

Trypsin-like protease of mites: purification and characterization of trypsin-like protease from mite faecal extract *Dermatophagoides farinae*. Relationship between trypsin-like protease and *Der f* III

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Summary

A serine protease from mite faecal extract, *Dermatophagoides farinae*, was purified using DEAE-Sephacel anion exchange chromatography and Superdex 75 pg gel chromatography. The molecular weight of this protease was 34 kD on SDS-PAGE under reducing conditions. The optimal pH and temperature of the protease were 8.0 and 47°C, respectively. In addition, this protease cleaved arginyl or lysyl residue containing substrates selectively and was only inhibited by aprotinin, FUT-175, and soy bean trypsin inhibitor and not by chymostatin, E-64 and iodoacetic acid. These results show that our purified serine protease belongs to the trypsin-type. Purified trypsin-like protease was shown to be allergenic by enzyme-linked immunosorbent assay. Antigenicity of trypsin-like protease was completely different from those of *Der f* I and *Der f* II. Both, 20 N-terminal amino acid sequence and amino acid compositions of the purified protease were very similar to those of *Der f* III. Good similarities were found between trypsin-like protease and *Der f* III concerning physicochemical properties such as molecular weight on SDS-PAGE and ammonium sulphate solubility. Summarizing the above data, it can be concluded that a trypsin-like protease from mite faecal extract is actually the *Der f* III allergen and that it may be involved in the digestive process of the mite as it was found not in mite body but in mite faeces.

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Introduction

Two species of mites, *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae* have been reported to be the most important source of allergen in house dust [1,2]. These species of mites were intensively studied and, at present, two groups of major allergens, *Der* I group (*Der p* I, *Der f* I) and *Der* II group (*Der p* II, *Der f* II), have been identified by several authors [3]. Recent reports have shown that some kinds of mite allergens are proteases. Dilworth *et al.* also reported that *Der f* I's cDNA sequence

had about 20% homology with several cysteine proteases such as cathepsin B and actinidin [4]. Recently, Heymann *et al.* isolated the third mite allergen, *Der f* III [5], and Stewart *et al.* purified trypsin-like protease from faecally enriched extracts of *D. pteronyssinus* and indicated it as *Der p* III allergen [6,7]. Furthermore, Yasueda *et al.* purified the fifth mite allergen (DP5, DF5) with chymotryptic activity [8]. Other biochemical studies also have shown that extracts of *D. pteronyssinus* and *D. farinae* contain both trypsin- and chymotrypsin-like proteases and these serine protease activities are also detected in house dust samples [6,9,10].

Recently, we reported the existence of two kinds of

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proteases, cysteine protease and trypsin-like protease, in the extract from the body of the mites (*D. farinae*) and the former protease was identified as *Der f* I allergen [11,12]. We detected the potent trypsin-like protease in the extract from the faeces of the mites as well as the bodies of them. Thus the detailed allergenic and biochemical properties of trypsin-like protease from mite faecal extract, *D. farinae*, have been examined and its relationship with *Der f* III has been explored in this study.

Materials and methods

Mite extracts

D. farinae was cultivated in a flat type flask containing animal food at 25°C and 75% humidity for 1 month. After cultivation, faeces were collected by sieving (250 mesh). Faeces-rich material (32.7 g) was extracted for 30 min in 655 ml saline and centrifuged (10 000 *g* for 60 min at 4°C). The supernatant was then filtered through a membrane filter (pore size 0.45 µm). The filtrate was used as the mite faecal extract (MFE).

Purification of mite trypsin-like protease

MFE (655 ml) was concentrated to 200 ml using polyethylene glycol (MW 50 kD), dialysed against 20 mM phosphate buffer (pH 6.0) and applied to a DEAE-Sephacel (Pharmacia, Sweden) column equilibrated with the same phosphate buffer. Elution was performed with a linear gradient concentration of NaCl from 0 to 0.4 M with the same buffer. The 0.15 M NaCl eluted fractions containing the potent trypsin-like protease activity were pooled, concentrated to 10.1 ml by polyethylene glycol and the precipitate obtained by 60–80% saturation of ammonium sulphate was collected. After being dissolved in 1 ml of 50 mM phosphate buffer, pH 7.4, the sample solution was applied to a column of Superdex 75 pg (Pharmacia, Sweden) and the fractions containing the potent protease activity were pooled. *Der f* I and *Der f* II were purified by column chromatography as reported previously [12,13].

