

Molecular Cloning and Immunochemical Characterization of a Novel Major Japanese Cedar Pollen Allergen Belonging to the Aspartic Protease Family

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Key Words

Allergen · Allergenome · Aspartic protease · Japanese cedar pollen · Pollinosis

Abstract

Background: Japanese cedar (*Cryptomeria japonica*) pollen is a major cause of seasonal pollinosis in Japan. Protease activity in the pollen grains may trigger pro-allergic responses but no such proteases have yet been identified as pollen allergens. **Objectives:** We report the molecular cloning and immunochemical characterization of a novel *C. japonica* pollen allergen belonging to the aspartic protease family. **Methods:** We focused on the *C. japonica* pollen allergen spot No. 63 (CPA63, 47.5% IgE binding frequency) on our 2-dimensional IgE immunoblot map. The internal amino acid sequences were determined using time-of-flight mass spectrometry. Full-length *cpa63* cDNA was cloned by rapid amplification of cDNA ends (RACE)-PCR. Recombinant CPA63 (r-CPA63) was expressed using the baculovirus-insect cell culture system and its IgE binding capacity was analyzed by enzyme-linked immunosorbent assay (ELISA). The proteo-

lytic activity of r-CPA63 was also assessed using a putative mature enzyme produced upon autolysis. **Results:** *cpa63* cDNA encoded a 472 amino acid polypeptide showing about 40% sequence identity to members of the plant atypical aspartic protease family. ELISA showed that r-CPA63 was recognized by IgE antibodies in the serum of 58% (18/31) of Japanese cedar pollinosis patients. We also demonstrated an aspartic protease-like enzyme activity of the putative mature r-CPA63. **Conclusions:** We have identified the first plant aspartic protease allergen from Japanese cedar pollen. The availability of the CPA63 sequence and its recombinant allergen production system are useful not only for pharmaceutical applications but also for further examination of the role of protease activity in the pathogenesis of cedar pollinosis.

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The sequence data reported in this paper have been deposited in the DDBJ/EMBL/Genbank Sequence Database under accession number AB510538.

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Introduction

Seasonal pollinosis is the most serious allergic disease in Japan. Japanese cedar (*Cryptomeria japonica*) pollen represents a major environmental factor triggering rhinitis and conjunctival congestion accompanied by pollen-specific IgE production [1]. The prevalence of pollinosis caused by Japanese cedar pollen has increased by 10% in the last decade to 26.5% [2], raising cedar pollinosis as a nationwide social issue to be solved urgently. In order to develop effective diagnostic and therapeutic agents, the identification and characterization of clinically important pollen allergens is essential. Thus far, however, only 2 *C. japonica* pollen antigens have officially been registered as major allergens by the International Union of Immunological Societies' Allergen Nomenclature Subcommittee, and extensively studied. One is Cry j 1 [1, 3], which has pectate lyase activity and is homologous to the major short ragweed allergen Amb a 1 family. The other is Cry j 2 [4, 5] possessing polymethylgalacturonase activity. Our group has shown that many of the IgE-reactive antigens in *C. japonica* pollen extract, other than Cry j 1 and Cry j 2, remain uncharacterized [6]. Among them, we have also identified the third major allergen, CJP-6 [6], which is structurally homologous to the isoflavone reductase family that includes the birch pollen allergen Bet v 5 [7] and the pear allergen Pyr c 5 [8].

We have recently elucidated the full IgE-binding spectrum of *C. japonica* pollen allergens using proteomic approaches (allergenome analysis), and isolated 131 IgE-reactive spots on a 2-dimensional (2-D) immunoblotting map of a *C. japonica* pollen extract [9]. We also found 31 unidentified spots that had a higher IgE-binding frequency than that of Cry j 2 (40%), strongly suggesting that these putative major allergens should be characterized to establish component-resolved diagnosis and effective immunotherapy. Among these IgE-binding spots, we identified a fourth allergen, CJP-4 [10], a class IV chitinase homologue showing a potent IgE-binding frequency (100%, 31 patient samples tested) as well as cross-reactivity with latex C-serum. Futamura et al. [11] have isolated 3 thaumatin-like cDNA isoforms termed Cry j 3 homologous to mountain cedar pollen allergen Jun a 3, but their allergenicities were not known. At present, Cry j 3 seems to be a minor allergen, because purified native Cry j 3 shows a low IgE-binding frequency (27%, 100 patient samples tested) [12].

The response to exogenous protease allergens appears to play an important role in allergen-induced inflammation. Proteases from several sources such as *Dermatophaga*

goides farinae and *D. pteronyssinus* (group 1, group 3, group 6 and group 9), *Periplaneta americana* (Per a 10), *Aspergillus fumigatus* (Asp f 2 and Asp f 10), *Epicoccum purpurascens* (Epi p 1), *Penicillium chrysogenum* (Pen ch 13), *P. citrinum* (Pen c 1), *P. oxalicum* (Pen o 18) and *Curvularia lunata* (Cur l 1) have been reported to be important allergens having proteolytic activity [13–20]. Evidence suggests that allergens with protease enzyme activity can induce pro-allergic inflammatory responses totally independent of their IgE-binding capacity. The best-characterized mite cysteine protease allergen Der p 1 and/or Der f 1 is thought to exacerbate allergic inflammation via multiple pathways, e.g. increasing the permeability of the respiratory mucosa [21, 22], promotion of IgE synthesis through the cleavage of CD23 on B cells [23–25], augmentation of Th2 response through the T cell cleavage of CD25 [26], induction of pro-inflammatory cytokine release from airway epithelium [27], disruption of epithelial tight junctions [28], or induction of epithelial apoptosis [29]. These synergistic effects of protease allergens may further promote IgE synthesis [14] and have direct inflammatory effects on the epithelium [27], which could explain why protease activity of airborne allergens is associated with the elicitation of pro-allergic responses. Pollen grains also contain significant amounts of proteases [30], but the pathophysiological role of proteases in atopic conditions is still unclear and protease allergens remain to be isolated and identified from the pollen.

