

# Generation of anaphylatoxins through proteolytic processing of C3 and C5 by house dust mite protease

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**Background:** The group 3 allergen of *Dermatophagoides* species (Der p 3 and Der f 3) has been identified as a 30 kd trypsin-like protease of the house dust mite. We previously showed that the 30 kd protease from *Dermatophagoides farinae* (Df-protease) could activate the bradykinin-generating cascade and exacerbate inflammatory reactions.

**Objective:** The purpose of this study was to determine whether Df-protease could enzymatically generate anaphylatoxins from complement components C3 and C5.

**Methods:** Df-protease was incubated with human serum C3 or C5 in a purified system, and the anaphylatoxin activity produced was assayed by measuring enhancement of vascular permeability and release of histamine from mast cells triggered by C3a and by assessing chemotaxis of polymorphonuclear cells caused by C5a. We also attempted to determine whether protease isolated from house dust could cause release of C5a from C5.

**Results:** Df-protease showed strong activation of C3 and C5, producing C3a and C5a by proteolytic cleavage of the complements. An appreciable amount of Df-protease was recovered in the house dust extract, and the house dust protease caused C5a release from C5.

**Conclusion:** Df-protease activated the complement system to produce anaphylatoxins. Thus it is suggested that house dust mite proteases may contribute to the pathogenesis of allergic and inflammatory diseases caused by house dust allergens. (J Allergy Clin Immunol 1997;100:253-60.)

**Key words:** *Dermatophagoides farinae*, house dust protease, Df-protease, C3, C5, anaphylatoxins

Two different species of mites, *Dermatophagoides farinae* and *D. pteronyssinus*, are reported to be the most important sources of allergen in house dust.<sup>1,2</sup> Recent reports show that the enzymatically active allergens are found in mite fecal pellets; and those allergens include a cysteine protease,<sup>3</sup> several serine proteases,<sup>4-6</sup> and glutathione S-transferase.<sup>7</sup> We previously demonstrated that a 30 kd protease from the house dust mite *D. farinae*

## Abbreviations used

|              |                                                          |
|--------------|----------------------------------------------------------|
| BSA:         | Bovine serum albumin                                     |
| Df-protease: | 30 kd protease from <i>Dermatophagoides farinae</i>      |
| MCA:         | 4-Methylcoumaryl-7-amide                                 |
| PBS:         | Phosphate-buffered saline                                |
| PMNs:        | Polymorphonuclear leukocytes                             |
| SBTI:        | Soybean trypsin inhibitor                                |
| SDS-PAGE:    | Sodium dodecylsulfate-polyacrylamide gel electrophoresis |

(Df-protease) could activate the bradykinin-generating cascade and that the bradykinin generated exacerbates inflammatory reactions in some pathologic conditions.<sup>6, 8, 9</sup>

The complement system plays an important role in host defense against infectious agents and in the inflammatory process. Complement activation results in the formation of the anaphylatoxins C3a, C4a, and C5a.<sup>10</sup> These anaphylatoxins are biologically active peptides that are defined functionally by their actions on small blood vessels, smooth muscle, mast cells, and peripheral blood leukocytes. They may contribute to the inflammatory reaction observed in a variety of allergic diseases.

Several investigators have shown that the complement system can be activated by allergens via the classical or the alternative complement pathway.<sup>11, 12</sup> Nagata and Glovsky<sup>13</sup> previously reported that anaphylatoxins were generated during in vitro incubation of serum with extracts from different airborne allergens. Furthermore, Castro et al.<sup>14</sup> demonstrated that house dust extract as a whole produced potent complement activation in the serum of both asthmatic and healthy subjects, although serum complement from patients with asthma was less affected than that from subjects without asthma. However, the detailed mechanisms of complement activation by allergen extracts have not been clarified.

In this study we tested the potential of Df-protease and house dust protease to generate anaphylatoxins from human C3 or C5 in a purified system. Our results indicate that house dust protease and Df-protease are potent generators of C3a from C3 and C5a from C5, suggesting possible involvement of house dust proteases in the pathogenesis of allergic diseases.

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## METHODS

### Reagents

Df-protease was purified to homogeneity from cultured mites as described previously.<sup>6</sup> Purified Df-protease showed a purity of more than 95% on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and possessed a fully active serine site in its protein molecule, as judged by active site titration as described in a previous report.<sup>9</sup> The benzamidine-agarose affinity column was obtained from Organo Co. Ltd. (Tokyo, Japan). 4-Methylcoumaryl-7-amide (MCA) peptidyl substrates were purchased from Peptide Institute (Osaka, Japan).