Measurement of protease activity

A synthetic substrate of trypsin, *t*-butyloxycarbonyl-L-phenyl-L-seryl-L-arginine 4-methylcoumaryl-7-amide (Boc-Phe-Ser-Arg-MCA) and other synthetic substrates for protease were purchased from the Peptide Institute Inc. (Osaka, Japan). The protease activity was determined by the method reported previously [14]. Briefly, 1 ml of reaction mixture consisting of 0.1 ml of sample (each column fraction or purified protease) and 0.9 ml of substrate (10 µmol) in 0.1 M Tris-HCl (pH 8.0) was incubated at 37°C for 30 min. Two millilitres of 15% (v/v)

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. Protease inhibitors were purchased from the Peptide Institute Inc. (Osaka, Japan) except aprotinin (Trasyol®, Bayer, Germany), FUT-175 (Futhan®, Torii & Co. Ltd, Japan) and iodoacetic acid (Wako Pure Ltd, Japan).

Measurement of allergenic activity

Sera (RAST score > 3 to *D. farinae*) from 23 mite-allergic patients were obtained (13 male and 10 female, aged 16–65 years with a positive prick test to *D. farinae*) with asthma who gave an immediate skin response (≥ 9 mm weal or ≥ 20 mm flare in diameter after 15 min) to prick test with 1:20 w/v of *D. farinae* (Torii & Co. Ltd, Japan) (containing 14.3 µg/ml of *Der f* I and 10.6 µg/ml of *Der f* II) glycerinated extract. Measurement of allergenic activity was performed according to the method of fluorometric ELISA reported by Sakaguchi *et al.* [15]. Briefly, a microplate (Dynatech, Immulon II®) was coated overnight with 0.05 ml of purified trypsin-like protease, *Der f* I or *Der f* II at concentration of 5 µg/ml in 0.1 M bicarbonate buffer, pH 9.5. After washing with 0.3 ml of PBS, pH 7.4/0.05% Tween 20, three times, excess absorption sites of the plate were blocked for 1 hr at room temperature by 0.3 ml of 3% gelatin in PBS. The plate was then washed three times and added with 0.05 ml of fivefold-diluted serum samples. After incubation for 3 hr at 25°C, the microplate was washed four times and added with 0.05 ml of tenfold-diluted β -D-galactosidase conjugated anti-human IgE (Phadezyme RAST kit®, Pharmacia, Sweden). After incubation for 16 hr at 25°C, 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 hr at 25°C, 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 substrates was measured at excitation, 365 nm, and emission, 450 nm on a fluorometer. Fluorescence intensity of 10 µM 4-methylumbelliferone was expressed as 1000 fluorescence intensity (F.I.).

Electrophoretic and chemical analysis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 4–20% gradient gel was carried out according to the method of Laemmli [16]. Protein content was determined by the method of Bradford [17] using bovine serum albumin as the standard.

Amino acid analysis

Amino acid analysis was carried out by Waters Pico-Tag system (Waters, Massachusetts, U.S.A.). The amino acid compositions of the purified trypsin-like protease and *Der f* III were compared with SAQ system [18].

Sequencing of amino acid of trypsin-like protease

The final preparation of trypsin-like protease was accomplished by HPLC (Model 130A, Applied Biosystem Co; Column, C8 300A, 2.1 ID $\times$ 30 mm): buffer A, 0.1% trifluoroacetic acid (TFA) in H_2O ; buffer B, 0.1% TFA in 70% acetonitrile; gradient, 0% buffer A to 100% buffer B at 2.2%; flow rate, 200 μ l/min. Determination of the

amino acid sequence of the purified trypsin-like protease was carried out employing a gas phase protein sequencer (Model 471A, Applied Biosystem Co., California, U.S.A.).

Statistical analysis

Student's *t*-test was used for statistical analysis.

