

# Mapping of *Dermatophagoides farinae* mite allergens by two-dimensional immunoblotting

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**Background:** Allergens from the house dust mite *Dermatophagoides farinae* are responsible for frequent respiratory allergic disorders, but only 3 groups of these allergens are well characterized.

**Objective:** This study was performed to complete the repertoire of *D farinae* allergens using two-dimensional (2-D) electrophoresis.

**Methods:** *D farinae* mite allergens, extracted from whole cultures in the presence of a mild detergent, were separated by 2-D electrophoresis with subsequent immunoblotting. IgE-binding proteins were detected with individual mite-sensitive patient sera and the anti-*D pteronyssinus* human serum pool. Allergens were identified by an inhibition immunoblot test, by means of specific mAbs, or by biochemical characterization. The internal peptides of 2 allergens were microsequenced.

**Results:** 2-D immunoblotting with individual patient sera showed a marked heterogeneity in the isoelectric point of the allergens, as well as differences in the individual IgE-binding patterns. In addition to identification of allergens Der f 1, Der f 2, and Der f 3, new allergens have been characterized as Der f 4, Der f 5, and 2 high molecular mass allergens. Microsequencing of peptides from the latter allergens revealed significant homologies with allergen Mag 3 from *D farinae* and with a chitinase from prawn *Penaeus japonicus*.

**Conclusion:** 2-D electrophoresis with subsequent immunoblotting and protein microsequencing allowed characterization of a more complete repertoire of *D farinae* allergens and their multiple isoforms, and identification of six new allergens. (J Allergy Clin Immunol 1998;102:631-36.)

**Key words:** Mite allergy, *Dermatophagoides farinae*, isoallergens, two-dimensional electrophoresis, IgE-immunoblotting, protein microsequencing

Exposure to house dust mites induces allergic disorders such as asthma, rhinitis, dermatitis, or conjunctivitis. House dust mites of the Pyroglyphidae family occur in most homes in temperate climates, and 2 species, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* and their allergens, are frequently present in European indoor environments. Characterizing these allergens is therefore an important step towards understanding, diagnosis, and treatment of allergic disease.

## Abbreviations used

|          |                                                              |
|----------|--------------------------------------------------------------|
| 1-D:     | One-dimensional                                              |
| 2-D:     | Two-dimensional                                              |
| IEF:     | Isoelectric focusing                                         |
| Mr:      | Molecular mass                                               |
| MS pool: | Anti- <i>Dermatophagoides pteronyssinus</i> human serum pool |
| pI:      | Isoelectric point                                            |
| SB 3-14: | 3-(N,N-Dimethylmyristyl ammonio) propanesulfonate            |

Basic approaches to allergen characterization, such as immunochemical and molecular biologic methods, have resulted in the classification of *D pteronyssinus* allergens into 10 groups.<sup>1</sup> Biochemically, some of these allergens have similar molecular mass and some are heterogeneous in their isoelectric forms. At the molecular level, a significant degree of sequence polymorphism has been described for groups 1, 2, and 3.<sup>2-4</sup> Only 3 groups of *D farinae* allergens have been well defined, although new allergens have been recently described.<sup>1</sup>

In this study we further characterize *D farinae* allergens by means of high-resolution two-dimensional (2-D) electrophoresis, which is able to separate complex protein mixtures. This protein separation method combined with immunodetection allowed completion of the repertoire of *D farinae* allergens. This method revealed the IgE-binding heterogeneity of these allergens. Two new high molecular mass allergens were identified by microsequencing of internal peptides.

## METHODS

### Allergens

***D farinae* extracts.** *D farinae* whole mite culture (200 mg)<sup>5</sup> was incubated for either 1 or 16 hours at 4°C, with 0.5 mL of distilled water or a 1% aqueous solution of sulfobetaine, SB 3-14 (Fluka Chemika-BioChemika, Switzerland), a detergent that combines a very high solubilizing power and a weak denaturing effect.<sup>6</sup> Extractions were performed in the presence of serine protease inhibitors (Sigma-Aldrich Chimie, France), 1 mmol/L phenylmethylsulfonyl fluoride, and aprotinin (1 mg/mL). After centrifugation (9000 g for 20 minutes), the supernatant was dialyzed against distilled water, treated with RNase A (0.25 mg/mL) and DNase I (1 mg/mL) (Sigma-Aldrich Chimie, France) and stored at -20°C. Protein determination was performed by means of the Micro BCA protein kit (Pierce, Rockford, Ill).

