

Vacuolar Serine Protease Is a Major Allergen of *Cladosporium cladosporioides*

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Key Words

Cladosporium cladosporioides • Vacuolar serine protease • Cla c 9 allergen

Abstract

Background: *Cladosporium* is an important allergenic fungus worldwide. We report here a major allergen of *C. cladosporioides*. **Methods:** Major *C. cladosporioides* allergens were characterized by immunoblotting, N-terminal amino acid sequencing, protein purification and cDNA cloning. **Results:** Seventy-four sera (38%) from 197 bronchial asthmatic patients demonstrated IgE binding against *C. cladosporioides* extracts. Among these 74 sera, 41 (55%) and 38 (51%) showed IgE binding against a 36- and a 20-kDa protein of *C. cladosporioides*, respectively. Both IgE-reacting components reacted with FUM20, a monoclonal antibody against fungal serine proteases. N-terminal amino acid sequencing results suggest that they are vacuolar serine proteases, and the 20-kDa component is possibly a degraded product of the 36-kDa allergen. A corresponding 5'-truncated 1,425-bp cDNA fragment was isolated. The mature protein after N-terminal processing starts with an N-terminal serine that is the ninth residue encoded by the 5'-truncated cDNA. The protein sequence deduced shares 69–72% sequence identity with *Penicillium* vacuolar serine proteases and was designated as Cla c 9. The purified 36-kDa Cla c 9 allergen showed proteolytic activity with peptide Z-Ala-Ala-Leu-pNA as sub-

strate. IgE cross-reactivity was detected between the purified Cla c 9 and serine protease allergens from *Aspergillus fumigatus* and *Penicillium chrysogenum*. **Conclusion:** We identified a vacuolar serine protease as a major allergen of *C. cladosporioides* (Cla c 9) and a major pan-allergen of prevalent airborne fungi. IgE cross-reactivity among these highly conserved serine protease pan-fungal allergens was also detectable.

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Introduction

About 10–20% of the general population worldwide suffer from atopic respiratory disorders [1, 2]. Understanding the cause of inhalant allergic reactions plays an important role in disease treatment and prevention. Hence, characterization of allergens is of fundamental importance in improving the diagnosis and treatment of allergy [3, 4].

Results from 19 random surveys show that among the more than 80 different airborne fungal genera associated with atopic asthma, *Cladosporium* is the most common fungus in the air, followed by *Alternaria*, *Penicillium*, *Aspergillus*, *Fusarium* and *Aureobasidium pullulans* [5]. At the residences of atopic and control children in the Taipei area, *Aspergillus*, *Cladosporium* and *Penicillium* are the most common microfungi, both indoors and outdoors

[6]. We have also identified previously that the alkaline and/or vacuolar serine proteases are major allergens of prevalent *Penicillium* and *Aspergillus* species [7].

Several *Cladosporium* species are important allergens, including *C. cladosporioides* and *C. herbarum* [8]. About 60 antigens from *C. herbarum* have been identified by crossed immunoelectrophoresis, and 36 of them react with IgE antibodies from patients' sera by crossed immunoelectrophoresis [9]. Eleven *C. herbarum* allergens have been cloned and characterized [10–17]; among them is an NADP-dependent mannitol dehydrogenase (Cla h 8) that can be recognized by IgE antibodies from 57% of all *Cladosporium*-allergic patients [14]. The other 10 are minor allergens and recognized by less than 20% of the *C. herbarum* allergic patients [13, 15–17]. However, information on allergens of the prevalent and allergenic *Cladosporium* species, *C. cladosporioides* [18, 19], is exiguous. Bouziane et al. [20] have identified, by a nitrocellulose electroblotting technique, allergens of *C. cladosporioides* with molecular masses ranging from 16 to 88 kDa. These allergens have not been further characterized. Thus, much has to be done on *C. cladosporioides* allergens.

In this study, major IgE-reacting *C. cladosporioides* proteins were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and 2D immunoblotting. The N-terminal amino acid sequence of the 20-kDa major allergen has been determined and the corresponding cDNA clone was isolated. We present evidence that the Cla c 9 major allergen of *C. cladosporioides* is a vacuolar serine protease. In addition, we demonstrate the presence of IgE cross-reactivity among the highly conserved serine protease pan-fungal allergens.

Materials and Methods

Serum Samples

Serum samples from 197 adult patients with bronchial asthma were collected at the allergy clinics of the Taipei Veterans General Hospital. Patients with asthma were diagnosed according to the statement provided by the American Thoracic Society [21]. After collection, serum samples were stored in aliquots at -70°C . This study has been approved by the Institutional Review Board of Taipei Veterans General Hospital.

Preparation of Allergenic Extracts

C. cladosporioides strain BCRC 30812 was isolated from the air of Taiwan and obtained from the Food Industry Research and Development Institute, Hsinchu, Taiwan. The *Cladosporium* fungus was grown in a liquid medium (CYA) containing Czapek concentrate (10 ml/l) [22], K_2HPO_4 (1.0 g/l), yeast extract (5 g/l; Difco, Detroit, Mich., USA) and sucrose (30 g/l) at 26°C , without agitation, for 6 days. The fungal mat containing both spores and my-

celia was collected. The allergenic extracts of *C. cladosporioides* were prepared essentially as described [23]. The allergenic extracts of *Penicillium chrysogenum* and *Aspergillus fumigatus* used in cross-reactivity studies were prepared as previously described [23, 24]. The protein content of the extracts was determined with a dye-binding method according to the manufacturer's instructions (Bio-Rad, Richmond, Calif., USA).

