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Original Article

Purification and characterization of M-177, a 177 kDa allergen, from the house dust mite *Dermatophagoides farinae*

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Abstract

A high molecular weight allergen, M-177, was recently discovered in the house dust mite, *Dermatophagoides farinae* (*D. farinae*). The aims of this study were to develop a conventional purification procedure for M-177 and then to analyze some of the immunochemical properties of M-177. Mite extracts obtained from purified mite bodies were a suitable material for preparing M-177, because the purified mite extract contained large amounts of M-177. The purification of this allergen from the extract was achieved by ethanol fractionation, anion exchange chromatography, and gel filtration chromatography. The purified antigen was immunochemically equivalent to that of a preparation obtained by a previous affinity method using an anti-Mag 3-immobilized column. The yield of this purification procedure was 36.8% of the initial amount of M-177 in the extract, 40-fold greater than that of the previous immunoaffinity method. Our purification method was useful for preparing this allergen. The purified M-177 reacted in skin tests in 11 of 16 mite-allergic patients, compared to 10 of 16 with Der f 2. The amount of M-177 in the purified mite extract determined by enzyme-linked immunosorbent assay inhibition was as much as 0.95% of the total protein, which was higher than the amounts of Der f 1 (0.52%) and Der f 2 (0.32%). The potent allergenic activity and large amount of M-177 in the mites indicate that it is an important mite allergen.

Key words: chromatography, *Dermatophagoides farinae*, high-molecular-weight allergen, histamine, IgE, M-177, Mag 3, purification, skin test.

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Introduction

House dust mites (*Dermatophagoides* species) are the major causative agents of allergic diseases, such as bronchial asthma, perennial rhinitis and atopic dermatitis.[1] Immunochemical analyses using the immunoglobulin E (IgE) of patients allergic to mites have detected a large number of antigenic components with a wide range of molecular sizes, from 10 kDa to > 110 kDa, in two closely related house dust mite species, *Dermatophagoides farinae* (*D. farinae*) and *Dermatophagoides pteronyssinus* (*D. pteronyssinus*).[2,3] Approximately 10 mite antigens have been isolated and well characterized as major or important allergens; these are considerably smaller molecules with sizes of 14 kDa to 70 kDa.[4] The partial purification of some larger allergens, Con A-reactive allergen (174 kDa), HM-I (150–155 kDa), and Dpt 4 (144–274 kDa) has been reported, but the characterization of these high-molecular-weight allergens still remains unresolved.[5–7]

Recently, we isolated a partial sequence of an allergen gene, *mag* 3, by immunoscreening its expression product (Mag 3) from a *D. farinae* cDNA library with rabbit antisera to the crude mite extracts and IgE of patients allergic

to mites. No start and stop codons were found in the nucleotide sequence of *mag* 3 (1049 b.p., corresponding to a 41 kDa protein). Neither genes homologous to *mag* 3 nor the corresponding deduced proteins were present in the EMBL/GenBank/DDBJ databases as of April 1998.

In order to find the natural form of Mag 3 in extracts of *D. farinae*, immunoblotting analysis using a specific antibody for the recombinant Mag 3 was performed. A 177 kDa protein detected by the immunoblotting analysis was isolated using an anti-Mag 3 antibody-immobilized column. The IgE reactivity of the high-molecular-weight antigen was comparable to that of Der f 2 in enzyme-linked immunosorbent assay (ELISA) using the sera of 23 patients who were allergic to mites.[8] However, there were a few problems in the preparation of this allergen.

First, most of our conventional and commercial preparations of mite body extracts were not suitable as a starting material for the immunoaffinity purification of this allergen because they contained large amounts of various cross-reactive antigens which were copurified by an anti-Mag 3-immobilized column. Second, the column lost the capacity to capture the specific antigens after 10 runs. Third, the average recovery of M-177 was less than 1% of the total amount in the starting material.

As for the first problem, freshly purified mite bodies were found to be a suitable starting material. An improvement in a purification procedure of this antigen with high recovery, using conventional chromatographic techniques, is desirable in order to resolve the other problems.

In this study, we improved the procedure for purifying mite bodies by using a simple centrifugation procedure, and designed a new purification protocol by using conventional chromatographic techniques to obtain a purified 177 kDa allergen with a high yield. Further, we reported that the allergen made up as much as 0.95% of the total protein in a purified mite extract, and that the potent *in vivo* allergenic activity of this allergen was comparable to that of Der f 2.

