Five disulfide bridges stabilize a hevein-type antimicrobial peptide from the bark of spindle tree (*Euonymus europaeus* L.)

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Abstract **A small 45 amino acid residue antifungal polypeptide was isolated from the bark of spindle tree (*Euonymus europaeus* L.). Though the primary structure of this so-called *E. europaeus* chitin-binding protein or Ee-CBP is highly similar to the hevein domain, it distinguishes itself from most previously identified hevein-type antimicrobial peptides (AMP) by the presence of two extra cysteine residues that form an extra disulfide bond. Due to these five disulfide bonds Ee-CBP is a remarkably stable protein. Agar diffusion and microtiterplate assays demonstrated that Ee-CBP is a potent antimicrobial protein. IC_{50}-values as low as 1 μg/ml were observed for the fungus *Botrytis cinerea*. Comparative assays further demonstrated that Ee-CBP is a stronger inhibitor of fungal growth than Ac-AMP2 from *Amaranthus caudatus* seeds, which is considered one of the most potent antifungal hevein-type plant proteins.**

Key words: Antimicrobial peptide; Bark; Hevein; Spindle tree; *Euonymus europaeus* L.

1. Introduction

Plants accumulate many types of defense proteins in their most vulnerable tissues to anticipate and cope with attacks from different pests and pathogens. Some of these proteins are induced by specific biotic or abiotic agents, whereas others are constitutively expressed. A particular group of such defense-related proteins are the small cysteine-rich peptides with antimicrobial properties. This group of so-called antimicrobial peptides (AMPs) comprises different protein families like thionins, plant defensins, lipid transfer proteins, hevein-type peptides and knottin-type peptides [1–3]. Almost all these AMPs possess four to eight cysteine residues forming two to four disulfide bonds that stabilize the overall fold of the polypeptide and contribute to the marked stability of the proteins. Only snakin-1 (St-SN1), a recently discovered AMP of 63 amino acids from potato tubers [4] contains 12 cysteines that form six disulfide bridges. The number of disulfide bonds is not directly linked to the family to which the AMPs belong but varies even within a single family. For example, within the family of chitin-binding hevein-type polypeptides, proteins have been identified with three (e.g. Ac-AMP), four (e.g. hevein) or five (e.g. *Eucommia ulmoides* AMPs) disulfide bonds [5–7].

This paper describes a novel hevein-type AMP from the bark of spindle tree (*Euonymus europaeus* L.), a shrub of the family Celastraceae, which is indigenous in Europe and is characterized by green twigs and typical spindle-shaped red fruits. The so-called *E. europaeus* chitin-binding protein, Ee-CBP, is a hevein-type protein with a unique structure characterized by the presence of 10 disulfide bridge-linked cysteine residues and an unusually high antifungal activity.

2. Materials and methods

2.1. Isolation and purification of Ee-CBP

A single spindle tree growing in a local forest (Egenhovenbos, Heverlee, Belgium) was chosen for the collection of bark tissue. Bark was removed (in February) from stems and twigs, chopped into small pieces, freeze-dried and powdered in a coffee mill. The powder obtained from 100 g of lyophilized bark was extracted in 1 l of 0.2 M NaCl. The homogenate was cleared by centrifugation (8000×*g*; 10 min) and the supernatant loaded onto a column (5 cm×10 cm; approximately 200 ml bed volume) of chitin (Sigma-Aldrich, St. Louis, MO, USA) pre-equilibrated with 0.2 M NaCl. After loading the extract, the column was washed with 0.2 M NaCl until the A_{280} fell below 0.01 and the bound chitin-binding proteins eluted with 20 mM acetic acid. The affinity-purified fraction (which contains besides Ee-CBP several other chitin-binding proteins) was dialyzed against 20 mM acetic acid for 48 h. Under these conditions the small-sized Ee-CBP peptide migrates through the dialysis membrane (molecular weight cutoff of 12–14 kDa, Medicell International, London, UK) leaving behind the contaminating larger chitin-binding proteins. The Ee-CBP fraction present in the dialysate was essentially pure and was concentrated by affinity chromatography on a smaller column (2.5 cm×5 cm; approximately 25 ml bed volume) of chitin. Approximately 10 mg of purified peptide was obtained from 100 g of lyophilized bark tissue. Chitinase activity was assayed using carboxymethyl-chitin-Remazol-brilliant-violet 5R as a substrate [8].