Results

Purification of mite trypsin-like protease

In order to study the distributions of trypsin-like protease, protease activities in mite body extract (MBE),

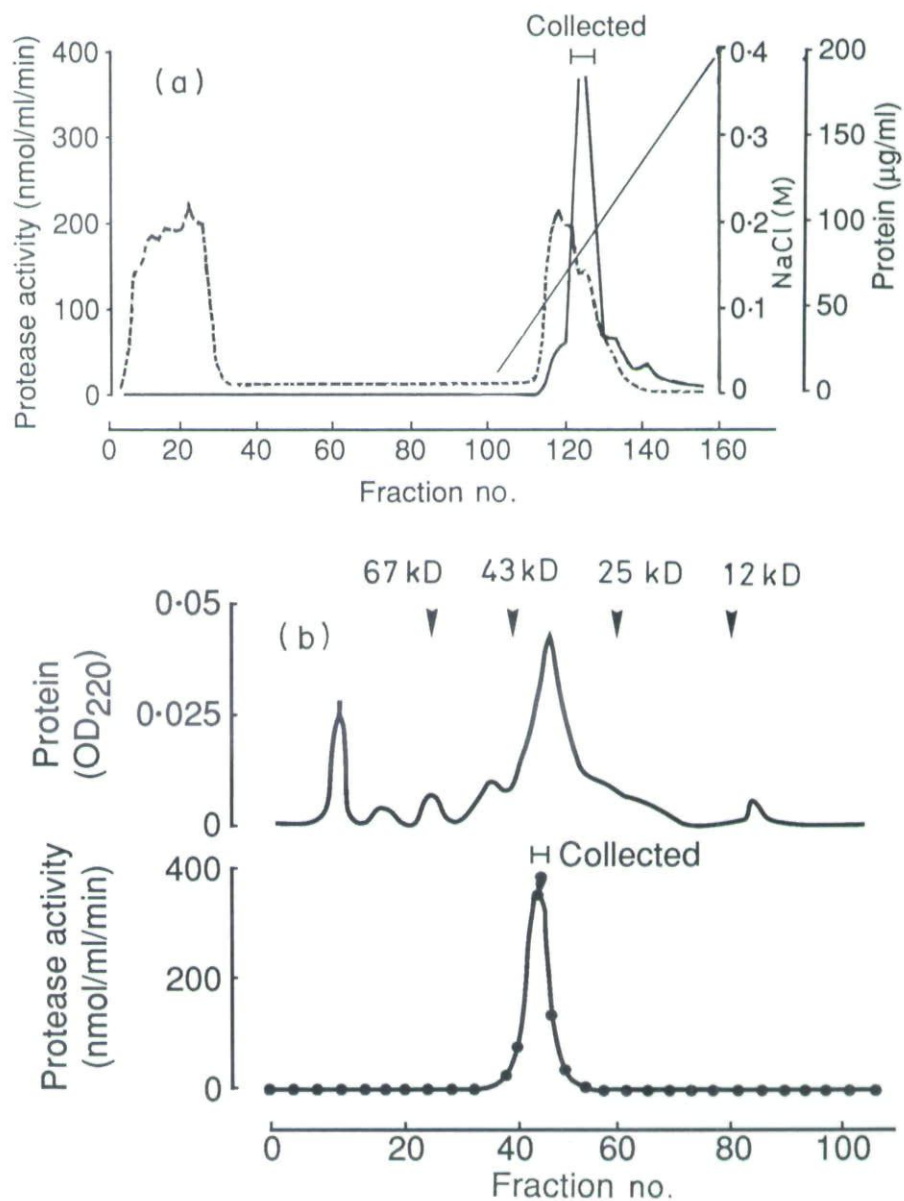


Fig. 1. Chromatographic patterns in purification steps of trypsin-like protease from *D. farinae*. (a) Anion exchange chromatography on DEAE-Sephacel (1.5 $\times$ 15 cm) equilibrated with 0.02 M phosphate buffer (pH 6.0). The linear gradient elution was performed with 0.02 M phosphate buffer (pH 6.0) containing sodium chloride from 0.04 M (3 ml/tube). Fractions from 122 to 129 were collected to obtain the trypsin-like protease. —, Protease activity. ---, Protein. (b) Gel chromatography on Superdex 75 pg (2.6 $\times$ 80 cm; 3 ml/tube). Fractions from 40 to 44 were collected.

