Two novel antifungal peptides distinct with a five-disulfide motif from the bark of *Eucommia ulmoides* Oliv

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Abstract Two antifungal peptides, named EAFP1 and EAFP2, have been purified from the bark of *Eucommia ulmoides* Oliv. Each of the sequences consists of 41 residues with a N-terminal blockage by pyroglutamic acid determined by automated Edman degradation in combination with the tandem mass spectroscopy and the C-terminal ladder sequencing analysis. The primary structurs all contain 10 cysteines, which are cross-linked to form five disulfide bridges with a pairing pattern (C1–C5, C2–C9, C3–C6, C4–C7, C8–C10). This is the first finding of a plant antifungal peptide with a five-disulfide motif. EAFP1 and EAFP2 show characteristics of hevein domain and exhibit chitin-binding properties similar to the previously identified hevein-like peptides. They exhibit relatively broad spectra of antifungal activities against eight pathogenic fungi from cotton, wheat, potato, tomato and tobacco. The inhibition activity of EAFP1 and EAFP2 can be effective on both chitin-containing and chitin-free fungi. The values of IC_{50} range from 35 to 155 μg/ml for EAFP1 and 18 to 109 μg/ml for EAFP2. Their antifungal effects are strongly antagonized by calcium ions. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Antifungal peptide; Amino acid sequence; Disulfide motif; *Eucommia ulmoides* Oliv

1. Introduction

Though plants are constantly exposed to various microbial pathogens, infections remain rare events. This is because plants have evolved several effective defense mechanisms to protect themselves from pathogenic attacks. A wide array of antimicrobial peptides and proteins are believed to be involved in such mechanisms [1,2]. Among them the cysteine-rich peptides form a large group of the antimicrobial proteins, in which a certain disulfide-motif stabilizes the molecular scaffold [2]. Accordingly, these plant antimicrobial peptides are usually classified by their distinct disulfide motif and sequence homologies (e.g. [2]). Thionins [3–5], plant defensins [6–8], hevein-like [9–12] and knottin-like [13–15] peptides belong to these classes. So far at most eight cysteines pairing into four disulfide bridges have been found in these peptides.

In this paper we report two novel antifungal peptides, EAFP1 and EAFP2, distinguished by 10 cysteines pairing into five disulfide bonds from the bark of *Eucommia ulmoides* Oliv (Garryales order, Eucommiaceae family), whose bark, called Du-Zhong in Chinese, is a traditional Chinese medicine mainly used as a tonic for reducing blood pressure and contains a kind of latex similar to that of rubber tree (*Hevea brasiliensis*) [9]. These two peptides are also N-terminal blockage by pyroglutamic acid. To our knowledge this is the first finding of a plant antifungal peptide with a five-disulfide motif. The purification, complete amino acid sequence, bioassay and comparison with hevein-like peptides will be described.

2. Materials and methods

2.1. Materials

The bark of *E. ulmoides* Oliv was obtained from the Kunming mountain area of the Yun-Nan province in China. Eight strains of plant pathogenic fungi, *Phytophthora infestans*, *Ascochyta lycopersici*, *Verticillium dahliae*, *Gibberella zeae*, *Alternaria nicotianae*, *Fusarium moniliforme*, *Fusarium oxysporum* and *Colletotrichum gossypii*, and two bacterial strains, *Bacillus megaterium* (Gram-negative) and *Pseudomonas syringae* (Gram-Positive), were used in the bioassay.

Dithiothreitol (DTT), iodoacetamide (IAA), α-cyano-4-hydroxycinnamic acid (CHCA), TPCK-treated trypsin and carboxypeptidase Y (CPY) were purchased from Sigma. All other reagents were of analytical grade or high performance liquid (HPLC) grade.

2.2. Isolation and purification of EAFPs

The washed bark of *E. ulmoides* Oliv was cut into pieces that were ground to power and extracted with 2 volumes of 0.5 mol/l NaCl with pH 6.0. The supernatant was precipitated with 95% saturated solution of $(NH_4)_2SO_4$, and then isolated by CM-cellulose-52 column, gel filtration column of Bio-gel-p-10 and Waters YWG-Pak C18 column, successively.

