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Isolation of Cysteine Protease in the Crude Mite Extract, *Dermatophagoides farinae*

Tohru Ando^a, Yoshitaka Ino^a, Michiko Haida^b, Reiko Honma^a, Hidenori Maeda^a, Hiroshi Yamakawa^a, Masahiro Iwaki^a, Hirokazu Okudaira^b

^a Research Laboratories, Torii & Co., Ltd., Ichikawa, Japan, and ^b Department of Medicine and Physical Therapy, Faculty of Medicine, University of Tokyo, Japan

Key Words. Allergenic activity · Cysteine protease · *Der f I* · Mites

Abstract. In order to study the relationship between cysteine protease and *Der f I*, which is one of the major allergens in the mite, *Dermatophagoides farinae*, isolation of cysteine protease was attempted using various column chromatographies. Both the potent cysteine protease activity and the allergenic activity were detected in the same fractions by anion exchange chromatography on a DEAE-Sephacel, gel chromatographies and chelating Sepharose 6B chromatography. In the double immunodiffusion test, the finally isolated fraction and rabbit anti-*Der f I* sera reacted to give a single precipitation line which fused completely with the precipitation line formed by *Der f I* and anti-*Der f I* sera. Sequence analysis for the first 10 N-terminal amino acids from cysteine protease and *Der f I* were identical. These results strongly suggest that cysteine protease of mites may be *Der f I* allergen and that measuring cysteine protease activity may possibly become a beneficial method for detecting *Der f I* allergens.

Introduction

House dust is a well-known typical allergen causing atopic asthma and allergic rhinitis. Among many allergens contained in house dust, two species of mites, genus *Dermatophagoides pteronyssinus* (*Dp*) and *Dermatophagoides farinae* (*Df*) are known to be dominant in Japan [1], Europe and the west coast of the United States [2]. Many efforts have been made to define the major allergen existing in house dust mites, and two groups of allergens, the *Der I* group (*Der p I*: P1, Dp42, Dpt12; *Der f I*: F1, Ag11, Df6) and the *Der II* group (*Der p II*: DpX; *Der f II*: Ag19/20, DF2) have been identified by several groups of workers [3]. Previously, we also attempted to purify the allergenic components designated as Me1, Me2, Me3, and Me4 [4–6] where Me1 corresponded to *Der f II* and Me2 to *Der f I* [7]. Further, we detected a trypsin-like protease and cysteine protease in the crude mite extract (CME) and suggested that cysteine protease might be *Der f I* allergen [8].

In order to purify the major allergens from mites, many methods have been used to detect allergenic ac-

tivity [9, 10], though these procedures are complicated and time-consuming. If *Der f I* is cysteine protease, it can be purified by tracing cysteine protease activity which is easily detectable. In the present study, we tried to isolate the *Der f I* allergen by monitoring cysteine protease activity.

Materials and Methods

Extraction

A crude extract of *Df* was prepared according to the method previously described [6]. Briefly, the cultured mites were defatted with ether, 300 ml of phosphate-buffered saline (PBS, pH 7.4) was added to 15 g of defatted mite, and the mixture was then stirred for 48 h at 4°C. The mixture was centrifuged (9,500 rpm for 30 min at 4°C) and the supernatant was dialyzed against distilled water for 48 h at 4°C. After filtration by membrane filter (pore size 0.45 µm), the filtrate was lyophilized and used as the CME.

Preparation of Cysteine Protease

The isolation procedure is summarized in figure 1. One point five grams of CME was dissolved in 50 ml of 0.01 M PBS (pH 7.0) and stirred for 1 h at 4°C, followed by centrifugation at 9,500 rpm for 1 h at 4°C. The supernatant was collected and saturated ammonium sulfate was added to 60% saturation. The precipitate was

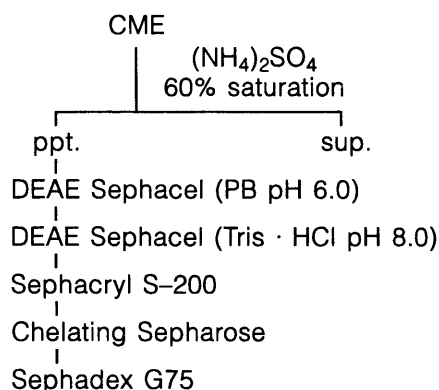


Fig. 1. Diagram of the isolation procedure for cysteine protease. sup = Supernatant; ppt = precipitate.

