of the *D. farinae* Proteins Identified by 1DE and LC–ESI–MS/MS Based on Their Functions

group	protein func- tion	protein name
1.	allergen	apolipoprotein (Der f 14/Mag3/vitellogenin), arginine kinase (Der f 20), chitinase (98 kDa HDM allergen/glycosylated-98/105 kDa), cysteine proteinase (Der f 15), Der f Alt a 10, elongation factor-1- α (Der f 17/calcium binding protein), ferritin (Der f 30), enolase- α , fructose biphosphate aldolase, group 2 allergen (<i>Euroglyphus maynei</i> ; Eur m 2/Der f 2/Niemann-Pick type C2), glutathione-S-transferase (Der f 8), NADH dehydrogenase, paramyosin (Der f 11), sarcoplasmic calcium-binding protein (shrimp allergen), serpin peptidase inhibitor (Der f 27), serum albumin (Bos d 6), triose phosphate isomerase (Der f 25), tropomyosin (Der f 10), and trypsin (Der f 3)
2.	structural protein	actins, chain B structure of Phactr1 Rpel-2 domain, and intermediate filament
3.	enzyme	aldolases including fructose biphosphate aldolase, alpha-enolase, AMP deaminase 3-like isoform X2, arginine kinase, ATPases, ATP synthases, beta-galactosidase-1-like protein 3, calcium transporting ATP synthase α and β subunits, carbonic anhydrase, chitinase, cysteine proteinase, cytochrome <i>b</i> -245 (membrane-bound oxidase), glutamine synthetase-2, glutathione-S-transferase, kinase, leucine aminopeptidase, NADH dehydrogenase, pappalysin-1-like, serpin B4 isoform-1 (peptidase), triose phosphate isomerase, and trypsin
4.	enzyme in- hibitor	serpin peptidase inhibitor
5.	receptor ac- tivity	exocyst complex component-1, glutamate receptor-1-like, POTE ankyrin domain family, and zinc finger protein 182 isoform XI
6.	transporter	peroxisome biogenesis factor, and transferrin
7.	binding/reg- ulatory/ cell signal- ing protein	calmodulin-like, heat shock protein-60, interphotoreceptor retinol-binding protein, elongation factor-1- α , and sarcoplasmic calcium-binding protein

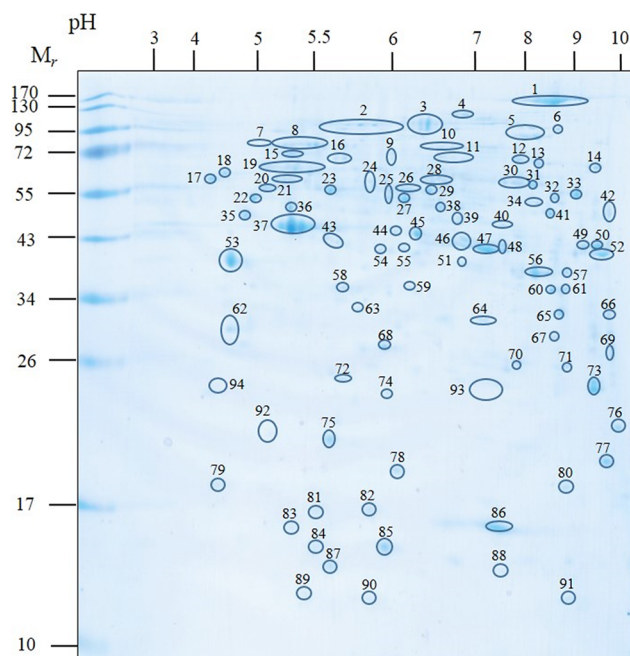


Figure 2. Coomassie Brilliant Blue G-250-stained proteins of *D. farinae* crude extract after 2D gel electrophoresis.