2.3. MALDI-TOF mass spectrometric analysis

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra were recorded on a Bruker Proflex III mass spectrometer. The matrix was used as CHCA dissolved in 50% ACN solution containing 0.1% trifluoroacetic acid (TFA) to a saturated solution. 2 μl (about 1–5 pmol/μl) of peptide dissolved in a 0.1% TFA solution in water was mixed with 20 μl of the CHCA-saturated matrix solution. 1 μl of this mixture was deposited on the target and dried. The spectra were calibrated with huwentoxin-I (MW 3750.45 Da, presented by S.P. Liang [16]).

2.4. Bioassay

Antimicrobial activity was determined by agar diffusion assay as described by Bormann et al. [17]. Test plates (85 mm in diameter) containing 17.5 ml of potato dextrose (PD) medium were seeded with test organisms, either spores of filamentous fungi or cells of

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Abbreviations: EAFP, *Eucommia* antifungal peptide; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; HPLC, high performance liquid chromatography; PD, potato dextrose; CHCA, α-cyano-4-hydroxycinnamic acid; CPY, carboxypeptidase Y; CAM, carboxyamidomethyl

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bacteria. After recovering overnight at 25°C, the solution containing the EAFPs was applied into wells (6 mm in diameter punched into the agar). The antifungal activities were shown by the occurrence of a visible zone of inhibited mycelia growth after incubation at 25°C for 48 h.

To assay the inhibition of hyphal growth, an agar plug (6 mm in diameter) containing mycelia of the test fungus was placed in the center of a petri dish containing 17.5 ml of PD medium and incubated at 25°C until the colony reached a diameter of 3–4 cm. A paper disk containing 20 μl of peptide was placed at the growing front of the hyphae and the test plates were further incubated.

The percentage of growth inhibition was assayed as described by Broekaert et al. [12]. 20 μl aliquots of two-fold serial diluted peptide solution were added to a 96-well plate and mixed with 80 μl of PD liquid medium containing ca 1000 fungal spores. The growth was recorded after incubating for 36 h at 25°C. The absorption at 595 nm served as a measurement for fungal growth.

2.5. Amino acid sequencing

The purified peptides were reduced and carboxyamidomethylated according to the method described by Liang [16] and digested with trypsin. The tryptic peptides were purified by reverse phase HPLC.

The Edman degradation was performed on an Applied Biosystem 491 gas-phase sequencer with a normal automatic cycle program. Tandem mass spectrometry for peptide sequencing were performed on an APEX™ II Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics). The MS/MS fragment ions are generated by introducing a 3500 μs argon pulse with 1 mbar pressure and interpreted according to the nomenclature proposed by Roepstorff and Johnson [18,19]. C-terminal sequence analysis was performed by MALDI-TOF mass spectrometry combined with CPY time-dependent and concentration-dependent digestion as described by Patterson [20].

3. Results

3.1. Isolation and purification of EAFPs

Two kinds of antifungal peptides were purified from the bark of *E. ulmoides* Oliv by the following steps: ammonium sulfate precipitation, CM-cellulose cation exchange chromatography, gel filtration and reverse-phase HPLC, successively. The effluents were monitored for absorbance at 280 nm and the active fraction was traced by the antifungal assay. In the reverse phase chromatogram two main peaks with antifungal activity appeared and were named EAFP1 and EAFP2, respectively, according to the retention time. The yield of EAFP2 was higher than that of EAFP1. The purities of EAFP1 and EAFP2 were evidenced by MALDI-TOF mass spectra. The molecular weights of the native EAFP1 and EAFP2 were determined as 4201.4 Da and 4158.9 Da, respectively, by the mass spectrometry.