separated from the supernatant by centrifugation at 9,500 rpm for 1 h at 4 °C. The precipitate was dissolved in 10 ml of 0.02 M phosphate buffer (PB, pH 6.0), then dialyzed against the same buffer and applied to a DEAE-Sephacel (Pharmacia) column equilibrated with the same PB. The column was then eluted with a linear sodium chloride gradient from 0 to 0.3 M, and aliquots of 5 ml were collected. The fractions containing high cysteine protease activity were pooled (Fr. 1) and dialyzed against 0.05 M Tris-HCl (pH 8.0) and applied to a DEAE-Sephacel column equilibrated with the same Tris buffer. The stepwise elution was then performed with 0.05 M Tris-HCl (pH 8.0), 0.05 M Tris-HCl containing 0.2 M sodium chloride (pH 7.4), and 0.05 M Tris-HCl containing 0.5 M sodium chloride (pH 7.4). Aliquots of 5 ml were collected. The fraction eluted with 0.05 M Tris-HCl containing 0.2 M sodium chloride (pH 7.4, Fr. 2) was concentrated from 45 to 6.5 ml by ultrafiltration through a PM-10 membrane (Amicon), and fractionated on a Sephacryl S-200 (Pharmacia) column equilibrated with 0.02 M PBS (pH 6.0). The fractions containing cysteine protease (Fr. 3) were concentrated in volumes from 90 to 20 ml, dialyzed against 0.05 M Tris-acetate containing 0.5 M sodium chloride (pH 7.5), and subjected to chelate chromatography on chelating Sepharose 6B (Pharmacia) which was previously chelated with zinc ion and equilibrated with the Tris-acetate buffer. The sample was applied to the column and the stepwise elution was performed by decreasing the pH of 0.05 M Tris-acetate buffer from 7.5 to 6.5, 5.5 and 4.5. Five milliliters of each eluate was collected. The fraction with cysteine protease activity, eluted with 0.05 M Tris-acetate containing 0.5 M sodium chloride at pH 5.5 (Fr. 4), was concentrated to 3 ml and applied to a Sephadex G-75 (Pharmacia) column equilibrated with 0.02 M PBS (pH 6.0). Three milliliters were collected. In all isolation procedure, the protein content of each fraction was monitored by measuring absorbance at 280 nm.

Measurement of Cysteine Protease Activity

Succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine 4-methylcoumarin-7-amide (Suc-Leu-Leu-Val-Tyr-MCA) was purchased from the Peptide Institute and used as the substrate for cysteine protease. Protease activity was determined by the method described previously [11]. Briefly, 1 ml of reaction mixture consisting of 0.1 ml of each column fraction, 0.8 ml of substrate (10 μ mol) in 0.1 M PB

(pH 6.0), both 0.05 ml of 0.02 M cysteine and 0.02 M disodium EDTA as activators, was incubated at 37 °C for 30 min. Two milliliters of 15% acetic acid solution were then added to the reaction mixture and the fluorescence of aminomethylcoumarin released from the substrate was measured at excitation, 380 nm, and emission, 460 nm, on a fluorometer.

