

Characterization of the Proteases in the Crude Mite Extract

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Abstract. Characterization of the proteases was performed in the crude mite extract fractionated by Sephacryl S-200 gel filtration. Three peaks of protease activities were detected in the fractions. From the results of substrate specificity and susceptibility to the inhibitors, PK.1 protease (about 60 kD) is suggested to be a trypsin-like protease of mites. From the results of susceptibility to various agents, PK.2 (about 30 kD) and PK.3 (about 20 kD) proteases may be cysteine proteases, e.g., papain and cathepsin B. PK.3 protease existed in the precipitate of 60% ammonium sulfate fractionation. The data in the present study suggest the possibility that *Dermatophagoides farinae* I allergen might be a cysteine protease probably derived from the gastrointestinal tract of the house dust mite.

Introduction

It is known that house dust is one of the most important respiratory allergens as a result of skin tests and clinical observations. House dust contains many allergens, e.g., mites, animal danders, and fungal spores. Among these allergens, the mites are suggested to be a major causative allergen that is known for its allergenicity in atopic asthma and other atopic diseases. Two house dust mite species, *Dermatophagoides farinae* (Df) and *Dermatophagoides pteronyssinus* (Dp), are reported as frequent inhabitants of house dust in many parts of the world [1]. Thus, many studies have been performed to characterize and identify the allergen in extracts from Df and Dp. Two groups of allergens, the Der I group (Der p I: P1, Dp 42, Dpt 12; Der f I: F1, Ag 11, Df 6) and the Der II group (Der p II: Dp X; Der f II: Ag 19/20, DF 2), have been purified from Df and Dp [2]. Previously, we have also attempted to purify the allergenic component of house dust mite Df by use of a variety of immunochemical methods and detected the allergen components designated as Me1, Me2, Me3, and Me4 [3-5]. Me1 corresponded to Der f II and Me2 to Der f I [6].

In order to purify the major allergens, we often use the gel filtration method. In the gel filtration proce-

dure, we have usually used the buffer containing aprotinin, a protease inhibitor, to abolish the protease activity existing in mite extract which may autodigest the mite allergens. However, we have not clarified the details of proteases. In the present report, we studied the protease activities in the extract of the house dust mite Df.

Materials and Methods

Extraction

Crude extract of Df was prepared according to a method previously described [5]. Briefly, the cultured mites were defatted with ether, 20 ml of phosphate-buffered saline (PBS; 2 mM phosphate buffer containing 85 mM NaCl, pH 7.4) was added to 1 g of defatted mite, and the mixture was then stirred or rotated for 48 h at 4°C. The mixture was centrifuged (9,500 rpm for 30 min at 4°C) and the supernatant dialyzed against distilled water for 48 h at 4°C. After filtration by membrane filter (pore size 0.45 µm), the filtrated solution was lyophilized and used as the crude mite extract (CME).

Gel Filtration of CME

One hundred milligrams of CME dissolved in 3 ml of phosphate buffer (100 mM, pH 7.4) was applied to a column (25 × 850 mm) of Sephacryl S-200 (Pharmacia) which was previously equilibrated with the phosphate buffer (100 mM, pH 7.4), and 5 ml of the eluate was collected. The protein content of the fraction was measured for optical density at 280 nm.