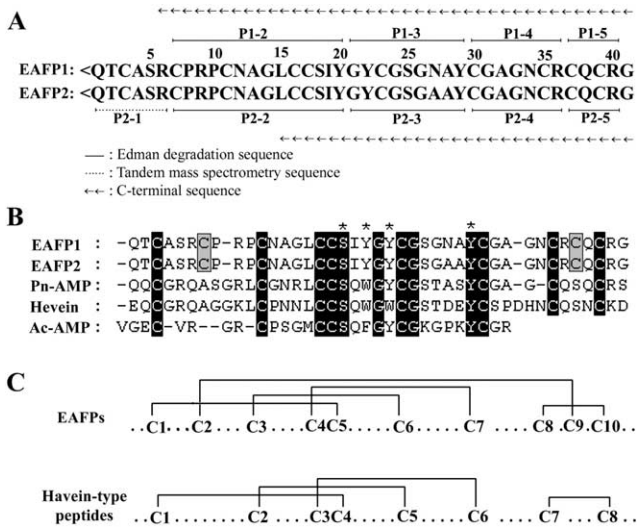

Fig. 1. A: The summary of the proof of the amino acid sequences in EAFP1 and EAFP2. B: Comparisons of the sequences of EAFP1 and EAFP2 with those of hevein-type peptides. Sequence identities to EAFPs are shaded in black, the additional two cysteines of EAFPs in gray, and the activity-related residues are indicated by an asterisk at the top. Pn-AFP, antifungal peptide from *Pharbitis nil* L. [22]; hevein, rubber latex hevein [9]; Ac-AMP, antimicrobial peptides from *Amaranthus caudatus* [12]. C: Cysteine connectivities of EAFPs in comparison with those of hevein-type peptides. Cysteine connectivities are shown by full lines. EAFPs are unique with a five-disulfide motif with pairing pattern (C1–C5, C2–C9, C3–C6, C4–C7, C8–C10).

3.2. Amino acid sequences of EAFPs

The molecular weights of carboxyamidomethyl (CAM) EAFP1 and EAFP2 were determined by MALDI-TOF mass spectrometry as 4782.4 and 4739.2 Da, respectively. The masses of CAM-EAFPs are ca. 580 Da higher than those of the native forms, indicating that EAFP1 and EAFP2 both contain 10 cysteines forming five pairs of disulfide, for the molecular weight will increase by 58 Da when half of the cysteine is carboxyamidomethylated.

Absence of PTH peaks when the native and CAM-EAFPs

Table 1
The sequences and masses of the tryptic fragments

EAFP1				EAFP2			
Retention time (min)	Sequence	Molecular weight		Retention time (min)	Sequence	Molecular weight	
		Exp. $[MH]^+$	Theor. [M]			Exp. $[MH]^+$	Theor. [M]
9.7	CQCR	608	622.7	9.4	CQCR	ND	622.7
11.0	CGAGNCR	796	794.3	10.3	CGAGNCR	792	794.3
12.9	ND[b]	705	704.3	12.3	<QTCASR[a]	705	704.3
15.1	CPRPC	692	689.3	13.6	CPRPC	689	689.3
24.7	GYCGSGNAY	947	949.4	25.7	GYCGSGAAY	904	905.4
39.8	CPRPCNAGLCCSIY	1727	1727.7	39.4	CPRPCNAGLCCSIY	1728	1727.7
41.1	NAGLCCSIY	1057	1057.4	40.2	NAGLCCSIY	1057	1057.4

<Q, the pyroglutamic acid; ND, not detected.
[a]The sequence is deduced from the tandem mass spectra and other sequences are determined by Edman degradation.
[b]The sequence is believed to be the same as the N-terminal sequence of EAFP2 though not determined experimentally, for the peptide with the molecular weight of 704 Da is also found in the peptide map of EAFP1, which has the same mass and same retention time in the peptide map.