Measurement of Allergenic Activity

The allergenic activity was determined by fluorometric enzyme-linked immunosorbent assay (ELISA) according to the method of Sakaguchi et al. [12]. Briefly, each column eluate was diluted ($\times 100$) using 0.1 M bicarbonate buffer (pH 9.5), and 0.05 ml of a diluted sample was added to each well of a polystyrene microtiter plate (Dynatech, Immulon II). The plate was incubated overnight at room temperature. After washing (0.3 ml PBS, pH 7.4/0.05% Tween 20, three times), excess absorption sites were blocked for 1 h at room temperature by 0.3 ml of 3% gelatin in PBS. The plate was then washed three times and 0.05 ml of five fold-diluted anti-mite pooled human sera (radioallergosorbent test score > 4) were added. After incubation for 3 h at room temperature, the microplate was washed four times and added with 0.05 ml of tenfold-diluted β -D-galactosidase-conjugated anti-human IgE (Phadezyme kit, Pharmacia). After incubation for 16 h at room temperature, the microplate was washed four times, and added with 0.1 ml of 0.1 mM 4-methylumbelliferyl- β -D-galactoside. After incubation for 2 h at room temperature, the enzyme reaction was stopped by adding 0.1 ml of 0.1 M glycine-NaOH (pH 10.3), and the fluorescence of 4-methylumbelliferone released from the substrate was measured at excitation, 365 nm, and emission, 450 nm, on a fluorometer.

Rabbit Antisera

Antisera to CME were prepared by immunizing New Zealand white rabbits with a mixture of CME (4 mg in 1 ml saline) and 1 ml of complete Freund's adjuvant (Difco). The rabbits were injected subcutaneously with the above mixture six times at 1-week intervals and bled 10 days after the last injection. Antisera to *Der f I* were kindly provided by Dr. Hiroshi Yasueda, National Sagami-hara Hospital, Japan.

Double Immunodiffusion

Double immunodiffusion was performed according to the method of Ouchterlony [13].

Sequencing of Amino Acid of Cysteine Protease

The cysteine protease of the final preparation was accomplished by high performance liquid chromatography (Model 130 A, Applied Biosystems Co.; Column, C8 300A, 2.1 ID $\times$ 30 mm): buffer A, 0.1% trifluoroacetic acid in H_2O ; buffer B, 0.1% trifluoroacetic acid in 70% acetonitrile; gradient, 0% buffer B to 100% buffer B at 2.2%; flow rate, 200 μ l/min. Determination of the amino acid sequence of the purified cysteine protease was carried out employing a gas phase protein sequencer (Model 471A, Applied Biosystems Co.).

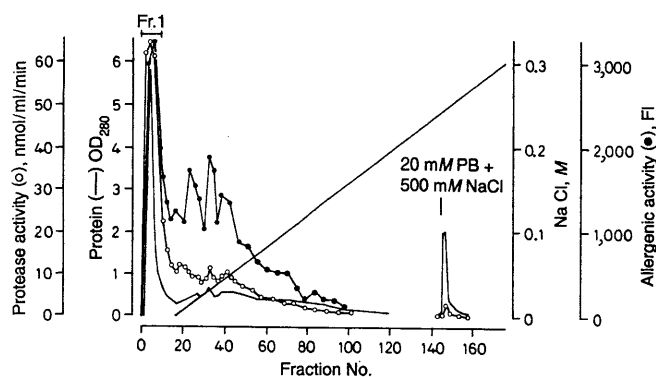


Fig. 2. Chromatography of the 60% ammonium sulfate precipitate of mites on a DEAE-Sephacel column (1.5×10 cm) equilibrated with 0.02 M PB (pH 6.0). The linear gradient elution was performed with 0.02 M PB (pH 6.0) containing sodium chloride from 0 to 0.3 M, and 5 ml of each eluate was collected. FI = Fluorescence intensity.

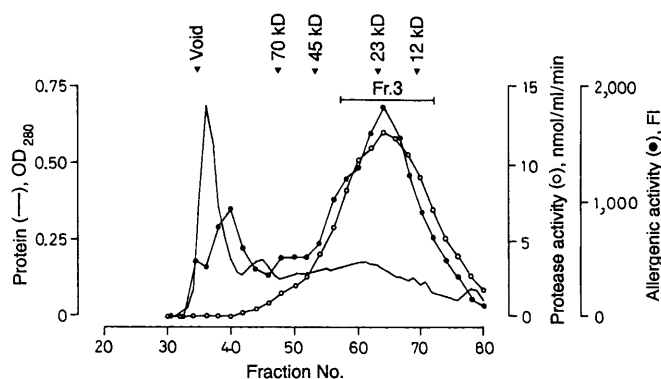


Fig. 4. Chromatography of Fr. 2 (compare with fig. 3) on a sephacryl S-200 column (2.5×88 cm) equilibrated with 0.02 M PBS (pH 6.0), and 5 ml of each eluate was collected. FI = Fluorescence intensity.

