

Acid-Induced Polymerization of the Group 5 Mite Allergen from *Dermatophagoides pteronyssinus*

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House dust mites are the most important source of indoor allergens and cause allergic diseases. Our studies here suggest that the group 5 allergen from *Dermatophagoides pteronyssinus* (Der p 5) is monomeric at neutral pH, but forms filaments at low pH. Circular dichroism measurements show Der p 5 is a helical protein, and the protein sequence reveals Der p 5 contains coiled-coil helices. The acid-induced filament assembly could be explained in part by the high content of charged residues (40%) in the coiled-coil structure. Interestingly, some of the known *Dermatophagoides* allergens also contain a heptad repeat, which could potentially form coiled coils. Therefore, coiled-coil helices may be one of the common structural motifs of mite allergens that contribute to their allergenicity. © 2001 Academic Press

Key Words: mite allergen; Der p 5; acid-induced polymerization; coiled coil.

Allergic diseases such as atopic dermatitis, rhinitis and asthma afflict about 20% of the world population. House dust mites have been regarded as one of the most important sources of indoor allergens since the 1960s. Mites of *Dermatophagoides* (*D.*) species (family *Pyroglyphidae*) are the most clinically significant. More than ten groups of mite allergens have been identified and characterized (1–4). However, the biological functions of some allergens including groups 2, 5, and 7 remain unknown.

Both the group 5 allergens from *D. pteronyssinus* (Der p 5) and *B. tropicalis* (Blo t 5) were cloned using IgE plaque radioimmunoassay (5, 6). Both recombinant group 5 allergens have been produced *in vitro* and

showed to be clinically significant. Approximately 50–70% of the mite allergic patients tested positive with stronger IgE-binding than other major allergens. Der p 5 specific IgE accounted for one quarter of the total house dust mite-specific IgE (5–9). Der p 5 is made up of a 19-residue leader peptide and a 113-residue mature protein, whereas Blo t 5 is made up of a 17-residue leader peptide and a 117-residue mature protein. Der p 5 shares 40% sequence identity with Blo t 5. Recently, a partial mRNA from the mite *Lepidoglyphus destructor* which encoded a group 5-like allergen (AJ250278) with 37% protein sequence identity to Der p 5, was identified. All these three sequences have unusual amino acid compositions. They have a high content of Ile and Leu (~20%), a high content of positively charged residues (~20%), and a high content of negatively charged residues (~20%). There are, however, no tryptophan residues and a low content of proline, cysteine, and glycine residues (0–2%).

Although the amino acid sequences of many mite allergens are known, there is limited biochemical and structural information to facilitate understanding of the molecular mechanism of the allergic reaction and to help the development of effective diagnostic and therapeutic agents. Our preliminary X-ray diffraction results suggested that acidic conditions may induce Der p 5 aggregation (10). In this report, additional methods including cross linking, circular dichroism, electron microscopy, and sequence analysis are used to characterize this allergen.

MATERIALS AND METHODS

Gel filtration chromatography. The recombinant Der p 5 was expressed in *E. coli* as a fusion protein with glutathione-S-transferase, and isolated through a glutathione agarose column (Sigma). The fused protein was then cleaved by thrombin overnight and further purified by gel filtration on a HiLoad 26/60 Superdex 75 column (Pharmacia) as described previously (10). In order to determine the oligomeric state of Der p 5 by gel filtration chromatography, standard molecular weight markers were loaded into the column and their individual retention time were measured.

Abbreviations used: Der p 5, the group 5 allergen from *Dermatophagoides pteronyssinus*; Blo t 5, the group 5 allergen from *Blomia tropicalis*.

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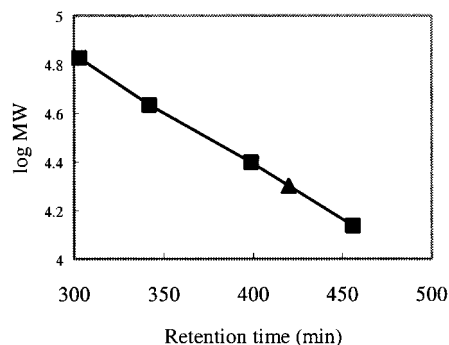


FIG. 1. The calibration curve used to estimate the molecular weight of Der p 5 (▲) by molecular exclusion chromatography. The standards (■) are bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa) (Pharmacia).