Materials and methods

Mite bodies and extract

Mites (*D. farinae*) were cultivated on a mixture of powdered commercial rat and hamster animal feed (Oriental Yeast Co. Ltd, Tokyo, Japan) and wheat bran, in a ratio of 7:3 at 25°C in 75% humidity for a month. The mites were obtained from Fumakilla Ltd (Hiroshima, Japan). The culture was suspended in at least 10 volumes (w/v) of NaCl-saturated water, and then centrifuged at 6000 *g* for 15 min. The floating particles (Df-I) were then collected on filter paper. This step was repeated twice. Df-I was then centrifuged with 10 volumes (v/w) of 30% ethanol at 6000 *g* for 15 min. The floating particles and the looser precipitate above the denser layer of precipitate were carefully discarded by decanting. The precipitate was then centrifuged with 10 volumes (v/w) of NaCl-saturated water at 6000 *g* for 15 min, and the floating particles (Df-II), which were pure mite bodies, were collected on filter paper.

Two mite body extracts, Dfb-I and Dfb-II, were prepared by grinding Df-I and Df-II, respectively, with twice weights (w/w) of fine quartz powder, in about 4 volumes (v/w) of 20 mmol phosphate buffered saline (PBS), pH 7.3, containing 0.1 mmol phenylmethylsulfonyl fluoride (PMSF), 5 mmol disodium dihydrogen ethylenediaminetetraacetate dihydrate (EDTA), 1 mmol monoiodoacetic acid, and 5 mmol 1,2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP), and stored at –85°C until use.

Purification of M-177

Fifteen mL of a Dfb-II preparation (157.2 mg total protein) was fractionated with 30–50% ethanol for 1 h at –20°C. The 30–50% ethanol precipitate was collected by centrifugation at 10 000 *g* at 15 min. After briefly drying for up to 15 min at room temperature, the precipitate was dissolved in 5 mL of 50 mmol Tris-HCl, pH 8.0, containing 0.2% sodium dodecylsulfate (SDS), 0.5% deoxycholic acid, and 1% Triton X-100, and then separated with an FPLC system (Amersham Pharmacia Biotech, Uppsala, Sweden) in a chamber kept at a temperature of 4°C. The elution profiles were monitored by ELISA with anti-Mag 3 antibody, and the anti-Mag 3-reactive fractions were collected.

The pooled fraction diluted with 5 volumes (v/v) of 50 mmol Tris-HCl, pH 8.0 was applied to a RESOURCE Q column (Amersham Pharmacia Biotech) at a flow rate of 3.0 mL/min. Gradient elution was carried out at a flow rate of 5.0 mL/min with 50 mmol Tris-HCl, pH 8.0, with an increasing concentration of NaCl (0 mol to 1.0 mol) after the absorbance of the effluent stabilized. The anti-Mag 3 positive fractions from 15 to 25 mL were pooled and concentrated approximately 20 times with > 100K cut micro-ultrafiltration units (Millipore Corporation, Bedford, MA, USA) and then separated on a Superdex 200 HR column (Amersham Pharmacia Biotech) with 20 mmol PBS, pH 7.3, at a flow rate of 0.75 mL/min. A positive single peak appeared at 11.3 mL and the fractions from 9 to 12 mL were pooled. The collected fraction containing M-177 was concentrated up to 500 μg/mL by

ultrafiltration, and stored at – 20°C in the presence of 50% glycerol.

Antigens and antibodies

Recombinant Mag 3, affinity-purified rabbit polyclonal anti-Mag 3 antibody, and affinity-purified 177 kDa natural allergen (M-177) were prepared as previously described.[8] Dr H Yasueda of the National Sagamihara Hospital, Kanagawa, Japan, provided us with Der f 1, Der f 2, and the corresponding rabbit antisera.

Human sera and subjects

The mite-allergic sera used were stored in our laboratory. The sera were collected from 22 newly diagnosed asthmatic patients who had a positive skin test to mites (mean diameter of erythema > 20 mm, Torii & Co. Ltd, Tokyo, Japan). Control sera were collected from two healthy volunteers (erythema < 9 mm). For the skin test, 16 subjects were selected randomly among newly diagnosed patients who had a positive skin test to mites (erythema > 10 mm, Torii & Co. Ltd), along with 6 volunteers with no history of asthma and allergic rhinitis. These experiments were performed on the patients and volunteers after information was given on the purpose of this study, the treatment and risk of anaphylaxis. Informed consent was obtained from all patients and volunteers.