**Der f 1.** This allergen was purified from a partially purified extract of *D farinae* by means of a combination of ammonium sulfate precipitation and ion-exchange chromatography.<sup>7</sup>

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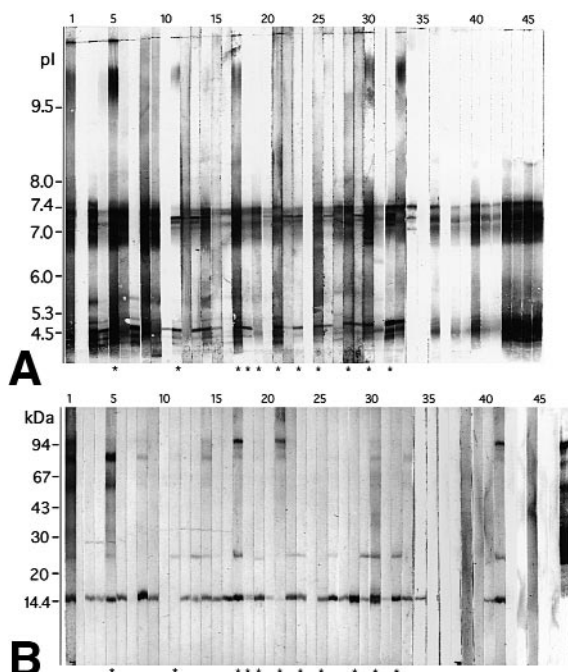


**FIG 1.** 1-D immunoblotting of *D farinae* allergens. Binding patterns of mite-sensitive patient IgE, mAbs anti-Der p 2, and anti-Der f 1 on **(A)** immunoprints after IEF and **(B)** Western blots after SDS-PAGE. *Strip 1*, MS pool; *strips 3-23 and 25-33*, individual mite-sensitive patient sera; *strip 34*, mAb anti-Der p 2; *strips 36-46*, different mAbs anti-Der f 1: 4C1B8, 6A8B10, and MAF 1 - MAF 9. For Western blots **(B)**, *strip 40* reincubated with mAb anti-Der p 2, *strip 41* with serum 17, and *strip 47* proteins stained with Indian ink. *Strip 2*, serum from nonallergic patient; *strips 24 and 35*, buffer control; *pI*, isoelectric point markers; *kDa*, molecular mass markers. \*Sera used in 2-D immunoblotting.

## Sera and mAbs

Sera were obtained from 30 house dust mite-sensitive patients (Pasteur Institute Hospital, Paris, France), selected on the basis of perennial symptoms, positive skin prick test results, and positive values for serum-specific IgE (RAST class, >4). The anti-*D pteronyssinus* human serum pool 82/528 from 10 mite-sensitive patients mainly exposed to *D pteronyssinus* was obtained from the National Institute for Biological Standards and Control (Hertfordshire, UK). This pool presented RAST class 6 for mites in agreement with the previously reported results<sup>8</sup> and is called MS pool.

The nine biotinylated anti-Der f 1 mAbs, MAF 1 through MAF 9, have been described previously.<sup>9</sup> MAF 6 and MAF 9 were used for quantification of Der f 1.<sup>10</sup> The anti-Der f 1 mAbs, 4C1B8 and 6A8B10, and one anti-Der f 3 mAb, 5A12, were a gift from Dr M. Chapman (University of Virginia). 6A8B10 and 5A12 were purified and biotinylated as published.<sup>9</sup> Biotinylated anti-Der p 2 (mAb 296) was produced and kindly provided by Dr J. M. Saint-Remy (Center for Molecular and Vascular Biology, Leuven, Belgium).