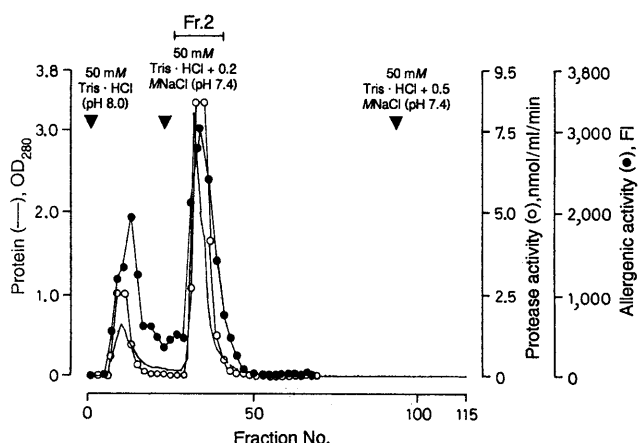


Fig. 3. Chromatography of Fr. 1 (compare with fig. 2) on a DEAE-Sephacel column (2.5×7.5 cm) equilibrated with 0.05 M Tris-HCl (pH 8.0). The stepwise elution was performed with 0.05 M Tris-HCl, containing 0.2 M NaCl (pH 7.4), and 0.05 M Tris-HCl containing 0.5 M NaCl (pH 7.4). Aliquots of 5 ml were collected. FI = Fluorescence intensity.

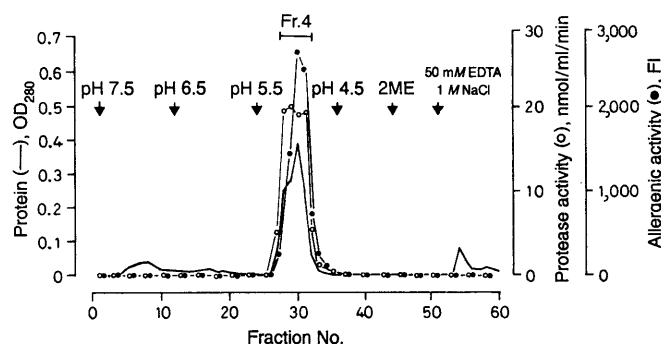


Fig. 5. Chromatography of Fr. 3 (compare with fig. 4) on chelating Sepharose 6B. The column (1.5×8 cm) was chelated with zinc ion and equilibrated with 0.05 M Tris-acetate containing 0.5 M sodium chloride (pH 7.5). The sample was eluted with a stepwise decrement in pH of the Tris-acetate buffer; pH 6.5, pH 5.5, pH 4.5. Aliquots of 5 ml were collected. FI = Fluorescence intensity.

Results

Isolation of Cysteine Protease

The precipitate of the 60% saturated ammonium sulfate fractionation of CME was applied to a DEAE-Sephacel column equilibrated with 0.02 M PB (pH 6.0; fig. 2). Both the potent cysteine protease activity and the allergenic activity were detected in the unad-

sorbed fractions in which a large amount of protein was detected. The unadsorbed fractions having an absorption of more than 1.0 at 280 nm (Fr.1) were pooled and then applied to a DEAE-Sephacel column equilibrated with 0.05 M Tris-HCl (pH 8.0, fig. 3). Both the potent cysteine protease activity and the allergenic activity were detected in the fractions eluted with 0.05 M Tris-HCl containing 0.2 M sodium chlo-

