Identification of Cecropin A Proteolytic Cleavage Sites Resulting from *Aspergillus flavus* Extracellular Protease(s)

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Cecropin A (CA) has wide-spectrum antifungal properties except for the nongerminated conidial form of *Aspergillus* spp. A possible reason for this difference in activity has been explored. This study demonstrated that CA was being degraded enzymatically by proteases present in the culture supernatant. As a first step in the characterization of the type of protease responsible for the degradation of CA and for the prevention of degradation of gene-encoded antimicrobial peptides based on CA, the amino acids surrounding the cleavage sites were determined. On the basis of the molecular weights of the fragments, the cleavage sites were determined to be near the N terminus of the peptide at the Lys^3−Leu^4, Phe^5−Lys^6, and Lys^7−Ile^8 bonds.

Keywords: *Cecropin; Aspergillus flavus; protease; antifungal; reversed-phase high-performance liquid chromatography; electrospray mass spectrometry*

INTRODUCTION

The cecropins are a class of antibacterial peptides produced by the humoral immune response of certain insects (Boman and Hultmark, 1981, 1987). They have a broad range of susceptible pathogens including both Gram-negative and Gram-positive bacteria. They are being used as the basis of synthetic antimicrobial peptides. These peptides function by forming ion channels in the lipid membranes of bacteria, causing the cells to lyse (Christensen et al., 1988), but they do not lyse eukaryotic cells such as Chang liver cells or sheep erythrocytes (Steiner et al., 1981). Because the synthetic all-D form of cecropin was also shown to be antibacterial, this mechanism does not involve stereoselective recognition by any type of target receptor (Wade et al., 1990).

Cecropin A (CA) was one of the first cecropins to be characterized. It contains 37 amino acid residues (Figure 1), with the N-terminal region having many basic, hydrophilic residues and the C-terminal region containing many hydrophobic residues. The C-terminal amino acid is also modified with an amide function. Biological activity is associated with the helix−hinge−helix conformation of the peptide (Christensen et al., 1988).

We have recently described the antifungal properties of CA against *Aspergillus* and *Fusarium* spp. (De Lucca et al., 1997). It was found from experiments testing the lethality of CA to various nongerminated and germinating fungal conidia that the nongerminated form of *Aspergillus* spp. was not susceptible to CA. This was in contrast to the susceptibility of the germinating form of *Aspergillus* spp. or either conidial form of *Fusarium* spp. To determine a possible reason for this exception, the fate of CA incubated with conidia of *A. flavus* was explored. We chose *A. flavus* because of its prominent role in the production of potent mycotoxins in agricultural products (Ciegler, 1975; Palmgren and Ciegler, 1983). The conidia of *A. flavus* could either contain an inhibitor to CA pore formation, lack the availability of a required membrane component that is produced only after germination, or contain a protease that degrades CA before antifungal activity can be elicited. Because the objective of our studies is to produce a crop species containing a gene-encoded antifungal peptide related to cecropin, the knowledge of how CA is degraded would help in its development. This paper describes the role of *A. flavus* extracellular protease in the degradation of CA. The sites within CA that are attacked by the protease are identified by high-performance liquid chromatography (HPLC) and electrospray mass spectrometry (ESMS).

1 10 20 30 37
KWKLFKKIEK VGQNIRDGII KAGPAVAVVG QATQIAK

Figure 1. Amino acid sequence of CA. The one-letter abbreviations for the amino acids have been used.

EXPERIMENTAL PROCEDURES

Materials. Potato dextrose agar (PDA) and potato dextrose broth (PDB) were obtained from Difco (Detroit, MI). CA (100 mg, 79% peptide content, 99% peptide purity) was purchased from Sigma Chemical Co. (St. Louis, MO).