Histamine release assay

The histamine release test has been described previously.[9] Peripheral blood cells were prepared from nine patients and two healthy donors whose profiles were determined in the same manner. Briefly, peripheral blood cells were collected from heparinized peripheral blood by centrifugation at 1500 *g* for 10 min. The cells were suspended in Hanks' buffer and incubated with 0.1 μg/mL of M-177 at

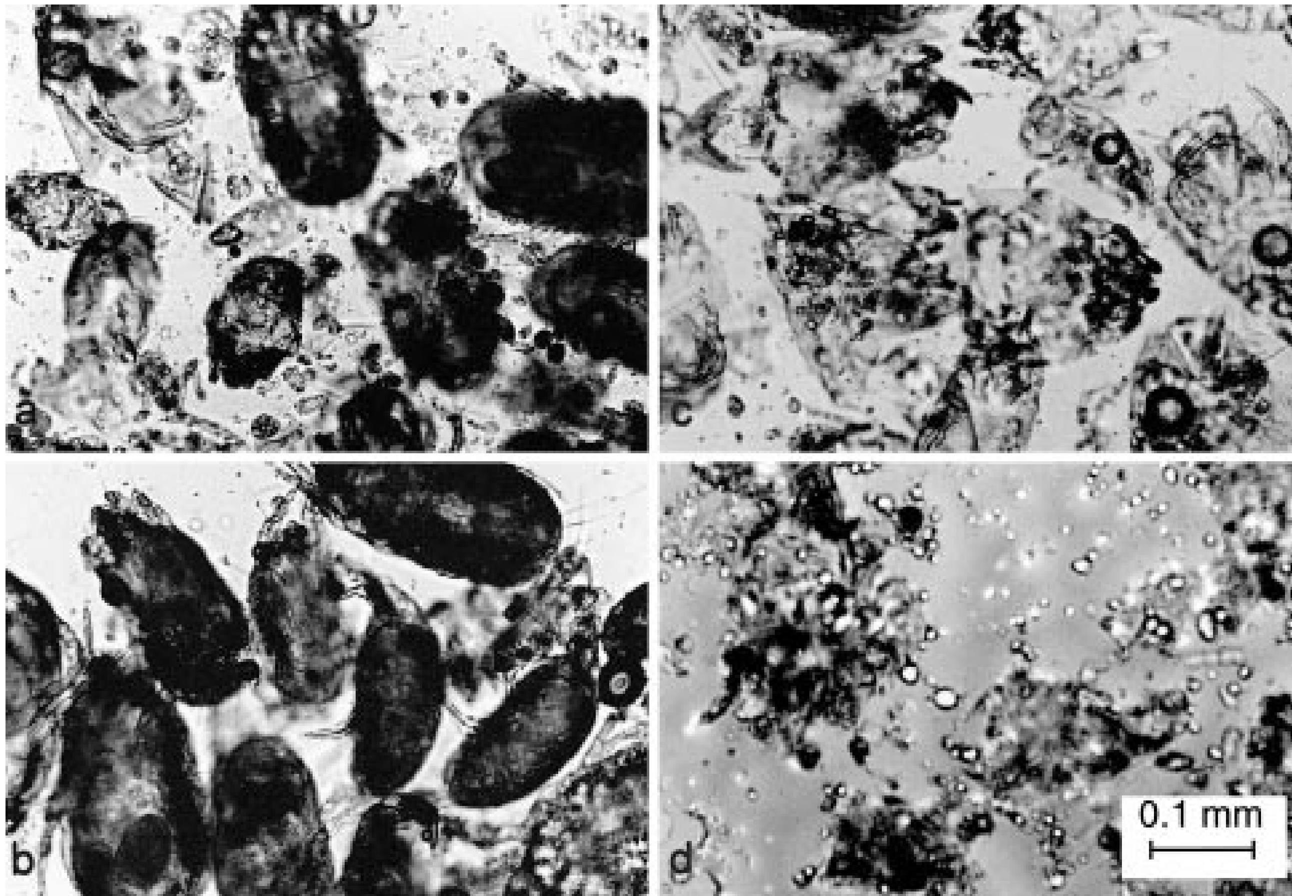

Fig. 1 Separation of mite bodies. The mite body fractions, (a) Df-I and (b) Df-II , and (c) the floating particles after centrifugation with 30% ethanol and (d) the precipitate after a final centrifugation with NaCl saturated water.