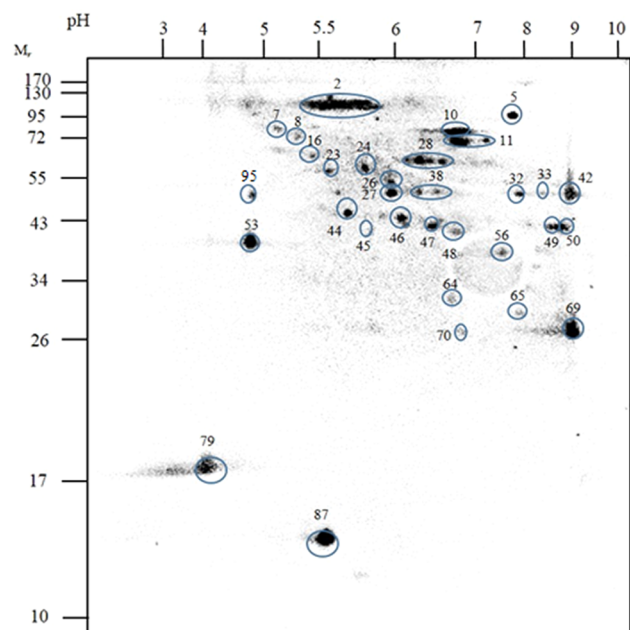


Figure 3. Representative results of 2DE-IgE immunoblotting. The *D. farinae* extract was separated by 2DE and probed with serum of allergic patient no. P8. IgE in serum of this patient reacted to 31 protein spots of the mite extract.

condition in our system is under the temperature in range of 25–30 °C with 75–80% humidity. With the indicated ambient, mite population becomes mature in approximately 4–6 weeks, which is rather shorter when compared with those of the conditions in the western countries.^{12–15} It has been documented that maximum growth phase provides not only the highest amount of live mites but also the allergen concentration,^{16,17} and hence we use mature mites for source of material for allergenic extract.

SDS-PAGE-separated crude extract of purified Df revealed the mite proteins ranged in molecular masses from ~10 to >170 kDa.

The protein banding pattern in the 1DE gel was, more or less, similar to the data previously reported, although different sources and perhaps different ages of the mites were used.^{18,19} One hundred and five orthologous proteins of the NCBI database retrieved on 2014-12-11 contained peptides matched with the *D. farinae* peptides derived from the 1DE gel. It was noticed that the intensities of the CBB-stained bands and the sizes of the 15 gel pieces did not correlate with the numbers of proteins contained in them. For examples, gel piece no. 1 contained as many as 24 proteins (MW > 170 kDa) (Supplementary Table S-2), while gel piece no. 7 (MW ~45 kDa) which was similar in gel size to the no. 1 but with higher protein band intensity revealed only one protein (Der f 17). Gel piece no. 3 stained faintly but contained as many as 16 proteins. Most of the previously reported mite allergens are in the MW range of 60 to 14 kDa,²⁰ with the exceptions of chitinases (Der f 15 and Der f 18),²¹ alpha-actinin (90 kDa; Der f 24),²⁰ and two minor allergens (one was MW 83 to 80 kDa, which was similar to Der f 14 or Mag3, and another was 101 to 95 kDa, which is most likely a protein of the Chitinase family with a glycanohydrolytic activity essential for mite development).²² Gel pieces 1, 2, and 3 (MW 72 to <170 kDa) contained Df proteins that their peptides matched with several of the previously reported high MW allergens of the mites and other organisms, such as group 14 allergen (apolipoprotein/Mag3), serum albumin (Bos d 6), and Chitinase (Der f 15; glycosylated 98 kDa or 63 kDa polypeptide).^{23,24} Nevertheless, alpha-actinin (actin binding protein of the spectrin gene superfamily) was not identified in the three gel pieces, although peptides of actins have been revealed in the gel piece no. 1. The allergen was revealed later in 2DE-IgE immunoblotting of spot no. 9 of Table 2, in which it was reacted by IgE of five allergic patients. (The spot no. 9 was located at about 65 to 70 kDa in Figure 2.) Groups 1 (cysteine protease), 2 (Niemann-Pick type C2), and 3 (trypsin/serine protease) allergens were found in gel pieces 13–15 with molecular size range of <26 to ~10 kDa, which conformed to the previous information.^{25–30} From the 1DE proteomic data, it can be concluded that not only are our findings in agreement with the annotated mite allergen archive but also several functionally different groups of the Df proteins are revealed, many of which have been known, some have not been previously reported, and several are to be characterized.

