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ORIGINAL ARTICLE

Protease Activity of Allergenic Pollen of Cedar, Cypress, Juniper, Birch and Ragweed

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ABSTRACT

Background: Pollen is an important trigger of allergic rhinitis, conjunctivitis, and/or asthma, and an exacerbating factor in atopic dermatitis. Although it is proposed that protease activity from allergen sources, such as mites, enhances allergenicity, little information is available on that from relevant allergenic pollens such as Japanese cedar and Japanese cypress pollens, which are the major cause of pollinosis in Japan.

Methods: We analyzed the protease activities derived from allergenic pollen of Japanese cedar, Japanese cypress, and Rocky mountain juniper, which belong to the *Cupressaceae/Taxodiaceae* family, and white birch and short ragweed, using synthetic substrates and class-specific inhibitors.

Results: We found that the pollen of the three members of the *Cupressaceae/Taxodiaceae* family contained serine protease activity, that the pollen of white birch and short ragweed contained not only serine protease activity but also cysteine protease activity, that all five types of pollen tested contained at least one other type of serine protease, whose sensitivity to a serine protease-specific inhibitor was relatively low, and that the content and releasability of the pollen-derived proteases differed according to the plant families.

Conclusions: Clinically relevant allergenic pollens tested in the present study can release serine and/or cysteine endopeptidases. Information on the spectrum of the endopeptidase activities from these allergenic pollen grains will be useful for investigating their contribution to the pathogenesis of allergies.

KEY WORDS

birch, cysteine protease, Japanese cedar, Japanese cypress, juniper, pollen allergy, protease inhibitor, proteolytic activity, ragweed, serine protease

INTRODUCTION

Enzymes,[1-4] lipids,[5-7] and chitin[8] produced by allergen-producing organisms have been suggested to be involved in the pathogenesis of allergic diseases through IgE-independent innate immunity. Proteolytic activity from allergen sources has been one of the candidates proposed to lead to enhanced allergenicity since initial studies.[1-3] Proteases derived from house dust mites have been suggested to be involved in the pathogenesis of allergies in sensitization and/or exacerbation by facilitating the passage of their own and other allergens across tissue barriers, cleaving various molecules, and modulating the functions of various cells and immune responses.[1,3,9-21] Some reports suggested that proteases derived from cockroaches,[22] molds,[23] and pollens[24] also contribute to the pathogenesis of allergies.

Pollen is an important trigger of allergic rhinitis, conjunctivitis, and/or asthma, and an exacerbating factor in atopic dermatitis.[25-27] In Europe and North America, birch of *Betula* species is the most important allergenic tree.[28] One report described the substrate specificity of birch pollen-derived protease activity.[29] Pollen of trees of the *Cupressaceae/Taxodiaceae* family, such as Japanese cedar (*Cryptomeria japonica*), Japanese cypress (*Chamaecyparis obtuse*), *Juniperus* species, and *Cupressus* species, are relevant allergens.[25,26,28] In Japan, Japanese cedar pollinosis is a common seasonal allergic disease posing a major public health problem caused by inhalation of pollen of Japanese cedar and Japanese cypress.[25,30] Aller-

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gens from Japanese cedar share IgE-epitopes with homologous allergens from Japanese cypress. Almost no information is available on endopeptidases from pollen of the *Cupressaceae/Taxodiaceae* family although a research group reported on purification and characterization of an aminopeptidase from Japanese cedar pollen.[31,32] Ragweed pollen of *Ambrosia* species is one of the most clinically relevant allergenic pollen, and serine proteases have been purified from ragweed pollen and characterized.[33,34] Grass pollen also contain protease activity, which causes detachment of murine airway cells *in vitro*.[24]

Here, we compare the strength and substrate specificity of proteolytic activities derived from allergenic pollen of Japanese cedar, Japanese cypress, Rocky mountain juniper, white birch, and ragweed using synthetic substrates and class-specific inhibitors.

METHODS

POLLEN

Pollen of Japanese cedar (*Cryptomeria japonica*) was purchased from Wako (Osaka, Japan) or kindly provided by Torii Pharmaceutical Co., Ltd. (Tokyo, Japan). Pollen of Japanese cypress (*Chamaecyparis obtusa*) was purchased from Wako. Pollen of Rocky mountain juniper (*Juniperus scopulorum*) and white birch (*Betula alba*) were purchased from Sigma (St. Louis, MO, USA). Pollen of short ragweed (*Ambrosia artemisiifolia*) was purchased from Polysciences (Warrington, PA, USA).

