

Recombinant Der p 1 and Der f 1 exhibit cysteine protease activity but no serine protease activity

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Abstract

Although mite major group 1 allergens, Der p 1 and Der f 1, were first isolated as cysteine proteases, some studies reported that natural Der p 1 exhibits mixed cysteine and serine protease activity. Clarifying whether the serine protease activity originates from Der p 1 or is due to contamination is important for distinguishing between the pathogenic proteolytic activities of group 1 allergens and mite-derived serine proteases. Recombinant mite group 1 allergens would be useful tool for addressing this issue, because they are completely free from contamination by mite serine proteases. Recombinant Der p 1 and Der f 1, and highly purified natural forms exhibited only cysteine protease activity. However, commercially available natural forms exhibited both activities, but the two activities were eluted into different fractions in size-exclusion column chromatography. The substrate specificity associated with the serine protease activity was similar to that of Der f 3. These results indicate that the serine protease activity does not originate from group 1 allergens.

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House dust mites of the *Dermatophagoides* species (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*) are associated with various allergic diseases such as bronchial asthma, rhinitis, and atopic dermatitis, and are the most important causative factor of in-door allergens [1–3]. Group 1 (Der p 1 and Der f 1) and group 2 (Der p 2 and Der f 2) allergens are considered to be the major allergens derived from house dust mites based on the frequency of patients sensitized by them, amount of specific IgE produced against them, and their content in

mite extract [4]. Group 1 allergens exist in abundance in mite fecal pellets and account for over 50% of the IgE antibodies produced against total mite extract. Der p 1 of *D. pteronyssinus* and Der f 1 of *D. farinae* have 82% sequence homology in the amino acid sequences of their mature forms, which corresponds to their natural allergen state, and the sequences are homologous to those of papain-like cysteine proteases [5,6]. Recently, the cysteine protease activity of Der p 1 was suggested to be involved in the pathogenesis of allergy [7–15].

Although Der p 1 and Der f 1 were first isolated as cysteine proteases, some studies reported that natural Der p 1 exhibits mixed cysteine and serine protease activity [11,16,17]. The specificity for substrates and

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inhibitors associated with such serine protease activity within their preparations was different from that of the cysteine protease activity. However, house dust mites produce at least three groups of serine proteases, group 3, 6, and 9 allergens [18–21], and therefore, the possibility of contamination of cysteine protease Der p 1 preparations with these serine proteases cannot be excluded. Clarifying whether the serine protease activity originates from Der p 1 or is due to contamination is important for distinguishing between the pathogenic proteolytic activities of mite major group 1 allergens and mite-derived serine proteases. Recombinant mite group 1 allergens as recently reported [22–24] would be useful tool for addressing this issue, because they are completely free from contamination by mite serine proteases.

Using synthetic substrates and irreversible class-specific inhibitors, we compared the specificity of four types of preparation of group 1 allergens: recombinant Der p 1 and Der f 1 expressed in yeast *Pichia pastoris* with yeast-derived N-glycosylation, those without N-glycosylation, commercially available natural types, and natural types purified using a different method. Consequently, we demonstrate that the serine protease activity does not originate from group 1 allergens and that the substrate specificity of the contaminated serine protease is similar to that of a mite group 3 allergen Der f 3.

Materials and methods

Natural Der p 1 and Der f 1. Commercially available natural Der p 1 and Der f 1 were purchased from Indoor Biotechnologies (Charlottesville, VA, USA) and Asahi Breweries (Tokyo, Japan), respectively. Purification of natural Der p 1 and Der f 1 from whole culture extracts of house dust mites by the method of Yasueda et al. [25] was performed. Protein concentration was determined by the Bradford procedure using a protein assay kit (Bio-Rad, Richmond, CA, USA) with bovine IgG (Bio-Rad) as the standard.

Recombinant Der p 1 and Der f 1. Recombinant Der p 1 and Der f 1 were prepared as previously described with some modifications [22–24]. Briefly, proforms of four recombinant types, Der p 1-N52Q, Der p 1-WT, Der f 1-N53Q, and Der f 1-WT, were secreted into the culture supernatant of yeast *P. pastoris* and converted into their prosequence-removed mature forms. The mature forms were then purified by anion exchange column and size-exclusion column chromatography.