Gel Electrophoresis and Immunoblotting

Proteins in the crude fungal extracts were separated by SDS-PAGE or 2D gel electrophoresis as described [24, 25]. The proteins were then transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (0.45 μm ; Millipore, Bedford, Mass., USA). The membranes were blocked with 1% skimmed milk. Immunoassay of protein components that react with IgE or monoclonal antibodies (MoAbs) against fungal serine proteases (PCM8, PCM10, PCM16, PCM39, 55A and FUM20) was carried out as described [24, 25]. For controls, serum of a healthy control and MoAb WH9 against house dust mite allergen Der p 7 [26] were used.

N-Terminal Amino Acid Sequence Analysis

Protein components of crude *C. cladosporioides* extracts were separated by 2D gel electrophoresis then transferred electrophoretically onto PVDF membranes. The proteins were visualized by Coomassie blue or immunostaining. Protein spots that reacted with MoAb FUM20 and IgE antibodies analyzed by immunoblotting were excised from the membrane and inserted into an ABI Procise Model 491 sequencer (Applied Biosystems, Inc., Foster City, Calif., USA) for automated cycles of Edman degradation.

Purification of nCla c 9

Chromatographic separation media were purchased from Pharmacia (GE Health Care, Uppsala, Sweden). Lyophilized *C. cladosporioides* extracts were dissolved in buffer A (20 mM Tris-HCl, 0.5 M NaCl, 1 mM CaCl_2 , 1 mM MnSO_4 , pH 7.2) then loaded onto a Con A Sepharose column that had been pre-equilibrated in buffer A. The column was washed extensively with buffer A then eluted with a step gradient of 0.1, 0.2 and 0.5 M NaCl in buffer A. Fractions with positive immunoblot reactivity against MoAb FUM20 were pooled and dialyzed against buffer B (50 mM Tris-HCl, pH 7.0) before loading onto a CM Sepharose Fast Flow ion exchanger that had been equilibrated in buffer B. Cla c 9 eluted off the column in the unbound fraction was identified according to its positive immunoblot reactivity against MoAb FUM20. The concentration of the protein preparation (nCla c 9) was determined by a dye-binding assay of Bio-Rad.

Proteolytic Activity of nCla c 9

The proteolytic activity of the purified nCla c 9 allergen was measured spectrophotometrically with Z-Ala-Ala-Leu-pNA (benzyloxycarbonyl-L-alanyl-L-alanyl-L-leucine-*p*-nitroanilide; Peptide Institute, Inc., Minoh-Shi, Osaka, Japan; code 3127) as a substrate [27]. The stock solution contained 5 mg/ml of the substrate in dimethylformamide. The assay mixture consisted of 0.1 or 1.0 μg of the purified nCla c 9, 15 μl (238 $\mu\text{g}/\text{ml}$ or 0.45 μM) of the substrate and 20 mM Tris-HCl (pH 7.0) in a total volume of 315 μl . After incubation at 37°C for 30 min, the absorbance of the liberated *p*-nitroanilide at 410 nm was determined and represented the proteolytic activity of the enzyme. As a negative control,

Table 1. Primers used in PCRs

Name	Nucleotide sequence in 5'- to 3'-end orientation
VSP-F-1	AA(G/A)A(A/G)(C/T)GC(T/C)CC(T/C/A)TGGGG
VSP-R-2	CC(A/G)GC(A/G)AT(A/G)TG(A/T/G)GG(A/G)GA(A/C/G)GCCAT
AP-V	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTTT(A/G/C)
AAP	GGCCACGCGTCGACTAGTAC GGGIIGGGIIGGGIIG
VSP-GSP-F1	CCGGTACCTCGATGGCGTCTCC
VSP-GSP-R2	CGCCCTCGCCACCGTCCTCGGTGTAGAGGTAC
AUAP	GGCCACGCGTCGACTAGTAC
VSP-Clone-F1	CGGGATCCTCTGAGTCCGAGGTGAGAGAAGAACGC
VSP-Clone-R2	AACTGCAGTTAGTAGCCACCCTTCTCAACGATGGCG

the purified nCla c 9 was replaced with bovine serum albumin (BSA; Pierce, Rockford, Ill., USA) in the proteolytic assay.

cDNA Cloning

The cDNA encoding the *C. cladosporioides* vacuolar serine protease was isolated with a ProSTAR™ First-Strand RT-PCR kit (Stratagene, La Jolla, Calif., USA) as previously described [23, 28, 29]. Primers used in the cloning experiments are listed in table 1. Primers VSP-F-1 and VSP-R-2 were used in the first set of polymerase chain reaction (PCR). They encode conserved amino acid sequences (KNAPWG and MASP HVAG) near the N and C termini of fungal serine proteases. The PCR product (the first-strand cDNA) was purified electrophoretically on agarose gel then subcloned into the pGem-Teasy vector (Promega, Madison, Wisc., USA) and transformed into *Escherichia coli* Top10F' competent cells. The plasmid DNA was purified and the nucleotide sequence of the cDNA insert was determined with an automatic sequencer (Applied Biosystems).

The 5'- and 3'-end RACE (rapid amplification of cDNA ends) reactions were used in order to obtain the full-length cDNA of Cla c 9. The primers used were the anchor primers (AP-V and AAP), together with the gene-specific primers (VSP-GSP-F1 and VSP-GSP-R2) that were synthesized according to the internal sequences of Cla c 9 obtained in the previous PCR reaction. VSP-GSP-F1 (for 3'-end RACE) and VSP-GSP-R2 (for 5'-end RACE) cover nucleotides 746–767 and 111–142, respectively, of the truncated Cla c 9 cDNA. Primer AP-V used in the 3'-end RACE reaction contains the sequence of primer AUAP plus a stretch of oligo-dT. The primer AAP used in the 5'-RACE reaction contains the sequence of primer AUAP and a stretch of oligo-dG. The first-strand cDNA isolated was used as a template in the 3'-end RACE reaction. An oligo-dC was added to the end of the first-strand cDNA with terminal deoxynucleotidyl transferase (Promega) before used as a template in the 5'-RACE reaction. Products of the RACE reactions were purified individually, subcloned, transformed and sequenced as described above.

