

Characterization of Folded Recombinant Der p 5, a Potential Diagnostic Marker Allergen for House Dust Mite Allergy

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Key Words

Dermatophagoides pteronyssinus · Der p 5 · Cross-reactivity · Diagnosis · Asthma · Therapy

Abstract

Background: Der p 5 was reported as an important allergen in *Dermatophagoides pteronyssinus*, which is particularly recognized by patients suffering from asthma. The aim of this study was to produce, by recombinant DNA technology, a folded Der p 5 allergen for diagnostic, therapeutic and preventive purposes. **Methods:** Der p 5-encoding cDNA was isolated from a λ gt11 *D. pteronyssinus* expression cDNA library and expressed in *Escherichia coli*. rDer p 5 was purified to homogeneity and characterized by mass spectroscopy and circular dichroism. IgE reactivity was tested with sera from 117 mite-allergic patients and in a basophil histamine release experiment. Der p 5-specific rabbit IgG antibodies were produced for the ultrastructural localization of the allergen in mites by immunogold electron microscopy as well

as for cross-reactivity studies. **Results:** rDer p 5 is a heat-stable protein with predominantly α -helical secondary structure which reacted with IgE from 31% of mite-allergic patients' sera and showed no relevant cross-reactivity to group 5 allergens from storage mites and tropical mites. rDer p 5-specific rabbit IgG antibodies inhibited mite-allergic patients' IgE binding to Der p 5 and allowed to localize the allergen in secretory granules of midgut epithelial cells of house dust mites. **Conclusions:** The described rDer p 5 molecule may be useful for diagnosis and immunotherapy of house dust mite-allergic patients.

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Introduction

House dust mites present one of the most potent allergen sources worldwide with an overall prevalence of sensitization of more than 20% and the allergens of house dust mites are, beside cat allergens, strongly associated

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with asthma [1]. House dust mites contain several allergenic molecules, among which Der p 5 has been identified as an important allergen [2, 3]. The cDNA coding for the Der p 5 allergen was one of the first allergen-encoding cDNAs isolated by recombinant DNA technology [4]. Der p 5 appears only in small amounts in whole mite body extracts, but serological IgE reactivity testing and skin testing with a recombinant Der p 5 (rDer p 5) molecule expressed as glutathione S-transferase fusion protein provided evidence that Der p 5 is an important allergen and more frequently recognized by patients suffering from allergic asthma than by patients with only rhinoconjunctivitis [5, 6]. Group 5 allergens have also been detected in other mite species besides house dust mites, such as tropical mites (for example *Blomia tropicalis*) and storage mites [7, 8]. However, little information regarding potential cross-reactivity between group 5 allergens from storage mites and house dust mites is available [9–11], and controversial information exists regarding cross-reactivity between *Dermatophagoides pteronyssinus* and *B. tropicalis* [12–17]. Although a considerable cross-reactivity between Der p 5 and *B. tropicalis* extract has been found [14], studies with Der p 5 and Blo t 5 demonstrated that Der p 5 shows low levels of IgE cross-reactivity with the homologous Blo t 5 [15–17]. The results suggest that the group 5 allergens of *D. pteronyssinus* and *B. tropicalis* are species-specific.

The aim of the present study was to obtain, by recombinant DNA technology, an authentic, structurally folded rDer p 5 molecule for diagnostic, therapeutic and preventive clinical use. The rDer p 5 molecule was studied by physicochemical technology regarding fold, tested for IgE reactivity in more than 100 mite-allergic patients and used as a probe to generate Der p 5-specific antibodies to study cross-reactivity among group 5 allergens from various mite species. Furthermore, the allergen was localized by immunogold electron microscopy at the ultrastructural level in house dust mites to gain information about the mode of sensitization to Der p 5.

Materials and Methods

Allergic Patient Sera, Allergens and Rabbit Antibodies

Sera were from 117 house dust mite (*D. pteronyssinus*)-allergic patients with positive case history indicative for house dust mite allergy and house dust mite-specific IgE antibodies (>0.7 kU_A/l) as determined with the ImmunoCAP System (Phadia AB, Uppsala, Sweden) [18]. Sera from nonallergic subjects and nonmite-allergic patients (n = 13) were also included.

Natural Der p 1 was isolated by affinity chromatography using the monoclonal antibody 4C1 [19]. Lep d 5 was expressed in *E. coli*

and purified as described [8]. Blo t 5 was a gift from Kaw Y. Chua, Department of Pediatrics, National University of Singapore.

Rabbit anti-rDer p 5 antibodies were obtained by immunization of a rabbit 3 times with 200 µg of purified rDer p 5 per injection using once Freund's complete and twice Freund's incomplete adjuvants (Charles River, Kisslegg, Germany). Preimmune serum was obtained from the rabbit before immunization.