## One-dimensional (1-D) electrophoresis

Isoelectric focusing (IEF) was performed in acrylamide gel (4% T, 4% C). Before focusing, the dried gel was reswollen in a 2% (wt/vol) mixture of carrier ampholytes (Serva Feinbiochemica, Heidelberg, Germany), with a pH range of 2 to 11. The *D farinae* extract loaded on the cathode side revealed the best IEF pattern. After IEF,

**TABLE I.** Determination of optimal conditions of *Dermatophagoides farinae* mite extraction

| Extraction conditions (4°C) | Proteins (mg/mL) | Der f 1 (μg/mL) |
|-----------------------------|------------------|-----------------|
| Water, 1 hr                 | 4.0              | 75              |
| Water, 16 hr                | 4.2              | 281             |
| 1% SB 3-14, 1 hr            | 4.5              | 69              |
| 1% SB 3-14, 16 hr           | 6.6              | 406             |

For protein assay, a Micro BCA protein kit was used with bovine serum albumin as protein standard and an ELISA protocol was used to quantify Der f 1. SB 3-14: 3-(N,N-Dimethylmyristyl-ammonio) propanesulfonate, detergent compatible with the protein assay used.

the gel was blotted on a cyanogen bromide (CNBr)-activated nitrocellulose sheet cut into strips, as published.<sup>11,12</sup>

For SDS-PAGE, the extract was diluted twice in a 38 mmol/L Tris-HCl (pH 6.8), 4% SDS buffer, heated for 10 minutes at 60°C, and applied onto acrylamide gel (gradient 8% to 22% T, 4% C).<sup>13</sup> After the run, proteins were electrotransferred onto a CNBr-activated nitrocellulose sheet by means of the semidry electroblotting technique and cut as described above.

## 2-D electrophoresis

A 2-D separation was performed as previously described.<sup>13</sup> Briefly, for the first separation, 80 μL of *D farinae* extract were applied to an IEF gel containing ampholytes forming a pH range of 3.5 to 9.5 (Pharmacia Biotech, Uppsala, Sweden). The gel was then cut into 50 strips (3 mm) and stored at -20°C for further experiments. Strips were equilibrated in a 38 mmol/L Tris-HCl pH 6.8, 4% SDS buffer, for 10 minutes at 60°C and layered onto the surface of the SDS-PAGE gel for the second separation. The 2-D separation gels were electrotransferred onto a CNBr-activated nitrocellulose sheet.

## Immunoblotting

Immunoblot analysis was performed according to the procedure previously described.<sup>14</sup> The strips and sheets were incubated overnight with 10-fold diluted patient sera. IgE antibodies were detected by biotinylated human anti-IgE equine antibodies diluted to 1:3000 (Genetic Systems Corp, Redmond, Wash). Biotinylated mAbs were used at a dilution of 1:1000 for anti-Der f 1, 1:1500 for anti-Der p 2, and 1:500 for anti-Der f 3. Anti-IgE and anti-IgG bound antibodies were revealed by means of alkaline phosphatase-conjugated streptavidin (Amersham, UK).

For immunoblot inhibition, patient serum 30 diluted to 1:10 was mixed with either purified Der f 1 (5 μg) or buffer solution for 1 hour.

## Amino acid sequence analysis

Proteins from *D farinae* extract, separated by 2-D gel electrophoresis, were visualized by amido black staining of the gel (Serva, France). Two spots of high molecular mass proteins reacting with IgE from mite-sensitive patient sera were cut out and subjected to in situ hydrolysis by endoproteinase Lys-C. After separation on a diethylaminoethyl-C18 column by high-pressure liquid chromatography, peptides were microsequenced by Edman degradation with an Applied Biosystems sequencer (model 473A). The partial amino acid sequences were compared for homologies to already known proteins by using the Basic Local Alignment Search Tool (BLAST) algorithm<sup>15</sup> (PIR, Swiss-Prot and Genpept databases).

