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Isolation and characterization of the 68 kD allergen from house dust mite *Dermatophagoides pteronyssinus*

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Abstract: House dust mites (HDM) represent a major source of allergens, contributing to the increasing incidence of type I hypersensitivity disease worldwide. Over 30 different IgE-binding proteins from the HDM extract were detected. Although group 1 and 2 have been identified as major allergens, due to the safety and efficacy of allergy diagnosis and immunotherapy, there is a need to carefully evaluate the clinical relevance of other allergens present in the HDM extract. In regard to this, a high molecular mass allergen of about 68 kD was purified from the HDM extract using a combination of gel permeation chromatography and reversed-phase chromatography. The IgG and IgE reactivity of the purified protein were preserved during the purification process, as confirmed by Western blot analysis with polyclonal rabbit antibodies and dot blot analysis with a pool of sera from subjects with house dust mite allergy, respectively. In addition, the IgE reactivity was confirmed using ELISA testing with nine patient sera. The biological potency of the 68 kD allergen was confirmed by skin prick testing in five allergic subjects, suggesting that the high molecular mass allergen is a good candidate for component-resolved diagnosis of house dust mite allergy and eventual therapeutic treatment.

Keywords: HDM; *Dermatophagoides pteronyssinus*; allergens; isolation.

INTRODUCTION

House dust mites (HDM) represent a major source of aeroallergens contributing to the increasing incidence of type I hypersensitivity disease worldwide.[1,2] More than 50 % of allergic patients and up to 80 % of asthmatic children are sensitized to mite allergens.[3] The HDM extract used in allergy diagnosis and therapy is a complex mixture of allergens and non-allergen components from mite

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bodies, fecal pellets and eggs.[4] Over 30 different proteins from mite extract bind IgE and 21 of them have been characterized.[5] Major IgE binding was reported for the group 1–3, 9, 11, 14 and 15 allergens.[6] One of the problems in standardization of HDM extracts is the presence of proteolytic enzymes, which are important allergens, the presence of which can, nevertheless, compromise the quality of the allergen extract. Allergens with proteolytic activity which have been found in the HDM extract are Der p1 – a cysteine protease, Der p3 with trypsin activity, Der p6 chymotrypsin, and Der p9 a collagenolytic serine protease. Group 1 and 2 represent the most important allergens as they are recognized by the majority of mite-allergic patients.[7] Nevertheless, about 20 % of mite allergic subjects do not produce IgE to the group 1 and 2, and given the high frequency of mite allergy, this constitutes a respectable number of patients.[6] In addition, sensitivity to one of the major allergens can be under- or over-represented in a limited group of patients,[8] and very often, it is dependent on the geographical region and environmental exposure to the allergen source. Moreover, the sequence polymorphisms of the major group 1 and 2 allergens produced by environmental mites can have an impact on the T-cell response to peptides containing different amino acid substitutions.[9] Therefore, structural features, as well as immunoreactivity of all allergens present in the HDM extract have to be considered and carefully evaluated.

Novel approaches based on a defined content of structurally defined individual allergens that can be applied in diagnosis and treatment of allergies are termed component-resolved diagnosis (CRD) and component-resolved immunotherapy[10] (CRI), respectively. Such an immunotherapeutic approach enables patient-tailored specific allergy treatment without risk of side effects and additional sensitization, especially in the pediatric population.[11]

The aim of this study was to isolate and examine the diagnostic potential of a high molecular mass protein of about 68 kD from house dust mite *Dermatophagoides pteronyssinus* in terms of its IgE-binding properties and biological activity in a group of Serbian HDM allergic persons.

EXPERIMENTAL

HDM allergen extract preparation

Dried house dust mites (*Dermatophagoides pteronyssinus*), 5 % (w/v), were extracted in 0.15 M phosphate-buffered saline (PBS), pH 7.6, by stirring overnight at 4–8 °C. After centrifugation at 6000 rpm for 30 min and filtration through a 0.22 μm membrane filter (Pall Europe Limited Portsmouth, England), the extract was lyophilized and stored at –70 °C. The protein concentration of the extract was determined by the Bradford method[12] using bovine serum albumin (BSA) as the standard.