*N*-Hydroxysuccinimidyl-6-(biotinamido)hexanoate and agarose-avidin D were purchased from Vector Laboratories Inc. (Burlingame, Calif.). Human C3, human recombinant C5a, *N*-formyl-methionyl-leucyl-phenylalanine, and bovine pancreatic trypsin were purchased from Sigma Chemical Company (St. Louis, Mo.). Human C5 was obtained from Calbiochem (San Diego, Calif.). RPMI-1640 medium was obtained from Gibco (Grand Island, N.Y.). OK-432, a penicillin-killed streptococcal preparation, was obtained from Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan). Soybean trypsin inhibitor (SBTI) (Kunitz type) was a gift from Fuji Seiyu (Osaka, Japan). All other reagents were of the highest analytic grade commercially available.

### Isolation of house dust protease

House dust was collected from houses of healthy volunteers by using a vacuum cleaner for household use with a built-in dust trap with a 0.6  $\mu$ m mesh (Hitachi Fuzzy 310, CV-AX 7600; Hitachi Ltd., Tokyo, Japan). The dust (100 gm) was defatted with ether (750 ml) near 0° C and was further dissolved in 1.5 L of 50 mmol/L Tris-HCl buffer (pH 7.4) containing 0.05% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>, followed by precipitation with ammonium sulfate at 80% saturation as described previously.<sup>6</sup> The precipitates were suspended in 60 ml of 10 mmol/L Tris-HCl buffer (pH 7.4). The crude extracts of house dust were applied to the benzamidine-agarose affinity column to absorb serine-type proteases (putative Df-protease). After elution with 0.15 mol/L *p*-aminobenzamidine, *p*-aminobenzamidine was completely removed from the eluted samples by dialysis against 1000 times excess volume of 5 mmol/L Tris-HCl (pH 7.4) for 8 hours; and the dialysis solution was changed two times. A fraction containing protease activity, which was determined as described below, was obtained (6 mg).

### Assay of protease

Specificity of proteolytic activity of Df-protease and putative protease in house dust extracts was determined fluorometrically by using fluorogenic MCA peptides as reported previously.<sup>8,9</sup> In this protease assay either the purified Df-protease (0.1  $\mu$ g/ml) or the protease (10  $\mu$ g/ml) from house dust extract (isolated as described) was incubated at 25° C for 30 minutes with a series of MCA substrates (final concentration, 50  $\mu$ mol/L) in 50 mmol/L Tris-HCl buffer containing 0.1% bovine serum albumin (BSA) and 0.02% Na<sub>2</sub>S<sub>2</sub>O<sub>5</sub>. Activity was expressed as a percentage of amidolytic activity of different protease preparations against 10 different peptide substrates. We define "100% of the activity" as the amidolytic activity against the hydrolysis of Boc-Gln-Gly-Arg-MCA (Boc-QGR-MCA), which is the most preferential substrate for Df-protease. Protein content was determined with BSA as a standard by the method of Lowry et al.<sup>15</sup>

### Preparation of anti-Df-protease antibody and its affinity column and immunologic detection of Df-protease in house dust extracts

Anti-Df-protease antibody (IgG) was raised in a rabbit by intradermal administration, once every 2 weeks, of 42  $\mu$ g of purified Df-protease, which was obtained from *D. farinae* cultured on solid yeast together with complete Freund's adjuvant. The IgG antibody was purified on a protein-A Sepharose column (Pharmacia, Uppsala, Sweden). The antibody was used for an agar gel diffusion assay and for an immunosorbent column.

The specificity of anti-Df-protease IgG antibody was verified by testing its neutralizing activity against Df-protease and well-known serine proteases such as trypsin and chymotrypsin. In addition, the specificity of the antibody was examined by using antibody-bound immunoaffinity column chromatography as described below. The results showed that our IgG antibody for Df-protease inhibited more than 80% of the protease activity of the pure Df-protease preparation, but it did not appreciably affect the enzyme activity of other serine proteases. Similarly, Df-protease, but not trypsin and chymotrypsin, was adsorbed on the immunoaffinity column.

Immunoabsorption of the putative Df-protease was performed to determine the presence of Df-protease in the house dust. Protease activity was quantified by using Boc-QGR-MCA as previously described. To prepare the affinity column, the purified anti-Df-protease IgG was biotinylated with *N*-hydroxysuccinimidyl-6-(biotinamido)hexanoate in sodium bicarbonate buffer (pH 8.5). The biotinylated IgG was then bound to agarose-avidin D to produce anti-Df-protease IgG-coupled agarose.

After protease-containing samples (i.e., 1.0  $\mu$ g of Df-protease or 100  $\mu$ g of protein from house dust extract purified on the benzamidine-agarose column as described above) were applied to the affinity column (bed volume, 0.5 ml), they were eluted with 2 ml of phosphate-buffered saline (PBS). The amounts of nonadsorbed protease activity in the eluate were determined by fluorometric assay as described above. The adsorbed enzyme activity was considered to be the protease closely related immunologically to Df-protease.