37°C for 30 min. The histamine released in the supernatant was analyzed by high performance liquid chromatography after deproteinization with perchloric acid.[10]

SDS-PAGE and immunoblotting analysis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions and immunostaining was performed on antigens electroblotted on polyvinylidene fluoride membranes (Immobilon-P membrane, Millipore Corporation). The membrane was blocked with 20 mmol of PBS, pH 7.3, containing 2% skim milk and 0.05% Tween 20, and reacted for 1 h with rabbit anti-Mag 3 antibody (1 μg/mL), rabbit anti-Der f 1 antisera (× 10 000), or rabbit anti-Der f 2 antisera (× 5000). Reactivity was detected for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (× 3000, Cappel Labs, West Chester, PA, USA), followed by staining with 3,3-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 within 10 min.

ELISA and ELISA inhibition

The IgE reactivity of M-177 was analyzed by ELISA. Falcon 96-well microtiter plates (Becton Dickinson & Co., Oxnard, CA, USA) were coated with antigen (0.2 μg) and blocked with 20 mmol PBS, pH 7.3, containing 3% dextran and 0.05% Tween 20. The plates were incubated with human sera (× 50) overnight at 4°C and detection was performed with biotin-conjugated goat anti-human IgE antibody (× 1000, for 3–5 h) (BioSource, Camarillo, CA, USA), alkaline phosphatase-conjugated streptavidin (× 1000, 2 h) (BioSource) and AttoPhos™ (Boehringer Mannheim, Mannheim, Germany). Fluorescence intensity was measured using a CytoFluorII™ (PerSeptive Biosystems, Framingham, MA, USA). The amount of specific IgE was estimated in 'units' from a standard IgE curve on the plate described previously.[8] One unit is equivalent to 1 ng of standard IgE (Yamasa Shoyu Co. Ltd, Chiba, Japan).

The amounts of antigen were estimated by ELISA inhibition. For the assay of M-177, affinity-purified M-177 was used as standard antigen. ELISA plates were coated with M-177 (0.1 μg), Der f 1 (0.1 μg) and Der f 2 (0.1 μg). Rabbit anti-Mag 3 antibody (1 μg/mL), rabbit

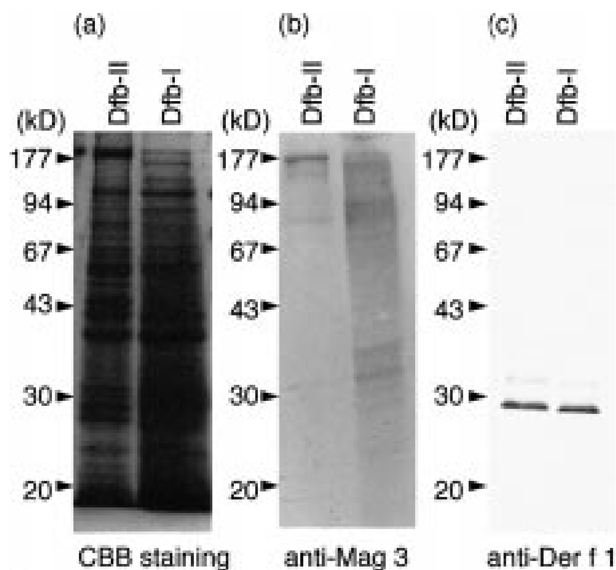

Fig. 2 Comparison between two different mite extracts, Dfb-I and Dfb-II, on an SDS-polyacrylamide gel and immunoblots. Dfb-I and Dfb-II were separated on a 10% SDS-polyacrylamide gel, followed by Coomassie brilliant blue (CBB) staining (100 μg, a), or immunoblotting with anti-Mag 3 antibody (50 μg, b), and anti-Der f 1 antiserum (50 μg, c).