Size-exclusion chromatography

The HDM extract was resolved by size-exclusion chromatography using a Superdex 200 column in 20 column volumes (850 mm×16 mm, Pharmacia, Uppsalla, Sweden), after equilibration of the column with 20 mM PBS, pH 8.0. The protein fractions were pooled and analyzed by SDS-PAGE.

Reversed-phase chromatography

Following size-exclusion chromatography, the pooled fractions containing a high-molecular mass allergen were further purified by reversed-phase chromatography (100 mm×4.6 mm C5 HPLC column, Supelco, Bellefonte, PA, USA), and analyzed by SDS-PAGE.

SDS-PAGE

The quality of the HDM extract was analyzed by 12 % SDS-PAGE and the resolved proteins were stained with Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany), as outlined by Laemmli *et al.*[13]

Production of the polyclonal antibodies to HDM extract

Two rabbits were immunized according to the protocol described by Harlow and Lane.[14] In brief, 0.25 mL of the HDM extract (0.50 mg mL^{-1}) was mixed with 0.25 mL FCA (Freund's complete adjuvant) for the first immunization. Every 15 days, for six months, the rabbits were boosted with a mixture of 0.25 mL of the HDM extract and 0.25 mL of FCA. Each rabbit was subcutaneously immunized with 0.50 mL of the emulsion. After six months, sera were collected and the antibodies were purified using Protein A Sepharose[15] (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

Patients' sera

Patients allergic to house dust mites (n = 17; 10 male and 7 female) were selected according to their case history, positive skin prick test and specific IgE for *D. pteronyssinus* extract (CAP-FEIA System, Unicap 100; Phadia, Uppsala, Sweden). Sera from three non-allergic individuals were pooled and used for control purposes.

Western blot

Proteins of the HDM (*D. pteronyssinus*) extract or isolated 68 kD protein were resolved by SDS-PAGE and electro-transferred onto a nitrocellulose membrane (0.45 μm Serva, Heidelberg, Germany).[16] The pattern of the IgE reactivity was determined with individual patient's sera (dilution 1:5, in 50 mM sodium phosphate, pH 7.4, containing 100 mM NaCl, 0.50 % (v/v) Tween-20; 0.50 % (w/v) bovine serum albumin (BSA) and alkaline-phosphatase labeled monoclonal anti-human IgE (Sigma Chemical Co., St. Louis, MO, USA) as the secondary antibody. Rabbit polyclonal anti-HDM antibodies were used to evaluate the IgG reactivity profile and alkaline-phosphatase labeled goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO, USA) was used as the secondary antibody. The IgE and IgG binding patterns were visualized with a substrate solution of 1.5 mg BCIP (5-bromo-4-chloro-3-indolyl phosphate, Serva, Heidelberg, Germany) and 3.0 mg NBT (nitro blue tetrazolium, Serva, Heidelberg, Germany) in 10 mL of 100 mM Tris buffer containing 150 mM NaCl and 5 mM $MgCl_2$, pH 9.6, according to Harlow and Lane.[14]

Dot blot

Purified 68 kD allergen or HDM extract were dotted (2.0 μL, c = 0.50 mg mL^{-1}) onto nitrocellulose membrane strips (0.45 μm Serva, Heidelberg, Germany). Subsequently, the membrane was blocked with buffer A (50 mM sodium phosphate, pH 7.4, 100 mM NaCl, 0.50 % (v/v) Tween-20, 0.5 % (w/v) bovine serum albumin (BSA) and 0.05 % (w/v) sodium azide) for one hour at room temperature and incubated with patients sera at a 1:5 dilution in buffer A for 3 h at room temperature. The bound IgE was detected with alkaline-phosphatase labeled monoclonal anti-human IgE antibodies (Sigma Chemical Co., St. Louis, MO, USA). The binding patterns were visualized with a BCIP/NBT substrate solution.