From the 2DE, 94 Df protein spots appeared on the CBB stained gel and 63 spots were bound by the IgE in sera of the Df allergic patients indicating that the proteins in the latter are allergens. Nevertheless, proteins in 23 of the 63 spots could not be identified due to limitation of the orthologous proteins currently available on the database. One more protein spot that was not revealed by the 2DE and staining reacted with IgE in 2 allergic patient sera. This protein was not identified. The less abundant components in the whole Df body extract (which might be allergens) may be missed out by the 2DE by two possible reasons; one is the abundant protein masking and another is the limited loading capacity of each IPG strip. In this study, some of the Df proteins that could be detected by the 1DE proteomics could not be revealed by the 2DE method. One of them is the group 1 major allergen (cysteine proteinase), which was detectable in gel piece no. 13 of the 1DE but not by the 2DE-IgE immunoblotting. The group 1 allergen is known to be more abundant in the mite feces than in the pure mite bodies;²³ thus the group 1 missing out by the 2DE-IgE immunoblotting should be due to the fact that the allergen amount in the 2DE-separated extract was lower than the lowest detectable limit of the assay. Presently, more than 30 allergens of *D. farinae* have been

Table 2. Allergenic Components of *D. farinae* Identified by 2-DE IgE-Immunoblotting

spot no.	<i>D. farinae</i> protein	patient no.																				frequency	% allergenicity
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
53	tropomyosin (Der f 10)		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	15	75
5	aconitate hydratase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	14	70
2	uncharacterized protein		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	11	55
11	apolipoprotein (Der f 14)			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	10	50
47	arginine kinase (Der f 20)			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	10	50
24	chitin-binding protein/chitinase (Der f 18)		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	10	50
7, 8	heat shock protein-70 (Der f 28)		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	10	50
34, 70	tri- α -phosphate isomerase (Der f 25)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	10	50
3	deoxyribonuclease-2-beta		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	9	45
27	uncharacterized protein		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	9	45
25, 38	alpha-enolase		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	8	40
87	Der f 5 allergen	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	8	40
45	fructose-bisphosphate aldolase		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	8	40
56	glyceraldehyde 3-phosphate dehydrogenase		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	8	40
14, 41, 88	NADH dehydrogenase subunit 6	+	+			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	8	40
86	Niemann-Pick type C2 (NPC2) family protein (Der f 2)					+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	8	40
54	translation elongation factor-2 (Der f 26)					+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	8	40
49	uncharacterized protein					+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	8	40
28	alpha-amylase (Der f 4)					+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	7	35
62	cytochrome <i>b</i> (mitochondrion)					+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	7	35
42	elongation factor calcium-binding protein (Der f 17)		+			+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	7	35
12	phosphoenolpyruvate carboxykinase					+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	7	35
46	uncharacterized protein					+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	7	35
93	uncharacterized protein					+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	6	30
37	actin	+	+	+					+	+	+	+	+	+	+	+	+	+	+	+	+	6	30
23	aldehyde dehydrogenase (Alt a 10)		+	+					+	+	+	+	+	+	+	+	+	+	+	+	+	6	30
32	serine hydroxymethyltransferase		+						+	+	+	+	+	+	+	+	+	+	+	+	+	6	30
1	uncharacterized protein					+		+	+	+	+	+	+	+	+	+	+	+	+	+	+	6	30
15	uncharacterized protein			+					+	+	+	+	+	+	+	+	+	+	+	+	+	6	30
44	uncharacterized protein								+	+	+	+	+	+	+	+	+	+	+	+	+	6	30
64	uncharacterized protein								+	+	+	+	+	+	+	+	+	+	+	+	+	6	30
94	uncharacterized protein					+			+	+	+	+	+	+	+	+	+	+	+	+	+	6	30
9	alpha-actinin (Der f 24)					+			+	+	+	+	+	+	+	+	+	+	+	+	+	5	25
26	gelsolin (Der f 16)		+			+			+	+	+	+	+	+	+	+	+	+	+	+	+	5	25
30	glutamate dehydrogenase		+			+			+	+	+	+	+	+	+	+	+	+	+	+	+	5	25
19	heat shock protein-60	+				+			+	+	+	+	+	+	+	+	+	+	+	+	+	5	25
10	uncharacterized protein		+			+			+	+	+	+	+	+	+	+	+	+	+	+	+	5	25
33	elongation factor 1-alpha		+			+			+	+	+	+	+	+	+	+	+	+	+	+	+	4	20
35	uncharacterized protein		+			+			+	+	+	+	+	+	+	+	+	+	+	+	+	4	20
48	uncharacterized protein								+	+	+	+	+	+	+	+	+	+	+	+	+	4	20
79	uncharacterized protein		+						+	+	+	+	+	+	+	+	+	+	+	+	+	4	20
20	alpha tubulin (Der f 33)		+						+	+	+	+	+	+	+	+	+	+	+	+	+	3	15