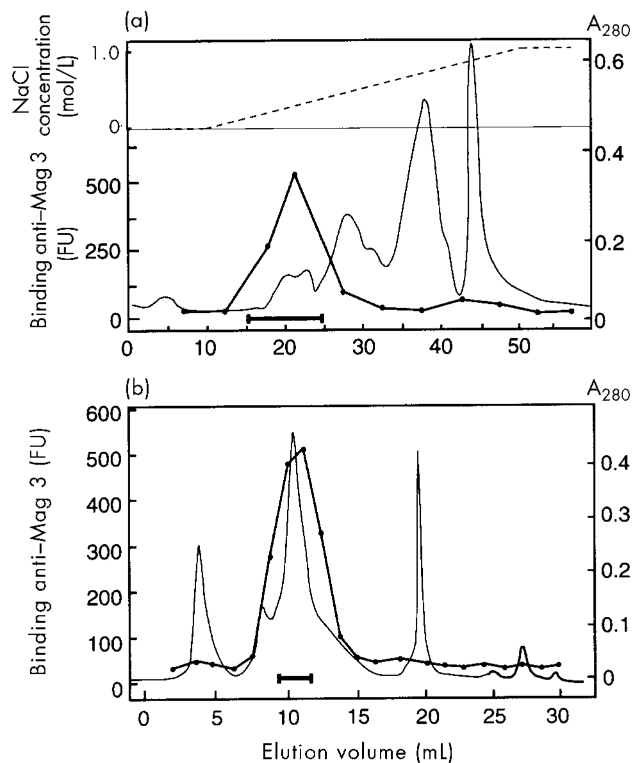

Fig. 3 Purification of M-177. The chromatographic profiles on (a) a RESOURCE Q column and (b) a Superdex 200 HR 10/30 column are shown. 50 μL of each fraction were coated on an ELISA plate and the reactivity to anti-Mag 3 antibody was measured (●). The dotted and thick lines show the linear gradient of the NaCl concentration and pooled fractions, respectively. FU, a tentative fluorescence unit.

anti-Der f 1 antiserum (× 10 000), and rabbit anti-Der f 2 antiserum (× 5000) were preincubated for 30 min at 37°C with a dilution series of Dfb-I or Dfb-II, and detection was performed with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (× 3000, for 1 h) (Cappel Labs) and AttoPhos™ (within 15 min) in the same manner. The antigen content of the mite extracts was estimated from the 50% inhibition value using a standard antigen curve on the same plate.

Results

Separation of mite bodies

A mite body fraction, Df-I, from a whole mite culture contained mite bodies, mite skeletons, and other impurities that were visible under a microscope (Fig. 1a). These impurities were unable to be removed by three additional centrifugational runs with NaCl-saturated water (data not shown). The mite skeletons and part of the impurities were separated into a floating fraction by centrifugation with 30% ethanol (Fig. 1c). The remaining impurities were then precipitated by the final separation with NaCl-saturated water (Fig. 1b,d). The yields of Df-I and Df-II

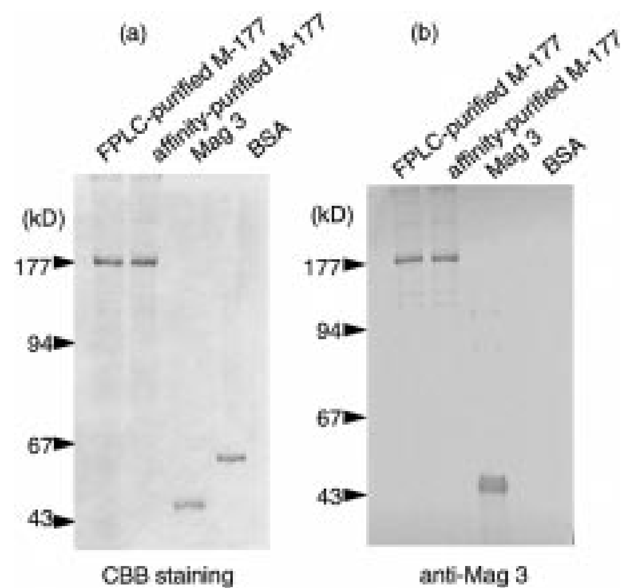

Fig. 4 Sodium dodecylsulfate-polyacrylamide gel electrophoresis and immunoblotting analysis of FPLC-purified and affinity-purified M-177. Both purified M-177 preparations were separated on a 10% sodium dodecylsulfate-polyacrylamide gel, followed by Coomassie brilliant blue (CBB) staining (2 μg, a), or immunoblotting with anti-Mag 3 antibody (1 μg, b).