Measurement of proteolytic activity. Cysteine protease activity was measured as previously described with modifications [23,26]. Enzymes (400 nM) were incubated with or without 1 mM dithiothreitol (DTT) for 5 min at 37 °C in 25 μ l. After further incubation with or without E-64 (50 μ M) (Peptide Institute, Osaka, Japan) and/or 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) (500 μ M) (Sigma, St. Louis, MO, USA) for 15 min in 50 μ l, assays were conducted in reaction buffer with substrates (0.1 mM) at 37 °C in 100 μ l scale. The final concentrations of the enzymes were 100 nM, and those of the inhibitors were 25 μ M for E-64 and 250 μ M for AEBSF. The substrates used were: butyloxycarbonyl-Gln-Ala-Arg-MCA (Boc-QAR-MCA), succinyl-Leu-Leu-Val-Tyr-MCA (Suc-LLVY-MCA), succinyl-Ala-Ala-Ala-MCA (Suc-AAA-MCA), butyloxycarbonyl-Phe-Ser-Arg-MCA (Boc-FSR-MCA), butyloxycarbonyl-Gln-Gly-Arg-MCA (Boc-QGR-MCA), butyloxycarbonyl-Val-Leu-Lys-MCA (Boc-VLK-MCA),

benzoyl-Arg-MCA (Bz-R-MCA), and succinyl-Ala-Ala-Pro-Phe-MCA (Suc-AAPF-MCA) (Peptide Institute). The fluorescence of aminomethylcoumarin released from the substrate was measured.

Protein sequencing. Samples of commercial natural Der p 1 and Der f 1 before and after SDS-PAGE and electroblotting onto membranes were subjected to N-terminal amino acid sequencing using protein sequencers [23,27].

Size-exclusion column chromatography. Recombinant and natural Der p 1 and Der f 1 were subjected to size-exclusion column chromatography on Protein-Pak 125 (Waters, Milford, MA, USA), and the absorbance at 280 nm was monitored. The eluted fractions were subjected to sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) and silver stained (Silver stain II kit wako; Wako, Osaka, Japan).

Results

Substrate specificity of recombinant and commercial natural Der p 1 and Der f 1

Measurement of the proteolytic activity using eight synthetic substrates revealed that the activity of recombinant mite group 1 allergens with yeast-derived hyperglycosylation (Der p 1-WT and Der f 1-WT) and that of commercial natural types were different from each other in terms of substrate specificity and DTT-dependence (Fig. 1). The patterns of substrate specificity and DTT-dependence were similar between Der p 1-WT and Der f 1-WT, and also between commercial Der p 1 and Der f 1.

Der p 1-WT and Der f 1-WT showed DTT-dependent proteolytic activity against six synthetic substrates (Figs. 1A–F) and almost no activity against two (Figs. 1G and H). This DTT-dependency suggested that the activity of Der p 1-WT and Der f 1-WT was that corresponding to a cysteine protease. On the other hand, the commercial natural types showed proteolytic activity and high DTT-dependence against three substrates (Figs. 1A–C); that with low or no DTT-dependence against three substrates (Figs. 1D–F), and little or no activity against two substrates (Figs. 1G and H). This suggested that commercial natural Der p 1 and Der f 1 possessed the other class of proteases as well as that of cysteine protease activity.

Activity of recombinant and natural Der p 1 and Der f 1 treated with class-specific irreversible protease inhibitors

To identify the class of proteases exhibiting DTT-independent activity among the commercial natural allergens (Figs. 1D–F, right two panels) and to reveal whether the difference between recombinant and commercial natural allergens was caused by the hyperglycosylation of recombinant allergens and/or by the method used for purification, we analyzed recombinant allergens without glycosylation (Der p 1-N52Q and Der f 1-N53Q) and natural allergens purified by the method