IgE ELISA

A MaxiSorp ELISA plate (Sigma-Aldrich, Steinheim, Germany) was coated with the purified 68 kD protein (10 μg mL^{-1}, 100 μL *per* well) in 15 mM Na_2CO_3/35 mM $NaHCO_3$, pH 9.5, overnight at 4 °C. The plate was washed (3×10 min) with TBS containing 0.010 % (v/v) Tween 20 (TTBS). After blocking for 2 h at room temperature with 1.0 % BSA in TTBS, the individual patient sera (1:5 dilution in 0.10 % (w/v) BSA in TTBS) were added to the plate and incubated overnight at 4 °C. The bound IgE was detected using alkaline-phosphatase labeled anti-human IgE (1:1000 Sigma Chemical Co., St. Louis, MO, USA). Following a 2 h incubation, the plate was subsequently incubated for 1 h with a substrate solution (1.0 mg mL^{-1} *p*-nitrophenyl phosphate in 100 mM diethanolamine buffer, pH 9.6), and the absorbance was measured at 405 nm. Absorbance values were considered positive if they exceeded the mean $A_{405\ nm}$ of the negative control by >3 *SD*. Due to the lack of sufficient sera, patients No. 7, 9, and 17 were not tested using ELISA.

Skin prick testing

For skin prick test (SPT), the 68 kD allergen (20 μg mL^{-1}) was prepared in PBS, as previously described.[17] Histamine phosphate at 1.0 mg mL^{-1} and PBS were used as the positive and negative control, respectively. The results of the SPTs were evaluated after 20 min and a wheal of at least 3 mm was considered positive. The skin prick testing was performed with the approval of the Ethics Committee of the University Children's Hospital and informed written consent was given by all patients.

RESULTS AND DISCUSSION

IgE reactivity of house dust mite extract

To examine the pattern of IgE reactivity in the Serbian population, seventeen individuals with a positive clinical history of HDM allergy were selected. Their clinical data are presented in Table I. A specific IgE to house dust mite allergens

TABLE I. Clinical and serological characterization of the house dust mite allergic subjects; ND – not determined

Patient	Sex	CAP class	Symptoms and diagnosis	Sensitization	ELISA 68 kD allergen
1. JM	M	6	Rhinoconjunctivitis	Dermatophagoides, g×1	+
2. NN	F	6	Asthma	Dermatophagoides	+
3. TS	M	6	Atopic dermatitis	Dermatophagoides	–
4. SM	F	6	Asthma	Dermatophagoides	+
5. SB	M	6	Rhinitis	Dermatophagoides, w3	+
6. TM	M	6	Asthma	Dermatophagoides	+
7. MM	M	5	Rhinitis, Asthma	Dermatophagoides	ND
8. ČS	F	5	Rhinitis	Dermatophagoides	–
9. ĆI	F	5	Asthma	Dermatophagoides	ND
10. MF	M	5	Rhinitis	Dermatophagoides	–
11. MD	F	5	Rhinitis	Dermatophagoides	–
12. KD	M	5	Rhinitis	Dermatophagoides, w6	+
13. KI	M	5	Asthma	Dermatophagoides	+
14. ĐB	M	4	Rhinitis	Dermatophagoides, g×1, w×1	ND
15. SJ	F	4	Rhinosinusitis	Dermatophagoides, w×1, w×3	+
16. MG	M	4	Rhinitis	Dermatophagoides, g×1, w×1, t×5	+
17. PS	F	4	Asthma	Dermatophagoides	ND

determined by ImmunoCAP was scored as: class 6 in six patients, class 5 in seven patients and class 4 in four patients. From the seventeen HDM allergic patients, six were co-sensitized to weed, grass and/or tree pollen. The pattern of the IgE reactivity in Western blot analysis with individual patient sera is shown in Fig. 1. The HDM extract showed a complex IgE-binding pattern with bands of various molecular mass ranging from 116–10 kD. The major allergens in this population of HDM allergic subjects with IgE reactivity by more than 50 % were proteins with molecular mass of about 14, 27, 47, 68 and 116 kD. The band at about 14 kD showed the strongest intensity in the IgE binding, probably representing Der p 2, and/or Der p 5.[2,18] The IgE binding in the range of 20–35 kD may represent Der p 6, Der p 7, Der p 8, and Der p 9. A strong IgE binding of the 68 kD protein was observed in four patients with a high specific IgE (patients 1, 2, 5, and 6).