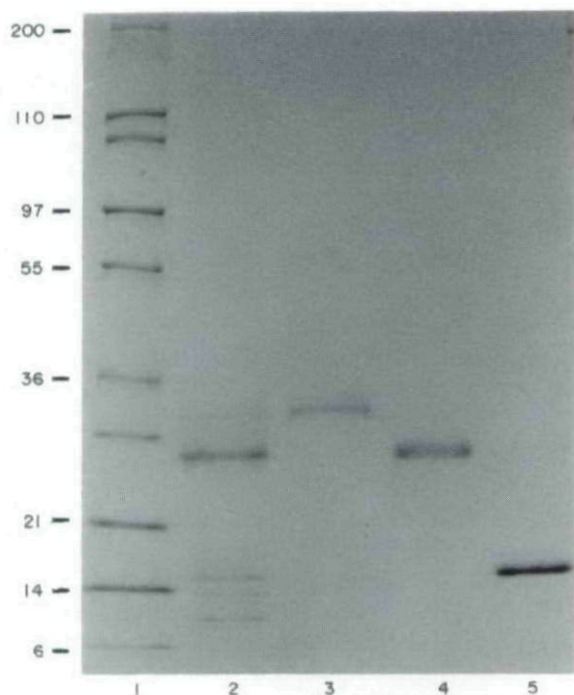


Fig. 2. SDS-PAGE pattern of purified trypsin-like protease from *D. farinae*. Samples were analysed on 4–20% gradient acrylamide gel under reducing conditions. Lane 1, marker protein. Lane 2, starting material (MFE). Lane 3, purified trypsin-like protease. Lane 4, *Der f* I. Lane 5, *Der f* II.

whole culture extract (WCE) and mite faecal extract (MFE) from *D. farinae* were examined. The trypsin-like protease activities of MFE and WCE were 2000- and 1000-fold stronger than that of MBE. In addition, the number of protein bands of MFE on SDS-PAGE was less

Table 1. Purification of mite trypsin-like protease

Purification step	Specific activity	Purification factor
	u/ μ g protein	
Extraction	1817	1.0
DEAE-Sephacel	14677	8.0
Superdex 75 pg	24354	13.5

compared with that of WCE. MFE was, therefore, chosen as the starting material to purify the protease.

MFE was applied to a DEAE-Sephacel column equilibrated with 20 mM phosphate buffer, pH 6.0, and the linear gradient elution was performed with 20 mM phosphate buffer (pH 6.0) containing sodium chloride from 0–0.4 M. The trypsin-like protease activity was detected in the fractions eluted by 0.15 M sodium chloride solution (Fig. 1(a)). These fractions were pooled, concentrated and fractionated by ammonium sulphate precipitation at 60–80% saturation. The fractions having high trypsin-like protease activity were collected and applied to Superdex 75 pg column. The major peak of protein was in good correspondence with the single peak of the protease activity as shown in Fig. 1(b). The molecular weight of the protease activity by Superdex 75 pg was estimated to be 34 kD. Fractions containing potent protease activity (Fr 40–44) were pooled and analysed on 4–20% gradient acrylamide gel under reducing conditions (Fig. 2). The trypsin-like protease gave a

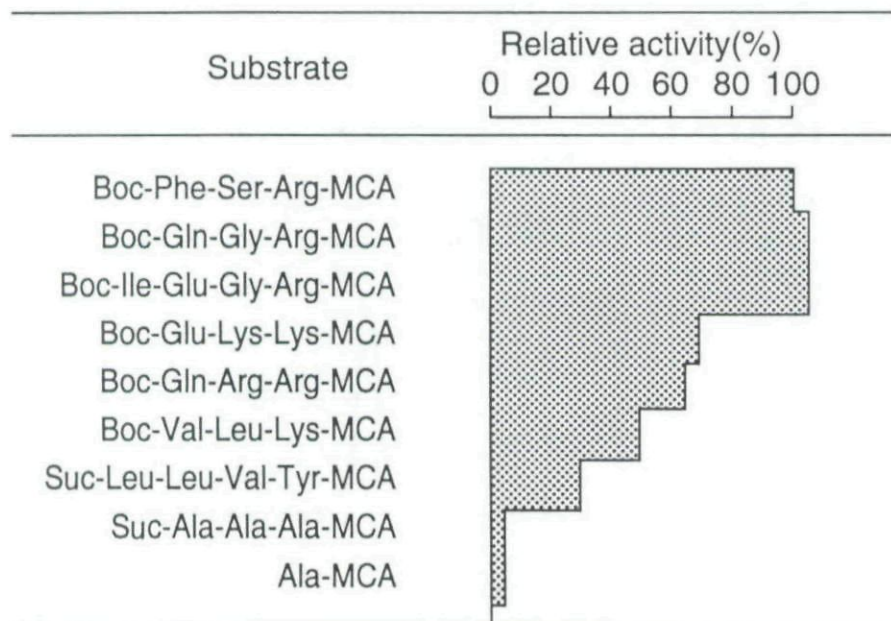


Fig. 3. Substrate specificity of purified trypsin-like protease. Trypsin-like protease was incubated with various synthetic substrates, and the protease activities were compared with that obtained by using Boc-Phe-Ser-Arg-MCA.