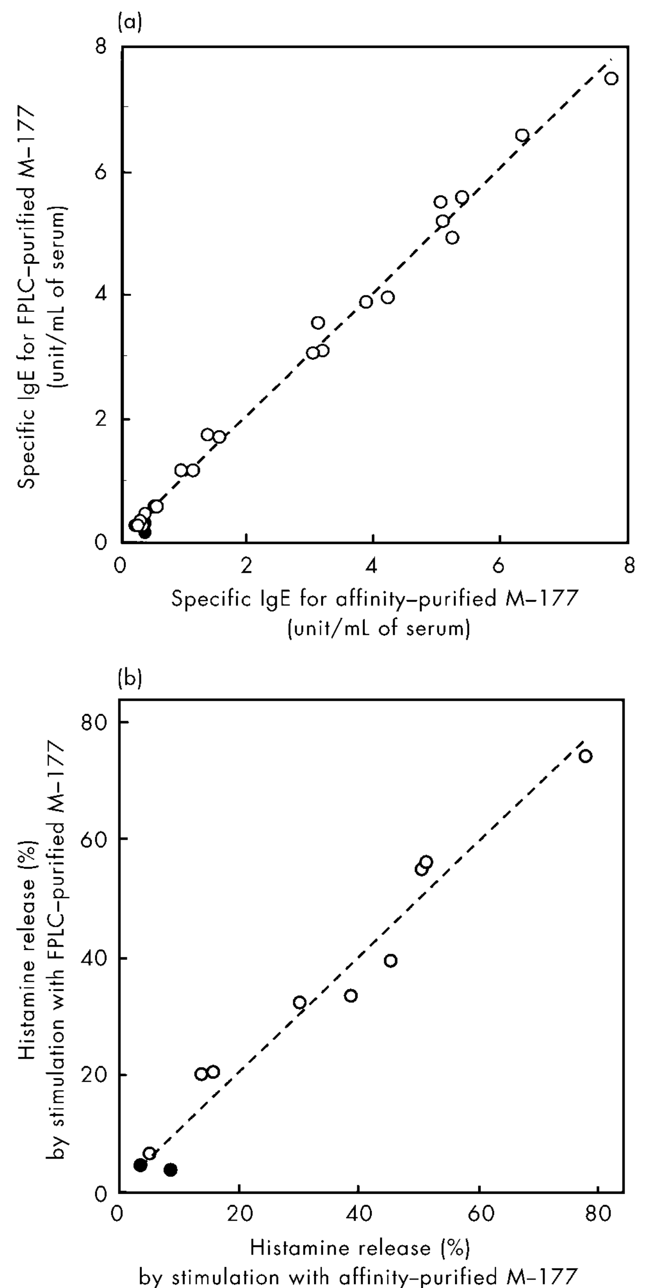

Fig. 5 Comparison of the *in vitro* allergenic activities of FPLC-purified and affinity-purified M-177. (a) The IgE reactivity of the two purified antigens was measured by ELISA. Open and solid circles indicate the specific IgE levels of individual subjects allergic to mites (n = 22) and healthy donors (n = 2), respectively. The correlation coefficient was 0.98 (n = 24). Values represent the mean of two experiments. (b) Histamine release from individual basophils by stimulation with the purified antigens was analyzed by HPLC. Open and solid circles indicate histamine release by individual subjects allergic to mites (n = 9) and healthy donors (n = 2), respectively. The correlation coefficient was 0.96 (n = 11).

Table 1. Amounts of major antigens in Dfb-I and Dfb-II evaluated by ELISA inhibition

Extract (Protein[a])	Dfb-I (128.5 mg)		Dfb-II (69.3 mg)	
Antigens	Total[b] amount (μg)	Antigen content (%)	Total[b] amount (μg)	Antigen content (%)
M-177	674.6[c]	0.53	660.4	0.95
Der f 1	922.6	0.72	362.4	0.52
Der f 2	621.9	0.48	219.0	0.32

[a]Protein contents of mite extract obtained from 100 g of whole mite culture are indicated. Protein content was measured by the Lowry method using bovine serum albumin as a standard; [b]the amount of antigen was measured by ELISA inhibition using purified antigens as a standard; [c]this value included both the M-177 and the cross-reactive antigens.