Expression and Purification of rDer p 5

D. pteronyssinus mites were purchased from Allergon (Vällinge, Sweden). A *D. pteronyssinus* cDNA library was constructed in λgt11 as described [20]. *E. coli* Y1090 were infected with 3×10^5 plaque-forming units, plated onto Luria-Bertani plates containing 100 µg/ml ampicillin, and recombinant protein expression was induced by overlaying the plates with nitrocellulose filters (Schleicher & Schuell, Dassel, Germany) soaked in 10 mM isopropyl β-D-thiogalactopyranoside after plaques became visible. IgE-reactive phage clones were identified with pooled serum IgE from 5 mite-allergic patients and isolated as described [21]. The DNAs of the phage clones were polymerase chain reaction (PCR) amplified using λgt11 primers [21], sequenced (MWG, Ebersberg, Germany) and compared with sequences submitted to the GenBank database at the National Center for Biotechnology Information. A cDNA coding for the mature protein of Der p 5 was identified and amplified by PCR using the following primers and PCR conditions: forward, 5'-GGAATTCCATATGGAAG-ATAAAAAACATGAT-3'; reverse, 5'-GAATTCCTTAACTTC-AATCTTTTAAAC-3'; 94°C for 30 s, 50°C for 30 s and 72°C for 30 s for 35 cycles. The PCR product was subcloned via the *Eco*RI (italics) and *Nde*I (underlined) restriction sites into the plasmid pET 17b (Novagen, Madison, Wisc., USA). Plasmid pET 17b containing the Der p 5 DNA was transformed into *E. coli* BL21 (DE3) (Stratagene, La Jolla, Calif., USA) and cells were grown to an OD₆₀₀ of 1.2 in Luria-Bertani medium containing 100 mg/l ampicillin. The expression of the recombinant protein was induced by addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.5 mM and cultured for additional 3 h at 37°C. *E. coli* cells from 1 liter of culture were harvested by centrifugation, resuspended in 10 ml 25 mM imidazole pH 7.4, 0.1% (v/v) Triton X-100 and treated with 100 µg lysozyme for 20 min at room temperature. The lysate of the bacterial cells was frozen in liquid nitrogen and defrosted in a 50°C water bath. Genomic DNA was digested by addition of 1 µg DNase I for 10 min at room temperature and subsequently 200 µl 5 M NaCl were added. A fraction containing the soluble proteins was obtained after centrifugation of the homogenate in an SS34 rotor (RC5C Sorvall; DuPont, Boston, Mass., USA) at 18,000 rpm at 4°C for 20 min. rDer p 5 was enriched by adding ammonium sulfate to 70% saturation at 0°C. After centrifugation (18,000 rpm, 4°C, 20 min), the supernatant containing the protein was dialyzed against buffer A (10 mM Tris-Cl pH 7.0, 4% isopropanol, 10 mg/l phenylmethylsulphonylfluoride) and applied to a DEAE Sepharose Fast Flow column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). rDer p 5-containing fractions were eluted by a 0–500 mM NaCl gradient, pooled, dialyzed against buffer B (10 mM Na₂HPO₄ pH 4.0, 5 mM β-mercaptoethanol, 10 mg/l phenylmethylsulphonylfluoride) and applied to an SP Sepharose Fast Flow column (GE Healthcare Bio-Sciences AB). rDer p 5 was eluted by a combined pH 4.0–7.0 and 0–500 mM NaCl gradient. Fractions containing more than 90% pure Der p 5 were pooled, dialyzed against 10 mM Na₂HPO₄ pH

7.0 and stored at -20°C . Protein samples were analyzed for purity by Coomassie blue-stained SDS-PAGE [22]. The protein concentration was determined with the Micro BCA Protein Assay Kit (Pierce, Rockford, Ill., USA).

Matrix-Assisted Laser Desorption Ionization Mass Spectrometry

rDer p 5 was analyzed by matrix-assisted laser desorption ionization mass spectrometry using a Kratos Axima CFR mass spectrometer in the linear mode (piChem, Graz, Austria). rDer p 5 was dissolved in 10% acetonitrile/0.1% trifluoroacetic acid, and α -cyano-4 hydroxycinnamic acid (dissolved in 60% acetonitrile, 0.1% trifluoroacetic acid) was used as a matrix. For sample preparation, a 1:1 mixture of protein solution and matrix solution was deposited onto the target and air dried.

Circular Dichroism Spectroscopy and Secondary Structure Determination

rDer p 5 was dissolved in MilliQ H_2O to a final concentration of 0.265 mg/ml. Circular dichroism (CD) measurements were performed on a J-715 spectropolarimeter (Jasco, Tokyo, Japan) using 0.1-cm path length cylindrical cells. Far ultraviolet (UV) CD spectra of rDer p 5 were recorded at 20°C at wavelength ranges from 250 to 185 nm. Each spectrum was recorded as an average of 3 scans, with 0.2 nm resolution at a scan speed of 50 nm/min. Temperature scans were performed according to a step-scan procedure, where the sample was heated to 95°C with a heat rate of $1^{\circ}\text{C}/\text{min}$ and then cooled to 20°C with the same rate. Temperature scans were recorded at 222 nm with a step resolution of 0.5°C . Every 5°C , continuous wavelength spectra were recorded with the specified parameters. Results were expressed as the molar mean residue ellipticity (θ_{MRE}) at a given wavelength. The secondary structure content of rDer p 5 was calculated using the secondary structure estimation program CDSSTR [23, 24]. The normalized root mean square deviation parameter was used to determine the correlation between the best calculated structure and the experimental data and is defined as: $\text{sum}[(\text{exp} - \text{cal})^2/(\text{exp}^2)^{1/2}]$ summed over all wavelengths, where exp is the experimental ellipticity and cal the ellipticity of the back-calculated spectra for the derived structure [25].