EXTRACTION OF PROTEASES FROM POLLEN GRAINS

Pollen grains were suspended in Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS, pH 7.4) (10 mg-grains/1 ml in 1.5 ml-tubes). The suspension was shaken gently at 37℃ for 15 minutes and centrifuged for 5 minutes at 2,000 *g*. The supernatant was collected and filtered (0.22 μm) (Sup 1). The precipitate was resuspended with fresh PBS. The procedure for extraction described above was repeated twice to prepare Sup-2 and Sup-3. The third precipitate was resuspended with fresh PBS. The suspension was sonicated once for 15 minutes (10 cycles of 1 minute of sonication at 30-second intervals) (Olympus, Tokyo, Japan). After centrifugation, the supernatant was filtered (Sup-S). The filtered supernatants were stored at −80℃ until used. These samples or those prepared with the modification of skipping the preparation of Sup-3 were used to obtain the data shown in Figure 1B, Fig. 1C, respectively.

Another procedure for extraction was also applied as follows. Pollen grains were suspended in saline, PBS (pH 7.4), or 50 mM Tris-Cl (pH 9.0) (10 mg-grains/1 ml in 1.5 ml-tubes). The suspension was shaken gently at 37℃ for 2 hours, sonicated twice, and incubated overnight on ice. The next day, the suspension was sonicated twice again. After centrifugation, the supernatant was filtered and stored at −80℃ until used. These samples were used to obtain the data shown in Figure 2B. Samples extracted with the Tris buffer were used to obtain the data shown in Figures 3–5 and Table 1.

MEASUREMENT OF PROTEASE ACTIVITY

Protease activity was measured as previously described[35] with minor modifications as follows. A 5-μl volume of each sample was used for each assay. After incubation with or without 1 mM dithiothreitol (DTT) and further incubation with or without E-64 (50 μM) and/or 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (500 μM), or EDTA (20 mM)in 50 μl, 50 μl of substrate solution was added. Assays were conducted in PBS containing DTT with substrates (0.1 mM) at 37℃ in 100 μl. The final concentrations of E-64, AEBSF, and EDTA were 25 μM, 250 μM, and 10 mM, respectively. The substrates used were butyloxycarbonyl-Gln-Gly-Arg-MCA (Boc-QGR-MCA), benzoyl-Arg-MCA (Bz-R-MCA), butyloxycarbonyl-Gln-Ala-Arg-MCA (Boc-QAR-MCA), butyloxycarbonyl-Phe-Ser-Arg-MCA (Boc-FSR-MCA), butyloxycarbonyl-Val-Leu-Lys-MCA (Boc-VLK-MCA), succinyl-Ala-Ala-Pro-Phe-MCA (Suc-AAPF-MCA) succinyl-Leu-Leu-Val-Tyr-MCA (Suc-LLVY-MCA), and succinyl-Ala-Ala-Ala-MCA (Suc-AAA-MCA). The fluorescence of aminomethylcoumarin released from the substrate was measured on a fluorometer SpectraMAX-GeminiEM (Molecular Devices, Sunnyvale, CA, USA).[16] Kinetic data were collected in all the experiments. The data shown are representatives of three or more independent experiments.

PROTEIN ASSAY

Protein concentrations were determined by the Bradford procedure with a protein assay kit (Bio Rad, Richmond, CA, USA), with bovine serum albumin (Bio Rad).

RESULTS

TIME COURSE FOR RELEASE OF PROTEASE ACTIVITY FROM POLLEN GRAINS AND EFFECT OF SONICATION

According to the procedure shown in Figure 1A, three supernatants, Sup-1, Sup-2, and Sup-3, were extracted from pollen grains under mild conditions without sonication. Sup-S was prepared with sonication. Protease activity against a synthetic substrate, Boc-QGR-MCA, in the presence of DTT was tested (Fig. 1B). In Japanese cedar, Japanese cypress, and Rocky mountain juniper, which belong to the *Cupressaceae/Taxodiaceae* family, Sup-S prepared with sonication showed particularly stronger activity than the supernatants prepared without sonication. Enhancement of the protease activity against Boc-QGR-MCA by sonication was statistically significant (Fig. 1C).