Preparation of Recombinant Protein rCla c 9

Fungal vacuolar serine proteases were hypothesized to be synthesized as a larger precursor that undergoes both N- and C-terminal cleavage upon maturation [23, 29–32]. The N terminus of the recombinant Cla c 9 (rCla c 9) allergen was constructed according to the experimentally determined sequence (SESEVE-

KNAPWGLARI) of the mature protein. The *C. cladosporioides* vacuolar serine protease was expressed as 6 × His-tagged protein according to the manufacturer's instructions (Qiagen Inc., Valencia, Calif., USA). The cDNA of Cla c 9 was amplified through PCR. The forward primer (VSP-Clone-F1; table 1) used in the reaction contains the *Bam*HI restriction site in addition to the cDNA sequence (nucleotides 25–50) encoding the experimentally determined N terminus of the mature Cla c 9. We hypothesize that the Cla c 9 precursor is cleaved at or near the Tyr (Y) residue encoded by nucleotides 985–987 of the isolated clone, analogous to the proposed C terminus of the vacuolar serine protease (pepC) of *Aspergillus niger* [32]. Thus, the reverse primer VSP-Clone-R2 (table 1) contains a *Pst*I restriction site, a newly added stop codon (TAA) and the sequence encoding the hypothetical C terminus (AIVEKGGY, nucleotides 963–987) of Cla c 9. The PCR product was restricted then ligated into the pQE-30 vector and transformed into *E. coli* M15 for protein expression. The recombinant protein of putative mature Cla c 9 (rCla c 9) was affinity purified with a Ni-NTA resin column (Qiagen) under denaturing condition according to instructions provided by the manufacturer. Immunoreactivity of rCla c 9 against IgE antibodies and MoAb FUM20 was analyzed by SDS-PAGE immunoblot.

Immunoblot Inhibition

For immunoblot inhibition studies, IgE-containing serum samples or hybridoma culture supernatants containing MoAb FUM20 were firstly incubated with purified nCla c 9 in solution or rCla c 9 that has been immobilized on PVDF membranes at room temperature for 1 h. The resulting serum samples or culture supernatants were then incubated with PVDF blots containing crude fungal extract proteins at 4°C for 16 h or at room temperature for 1 h. As control experiments, the blots were incubated with similar serum samples or hybridoma culture solutions that have been preincubated with equivalent amounts of BSA (Pierce). The blots were then washed and incubated with alkaline phosphatase-conjugated monoclonal anti-human IgE antibodies (Pharmin-gen, San Diego, Calif., USA) or with horseradish peroxidase-labeled goat anti-mouse IgG (H+L; Jackson ImmunoResearch Laboratories, West Grove, Pa., USA). They were developed with enzyme substrates as described previously [23–25].

Fig. 1. a Dye-stained protein profile of *C. cladosporioides* extract on a PVDF membrane. Immunoblot reactivity of MoAb FUM20 (**b**) and IgE antibodies in 23 serum samples from asthmatic patients (**c**) against components of *C. cladosporioides*.

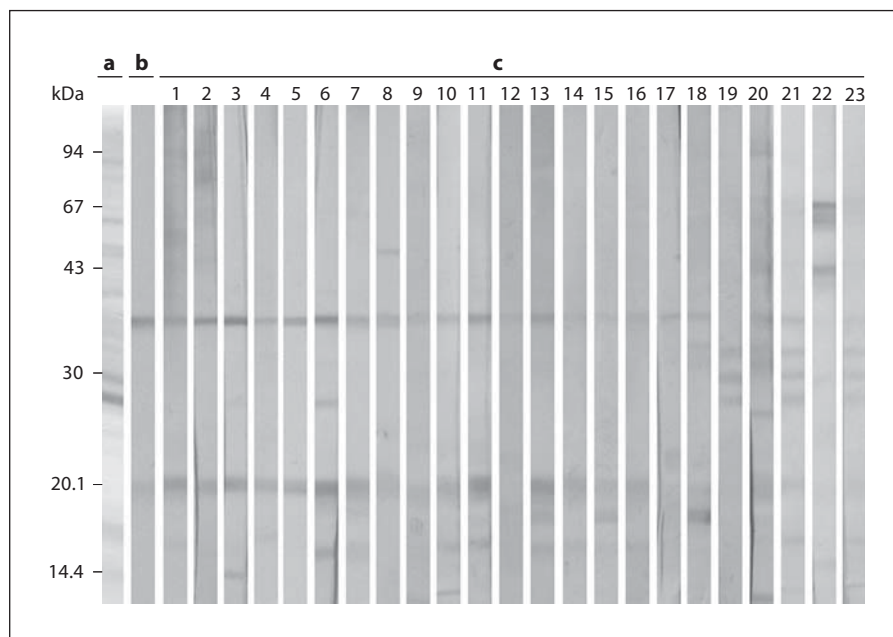
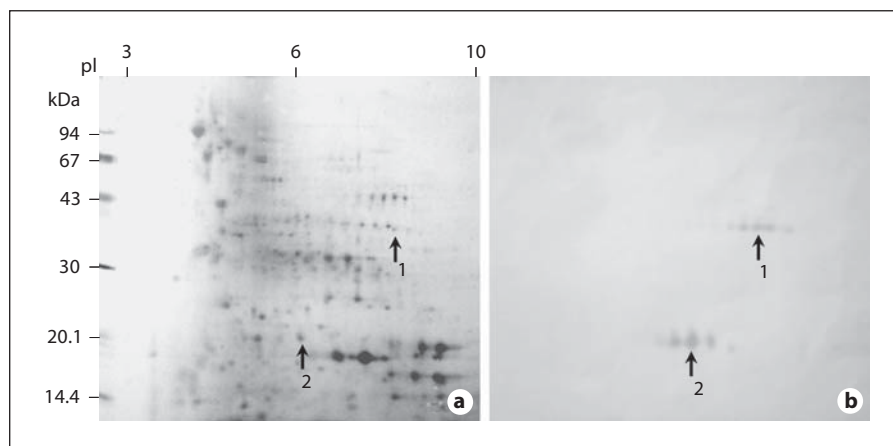