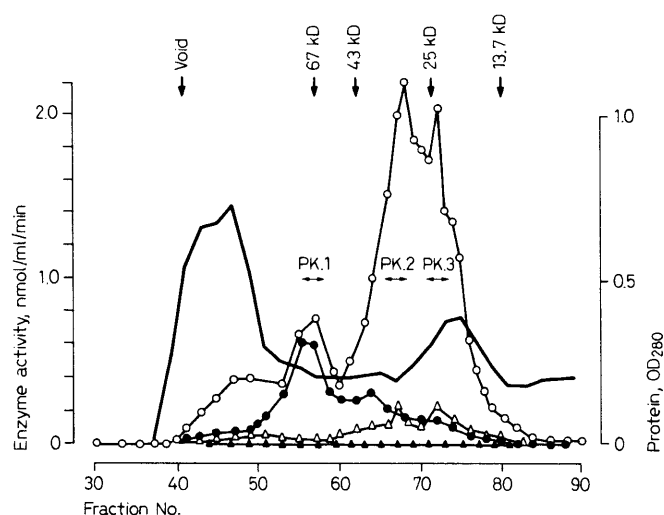


Fig. 1. Protease activities in the gel-filtrated CME. CME was gel filtrated using Sephacryl S-200. Five milliliters of each eluate was collected. The protein content (—) of the fraction was measured for optical density at 280 nm. The protease activities in the fractions were measured using Boc-Val-Leu-Lys-MCA (○), Boc-Phe-Ser-Arg-MCA (●), Suc-Leu-Leu-Val-Tyr-MCA (△), and Suc-Ala-Pro-Ala-MCA (▲) as substrates.

Measurement of Protease Activities

t-Butyloxycarbonyl-L-phenylalanyl-L-seryl-L-arginine 4-methylcoumaryl-7-amide (Boc-Phe-Ser-Arg-MCA), t-butyloxycarbonyl-L-valyl-L-leucyl-L-lysine 4-methylcoumaryl-7-amide (Boc-Val-Leu-Lys-MCA), succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine 4-methylcoumaryl-7-amide (Suc-Leu-Leu-Val-Tyr-MCA), and succinyl-L-alanyl-L-prolyl-L-alanine 4-methylcoumaryl-7-amide (Suc-Ala-Pro-Ala-MCA) were purchased from the Peptide Institute and used for the substrate of proteases. The protease activities were determined as described previously [7]. Briefly, 1 ml of reaction mixture consisting of 0.1 ml of each mite fraction and 0.9 ml of substrate (10 μ mol) in 100 mM phosphate buffer (pH 7.0) was incubated at 37°C for 15 min. Two milliliters of a 15% acetic acid solution was added to the reaction mixture and the fluorescence of aminomethylcoumarin released from the substrate measured at excitation 380 nm and emission 460 nm on a fluorometer.

Optimum pH of Proteases

Glycine-HCl buffer (100 mM, pH 2.0 and 4.0), citrate buffer (100 mM, pH 5.0), phosphate buffer (100 mM, pH 6.0 and 7.0), and Tris-HCl buffer (100 mM, pH 8.0 and 9.0) were used. Boc-Phe-Ser-Arg-MCA for PK.1 protease and Boc-Val-Leu-Lys-MCA for PK.2 and PK.3 proteases were used as substrate. The enzyme activities were measured as described for Measurement of Protease Activities.

Heat Stability of Proteases

The PK.1., PK.2., and PK.3 proteases were incubated at 37, 45, and 55°C for variable time intervals (min) in 100 mM phosphate buffer (pH 7.0). The remaining protease activities were detected us-

ing Boc-Phe-Ser-Arg-MCA in 100 mM phosphate buffer (pH 7.0) for PK.1 protease and Boc-Val-Leu-Lys-MCA in 100 mM citrate buffer (pH 5.0) for PK.2 and PK.3 proteases.

Effects of Various Agents

Aprotinin (Trasylol®, Bayer), MR-20 (Miracid®, Mochida Pharmaceuticals), soybean trypsin inhibitor (SBTI, Sigma), FUT-175 (Futhan®, Torii & Co.), N-tosyl-L-lysine chloromethylketone (TLCK, Peptide Institute), N-tosyl-L-phenylalanine chloromethylketone (TPCK, Peptide Institute), leupeptin (Peptide Institute), and iodoacetic acid (Wako Pure Ltd.) were used as the protease inhibitors. Other agents used were L-cysteine monohydrochloride (Cysteine, Nippon Rikagaku), ethylenediaminetetraacetic acid (EDTA, Nakarai Chemical), and ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA, Nakarai Chemical). The effects of various agents on the proteases are determined as follows: One milliliter of reaction mixture consisting of 0.1 ml of each fraction and substrate solution (10 μ mol) was incubated at 37°C for 15 min in the presence or absence of test agents. After the reaction was stopped by adding acetic acid solution, the intensity of fluorescence was measured as described for Measurement of Protease Activities. Phosphate buffer (100 mM, pH 7.0) and Boc-Phe-Ser-Arg-MCA were used for PK.1 protease. Citrate buffer (100 mM, pH 5.0) and Boc-Val-Leu-Lys-MCA were used for PK.2 and PK.3 proteases.