Table 2. Effects of various inhibitors on the activity of the purified trypsin-like protease

Material	Concentration (μ M)	Inhibition (%)
Aprotinin	1 KIU/ml	99.6
SBTI	1	99.3
TLCK	100	50.0
<i>p</i> -aminobenzamidine	1000	57.9
FUT-175	1	99.4
Leupeptin	1	98.0
Antipain	10	91.8
TPCK	10	0
Chymostatin	10	0
Iodoacetic acid	100	0
E-64	10	0
Pepstatin	10	0

* KIU, Kalligenase inhibitory unit.

single band with a molecular weight of 34 kD. Purification procedure was summarized in Table 1. The specific activity of the purified mite trypsin-like protease increased 13.5-fold in comparison with that of MFE. The yield of the trypsin-like protease was 300 μ g.

Enzymological properties of the purified mite trypsin-like protease

The optimum pH and the optimum temperature of the purified protease were 8.0 and 47°C. The protease activity

was reduced to approximately 40% during 60 min incubation at 37°C. The protease activity was completely lost by heating for 20 min at 56°C. The substrate specificity of the purified protease was tested with various synthetic substrates of serine proteases and other proteases (Fig. 3). The activity was relatively specific for hydrolysis of arginyl or lysyl residue containing substrates. Effects of various inhibitors on the protease activities are shown in Table 2. The protease activity was inhibited by trypsin-like serine protease inhibitors such as aprotinin, SBTI, leupeptin and FUT-175, but not by cysteine protease inhibitors such as E-64 and iodoacetic acid and chymotrypsin inhibitor, chymostatin.

Allergenic activity of the trypsin-like protease

The allergenic activity of purified trypsin-like protease was examined using 23 mite sensitive asthmatic patients' sera. As shown in Fig. 4, the frequency of reactivity seen with trypsin-like protease (70%) was as high as that observed with *Der f* I (79%) or *Der f* II (83%) and the reactivity of each serum to the above antigens differed depending on the patients. There was no significant difference in relative allergenic potencies among these three purified allergens statistically (means $\pm$ s.e. of the allergenic activity from the patients who showed a positive response: trypsin-like protease 360 ± 51 , *Der f* I 445 ± 69 , *Der f* II 347 ± 63).

Amino acid composition and N-terminal amino acid sequence of purified trypsin-like protease

The amino acid compositions of the purified trypsin-like protease and *Der f* III were compared. The two molecules

Fig. 4. Allergenic activity (allergenicity) of trypsin-like protease to 23 mite allergic patients' sera. Allergenic activity of trypsin-like protease was measured by fluorometric ELISA. The hatched line means cut off value. Cut off value is 100 fluorescence intensity (F.I.) It was determined using sera from eight skin test negative control subjects.

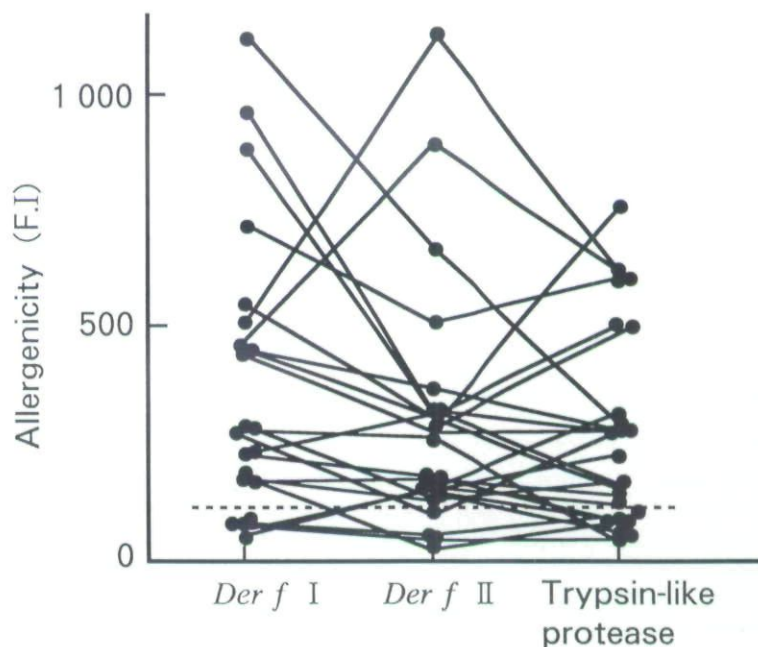


Table 3. Amino acid analysis of trypsin-like protease and *Der f* III. Data of amino acid composition of *Der f* III were obtained from Heymann *et al.* [5]