Because of the lack of information about protease allergens in seasonal cedar pollinosis, we searched for proteolytic enzyme allergens among the unidentified allergen spots on our 2-D IgE immunoblotting map [9]. We found that spot No. 63 (referred to as CPA63), which showed an IgE-binding frequency of 47.5% (19/40 patient samples tested), contained internal amino acid sequences homologous to peptidases.

In this study, we report the cDNA cloning and immunochemical characterization of the CPA63 polypeptide, which is homologous to members of the plant aspartic protease/nucleoid DNA binding protein family. We also show that the r-CPA63 produced using an insect cell culture expression system has a high IgE-binding capacity as well as aspartic protease-like enzyme activity.

Materials and Methods

C. japonica Pollen

C. japonica pollen was collected on the Mikado Island (Hiroshima, Japan) and was stored at –20°C until required.

Human Sera

C. japonica pollen-allergic sera were obtained from 31 patients selected on the basis of clinical history, seasonal symptoms and a positive score against *C. japonica* pollen-specific IgE (RAST score = 3 or higher). Non-allergic sera from 4 healthy volunteers were used as a negative control. These sera were stored at -30°C until needed. All experiments were approved by the institutional review board of Takanobashi Central Hospital and were described in detail to all participants before they provided their informed consent.

Time-of-Flight Mass Spectrometry

Comprehensive 2-D immunomapping of *C. japonica* allergen was performed in our previous study [9] and spot No. 63 (CPA63) was found to be one of major allergens with high IgE-binding frequency (47.5%, 19/40 patient samples tested). Excision of the CPA63 protein and its tryptic in-gel digestion were performed as previously described [9, 10]. MALDI-TOF MS and tandem MS (MS/MS) sequencing analyses were then carried out using an Ultraflex TOF/TOF spectrometer (Bruker Daltonics, Billerica, Mass., USA).

cDNA Cloning

Total RNA was extracted from frozen *C. japonica* anther (5 g, crushed in liquid nitrogen) using Concert Plant RNA reagent (Invitrogen, Carlsbad, Calif., USA), and mRNA was selected using the Oligotex-dT30 Super mRNA purification kit (TaKaRa Bio, Ohtsu, Japan). cDNA templates for Rapid Amplification of cDNA Ends (RACE)-PCR were synthesized using the BD Smart RACE cDNA amplification kit (BD Biosciences, San Jose, Calif., USA). 5'- and 3'-RACE PCR was then carried out using the BD Advantage 2 PCR kit (BD Biosciences) with *cpa63* gene-specific primers for 5'-RACE (5'-CTCCACCAGCCTAGTGATGACTGTTCC-3', complementary to the nucleotide No. 1073–1099, amino acid residues 348–356) and 3'-RACE (5'-CGGCGACGTGGAGTTCC-TCTCATC-3', corresponding to the nucleotide No. 1201–1225, amino acid residues 391–399). The resulting PCR fragments were sub-cloned into the pGEM-T Easy Vector (Promega, Madison, Wisc., USA) and their nucleotide sequences determined using an ABI PRISM 3100 Avant genetic analyzer (Applied Biosystems, Foster City, Calif., USA).

Expression and Purification of Recombinant CPA63

Expression of recombinant CPA63 (r-CPA63) was achieved using a baculovirus-insect cell culture system. The full-length *cpa63* cDNA was inserted into a baculovirus transfer plasmid, pVL1393 (BD Biosciences), and a (His)₆-tag sequence was introduced into its 3'-terminus to enable the detection and purification of the recombinant protein. To make the recombinant baculovirus, Sf21 insect cells were co-transfected with the CPA63/pVL1393 expression construct and baculovirus genomic DNA using the BD BaculoGold Transfection Kit (BD Biosciences), and cultured in Sf900II serum-free medium (Invitrogen) at 27°C for 3 days. Fresh Sf21 cells were infected with the resulting CPA63 baculovirus and cultured for 3 days. This infection procedure was repeated in a total of 6 times for amplification of viral titer. For the expression of r-CPA63, the amplified recombinant virus was allowed to infect fresh Sf21 and cultured for 4 days. The culture supernatant (500 ml) was concentrated using an Amicon Ultra 15 centrifugal filter (30 kDa molecular mass cutoff; Milli-

pore, Billerica, Mass., USA) followed by buffer exchange into 50 mM sodium phosphate (pH 7.4) containing 20 mM imidazole. The sample was then loaded onto a HisTrap HP metal chelate affinity column (GE Healthcare Bio-Sciences, Uppsala, Sweden), and the r-CPA63-containing fraction was obtained by elution with 200 mM imidazole/phosphate buffer. The semi-purified sample was then loaded onto a HiTrap Q HP column (GE Healthcare Bio-Sciences) pre-equilibrated with 20 mM Tris-HCl (pH 8.4), and eluted using a stepwise gradient up to 1 M NaCl with 20 mM Tris-HCl. The r-CPA63 protein was eluted at a NaCl concentration of 200 mM.

SDS-PAGE and Western Blotting

Protein samples (both culture supernatant and purified samples) were analyzed by SDS-PAGE on a 12.5% slab gel and the protein bands were visualized by silver staining. For Western blotting, protein samples were transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore). After blocking with phosphate-buffered saline (PBS) supplemented with 0.05% Tween 20 (PBST) and 5% skim milk, the blot was probed with an anti-His monoclonal antibody (GE Healthcare Bio-Sciences) at a dilution of 1:10,000 in the blocking buffer. After washing, the blot was reacted with a horseradish peroxidase-conjugated rabbit anti-mouse IgG polyclonal antibody (Sigma, St. Louis, Mo., USA). The protein bands were visualized on an X-ray film (Konica Minolta, Tokyo, Japan) using an ECL-Plus Western blotting detection kit (GE Healthcare Bio-Sciences).