Ammonium Sulfate Fractionation and Gel Filtration

CME (5 g) was dissolved in 200 ml of PBS (2 mM phosphate buffer containing 85 mM NaCl, pH 7.4). The CME solution was cooled to 4°C, and saturated ammonium sulfate solution was added to 60% saturate. The precipitate and the supernatant were separated by centrifugation (9,500 rpm for 1 h at 4°C). The sediment was dissolved in 30 ml of PBS and dialyzed against the same buffer at 4°C overnight. The ammonium sulfate supernatant was concentrated using polyethylene glycol to 20 ml and dialyzed against PBS at 4°C overnight. Then, both ammonium sulfate fractionated preparations were applied to a column (50 $\times$ 920 mm) of Sephacryl S-200 (Pharmacia) previously equilibrated with PBS, and 10 ml of each eluate was collected. The protease activity in each fraction was measured as described for Measurement of Protease Activities.

Allergenic Activity

The allergenic activity was determined by enzyme-linked immunosorbent assay (ELISA). Sephacryl S-200 eluate was diluted ($\times$ 100) using PBS, and 0.1 ml of diluted sample was applied to a CBrN-activated paper disk. After incubation at room temperature overnight, the allergen-binding paper disk was washed with 0.1 M NaHCO₃ solution. β -Ethanolamine (1 M dissolved in 0.1 M NaHCO₃) was added to a paper disk and incubated at room temperature for 3 h. The paper disk was washed once with 0.1 M NaHCO₃ and three times with 0.1 M acetate buffer (pH 4.0). Then, 50 μ l of antimite pooled human serum (radioallergosorbent test score > 4) was added to the paper disk. After incubation at 37°C for 3 h, the quantity of antimite human IgE bound to the paper disk was measured using the Phadezyme RAST® kit (Pharmacia). Briefly, the antimite human IgE-binding paper disk was washed three times with phosphate buffer. Fifty microliters of β -D-galactosidase-conjugated antihuman IgE serum was added to a paper disk. After incubation at room temperature for 16 h, the paper disk

was washed three times with phosphate buffer, and 0.2 ml of substrate solution (ortho-nitrophenyl- β -D-galactopyranoside and glutathione dissolved in phosphate buffer) was added to a paper disk. After incubation at 37°C for 2 h, the enzyme reaction was stopped by adding 1.5 ml of NaCO₃ solution, and the absorbance of the reaction mixture was measured at 420 nm.

Results

Protease Activities in the Gel-Filtrated CME

The protease activities in the gel-filtrated fractions are shown in figure 1. Boc-Phe-Ser-Arg-MCA- and Boc-Val-Leu-Lys-MCA-cleaving protease activities were measured in the fractions with a molecular weight of about 60 kD. Boc-Val-Leu-Lys-MCA- and Sac-Leu-Leu-Val-Tyr-MCA-cleaving protease activities were obtained in the fractions with a molecular weight of about 30 kD and 20 kD, respectively. Suc-Ala-Pro-Ala-MCA-cleaving protease activity was not observed in all fractions. The fractions 57 and 58, 66–68, and 71–73 were pooled and designated as PK.1, PK.2, and PK.3, respectively.

pH Dependency of PK.1, PK.2, and PK.3 Proteases

The effects of the pH value on the activity of each protease are shown in figure 2. PK.1 protease showed a maximum activity between pH 7.0 and 8.0. On the other hand, the optimum pH for PK.2 and PK.3 proteases measured using Boc-Val-Leu-Lys-MCA was 5.0.