Table 2. *In vivo* allergenic activities of M-177 and the other mite allergens

Case *n*	aM-177	fM-177	Der f 2	Mite	Age	symptom
1	–	–	5	3	22	Allergenic rhinitis
2	1	2	±	2	23	Allergenic rhinitis
3	3	3	3	4	24	Allergenic rhinitis
4	5	4	2	3	24	Allergenic rhinitis
5	6	5	6	3	23	Allergenic rhinitis
6	–	–	1	6	24	Atopic dermatitis
7	8	8	6	6	23	Atopic dermatitis
8	4	2	8	5	38	Bronchial asthma
9	4	3	–	5	35	Bronchial asthma
10	5	5	3	5	42	Bronchial asthma
11	±	±	±	5	68	Bronchial asthma
12	±	±	–	7	67	Bronchial asthma
13	–	–	6	4	58	Bronchial asthma
14	6	7	6	6	24	Bronchial asthma
15	1	2	–	1	59	Bronchial asthma
16	3	2	±	3	67	Bronchial asthma
17	–	–	–	–	28	No symptoms
18	–	–	–	–	22	No symptoms
19	–	–	–	5	26	No symptoms
20	–	–	–	4	27	No symptoms
21	±	±	±	3	22	No symptoms
22	±	–	–	1	55	No symptoms
MS	2.9	2.7	2.9	4.3		
N	11	11	10	16		

Allergenic activity was evaluated with *in vivo* skin test. Twenty μL of antigen solution (1 μg/mL) was injected subcutaneously. The erythema was measured after 15 min. The score is based on mean diameter as follows: 0–4 mm (–), 5–9 mm (±), 10–14 mm (1), 15–19 mm (2), 20–24 mm (3), 25–29 mm (4), 30–34 mm (5), 35–39 mm (6), 40–44 mm (7), and > 45 mm (8). aM-177, affinity-purified M-177; fM-177, FPLC-purified M-177; MS, mean score for 16 patients; N, number of positive patients.

from 100 g of the same whole mite culture were 8.4 g and 2.6 g (wet weight), respectively.

Two extract preparations from the same mite culture showed different electrophoretical behaviors (Fig. 2a,b). Most of the antigen cross-reacted with anti-Mag 3 antibody in Dfb-I disappeared in Dfb-II except for a 177 kDa molecule (M-177), and relative content of M-177 increased with purification of the mite bodies. However, the same binding pattern to Der f 1 was observed in both of the extracts (Fig. 2c), and anti-Der f 2 also showed a quite similar binding pattern to a 14 kDa molecule (data not shown). Therefore, the purification of mite bodies was a prerequisite for the efficient enrichment of this allergen.

Purification of M-177

M-177 was purified from Dfb-II by ethanol fractionation, anion exchange chromatography (Fig. 3a), and gel

filtration chromatography (Fig. 3b). The yield of M-177 was 551.3 μg from 157.2 mg of Dfb-II. The amount, the specific activity (total M-177/total protein) and the recovery of M-177 in each purification step were as follows: Dfb-II: 660.4 μg, 0.01, and 100%; the 30%–50% ethanol fractionation: 447.2 μg, 0.061, and 67.7%; the RESOURCE Q fraction: 302.7 μg, 0.592, and 45.8%; and the Superdex 200 HR fraction: 243.0 μg, 1.015, and 36.8%. By this method, M-177 was purified practically to be a homogeneous molecule and its antigenic activity was equivalent to that of a previous immunoaffinity-purified antigen.

In order to confirm that the purified M-177 was identical to that purified by the affinity method using an anti-Mag 3-immobilized column, we analyzed immunochemically both preparations of M-177. The same patterns of both purified preparations were observed on a SDS-polyacrylamide gel and an immunoblot (Fig. 4a,b). The conventional chromatographic preparations still contained some minor antigens that were reacted specifically with anti-Mag 3, and corresponded to 240 kDa, 140 kDa, and 120 kDa in size. Repeated gel filtration failed to remove these minor antigens (data not shown). These cross-reactive antigens were also observed in the affinity-purified preparation (Fig. 4b), and the specific antibody binding to these antigens in both preparations of M-177 were absorbed completely with both of them on immunoblots (data not shown). From these results, both preparations of M-177 were electrophoretically identical.

The IgE reactivity of the FPLC-purified M-177 was quite similar to that of the immunoaffinity-purified preparation by ELISA ($n = 24$, $r = 0.98$; Fig. 5a). The histamine release profiles were also similar ($n = 11$, $r = 0.96$; Fig. 5b). These results suggested that the FPLC-purified M-177 was immunochemically the same antigen as the immunoaffinity-purified preparation.

Antigen contents in mite bodies extracts

The amount of M-177 and the other major antigens in the two extracts was estimated by ELISA inhibition (Table 1). The relative amounts of M-177 and its cross-reactive substances increased 1.8 times with the purification of mite bodies (0.53 to 0.95%). In contrast, the relative amounts of Der f 1 and Der f 2 in Dfb-II decreased from 0.72 to 0.52% and from 0.48 to 0.32%, respectively. The total amount of M-177 accounted for as much as 0.95% of the total protein, which was the largest content among the three mite antigens in Dfb-II.