Cross linking. A few micrograms of protein in a PBS buffer were incubated with various amounts of glutaraldehyde (Merck) or with dithiobis(succinimidylpropionate) (DSP, Pierce) in a total volume of 10–200 μ l (11). After 30 to 240 min, 50 mM Tris-HCl (pH 7.5) was added to stop the DSP reaction, and the SDS-PAGE sample buffer was added to stop the glutaraldehyde reaction. The reaction samples were then separated on a 7.5% Tricine-SDS-PAGE and the protein bands visualized using Coomassie brilliant blue R-250 or silver staining.

Electron microscope and light scattering. The protein solution was pipetted onto an EM grid carbon-coated by glow-discharge. After 10–15 s, the grid was negatively stained by washing with 4–5 drops of 1% uranyl acetate, air dried and then examined using a Zeiss 109 electron microscope operated at 80 KV. For light scattering, 2 mm of protein solutions in the PBS buffer were added into a glass tube, and measured for dynamic light scattering (model DLS 7000, Otsuka). Various Der p 5 samples in the PBS buffers at pH 7.5 or 4.6 were scanned.

Circular dichroism. Circular dichroism (CD) studies were performed in a 1 mm cuvette using a Jasco CD spectropolarimeter (model J720) equipped with constant liquid nitrogen flush. The solvents used for all CD experiments were 10 mM sodium phosphate or distilled water. The results were presented as mean residual ellipticities $[\theta]$ in degree $\text{cm}^2 \text{dmole}^{-1}$. The CD spectra were measured over the range of 190–270 nm, each for three scans, with a scan rate of 20 nm/min.

RESULTS AND DISCUSSION

Acid-Induced Polymerization

The recombinant Der p 5 was confirmed by the N-terminal amino acid sequencing, containing 115 amino acid residues with two extra amino acid residues at the N-terminus, resulting from the design of the thrombin cleavage site. The protein concentration was determined by Pico-Tag quantitative amino acid analysis (Waters). The mean extinction coefficient of Der p 5, (280 nm, 1 cm cuvette) calculated for 6 samples was unusually low, 0.21 OD/mg/ml, due to the lack of tryptophan residues. From this, the protein concentration could be determined easily from its UV absorbance.

The gel filtration chromatography showed that Der p 5 was eluted after the 25-kDa chymotrypsinogen A, but

before the 14-kDa ribonuclease A (Fig. 1). The estimated molecular weight of Der p 5 is 20 kDa, midway between monomer and dimer. Then Der p 5 may exist in solution as a mixture of monomers and dimers, or it contains an unusual structure at neutral conditions. However, no protein was eluted when Der p 5 was diluted in an acidic solution such as 10 mM phosphate buffer (pH 4.5) prior loading into the size column. Capillary electrophoresis (Beckman) showed that Der p 5 at 0.012 mg/ml or 0.12 mg/ml when injected gave a sharp absorbance peak at a retention time of 4.8 min in 10 mM phosphate buffer (pH 7.0). No such peak was observed if 10 mM phosphate buffer (pH 5.0) was used for elution, or if Der p 5 was diluted into this acidic solution.

For cross-linking studies, the dimeric 25-kDa *S. japonicum* glutathione-S-transferase (GST) and the monomeric 30-kDa bovine DNase were used as internal controls (Fig. 2). All GST molecules were cross-linked into dimers, tetramers, and higher oligomers in the presence of 0.2–5 mM DSP with a reaction time of 30 min. Some GST molecules were not cross-linked in the presence of glutaraldehyde unless higher concentrations and longer reaction times were used, such as 0.5% glutaraldehyde for 2 h. On the other hand, most DNase and Der p 5 in neutral solutions were not cross-linked by DSP or by glutaraldehyde. Minor bands of dimeric DNase may be due to random cross-linking at higher concentrations of protein solutions or reagents. Then most Der p 5 molecules seem forming monomers at neutral conditions. However, Der p 5 molecules in acidic solutions were cross-linked into a variety of different oligomers and this resulted in a very smeared band on the SDS-PAGE (data not shown). The cross-linking reagents could affect the mobilities and widths