2.2. Amino acid sequence analysis and mass spectrometry of Ee-CBP

Ee-CBP was treated with pyroglutamate aminopeptidase (Roche Diagnostics, Mannheim, Germany) to remove the blocked cyclic N-terminal pyroglutamic acid [9]. N-terminal amino acid sequencing was done on a pulsed liquid-phase 491 Procise-cLC protein sequencer (Applied Biosystems, Foster City, CA, USA). To complete the amino acid sequence of the peptide, reduced and alkylated (with iodacetamide) Ee-CBP was digested with the endoproteases Glu-C and trypsin (both from Roche Diagnostics) according to Walker [9] and the fragments separated by reverse-phase high-performance liquid chromatography (RP-HPLC) on a C8 column (Aquapore RP-300) connected

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with an electrospray ion trap mass spectrometer (Esquire-LC, Bruker Daltonic, Bremen, Germany). Peptide fragments were sequenced by Edman degradation or by fragmentation on the mass spectrometer. For relative molecular mass determination, native Ee-CBP and proteolytic fragments were desalted on ZipTip, if required (Millipore, Bedford, MA, USA) and injected on the ion trap mass spectrometer.

2.3. Determination of free thiol groups

To check the presence of free cysteines in Ee-CBP, 20 nmol of the peptide was labeled with a 10-fold molar excess of the fluorescent probe *N*-(1-pyrenyl)maleimide (PM) dissolved in *N*,*N*-dimethylformamide as described by Bhattacharyya and Roy [10]. After labeling, the protein was dissolved in sample buffer, separated by SDS–PAGE and the presence of fluorescent free thiol groups checked on an UV-transilluminator. An equal amount of reduced Ee-CBP served as a positive control together with GNA, a lectin from *Galanthus nivalis* L. with one free cysteine residue [11].

2.4. Assays for antimicrobial acivity

The antifungal activity of Ee-CBP was evaluated by agar diffusion assays (adapted from Schlumbaum et al. [12]). Mycelium plugs of five phytopathogenic fungi (*Alternaria brassicicola* MUCL 20297, *Botrytis cinerea* MUCL 6492, *Fusarium culmorum* IMI 180420, *Neurospora crassa* FGSC 2489 and *Phoma exigua* CBS 431.74) were placed under sterile conditions in the center of Petri dishes filled with water agar (1%). After 1–3 days (depending on the fungal growth rate at 22°C) different amounts of Ee-CBP were added into expunged holes equidistant from the mycelium plug.

In vitro microtiterplate assays (according to Broekaert et al. [13]) were used to compare the antimicrobial activity of Ee-CBP to that of Ac-AMP2. Test fungi were the five aforementioned fungal strains, *Fusarium oxysporum* f.sp. *cubense* and *Mycosphaerella eumusae* (two tropical fungi isolated from *Musa* sp.), *F. oxysporum* f.sp. *matthiolae* CBS 247.61, *Phytophthora cryptogea* CBS 418.71, *Pythium ultimum* MUCL 30159, *Rhizoctonia solani* CBS 207.84 and *Trichoderma hamatum* ATCC 20765. Each well contained two-fold serial dilutions of 20 μl filter-sterilized AMP (500 μg/ml) and 80 μl of 1.6×10^4 fungal spores/ml in half-strength PDB. The same protocol was followed for the yeasts *Pichia anomala* strain K MUCL 40563 and *Saccharomyces cerevisiae* strain Sp1 with 10^5 CFU. Bacterial strains tested were *Bacillus megaterium* ATCC 13632, *Sarcina lutea* ATCC 9341, *Erwinia carotovora* subsp. *carotovora* LMG 2458 and *Ralstonia solanacearum* strain 2644, applied in 80 μl of a soft agarose medium (tryptone 1%, low melting point agarose 0.5%). Microbial growth inhibition was quantified by measuring the A_{595}.