Histamine Release Assay

Peripheral blood mononuclear leukocytes were isolated from heparinized blood samples of a *D. pteronyssinus*-allergic individual by dextran sedimentation [26]. Cells were incubated with increasing concentrations (10^{-7} to 10^{-1} $\mu\text{g}/\text{ml}$) of nDer p 1 and rDer p 5. Histamine released in the cell-free culture supernatant was measured by radioimmunoassay (Immunotech, Marseille, France) and indicated as percentage of total cellular histamine release. Results are displayed as mean values of triplicate determinations.

Nondenaturing IgE-Binding Assay and IgE Competition Assay

Specific IgE reactivity of nondenatured, dot-blotted rDer p 5 and nDer p 1 were analyzed by nondenaturing dot blot assays. 0.5 μg of each protein (0.25 $\mu\text{g}/\mu\text{l}$) was dotted onto nitrocellulose strips (Schleicher & Schuell). The strips were blocked with buffer C [50 mM sodium phosphate pH 7.4, 0.5% (v/v) Tween-20, 0.5% (w/v) BSA and 0.05% (w/v) sodium azide] and exposed to sera

from mite-allergic patients at a 1:10 dilution in buffer C. Bound IgE antibodies were detected with ^{125}I -labeled anti-human IgE antibodies (Phadia AB) diluted 1:10 in buffer C, and visualized by autoradiography (Kodak XOMAT film; Kodak, Heidelberg, Germany). Thirteen nonmite-allergic and 13 mite-allergic patients (Der p 5 positive) were tested for IgE reactivity to Der p 5 in an ELISA assay as described [18].

The ability of rabbit antibodies raised against purified rDer p 5 to inhibit the binding of mite-allergic patients' IgE to rDer p 5 was examined in ELISA inhibition assays [27]. The degree of inhibition was calculated as follows: % inhibition of IgE binding = $100 - \text{OD}_{\text{anti-Der p 5 serum}} \times 100/\text{OD}_{\text{preimmune serum}}$.

IgE Inhibition Experiments

Inhibitions were performed with mite-allergic patients' sera which were double positive to Der p 5 and Lep d 5 or Blo t 5 by preadsorbing 1:10 in buffer C-diluted sera with 10 $\mu\text{g}/\text{ml}$ protein (Der p 5, Blo t 5, Lep d 5 or BSA) at 4°C overnight. 0.5 μg aliquots of the proteins (0.25 $\mu\text{g}/\mu\text{l}$) were dotted onto nitrocellulose strips and then incubated with the preadsorbed sera. Bound IgE antibodies were detected as described before.

Mite Extracts and Immunoblots

Mite extracts from *Glycyphagus domesticus*, *Lepidoglyphus destructor*, *B. tropicalis*, *Tyrophagus putrescentia*, *D. pteronyssinus* and *Dermatophagoides farinae* (Allergon, Vällinge, Sweden) were prepared by homogenization of 0.3 g mites in 5 ml SDS buffer [62 mM Tris, 200 mM SDS, 10% (v/v) glycerol, 5% (v/v) β -mercaptoethanol, 2.5% (v/v) bromphenolblue dissolved in 1% methanol, pH 6.8] using an Ultra-Turrax T25 Basic disperser (IKA, Staufen, Germany). After incubation at 95°C for 10 min, the debris was removed by centrifugation at 4,000 rpm and 4°C for 10 min. The quality of the extracts was analyzed by SDS-PAGE and Coomassie Brilliant Blue (BioRad, Hercules, Calif., USA) staining [22]. Equal amounts of each extract according to Coomassie blue staining were separated by 14% SDS-PAGE and blotted onto nitrocellulose (Schleicher & Schuell) [28]. Membranes were blocked in buffer C twice for 10 min and once for 30 min and incubated with rabbit anti-rDer p 5 serum and the corresponding preimmune serum, diluted 1:1,000 in buffer C overnight at 4°C . Bound antibodies were detected with 1:1,000 diluted ^{125}I -labeled anti-rabbit antibodies from donkey (GE Healthcare Bio-Sciences AB) and visualized by autoradiography (Kodak XOMAT film).

Immunogold Electron Microscopy

Anti-Der p 5 IgG antibodies were isolated by affinity chromatography (ImmunoPure IgG Purification Kit; Pierce) from the serum of a Der p 5-immunized rabbit and further purified via affinity to column-coupled Der p 5 (AminoLink Kit; Pierce). IgG antibodies that did not bind to immobilized Der p 5 were used for control experiments. IgG concentrations of the anti-Der p 5 antibodies and the control antibody preparation were adjusted by photometry at 280 nm.