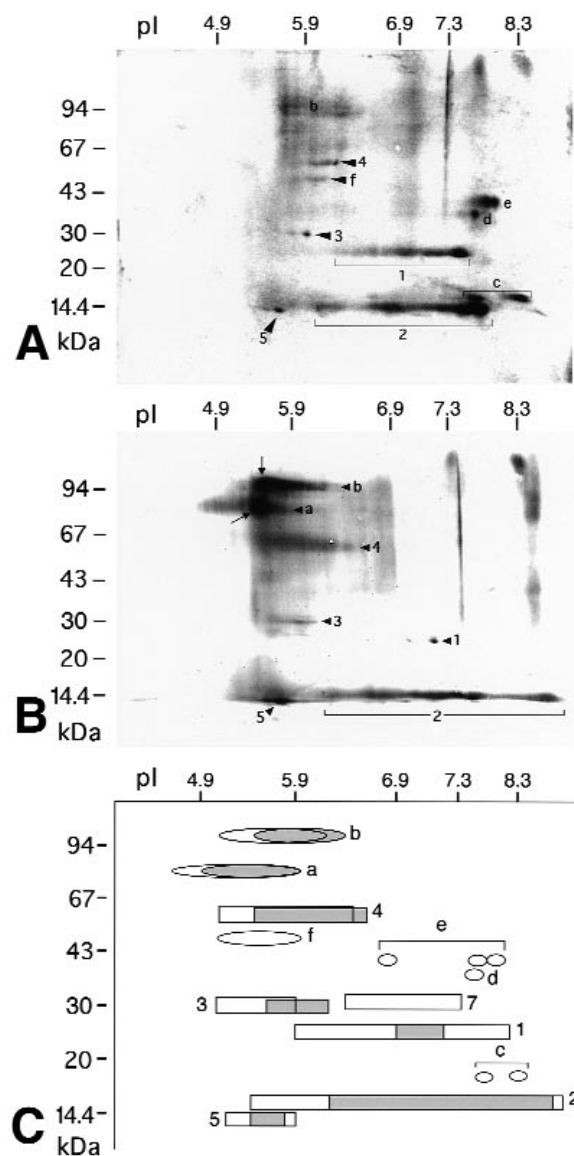


**FIG 2.** 2-D immunoblotting of *D farinae* allergens: **A**, IgE reactivity of representative mite-sensitive patient serum (no. 30); **B**, IgE reactivity of MS pool; arrows mark protein spots excised for amino acid sequence analysis. **C**, schematic drawing of Der f allergens. Numbers and lower case letters indicate allergens. Allergens revealed with anti-*D pteronyssinus* human serum, 82/528 (MS pool) (shaded boxes); allergens revealed with individual patient sera (open boxes); allergens not yet described (open circles).

## RESULTS

### Mite allergen solubilization

To define the best conditions for protein solubilization and antigenicity preservation, *D farinae* mite culture extractions were performed under various conditions. As shown in Table I, the maximum yields of total protein and Der f 1 concentrations were obtained with an incu-

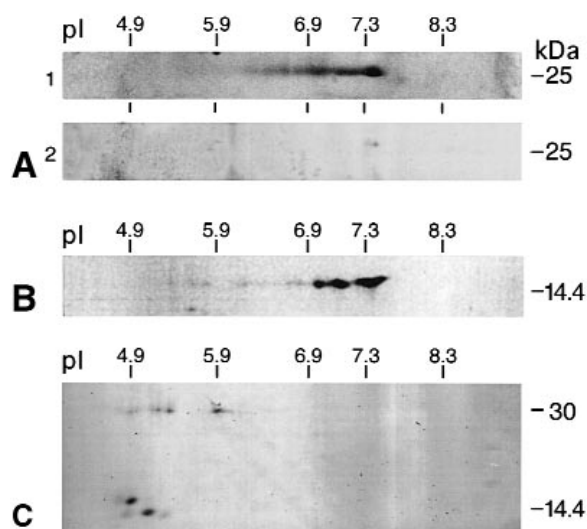


**FIG 3.** 2-D immunoblot analysis for identification of main *D farinae* allergens: **A**, Der f 1 recognition by IgE from patient serum 30: panels 1 and 2 serum preincubated without and with purified Der f 1. **B**, Der f 2 recognition by mAb anti-Der p 2 (296); **C**, Der f 3 recognition by mAb anti-Der f 3 (5A12).

bation time of 16 hours at 4°C in a 1% aqueous solution of SB 3-14. These conditions were selected to study the allergen content in *D farinae* culture.

### Mite allergens revealed after 1-D electrophoresis (IEF or SDS-PAGE)

Proteins from *D farinae* extract were separated by IEF (Fig 1, A). After blotting, incubation with the MS pool and 30 mite-sensitive patient sera displayed 15 to 16 IgE-binding proteins, mainly around isoelectric points (pI) 4.5 and 7.2 (lanes 1, 3 through 23, and 25 through 33). The strips incubated with 10 anti-Der f 1 mAbs revealed protein isoforms similar to the IgE pattern (lanes 36 and 38 through 46). The anti-Der p 2 mAb recognized only 3 protein bands, with pI 7.0, 7.2, and 7.4 (lane 34).