CPA63-Specific Antisera and 2-D Immunoblotting

Purified r-CPA63 (10 μg) was emulsified with Titre-Max Gold adjuvant (CytRx Corp., Norcross, Ga., USA) and used for immunization. BALB/c mice (4 weeks old; Charles River, Yokohama, Japan) were immunized intraperitoneally with r-CPA63 every 2 weeks for 4 weeks. CPA63-specific antisera were collected 2 weeks after the final immunization. For 2-D immunoblotting, crude *C. japonica* pollen extract (50 μg) was applied to 2-D SDS-PAGE (mini gel scale, 12.5% slab gel) as essentially described [9]. Proteins were then blotted onto an Immobilon P transfer membrane (Millipore). After blocking with 5% skim milk/PBST, the blot was probed with CPA63-specific antisera (diluted 1:5,000 in blocking buffer) followed by incubation with a secondary horseradish peroxidase-conjugated anti-mouse IgG antibody (Sigma-Aldrich). For protein markers for isoelectric focusing and SDS-PAGE, we used the Broad pI Kit, pH 3.5–9.3 and the LMW Marker Kit, respectively (GE Healthcare Bio-Sciences). The ECL-based detection of the protein bands was carried out as described above. The animal experiments were carried out in accordance with protocols reviewed and approved by the committee of animal experimentation at Hiroshima University.

In vitro Maturation of r-CPA63

For proteolytic automaturation of the CPA63 zymogen, r-CPA63 (putative proform) was incubated in 0.1 M acetate buffer (pH 3.3) at room temperature for 24 h. To confirm successful maturation, the reaction mixture was applied to a 12.5% SDS-PAGE gel and the protein bands were visualized by silver staining. To determine a cleavage site on the CPA63 zymogen, the proteins separated in SDS-PAGE gels were electroblotted onto the Immobilon P membrane (Millipore). After staining the membrane with

Coomassie brilliant blue R250, the putative mature CPA63 protein band was excised and its N-terminal amino acid sequence was determined using a 492 Protein Sequencer (Applied Biosystems).

Enzyme-Linked Immunosorbent Assay

The IgE-binding frequency of purified mature r-CPA63 was evaluated by ELISA as previously described [6] with minor modifications. In brief, protein sample (50 µl; 500 ng/ml in 100 mM bicarbonate buffer, pH 9.4) was coated onto a 96-well microtiter plate (NUNC-Immuno Plate Maxisorp F96; Nalge Nunc International, Roskilde, Denmark). After blocking with 3% skim milk/1% bovine serum albumin/PBS, diluted Japanese cedar pollinosis patients' sera (50 µl; diluted 1:50 with blocking buffer) was added and incubated overnight at 4°C. Secondary biotinylated goat anti-human IgE (1:10,000; Biosource International, Camarillo, Calif., USA) was then added and the plate was incubated for 2 h at room temperature, followed by an incubation with 50 µl alkaline phosphatase-conjugated streptavidin (1:10,000; Jackson ImmunoResearch Laboratories, West Grove, Pa., USA) for 1 h. Finally, AttoPhos substrate solution (Promega) was added and incubated at room temperature for 30 min. The fluorescence intensity of each well was analyzed using a Wallac 1420 ARVOsx Multilabel Counter (Perkin Elmer Life Sciences, Boston, Mass., USA).

Protease Assay

The proteolytic activity of the mature r-CPA63 was assessed as previously described [31, 32] with minor modifications. First, we prepared a fluorescein isothiocyanate (FITC)-labeled hemoglobin (FITC-Hb) as a substrate using bovine blood Hb (Sigma-Aldrich) and the EZ-label FITC protein labeling kit (Thermo Scientific, Rockford, Ill., USA). For the protease assay, purified mature r-CPA63 (0.3 µM, final reaction volume of 25 µl in PBS) was mixed with FITC-Hb (0.5 mg/ml) on ice and incubated at 37°C for 90 min. After stopping the reaction by addition of 75 µl of 5% tri-chloroacetic acid and centrifugation (10,000 g for 10 min at 4°C), 2 µl of the supernatant was diluted with 200 µl of 500 mM Tris-HCl (pH 8.5) and its fluorescence intensity measured using a Wallac 1420 ARVOsx Multilabel Counter. To test the inhibitors specificity of r-CPA63, the proteolytic enzyme reaction was carried out in the presence of the following protease inhibitors: 10–100 µg/ml pepstatin A (an aspartic protease inhibitor; Sigma-Aldrich), 3 µM E64 (a cysteine protease inhibitor; Sigma-Aldrich), 100 µM phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor; Nacalai Tesque, Kyoto, Japan), 10 µg/ml leupeptin (a serine and cysteine protease inhibitor; Sigma-Aldrich) and 5 mM EDTA (a metalloprotease inhibitor; Nacalai Tesque).

We determined the above concentrations of inhibitors in reference to their optimal ranges as follows: pepstatin A (1–10 µM = 0.67–6.7 µg/ml) [33–35], E64 (1–10 µM) [36, 37], PMSF (100–1,000 µM) [38, 39], leupeptin (1–100 µM = 0.5–50 µg/ml) [33, 40–42], and EDTA (1–10 mM) [43, 44]. Pepsin from porcine stomach mucosa (Sigma-Aldrich) was used as a positive control for the aspartic protease.

Statistical Analysis

The statistical analysis was performed using Student's t test. $p < 0.05$ was accepted as the level of significance.

Results

Molecular Cloning of a New C. japonica Pollen Allergen CPA63, Structurally Homologous to Aspartic Protease/Nucleoid DNA Binding Protein Family Members