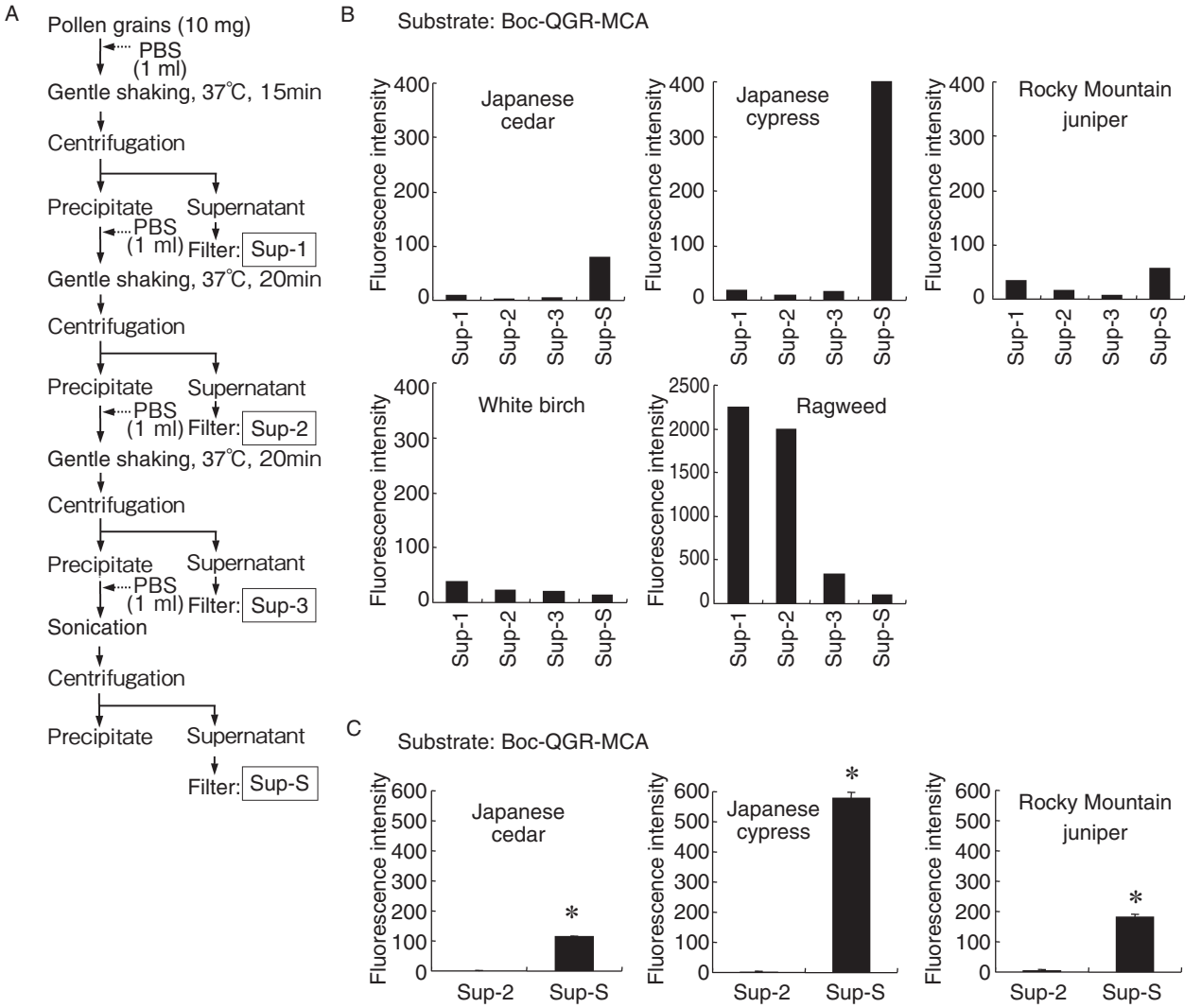

Fig. 1 Time course for release of protease activity from pollen grains and effect of sonication. (**A**) Schematic representation of the procedure used to prepare samples. (**B**) and (**C**) Protease activity against the Boc-QGR-MCA substrate in the presence of DTT. The fluorescence intensity at 120 minutes after the beginning of the protease reaction is shown. The vertical scale is the same among the different types of pollen except for ragweed. In **B**, Sup-S was prepared after the preparation of Sup-3 according to the procedure shown in **A**. In **C**, Sup-S was prepared after the preparation of Sup-2 by skipping the preparation of Sup-3, and means and SD for triplicated wells are shown. * $p < 0.05$ compared with Sup-2 without sonication by unpaired *t*-test (two-tailed) in **C**.