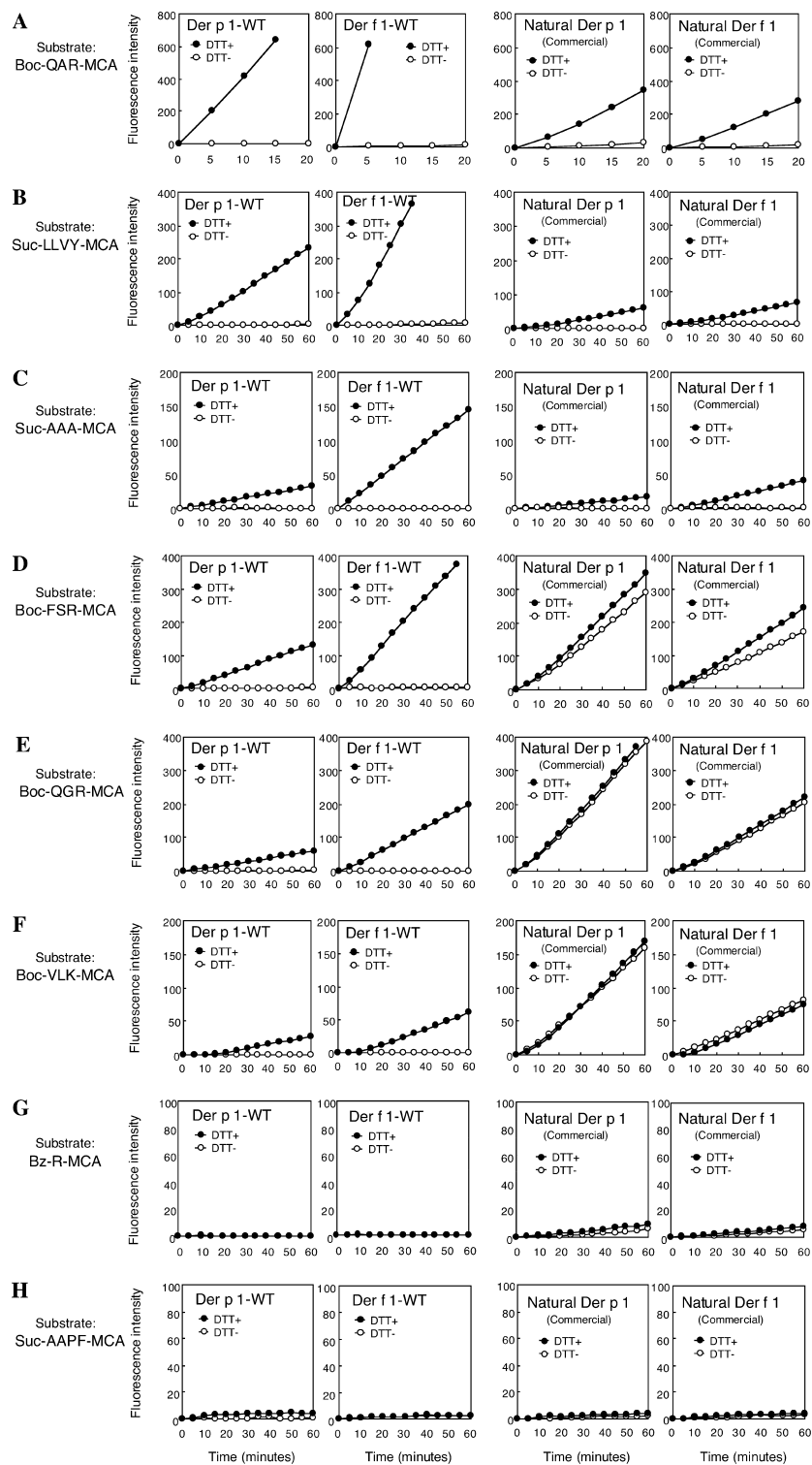


Fig. 1. Hydrolysis of synthetic substrates by recombinant and commercial natural Der p 1 and Der f 1. Eight substrates (A–H) were used. WT: recombinant forms with N-glycosylation. Commercial: commercial natural allergens. The reaction was performed in the presence (DTT+) or absence of DTT (DTT–).