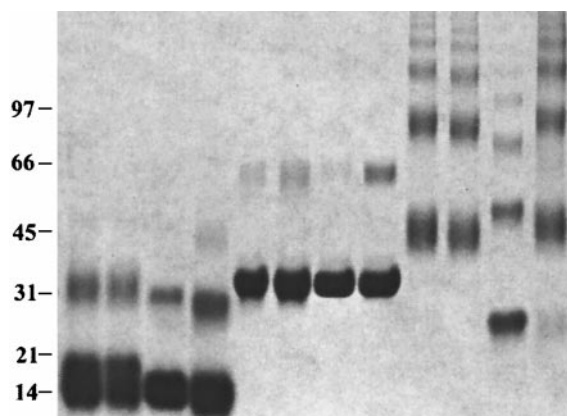


FIG. 2. Cross-linking studies of Der p 5. The position of molecular size standards (in kDa) are indicated. The protein bands were visualized with Coomassie blue. From left to right, lanes 1–4, 5–8, 9–12: 5 μ g Der p 5, 4 μ g bovine DNase, and 8 μ g GST reacted with 1 mM, 4 mM DSP, and 0.1%, 0.5% glutaraldehyde for 2 h, respectively. Most Der p 5 molecules seem forming monomers at neutral conditions.

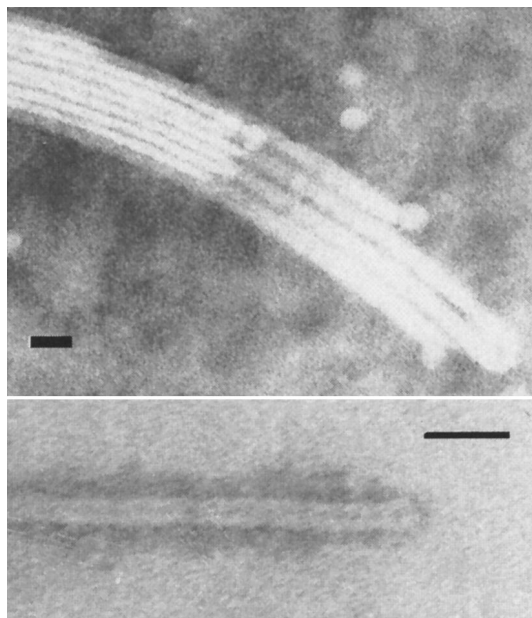


FIG. 3. Electron micrograph of Der p 5 filaments. The bar represents 30 nm. The filaments have a characteristic line of faint dark spots along their axis. They are thus probably composed of two subfilaments coiling around each other.

of the protein bands on SDS-PAGE as observed previously (11).

The electron micrography images showed that Der p 5 forms long filaments in 10 mM phosphate buffer (pH 4.0) (Fig. 3). These filaments are probably composed of two subfilaments that coil around each other and had a width of approximately 8.0 nm. Dynamic light scattering could not detect any protein of significant size at protein concentration of 5 mg/ml in PBS buffer (pH 7.5), perhaps because the monomeric proteins are too small to be observed by this method. However, two aggregated particle sizes appeared after mixing 1.5 ml of 5 mg/ml Der p 5 in PBS buffer (pH 7.5) in a 1.5 ml crystallization reservoir with 100 mM citrate acid-sodium phosphate (pH 3.4) and 5% PEG 6000. One size was consistently 8.6 ± 2.2 nm, whereas the other size kept increasing (data not shown). These two aggregated peaks can not be eliminated by the addition of 1 M Na_2HPO_4 returning the solution to neutral pH. The acid-induced polymerization would seem to be irreversible, and the transient pH is between 5 and 6 because the Der p 5 crystals dissolve when pH is increased to 5.3. Therefore, we conclude that Der p 5 forms monomers at neutral pH, but assemblies into filaments at acidic pH.

Both the cDNA sequences of Der p 5 and Blo t 5 code for leader peptides, and the program PSORT (12) predicted that these two allergens would be secreted extracellularly through the acidic secretory vesicles. Thus the irreversible acid-induced polymerization of

Der p 5 may occur during protein secretion and be part of normal protein function.