Sup-1 from white birch pollen showed stronger activity than Sup-2, Sup-3, and Sup-S. Ragweed pollen showed the strongest protease activity among the pollens tested, and the majority of its activity was released in Sup-1 and Sup-2 without sonication. These results indicated that all the pollen tested contained protease activity but their content and releasability differed among plant families.

EFFECT OF BUFFERS ON EFFICIENCY OF EXTRACTION OF PROTEASE ACTIVITY

Pollen extracts were prepared with three buffers, saline, PBS (pH 7.4), and Tris buffer (pH 9.0) according to the procedure shown in Figure 2A, which has a longer time for extraction and repeated sonications compared with that shown in Figure 1A. Protease activity against the synthetic substrate Boc-QGR-MCA in the presence of DTT was tested (Fig. 2B). In Japanese cedar, Japanese cypress, and Rocky mountain juniper, PBS and the Tris buffer showed equivalent efficiency but little or no activity was extracted when using saline. In white birch, activity could be extracted with the three buffers, and the ratio of efficiency among the Tris buffer, PBS, and saline was approximately 4 : 2 : 1. In ragweed pollen, PBS and the Tris buffer showed equivalent efficiency, and saline

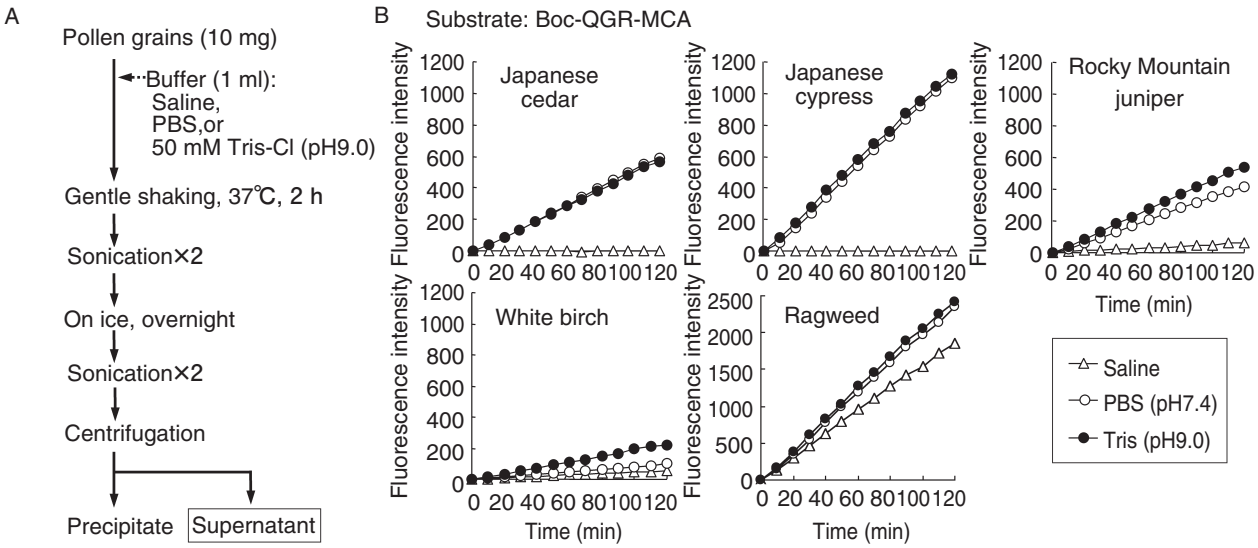

Fig. 2 Effect of buffers on efficiency of extraction of protease activity. (**A**) Schematic representation of the procedure used to prepare samples. (**B**) Protease activity against the Boc-QGR-MCA substrate in the presence of DTT. The vertical scale is the same among the different types of pollen except for ragweed.