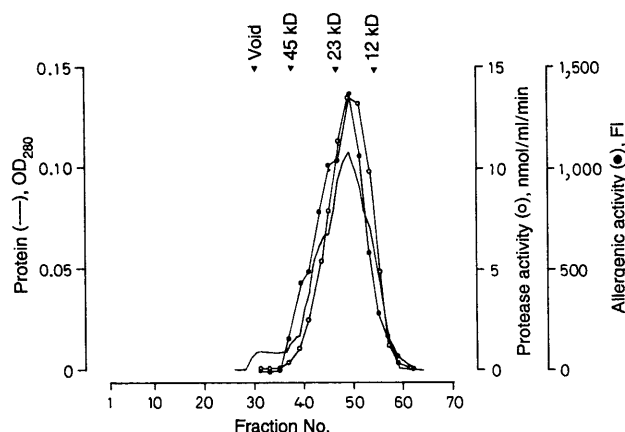


Fig. 6. Chromatography of Fr. 4 (compare with fig. 5) on a Sephadex G-75 column (1.9 × 77 cm) equilibrated with 0.02 M PBS (pH 6.0). Aliquots of 3 ml were collected. FI = Fluorescence intensity.

ride (pH 7.4, Fr. 2). The Sephacryl S-200 gel filtration profile of these fractions is shown in figure 4. A single peak of protease activity was present in the fraction with a molecular weight of about 20 kD (Fr. 3), while the protein peak was observed in the void volume fractions. Fr. 3 was then applied to a chelating Sepharose 6B column using zinc as chelating ion (fig. 5). Both the potent cysteine protease activity and the allergenic activity were found in the fractions eluted with 0.05 M Tris-acetate containing 0.5 M sodium chloride (pH 5.5, Fr. 4). The Sephadex G-75 gel filtration profile of Fr. 4 is shown in figure 6. The cysteine protease activity, the allergenic activity and the protein were observed as a single peak in the same fractions with a molecular weight of 20 kD. The peak fractions were collected and reprecipitated by ammonium sulfate at 55% saturation. This precipitate was regarded as the final preparation of our experiment and contributed to the following experiment. The cysteine protease activity of the final preparation (153 nmol/min/mg protein) was increased by about 6.5 times in comparison with that of CME (24 nmol/min/mg protein).

Double Immunodiffusion

The purity of the final preparation was examined by double immunodiffusion. The results obtained are shown in figure 7. The final preparation and anti-*Der f I* sera reacted to give a single precipitation line which fused completely with the precipitation line

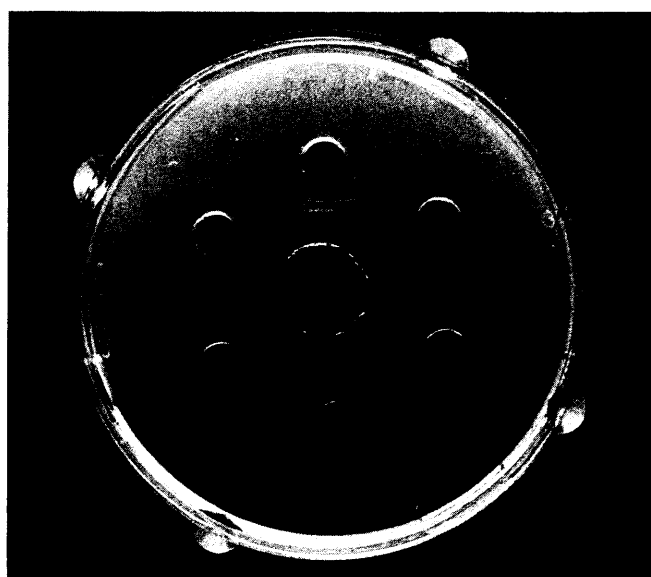
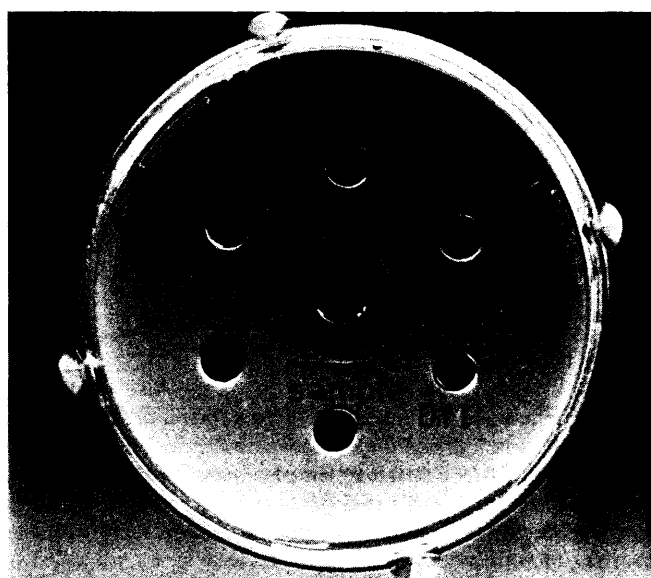