Fig. 2. 2D immunoblot analysis. *C. cladosporioides* crude extracts were separated by 2D SDS-PAGE and transferred onto PVDF membranes. **a** Dye-stained protein profile. **b** A similar blot was reacted against IgE antibodies of mixed serum samples from 5 asthmatic patients (cf. serum No. 1–5 in fig. 1).



Results

Identification of a Major Allergen from *C. cladosporioides*

In this study, 74 (38%) of the 197 sera tested showed IgE immunoblot reactivity against components in the crude *C. cladosporioides* extracts (data not shown). Among these 74 positive sera, 41 (55%) demonstrated IgE binding to a 36-kDa component. Representative results from 23 of these 41 sera are shown in figure 1. A major 36-kDa IgE-reacting band was observed for most of these 23 serum samples. In addition, IgE-binding against a 20 kDa *C. cladosporioides* component was also observed for 38 (51%) of these positive serum samples (data not shown).

In addition, IgE antibodies in sera 6, 8 and 18–23 reacted with numerous more components from *C. cladosporioides* (fig. 1). By immunoblotting, both the 36- and 20-kDa IgE-reacting components also reacted with MoAb FUM20 (fig. 1b). These 2 IgE-reacting components did not react with the other 5 MoAbs tested (PCM8, PCM10, PCM16, PCM39 and 55A; data not shown). Therefore, these 2 components might be structurally closely related. For controls, serum obtained from a healthy laboratory control and MoAb WH9 against mite allergen Der p 7 [26] showed negative reactivity against *C. cladosporioides* (data not shown).

The IgE-reacting components from *C. cladosporioides* were further characterized with 2D immunoblotting.

Sera 1–5 in figure 1 were pooled for the experiment, and the result is shown in figure 2b. A similar profile was obtained with MoAb FUM20 as probe (data not shown). Spot 2 on the 2D gel (fig. 2a) has an N-terminal amino acid sequence of SESEVEKNAPWGLARI and homology to those of the vacuolar serine proteases from different fungal species [23, 24, 28–30, 32]. Sequencing of the 36-kDa component was unsuccessful due to the lack of sufficient samples. Since both 36- and 20-kDa components react with FUM20, we tentatively conclude that they are of the same origin. Probably, the 20-kDa IgE-reacting component is a degraded product of the 36-kDa major serine protease allergen.

Proteolytic Activity of the Purified nCla c 9 Allergen

In the enzymatic assay, the absorbance at 410 nm reflects the proteolytic cleavage of pNA from the peptide substrate by nCla c 9. The mean values of A_{410} for 0.1 and 1.0 μ g of purified nCla c 9 were 0.17 ± 0.04 and 0.29 ± 0.02 , respectively. An A_{410} of 0.14 ± 0.04 was obtained when BSA was used as substrate. The results indicate that the purified nCla c 9 is proteolytically active with the peptide Z-Ala-Ala-Leu-pNA as substrate.

cDNA Cloning

The isolated cDNA clone has 1,425 bp (GenBank accession No. EF407520). It is truncated at the 5'-end, and the typical polyadenylation signal (AATAAA) was not found from the upstream of the poly A tail. It is shown in figure 3 together with the deduced amino acid sequence of the putative open reading frame (from bases 1 to 1,164). The cDNA encodes a polypeptide of at least 388 residues. Residues 9–24 of the deduced amino acid sequence are identical to that of the N terminus of the IgE-reacting spot 2 in the 2D gel (fig. 2). Thus, the cDNA isolated corresponds at least to the 20-kDa IgE-binding components of *C. cladosporioides*.

Sequence alignment suggests that this cDNA from *C. cladosporioides* encodes a vacuolar serine protease. Assuming the mature protein starts from Ser9, then a protein of 39,913 Da can be translated from this cDNA. Therefore, the 36-kDa spot we observed on the 2D gel (fig. 2) can be derived from this cDNA. There are 3 cysteines (Cys90, Cys193, Cys224), 4 putative N-glycosylation sites (^{116} NGS ^{118} , ^{156} NMS ^{158} , ^{194} NYS ^{196} and ^{319} NYS ^{321}) and 3 active-site residues for serine proteases (Asp54, His86, Ser252) on the N-terminally processed mature protein (fig. 3). The tripeptide recognition sequences for N-glycosylation are Asn-X-Ser or Asn-X-Thr, where X is any amino acid except proline. The encoded protein has