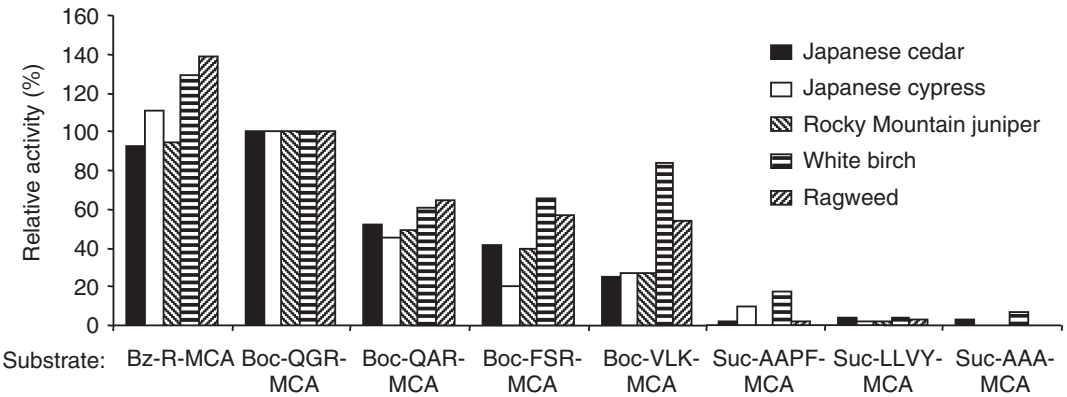

Fig. 3 Substrate specificity of protease activity within pollen extracts obtained with the Tris buffer. The activity measured in the presence of DTT for each substrate is shown as the relative fluorescence intensity to that for the substrate Boc-QGR-MCA at 120 minutes after the beginning of the protease reaction.

showed approximately 80% efficiency compared with the two buffers.

Approximate ranges of pH of the supernatants prepared according to the procedure shown in Figure 2A were estimated with the use of pH test paper to be pH 5.0–5.5 for Japanese cedar, Japanese cypress, and Rocky mountain juniper and pH 5.5–6.0 for white birch and ragweed when extracted with saline, pH 6.5–7.0 for the five types of pollen when extracted with PBS (pH 7.4), and pH 8.5–9.0 for the five types of pollen when extracted with the Tris buffer (pH 9.0) (unpublished data), suggesting that pollen extracts contained acidic materials and that pH affected the efficiency of extraction of protease activity. Additionally, pH in measuring protease activity was considered to be similarly neutral because the assay was conducted after 1/20-dilution of the supernatants. Pollen extracts obtained with the Tris buffer by the procedure shown in Figure 2A were used for all the subsequent assays. Protein concentrations of pollen extracts with the Tris buffer of Japanese cedar, Japanese cypress, Rocky mountain juniper, white birch, and ragweed were 150, 240, 50, 130, and 410 μg/ml, respectively.

SUBSTRATE SPECIFICITY

The substrate specificity of protease activity in the pollen extracts in the presence of DTT was compared (Fig. 3). Although the overall pattern was not so different among the plants, some differences were ob-