Goat anti-rabbit IgG antibodies coupled to colloidal gold particles with a diameter of 10 nm were supplied by British BioCell (Plano, Wetzlar, Germany). House dust mites (*D. pteronyssinus*) were cultivated at Leti (Madrid, Spain). The mites were collected from the culture using a fine moist needle and placed on the surface of a vessel filled with tap water in order to clean them from adhering particles of the culture substrate. They were put into a

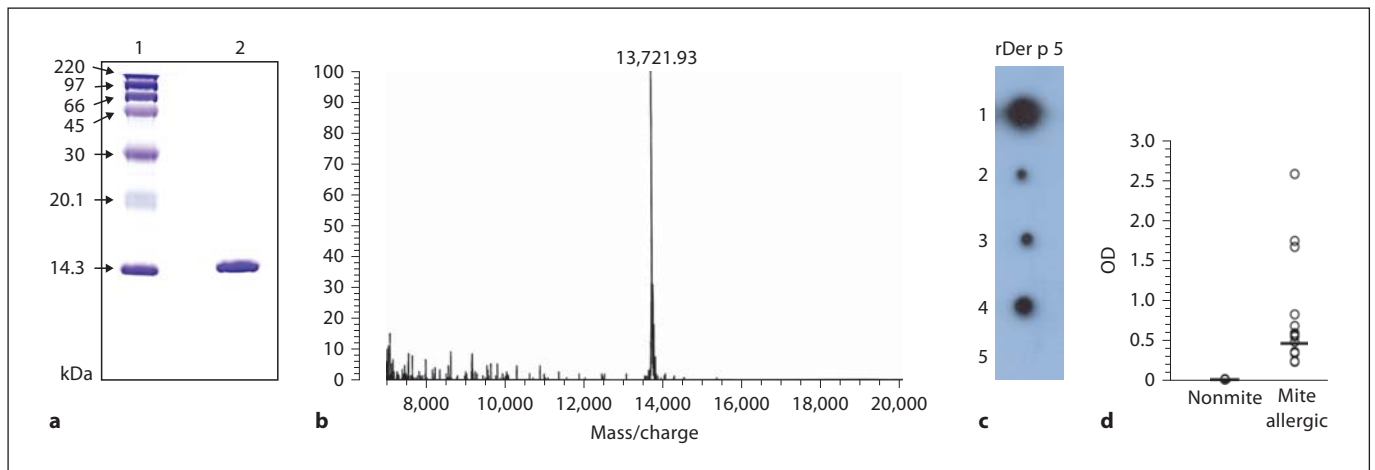


Fig. 1. Characterization of purified rDer p 5. **a** Coomassie-stained SDS-PAGE containing a molecular weight marker (lane 1) and 3 μ g of purified rDer p 5 (lane 2). **b** Mass spectroscopic analysis of rDer p 5. **c** Specific IgE reactivity of dot-blotted rDer p 5 deter-

mined with sera from 4 mite-allergic patients (1–4) and 1 nonallergic person (5). **d** OD values of Der p 5-specific IgE antibodies of sera from nonmite-allergic ($n = 13$) and Der p 5-positive patients ($n = 13$) determined in an ELISA assay.

mixture of 6% p-formaldehyde/0.5% glutaraldehyde in 0.3 M PIPES buffer, pH 7.3, and fixed at 4°C for 3 days. Fixed specimens were dehydrated in an ascending series of ethanol at 4°C, embedded in Lowicryl K4M at 4°C and finally polymerized at –35°C. Ultrathin sections were cut and placed on silver grids for immunocytochemical detection of the allergen.

The labeling protocol for the detection of the mite allergen was as follows (room temperature, moist chamber, PBS buffer/0.1% BSA, pH 7.4/Tris buffer/0.1% BSA, pH 8.2): (1) 5% BSA in PBS buffer, 15 min; (2) rabbit anti-Der p 5 antibodies or control rabbit IgG antibodies, diluted 1:30 in PBS buffer, 2 h; (3) PBS buffer, 1 $\times$ 5 min, Tris buffer, 2 $\times$ 5 min; (4) Goat anti-rabbit IgG antibodies, coupled to 10-nm colloidal gold particles, diluted 1:20 in Tris buffer, 1 h; (5) Tris buffer, 1 $\times$ 5 min, distilled water, 2 $\times$ 5 min. After immunolabeling, sections were stained with uranyl acetate and lead citrate. The sections were examined in a Philips EM 410 (FEI, Eindhoven, Netherlands) at 80 kV. Photographs were taken using 23D56 films (Agfa Gevaert, Mortsel, Belgium).