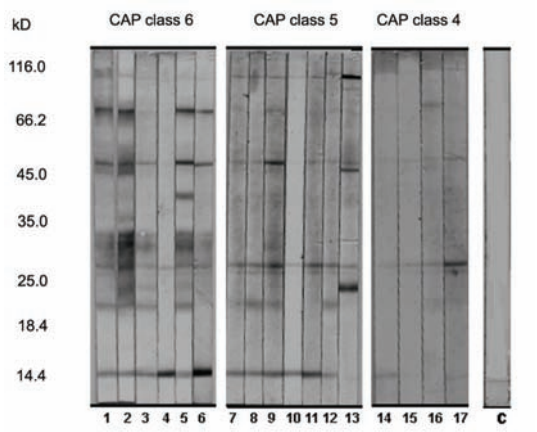
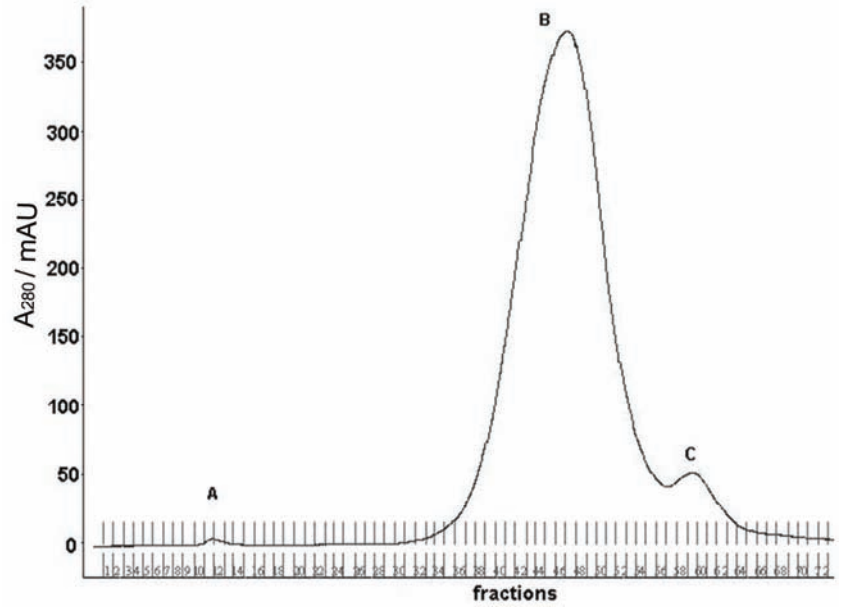


Fig. 1. IgE reactivity profile to HDM allergen extract obtained with HDM patients' sera.