Fig. 7. Double immunodiffusion. **a** Result between sample (the final preparation) and *Der f I* (Df I) with anti-*Der f I* (aDf I) sera. **b** Result between sample (the final preparation) and *Der f I* with anti-crude mite extract (aCME) sera.

formed by *Der f I* (DfI) and anti-*Der f I* sera (fig. 7a). The final preparation and anti-CME sera also reacted to give a single precipitation line which fused completely with the precipitation line formed by *Der f I* and anti-CME sera. On the other hand, several precipitation lines were formed between CME and anti-CME sera (fig. 7b).

Amino Acid Sequence of the Cysteine Protease

Sequence analysis for the first 10 N-terminal amino acids from the cysteine protease and *Der f I* were compared. As shown in figure 8, both amino acid sequences were identical.

Discussion

Proteases are classified into two groups, exopeptidases and endopeptidases. Endopeptidases hydrolyze the intrapeptide chain bond and exopeptidases hydrolyze the C- or N-terminal amino acid residue. Endopeptidases are further classified into four groups according to their active sites: serine proteases, cysteine proteases, carboxyl proteases, and metalloproteases [14]. Previously, we reported that two groups of proteases were detected in the extract of *Df* [8]. One was a serine protease which cleaved arginyl and lysyl residue-containing substrates and was inhibited by various serine protease inhibitors. The other was a cysteine protease which showed optimum pH in the acid range and was activated by cysteine and disodium EDTA. Furthermore, we indicated that cysteine protease possessed some points of similarity to *Der f I* in their allergenic and biochemical specificity. Namely, this cysteine protease was detected in the precipitate of the 60% saturation of ammonium sulfate, and the gel filtration profile coincided with the 20-kD allergenic activity of mite extract. This cysteine protease easily lost its enzyme activity by heating and *Der f I* is known to be a heat-labile allergen. From this point of view, we previously suggested the possibility that *Der f I* was cysteine protease. We therefore tried to clarify the relation between cysteine protease and *Der f I* in the present paper.

This cysteine protease cleaves lysyl and tyrosyl residue-containing substrates. It is known that trypsin cleaves the lysyl residue-containing substrate and chymotrypsin cleaves the tyrosyl residue-containing substrate. In the preliminary experiment in order to eliminate the influence of other protease activities, we searched for a method to detect this specific cysteine protease activity. The best result was obtained using a tyrosyl residue-containing substrate such as Suc-Leu-Leu-Val-Tyr MCA at pH 6.0, and by adding cysteine and disodium EDTA to the reaction mixture as activators. Using this method, we tried to purify cysteine protease in the extract of *Df*. 60% ammonium sulfate precipitation was chosen as the starting method ac-

Cysteine protease

1 10
NH₂-Thr-Ser-Ala-Cys-Arg-Ile-Asn-Ser-Val-Asn-

Der f I

1 10
NH₂-Thr-Ser-Ala-Cys-Arg-Ile-Asn-Ser-Val-Asn-

Fig. 8. Sequence of N-terminal amino acids comparing cysteine protease and *Der f I*. *Der f I* sequence data were obtained from Heymann et al. [9].