Results

Expression and Purification of Folded IgE-Reactive rDer p 5

The cDNA of Der p 5 was isolated with IgE from mite-allergic patients out of a *D. pteronyssinus* λ gt11 cDNA library. The sequence of the isolated Der p 5 clone was identical to the Der p 5 sequence deposited in GenBank (S76337). The DNA sequence coding for the 113 amino acid mature protein was expressed using the plasmid pET 17b in *E. coli* and purified by ion exchange chromatography to homogeneity (fig. 1a). The yield of purified rDer

p 5 was between 35 and 45 mg protein/liter of *E. coli* culture. The molecular weight of rDer p 5 determined by mass spectroscopic analysis was 13.72 kDa (fig. 1b), which is in agreement with the calculated molecular weight of 13.72 kDa. Purified rDer p 5 was examined for specific IgE reactivity with sera from 117 house dust mite-allergic patients. Of these 117 sera, 36 (31%) showed IgE reactivity to Der p 5. In comparison, 100 of these sera were also tested for IgE reactivity to the mite allergen Der p 1 and 88% of them were Der p 1 positive. In figure 1c, serum IgE reactivity to rDer p 5 of 4 representative Der p 5-positive patients is exemplified. Serum from a nonallergic individual showed no IgE reactivity to Der p 5 (fig. 1c, lane 5). In figure 1d, 13 nonmite-allergic and 13 mite-allergic patients were tested for IgE reactivity to Der p 5. Only mite-allergic patients showed strong IgE reactivity to Der p 5 (median OD 0.478), whereas nonmite-allergic individuals showed no IgE reactivity to Der p 5 (median OD 0.036).

rDer p 5 Represents a Folded Protein with Predominant α -Helical Structure

Analysis of the far UV CD spectrum of purified rDer p 5 at room temperature showed a typical α -helical spectrum with 2 minima, at 208 and 222 nm, and a large maximum at 192.5 nm (fig. 2a). The program CDSSTR with the reference dataset 3 was used to calculate the fractions of secondary structures and yielded 86% α -helix, 0% β -turns, 5% turns and 8% random coil structures. The cal-

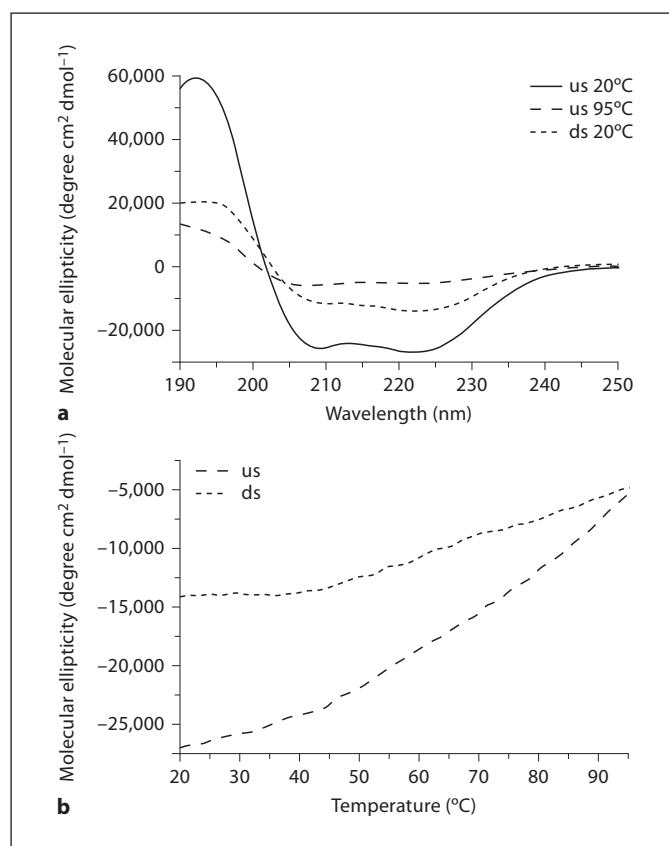


Fig. 2. Far UV CD analysis of Der p 5. **a** CD spectra recorded at +20°C (us 20°C), after heating to +95°C (us 95°C) and cooling to +20°C (ds 20°C). Results are expressed as molecular ellipticity (y-axis) at different wavelengths (x-axis). **b** Thermal unfolding (us) and refolding curves (ds). Molecular ellipticities (y-axis) were recorded at 222 nm at different temperatures (x-axis).

culated spectrum was compared to the experimental spectrum and yielded a very good fit with a normalized root mean square deviation value between the experimental and calculated spectrum of 0.03. Upon heating to 95°C, the protein was largely unfolded (fig. 2) and cooling to 20°C restored approximately 50% of the fold (fig. 2a). An unusually high transition temperature was estimated (95–100°C) according to the shape of the unfolding curve.

rDer p 5 Induces Specific Basophil Histamine Release

The allergenic activity of rDer p 5 was evaluated in a basophil activation experiment. In a representative experiment, rDer p 5 induced specific and dose-dependent histamine release from granulocytes of a mite-allergic patient (fig. 3). In this patient, an induction of more than