We have previously established the 2-D IgE immunoblotting map of *C. japonica* allergens and found many uncharacterized allergen spots in addition to the major allergens Cry j 1 and Cry j 2 [9]. Of these, we focused on spot No. 63 (CPA63), which showed a high IgE-binding frequency (47.5%, 19/40 patient samples tested). TOF-MS analysis of the CPA63 spot revealed 2 internal amino acid sequences (FTPLLSNSR and LVEPAYNAMR). A subsequent BLAST database search identified a *C. japonica* expressed sequence tag (BP173910) that contained both of the amino acid sequences, and these showed significant homology to those of aspartic protease-like peptidases. Based on this sequence information, we cloned a full-length cDNA encoding CPA63 using 5' and 3' RACE-PCR. Nucleotide sequencing and BLAST analysis revealed that cpa63 cDNA comprises 1,544 nucleotides that encode 472 amino acids (fig. 1), and has about 40% sequence identity to members of the plant aspartic protease/nucleoid DNA-binding protein family. Its calculated molecular weight and isoelectric point are 51,128.75 Da and 4.69, respectively. The CPA63 polypeptide contains the 2 catalytic sequence motifs DTG and DSG (shown open boxed in fig. 1), both of which are responsible for the aspartic protease-like proteolytic activity [45]. The plant aspartic protease family A1 is classified into 3 subfamilies: typical, nucellin-like and atypical subfamilies [45]. The phylogenetic analysis of CPA63 against other plant aspartic proteases using the ClustalW software showed that CPA63 was a member of the atypical subfamily of plant aspartic proteases that includes CND41 (Genbank accession number; BAA22813, *Nicotiana tabacum*), CDR1 (AAP72988, *Arabidopsis thaliana*) and Nepenthesin 1 (Q766C3, *Nepenthes gracilis*) (fig. 2). Indeed, the CPA63 polypeptide sequence contains the following common features of this subfamily [46]: (1) the absence of an internal segment termed the 'plant-specific insert'; (2) a high number of cysteine residues between the first catalytic aspartic acid residue 142 and a conserved tyrosine residue 204, and (3) a relatively conserved active site motif in the first catalytic center (V-L-DTG-S-N, as shown in fig. 1; the consensus motif sequence is 'hydrophobic-hydrophobic-DTG-Ser-acidic').

Fig. 1. Nucleotide and deduced amino acid sequence of CPA63. Two putative catalytic sequence motifs (DTG and DSG) are highlighted in open boxes. The sequence around the first catalytic motif (V-L-DTG-S-N) is shown underlined. An invariant tyrosine residue (No. 204) in the flap region that covers the active site cleft is double underlined. The high number of cysteine residues between the first catalytic aspartic acid 142 and the conserved tyrosine 204 are highlighted by grey boxes. Internal amino acid sequences obtained by the TOF-MS analysis are underlined with dashed lines. A putative signal peptide sequence (Nos. 1–20; predicted by SignalP 3.0 Server) is also underlined. An arrowhead indicates a putative cleavage site of the CPA63 proform as assessed by N-terminal amino acid sequencing of mature CPA63. Obtained N-terminal amino acid sequence of mature CPA63 ('SIKGD') is also underlined.

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-31 GGATCATTGCCTGTGCTTCTCATAGACGACA -1
1 ATGGCAAGCGCTCTATGCTCATTCTTCTTTCTAATAATTTCTGTGTGGGCA 60
1 M A R R L C S F L L S F L I I V S V W A 20
61 GAAACAGTAAATTCGCCGACTAAACCTTGCATCATTACCTGGAAGATGCAGAGGAT 120
21 E N S K F A R L N L A S F T W K D A E D 40
121 AACAAGAACTGCTCTGCGGGTGAATTAGAAACATCAAGCTTGAGCGTGATGCACATTCAG 180
41 N K N C S A G E L E T S S L S V M H I Q 60
181 GGCAAGTGTCTCCTTTCCGCCCTCAATTCATCATGGTGGACAGCGGTATCCGAGTCA 240
61 G K C S P F R L L N S S W W T A V S E S 80
241 ATTAAGGCGACACTGCGCGCTACAGAGCAATGGTGAAGGGGATGGAGCGCCGGGAAA 300
81 I K G D T A R Y R A M V K G G W S A G K 100
301 ACTATGGTTAATCCCAAGAGGACGCGGACATCCCGCTGGCTTCGGGACAGGCCATCAGC 360
101 T M V N P Q E D A D I P L A S G Q A I S 120
361 TCGTCAAACATACATTATCAAGCTGGGTTTCGGCACGCTCCGCAGAGCTTCTACACTGTC 420
121 S S N Y I I K L G F G T P P Q S F Y T V 140
421 CTCGATACCGGCAGCAATATCGCTTGGATTCGGTCAACCCATGCTCGGTTGCTCAAGT 480
141 L D T G S N I A W I P C N P C S G C S S 160
481 AAGCAACACCGTTTGGAGCATCCAAGTCGTCTACGTATAACTACCTACCTCGCGTTCT 540
161 K Q Q P F E P S K S S T Y N Y L T C A S 180
541 CAGCAGTGTGAGCTTCTGCGTGTCTGTACGAAGAGCGATAACAGTGTGATGACCTC 600
181 Q Q C Q L L R V C T K S D N S V N C S L 200
601 ACTCAGCGGTACGGCGACAGTCGGAGGTGGACGAGATCTTGTCTGTGAGACACTCTCT 660
201 T Q R Y G D Q S E V D E I L S S E T S 220
661 GTCGGCTCTCAGCAAGTGAAAATTTGTGTTCCGATGTTCAATCGGCAAGGGGACTC 720
221 V G S Q Q V E N F V F G C S N A A R G L 240
721 ATCCAGAGGACGCCAGCTTGGTTGGGTTGGGAGGAATCCTCTCTCTCTCTCTCTCAG 780
241 I Q R T P S L V G F G R N P L S F V S Q 260
781 ACGGCAACGTTATATGACAGTACGTTCTCGTATTCGCTTCCCTCCCTCTCTCTCTGCT 840
261 T A T L Y D S T F S Y C L P S L F S S A 280
841 TTCACAGGCTCTCTCTCTTGGGAAAGAAGCTCTTCTGCGCAGGGCTTGAAGTTCACG 900
281 F T G S L L L G K E A L S A Q G L K F T 300
901 CCTCTCTTGTCCAACCTAGGTACCCTTCTTTTATTATGTGGGTTAAACGGAATCTCT 960
301 P L L S N S R Y P S F Y Y V G L N G I S 320
961 GTGGGAGAAGAGCTTGTATCCATCCAGCGGGGACATTGAGCTTGGATGAGTCGACGGGA 1020
321 V G E E L V S I P A G T L S L D E S T G 340
1021 AGAGGGACTATCATAGACTCGGGAACAGTCATCACTAGGCTGGTGGAGCCCGCTTACAAT 1080
341 R G T I I D S G T V I T R L V E P A Y N 360
1081 GCCATGCGAGATCTTTTCGAGTCAGCTCTCTAATCTGACTATGCTAGTCCACAGAT 1140
361 A M R D S F R S Q L S N L T M A S P T D 380
1141 CTTTTTGATACGTGCTACAACAGGCCGTCGGCGAGCTGGAGTTTCTCTCATCAGTTG 1200
381 L F D T C Y N R P S G D V E F P L I T L 400
1201 CATTTGACGATAATCTGACTTGACTCTGCCCTGGATAACATTTATACCTGGGAAT 1260
401 H F D D N L D L T L P L D N I L Y P G N 420
1261 GACGACGGTCCGTGTTGTGCTTGTCTTTCGGTCTTCCACCGGAGGAGGGGACGATGTA 1320
421 D D G S V L C L A F G L P P G G G D V 440
1321 TTGTCAACATTTGGGAACATATCAGCAGCAGAAGTTGCGAATAGTGATGATGTTGCAGAG 1380
441 L S T F G N Y Q Q Q K L R I V H D V A E 460
1381 TCTAGGCTTGGAAATGTAGCAGAGAAGTGCAGTGGTTAGTTGGTTGCTGAGTGTGTTTA 1440
461 S R L G I A S E N C D G * 472
1441 TTCACCGTATTGTGCGCTATAATACCACACCAACGTCCTCTGCTTCACTATAATACC 1500
1501 AGAATGATAACGT 1513