### Cell preparation

Mouse polymorphonuclear leukocytes (PMNs) were obtained from the peritoneal exudate after induction by injection of OK-432 as reported previously.<sup>16</sup> Briefly, male ddY mice were injected intraperitoneally with 1 U, defined as 1 KE (klinisch einheit) by the manufacturer, in 0.1 ml of saline solution. The elicited peritoneal cells, which were more than 80% PMNs, were harvested by peritoneal lavage at 16 hours after injection of OK-432 and were used without further purification throughout the experiment. Rat peritoneal mast cells were obtained as described previously.<sup>17</sup> The peritoneal cavity was lavaged with 30 ml of ice-cold Tyrode's buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 1.0 mmol/L MgCl<sub>2</sub>, 1.0 mmol/L CaCl<sub>2</sub>, 0.4 mmol/L NaH<sub>2</sub>PO<sub>4</sub>, 1.0 mmol/L glucose, 10 mmol/L *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, and 0.1% BSA) containing heparin (10 U/ml) and 0.1% gelatin. The cell suspension was centrifuged at 500 g for 5 minutes at 4° C, and the mast cells were then isolated by centrifugation (500 g, 5 minutes, 4° C) after the cell suspension was overlaid on 38% BSA in Tyrode's buffer, yielding a final population of more than 90% mast cells with viability of more than 95% as determined by the trypan blue exclusion method.

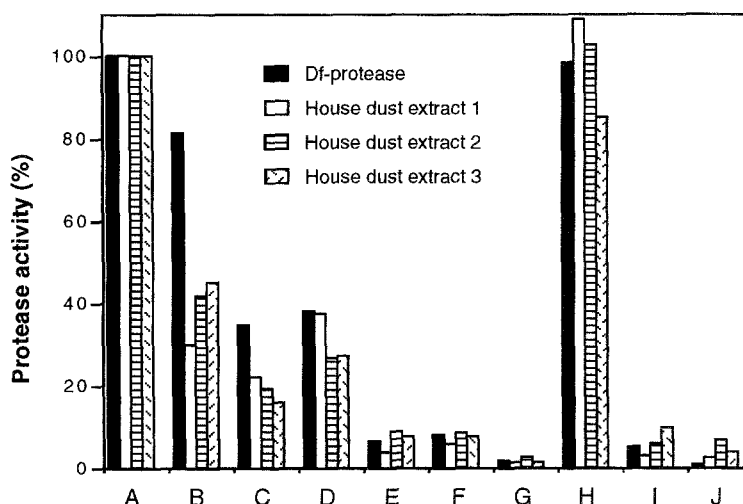


**FIG. 1.** Substrate specificities of Df-protease and crude protease from house dust extracts from houses of three different individuals. Substrates included are: A, Boc-QGR-MCA (for factor XIIa); B, Boc-LEGR-MCA (for factor Xa); C, Boc-EKK-MCA (for plasmin); D, Boc-VLK-MCA (for plasmin); E, Z-FR-MCA (for plasma kallikrein); F, PFR-MCA (for tissue kallikrein); G, Bz-R-MCA (for trypsin); H, Boc-FSR-MCA (for trypsin); I, EGR-MCA (for urokinase); and J, Suc-AAPF-MCA (for chymotrypsin). Boc, *t*-Butyloxycarbonyl; Z, carbobenzoxyl; Bz, benzoyl; Suc, succinyl. All other capital letters refer to amino acids expressed as single letters. See text for details.

### Treatment of C3 and C5 with Df-protease

Human C3 or C5 at a concentration of 5.0  $\mu\text{g/ml}$  (0.25  $\mu\text{mol/L}$ ) was incubated with varied concentrations of Df-protease (2.5 ng/ml, or 0.08 nmol/L, to 7.5  $\mu\text{g/ml}$ , or 0.25  $\mu\text{mol/L}$ ); molar ratios of Df-protease to C3 or C5 ranged from 0.0003 to 1. Incubations were carried out in 100  $\mu\text{l}$  of 0.01 mol/L phosphate-buffered 0.15 mol/L saline (PBS, pH 7.4) at 37° C. We also examined the time profile of C5a release from C5 after treatment with Df-protease at a molar ratio of 0.01 (1:100, Df-protease to C5) in PBS at 37° C. The reaction was stopped by inhibition of the protease by incubation with 5  $\mu\text{mol/L}$  SBTI for 30 minutes, after which C3a and C5a produced in the reaction mixture were measured as described below. Similarly, we examined the ability of protease isolated from house dust to cause release of C5a from C5.

### Vascular permeability assay

The test to measure enhanced vascular permeability in guinea pig skin was performed according to the method for anaphylatoxin activity described previously<sup>18</sup> with a slight modification. Briefly, 2.5% Evans blue dye in saline solution (12.5 mg of Evans blue dye/kg body weight) was injected intravenously into an anesthetized (pentobarbital) guinea pig. Then, 100  $\mu\text{l}$  of the reaction mixture was immediately injected intradermally into the guinea pig. After 30 minutes, the animals were anesthetized with pentobarbital and exsanguinated, and the skin was removed. The stained spot was then removed and incubated with dimethylformamide at 60° C for 48 hours to extract Evans blue dye. The Evans blue dye content of these samples was determined by measuring optical density at 620 nm and correlating this result with the standard curve of known concentrations of Evans blue dye in formamide.