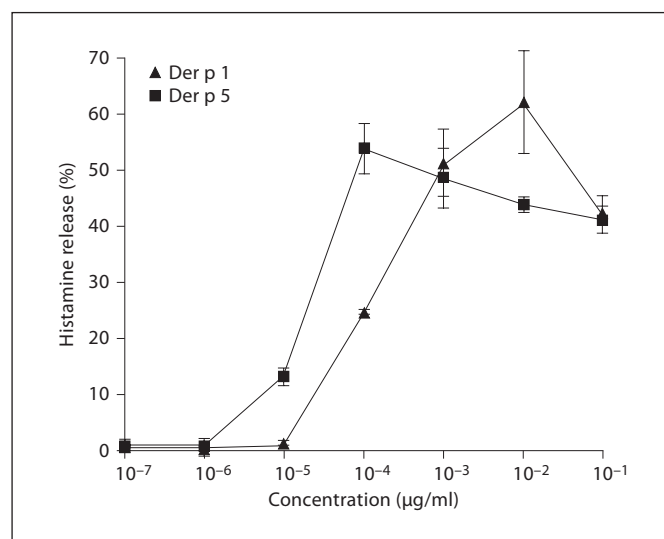


Fig. 3. rDer p 5 induces basophil histamine release. Peripheral blood mononuclear leukocytes of a *D. pteronyssinus*-allergic patient were incubated with various concentrations of nDer p 1 and rDer p 5 (x-axis). The percentage of total histamine released into the cell-free culture supernatant is shown on the y-axis.

50% histamine release was observed already at very low concentrations of rDer p 5 (10⁻⁴ µg/ml). Natural Der p 1, the major allergen of *D. pteronyssinus*, showed 50% histamine release at 10⁻³ µg/ml in this patient.

Der p 5 Shows No Relevant IgE Cross-Reactivity with Lep d 5 and Blo t 5

In order to study whether there is IgE cross-reactivity between Der p 5 and group 5 allergens from storage mites and tropical mites, IgE cross-reactivity experiments were performed with sera that were double positive to Der p 5 and Lep d 5 or Blo t 5. Only few of the Der p 5-positive sera also showed weak IgE reactivity to Lep d 5 and/or Blo t 5 (patients 15, 57, 87 and 110 with Der p 5 and Lep d 5; patients 15 and 71 with Der p 5 and Blo t 5; fig. 4). Neither Lep d 5 nor Blo t 5 induced a relevant inhibition of IgE binding to Der p 5 and Der p 5 induced inhibition of IgE binding to Lep d 5 and Blo t 5 only in 1 patient (patient 15).

Immunization with rDer p 5 Induces IgG Antibodies Which Block Patients' IgE Reactivity to Der p 5

An ELISA competition assay was performed to test whether IgG antibodies induced by immunization with rDer p 5 are able to block the binding of allergic patients' IgE to Der p 5. ELISA plate-bound Der p 5 was preincu-

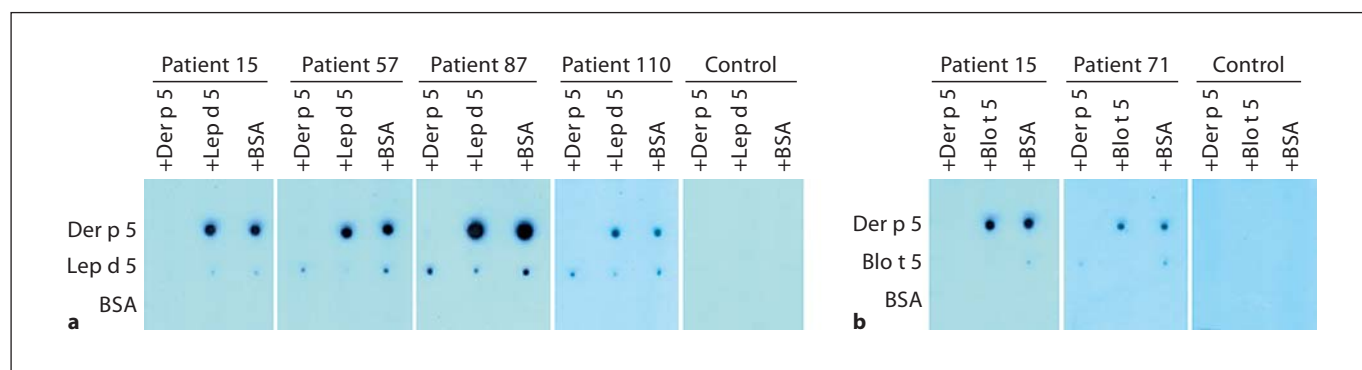
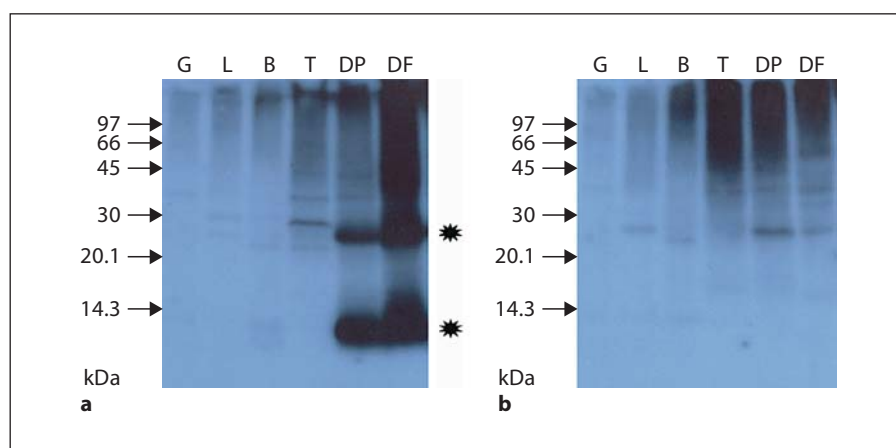