3. Resuls and discussion

3.1. Isolation and characterization of Ee-CBP

A simple purification procedure based on affinity chromatography on chitin and selective permeation through a dialysis membrane allowed purifying reasonable amounts of Ee-CBP. The native protein yielded no signal upon N-terminal sequencing, indicating that it is N-terminally blocked. After deblocking with pyroglutamate aminopeptidase a single sequence of 36 residues was obtained which shares a high similarity/identity with previously sequenced or cloned hevein-type AMPs. Calculations based on the difference in molecular mass between native Ee-CBP (4992.5 Da) and the polypeptide comprising the first 36 residues (3973.4 Da) indicated approximately 10 residues were still missing at the C-terminus. To complete the sequence, reduced and alkylated Ee-CBP was digested with Glu-C and the resulting fragments separated by RP-HPLC. Two fragments were recovered. The peptide eluting in the second peak corresponded to the first 29 residues, whereas that in the first peak yielded a unique sequence of 16 residues starting with YCCTSQG (corresponding to residues 30–45 of the intact polypeptide chain). Using this seven-residue overlap the complete sequence of Ee-CBP could be tentatively reconstructed. Sequencing of a peptide obtained

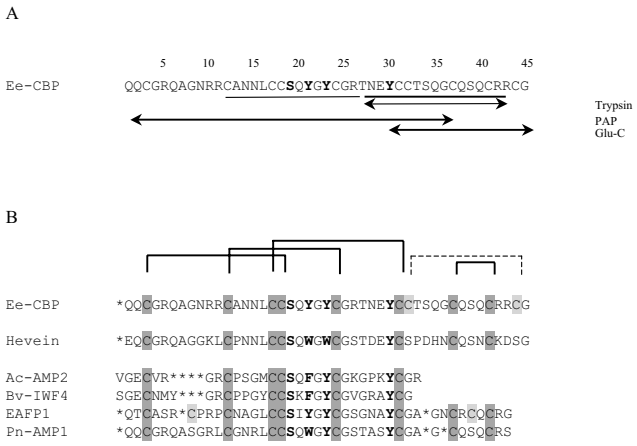

Fig. 1. A: Complete amino acid sequence of the mature Ee-CBP obtained after digestion with pyroglutamate aminopeptidase and the endoproteases Glu-C and trypsin. Fragments identified on the mass spectrometer are underlined, whereas arrows indicate sequenced peptide fragments. B: Sequence alignment of Ee-CBP in comparison with other hevein-type antimicrobial peptides. The position of the conserved cysteine residues (compared to hevein) is shaded in black. Gray-shaded residues indicate the extra cysteine residues present in Ee-CBP and EAFP1. The well-conserved amino acids shown in bold are involved in chitin-binding activity. The disulfide bridges are indicated with full or dashed lines (Ee-CBP). Proteins shown in (B) are hevein (from *Hevea brasiliensis* L. [6]); Ac-AMP2 (from *Amaranthus caudatus* L. [5]); Bv-IWF4 (from *Beta vulgaris* L. [15]); EAFP1 (from *Eucommia ulmoides* Oliv [7]) and Pn-AMP2 (from *Pharbitis nil* L. [17]).

by tryptic digestion of reduced and alkylated Ee-CBP confirmed that the sequence in the overlap region between the two Gly-C fragments was correct (Fig. 1A). Additional evidence for the accuracy of the sequence was provided by mass spectrometry of Ee-CBP. As shown in Fig. 2, Ee-CBP yielded a minor component of 4992.9 Da, which matches exactly the calculated molecular mass of the presumed 45 amino acid polypeptide (taking into account the N-terminal pyroglutamic acid) (4992.5 Da). The major peak in the mass spectrum (representing 85% of the protein) corresponds to native Ee-CBP without the C-terminal glycine residue. Based on these data, one can reasonably assume that the sequence is correct. Ee-CBP has a calculated iso-electric point of 11.81 and is not glycosylated. The extinction coefficient at 280 nm for a 1 mg/ml solution is 1.004.