When biologically active Df-protease was injected into guinea pig skin, a vascular permeability reaction was observed. As we reported previously,<sup>9</sup> this permeability is triggered through activation of the kinin generation cascade by the protease. Thus in the vascular permeability assay for C3a, we

were particularly careful to inhibit the Df-protease activity completely, and each reaction mixture containing Df-protease was treated with a great excess of a protease inhibitor for Df-protease (SBTI) before injection into the skin.

### PMN chemotaxis assay

Generation of C5a was assayed by using the PMN chemotaxis assay. Chemotactic activity of C5a generated in the reaction of C5 with Df-protease was measured by the modified Boyden chamber method with Nuclepore filters (Nuclepore Corp., Pleasanton, Calif.) and blind well chambers (Labo Science Inc., Tokyo, Japan), which were made of Lucite (polyacrylate) plastic, as described previously.<sup>16</sup> The filter pore size for the PMN experiments was 2.0  $\mu\text{m}$ . A total volume of 250  $\mu\text{l}$  of cell suspension in RPMI-1640 medium containing 0.5% BSA, pH 7.4, was placed in the upper chamber; 113  $\mu\text{l}$  of sample was placed in the lower compartment, over which a membrane filter was placed. After incubation for 90 minutes at 37° C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air, the filters were removed and cells were stained with Giemsa solution. The numbers of cells that had migrated completely through the filter were determined by observing five random fields at a magnification of  $\times 400$ . Results are expressed as a ratio to control on the basis of the total cell migration induced by  $10^{-8}$  mol/L *N*-formyl-methionyl-leucyl-phenylalanine (positive control = 1.0).

### Histamine release assay

Peritoneal mast cells isolated from rats ( $0.5$  to  $1.0 \times 10^5$  mast cells per tube) were incubated with C3 treated with various concentrations of Df-protease at 37° C for 30 minutes. After incubation, reactions were stopped by addition of ice-cold Tyrode's buffer. Cell samples were immediately centrifuged at 4° C for 5 minutes at 500 *g* to obtain supernatants. After precipitation of protein with 6% perchloric acid, the histamine concentration in the supernatant was measured by fluorometric assay as described previously.<sup>19</sup> Histamine release in situ from

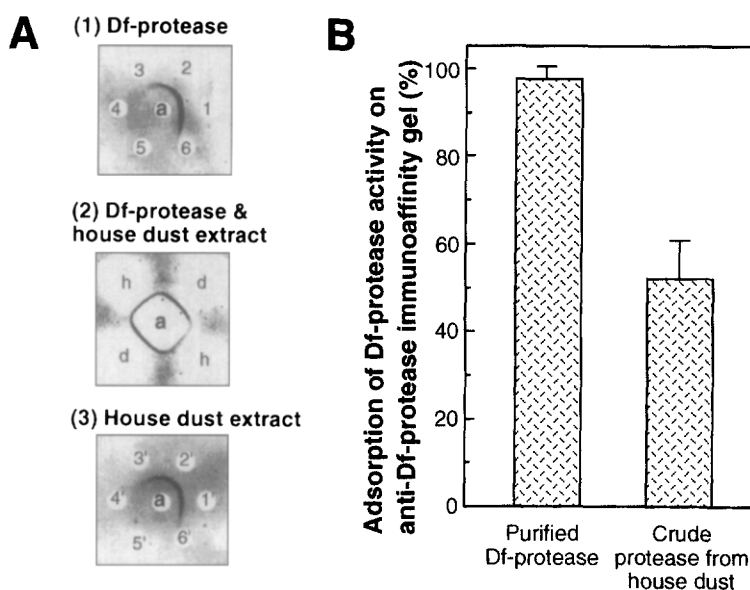


**FIG. 2.** Immunologic identity of purified Df-protease from cultured mites and protease in house dust extracts. **A**, Ouchterlony's immunodiffusion of Df-protease and a house dust extract against anti-Df-protease antiserum. (1) *a*, Anti-Df-protease antiserum: 1, 2, 3, 4, 5, and 6 indicate 700, 350, 175, 87.5, 43.8, and 21.9 ng of Df-protease, respectively. (2) *d*, Df-protease (0.7  $\mu$ g); *h*, a house dust extract (70  $\mu$ g). (3) 1', 2', 3', 4', 5', and 6' indicate 70, 35, 17.5, 8.75, 4.38, and 2.19  $\mu$ g of protein from house dust extract, respectively. **B**, Immuno-adsorption/elution profile. IgG antibody purified was biotinylated and attached to avidin D-agarose, to which a Df-protease-containing sample was applied. After washing, protease activity in the eluate was measured. Data are expressed as means  $\pm$  SD of three different experiments. See text for details.

the mast cells in the reaction mixture was expressed as a percentage of the total amount of histamine initially present in the cells, according to the following formula: % Histamine release = (Histamine in supernatant after treatment  $\div$  Histamine in supernatant + Histamine in cells)  $\times$  100.