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Expression and Purification of r-CPA63 Using the Baculovirus-Insect Cell Culture System

To analyze the allergenicity and immunochemical properties of CPA63, we expressed the protein using the baculovirus-insect culture system. Infection of Sf21 insect cells with the recombinant CPA63 baculovirus resulted in the successful secretion of the 52-kDa r-CPA63 protein into the culture supernatant as confirmed by Western blot analysis using the anti-His antibody (lane 2; fig. 3a). We next tried to purify r-CPA63 first using

HisTrap HP chelate affinity chromatography. We found that r-CPA63 was eluted by 200 mM imidazole (lane 3; fig. 3a). The resulting semi-purified sample was then loaded onto a HiTrap Q anion exchange column to yield a r-CPA63 protein as confirmed by silver staining and Western blot analysis (lane 4; fig. 3a).

2-D Immunoblotting

We then sought to test for the presence of the CPA63 isoforms in *C. japonica* pollen extract. Murine CPA63-

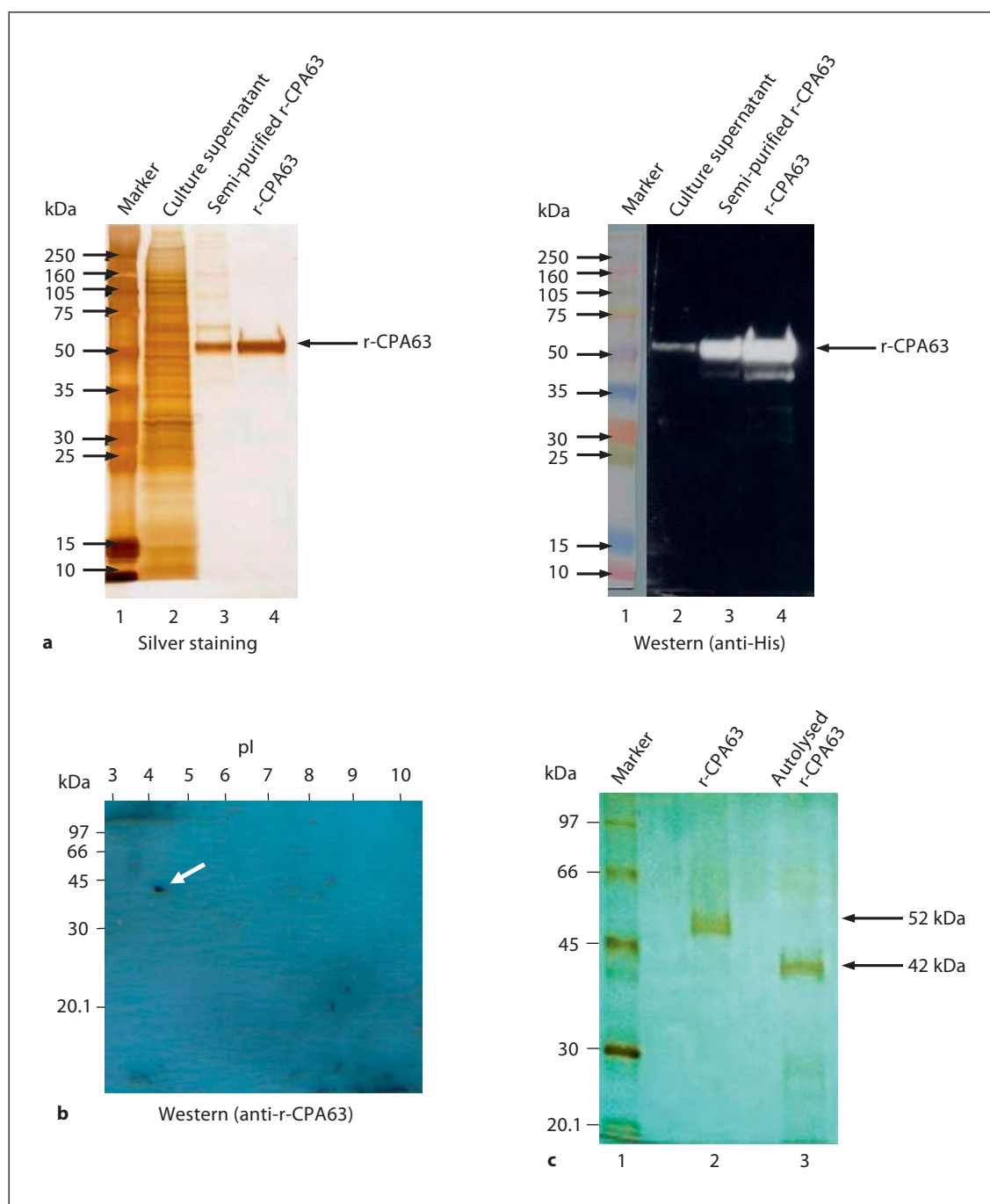
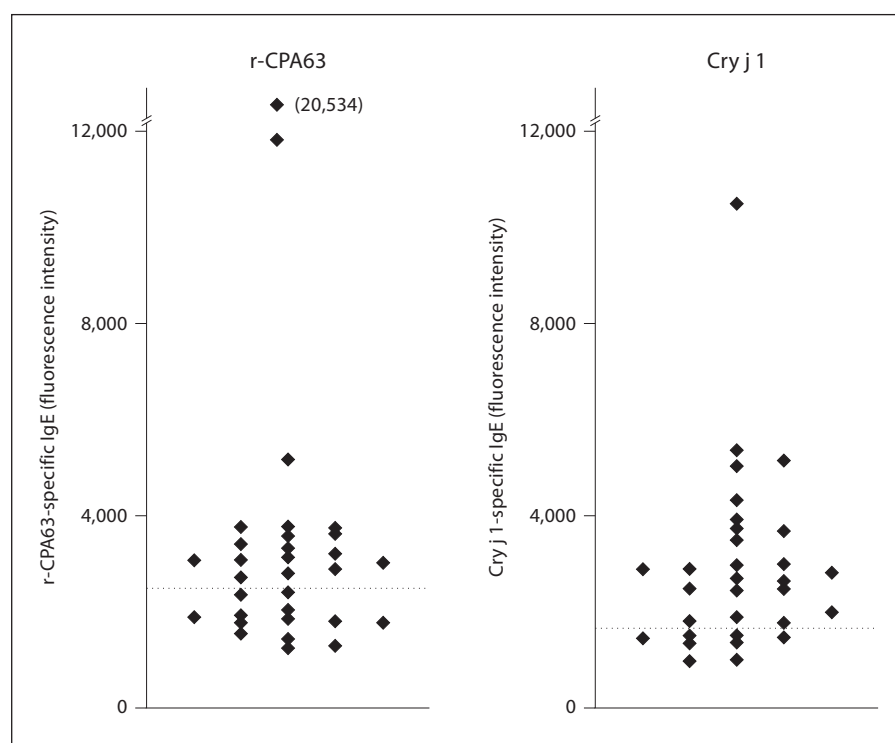