Ee-CBP shares a high sequence identity/similarity with hevein and other hevein-type antifungal proteins (Fig. 1B) and accordingly can be classified as a hevein-type AMP. All eight cysteine residues present in hevein are conserved in Ee-CBP. On the homology of hevein, one can reasonably assume that these eight cysteine residues form four disulfide bridges (between Cys^3 and Cys^{18}, Cys^{12} and Cys^{24}, Cys^{17} and Cys^{31}, and Cys^{37} and Cys^{41}, respectively). Surprisingly, Ee-CBP contains two additional cysteine residues in its sequence at positions 32 and 44, respectively. To check whether these two cysteine residues also form a disulfide bond the possible presence of free thiol groups was corroborated. Unreduced Ee-CBP was treated with the fluorescent probe PM and subsequently analyzed by SDS–PAGE. The resulting polypeptide band yielded no fluorescent signal upon exposure to UV light, whereas the positive control proteins GNA and reduced Ee-CBP lighted up as bright bands. It can be concluded, therefore, that no free thiol groups are present in native Ee-CBP, and accord-

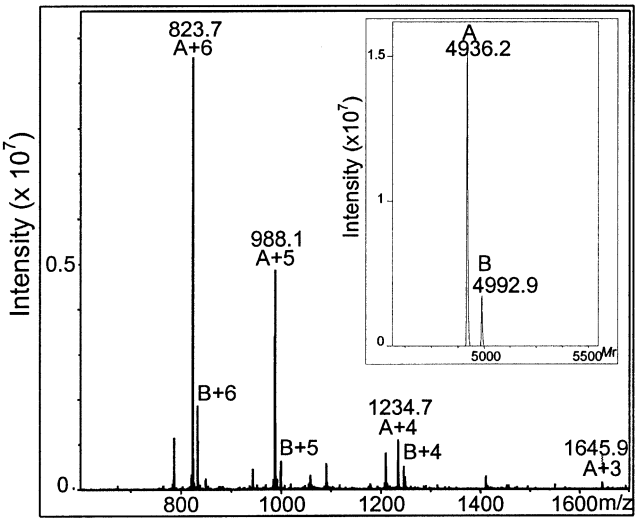

Fig. 2. Mass spectrum of Ee-CBP and charge deconvolution diagram indicating the total relative molecular mass of mature Ee-CBP. The minor peak (15%) represents the intact protein, whereas the major peak with molecular mass of 4936.2 Da (85%) corresponds to a processing product lacking the C-terminal glycine residue.

ingly Cys^{32}–Cys^{44} form a fifth disulfide bridge (Fig. 1B). Taking into consideration the results of structural analysis of hevein [6], which demonstrated that Cys^{32} is located close to the C-terminus, the formation of an extra disulfide bridge between Cys^{32} and Cys^{44} of Ee-CBP is sterically allowed. The absence of free thiol groups also explains the difference of 10 Da between the calculated molecular mass of reduced Ee-CBP (5002.5 Da) and the measured value (4992.9. Da).

Enzymatic assays demonstrated that Ee-CBP is devoid of both exo- and endochitinase activity, which indicates that the purified protein is not contaminated by chitinases that may interfere with the antifungal assays described below. Like all hevein-type AMPs, Ee-CBP has a high affinity for chitin and accordingly can be purified by affinity chromatography on crude chitin.

3.2. Antimicrobial activity

To check whether Ee-CBP exhibits similar biological activities as the related hevein-type AMPs from other species the antimicrobial activity of the novel spindle tree protein was investigated. Agar diffusion assays in Petri dishes indicated that Ee-CBP possesses a very strong antifungal activity. The growth of the phytopathogenic fungi *B. cinerea* and *N. crassa* was inhibited at concentrations as low as 5 μg/ml (Fig. 3A), and that of the ascomycetes *A. brassicicola* and *F. culmorum* at 10 μg/ml. For the fungus *P. exigua*, a slightly higher concentration of Ee-CBP (25 μg/ml) was required for growth inhibition (Fig. 3A). To better quantify the results, the inhibitory activity of Ee-CBP was tested against a series of fungi in a microtiterplate assay. As shown in Table 1, the results of these microtiterplate assays are in good agreement with those of the agar diffusion assays (Fig. 3A). Microtiterplate assays were also used to compare the antifungal activity of Ee-CBP to that of Ac-AMP2 which is considered as one of the most potent hevein-type antifungal polypeptides. According to the data summarized in Table 1, Ee-CBP exhibits a stronger antimicrobial activity than Ac-AMP2 towards most tested fungi. For example, the IC_{50} of Ee-CBP is 16 and six-fold lower than that of Ac-AMP2 for *A. brassicicola* and *F. oxysporum* sp., respectively. Only for the Deuteromycete *P. exigua* Ee-CBP was slightly less active than Ac-AMP2. It is also noteworthy that the IC_{50} for *T. hamatum*, an antagonistic fungus used in biological control, was relatively high for Ee-CBP (as well as for Ac-AMP2).