All data obtained for histamine released after C3a stimulation are subtracted from the histamine released spontaneously without specific stimulation.

#### SDS-PAGE analysis of proteolytic cleavage of C3 by Df-protease

To verify that C3a is produced by the specific proteolysis of C3 by Df-protease, SDS-PAGE was carried out with C3 and with or without Df-protease. Specifically, C3 was treated with Df-protease at a molar ratio of 0.03 (Df-protease to C3) in PBS (pH 7.4) at 37°C in the same manner as described above. After various incubation periods, aliquots of the reaction mixture (10  $\mu$ g of C3) were analyzed by SDS-PAGE (10% to 20% gradient gel) under reducing conditions with dithiothreitol according to the method of Laemmli.<sup>20</sup> After electrophoresis, the protein band was stained with Coomassie Brilliant Blue (Wako Pure Chemical, Osaka, Japan) as described elsewhere.<sup>9</sup>

#### Statistical analysis

All data are expressed as means  $\pm$  SD of three different experiments. Statistical analyses were performed by two-tailed *t* test for unpaired data.

### RESULTS

#### Purification of house dust protease

Our environmental studies showed that house dust collected from houses of healthy volunteers contained a

trypsin-like protease with the same substrate specificities as purified Df-protease obtained from cultured *D. farinariae* (Fig. 1). The trypsin-like protease in house dust extract was isolated on a benzamidine-agarose affinity column.

When the amidolytic activity for Boc-QGR-MCA of the protease from house dust was compared with that of Df-protease, the relative protease activity of the house dust protease preparation was found to be 0.59% (mean value) of the purified Df-protease on the basis of total protein content. Furthermore, the protease from house dust seems to be immunologically identical to the purified Df-protease, as revealed by immunodiffusion (Fig. 2). About 50% of the house dust extract protease was adsorbed on an anti-Df-protease column (Fig. 2, B); thus, at least 50% of the house dust protease is Df-protease.

#### C3a generation from human C3 induced by Df-protease

Df-protease was incubated with C3 in PBS at various molar ratios. C3a generated in the reaction mixture was quantitated by its vascular permeability-enhancing activity, which was assessed by extravasation of Evans blue dye at the site of injection. As shown in Fig. 3, A, Df-protease caused generation of C3a from C3 in a dose-dependent manner with respect to the amount of Df-protease. Furthermore, Df-protease activated C3 to express the appreciable activity of the anaphylatoxin

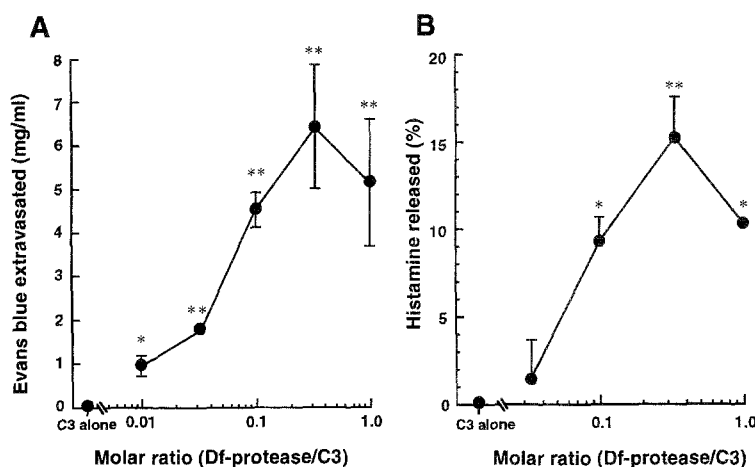


**FIG. 3.** C3a release from C3 after treatment with Df-protease. C3 (5  $\mu$ g/ml) was incubated with Df-protease at various molar ratios for 30 minutes at 37° C in PBS, pH 7.4. The molar ratio of Df-protease to C3 was from 0.01 to 1. After the reaction was stopped, C3a activity was measured by the vascular permeability assay (**A**) or by histamine release from rat mast cells (**B**) as described in Methods. Data are expressed as means  $\pm$  SD of three different experiments. \*Significant difference from value for C3 alone at  $p < 0.05$ . \*\*Significant difference from value for C3 alone at  $p < 0.01$ .

(C3a) at molar ratios of more than 0.01 (Df-protease to C3) under our reaction conditions. Very little vascular permeability was observed with C3 alone without Df-protease treatment (amount of Evans blue dye extravasated,  $0.10 \pm 0.01$  mg/ml). Similarly, Df-protease alone treated with SBTI showed no appreciable vascular permeability reaction. This suggests a potent and specific action of Df-protease in generation of the active peptide C3a from C3.