Degradation of CA. A culture of *A. flavus* was grown on PDA slants for 1 week (30 °C). One percent PDB (4 mL) was added to the slant culture. Conidia were suspended by gentle agitation using a sterile pipet tip. The conidial suspension was quantitated with a hemocytometer. Visual observation confirmed the absence of hyphae. A 10^6 conidia/mL suspension was prepared in 1% PDB. The conidial sample was then divided into two aliquots. For one sample, conidia were removed by centrifugation (1700*g*, 10 min, 4 °C), whereas conidia remained in the second sample. Aliquots (250 μL) of the conidia-free supernatant and conidial suspension were added separately to vials containing freeze-dried peptide (100 μg). The contents were gently mixed by hand and incubated (30 °C, 30 min). A peptide control was prepared by adding 250 μL of 1% PDB to CA (100 μg) and incubated as before. After the incubation period, 25 μL of 10% trifluoroacetic acid (TFA) was added to each sample.

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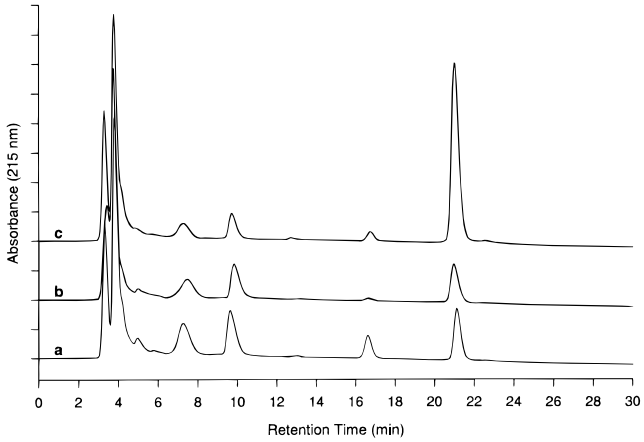

Figure 2. RP-HPLC analysis of CA incubated with (a) nongerminating conidia of *A. flavus*, (b) the supernatant of nongerminating conidia of *A. flavus*, or (c) germinating conidia of *A. flavus*: (a) conidia were incubated for 30 min at 30 °C in a solution of CA; (b) prior to incubation with CA, conidia were removed from the supernatant by centrifugation; **(**c**)** prior to incubation with CA, conidia were incubated at 30 °C for 8 h. An HPLC gradient elution of 26−41% acetonitrile in 0.1% TFA over 15 min was used at a flow rate of 0.2 mL/min on a C18, 5 μm, 2.1 × 250 mm column. Standard CA has a retention time of 21 min.

HPLC Analyses. Ten milliliter centrifuged samples were injected onto a reversed-phase (RP)-HPLC column (Vydac C18, 5 μm, 2.1 × 250 mm), which was equilibrated with 26% acetonitrile in 0.1% TFA (aqueous) at a flow rate of 0.2 mL/min. A gradient of 26−41% acetonitrile over 15 min was used to separate the peptides. Peaks were monitored at 215 ± 2 and 270 ± 10 nm using a diode array detector. Fractions corresponding to peaks were collected manually.

ESMS Analyses. ESMS was performed using a Vestec 201 ESMS. Mass spectral scans from *m*/*z* 100 to 2000 were employed for full-scan studies. HPLC peaks were analyzed by both on-line liquid chromatography/mass spectrometry (LC/MS) and direct infusion of collected HPLC peaks. For on-line LC/MS, HPLC column eluant was split to reduce the flow rate into the MS to ~5 μL/min. For isolated HPLC samples, the peak fraction was directly injected into the MS at a flow rate of 1 μL/min. Deconvolution of multiply charged peaks was performed manually. The peptide sequence possibilities of the ESMS peaks were determined by a self-written Basic computer program, based on the fragment mass and parent sequence.

RESULTS

HPLC conditions were developed that gave a retention time of 21 min for the elution of CA. The culture medium (1% PDB) peaks eluted with the solvent front and did not interfere with the study. A sample of CA incubated with conidia of *A. flavus* showed a reduced amount of the CA peak and the addition of three new peaks at 7, 10, and 17 min (Figure 2a). When conidia of *A. flavus* were pregerminated for 8 h and the resulting germlings were incubated with CA for 30 min at 30 °C, the same three degradation peaks were observed by HPLC, although at a much smaller concentration, accompanied by a smaller decrease in the parent peak (Figure 2c). When conidia of nongerminated *A. flavus* were washed prior to incubation with CA, no proteolytic HPLC peaks were observed. The supernatant obtained from the *A. flavus* conidia wash was also incubated with CA and found to produce the three proteolytic breakdown HPLC peaks seen previously, although the 17 min peak was very diminished from that seen with the direct conidial incubation (Figure 2b). A time course study (Figure 3) of the proteolytic breakdown of CA by conidial supernatant showed CA to be depleted in 1 h. After 2.5 h, a new peak with a retention time of 5 min was observed, and after 5 h, the 5 min peak and most of the 10 min peak were lost.