*D farinae* extract was analyzed by SDS-PAGE and Western blotting (Fig 1, B) with the same panel of antibodies as described above. Two IgE-binding proteins with a relative molecular mass (Mr) of 14 to 15 and 24 to 25 kD were recognized by at least 50% of the patient sera, and allergens with Mr of 80 to 83 and 95 to 101 kD were recognized by 37% of sera (lanes 1, 3 through 23, and 25 through 33). Other allergens with Mr of 30, 37, and 58 kD were revealed with some of the sera. The anti-Der p 2 mAb recognized a protein band with Mr of 15 kD (lane 34). No antigens could be detected using the 11 anti-Der f 1 mAbs (lanes 36 through 46), although the presence of allergens on these blots was confirmed by reincubating lanes 40 and 41 with mAb anti-Der p 2 and serum 17, respectively.

**TABLE II.** Characteristics of *D farinae* allergens recognized by individual mite-sensitive patient sera and the anti-*D pteronyssinus* human serum pool (MS pool)

| Allergens    | b       | a        | 4       | f       | e       | d   | 7       | 3       | 1       | c       | 2       | 5       |
|--------------|---------|----------|---------|---------|---------|-----|---------|---------|---------|---------|---------|---------|
| Mr(kDa)      | 95-101  | 80-83    | 58-60   | 49-51   | 42      | 38  | 28-31   | 30      | 24-25   | 17.5    | 14-15   | 13-14   |
| Serum number |         |          |         |         |         |     |         |         |         |         |         |         |
| 5            | —       | 5.1-5.2* | 5.2-6.0 | 5.2-5.8 | 6.8     | —   | 6.6-7.1 | 5.0-5.2 | —       | —       | 5.7-7.3 | —       |
| 11           | —       | —        | —       | —       | —       | —   | —       | —       | 6.6-6.9 | —       | —       | —       |
| 17           | 5.2     | 4.9-5.2  | 5.2-5.9 | 5.2-5.8 | —       | —   | —       | 4.9-5.2 | 6.2-6.8 | —       | 6.8-7.2 | —       |
| 18           | —       | 5.8      | —       | —       | —       | —   | —       | —       | —       | —       | 6.7-8.8 | 5.9     |
| 19           | —       | 5.8      | —       | —       | —       | —   | —       | —       | —       | —       | 5.9-8.0 | —       |
| 21           | 5.3-6.2 | —        | —       | —       | —       | —   | —       | —       | —       | —       | —       | 5.4     |
| 23           | —       | —        | —       | —       | —       | —   | —       | —       | 7.5-8.0 | —       | 5.6-8.0 | —       |
| 25           | —       | 5.1-6.0  | —       | —       | —       | —   | 6.4     | —       | 6.6-7.3 | —       | 5.6-7.3 | 5.3     |
| 28           | —       | —        | —       | —       | —       | —   | —       | —       | 5.9-8.2 | —       | 6.9-8.2 | —       |
| 30           | 5.6-6.3 | —        | 5.9-6.2 | 5.5-6.0 | 7.7-7.9 | 7.6 | —       | 5.7-5.9 | 6.2-7.5 | 7.7-8.3 | 5.9-7.8 | 5.4-5.6 |
| 32           | —       | —        | 6.4     | —       | —       | —   | —       | —       | 7.7     | —       | 5.6-7.8 | 5.7     |
| MS pool      | 5.4-6.4 | 4.5-5.9  | 5.4-6.6 | —       | —       | —   | —       | 5.6-6.2 | 6.9-7.2 | —       | 6.3-8.7 | 5.4-5.7 |

\*Values indicate range of pI.

Mr and range of pI evaluated by 2-D immunoblots.

### Mite allergens revealed after 2-D electrophoresis

Immunoblotting was performed with the MS pool and with 11 individual sera selected for their representative IgE-binding patterns in IEF or SDS-PAGE. A typical result obtained with an individual serum is shown in Fig 2, A. Fig 2, B shows the allergens recognized by the MS pool, and Fig 2, C summarizes all *D farinae* allergens detected. A marked heterogeneity of pI was observed for each allergen, as shown in Table II.