Generation of C3a from C3 as caused by Df-protease was further substantiated by assessment of histamine release from mast cells stimulated with Df-protease plus C3 (Fig. 3, *B*). Specifically, a measurable level of histamine production was observed at the same molar ratios of Df-protease to C3 as those in the chemotaxis assay for C3a. In contrast, C3 not treated with Df-protease induced only negligible histamine release ( $0.09\% \pm 0.01\%$ ), and Df-protease alone did not show any measurable histamine release.

#### C5a generation from human C5 induced by Df-protease and house dust protease

Purified human serum C5 was incubated with various concentrations of Df-protease (molar ratios of Df-protease to C5: from 0.0003 to 1) for 30 minutes at 37° C. The aliquots of the incubation mixtures were diluted, and chemotactic activity for PMNs was evaluated by using a modified Boyden chamber assay (Fig. 4, *A*). C5 alone exhibited very little chemotactic response to PMNs ( $0.08 \pm 0.01$ ). Also, Df-protease in the absence of C5 had no chemotactic activity. The C5a activity based on chemotaxis became apparent in the reaction of Df-protease with C5 at a molar ratio of less than 0.003. Release of C5a was increased in a dose-dependent manner with Df-protease up to the molar ratio of 0.03 (Df-protease/C5) (Fig. 4, *A*). However, generation of

C5a activity was diminished as the molar ratio was increased over 0.03. Similarly, a time-dependent increase in C5a release from C5 was observed after treatment of C5 with Df-protease at a molar ratio of 0.03; again, during the 30-minute incubation period, the C5a-generating potency of Df-protease seemed to decrease (Fig. 4, *B*). Therefore it appears that C5a is susceptible to proteolysis by Df-protease and undergoes further degradation to inactive peptides.

When we compared the efficacies of Df-protease and bovine pancreatic trypsin in C5a generation from C5 (Fig. 5), Df-protease showed a stronger C5a-releasing action than did trypsin at a molar ratio of 0.01. Moreover, we noted a significant release of C5a from C5 treated with house dust protease, which was isolated from house dust extract (Fig. 5). The C5a-generating activity of the house dust protease was completely nullified by SBTI (data not shown), indicating involvement of a specific serine-type protease that cleaves C5.

These results suggest that house dust protease and Df-protease possess a strong potential to produce C5a anaphylatoxin from C5. To determine and identify C3a and C5a, we used ex vivo bioassay systems of different animal species (not human). However, these assays are routinely used methods, and this study is indirectly intended to examine the biologic activity of these anaphylatoxins in human beings. We understand that it is established that the activity of anaphylatoxins measured by the bioassay (even with different animal sources) contributes to the biologic action in human beings in vivo.<sup>18, 21-23</sup>

#### SDS-PAGE of proteolytic processing of C3 by Df-protease

As shown in Fig. 6, C3 is proteolytically processed by Df-protease (molar ratio of Df-protease to C3: 0.03) by

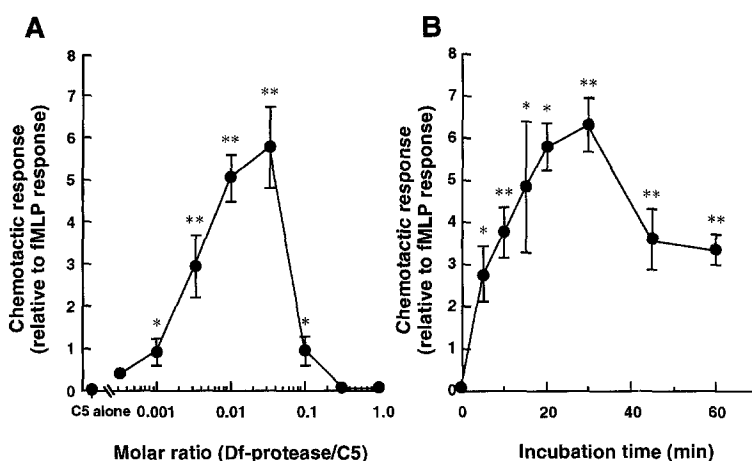


**FIG. 4.** C5a generation from C5 by Df-protease. **A**, C5 (5  $\mu$ g/ml) was incubated with Df-protease at several molar ratios for 30 minutes at 37° C in PBS. The molar ratio of Df-protease to C5 varied from 0.0001 to 1. **B**, Time profile of C5a generation from C5 by incubation with Df-protease at a molar ratio of 0.03 (Df-protease to C5) in PBS at 37° C. After the reaction was stopped, C5a activity was assayed by using PMN chemotaxis. Data are expressed as means  $\pm$  SD of three different experiments. \*Significant difference from value for C5 alone at  $p < 0.05$ . \*\*Significant difference from value for C5 alone at  $p < 0.01$ . fMLP, Formyl-methionyl-leucyl-phenylalanine. See text for details.

cleavage of a specific site. The molecular size of the  $\alpha$ -chain of C3 decreased within 5 minutes by 9 kd, producing cleavage forms of the subunit ( $\alpha'$ , a major product: 101 kd and  $\alpha''$ , a minor product: 95 kd). The quantity of molecular mass of C3 is consistent with the removal of the size of C3a (9 kd).<sup>23</sup> The  $\beta$ -chain of C3, however, was not affected by the treatment with Df-protease. This result suggests that Df-protease releases C3a from C3 through a specific limited proteolysis of C3.