by Yasueda et al. [25] and compared them with Der p 1-WT, Der f 1-WT, and commercial natural allergens using two irreversible class-specific protease inhibitors, cysteine protease-specific E-64 and serine protease-spe-

cific AEBSF (Figs. 2 and 3). We selected Boc-QAR-MCA and Boc-QGR-MCA for the substrates, because they are typical substrates suitable for measuring DTT-dependent and -independent activities of commer-

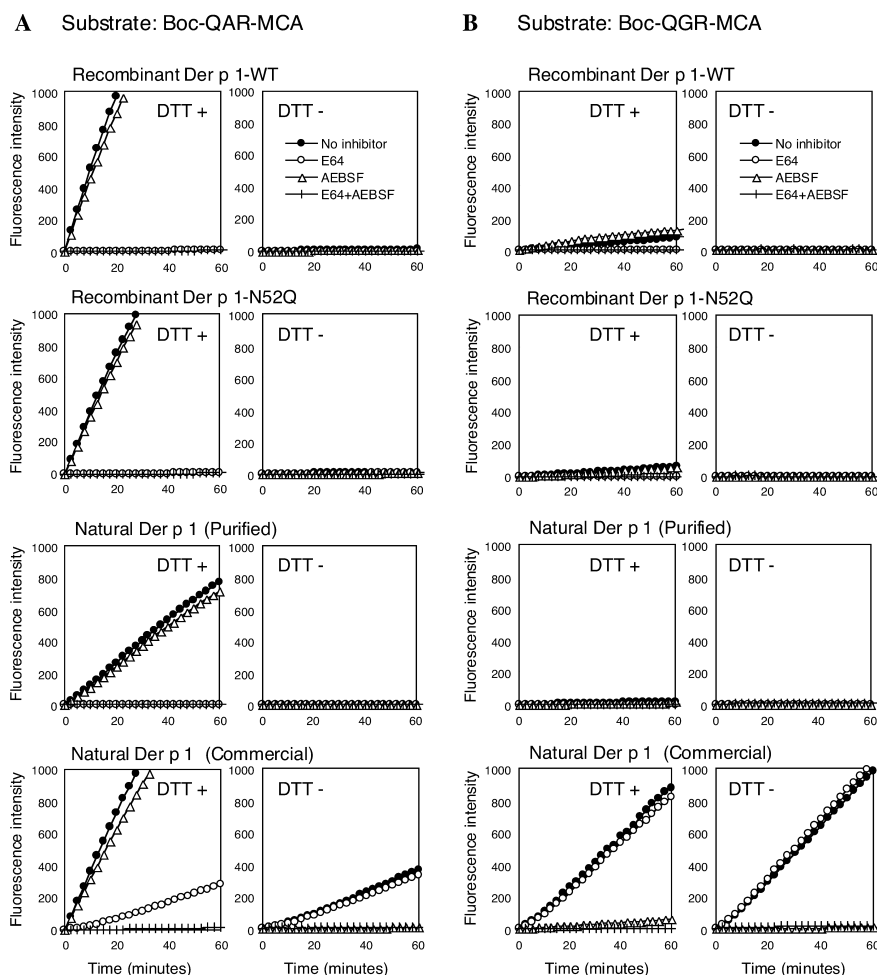


Fig. 2. Activity of recombinant and natural Der p 1 treated with irreversible class-specific protease inhibitors. Boc-QAR-MCA (A) and Boc-QGR-MCA (B) were used as substrates. Der p 1-WT: recombinant Der p 1 with yeast-derived N-glycosylation. Der p 1-N52Q: recombinant Der p 1 without N-glycosylation. Der p 1 (purified): natural Der p 1 purified by the method of Yasueda et al. [25]. Der p 1 (commercial): commercial natural Der p 1. The reaction was performed in the absence of inhibitors or in the presence of E-64, AEBSF or E-64 and AEBSF; and in the presence (DTT+) or absence of DTT (DTT-).

cial natural allergens, respectively (Figs. 1A and E, right two panels).

The activities of recombinant allergens and natural types purified by the method of Yasueda et al. against Boc-QAR-MCA (Figs. 2A and 3A, upper six panels) and Boc-QGR-MCA (Figs. 2B and 3B, upper six panels) were DTT-dependent, and E-64 completely inhibited the activities while AEBSF did not. This indicated that recombinant allergens and natural allergens purified by this method only possessed cysteine protease activity and that hyperglycosylation of recombinant allergens did not affect the activity.