Fig. 3. a Expression and purification of r-CPA63 using the baculovirus-insect cell culture system. r-CPA63 secreted into Sf21 culture supernatant (lane 2) was purified using HisTrap chelate affinity chromatography (lane 3) followed by HiTrap Q anion exchange chromatography (lane 4). Protein bands were visualized by SDS-PAGE (silver staining) and on western blots using anti-

His antibody. **b** 2-D immunoblotting of *Cryptomeria japonica* pollen extract with CPA63-specific antiserum. The white arrowhead indicates an anti-CPA63-reactive antigen spot. **(c)** In vitro autolysis of r-CPA63 after incubation in acidic conditions (pH 3.3). The molecular weight falls from 52 kDa (lane 2) to 42 kDa (lane 3).

Fig. 4. IgE-binding activity of r-CPA63. ELISA was performed using sera from 31 Japanese cedar pollinosis patients (RAST score ≥ 3). Sera from 4 healthy donors were used as a negative control. The dotted line indicates a cutoff value (mean ELISA value + $3 \times$ SD from normal control sera) for judging positive IgE-binding to r-CPA63. For comparison, specific IgE titer against native Cry j 1 (right panel) was also analyzed in the same donors.



cleavage of a propeptide region of about 10 kDa through autolysis. Protein sequencing revealed that the N-terminal amino acid sequence of the mature 42-kDa r-CPA63 was SIKGD (corresponding to amino acid residues 80–84, shown underlined in fig. 1), indicating that the *in vitro* cleavage site is located between glutamic acid residue 79 and serine residue 80 (shown by the arrowhead in fig. 1).

IgE-Binding Capacity of r-CPA63

We next used ELISA to test the IgE-binding activity of mature r-CPA63 using sera from 31 Japanese cedar pollinosis patients (RAST score ≥ 3). Sera from 4 healthy donors were used as a negative control. We found that r-CPA63 was recognized by IgE antibodies in the sera of 18/31 cedar pollinosis patients (58.1%; fig. 4, left panel), while the major allergen Cry j 1 was recognized by 23/31 of the patients IgE (74.2%; fig. 4, right panel). These results suggest that CPA63 is an additional major sensitizing allergen for Japanese cedar pollinosis patients.

CPA63 Has Aspartic Protease-Like Enzyme Activity

CPA63 contains all the necessary amino acid residues required for the proteolytic activity of an aspartic protease (fig. 1). Thus, we next tested the proteolytic activity

of both the 52-kDa r-CPA63 and the 42-kDa putative mature r-CPA63 using FITC-Hb as a substrate. We found that the mature r-CPA63, but not the 52-kDa r-CPA63 zymogen, has potent proteolytic activity at pH 5 (fig. 5a), indicating that r-CPA63 becomes enzymatically active upon *in vitro* maturation. Since the aspartic protease is an acidic protease, we next analyzed whether the proteolytic activity of mature r-CPA63 was dependent upon pH. As expected, the mature r-CPA63 showed significantly higher proteolytic activity under acidic conditions (pH 2.5) than at neutral and basic conditions (fig. 5b). At this acidic pH, the enzyme concentration-activity curve indicates a good linear correlation between mature r-CPA63 concentration and its protease activity (data not shown). Enzymatic activity of CPA63 (0.3 μ M) was less potent than that of pepsin (positive control), since one third of the pepsin concentration (0.1 μ M) resulted in about 3 times higher enzyme activity (fig. 6b). To further establish CPA63 as an aspartic protease, we examined the effect of a series of protease inhibitors on its proteolytic activity. We found that only aspartic protease inhibitor pepstatin A specifically suppresses the enzyme activity of CPA63 in a dose-dependent manner. None of other protease inhibitors tested [E64 (cysteine protease), PMSF (serine protease), leu-