cording to our previous report [8]. Chua et al. [15] carried out sequence analyses of the *Der p I* cDNA clone and demonstrated the similarity of their amino acid sequences between *Der p I* and a group of cysteine proteases such as papain and cathepsin B; thus, the precipitate obtained by 60% ammonium sulfate fractionation was first subjected to a DEAE-anion exchange column in accordance with the purification method of human and rat cathepsin B. Although Barrett et al. [16] reported that these cathepsin Bs were adsorbed with a DEAE-anion exchange column equilibrated with 0.02 M PB (pH 6.0), cysteine protease derived from CME was not adsorbed with this column. This indicated that cysteine protease of mites might be different from human and rat cathepsin B in their isoelectric points. The unadsorbed fractions showing the potent cysteine protease activity were again subjected to a DEAE-Sephacel chromatography by changing the pH of the eluate. The majority of cysteine protease was adsorbed with a DEAE-Sephacel column equilibrated with 0.05 M Tris-HCl (pH 8.0) and was eluted with 0.05 M Tris-HCl containing 0.2 M sodium chloride (pH 7.4, Fr. 2). Fr. 2 was further applied to a column of Sephacryl S-200 and the cysteine protease activity was detected as a single peak with a molecular weight of about 20 kD. Since the preliminary experiments indicated that the cysteine protease activity of mites was inhibited by zinc ion, a subsequent isolation step was therefore performed by chelate chromatography on chelating Sepharose 6B using zinc as the chelating ion. Cysteine protease was detected as a single peak in the fractions eluted with

0.05 M Tris-acetate containing 0.5 M sodium chloride (pH 5.5, Fr. 4). Fr. 4 was further applied to a column of Sephadex G-75 and the cysteine protease activity was detected as a single peak with a molecular weight of 20 kD. The peak fractions were collected and reprecipitated by ammonium sulfate at 55% saturation. The precipitate of ammonium sulfate fractionation was regarded as a final preparation of our experiment. As mentioned above, we traced the cysteine protease activity in various isolation procedures, and the final preparation of our experiment therefore might be a purified cysteine protease of *Df*. If *Der f I* is a cysteine protease, *Der f I* may exist in our final preparation. Thus, we examined the existence of *Der f I* in our final preparation by double immunodiffusion. The final preparation and anti-CME sera reacted to give a single precipitation line. The precipitation lines formed between anti-*Der f I* sera and the final preparation of our experiment and also *Der f I* fused in a reaction which indicated complete identity. These results indicate that our cysteine protease might be a *Der f I*. The N-terminal amino acid sequence of *Der f I* has already been reported by Heymann et al. [9]. We therefore examined the N-terminal amino acid sequence of our cysteine protease and compared it with that of *Der f I*. The first 10-terminal amino acids from the cysteine protease and *Der f I* were identical. The data obtained in the present study strongly suggest that *Der f I* is cysteine protease. Yasueda et al. [17] purified *Der f I* by monitoring the antigenic activities with a combination of various chromatographies. *Der f I* was adsorbed with a DE-52 anion exchange column equilibrated with 0.05 M Tris-HCl (pH 8.0) and eluted with 0.05 M Tris-HCl containing 0.2 M sodium chloride (pH 7.4). They also found that chelate chromatography using zinc as the chelating ion was a useful method for purification of *Der f I*. These chromatographies were also found useful in our present study to purify cysteine protease of mites. Furthermore, the peak fractions of the cysteine protease activity at various chromatographic steps contained a potent allergenic activity. These results strongly support the possibility that *Der f I* is cysteine protease.

Radioimmunoassay and radioimmunoelectrophoresis, etc., are used to purify *Der f I* allergen from the extract of mite bodies and whole culture [6, 9]. The antigen-antibody reaction was applied to all these methods, but these methods are complicated and time-consuming. For example, several reaction steps

and 2 or 3 days are needed to detect *Der f I* by ELISA or a radioimmunoassay method [18, 19]. The method to measure cysteine protease activity is the one-step enzyme reaction, and the reaction period required for detection is only 30 min. In the present study, we traced cysteine protease activity and obtained *Der f I*. Thus, the method measuring cysteine protease activity might be useful for the detection of *Der f I* allergen in the purification procedure of mite extract.

Acknowledgements

We are very grateful to Dr. Hiroshi Yasueda, National Sagami Hospital, Kanagawa, Japan, for providing authentic *Der f I* and rabbit antisera to *Der f I*.

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Received: May 3, 1991

Accepted after revision: June 3, 1991

Correspondence to: Dr. T. Ando
Research Laboratories
Torii & Co., Ltd.
3-14-3 Minamiyawata
Ichikawa 274 (Japan)