In vivo allergenic activity

Skin testing was used to compare the *in vivo* allergenic activity of M-177 with that of Der f 2 (Table 2). M-177 was reacted with 11 of the 16 patients by skin test. The frequency of this allergen was comparable to that of Der f 2 (10/16). In addition, both preparations of M-177 also showed quite similar profiles in all subjects by skin test. The potent allergenic activity and the highest amount in Dfb-II suggested that M-177 should be one of the major mite allergens.

Discussion

The improved method of mite separation presented in this paper is a simple and rapid way to obtain adequately purified mite bodies. The purification of mite bodies effectively decreased the amount of the other anti-Mag 3 cross-reactive antigens and increased the content of M-177 in the extract. These changes provided a suitable starting material for preparing this high molecular weight allergen. The amount of M-177 in the mite body extracts after purifying the mite bodies was estimated by ELISA inhibition to increase 1.8-fold (Table 1), although this estimate includes the other cross-reactive antigens in addition to M-177. When the bands of the 177 kDa molecule stained on an SDS-polyacrylamide gel were analyzed by densitometry, the content of M-177 in Dfb-II was estimated to be 29.8 times greater than that in Dfb-I.

Almost all of the mite skeletons were removed by centrifugation with 30% ethanol (Fig. 1c), but some impurities remained in the mite fraction. These were not removed by repeating the centrifugation with 30% ethanol. A final centrifugation with NaCl-saturated water was essential to completely remove these impurities (Fig. 2d). Microscopic observation suggested that some of these impurities were attached to the cuticle sensory hairs on the body surface. Once these impurities were stripped with ethanol and the ethanol was removed, they could be precipitated by centrifugation with NaCl-saturated solution.

Originally, this 177 kDa allergen, which was found as a cross-reacted antigen with the specific antibody for the recombinant Mag 3, was believed to be the natural form of Mag 3. The existence of other high-molecular-weight allergens has been reported, but these have not been fully characterized.[5–7] We confirmed that M-177 was degraded into various fragments by Der f 1 (cysteine protease) and other proteases, and that these fragments

were capable of reacting with patients' IgE and anti-Mag 3 antibodies (data not shown). Therefore, the large number of smaller cross-reactive antigens detected with anti-Mag 3 antibody in Dfb-I were probably degraded fragments of M-177. These antigens were also detected in most of our conventional and commercial preparations of mite extracts (data not shown).

These results suggest that M-177 should be a key allergen, responsible for the diversity of antigenic components observed in crude mite antigens. In order to analyze the molecular aspects of this allergen, the development of a new purification procedure in place of the immunoaffinity procedure was necessary so that this allergen could be prepared stably.

The recovery of the new procedure (36.8%) was approximately 40-fold higher than that of the immunoaffinity purification method (less than 1%). The higher recovery by this conventional chromatographic purification method and the greater durability of commercial columns provides economic advantages for large-scale preparation of the antigen.

M-177 was as much as 0.95% of the total protein in Dfb-II. This was a greater proportion than either Der f 1 (0.52%) or Der f 2 (0.32%) (Table 1). M-177 was found in all of the internal organs of mites.[8] This suggests that M-177 is a major constituent of mite bodies. The amount of M-177 in mite extracts increased with purification of the mite bodies, but amounts of Der f 1 and Der f 2 decreased (Table 1). Der f 1 and Der f 2 were detected in the digestive organs and were present in both mite body extracts and mite feces extracts.[11,12] M-177 was not detected in the feces extracts (data not shown). This implies that the removed impurities included antigen components derived from mite feces or digestive enzymes.

Der f 2 and Der f 1 are well known major *D. farinae* allergens. In a previous report, we used ELISA to show that the IgE reactivity of M-177 (69.6%) was equivalent to that of Der f 2 (56.5%) in 23 mite-allergic subjects.[8] Based on skin testing, the *in vivo* allergenic activity of M-177 is also comparable to that of Der f 2 (Table 2). This potent allergenic activity suggests that this allergen is useful for diagnosing mite allergies. M-177 should be a suitable standardization antigen to redress the heterogeneity of antigenic activity observed in crude mite antigen preparations. This heterogeneity still remains a major problem for analytical and clinical purposes.[13,14] In order to learn more about this phenomenon, we plan to undertake further studies of this allergen, obtained by the new preparation method.

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