Most AMPs not only inhibit fungal growth but also affect the morphology of germinating spores and/or growing hyphae. To check whether Ee-CBP causes similar effects the morphology of different fungi grown in the presence and absence of the protein was compared. Microscopic analyses re-

Table 1
Comparison of the antimicrobial activity of Ee-CBP with Ac-AMP2

	IC_{50}(μg/ml)		Relative activity
	Ee-CBP	Ac-AMP2	
Fungi			
Alternaria brassicicola[a]	3	50	16.7
Botrytis cinerea[a]	1	2	2
Fusarium culmorum[a]	3	6	2
Fusarium oxysporum f.sp. *cubense*[a]	15	100	6.7
Fusarium oxysporum f.sp. *matthiolae*[a]	5	30	6
Mycosphaerella eumusae[a]	6	8	1.3
Neurospora crassa[a]	2	3	1.5
Phoma exigua[a]	33	30	0.9
Phytophthora cryptogea[b]	25	50	2
Pythium ultimum[a]	33	95	2.9
Rhizoctonia solani[c]	25	100	4
Trichoderma hamatum[a]	100	100	1
Gram-positive bacteria			
Bacillus megaterium[d]	2	7	3.5
Sarcina lutea[d]	7	20	2.9

The relative antimicrobial activity is expressed as (IC_{50} Ac-AMP2)/(IC_{50} Ee-CBP).
Concentration of protein required for 50% growth inhibition after incubation:
[a]for 48 h;
[b]for 1 week;
[c]for 96 h;
[d]and for 24 h;