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GATCAGGAGGTCCACGTCCTCAAGTCTGAGTCCGAGGTCCGAGGAAGCCCTCCCTGGGGTCTGGCCGATATCTCA 75
D Q E V H V L K I S E S E V E K N A P W G L A R I S 25
CACCGTGACAGCGTGTGCTTGGCACTTCAACAGTACCTCTACACCGAGGACGGTGGCGAGGGGTTGATGTC 150
H R D S L S F G T F N K Y L Y T E D G G E G V D V 50
TATGTCTGTCGACCCGCCACCAAGTGTACCACTGTGAGTCTCGAGGTGCGCGCTTGGGCCAAGACCATCCCC 225
Y V V D T G T N V D H V D F E G R A S W G K T I P 75
CAGGGTGTATGACAGATGAGGATGGCAACGGACACGGAACACACTGCTTGGCACTGTGGCCGCAAGAAAGTACGGT 300
Q G D A D E D G N G H G T H C S G T V A G K K Y G 100
GTAGCCAAGAAGGCTCAAGTCAAGGCGCTCAAGTCTTGGCTTCAACGGCTCTGGCTCCATGTCCGAGCTTGT 375
V A K K A H V K A V K V L R S N G S G S M S D V V 125
AAGGGTGTGAGTACGCGCTGAGTCTCACTCGAGCAGGTGAGTCCCAAGAGAGGCAAGAGAAAGGGCTTC 450
K G V E Y A A E S H L E Q V S I A K K G K R K G F 150
AAGGGCTCTACTGCCAATGAGCTTGGCGGTGGCAATCTCCATCTCGACAAAGGCTGTCAACGCTGCTGCTC 525
K G S T A N M S L G G G K S P I L D K A V N A A V 175
GACCTGGCATCCACTTGGCTGTGGCGCGGTAAAGCAACGCTGACTCTTCTGCACTACTCTCTGCGCGCGCT 600
D A G I H F A V A A G N D N A D S C N Y S P A A A 200
GAGAAGCGCGTCCCGTGTGTGTAGCACTCTTCTGAGACGCGCTACTTCTCAACTAGCGGCAAGTCAAC 675
E N A V T G A S T L S D E R A G Y F N Y G K N 225
GACATCTTGTCTCCGGTCTTAACATCCAGTCCAGCTGGATGGCAGCAAGTACGCGCTCAACACCATCTCCGGT 750
D I F A P G L N I O S T W I G S K Y A V N T I S G 250
ACCTCGATGGCTCTCCCGACGTTGGCGGTCTCTTGTCTTCTTCTCCAGCCGCAAGGACTCTGCC 825
T S M A S P H V A G L L A Y L L S L Q P A K D S A 275
TTGCTGTTCGGATATTAGCCCAAGAGCTCAAGGCTAACTCATCTTATCGCCATCTTGGCGCTCTTACT 900
F A V A D I S P K K L K A N L I S I A T V G A L T 300
GATGTCCCAAGCAACGCGCAATCTCGCTGGAGCGTGGCGGAGGTCCAACACAGCGCCATGCTGAG 975
D V P S N T A N I L A W N G G G E S N Y S A I V E 325
AAGGCTGGCTACAGGCGCGTGTGGCGCCACCATCTCGAGGAGATGAGTCTGAGGCCAAGGTGCCCAAG 1050
K G G Y K A A V R P T M L E E I E S E A K V A S 350
AAGGTCTACTCGAGGTTGACGAGCTTGCCAAGAAGGTGGCGAGCTCACTGAGAAGATTGAGGATCTCATCTCC 1125
K V Y S E G D E L A K K V A E L T E K I E D L I S 375
GGTGACCTCAAGGACATGTTCGGTGAAGCTCAAGCGGAGTAAAGAGGTGGCTTGTGTCTACTTGACAAAGCAT 1200
G D L K D M F R D L K R E * 388
CTCACTGGGAAGCGGAGCGGACAGGTTGGCAATGTGCATAACAGAGCATGTTGGTTCCTCGATGAGACT 1275
GGATGTTCAGATGCGCTTTAGCATCTGTTGGATGGGAATCAITTCGAGGCGTGGTGGCTGACATTTGGCT 1350
TCTTGACAGACCCCTGGGCCACATATATACATGTACGAGAGTTTGTATCAGAGAAAAA 1425
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Fig. 3. The nucleotide and deduced amino acid sequences of Cla c 9 (GenBank accession No. EF407520). Numbers to the right indicate the positions of the nucleotides and the deduced amino acid residues of the sequences. The amino acid triad (D54, H86 and S252) that is characteristic of serine proteases is depicted in bold letters and shaded. Potential N-glycosylation sites are in bold letters and underlined. Amino acid residues (9–24) that are identical to those determined experimentally for the N terminus of the 20-kDa major IgE-binding component in the *C. cladosporioides* extracts are underlined. The vertical arrow marks the amino terminus of the mature vacuolar serine protease. The stop codon TAA is denoted with an asterisk. Nucleotides in gray correspond to those synthesized and used as primers for PCR in the cDNA cloning of Cla c 9 as shown in table 1. The sequences corresponding to primers VSP-Clone-F1 and VSP-Clone-R2 used in the preparation of rCla c 9 are boxed.

68–72% sequence identity with that of vacuolar serine proteases from *Penicillium* and *Aspergillus* species (Pen ch 18; accession No. AF263454; Pen o 18, accession No. AF243425; Pen c 18, accession No. AF245168, and Asp f 18, accession No. Y13338), plus 56 and 57% sequence identity with those from *Rhodotorula mucilaginosa* (Rho m 2, accession No. AY547285) [28] and *Saccharomyces cerevisiae* (protease B, accession No. M18097) [30], respectively. It was designated as Cla c 9 according to the Allergen Nomenclature Committee. All these mature fungal serine proteases contain 3 cysteine residues. The most likely candidate for the free cysteine of the *Saccharomyces* protease B is Cys 81, located near the active-site His77 [30]. The remaining 2 may be responsible for a di-