Effect of Heating on the Protease Activities

Heat stabilities of the proteases are shown in figure 3. Protease activities of PK.1, PK.2, and PK.3 were reduced during incubation for 40 min at 45°C to approximately 80, 40, and 50%, respectively. At 55°C, the remaining protease activity of PK.1 was about 40%, while those of PK.2 and PK.3 were completely diminished by heating for 20 min.

Effects of Various Agents on the Protease Activities

The effects of various agents on the activities of PK.1, PK.2, and PK.3 proteases are shown in table 1. The PK.1 activity was inhibited by aprotinin, MR-20, SBTI, FUT-175, TLCK, and leupeptin. Cysteine, EDTA, and EGTA did not influence the PK.1 protease activity. The PK.2 and PK.3 protease activities were not inhibited by aprotinin, MR-20, SBTI, FUT-175, and TPCK, but were inhibited by TLCK, leupeptin, and iodoacetic acid. Cysteine and EDTA activated the PK.2 and PK.3 protease activities.

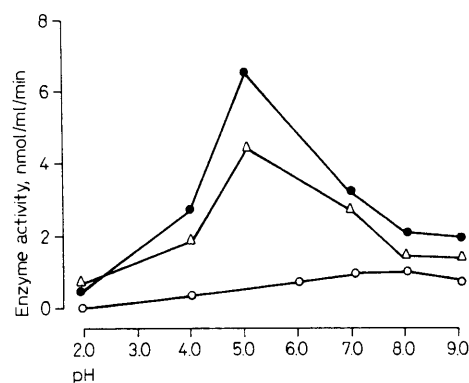


Fig. 2. Effect of pH on the activity of PK.1, PK.2, and PK.3 proteases. The PK.1 protease activity (○) was measured using Boc-Phe-Ser-Arg-MCA; PK.2 (●) and PK.3 (△) protease activities were determined using Boc-Val-Leu-Lys-MCA.

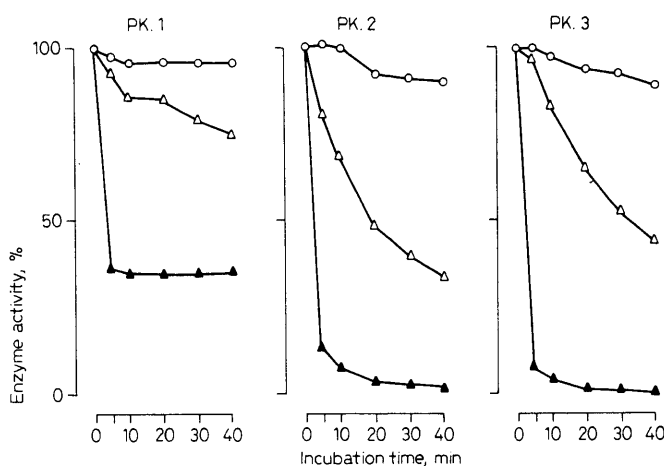


Fig. 3. Effects of heating on the activities of PK.1, PK.2, and PK.3 proteases. PK.1, PK.2, and PK.3 fractions were heated at 37 (○), 45 (△), and 55°C (▲) for 0–40 min in 100 mM phosphate buffer (pH 7.0). The remaining activity was measured using Boc-Phe-Ser-Arg-MCA (pH 7.0) for PK.1 protease and Boc-Val-Leu-Lys-MCA in 100 mM acetate buffer (pH 5.0) for PK.2 and PK.3 proteases.