## DISCUSSION

The house dust mites *D. farinae* and *D. pteronyssinus* have been regarded as the major allergens in some allergic diseases such as bronchial asthma, atopic dermatitis, and allergic rhinitis.<sup>1,2</sup> On the basis of immunologic and physicochemical similarities, mite allergens have been classified into more than seven groups by the World Health Organization/International Union of Immunological Societies Allergen Nomenclature Subcommittee<sup>24</sup> and by Stewart.<sup>25</sup> Group 3 allergens are 30 kd proteins that have been reported to be trypsin-like proteases according to their substrate specificity and amino acid sequence homology.<sup>24-28</sup> Hence, Df-protease is classified as a group 3 allergen. We also demonstrated in this study that a protease similar to Df-protease was found to exist in the crude extract of house dust and that it exhibited a proteolytic activity similar to that of Df-protease (Figs. 1 and 2). Group 3 allergens are characteristic of the *Dermatophagoides* genus, and they trigger an allergic reaction. The frequency of positive allergic responses to group 3 allergens in mite-allergic populations varies from 16% to 100%.<sup>5,25,26</sup> Furthermore, we showed earlier that Df-protease can activate the kinin-generating cascade and may exacerbate inflammatory reactions<sup>8,9</sup>; it is likely that Df-protease in house dust could stimulate inflammatory reac-

tions and promote an allergic reaction by means of potent proteolytic action.

Complement components C3, C4, and C5 constitute a family of proteins that play a central role in the complement system-mediated inflammatory response, and low-molecular-weight peptides derived from them exhibit anaphylatoxin activity, which is generated enzymatically.<sup>17,29-31</sup> C3, C4, and C5 are activated by proteolytic cleavage at structurally homologous sites in their  $\alpha$ -subunits, resulting in release of anaphylatoxins C3a, C4a, and C5a, which cause smooth muscle contraction, release of histamine from mast cells, and PMN chemotaxis.<sup>10</sup> The complement components C3 and C5 are activated by a complex plasma protease with trypsin-like specificities called convertase, as well as by noncomplement-related proteolytic enzymes (e.g., trypsin, thrombin, and elastase).<sup>31</sup>

Previous studies have demonstrated that the complement system can be activated by house dust and moldy hay dust allergens,<sup>11-14</sup> suggesting that the complement system may contribute to various inflammatory reactions in allergic diseases. Of relative importance is the finding by Regal et al.<sup>32</sup> that complement activation might be an important determinant of the severity of antigen-induced bronchoconstriction. Furthermore, a recent report shows that the anaphylatoxins C3a and C5a induced a rapid release of histamine from human skin mast cells.<sup>33</sup> Thus typical clinical features of allergic and inflammatory skin diseases, such as the wheal-and-flare reaction, may be attributable to mast cell activation by C3a and C5a.

In addition to Df-protease from *D. farinae*, the protease allergens are known to originate from different sources (e.g., Der f 1 from *D. farinae*; Der p 1, Der p 3, and Der p 6 from *D. pteronyssinus*; Bom p 4 from an

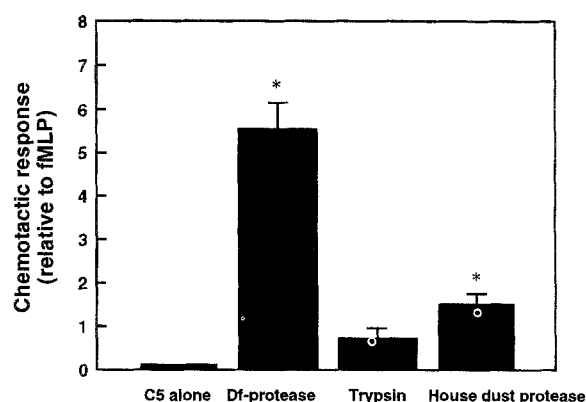


**FIG. 5.** C5a release from C5 after incubation with Df-protease, bovine pancreatic trypsin, or protease from house dust extract. C5 was incubated with Df-protease (7.5 ng), trypsin (6 ng), or the protease (crude) from house dust extract isolated on the benzamidine-agarose affinity column (75 ng). Protease from house dust had 0.6% relative protease activity compared with Df-protease used in this assay. After the reaction was stopped, C5a activity released was measured by using a PMN chemotaxis assay. Data are expressed as means  $\pm$  SD of three different experiments. \*Significant difference from value for C5 alone at  $p < 0.05$ . fMLP, Formyl-methionyl-leucyl-phenylalanine. See text for details.

insect *Bombus pennsylvanicus*; a protease from *Aspergillus fumigatus*; and the protease from pollens).<sup>3-5, 24, 34-36</sup> One of the most interesting findings on the biochemical characteristic of the protease allergen is detachment of epithelial cells by a serine-type protease from *A. fumigatus* as reported by Robinson et al.<sup>36</sup> In their report it is suggested that epithelial damage caused by the fungal protease could trigger the allergic immune response and contribute to the fungal protease's allergenicity by potentiating the accessibility of the various allergens to the immune system.