Filament Assembly through Putative Coiled-Coil Helices

Circular dichroism measurements showed that Der p 5 is an α -helical protein between pH 2 and pH 11 (Fig. 4). Different programs predicted different helical percentages in a range between 55 and 70% with excellent fitting curves. Various pH conditions change the protein absorbance but not the helical percentage. In addition to the unusual amino acid composition, the protein sequences of the three group 5 allergens show a heptad periodicity named a to g in which hydrophobic residues usually occur at positions a and d, a characteristic of coiled coils (Fig. 5). The PAIRCOIL and COILS programs (13, 14) showed high probabilities of forming coiled coils. The occurrence of multiple heptad repeats revealed the possibility that these regions fold into three coiled-coil helices similar to the single repeat of spectrin (Fig. 5B) (15). The presence of a proline residue after the first heptad repeats in Der p 5 might indicate the occurrence of a turn. Interestingly, the C-terminal residues (80–113) of Der p 5 share unusually high similarity to the coiled-coil region of the Hepatitis D virus antigen (Fig. 5A).

The acid-induced filament assembly of Der p 5 could be explained in part by the electrostatic interaction between coiled-coil helices. Studies of short coiled-coil peptides, and fibrous proteins such as spectrin and myosin reveal that electrostatic interactions play a critical role in protein aggregation. Based on the short

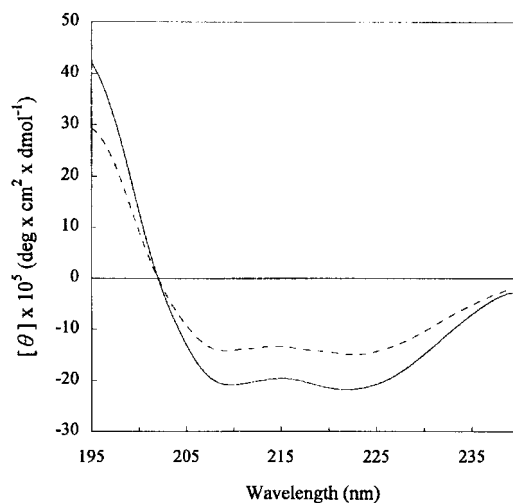


FIG. 4. Circular dichroic spectra of Der p 5, with a protein concentration of $3.85 \mu\text{M}$ ($55 \mu\text{g/ml}$). The same amount of concentrated proteins was diluted into 10 mM sodium phosphate buffer (pH 2.0–11.0). The protein solution at pH 4.8 is shown in solid lines, whereas that at pH 7.0 in dashed lines. The protein absorbance but not the helical percentage is various at different pH conditions.