**Der f 1, Der f 2, and Der f 3 allergens and their isoforms.** The IgE-binding protein, defined by a molecular mass of 24 to 25 kD, corresponded to Der f 1; the preincubation of patient's serum 30 with purified Der f 1 led to almost complete inhibition of IgE binding to this allergen, whereas this serum revealed 5 to 6 isoforms with pI ranging from 6.2 to 7.5 (Fig 3, A, panels 1 and 2). Patient serum 28 revealed at least 10 isoforms (pI: 5.9 to 8.2), whereas serum 32 only recognized 1 isoform with pI of 7.7 (Table II). The allergen with an Mr of 14 to 15 kD had the characteristics of Der f 2 and was revealed in 3 isoforms (pI: 7.0, 7.2, 7.4) by anti-Der p 2 mAb (Fig 3, B). For this allergen, patient serum 17 displayed the most restricted IgE response (ie, 2 isoforms with pI 6.8 and 7.2), whereas serum 23 showed a larger response, 10 to 11 contiguous isoforms with pI ranging from 5.6 to 8.0 (Table II). The isoforms recognized by most of the patient sera tested were considered to be immunodominant: Der f 1, pI: 6.6 to 6.8 and Der f 2, pI: 6.9 to 7.2. The allergen located at a Mr of 30 kD corresponded to Der f 3; it was recognized by anti-Der f 3 mAb in 4 isoforms with pI of 4.9, 5.2, 5.3, and 5.9 (Fig 3, C). Patient sera and MS pool revealed different isoforms of pI ranging from 4.9 to 6.2 (Table II). Anti-Der f 3 mAb also revealed additional antigens with Mr of 14 and 15 kDa and pI of 5.1 and 4.9, not recognized by IgE from the patient sera studied.

**New allergens.** The allergen located at an Mr of 58 to 60 kD and presenting up to 6 isoforms with patient sera 5 and 17 (Table II) and a smear with the MS pool (Fig 2, B) was associated with  $\alpha$ -amylase activity<sup>16</sup> (data not shown). This allergen presenting the molecular characteristics and biologic function of Der p 4<sup>17</sup> was therefore identified as Der f 4. Another allergen with Mr of 13 to 14 kD appeared as intensely stained spots with a major isoform at pI 5.6; this pI corresponds to the calculated pI of recombinant Der p 5.<sup>18</sup> It was called Der f 5 by analogy with this latter allergen and was revealed by 5 of the 11 sera.

Two protein groups with a high Mr were intensely recognized by the MS pool: one group with Mr of 80 to 83 kD and pI 4.5 to 5.9, and the other group with Mr of 95 to 101 kD and pI 5.4 to 6.4. These two allergens, called a and b respectively, presented multiple isoforms that were clearly revealed by individual sera (Table II).

Five allergens were only detected in the presence of 4 individual sera: 5, 17, 25, and 30 (Table II). Four of them have not been previously described and were called allergens c (Mr = 17.5 kD; pI 7.7 to 8.3), d (Mr = 38 kD; pI 7.6), e (Mr = 42 kD; pI 6.8 and 7.7 to 7.9), and f (Mr = 49 to 51 kD; pI 5.2 to 6.0). At Mr of 28 to 31 kD, patient serum 5 recognized 6 isoforms of pI ranging from 6.6 to 7.1 and patient serum 25 reacted with one isoform of pI 6.4. The latter allergen could correspond to Der f 7.<sup>19</sup>

### Microsequencing of high molecular mass allergens

N-terminal amino acid sequence analysis of allergens a and b (indicated by arrows on Fig 2, B) was performed, but no results could be obtained because of a blocked amino terminus. The sequence of 3 internal peptides of allergen a was therefore determined as P9: VTALLL-LK, P15: MHMDFPNVFQADLTG, and P16: EIGITR-

NIAREYGSYT. Sequence alignments of peptides P9 and P15 showed 100% homology with two peptides from a *D farinae* allergen, Mag 3.<sup>20</sup> Two internal peptides from allergen b were sequenced, P14: AFEPHGYLLTAASV and P15: SLVMDSEMPNEARIA. P14 showed 78% homology with peptide <sup>301</sup>AFKPHGLLLSAAVS<sup>314</sup> from a prawn *Penaeus japonicus* chitinase.<sup>21</sup>

## DISCUSSION

This work presents an extended repertoire of *D farinae* mite allergens recognized by specific IgE antibodies from human sera of patients with allergy and focuses on the characterization of high molecular mass allergens.