Protease Activity and Allergic Activity in the Supernatant of Ammonium Sulfate Fractionation

The supernatant of the 60% saturated ammonium sulfate fractionation of CME was applied to a Sephacryl S-200 column. The gel filtration profile is shown in figure 4. PK.1 and PK.2 protease activities were observed in the fractions with a molecular weight of about 60 and 30 kD, respectively, but the PK.3 protease activity was not detected in the fraction

Table 1. Effect of various agents on the activities of PK.1, PK.2, and PK.3 proteases

Agents	Concentration μM	Inhibition, %		
		PK.1	PK.2	PK.3
Aprotinin ^a	1	14.9	0	0
	10	21.9	0	0
MR-20 ^b	1	21.9	0	0
	10	29.2	0	0
SBTI	1	28.5	0	0
	10	36.7	0	0
FUT-175	1	33.1	0	0
	10	43.2	0	0
TLCK	10	70.2	72.1	67.5
	100	86.2	89.4	91.8
TPCK	1	6.0	0	0
	10	13.0	4.6	0
Leupeptin	1	86.9	93.8	93.0
	10	94.7	97.1	97.3
Iodoacetic acid	10	0	81.6	83.0
	100	9.7	92.5	96.9
Cysteine	10	0	-7.5	-103.1
	100	0	-276.7	-420.3
EDTA	10	0	-28.4	-13.1
EGTA	10	0	-5.9	0

^a KIU/ml.^b U/ml.

with a molecular weight of about 20 kD. The allergenic activity was observed in three peaks at 60, 30, and 20 kD.

Protease Activity and Allergenic Activity in the Precipitate of Ammonium Sulfate Fractionation

The gel filtration profile of the 60% ammonium sulfate precipitate is shown in figure 5. PK.2 and PK.3 protease activities were observed in the fractions with a molecular weight of about 30 and 20 kD, respectively. The potent allergenic activity was observed in the peak fractions which showed the potent protease activities.

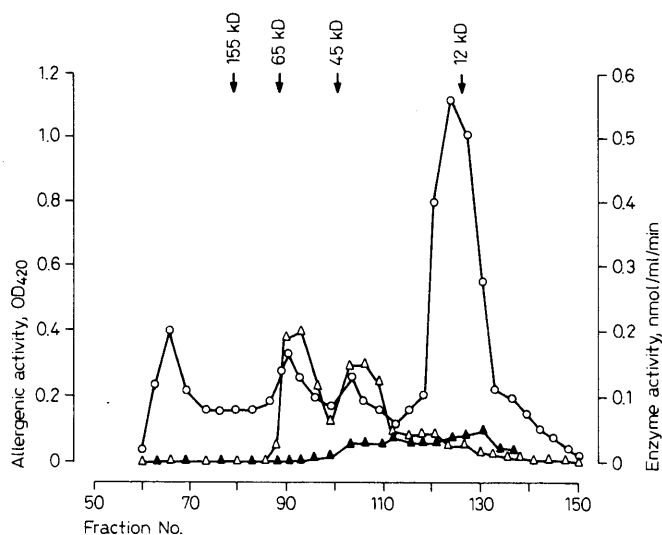


Fig. 4. Gel filtration pattern of supernatant. The supernatant of the ammonium sulfate fractionation (60% saturation) was applied to a Sephacryl S-200 column, and each 10 ml of the eluate was collected. The allergenic activity (○) against antimite human IgE was detected by ELISA, and protease activities were measured using Boc-Val-Leu-Lys-MCA (△) and Suc-Leu-Leu-Val-Tyr-MCA (▲).