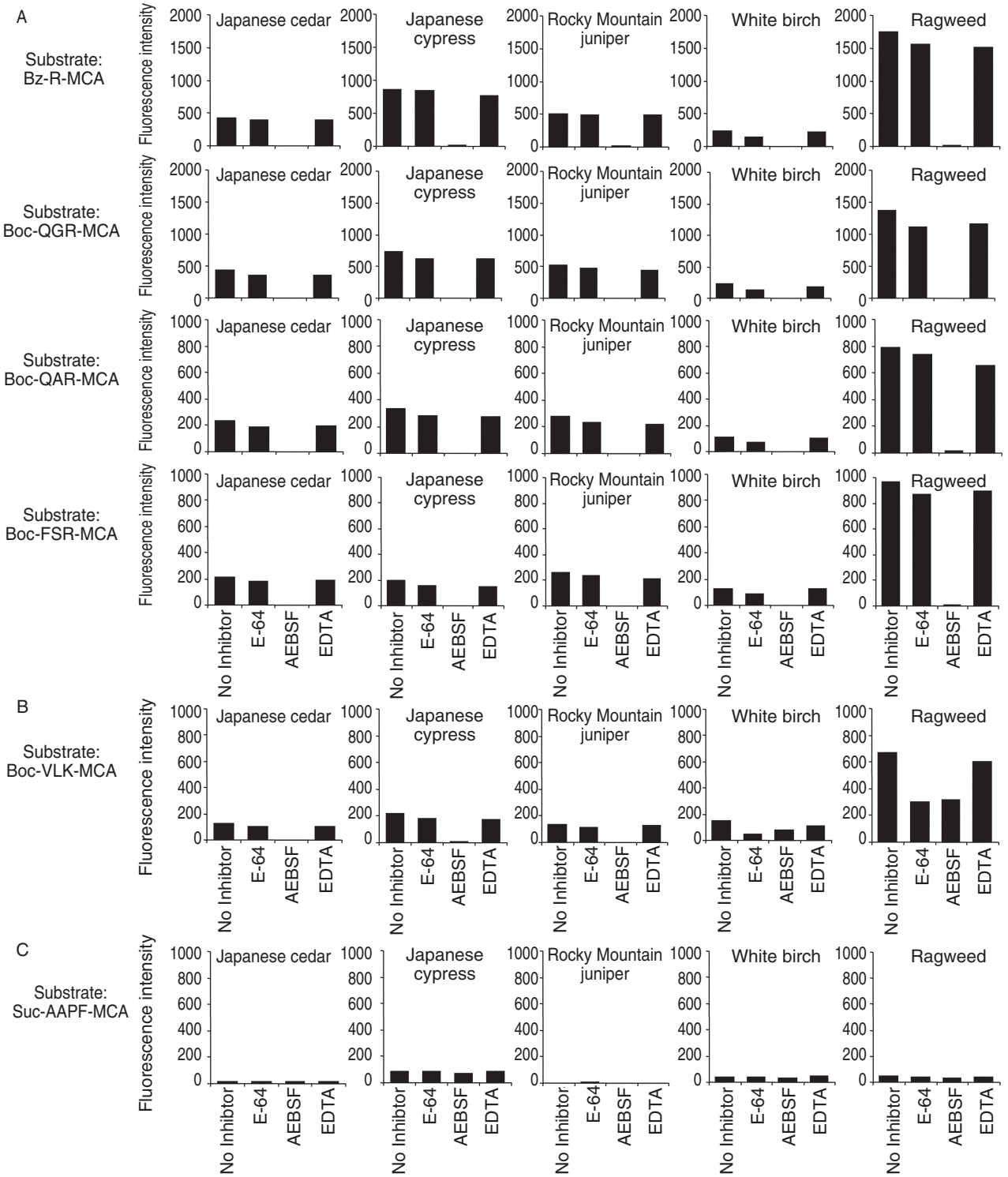

Fig. 4 Inhibition test using class-specific protease inhibitors. Pollen extracts obtained with the Tris buffer by the procedure shown in Figure 2A were used for the measurement in the presence of DTT with or without treatment with E-64, AEBSF, or EDTA. The fluorescence intensity at 120 minutes after the beginning of the protease reaction is shown. The vertical scale is the same among the different types of pollen.

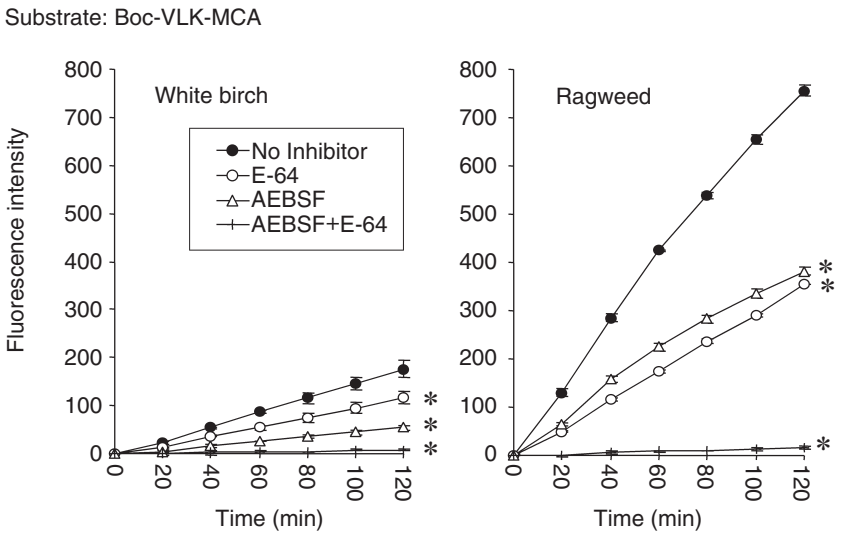

Fig. 5 Combination of E-64 and AEBSF almost completely inhibited protease activity within pollen extracts of white birch and ragweed against the Boc-VLK-MCA substrate. Pollen extracts obtained with the Tris buffer by the procedure shown in Figure 2A were used for the measurement in the presence of DTT. Means and SD for triplicated wells are shown. * $p < 0.05$ vs. the other groups (E-64 + AEBSF) and vs. no inhibitor (E-64 or AEBSF) at 120 minutes by the Tukey post hoc test followed by one-way ANOVA.

Table 1 DTT-dependency of protease activity within pollen extracts.