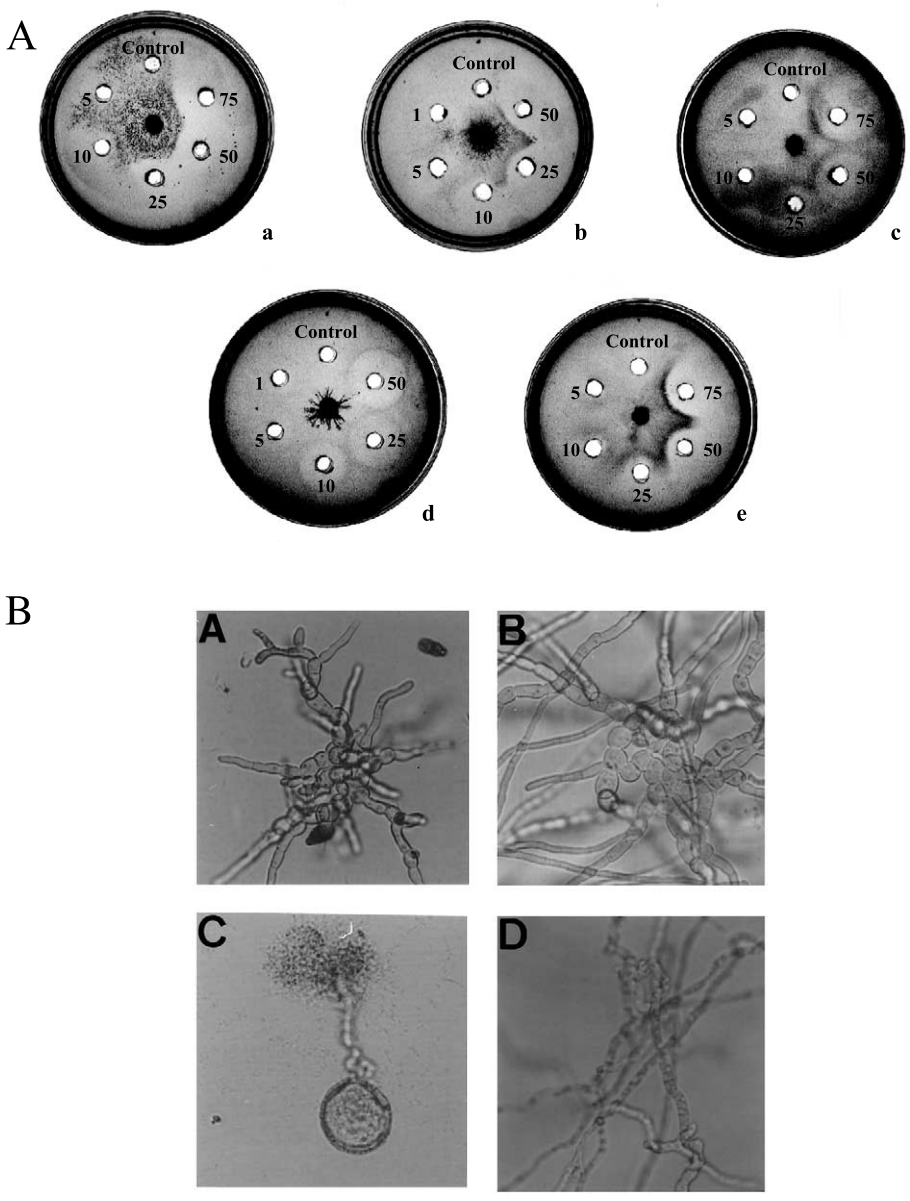

Fig. 3. Effect of Ee-CBP on fungal growth and morphology. A: Antibiosis of different fungi after incubation for 1 week at 22°C in the presence of different concentrations of Ee-CBP (1–75 μg/ml). Panels a–e show the effect of Ee-CBP on the growth of *A. brassicicola*, *B. cinerea*, *F. culmorum*, *N. crassa* and *P. exigua*, respectively. B: Effect of Ee-CBP on fungal morphology (magnification 200×). Panels A and B show stunted growth and hyperbranching of *A. brassicicola* and the normal growing fungus, respectively. Panels C and D display bulbed spores and spore release of *N. crassa* and the unaffected fungus, respectively.

vealed that Ee-CBP severely affects the morphology of the test fungi. Commonly observed effects are bulbing, spore release, hyphal branching and stunted growth (Fig. 3B). These microscopic observations are in good agreement with the results reported for hevein [14] and IWF4, an antifungal peptide from beet leaves [15]. Besides fungi, Ee-CBP also inhibited the growth of Gram-positive bacteria but not that of Gram-negative bacteria and yeasts. Addition of mono- and divalent cations like K^+ and Ca^{2+} to the medium resulted in a decrease of the antifungal activity of Ee-CBP. In this respect, Ee-CBP behaves like most of the previously described hevein-type AMPs.

Ee-CBP is a markedly stable protein. The antifungal activity is not affected by boiling the peptide for 10 min or by prolonged storage. Like most hevein-type AMPs, Ee-CBP is stable over a wide pH range (from pH 2 to pH 11) [5].

At present, the mode of action of hevein-type AMPs is still unclear. According to one hypothesis the compact folding of the AMPs allows migration through the pores of the fungal cell wall, enabling the peptides to interact with the plasma membrane and impede fungal growth [7,14,16]. Another hypothesis relates the antimicrobial activity to the highly basic iso-electric point of hevein-type AMPs [5,15,17]. It is true that Ee-CBP (pI 11.81), Ac-AMP1 (pI 10.25), Ac-AMP2 (pI 10.55), Bv-IWF4 (pI 9.76), both EAFPs (both with a pI of 11.14) as well as both Pn-AMPs (both with a pI of 12.01) are all very basic proteins compared to hevein (pI 4.44). It should be mentioned here that all iso-electric points have been calculated using the same software (http://www.up.univ-mrs.fr/cgi-

wabim/a-compo-p.pI) and assuming that all the cysteine residues are linked by disulfide bonds. These calculated pI values are in good agreement with the experimental values reported.

Since Ee-CBP possesses no exo-, nor endochitinase activity the antifungal activity of the protein has to be ascribed most likely to the high affinity of the protein for chitin and oligomers of β-1,4-*N*-acetyl-D-glucosamine. This chitin-binding activity apparently relies on the presence of well-conserved aromatic amino acids and the N-terminal pyroglutamate residue [18]. It should be mentioned, however, that the chitin-binding activity of Ee-CBP on its own cannot explain the inhibitory activity towards Oomycetes (which do not contain chitin in their cell wall) and Gram-positive bacteria [17]. Further research on the mode of action of Ee-CBP will be required prior to use of this AMP for genetically engineering purposes, either alone or in combination with other plant defensive proteins.

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