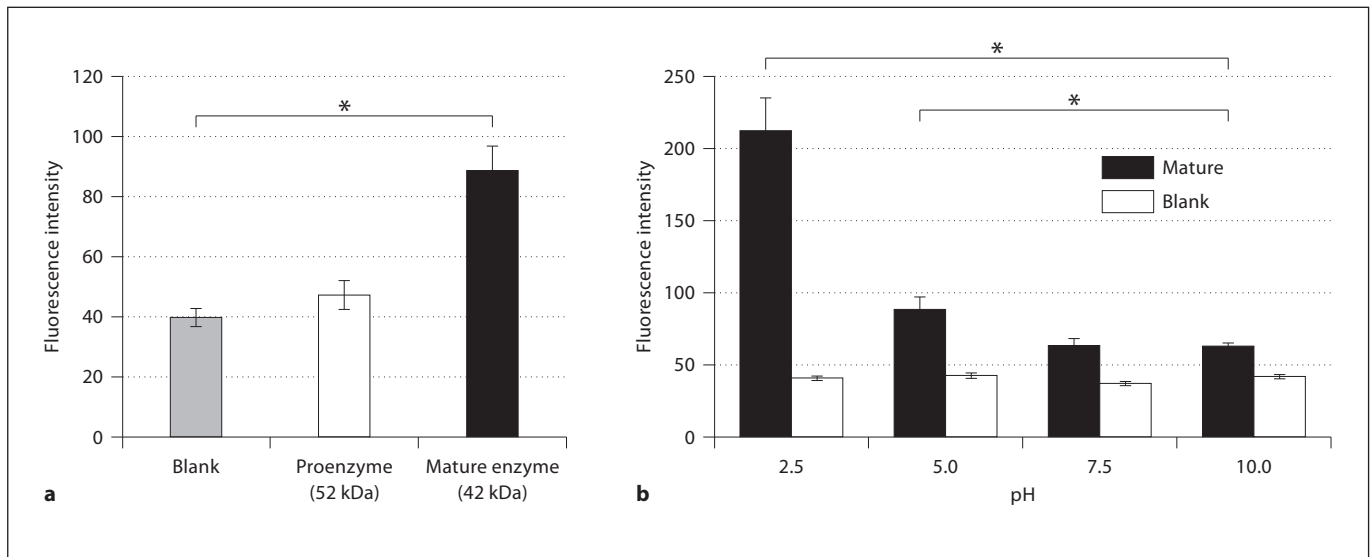


Fig. 5. Protease activity of r-CPA63. **a** Proteolytic activity of the mature 42-kDa CPA63. The proteolytic activities of the 52-kDa r-CPA63 proenzyme and putative mature form of the 42-kDa r-CPA63 (0.3 μ M each) were assessed using FITC-Hb as a substrate. * $p < 0.05$. **b** pH dependency of the proteolytic activity of mature r-CPA63. All data are expressed as fluorescence intensity and are representative of 3 independent experiments. * $p < 0.05$.

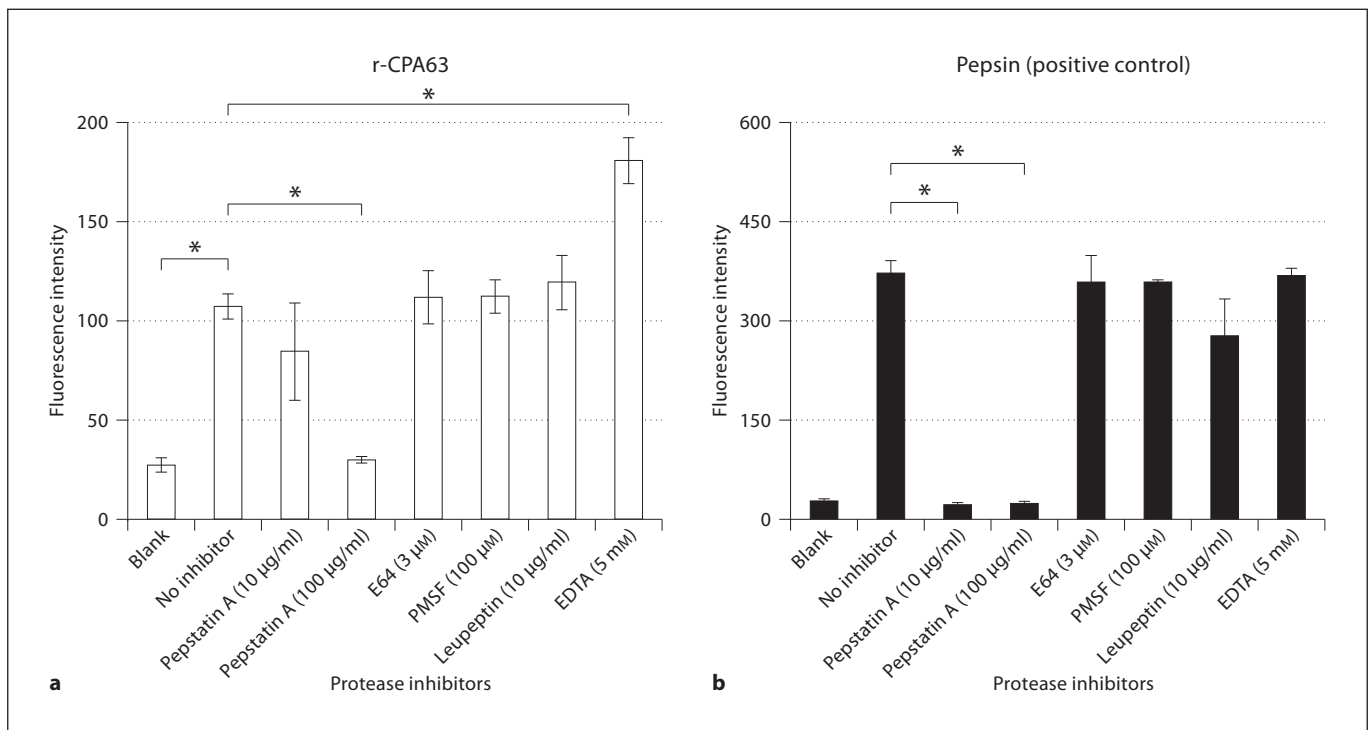


Fig. 6. Effect of protease inhibitors on the proteolytic activity of mature r-CPA63. **a** The 42-kDa mature r-CPA63 (0.3 μ M) was pre-incubated with a series of protease inhibitors at the indicated concentrations and then tested for proteolytic activity using FITC-Hb as a substrate. * $p < 0.05$. **b** Commercial porcine stomach pepsin (0.1 μ M) was used in the same experiment as a positive control. * $p < 0.05$.