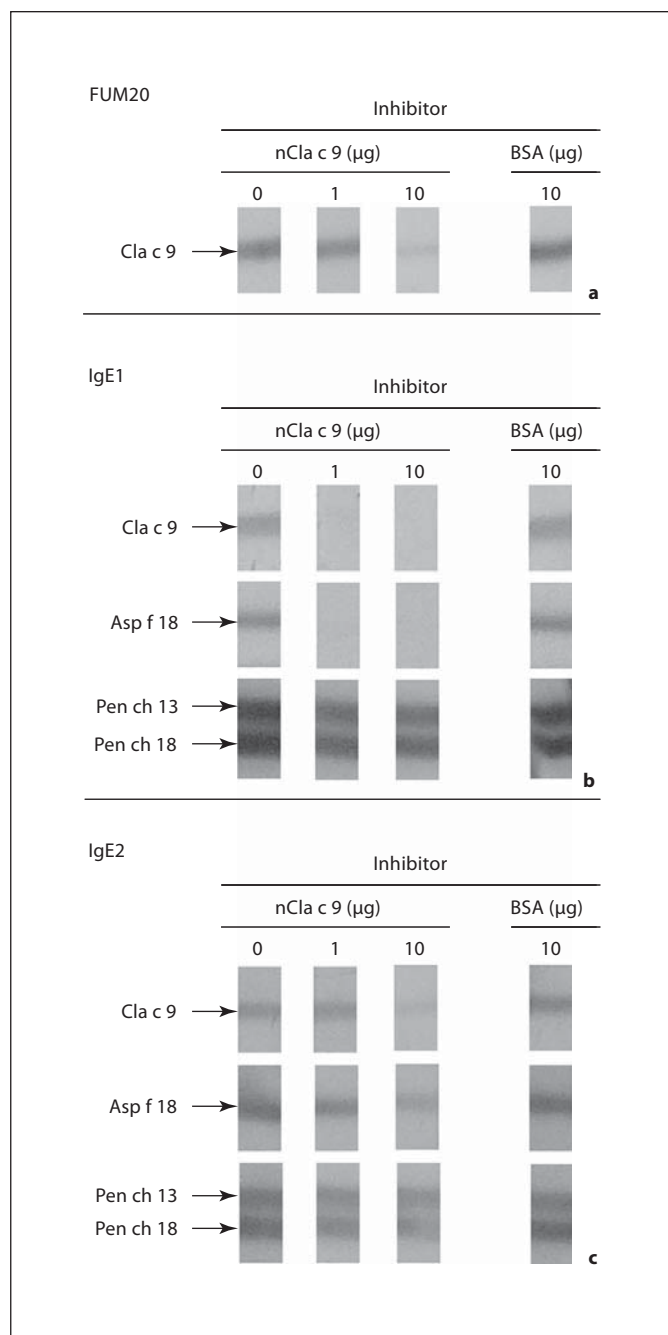


Fig. 4. Inhibition of MoAb FUM20 and IgE immunoblot reactivity against Cla c 9, Asp f 18 and Pen ch 18 in crude fungal extracts with purified nCla c 9 and BSA as inhibitors. **a** FUM20 bindings against Cla c 9 with and without inhibitors. **b, c** IgE binding of 2 different sera against Cla c 9, Asp f 18 and Pen ch 18 with and without inhibitors, using serum No. 16 in fig. 1 (**b**) and serum No. 14 in fig. 1 (**c**).

sulfide bond. The Cys90 on Cla c 9 locates in the proximity of the active-site histidine (His86). Analogous to protease B, the other 2 Cys (Cys193, Cys224) may form a disulfide bond.

Antigenic Cross-Reactivity

The nCla c 9 purified from the crude extracts of *C. cladosporioides* can react with MoAb FUM20 and IgE antibodies in serum samples from asthmatic patients (data not shown). Our results presented in figure 4a indicate that FUM20 binding against the 36-kDa component in the crude extracts of *C. cladosporioides* was inhibited dose-dependently by preabsorbing the FUM20-containing culture supernatant with 1 and 10 μg of purified nCla c 9. Similarly, 2 patient sera (IgE1 and IgE2; fig. 4b, c, top rows) lost the IgE-binding activity against the same 36-kDa component after the sera were preabsorbed with nCla c 9. Inhibition of FUM20 or IgE binding was not detected when the same culture supernatant or sera were preabsorbed with 10 μg of BSA.

The presence of IgE cross-reactivity between nCla c 9 and the vacuolar serine protease allergens from the crude extracts of *A. fumigatus* (Asp f 18) and *P. chrysogenum* (Pen ch 18) was also analyzed. By immunoblotting, MoAb FUM20 can recognize and bind Asp f 18 and Pen ch 18 in the crude *A. fumigatus* and *P. chrysogenum* extracts, respectively (data not shown) [25]. Preabsorption of serum IgE1 (fig. 4b) with 1 and 10 μg of nCla c 9 inhibited its IgE immunoblot reactivity against Asp f 18. Approximately 40 and 44% of inhibitions of IgE binding against Pen ch 18 were detected when the same serum was preabsorbed with 1 and 10 μg of nCla c 9, respectively (fig. 4b). The degree of inhibition was determined by measuring the immunoblot with the AlphaEaseFC™ software (version 4.0.0; Alpha Innotech Corporation, San Leandro, Calif., USA). Inhibition of IgE binding was not detected when the same serum was preabsorbed with 10 μg of BSA (fig. 4b).

Similarly, results also showed inhibition of IgE binding against Asp f 18 and Pen ch 18 when the serum was preabsorbed with nCla c 9 (fig. 4c). Inhibitions of IgE binding against Pen ch 18 of about 49 and 61% were detected when the serum was preabsorbed with 1 and 10 μg of nCla c 9. Inhibitions of IgE binding against Asp f 18 of about 43 and 75% were detected when the same serum was preabsorbed with 1 and 10 μg of nCla c 9, respectively. Preabsorption of the same serum with 10 μg of BSA did not inhibit its IgE binding against Asp f 18 and Pen ch 18 (fig. 4c).