Fig. 4. Lack of relevant IgE cross-reactivity between Der p 5 and Lep d 5 or Blo t 5. Sera from mite-allergic patients with IgE reactivity to Der p 5 and Lep d 5 (patients 15, 57, 87 and 110; **a**) and Der p 5 and Blo t 5 (patients 15 and 71; **b**) as well as serum from a

nonallergic individual (control) were preadsorbed with group 5 allergens or BSA (top) and then exposed to nitrocellulose-dotted group 5 allergens and BSA (left).

Fig. 5. Lack of reactivity of polyclonal rabbit anti-rDer p 5 IgG antibodies with storage mites. SDS-PAGE-separated *G. domesticus* (G), *L. destructor* (L), *B. tropicalis* (B), *T. putrescentiae* (T), *D. pteronyssinus* (DP) and *D. farinae* (DF) extracts were incubated with a rabbit anti-rDer p 5 antiserum (**a**) and the rabbit's preimmune serum (**b**). Asterisks indicate group 5 allergens (monomer, dimer).



bated with rabbit anti-Der p 5 IgG or preimmune IgG and then incubated with serum IgE from 13 mite-allergic patients. Inhibition of IgE binding ranging from 49 to 96% (mean 84%) was found (table 1). In 8 of the 13 patients, anti-Der p 5 IgG inhibited IgE binding to Der p 5 by more than 90% (table 1).

Der p 5 Is a Specific Marker Allergen for House Dust Mites

The polyclonal rabbit anti-Der p 5 IgG antibodies which had specifically blocked allergic patients' IgE reactivity to Der p 5 were used to search for cross-reactive group 5 allergens in the mites *G. domesticus*, *L. destructor*, *B. tropicalis*, *T. putrescentiae*, *D. pteronyssinus* and *D. farinae*. The anti-Der p 5 antiserum identified specifically nitrocellulose-blotted group 5 allergens (monomers:

13 kDa, dimers: 26 kDa) in *D. pteronyssinus* and *D. farinae*, but not in any of the storage mites or the tropical mite *B. tropicalis* (fig. 5).

Der p 5 Is Secreted by Midgut Epithelial Cells

The gut of mites is composed of foregut, midgut and hindgut [29, 30]. The midgut is divided into a large anterior portion with 2 diverticulae (caeca) and a posterior section. Figure 6a shows the anterior portion of the midgut lined by epithelial cells. The epithelial cells of this region have a single nucleus, numerous short microvilli, rough endoplasmatic reticulum and secretory vesicles (fig. 6b–d). The food in the lumen of the midgut is beginning to form a food ball and is surrounded by a band of material which constitutes the peritrophic membrane (fig. 6a, c).

Table 1. Rabbit anti-Der p 5 antibodies inhibit IgE binding of mite-allergic patients to Der p 5

Patient	Preimmune serum OD values	Anti-Der p 5 immune serum, OD values	Inhibition of IgE binding, %
1	0.720	0.058	95
2	0.292	0.061	86
3	2.042	0.145	94
4	0.986	0.105	91
5	1.210	0.069	96
6	0.086	0.040	71
7	0.129	0.032	91
8	0.074	0.048	49
9	0.077	0.038	71
10	0.096	0.044	70
11	0.247	0.042	91
12	0.198	0.030	96
13	0.176	0.034	92
Mean	0.487	0.057	84

Rabbit anti-rDer p 5 antibodies strongly bound to the secretory vesicles in the epithelial cells (fig. 6b), the emerging peritrophic membrane, the food ball (fig. 6c) and to fibrous structures enclosed in the food material (data not shown). Control IgG antibodies did not bind to the secretory vesicles in the midgut epithelial cells (fig. 6d) nor to structures of the gut lumen (that is, food ball; data not shown), indicating the specificity of the binding.

Discussion

Der p 5 is an important house dust mite allergen which is frequently recognized by patients suffering from allergic asthma [4, 5]. Here we produced, by recombinant DNA technology and expression as well as purification under nondenaturing conditions, a structurally folded rDer p 5 molecule. CD measurements showed that rDer p 5 is an α -helical protein with high heat stability. The experimentally derived secondary structure is in accordance with the calculated spectrum derived from the amino acid sequence of the protein [23].

rDer p 5 showed allergenic activity by inducing basophil histamine release and reacted with IgE from more than 30% of house dust mite-allergic patients. Der p 5-specific polyclonal rabbit IgG antibodies inhibited IgE reactivity of house dust mite-allergic patients almost completely and hence represented an accurate tool for cross-reactivity studies. Using the polyclonal rabbit IgG