On the other hand, the activities of commercial natural allergens against Boc-QAR-MCA consisted of major DTT-dependent and minor DTT-independent activities (Figs. 2A and 3A, panels at the bottom), and those against Boc-QGR-MCA possessed inverse major DTT-independent and minor DTT-dependent activities (Figs. 2B and 3B, panels at the bottom). The DTT-dependent and -in-

dependent activities of commercial natural allergens were inhibited by E-64 and AEBSF, respectively, and the addition of E-64 and AEBSF was necessary for complete inhibition in the presence of DTT. This indicated that the activity of commercial natural allergens consisted of cysteine protease and serine protease activity.

Substrate specificity of cysteine protease activity of recombinant and commercial natural Der p 1 and Der f 1

To compare the substrate specificity of the cysteine protease associated activities of recombinant and commercial natural allergens, DTT-dependent activities were calculated from the results shown in Fig. 1. The patterns of relative DTT-dependent activities were similar between recombinant and commercial natural allergens (Fig. 4A), although absolute DTT-dependent activity was higher for recombinant allergens than for commercial natural allergens (Fig. 1). This indicated

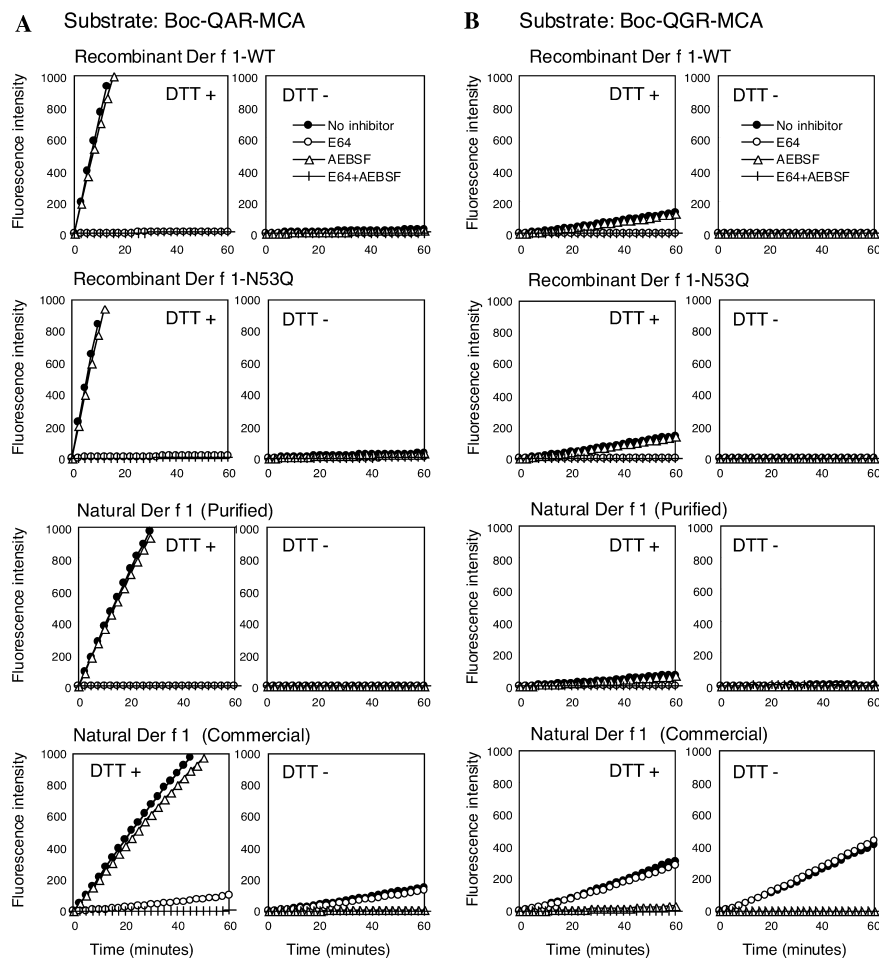


Fig. 3. Activity of recombinant and natural Der f 1 treated with class-specific irreversible protease inhibitors. The reaction was performed as that in Fig. 2, except that recombinant and natural Der f 1 were used.

that the substrate specificity of the cysteine protease associated activities was similar between recombinant and commercial natural allergens.