The three unknown peaks and CA, isolated by HPLC, were identified by ESMS. The 7 min peak gave a mass of 3301 (Figure 4a), corresponding to the Lys^{6}−Lys^{37} fragment of CA. This fragment results from the cleavage

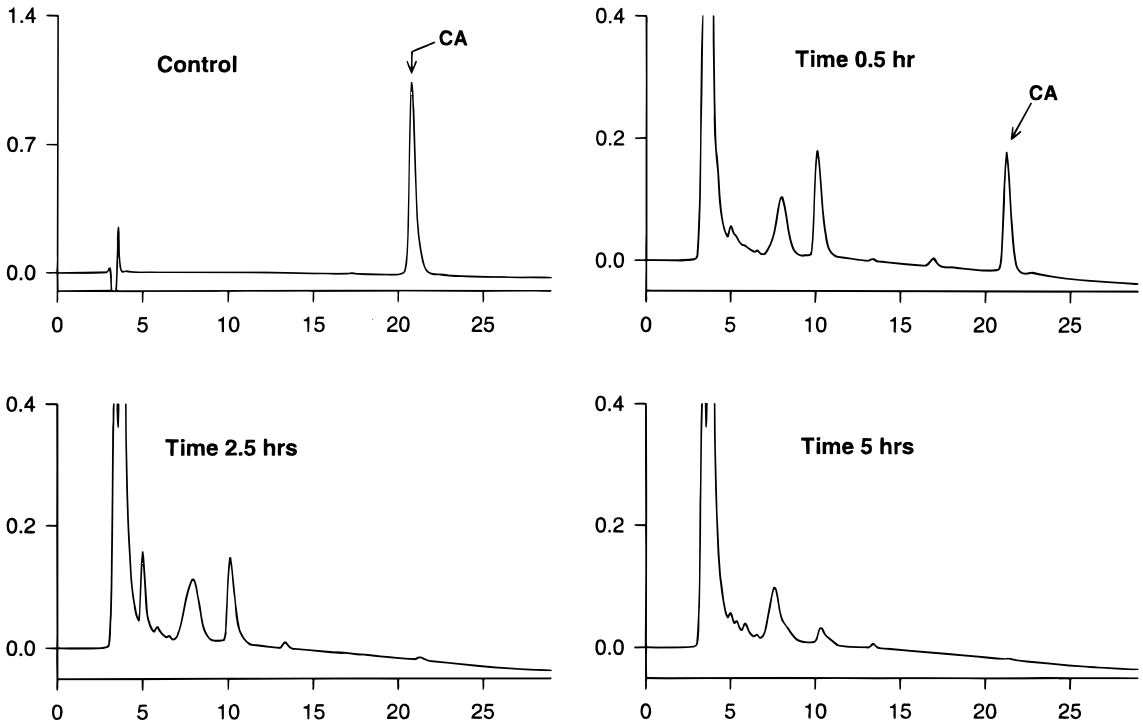

Figure 3. RP-HPLC time course analysis of CA incubated with the supernatant of nongerminating conidia of *A. flavus*. See Figure 2 for details.