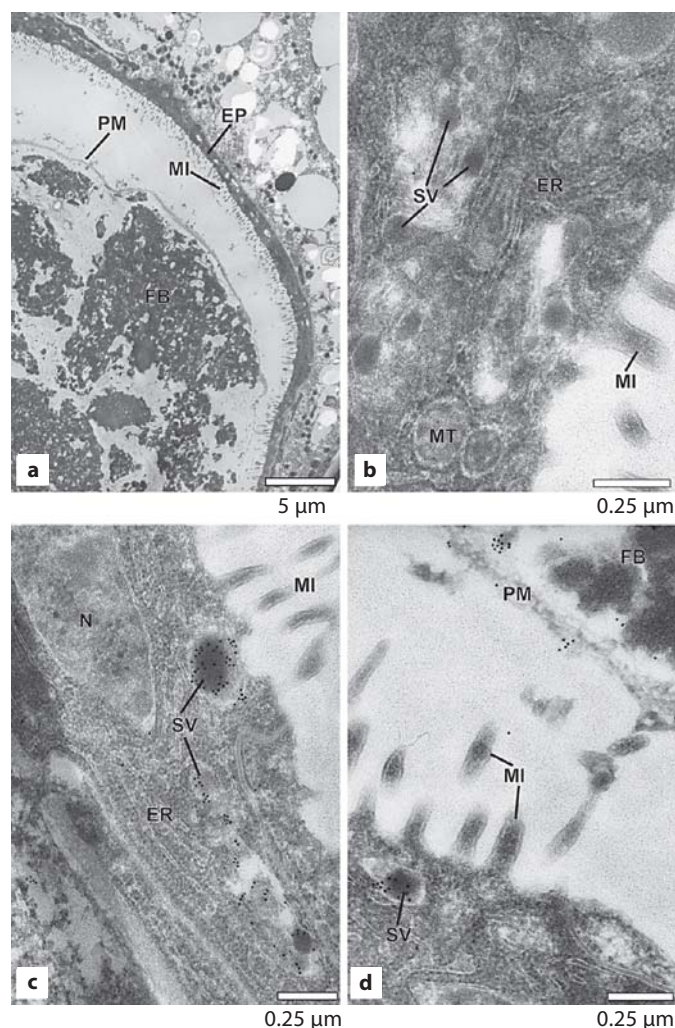


Fig. 6. Localization of Der p 5 in *D. pteronyssinus*. **a** Anterior midgut without staining. **b, c** Epithelial cells in the midgut after incubation with anti-Der p 5 antibodies. **d** Control with control Ig. Black dots correspond to sites of specific binding. EP = Epithelium; ER = endoplasmic reticulum; FB = food ball; MI = microvilli; MT = mitochondrion; N = nucleus; PM = peritrophic membrane; SV = secretory vesicle.

antibodies, we could demonstrate that Der p 5 is a specific marker allergen for house dust mites (*D. pteronyssinus*, *D. farinae*), whereas no relevant cross-reactivity was found with tropical mites and storage mites. IgE inhibition experiments performed with mite-allergic patient's sera indicated that there is also no relevant IgE cross-reactivity between Der p 5 and group 5 allergens from tropical mites and storage mites. The lack of cross-reactivity is in accordance with studies performed with *D. pteronyssinus* and *B. tropicalis*. Group 5 allergens from

D. pteronyssinus and *B. tropicalis* show 43% sequence homology, but no relevant cross-reactivity [7]. Another study assumed that the lack of cross-reactivity between Der p 5 and Blo t 5 is probably due to the fact that identical amino acids are scattered throughout the whole protein molecules and that there is no conserved peptide motif [16].

The Der p 5 molecule may therefore be a useful diagnostic marker to discriminate between allergic sensitization to house dust mites, tropical mites and storage mites in populations that are exposed to these allergen sources. A combination of *D. pteronyssinus*-specific marker allergens may then be useful for the differentiation between co- and cross-sensitization and represent a clinical aid for the accurate prescription of immunotherapy for house dust mite allergy.

Using Der p 5-specific antibodies, we could demonstrate that Der p 5 is a secretory product of gut epithelial cells. It is therefore likely that Der p 5 sensitizes patients because it becomes incorporated into respiratory fecal particles. This result is supported by the observation that the group 5 allergen Blo t 5 from *B. tropicalis* is also secreted in the gut and associated with the gut contents [31]. The fact that Der p 5 appeared to be very stable at least in thermal denaturation experiments would also explain why this protein can sensitize patients after excretion of the protein via feces in the environment.

The rDer p 5 molecule can now be used in clinical provocation studies investigating the clinical relevance of this protein and it may be considered to incorporate rDer p 5 as a relevant component in a vaccine against house dust mite allergy. In fact, we have shown that Der p 5 can induce in rabbits IgG antibodies which strongly inhibit house dust mite-allergic patients' IgE reactivity to Der p 5. Studies performed with recombinant allergens indicate that the induction of blocking IgG antibodies is an important mechanism in allergen-specific immunotherapy with recombinant allergens [32, 33]. It may therefore be expected that vaccination of house dust mite-allergic patients with rDer p 5 will also induce a robust Der p 5-specific and protective IgG response. In summary, the rDer p 5 molecule described may be a useful tool for the diagnosis and treatment of house dust mite allergy.

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