Substrate specificity of serine protease associated activity of commercial natural Der p 1 and Der f 1

The substrate specificity of commercial natural allergens with DTT-independent activity (Fig. 1) was compared with the previously described specificity of mite serine proteases, except group 9 allergens, whose substrate specificity was not well characterized, and was found to be similar to the mite group 3 allergen Der f 3 but not to group 6 allergens (Fig. 4B, Boc-FSR-MCA, Boc-VLK-MCA, Suc-AAPF-MCA, and Suc-LLVY-MCA).

N-terminal sequencing of commercial natural Der p 1 and Der f 1

To determine the content of the serine protease within the commercial natural allergens, they were subjected

to protein sequencing. N-terminal sequences for Der p 1 and Der f 1 were detected, but those for mite serine proteases that belong to three groups, group 3, 6, and 9 allergens, were not detectable. This indicated that contamination of mite serine proteases was minute if the serine protease activity was mite-derived. However, it was possible that isoforms of Der p 1 and Der f 1 exhibited the serine protease activity [16]. Therefore, to determine whether the cysteine and serine protease activities of the commercial natural allergens were from identical molecular species or not, we next analyzed the commercial natural allergens using size-exclusion column chromatography.

Size-exclusion column chromatography of recombinant and natural Der p 1 and Der f 1

The peak activity against Boc-QAR-MCA, which was used to detect cysteine protease activity in the presence of DTT, and that against Boc-QGR-MCA to detect serine protease activity in the absence of DTT were eluted into different fractions (Fig. 5, panels at

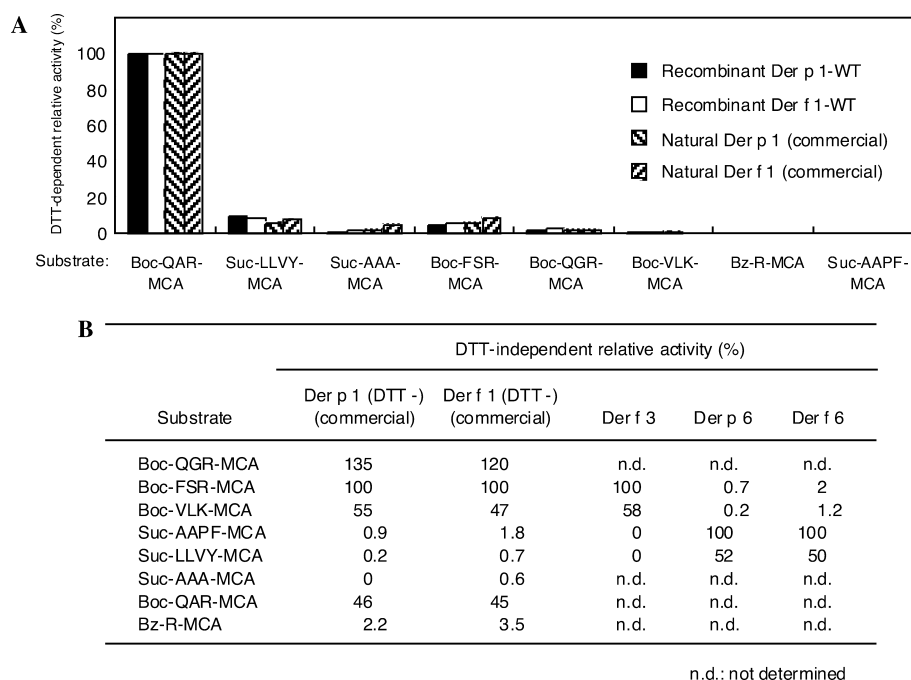


Fig. 4. Substrate specificity associated with the protease activity of recombinant and commercial natural Der p 1 and Der f 1. (A) Comparison of the specificity of cysteine protease activity. The activity for each substrate is shown as the relative DTT-dependent fluorescence intensity to that for the substrate Boc-QAR-MCA. The DTT-dependent fluorescence intensity used was calculated by subtracting the intensity in the absence of DTT from that in the presence of DTT. (B) Comparison of the specificity of serine protease activity. The activity for each substrate is shown as the relative DTT-independent fluorescence intensity to that for the substrate Boc-FSR-MCA in commercial natural Der p 1 and Der f 1, and natural Der f 3; and for Boc-AAPF-MCA in natural Der p 6 and Der f 6. The DTT-independent fluorescence intensity used was the intensity in the absence of DTT. The values for natural Der f 3, Der p 6, and Der f 6 were obtained from a previous report [20].