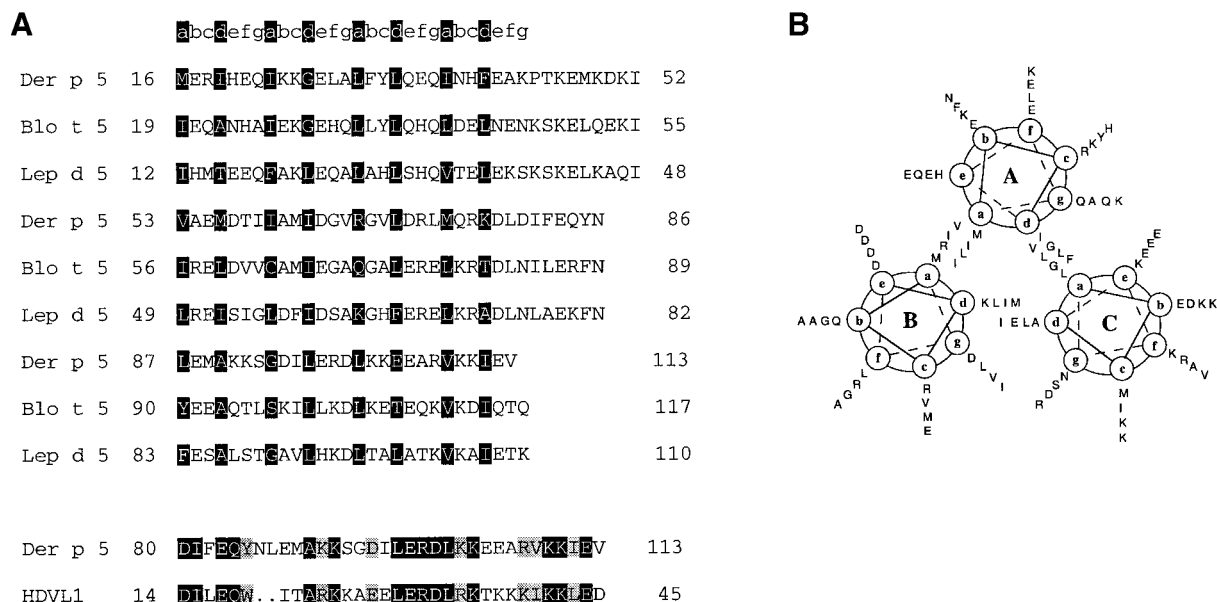


FIG. 5. (A) The multiple heptad repeats of the group 5 allergens from *D. pteronyssinus*, *B. tropicalis*, and *L. destructor* (Lep d 5, AJ250278). The hydrophobic positions a and d are shown in white with the black background. The C-terminal residues of the group 5 allergens shares high similarity to the coiled-coil region of the Hepatitis D virus antigen (sw:aant_hdvll). Identical amino acids are shown in white with the black background, whereas similar amino acids are shaded gray. (B) The putative helical wheel diagram of Der p 5 in cross section.

peptide studies, Kim and coworkers concluded that the favorable hydrophobic packing between hydrophobic residues at positions a and d, and avoidance of unfavorable electrostatic repulsion at the positions e and g are most important for coiled-coil package. The Fos leucine zipper peptide (16), and the influenza hemagglutinin LOOP-36 peptide (17) can form stable oligomers at low pH but not at neutral pH, revealing that protonation of the acidic residues relieves the destabilizing electrostatic repulsion, and enhances the possible interactions between acidic residues and hydrophobic residues. Similar unfavorable interactions could possibly form in the group 5 allergens at neutral pH, but would be relieved at acidic conditions. These are the alignment of three or four negatively charged residues at the positions e along one face of the helix, the interhelical electrostatic repulsion between e and g; and a glutamate residue at a position d (Fig. 5B).

The cytoskeletal spectrin superfamilies comprise largely tandem repeats, each of which consist of 100 to 120 amino acids. Recombinant polypeptides which represent single spectrin repeats exist in solution as a mixture of monomers and dimers (15). The crystal structures of the spectrin single repeats showed a bundle of three antiparallel coiled-coil helices (15, 18). During spectrin oligomerization, complementary electrostatic interactions are likely to play a pivotal role, ensuring proper docking of the correct lateral triple-helical bundle pairs (19).

During the filament assembly such that which occurs in myosin, oligomer could initially form through

the long coiled-coil helices, and then the interactions between oligomer surfaces drive the filament assembly. Charge complementation between coiled-coil oligomers has been postulated to determine the alignment of many filamentous proteins such as myosin and intermediate filament (20, 21). Remarkably, even single charge reversals (e.g., glutamate to lysine) could disrupt or restore the filament assembly of paramyosin (22). It is noteworthy that laminins, held together by coiled-coil interaction and disulfide bonds, can be induced to aggregation by negative charges in an acidic environment (23). In summary, the high content (40%) of the charged residues found in Der p 5 could contribute electrostatic interactions and result in the formation of higher order oligomers, and thus account for the pH-dependent filament assembly.

Implication in Allergenicity

The sensitization mechanisms by which the mite allergens influence immunogenicity are still unclear. The finding that many environmental allergens possess enzyme activities (principally hydrolytic), and the high degree of sensitization produced by enzymes in commercial, domestic and pharmaceutical use, suggested that an enzymatic activity may enhance or be required for an antigen to induce a persistent allergic response. However, many allergens do not possess any known enzymatic activity. These allergens might contain other immunogenic properties. Interestingly, some known mite allergens including groups 7, 10 (tro-

pomyosin), 11 (paramyosin), and Mag 29 (heat shock protein), are probably nonenzyme and contain similar long heptad repeats to group 5. Coiled-coil helices have been proposed to possess strong immunogenicity in addition to their contribution to protein oligomerization. Many coiled-coil fibrous proteins from different organisms have been identified as strong antigens, for example, myosin, tropomyosin, paramyosin, intermediate filaments, and nuclear lamins (24). Dohlman and coworkers used the COILS program to analyze some known sequences of human autoantigens in autoimmune diseases. Forty of the 109 known human autoantigens were predicted to contain coiled-coil helices with a probability of greater than 0.9. Therefore, mite allergens may have a number of common properties resulting in a common mechanism for allergic reactions; for instance enzymatic activity and coiled-coil helices.

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