

# 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<sub>50</sub>-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.

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## 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 snak-in-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<sub>280</sub> 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 iodoacetamide) 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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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<sup>3</sup> and Cys<sup>18</sup>, Cys<sup>12</sup> and Cys<sup>24</sup>, Cys<sup>17</sup> and Cys<sup>31</sup>, and Cys<sup>37</sup> and Cys<sup>41</sup>, 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-

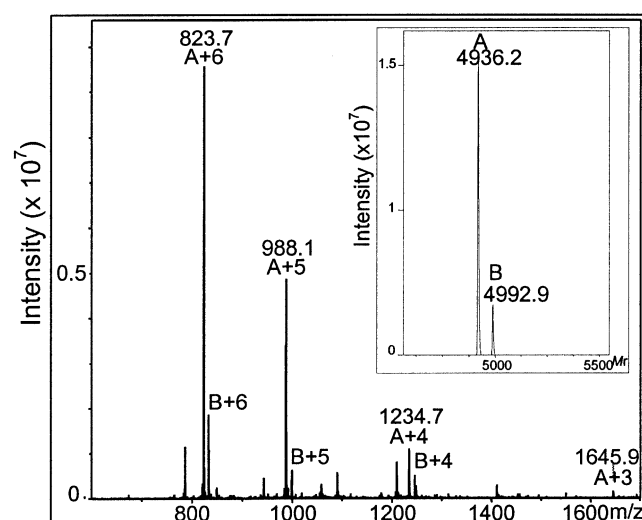


Fig. 2. Mass spectrum of Ee-CBP and charge deconvolution diagram indicating the total relative molecular mass of mature Ee-CBP. The minor peak (15%) represents the intact protein, whereas the major peak with molecular mass of 4936.2 Da (85%) corresponds to a processing product lacking the C-terminal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

ingly Cys<sup>32</sup>–Cys<sup>44</sup> form a fifth disulfide bridge (Fig. 1B). Taking into consideration the results of structural analysis of hevein [6], which demonstrated that Cys<sup>32</sup> is located close to the C-terminus, the formation of an extra disulfide bridge between Cys<sup>32</sup> and Cys<sup>44</sup> 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<sub>50</sub> 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<sub>50</sub> 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 <sub>50</sub> (µg/ml)		Relative activity
	Ee-CBP	Ac-AMP2	
Fungi			
<i>Alternaria brassicicola</i> <sup>a</sup>	3	50	16.7
<i>Botrytis cinerea</i> <sup>a</sup>	1	2	2
<i>Fusarium culmorum</i> <sup>a</sup>	3	6	2
<i>Fusarium oxysporum</i> f.sp. <i>cubense</i> <sup>a</sup>	15	100	6.7
<i>Fusarium oxysporum</i> f.sp. <i>matthiolae</i> <sup>a</sup>	5	30	6
<i>Mycosphaerella eumusae</i> <sup>a</sup>	6	8	1.3
<i>Neurospora crassa</i> <sup>a</sup>	2	3	1.5
<i>Phoma exigua</i> <sup>a</sup>	33	30	0.9
<i>Phytophthora cryptogea</i> <sup>b</sup>	25	50	2
<i>Pythium ultimum</i> <sup>a</sup>	33	95	2.9
<i>Rhizoctonia solani</i> <sup>c</sup>	25	100	4
<i>Trichoderma hamatum</i> <sup>a</sup>	100	100	1
Gram-positive bacteria			
<i>Bacillus megaterium</i> <sup>d</sup>	2	7	3.5
<i>Sarcina lutea</i> <sup>d</sup>	7	20	2.9

The relative antimicrobial activity is expressed as (IC<sub>50</sub> Ac-AMP2)/(IC<sub>50</sub> Ee-CBP).