Amino acid	Residues/100 residues	
	Trypsin-like protease	<i>Der f</i> III
Aspartic acid	11.8	11.6
Threonine	5.0	5.9
Serine	7.9	8.8
Glutamic acid	11.4	9.7
Proline	5.1	5.2
Glycine	12.2	11.4
Alanine	6.9	6.6
1/2 Cystine	NA	NA
Valine	8.0	6.7
Methionine	2.0	2.1
Isoleucine	6.3	6.3
Leucine	6.1	7.4
Tyrosine	4.9	3.8
Phenylalanine	1.5	2.3
Histidine	2.1	2.2
Lysine	5.5	6.6
Arginine	3.3	3.4

Table 4. N-terminal amino acids sequence of trypsin-like protease, *D. farinae* protease, *Der f* III, *Der p* III and other serine proteases

Protein	Sequence
	1 20
Trypsin-like protease	I V G G V K A Q A G D X P Y Q I X L Q X
<i>D. farinae</i> protease	I V G G V K A Q A G D X P Y Q I S L Q S
<i>Der f</i> III	I V G G V K A L A G D X P Y E I S L E V
<i>Der p</i> III	I V G S E K A L A G E X P Y Q I S
Hornet chymotrypsin	I V G G T D A P R G K Y P Y Q V S L R A
Bovine trypsin	I V G G Y T C G A N T V P Y Q V S L N S

One letter code used for amino acid sequences. *D. farinae* protease, *Der f* III, and *Der p* III sequence data were obtained from Kohmoto *et al.* [23], Heymann *et al.* [5], and Stewart *et al.* [6] respectively, whereas hornet chymotrypsin and bovine trypsin sequence data were obtained from Jany *et al.* [19] and James *et al.* [20] respectively.

differed by only 12 SAQ units and were judged to be closely related (Table 3). The first 20 N-terminal amino acid sequences of trypsin-like protease, *D. farinae* protease, *Der f* III, *Der p* III and other serine proteases such as hornet chymotrypsin and bovine trypsin are shown in Table 4. Trypsin-like protease has more than 50% homology with other proteins. Seventeen and 15 out of 20 N-terminal amino acid residues of the trypsin-like protease were identical to those of *D. farinae* protease and *Der f* III, respectively.

Discussion

We previously reported that *Der f* I allergen might be digestive protease secreted from the gastrointestinal tract of mite [12]. Tovey *et al.* also showed that *Der p* I allergen was closely related to faecal particle [21]. If a trypsin-like protease of mite is also digestive protease, it can be found in mite faeces. The potent trypsin-like protease activity was detected in whole culture extract (WCE) and mite faecal extract (MFE) from *D. farinae*. These results suggest that mite trypsin-like protease as well as *Der f* I might be the digestive proteases of mites. Thus, we chose MFE as the starting material for purification of trypsin-like protease because of its potent trypsin-like protease activity. DEAE-Sephacel anion exchange chromatography and Superdex 75 pg gel chromatography were used to purify trypsin-like protease, and the final preparation gave a single band with a molecular weight of 34 kD on SDS-PAGE and the same molecular weight was determined by Superdex 75 pg gel chromatography. Our previous report showed that molecular weight of trypsin-like protease existing in MBE was 60 kD [11]. The distinct difference in molecular weights between two trypsin-like proteases led us to consider that the mites might have two types of trypsin-like proteases at least.