peptin (cysteine and serine protease) or EDTA (metalloprotease)] showed such an inhibitory action at their optimum concentrations, while EDTA significantly up-regulated the proteolytic activity of CPA63 (fig. 6a). Collectively, these results confirm that CPA63 has an aspartic protease-like enzyme activity.

Discussion

A previous 2-D IgE immunoblotting analysis of Japanese cedar pollen extract demonstrated 31 new allergen spots showing higher IgE-binding frequency than that of major allergen Cry j 2 [9]. A complete assortment of clinically important allergen in *C. japonica* pollen is an important step towards the development of component-resolved diagnosis and effective tailor-made immunotherapy due to varied IgE-binding spectra among patients. As no information is available on protease allergen in seasonal cedar pollinosis, we searched for proteolytic enzyme allergen from our 2-D IgE immunoblotting map, and identified CPA63 as a novel *C. japonica* major allergen belonging to aspartic protease.

Structural and phylogenetic analyses revealed that CPA63 belongs to the family of atypical aspartic proteases that are characterized by the lack of the 'the plant-specific insert', which is found in the majority of typical plant aspartic proteases (fig. 2b). Other plant aspartic proteases that lack the plant-specific insert have been found in some plants such as rice [47], tobacco [48] and members of the *Arabidopsis* family [46]. CPA63 also possesses a putative signal peptide sequence (fig. 1). This signal peptide may have worked properly in our insect cell expression system to allow successful secretion of r-CPA63 (fig. 3b), as we did not add any extra signal peptides into our recombinant baculoviral expression construct.

ELISA demonstrated that IgE antibodies in the sera of 18/31 patients (58.1%) recognized r-CPA63 (fig. 4), which is roughly comparable to that of native Cry j 1 (74.2%, 23/31 patient samples tested). These results suggest that CPA63 is an additional major sensitizing allergen in Japanese cedar pollinosis. It is interesting to note that Bla g 2, one of the most potent cockroach allergens (which elicits IgE responses in 60–80% of the patients) [49], also shows homology to the aspartic protease family of enzymes.

Allergens from different sources may fulfill a variety of biological functions: acting as enzymes, enzyme inhibitors, ligand binding proteins (lipocalins) or structur-

al proteins [50–53]. To gain further insight into the biochemical function and pathophysiological role of CPA63, we sought to demonstrate its proteolytic activity and to test the ability of different protease inhibitors to inhibit its activity. We found that r-CPA63 gains proteolytic activity after in vitro processing of the putative propeptide sequence (fig. 3c and 5a). The propeptide at the N-terminus of aspartic proteases is thought to mask the substrate-binding cleft and prevent binding of the substrate to the catalytic center [45]. It is plausible, therefore, that the 52-kDa r-CPA63 proform is enzymatically inactive at pH 5.0, a condition that is not acidic enough to cleave the propeptide. The proteolytic activity of the 42-kDa r-CPA63 was specifically inhibited by the aspartic protease inhibitor pepstatin A, but not by other protease inhibitors (fig. 6a). We observed that the inhibitory action of pepstatin A against r-CPA63 was not as potent as it was against our positive control enzyme, pepsin (fig. 6a, b). Similarly, other plant atypical aspartic proteases are insensitive to pepstatin A when tested at low concentrations, as in the case of CDR1 [54] and CND41 [32], for example. This reflects the need for a higher effective concentration of pepstatin A to inhibit those plant atypical aspartic proteases including CPA63. We also observed a significant up-modulation of the CPA63 enzyme activity by EDTA (fig. 6a). Interestingly, the same positive regulatory effect of EDTA was also noted in the case of another plant atypical aspartic protease CND41 [32]. These results suggest a common regulatory mechanism of the atypical aspartic protease by EDTA, although its molecular basis is currently unknown. Interestingly, EDTA did not affect the enzymatic activity of porcine pepsin (fig. 6b), suggesting that the positive regulatory effect of EDTA is specific for the atypical subfamily of plant aspartic proteases.

German cockroach major allergen Bla g 2 structurally belongs to the family of aspartic proteases like CPA63, it is an enzymatically inactive protease with an unusual catalytic triad sequence [49, 55, 56]. By contrast, here we have clearly shown that CPA63 simultaneously carries both high IgE-binding activity and aspartic protease activity. This raises the possibility that CPA63 may have an additional proallergic potency that is directly related to its proteolytic activity. The CPA63 may become enzymatically active in the lumen of the stomach and in rare situations in the respiratory tract via gastroesophageal reflux [57] in humans. Our 2-D Western analysis indicated that native CPA63 seemed to present as a mature form in the pollens (fig. 3b), suggesting that it may exert proteolytic activity at low to semi-neutral pH ranges. The pathophys-

iological role of its protease activity in vivo should be addressed in future studies.

In conclusion, we have identified a novel major Japanese cedar pollen allergen, CPA63, as the first plant allergen homologous to aspartic proteases. We have also shown that CPA63 is the first active aspartic protease allergen. The identification of the CPA63 DNA sequence

and its expression in a recombinant allergen production system are a key to the development of component-resolved diagnoses and specific immunotherapies. Moreover, enzymatically active r-CPA63 is useful for testing the role of protease activity in the pathogenesis of Japanese cedar pollinosis.

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