Table 2. continued

spot no.	<i>D. farinae</i> protein	patient no.																				frequency	% allergenicity
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
13	transketolase-like protein	+								+									+		3	15	
16	uncharacterized protein				+			+												+	3	15	
43	uncharacterized protein												+					+			3	15	
55	uncharacterized protein		+				+		+												3	15	
58	uncharacterized protein			+				+	+						+				+		3	15	
65	uncharacterized protein					+		+	+												3	15	
85	uncharacterized protein							+	+			+					+				3	15	
22	ATP synthase subunit beta		+							+											2	10	
95 ^a	uncharacterized protein				+			+													2	10	
40	citrate synthase			+				+										+			2	10	
39	phosphoglycerate kinase					+				+											2	10	
36	beta actin													+							1	5	
17	cofilin (Der f 31)														+						1	5	
59	hexosaminidase								+							+					1	5	
66	uncharacterized protein								+												1	5	
69	uncharacterized protein							+													1	5	
89	uncharacterized protein													+							1	5	

^aThis protein spot was not present in the 2DE gel stained by CBB dye but was revealed in the 2DE-IgE immunoblotting by serum samples of two Df allergic patients.

characterized.²⁰ From combined results of 1DE and 2DE-IgE immunoblotting of this study, 20 of the previously reported Df allergens have been found in the Df extract including: Der f 1 (cysteine proteinase), Der f 2 (NPC2 family), Der f 3 (trypsin/serine protease), Der f 4 (α -amylase), Der f 5 (NCBI no. 6954; biochemical function is unknown), Der f 10 (tropomyosin), Der f 11 (paramyosin), Der f 14 (apolipoprotein/Mag3), Der f 16 (gelsolin), Der f 17 (elongation factor calcium binding protein), Der f 18 (chitin-binding protein), Der f 20 (arginine kinase), Der f 24 (α -actinin), Der f 25 (triose-phosphate isomerase), Der f 26 (translational elongation factor-2), Der f 27 (Serpin peptidase inhibitor), Der f 28 (heat shock protein-70), Der f 31 (cofilin), Der f 33 (α -tubulin), and Der f Alt a 10 (aldehyde dehydrogenase). Aconitate hydratase, an enzyme that catalyzes the stereospecific isomerization of citrate to isocitrate via *cis*-aconitate in the tricarboxylic acid cycle, was found to be a novel Df allergen that sensitized 70% of the allergic Thai patients. Several other proteins have been identified as minor Df allergens by using 20 Df allergic Thai patients. Allergens previously reported in other organisms such as fructose biphosphate aldolase (aldolase A) from *Candida albican* and *Asperillus* spp.,^{31,32} biting midge (*Forcipomyia taiwana*; For t 2) and Japanese oak silk moth (*Antheraea yamamai*),³³ *Dirofilaria immitis*/endosymbiont bacteria *Wolbachia* spp.),³⁴ and Asian wasp (*Vespa affinis*)³⁵ and alpha-enolase from *Cladosporium herbarum* (Cla h 6)³⁶ were found to be bound by IgE of the mite allergic patients of this study, indicating that the Df mite also shares the allergenic epitopes of the cross-reactive allergens.