Results in figure 4b and c also showed that IgE antibodies in both serum samples reacted with the 34-kDa alkaline serine protease (Pen ch 13) in the crude extracts of *P. chrysogenum*. The identification of Pen ch 13 in the crude *P. chrysogenum* extracts was confirmed by its immunoblot reactivity with MoAb PCM39 (data not shown) [25]. Inhibitions of IgE binding against Pen ch 13 of about 53 and 49% (fig. 4b) and of about 60 and 78% (fig. 4c) were detected when sera No. 1 and 2 in figure 4 were pre-absorbed with 1 and 10 μ g of nCla c 9, respectively (fig. 4b).

Recombinant Cla c 9

The purified rCla c 9 reacted with FUM20 and IgE antibodies in 16 sera of asthmatic patients tested (fig. 1, serum No. 1, 2, 4 and 6–18; data not shown). The rCla c 9 demonstrated dose-dependent inhibition of IgE-binding against nCla c 9 in crude *C. cladosporioides* extracts when serum No. 14 of figure 1 was preabsorbed with 2 and 10 μ g of rCla c 9 (fig. 5). Preabsorption of the same serum with 2 and 10 μ g of BSA did not inhibit its IgE binding against nCla c 9 (fig. 5). The rCla c 9 was also renatured according to recommendations provided by the manufacturer of the QIAexpress kit (Qiagen). However, the enzymatic activity of the renatured rCla c 9 was not detectable in parallel with the determination of the enzymatic activity of the isolated natural Cla c 9 (data not shown).

Discussion

Approximately 25–30% of atopic asthma cases are associated with airborne fungi [33]. In this study, IgE reactivity against *C. cladosporioides* was detectable in 38% of sera obtained from 197 adult asthmatic patients. The result is close to that obtained with *Penicillium* and *Aspergillus* species which sensitize about 24–34% of the atopic asthma patients [7, 24, 34]. Thus, similar to *Penicillium* and *Aspergillus* species, *C. cladosporioides* may also play an important role in human respiratory atopic disorders.

In the present study, a 36- and a 20-kDa major IgE-binding component of *C. cladosporioides* was identified. Among 6 MoAbs against fungal serine proteases tested, both IgE-reacting *C. cladosporioides* components reacted only with MoAb FUM20. FUM20 has been shown to react with vacuolar serine protease major allergens from *Penicillium citrinum* (Pen c 18), *P. chrysogenum* (Pen ch 18), *Penicillium oxalicum* (Pen o 18), *A. fumigatus* (Asp f 18) and alkaline serine proteases from *A. fumigatus* (Asp

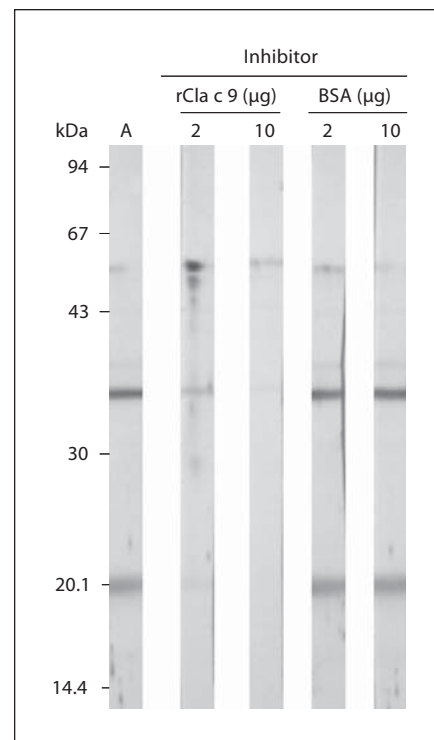


Fig. 5. Inhibition of IgE immunoblot reactivity against Cla c 9 in crude *C. cladosporioides* extracts with purified rCla c 9 and BSA as inhibitors. A = IgE binding against Cla c 9 using serum No. 14 of figure 1.

f 13) and *Aspergillus flavus* (Asp fl 13) [25]. It recognizes an epitope located at the N terminus of serine protease pan-fungal allergens [35]. Hence, the 36- and the 20-kDa IgE-binding components of *C. cladosporioides* probably share the same N-terminal amino acid sequence.

We determined the N-terminal sequence of the 20-kDa component, and a truncated cDNA encoding this sequence was isolated subsequently. Amino acid residues 9–24 deduced from the truncated cDNA were found to be identical to those of the N-terminal 16 amino acids of the 20-kDa major allergen of *C. cladosporioides*. Since this cDNA can translate into a 40-kDa vacuolar serine protease, it probably encodes the 36-kDa allergen (Cla c 9) we observed, and the 20-kDa component is the breakdown or C-terminal processed product of the 36-kDa Cla c 9.

To ensure that the 20- and 36-kDa allergens are derived from the same protein, we excised the 36-kDa IgE-reacting component from a 2D gel for peptide mapping analysis. The gel spot was digested with trypsin and the resulting peptides were extracted for mass analysis on a time-of-flight instrument with matrix-assisted laser de-

sorption ionization. The mass spectrum (data not shown) has 4 peaks with observed m/z of 884.37, 949.34, 1,115.58 and 1,831.15. These ions are possibly derived from Cla c 9 tryptic fragments. The inferred sequences of these fragments are: $^8\text{NAPWGLAR}^{15}$ (calculated m/z 884.47), $^{20}\text{DSLSFGTFNK}^{29}$ (calculated m/z 1,115.58), $^{119}\text{GVEY-AAESHLEQVSIK}^{135}$ (calculated m/z 1,830.92), and $^{208}\text{AYFSNYGK}^{215}$ (calculated m/z 949.43). The $^8\text{NAPWGLAR}^{15}$ fragment is part of the N terminus of the 20-kDa allergen as determined by Edman degradation. Hence, our results strongly suggest that the 36- and 20-kDa allergens share the same N-terminal sequence and that the 20-kDa component is a breakdown product of the 36-kDa Cla c 9.