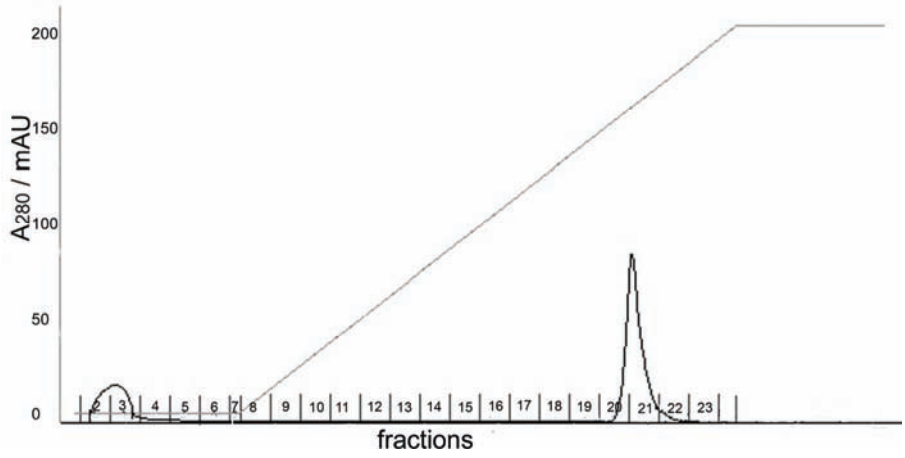
Isolation of the 68 kD allergen

The 68 kD allergen was isolated from the HDM extract by a combination of size-exclusion chromatography on a Superdex 200 column and subsequent reversed-phase chromatography on a C5 column. The HDM proteins were resolved into three peaks by gel permeation chromatography, pooled according to the chromatogram fractions: 11–12, 35–54 and 57–63 (Fig. 2a), and analyzed by SDS PAGE and Western blot (Fig. 3). Pool A contained high molecular mass proteins of about 60–116 kD and 30–40 kD, pool B contained a doublet of about 25–30 kD and low-molecular mass proteins of 15–20 kD and 25–40 kD, while pool C contained proteins with molecular masses of about 14 and 35 kD. Pool A was applied on the C5 column and a 68 kD protein was eluted with 79 % acetonitrile containing 0.10 % TFA (Fig. 2b), and was analyzed by SDS-PAGE (Fig. 3a) and Western blot (Fig. 3b). According to the literature, 21 allergens[5] from *Dermatophagoides pteronyssinus* house dust mite, with a wide range of molecular

masses (7–177 kD),[6,19] have been identified. Recently, two recombinant house dust mite allergens, Der p 15, with a molecular mass of about 60 kD, and Der p 18, of about 50 kD, were characterized.[20] However, there is no report in the literature on the properties of a 68 kD house dust mite protein.

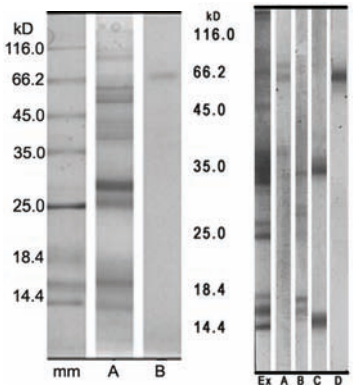


(a)



(b)

Fig. 2. Separation of the HDM extract by a) size-exclusion chromatography on a Superdex 200 column and b) reversed-phase chromatography on a C5 column.



(a) (b)

Fig. 3. Isolation of the 68 kD protein: a) SDS PAGE analysis: HDM extract (A), purified 68 kD protein (B); molecular mass proteins (mm) and b) Western blot: HDM extract (Ex), A pool of 11-12 fractions (A), B pool of 35-54 fractions (B), C pool of 57-63 fractions (C), A pool after C5 reversed-phase chromatography (D).

IgE and IgG reactivity of the 68 kD allergen

IgE and IgG reactivity of the 68 kD allergen was confirmed using dot blot analysis (Fig. 4) with a pool of patient sera and rabbit polyclonal antibodies, respectively. In addition, IgE reactivity was evaluated with sera from individual patients using ELISA (Fig. 5). These results suggest that the IgE binding epitopes of the isolated 68 kD allergen were preserved during the purification process and nine from fourteen tested patients (64 %) showed a specific IgE reactivity (Table I) to the 68 kD allergen.

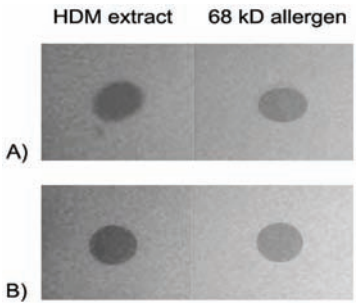


Fig. 4. IgG (A) and IgE (B) reactivity of dot-blotted HDM extract and 68 kD allergen.

For the evaluation of the biological activity, five patients were skin prick tested with a solution containing the 68 kD allergen (20 μg mL^{-1}). The positive results in all the tested patients (Table II) suggested that the 68 kD allergen is capable of bridging specific IgE antibodies on the mast cells and triggering the release of preformed biologically active mediators, such as histamine. It is noteworthy that the isolated allergen induced a larger skin prick test reaction compared to the HDM extract in three patients. Taking into consideration the prevalence of IgE reactivity in the tested group of HDM allergic subjects, the 68 kD allergen is a good candidate for component-resolved allergy diagnosis, or at least for patients from the Serbian climate.