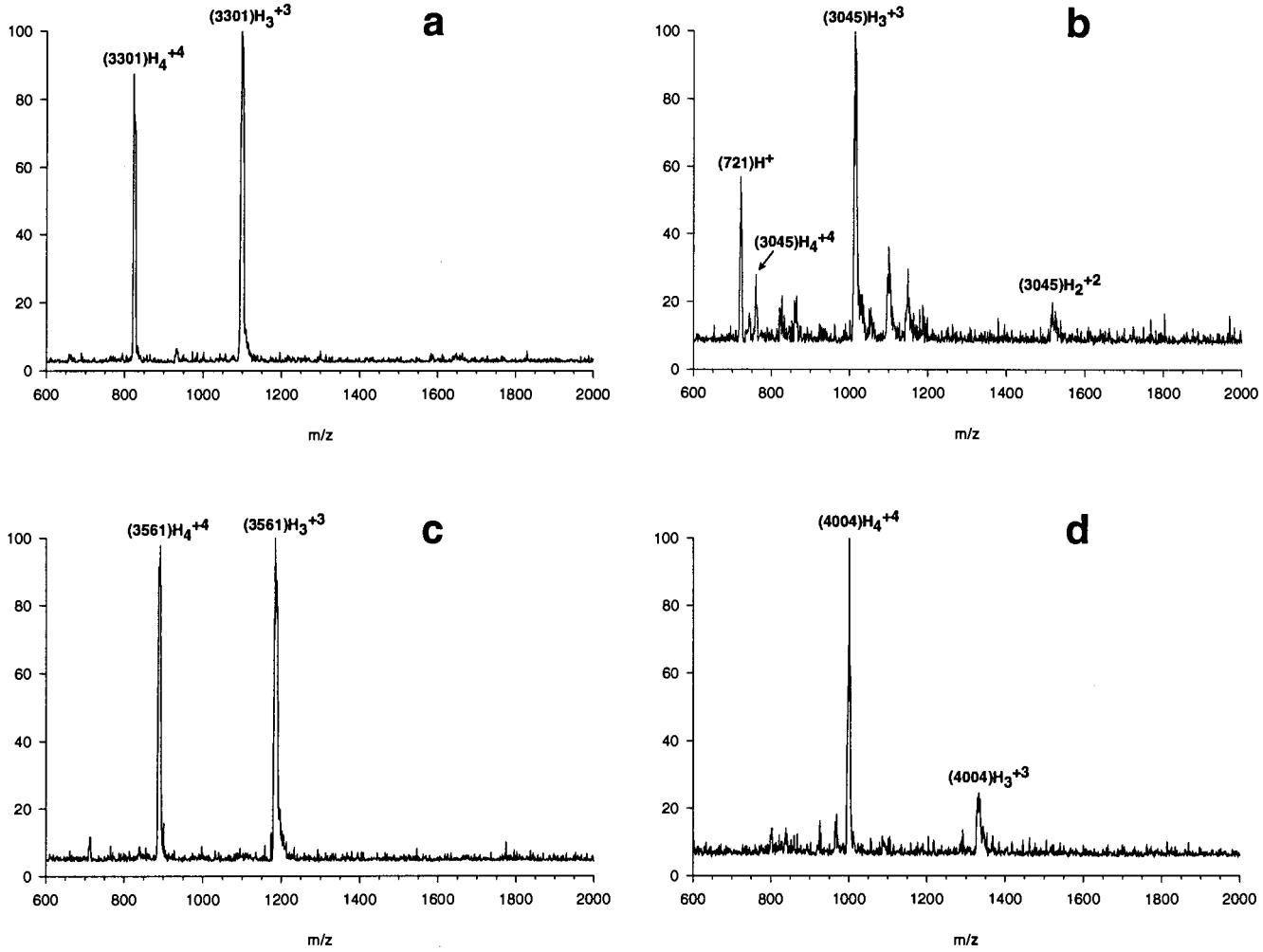

Figure 4. ESMS analysis of HPLC peaks (a) 7 min, (b) 10 min, (c) 17 min, and (d) 21 min. The HPLC sample was obtained from incubation of CA with nongerminating conidia of *A. flavus*. The parent mass is obtained by deconvolution of peaks resulting from multiply charged species. The peptide sequence is that with highest probability based on parent mass and sequence of CA: (a) 3301 = des-(Lys[1]-Trp[2]-Lys[3]-Leu[4]-Phe[5]) CA; (b) 3045 = des-(Lys[1]-Trp[2]-Lys[3]-Leu[4]-Phe[5]-Lys[6]-Lys[7]) CA; 721 = Lys[1]-Trp[2]-Lys[3]-Leu[4]-Phe[5]; (c) 3561 = des-(Lys[1]-Trp[2]-Lys[3]) CA; (d) 4004 = CA.

of the Phe[5]—Lys[6] bond. The 10 min peak gave masses of 721 and 3045 (Figure 4b), corresponding to the Lys[1]—Phe[5] and Ile[8]—Lys[37] fragments of CA, respectively. These fragments result from cleavage at the Phe[5]—Lys[6] and Lys[7]—Ile[8] bonds, respectively. The 17 min peak gave a mass of 3561 (Figure 4c), corresponding to the Leu[4]—Lys[37] fragment of CA. This fragment results from the cleavage of the Lys[3]—Leu[4] bond. The CA peak at 21 min was also isolated and identified by ESMS to have a mass of 4004 (Figure 4d).