Proteases are classified into two groups, exopeptidases and endopeptidases on the cleaving site of the substrate. Endopeptidases are further classified into four groups according to their active sites: serine proteases, cysteine proteases, carboxyl proteases, and metalloproteases. Serine proteases are classified into three groups from the substrate specificity: trypsin-like proteases, chymotrypsin-like proteases and elastases [22]. Trypsin, kallikrein, thrombin, plasmin, C1s, C1r, etc. are known to belong to the group of trypsin-like protease. However, these proteases show the slight differences to substrate specificity. In the present study, we used Boc-Phe-Ser-Arg-MCA as the substrate for detecting trypsin-like protease activity through various column chromatography steps. In the cases of mammalian trypsin-like proteases, this substrate is known to be cleaved by other proteases as well as trypsin. Thus we studied the enzymological properties of

mite faecal extract-derived trypsin-like protease. In order to clarify whether two types of trypsin-like proteases are existing in mite or not, substrate specificity, inhibitor susceptibility, pH optimum and other enzymological properties of trypsin-like proteases from MFE were measured, and compared with those of trypsin-like protease from MBE reported previously [11]. These two trypsin-like proteases had good enzymological similarity, suggesting that two kinds of trypsin-like proteases with the same enzymological properties and different molecular size were existing in mites.

Recently, Takahashi *et al.* purified a serine protease designated as *D. farinae* protease from whole culture extract of mites [9]. Kohmoto *et al.* pointed out the similarity between *D. farinae* protease and *Der f* III by comparing their *N*-terminal amino acid sequences [23]. However, *D. farinae* protease and *Der f* III were not concluded to be identical since their *N*-terminal amino acid sequences did not completely agree, and allergenic activity of *D. farinae* protease had not been examined. In the present paper, we aimed to clarify the relationship between trypsin-like protease, *D. farinae* protease and *Der f* III. Firstly, we compared amino acid compositions of trypsin-like protease and *Der f* III and found them to have a close resemblance. Secondly, we compared 20 *N*-terminal amino acid sequences of trypsin-like protease, *D. farinae* protease and *Der f* III. Similar *N*-terminal amino acid residues were observed between trypsin-like protease and *D. farinae* protease. This result strongly suggests that both proteases are the same molecule. In the case of trypsin-like protease and *Der f* III, a slight difference in amino acid sequences was measured. This discrepancy in *N*-terminal amino acid sequences may be attributable to the differences of *D. farinae* itself used to purify the above enzymes and allergen. A similar discrepancy was observed in *D. pteronyssinus*. Namely, the 20 *N*-terminal amino acid sequences of *Der p* III and trypsin from *D. pteronyssinus* were not completely identical [7]. Double immunodiffusion method revealed no precipitation line between trypsin-like protease and rabbit anti-*Der f* I sera or rabbit anti-*Der f* II sera (data not shown). This result suggested that antigenicity of trypsin-like protease was different from those of *Der f* I and *Der f* II. Thirdly, we investigated the allergenic activity of trypsin-like protease using 23 sera from mite allergic patients with a positive prick test to *D. farinae*. Sixteen out of 23 patients' sera (70%) reacted to the trypsin-like protease. Heymann *et al.* [4] reported IgE antibody to *Der f* III was demonstrated in eight of the 51 sera (16%) by antigen binding assay. On the other hand, Stewart *et al.* [7] reported that *Der p* III reacted with all the sera from 54 mite allergic patients by direct RAST. Our fluorometric ELISA studies were not consistent with either of them. As to the differences in

positive rates of IgE antibody to trypsin-like protease, *Der f* III and *Der p* III, there are some possibilities: (i) differences of patient's races; (ii) differences of crude mite antigen for prick test to select the patients; (iii) differences of methodology to measure IgE antibody to each allergen. As mentioned above, the positive rates of IgE antibody to trypsin-like protease and *Der f* III were not agreed. However, it is clarified that trypsin-like protease possessed similar physicochemical properties to that of *Der f* III. Namely, the molecular weight of trypsin-like protease was 34 kD on SDS-PAGE and it was detected in 60–80% SAS fraction of mite faecal extract. The molecular weight of *Der f* III was 29 kD on SDS-PAGE and it was contained in 50–80% SAS fraction of mite spent growth medium [5]. Trypsin-like protease possessed allergenic activity, and amino acid composition and *N*-terminal amino acid residues of trypsin-like protease and *Der f* III were almost the same. These results strongly suggest that trypsin-like protease is actually *Der f* III. In addition, we detected the trypsin-like protease activity in all house dust samples ($n=30$) and the substrate specificity experiment showed that the trypsin-like proteases of mites and house dust were very similar (data not shown). Further investigations of mite and house dust trypsin-like protease are required for a better understanding of their biological roles as protease in mite allergic patients.

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