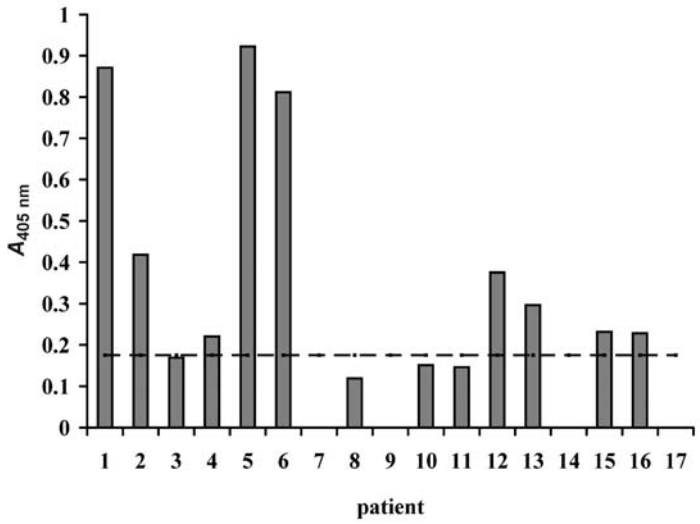


Fig. 5. Histogram: IgE ELISA to 68 kD allergen.

Table II. Skin prick test reactivity (wheal×flare, mm^2) to HDM extract and 68 kD allergen

Allergen	Patient No.				
	1	2	3	4	5
HDM extract	8×20	5×10	4×15	8×30	10×35
68 kD allergen	9×30	7×10	4×0	9×30	9×30
Histamine	5×20	5×10	5×25	4×0	–

CONCLUSIONS

A high-molecular mass allergen of about 68 kD was isolated from a HDM extract and its IgE reactivity was evaluated. Using a combination of two biochemical methods, gel permeation chromatography and reversed-phase chromatography, a 68 kD protein was isolated. The strong IgE binding of the 68 kD allergen was observed in patients with a high level of specific IgE (CAP class 6). IgE reactivity was confirmed in 64 % of the tested HDM allergic patients by ELISA and its biological activity was confirmed in five HDM allergic subjects. According to these results, the 68 kD allergen is a good candidate for component--resolved allergy diagnosis of house dust mite allergy. Further biochemical characterization, such as amino acid sequence determination, should provide more data on its structural features. Additional clinical studies should evaluate its allergenic potency in HDM allergic patients with lower level of specific IgE (CAP class < 4), as well as its diagnostic potential in the pediatric population.

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ИЗВОД

ИЗОЛОВАЊЕ И КАРАКТЕРИЗАЦИЈА 68 kD АЛЕРГЕНА ИЗ ЕКСТРАКТА КУЋНИХ ГРИЊА

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Грињe из кућне прашине представљају један од главних извора алергена који су у значајној мери допринели порасту првог типа преосетљивости. Преко 30 IgE-везујућих протеина из кућне прашине је детектовано до данас. Алергени групе 1 и 2 означени су као главни алергени кућне прашине. Међутим, да би се побољшала сигурност и ефикасност дијагнозе и терапије алергијских обољења изазваних грињама из кућне прашине, неопходно је одредити клинички значај свих алергена из овог алергенског извора. U овом раду изолован је алерген високе молекулске масе од 68 kD из екстракта кућне прашине комбиновањем гел-пермеационе хроматографије и реверсно-фазне хроматографије. IgG и IgE реактивност пречишћеног протеина је проверена у «Western blot»-у и «dot blot»-у са поликлонским зечијим антителима на екстракт кућне прашине и «pool»-ом серума особа алергичних на кућну прашину, редом. 64 % пацијената је показало IgE реактивност на пречишћени протеин у ELISA тесту. Биолошка реактивност пречишћеног алергена је потврђена у кожним пробама на пет пацијената, указујући да је пречишћен алерген добар кандидат за дијагнозу алергије на кућну прашину појединачним компонентама и евентуални терапеутски третман.

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