Concentration of protein required for 50% growth inhibition after incubation:

<sup>a</sup>for 48 h;

<sup>b</sup>for 1 week;

<sup>c</sup>for 96 h;

<sup>d</sup>and for 24 h;

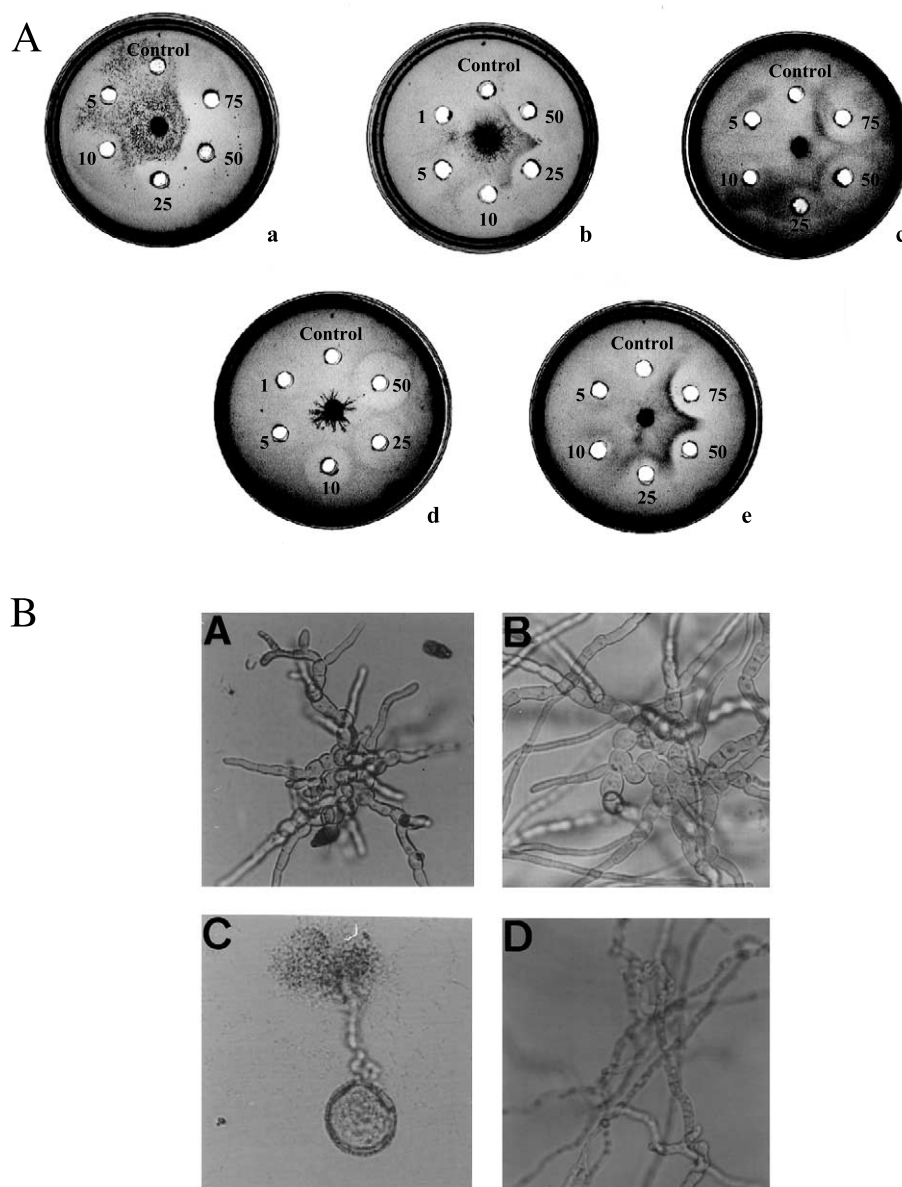


Fig. 3. Effect of Ee-CBP on fungal growth and morphology. A: Antibiosis of different fungi after incubation for 1 week at 22°C in the presence of different concentrations of Ee-CBP (1–75 µg/ml). Panels a–e show the effect of Ee-CBP on the growth of *A. brassicicola*, *B. cinerea*, *F. culmorum*, *N. 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.I) 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  $\beta$ -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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