Overlapping of allergens with similar pI or the same molecular mass was observed on IEF or SDS-PAGE (Fig 1) and showed the limitations of 1-D electrophoresis. These limitations were overcome by using 2-D electrophoresis, a method combining pI and molecular mass separations.

The major allergen, Der f 1, was further identified by means of an inhibition assay with purified Der f 1 in 2-D immunoblotting. The absence of mAbs binding with Der f 1 observed in Western blotting could be explained by heat denaturation<sup>22</sup> in SDS-containing buffer because the heating step was made before 2-D immunoblotting. It should be noted that anti-Der f 1 mAbs reacted with Der f 1 only in native conditions used for IEF (Fig 1, A). Specific recognition by anti-Der p 2 mAb and anti-Der f 3 mAb allowed us to precisely identify the corresponding *D farinae* allergens. The anti-Der f 3 mAb also recognized 2 antigens whose cross-reactivity with Der f 3 indicated that they may be either isoforms with different molecular mass or proteolytic products, although protease inhibitors were used. These antigens were not recognized by IgE from the patient sera studied, showing that the anti-Der f 3 mAb recognized an epitope that does not elicit an IgE response in the mite-sensitive patients studied.

By blotting with individual mite-sensitive patient sera, allergens Der f 1, Der f 2, and Der f 3 presented a marked heterogeneity of pI, revealing multiple isoforms. This variation of pI could be caused by few, but significant changes in amino acid sequences, in line with the sequence polymorphism recently described for Der p 1,<sup>2</sup> Der f 2,<sup>23</sup> and Der f 3.<sup>24</sup> Breiteneder et al<sup>25</sup> showed for a tree allergen that such changes could induce modifications in B-cell epitope recognition by allergic patient sera. These amino acid changes do not exclude the possibility of changes in glycosylation of these allergens. A few isoforms were immunodominant; they were recognized by the majority of patient sera tested. Similarly, Nishiyama et al,<sup>23</sup> using 3 variants of Der f 2, observed equal recognition by IgE from 14 mite-sensitive patient sera.

Because of the marked similarity between *D pteronyssinus* and *D farinae* allergens, the MS pool was used to reveal cross-reactive allergens between these two mite species, as for Der f 4, Der f 5, and high molecular mass allergens a and b. To date, Western blotting analysis has revealed some allergens with Mr ranging from 95 kd to 75 kd from either *D pteronyssinus*,<sup>26</sup> *D farinae*,<sup>27,28</sup> or *Lepidoglyphus destructor*.<sup>29</sup> It was therefore interesting

to focus on the characterization and identification of allergens a and b belonging to this Mr range in our 2-D analysis. These allergens were resolved into multiple isoforms corresponding to an acidic pI range, with Mr of 80 to 83 kd and 95 to 101 kd. As N-terminal amino acids of these allergens were blocked, internal sequence analysis was performed. Sequence alignments for peptides from allergen a exhibited a marked homology with Mag 3, part of an allergen recently cloned from *D farinae*,<sup>20</sup> suggesting partial identity between allergen a and Mag 3. For allergen b, one peptide shared a significant sequence homology with a chitinase from the prawn *Penaeus japonicus*,<sup>21</sup> an enzyme involved in molting. The new allergen b would belong to the chitinase family with a glycanohydrolytic activity essential in mite development.

Four new allergens (c, d, e, and f) not previously described in the literature were detected with several individual sera, but not with the MS pool. They were therefore *D farinae*-specific. They appeared as visible spots on 2-D immunoblotting, but they were only barely detected, or not detected at all, on 1-D Western blotting analysis.

In conclusion, the repertoire of *D farinae* allergens was well defined by the high-resolution 2-D technique combined with IgE immunodetection. The MS pool allowed us to visualize the cross-reactive allergens between *D pteronyssinus* and *D farinae*, in particular 2 high molecular mass allergens further identified by protein microsequencing. The 2-D map of *D farinae* allergens was completed by means of individual patient sera, revealing new allergens and leading to a large IgE recognition pattern of *D farinae* isoallergens.

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