The profile of serum IgE specific to the Df allergens of the 20 allergic patients of this study indicated that the major sensitizing allergens were tropomyosin (Der f 10), aconitate hydratase, and one uncharacterized high-molecular-weight protein (~95 kDa, spot 2 of Figure 2). Apolipoprotein (Der f 14/Mag3), arginine kinase (Der f 20), chitin-binding protein/Chitinase (Der f 18), heat shock protein-70 (Der f 28), and triose-phosphate isomerase (Der f 25) were reactive to IgE of 50% of the Df allergic patients. Allergenicity of the group 1 allergen, which has been reported as the mite major allergen,^{11,37,38} could not be determined among this group of patients as the body extract of pure mites contained too little of the Der f 1. The group 2 allergen, which is also known to be the mite major allergen,^{4,11} was reacted by IgE in sera of only 8 (40%) of this set of the allergic Thai patients, and thus it may be regarded as a minor allergen. The unconformity might be because the Der f 2 contained variants with amino acid substitutions that cause different IgE reactivity.^{39,40} For the mites collected in Thailand, Piboonpocanun et al. have previously reported the polymorphic sequence substitutions of Der f 2.⁴¹ Besides, genetics and environmental attributes of the patients in individual studies may be different, which impact upon the percent IgE reactivity of a particular allergen. This fact emphasizes the importance of component-resolved diagnosis (CRD) set forth by Valenta et al. since 1999.⁷ On the contrary, Der f 10, which was minor allergen among the Korean patients, gave IgE binding to sera of 75% of the 20 Thai patients. Several publications reported that the culturing temperature and culture media influences the rate of mite-population growth, allergen production, and the ratio of allergens in extracts made from cultured mites.^{12–15,42,43} This might explain the dissimilar results and immuno-proteomics. Moreover, identification and characterization of HDM allergens using new approaches like genomic and transcriptomic techniques could reveal 20 canonical allergens and 7 noncanonical allergen homologues including novel allergen, ubiquinol-cytochrome

c reductase binding protein-like protein designated Der f 24.⁴⁴ The overall findings emphasize the importance of CRD in designing a specific tailored allergen immunotherapy for individual patients to expect improved treatment efficacy and conform to the medical model of personalized medicine (PM).

CONCLUSIONS

By 1DE proteomics, *Dermatophagoides farinae* (Df) whole body extract contained 105 known and unknown proteins. The known ones could be designated to seven functionally different groups. By 2DE-IgE immunoblotting using sera of 20 Df allergic patients, tropomyosin (Der f 10), aconitate hydratase, and one unknown high-molecular-weight protein were identified as the mite major allergens. Aconitate hydratase is a novel Df major allergen unraveled in this study. Percent allergenicity of the group 1 allergen among the patients could not be assessed owing to the allergen scarcity in the mite body. The group 2 mite allergen was bound by IgE of only 40% of the patients. Several novel minor allergens have been found, for example, fructose biphosphate aldolase and alpha-enolase. The overall data emphasize the importance of component-resolved diagnosis for a tailor designed of allergen-specific immunotherapy.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b00663.

Table S-1. Demographic and clinical characteristics of the allergic patients and the normal controls. Table S-2. *D. farinae* proteins of in-gel tryptic digestions of *D. farinae* searching against NCBIInr and their accession numbers. Figure S-1. MS/MS Fragmentation of single peptide identification from SDS-PAGE. (PDF)

Table S-3. LC-MS/MS Mascot results of *D. farinae* searching against NCBIInr database from SDS-PAGE. Table S-4. LC-MS/MS Mascot results of *D. farinae* searching against NCBIInr database from 2D-gel. (XLSX)

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Notes

The authors declare no competing financial interest

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ABBREVIATIONS

CBB, Coomassie Brilliant Blue G-250; CE, crude extract; CRD, component-resolved diagnosis; CRIT, component-resolved

immunotherapy; Df, *Dermatophagoides farinae*; Dp, *Dermatophagoides pteronyssinus*; DTT, dithiothreitol; HDM, house dust mite(s); HRP, horseradish peroxidase; IAA, iodoacetamide; IgE, immunoglobulin class/isotype E; kAU/L, kiloallergy units per liter; kDa, kilodaltons; LC-ESI-MS/MS, liquid chromatography-electrospray ionization-tandem mass spectrometry; mA, milliamperes; N, normal (healthy) control; NC, nitrocellulose membrane(s); NCBIInr, National Center for Biotechnology Information-Nonredundant; NPC2, Neimann-Pick type C2; P, patient; PBS, 0.01 M of phosphate-buffered saline, pH 7.4; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS-T, tris buffered saline containing 0.05% Tween-20; 1DE, one-dimensional gel electrophoresis; 2DE, two-dimensional gel electrophoresis

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