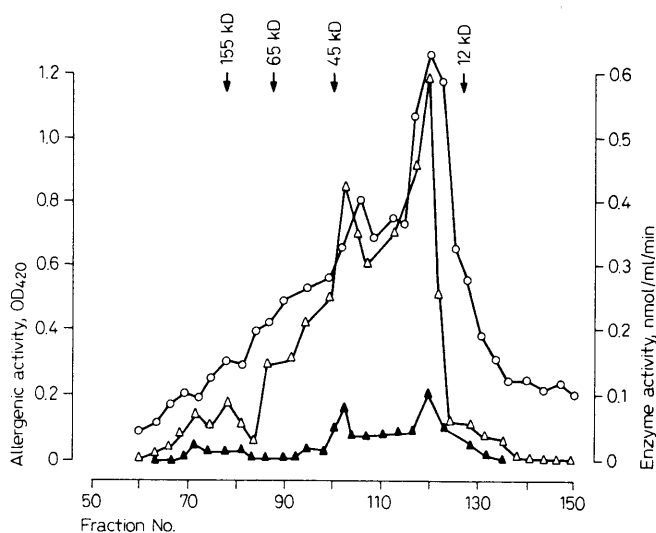


Fig. 5. Gel filtration pattern of precipitate. The precipitate of the ammonium sulfate fractionation (60% saturation) was applied to a Sephacryl S-200 column, and each 10 ml of the eluate was collected. The allergenic activity (○) against antimite human IgE was measured by ELISA, and protease activity was detected using Boc-Val-Leu-Lys-MCA (△) and Suc-Leu-Leu-Val-Tyr-MCA (▲).

Discussion

CME was gel filtrated using a Sephacryl S-200 column and the protease activities in the fractions measured. Three peaks of protease activities were detected. The first peak (PK.1) was observed in the fraction of about 60 kD. PK.1 protease cleaved arginyl and lysyl residue containing substrates such as Boc-Phe-Ser-Arg-MCA and Boc-Val-Leu-Lys-MCA. The second (PK.2) and the third (PK.3) peaks of protease activities were detected in the fractions of about 30 and 20 kD, respectively. PK.2 and PK.3 proteases cleaved lysyl and tyrosyl residue containing substrates such as Boc-Val-Leu-Lys-MCA and Suc-Leu-Leu-Val-Tyr-MCA. PK.1, PK.2, and PK.3 proteases did not cleave alanyl residue containing substrate such as Suc-Ala-Pro-Ala-MCA. PK.1 protease showed the pH optimum at 7.0–8.0. The pH dependence of PK.2 and PK.3 proteases showed a maximum at 5.0. PK.1 protease was more stable against heating at 45 or 55°C than PK.2 and PK.3 proteases.

Serine proteases are classified according to their substrate specificity into three groups: trypsin-like proteases, chymotrypsin-like proteases, and elastase. Trypsin-like protease has a specificity for arginyl and lysyl residue containing substrates, chymotrypsin-like serine protease for substrates containing tyrosyl and phenylalanyl residues, and elastase for alanyl residue containing substrates. It has been reported that aprotinin and MR-20 inhibit trypsin and chymotrypsin, and SBTI and FUT-175 inhibit trypsin [8]. PK.1 protease was inhibited by these serine protease inhibitors. These results and the substrate specificity suggest that PK.1 protease may be a trypsin-like serine protease of mites.

PK.2 and PK.3 proteases were inhibited by TLCK, leupeptin, iodoacetic acid, but not by serine protease inhibitors such as aprotinin, MR-20, FUT-175, and SBTI. Furthermore, PK.2 and PK.3 proteases were activated by cysteine. Cysteine proteases, e.g., papain and cathepsin B, are inhibited by TLCK, leupeptin, and iodoacetic acid and are activated by cysteine [9–12]. Cathepsin B is known to show a maximal activity at acid region [9]. The susceptibility to various agents and pH optimum of protease activity indicate that the PK.2 and PK.3 proteases may be cysteine proteases such as papain and cathepsin B.

Previously we reported that *Df* had two major allergens named Me1 (17 kD) and Me2 (27 kD) which correspond to *Derf* II and *Derf* I, respectively [6].