Substrate	Japanese cedar	Japanese cypress	Rocky mountain juniper	White birch	Ragweed
Bz-R-MCA	351 † (347 ‡)	1058 (837)	457 (396)	219 (191)	1785 (1225)
Boc-QGR-MCA	364 (330)	888 (706)	449 (421)	183 (148)	1322 (929)
Boc-QAR-MCA	196 (211)	376 (308)	230 (220)	112 (102)	770 (884)
Boc-FSR-MCA	199 (184)	223 (180)	188 (174)	139 (123)	785 (579)
Boc-VLK-MCA	88 (88)	249 (199)	129 (122)	163 (105)	711 (317)
Suc-AAPF-MCA	16 (9)	104 (133)	3 (10)	38 (47)	29 (209)

Pollen extracts obtained with the Tris buffer by the procedure shown in Figure 2A were used for the measurement. † and ‡ : Fluorescence intensity in the presence and absence of DTT at 120 minutes, respectively.

served. For example, with the Boc-VLK-MCA substrate, the pollen extracts of white birch and ragweed showed greater relative activity than those of Japanese cedar, Japanese cypress, and Rocky mountain juniper.

INHIBITION TESTS USING CLASS-SPECIFIC PROTEASE INHIBITORS

To identify the class of proteases within the pollen extracts, the effects of class-specific protease inhibitors, the cysteine protease-specific inhibitor E-64, the serine protease-specific protease inhibitor AEBSF, and the metalloprotease-specific inhibitor EDTA, were analyzed in the presence of DTT (Fig. 4). Protease activity against substrates containing arginine at the P1 position was almost completely inhibited with AEBSF in all the pollen samples tested (Fig. 4A). Protease activity against the Boc-VLK-MCA substrate was completely inhibited with AEBSF in Japanese cedar, Japanese cypress, and Rocky mountain juniper, and partially inhibited with E-64 and AEBSF in white birch and ragweed (Fig. 4B). The combination of E-64 and AEBSF almost completely inhibited protease activity within extracts of pollen of white birch and ragweed against this substrate (Fig. 5). Protease activity against the Suc-AAPF-MCA substrate was not inhibited at the inhibitor concentrations tested (Fig. 4 C), although a higher concentration of AEBSF inhibited the activity (unpublished data).

These results indicated that pollen extracts of Japanese cedar, Japanese cypress, and Rocky mountain juniper, which belong to the *Cupressaceae/Taxodiaceae* family, contained serine protease, that those of white birch and ragweed contained both serine pro-

tease and cysteine protease, and that the extracts from the five types of pollen contained another serine protease, activity of which was detected with the Suc-AAPF-MCA substrate and the sensitivity of which to AEBSF was lower.

DTT-DEPENDENCY OF PROTEASE ACTIVITY

Protease activities in the presence and absence of DTT were compared (Table 1). For the Boc-VLK-MCA substrate, significant enhancement in the presence of DTT was observed in pollen extracts of white birch and ragweed (Table 1, White birch, Ragweed). For Bz-R-MCA, Boc-QGR-MCA, and Boc-FSR-MCA, enhancement in the presence of DTT was observed in ragweed pollen extract. Protease activity against the Suc-AAPF-MCA substrate was reduced by addition of DTT most extensively in ragweed.

These results provided evidence that significant cysteine protease activity was contained in white birch and ragweed pollen extracts (Fig. 4B, White birch, Ragweed; and Fig. 5) and indicated that addition of DTT partially inactivated protease activity against the Suc-AAPF-MCA substrate in ragweed.

DISCUSSION

Although proteolytic activity from allergen sources is one of the first candidates proposed to lead to enhanced allergenicity,[1-3] little information is available on that from relevant allergenic pollens such as Japanese cedar and Japanese cypress pollen, which are the major cause of pollinosis in Japan. Here, we analyzed the protease activities derived from allergenic pollen of Japanese cedar, Japanese cypress, and Rocky mountain juniper, which belong to the *Cupressaceae/Taxodiaceae* family, and white birch and ragweed at neutral pH, using synthetic substrates and class-specific inhibitors. We demonstrated that (1) the pollen of the three members of the *Cupressaceae/Taxodiaceae* family contain serine protease activity, (2) the pollen of white birch and short ragweed contain not only serine protease activity but also cysteine protease activity, (3) all five types of pollen tested contain at least one other type of serine protease, and (4) the content and releasability of the pollen-derived proteases differ according to the plant families. Information on the spectrum of the endopeptidase activities from these allergenic pollen grains will be useful for investigating their contribution to the pathogenesis of allergies.