The fungal alkaline/vacuolar serine proteases are translated as a precursor with an N-terminal preproenzyme sequence [23, 29–32, 36]. The N-terminal prosequence of these proteases seems to be involved in protein folding and processing of the precursor into an active conformation [31]. Besides N-terminal cleavage, the mature vacuolar serine proteases may also undergo C-terminal processing [30–32]. The C-terminal prosequence is required for translocating the proteases across the cytoplasmic membrane [37]. After removing the N-terminal prosequence, the remaining open reading frame for Cla c 9 is 380 amino acids in length, and the polypeptide has a molecular mass of 39,913 Da which is much larger than the 36 kDa observed for the mature Cla c 9, as determined on SDS-PAGE and 2D gels. The protein can be cleaved at or near Tyr329 (fig. 3). The resulting polypeptide has a molecular mass of approximately 33,248 kDa and a C terminus homologous to that proposed for pepC [32]. Including possible glycosylations (nCla c 9 binds to a Con A Sepharose column), the protein with both N- and C-terminal processing can have a molecular mass close to that observed for the IgE- and MoAb-binding 36-kDa component in the crude extracts of *C. cladosporioides*. In addition, rCla c 9 dose-dependently inhibited IgE binding against the 36-kDa nCla c 9 in crude *C. cladosporioides* extracts. The observation supports the notion that the 36-kDa major allergen is a vacuolar serine protease of *C. cladosporioides* and much of the antigenicity of nCla c 9 is retained by rCla c 9.

FUM20 and IgE binding against the 36-kDa component in crude *C. cladosporioides* extracts were inhibited dose-dependently by the purified nCla c 9. Furthermore, nCla c 9 has a proteolytic activity against a subtilisin chromogenic substrate [27]. It confirms that the 36-kDa major allergen (Cla c 9) is a vacuolar serine protease of *C. cladosporioides*. The N-terminal lysine, proline and tryptophan residues on fungal serine proteases are critical

core amino acid residues recognized by FUM20 [35]. Our results obtained from this study confirm the previous finding by showing the corresponding K7, P10 or W11 at the N-terminus of the mature Cla c 9 (fig. 3). In addition, FUM20 reacts with the 20-kDa N-terminal fragment of Cla c 9.

In this study, IgE binding against Asp f 18 and Pen ch 18 was also inhibited dose-dependently by nCla c 9. It indicates the presence of IgE cross-reactivity between Cla c 9 and Asp f 18 and between Cla c 9 and Pen ch 18. We have also previously identified a vacuolar serine protease as a major allergen (Rho m 2) of *R. mucilaginosa*, the most frequently encountered species of yeasts in the environment [28]. In this study, purified nRho m 2 [28] also dose-dependently inhibited IgE binding against Cla c 9 and Asp f 18 in crude fungal extracts (data not shown). The presence of IgE cross-reactivity among the fungal vacuolar serine protease allergens including Pen ch 18, Pen c 18, Pen o 18, Asp f 18, Asp fl 18 and Rho m 2 has been reported [23, 24, 28, 29, 34]. Thus, results obtained before and from the present study suggest the presence of IgE cross-reactivity among major vacuolar serine protease pan-fungal allergens from at least 7 different prevalent fungal species.

In addition to vacuolar serine proteases, the group 13 alkaline serine proteases are also major allergens of prevalent *Penicillium* and *Aspergillus* species [7, 24, 33, 34]. In this study, inhibition of IgE-binding against Pen ch 13 by nCal c 9 was also detected. It indicates the presence of IgE cross-reactivity between Cla c 9 and Pen ch 13. IgE cross-reactivity between the group 18 (Pen ch 18) and the group 13 (Pen ch 13) major allergens from *P. chrysogenum* has been reported [23]. All these results obtained suggest that there may be allergenic cross-reactivity among the alkaline/vacuolar serine protease major allergens from prevalent airborne fungi including *Penicillium*, *Aspergillus*, *Rhodotorula* and *Cladosporium* species. Recently, a serine protease (Cur l 1) was also identified as a major allergen of the prevalent fungus *Curvularia lunata* [38]. IgE reactivity against Cur l 1 was detectable in sera from 80% of *C. lunata* hypersensitive patients [38]. The presence of IgE cross-reactivity between Cur l 1 and serine protease major allergens from various fungal species mentioned above needs further study.

In conclusion, the alkaline and/or vacuolar serine proteases have been identified previously as major allergens of prevalent *Penicillium*, *Aspergillus* and *Rhodotorula* species [7, 24, 28, 33, 34]. In this study, a vacuolar serine protease was also identified as a major allergen of

C. cladosporioides (Clas 9). In addition, IgE cross-reactivity among these highly conserved serine protease pan-fungal allergens was also detected. Although the pathophysiological significance of serine proteases as major fungal allergens is the subject of current research [39], our results have shown that the alkaline serine protease of *P. chrysogenum* (Pen ch 13) may contribute to asthma by degrading the airway epithelial barrier and stimulating the release of mediators from human bronchial epithelial cells that coordinate local immune responses and

the inflammatory process [40]. All of these results obtained provide valuable information in clinical fungal allergy.

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