Chua et al. [13] studied the sequence analyses of the *Derp* I cDNA clone and the similarity between its amino acid sequence and group of cysteine proteases. These authors suggested *Derp* I might be a cysteine protease. Thus, we purified the allergens from CME by a combination of ammonium sulfate precipitation and gel filtration and studied the relationship between allergenic activity of allergens against antimitic human IgE and protease activities of them. PK.1 protease activity was detected at the 60-kD fraction in the supernatant of the 60% ammonium sulfate fractionation. PK.2 protease activity was detected at the 30-kD fraction in both supernatant and precipitate of the 60% ammonium sulfate fractionation. These fractions, PK.1 and PK.2, showed potent allergenic activities. PK.3 protease was detected in the precipitate of the 60% saturation of ammonium sulfate, and the gel filtration profile coincided with that of the 20-kD allergenic activity of mite extract. The protease activity was not detected, but potent allergenic activity was observed at the 20-kD fraction in the supernatant of the 60% ammonium sulfate fractionation. Previously, we showed that the *Derf* II allergen, Me1, existed in the supernatant of the 60% ammonium sulfate fractionation and *Derf* I allergen, Me2, in the precipitate [6]. Thus, it is strongly suggested that *Derf* I is a kind of protease similar to cysteine proteases, e.g., papain and cathepsin B. PK.3 protease was more heat labile than PK.1 protease. These observations support the hypothesis that PK.3 protease is a *Derf* I allergen protein, since the *Derf* I group allergen is recognized to be a heat-labile glycoprotein [2]. It was pointed out recently [14] that *Der* I allergen was closely related to fecal particles. *Der* I allergen may be derived from digestive protease secreted from the gastrointestinal tract of the house dust mite.

References

- 1 Bronswijk JEMH: Hausstaubmilben: Vorkommen und Bedeutung. *Allergologie* 1978;1:55–60.
- 2 Platts-Mills TAE, Chapman MD: Dust mites: Immunology, allergic disease, and environmental control. *J Allergy Clin Immunol* 1987;80:755–775.
- 3 Miyamoto T, Kudo K, Nakagawa T, et al: Mite antigen. Allergy and clinical immunology. Proc IXth Int Congr of Allergology, Buenos Aires. Amsterdam, Excerpta Medica, 1976, Int Congr Ser No 414, p 173.
- 4 Nakagawa T, Kudo K, Okudaira H, et al: Characterization of the allergenic component of the house dust mite, *Dermatophagoides farinae*. *Int Arch Allergy Appl Immunol* 1977;55:47–53.

- 5 Haida M, Okudaira H, Ogita T, et al: Allergens of the house dust mite *Dermatophagoides farinae*. Immunochemical studies of four allergenic fractions. J Allergy Clin Immunol 1985;75: 686-692.
- 6 Yamashita N, Haida M, Suko M, et al: Allergens of the house dust mite *Dermatophagoides farinae*. II. Immunological characterization of four allergenic molecules. Int Arch Allergy Appl Immunol 1989;88:173-175.
- 7 Iwaki M, Ino Y, Motoyoshi A, et al: Pharmacological studies of FUT-175, nafamostat mesilate. Effects on the pancreatic enzymes and experimental acute pancreatitis in rat. Jpn J Pharmacol 1986;41:155-162.
- 8 Ino Y, Suzuki K, Sato T, et al: Comparative studies of nafamostat mesilate and various serine protease inhibitors in vitro. Folia Pharmacol Jpn 1986;88:449-455.
- 9 Barrett AJ: Human cathepsin B. Purification and some properties of the enzymes. Biochem J 1973;131:809-822.
- 10 Greenberg DM: Plant proteolytic enzymes. Methods Enzymol 1955;2:54-64.
- 11 Umezawa H: Structures and activities of protease inhibitors of microbial origin. Methods Enzymol 1976;45:678-694.
- 12 Ikegawa H, Aoyagi T, Takeuchi T, et al: Effect of protease inhibitors of actinomycetes on lysosomal peptide-hydrolases from swine liver. J Antibiot 1971;24:488-489.
- 13 Chua KY, Stewart GA, Thomas WR, et al: Sequence analysis of cDNA coding for a major house dust mite allergen, *Der p* 1. Homology with cysteine protease. J Exp Med 1988;167:175-182.
- 14 Lind P: Demonstration of close physiochemical similarity and partial immunochemical identity between the major allergens, Dp42, of the house dust mite, *D. pteronyssinus*, and corresponding antigen of *D. farinae* (Df 6) and *D. microceras* (Dm 6). Int Arch Allergy Appl Immunol 1986;79:60-65.

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