As demonstrated in our experiment, Df-protease showed strong and specific activation of C3 and C5, resulting in formation of anaphylatoxins C3a and C5a, respectively. It is well known that these anaphylatoxins are potent inducers of inflammatory responses, such as vascular permeability and recruitment of neutrophils and monocytes/macrophages to the tissue, as shown in this study. Moreover, our previous work clearly demonstrated that Df-protease also activates kinin-generating cascades consisting of high- and low-molecular-weight kininogens.<sup>8, 9</sup> Therefore like *A. fumigatus* protease, the mite protease may play a role in triggering the allergic responses, and the protease per se may act as an allergen. In this context it is conceivable that there is a unique entity of proteases as allergens, which will be critically involved in the pathogenesis of various allergic diseases. The anaphylatoxin generation and activation of the kinin-generating cascade by Df-protease are not simple pharmacologic phenomena, but these are very unique biochemical properties, and our report will provide an important insight into the molecular mechanism of the complicated allergic responses in human beings.

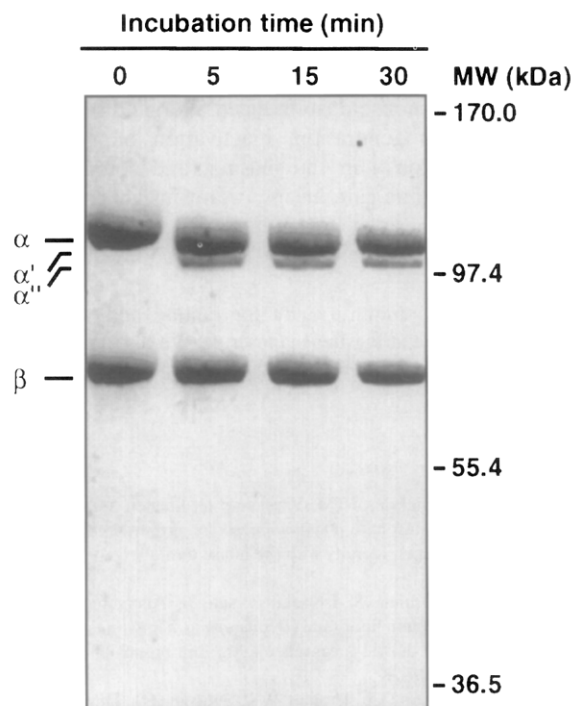


**FIG. 6.** SDS-PAGE analysis for proteolytic processing of C3 by Df-protease. C3 was treated with Df-protease at a molar ratio of 1:30 (Df-protease:C3) in PBS (pH 7.4) at 37°C. After various incubation periods, the reaction mixture containing 10  $\mu$ g of C3 was subjected to SDS-PAGE. See text for details. MW, Molecular weight.

House dust mites are widely distributed in every human habitation, particularly in the temperate zone. Therefore it is expected that inhabitants are frequently exposed to Df-protease through the respiratory tract, the skin, or the gastrointestinal tract. Airborne dust is estimated to have a concentration of about 50  $\mu$ g/m<sup>3</sup>,<sup>37</sup> which would contain 5 to 10 ng of house dust mite protease per cubic meter. In this study we demonstrate that at least 3.75 ng of Df-protease can generate anaphylatoxins (Figs. 3 and 4). Our results indicate that purified Df-protease obtained from cultured house dust mites efficiently activates the complement system by proteolytic cleavage of C3 and C5. Furthermore, the crude protease in house dust extract also generates C5a from C5 (Fig. 5). In addition, we reported earlier that the proteolytic activities of Df-protease were not completely inhibited by endogenous human plasma serine-type protease inhibitors.<sup>8</sup> Thus we speculate that the protease in house dust, Df-protease, exerts a significant biologic effect in the pathogenesis of allergic diseases.

We previously showed that Df-protease triggers vascular permeability enhancement by activation of the kinin-generating cascade<sup>8, 9</sup> and inactivates the cysteine-protease inhibitory activity of kininogen,<sup>9</sup> which plays an important role in some inflammatory conditions. We demonstrate here that Df-protease proteolytically generated anaphylatoxins C3a and C5a from human plasma C3 and C5, resulting in histamine release from mast cells

and chemotaxis of PMNs. On the basis of all these results, it is most reasonable that house dust mite protease, such as Df-protease, may contribute to the pathogenesis of house dust-induced allergic diseases by means of kinin generation, inactivation of cysteine-protease inhibitor(s) in the plasma, and complement activation. We thus gain an important insight into molecular pathogenesis of house dust-induced allergic diseases.

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