DISCUSSION

To answer the question, raised in previous studies (De Lucca et al., 1997), of why CA is lethal to conidia of *Fusarium* spp. (both nongerminating and germinating) and to germinating *Aspergillus* spp. but not to nongerminating *Aspergillus* spp., we looked at the fate of CA in the presence of *A. flavus* conidia. The proteolytic breakdown of a related peptide, cecropin B, by the intercellular fluid of peach leaves has been demonstrated previously (Mills et al., 1994). Proteolysis was associated with loss of toxicity toward the peach bacterial pathogen *Pseudomonas syringae* pv. *syringae*. The presence of extracellular proteases from *A. flavus* has also been noted previously (Zhu et al., 1990), but in no case has the sites of proteolysis been identified.

By HPLC it was observed that CA was converted into several new compounds when incubated with nongerminated conidia suspensions from *A. flavus*. When the conidia were allowed to germinate for 8 h before mixing with CA, a small amount of the new compounds was produced, but the majority of CA was still present. No breakdown of CA was observed with either conidial form of *F. moniliforme* (De Lucca et al., 1997). These results correspond with the bioassays which showed that nongerminating conidia of *A. flavus* were not susceptible to CA, whereas the amount of CA needed for lethality of germinating conidia of *A. flavus* was 10 times that needed for the *F. moniliforme* conidia (both nongerminating and germinating).

HPLC results also demonstrated that the agent causing the degradation of CA was not conidial wall associated but was a compound released into the culture medium. This compound was, itself, slowly degraded, as seen by the reduced degradation of CA with the 8-h-germinated *A. flavus*. This protease apparently was produced by the hyphae prior to harvesting of the conidia.

The peaks observed by HPLC were identified by ESMS to be proteolytic degradation products. Three sites of cleavage were detected, all near the N terminus of CA. The amino acid on the N-terminal side of the

cleavage site was either phenylalanine or lysine. The amino acids on the C-terminal side were lysine, leucine, or isoleucine. In two cases, the cleavage site contained a positively charged amino acid followed by a branched, hydrophobic amino acid. The third case was between an aromatic amino acid and a positively charged amino acid.

In conclusion we have shown by HPLC and ESMS that CA is proteolytically degraded by *A. flavus* by an extracellular protease. Protease activity was specific to three sites near the N terminus of the peptide. The sites of recognition all contain a lysine residue and a hydrophobic amino acid; however, their respective order is not consistent.

The standard fungicidal bioassay procedures involves a 48 h incubation of a suspension of fungal conidia with a potential antifungal compound. For studies looking strictly at compound activity, this procedure may be flawed when using peptides as the antifungal agent. The results of this study demonstrate that the use of this bioassay procedure with antifungal peptides needs to be reconsidered because of fungal proteases. If these proteases are not removed from the conidial suspension prior to the bioassay procedure, they can quickly degrade the antifungal peptide, resulting in an inaccurate assessment of activity.

It should be noted that although proteolysis of CA does occur with *A. flavus*, resulting in its inactivation, the removal of the protease did not restore the lethality of CA. We have since determined that the nonlethality of conidia of *A. flavus* incubated with CA may additionally be due to the unavailability or low concentration of the CA-binding ergosterol present in the conidial wall (membrane) (De Lucca et al., 1997). The structure of the nongerminating conidial wall of *A. flavus* may also play a role in its viability.

ABBREVIATONS USED

CA, cecropin A; HPLC, high-performance liquid chromatography; MS, mass spectrometry; PDA, potato dextrose agar; PDB, potato dextrose broth; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; ESMS, electrospray mass spectrometry; LC/MS, liquid chromatography/mass spectrometry.

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