Although the ragweed pollen extract exhibited the strongest protease activity, the other pollen extracts also exhibited significant protease activity (Table 1). The releasability of the proteases against Boc-QGR-MCA differs according to the plant family in buffers suitable for extraction (Fig. 2B) and necessity of sonication (Fig. 1B, Fig. 1 C). Without sonication, protease activity against this substrate was not effectively released from the pollen of the three members of the *Cupressaceae/Taxodiaceae* family. However, the possibility that the activity is releasable *in vivo* in mucosa or epidermis, where various biochemical active molecules exist, cannot be excluded. The highest protein concentration was detected in ragweed and it could explain the strongest protease activity. The differences in releasability of proteases and proteins might be due to differences in the pollen structure among plant families (unpublished data). The overall pattern of specificity against the synthetic substrates tested was not so different (Fig. 3).

In Japanese cedar, Japanese cypress, and Rocky mountain juniper, which belong to the *Cupressaceae/Taxodiaceae* family, protease activity against four substrates containing arginine at the P1 position and Boc-VLK-MCA were almost completely inhibited with AEBSF (Fig. 4A, Fig. 4B). This indicates that the pollen of the *Cupressaceae/Taxodiaceae* family contain serine protease. The serine protease activity detected in the present study is considered to be that of an endopeptidase and not to be derived from an aminopeptidase from Japanese cedar pollen reported by another research group,[31,32] because aminopeptidases hydrolyze at the unblocked N-terminal amino acid residue of substrates and generally cannot hydrolyze the substrates used in the present study, which have a chemically blocked N-terminus.

In white birch and ragweed, protease activity against four substrates containing arginine at the P1 position were almost completely inhibited with AEBSF (Fig. 4A), and activity against Boc-VLK-MCA was partially inhibited with either AEBSF or E-64 (Fig. 4B, Fig. 5) and almost completely inhibited with a combination of the two (Fig. 5). This indicates that the white birch and ragweed pollen contain both serine protease and cysteine protease. The significant decrease in activity against Boc-VLK-MCA in the absence of DTT supports the existence of a cysteine protease within the pollen extracts of white birch and ragweed (Table 1). Protease activity in white birch pollen, possibly of a serine protease, was reported,[29] and trypsin- and chymotrypsin-like serine proteases were purified from ragweed pollen and characterized.[33,34] The detection of cysteine protease activity in white birch and ragweed pollen has been demonstrated in the present study for the first time.

In the pollen of the five species tested, protease activity against Boc-AAPF-MCA in the presence of DTT was not inhibited with AEBSF, E-64, and EDTA at the concentrations tested in the present study (Fig. 4C). As a higher concentration of AEBSF inhibited the activity (unpublished data), the activity is suggested to be from another serine protease, the sensitivity of which to AEBSF was relatively low. Protease activity against this substrate was greater in the absence of DTT in the ragweed pollen extract (Table 1), indicating DTT partially inactivates at least one molecular species exhibiting serine protease activity against this

substrate. In our preliminary experiments, the protease activity against the substrate was released from the pollen of the three members of the *Cupressaceae*/*Taxodiaceae* family without sonication (unpublished data) unlike that against the Boc-QGR-MCA shown in Figure 1. Comparison of the releasability of the pollen-derived proteases is an important issue to be addressed in a future study, because their releasability could relate to exposure of human tissues to the protease activity in physiological conditions.

House dust mite group 1 allergens are cysteine proteases and group 3, 6, and 9 allergens are serine proteases.[35,36] Protease activities of these mite allergens have been suggested to be involved in the pathogenesis of allergies in sensitization towards IgE production and/or exacerbation by reducing physical and biochemical tissue barriers, cleaving various molecules, and modulating the functions of various cells.[1,3,9-21] Although no proteases have been reported as allergens recognized by patients' IgE for the types of pollen tested in the present study, enzymatic activities of the serine protease and/or cysteine protease detected in the present study might contribute to form a microenvironment with dysfunctional tissue barriers and inflammation toward sensitization or exacerbation as well as pollen-derived lipids,[5-7] and oxidase,[4] which have been suggested to be involved in the pathogenesis of allergic diseases through IgE-independent pathway. The pollen-derived protease activities should be examined for contribution to the pathogenesis of allergic diseases in future studies.

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