the bottom). The peaks for the cysteine protease activity of Der p 1-N52Q, Der f 1-N53Q, and the natural types purified by the method of Yasueda et al. were eluted into a fraction at an elution time equivalent to that associated with the cysteine protease activity of the commercial natural allergens (Fig. 5, middle four panels). Those for Der p 1-WT and Der f 1-WT were eluted earlier reflecting their larger molecular sizes (Fig. 5, panels at the top). The shapes of protein-elution curves and band density on SDS-PAGE of the eluted fractions were correlated well with the strength of the cysteine protease activity [22] but not with that of the serine protease activity (data not shown). These results indicated that the cysteine and serine protease activities associated with the commercial natural allergens originated from different molecular species and that the serine protease had an apparent molecular size different from those of Der p 1 and Der f 1.

Discussion

Several studies have found that cysteine and serine protease activities are associated with affinity-purified natural Der p 1 [11,16,17], and we obtained similar results using commercial natural Der p 1 and Der f 1. However, recombinant Der p 1 and Der f 1, and natural

allergens purified by the method of Yasueda et al. only exhibited cysteine protease activity (Figs. 1–3). We showed that the substrate specificities of commercial natural Der p 1 and Der f 1 are similar to those of recombinant forms for DTT-dependent cysteine protease activity (Fig. 4A), and similar to that of a mite group 3 allergen Der f 3 for DTT-independent serine protease activity (Fig. 4B). The cysteine and serine protease activities eluted into different fractions during size-exclusion column chromatography (Fig. 5), indicating that these different types of protease activity are due to different molecular species. These results indicate that the major house dust mite group 1 allergens have only cysteine protease activity and that the serine protease activity detected within some preparations of natural group 1 allergens is due to contamination by serine protease with similar substrate specificity to Der f 3.

Hewitt et al. [11] reported that natural Der p 1, which was affinity-purified using a column coupled with murine anti-Der p 1 monoclonal antibody (mAb), exhibited cysteine and serine protease activities even after further active-site affinity purification using a thiol column. They showed that different substrate specificities associated with the cysteine and serine proteases are present using insulin B chain as a model peptide [16]. Recently, Brown et al. [17] reported similar results using two synthetic substrates, Boc-QAR-MCA and Tosyl-Gly-Pro-Arg-MCA;

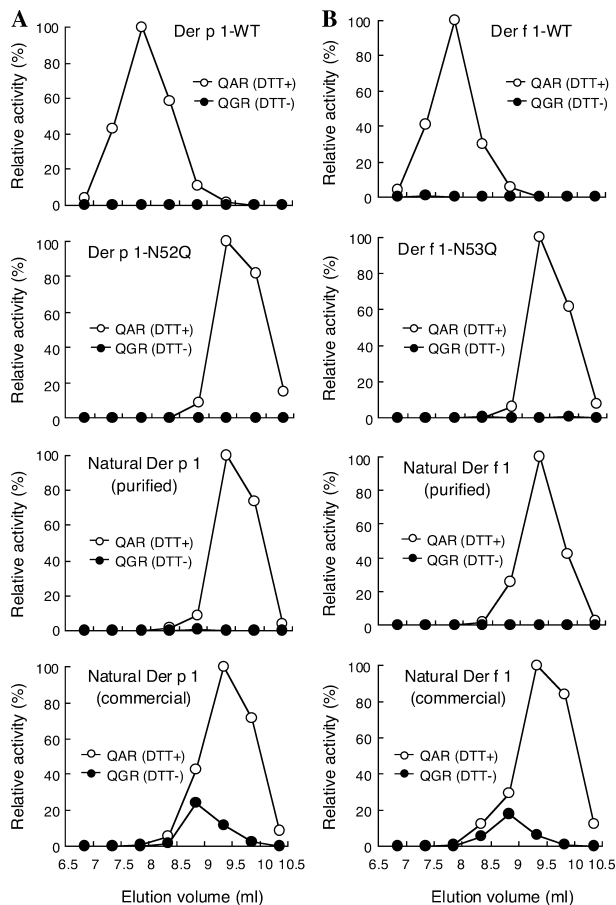


Fig. 5. Proteolytic activity associated with eluted fractions of recombinant and natural Der p 1 and Der f 1 separated by size-exclusion column chromatography. (A) Der p 1. (B) Der f 1. Eluted fractions were subjected to the measurement of proteolytic activity against the substrate Boc-QAR-MCA in the presence of DTT [QAR (DTT+)] and against Boc-QGR-MCA in the absence of DTT [QGR (DTT-)]. The relative fluorescence intensity to the peak intensity for Boc-QAR-MCA is shown.

and in the present study, we too observed similar behavior using several synthetic substrates of commercial natural Der p 1 and Der f 1, although the strength of the serine protease activity varied by lot and manufacturers (data not shown). Schulz et al. [26] and Brown et al. [17] reported that the serine protease found within the preparation of affinity-purified natural Der p 1 could be removed by passing it through a column coupled with soybean trypsin inhibitor (SBTI). However, whether such serine protease activity is associated with the group 1 allergens or contamination by mite-derived serine proteases [18–21] has not been clarified. We showed that recombinant Der p 1 and Der f 1 purified by a simple method [22,23] and natural allergens purified by another method which used many purification steps without mAb and SBTI [25] exhibited only cysteine protease activity. Furthermore, we indicated that the serine proteases within commercial Der p 1 and Der f 1 have molecular sizes different from that of the cysteine protease and

that their substrate specificities are similar to that of Der f 3 but not to those of Der p 6 and Der f 6 [20]. Therefore, the identity of the serine protease activity might be the group 3 allergens. The larger molecular sizes of the peaks associated with serine protease activity for commercial group 1 allergens compared to those of the peaks associated with cysteine protease activity for size-exclusion column chromatography do not conflict with the higher molecular weights of group 3 allergens Der p 3 and Der f 3 compared to those of the group 1 allergens estimated by SDS-PAGE [19,28].

Co-purification of serine protease activity with cysteine protease Der p 1 by both affinity purification using the mAb-coupled column and active-site affinity purification was reported by Hewitt et al. [11]. Possibilities that the residual serine protease activity is due to insufficient column washing after loading of the extract to the columns or that the serine protease have weak affinity for column materials or the murine mAb used are not to be excluded, as we have observed that some preparations of whole culture extracts of mites showed a much higher proteolytic activity for serine protease activity than cysteine protease activity. Der p 3 might bind to the thiol column via Cys12, which is predicted to be an unpaired cysteine residue based on its homology with trypsin proteins from various species [29].

The cysteine protease activity of Der p 1 is suggested to be involved in the pathogenesis of allergy [7–15]. Mite-derived serine protease activity also has biological activities including the ability to disrupt tight junctions [30], stimulate bronchial epithelial cells by cleaving the protease activated receptor-2 (PAR-2) [31], cleave an intrinsic protease inhibitor, elafin [17], and produce anaphylatoxins by cleaving complement components [32]. Although we considered that the amount of the serine proteases that contaminated the commercial group 1 allergens was small because no peaks associated with the group 3, 6, and 9 allergens were detected by protein sequencing analysis, their activities were not negligible. Therefore, recombinant Der p 1 and Der f 1, which can be easily prepared and exhibit only cysteine protease activity, will be useful for the accurate analysis of the pathogenicity of the cysteine protease activity of the major mite group 1 allergens, which are distinct from those of mite serine proteases. Additionally, further analysis of the contribution of serine protease activity including that by the group 3 allergens, which could have significant activities and different substrate specificities from the major group 1 allergens [17], to the pathogenesis of allergy may also be important.

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