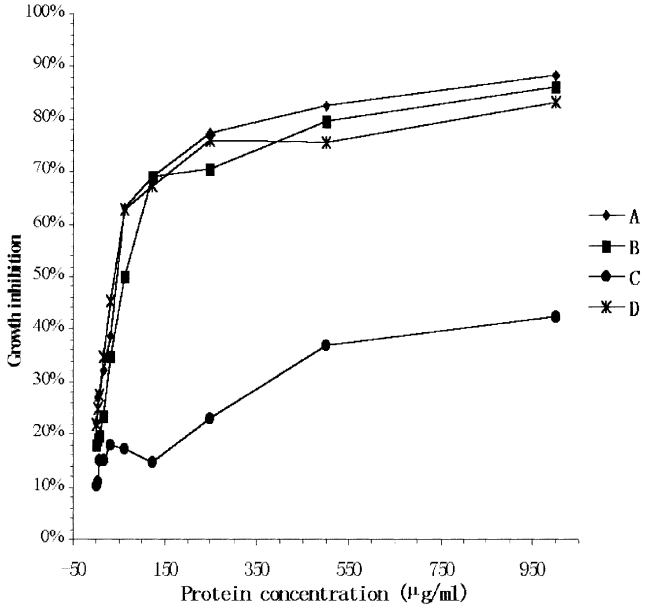

Fig. 2. A, B: The antifungal activity of EAFP1 (A) and EAFP2 (B) against fungus *C. gossypii*. C: The antagonistic effect of Ca^{2+} on the antifungal activity of EAFP2. D: The mixture of EAFP1 and EAFP2 in equal amounts.

are applied to Edman degradation indicated that the N-terminals of EAFPs were blocked. The following experiments were performed to obtain their sequence information. CAM-EAFPs were digested by trypsin and the resulted peptides were fractionated and identified by MALDI-TOF mass spectrometry, and then applied to Edman degradation sequencing or tandem mass analysis. The results are summed up in Table 1. C-terminal sequences were obtained by C-terminal ladder sequencing analysis [20]. The results showed that the C-terminal sequences are R CPRPC NAGLC CSIYG ΔΔΔSG NAYΔΔ AGNCR CQCRG and LCC SIYG YΔΔSΔ AAYCΔ ΔΔNCR CQCRG (Δ is the undefined residue in the mass spectra) for EAFP1 and EAFP2, respectively, which not only provide full sequence assembling information, but also confirm the results summarized in Table 1.

From the above results the sequences of the EAFPs can be completely deduced as shown in Fig. 1A, which are in good agreement with the results of the amino acid analysis (data not shown). The average molecular weights calculated from the sequences are 4211.80 Da and 4168.77 Da for EAFP1 and EAFP2, respectively, which are about 10 Da higher than the experimental value. This result further supports the supposition that each of the 10 cysteines of the EAFPs loses a hydrogen atom to form five pairs of disulfide bond.

3.3. Antifungal activity of EAFPs

The antifungal activity and the antifungal spectra of EAFPs were determined by Agar diffusion assay. The spectra of antifungal activity of EAFPs are relatively broad. The EAFPs can inhibit all examined phytopathogenic fungi including *P. infestans*, *A. lycopersici*, *V. dahliae*, *G. zeae*, *A. nicotianae*, *F. moniliforme*, *F. oxysporum*, *C. gossypii*. In contrast, EAFPs have no effect on the growth of the tested Gram-negative bacterium *B. megaterium* and Gram-positive bacterium *P. syringae*. In addition, EAFP1 and EAFP2 also significantly inhibit hyphal growth of the above fungi, of which *C. gossypii* is the most sensitive. The crescent-shaped zone of inhibition for *C. gossypii* can be stable for over two weeks. The spores of *A. lycopersici*, *F. moniliforme*, *F. oxysporum* and *C. gossypii* were collected for the percentage growth inhibition assay. IC_{50} values were derived from dose–response curves (percentage of growth inhibition versus protein concentration) and are listed in Table 2. EAFP1 and EAFP2 show similar antifungal activities with IC_{50}, ranging from 35 to 155 μg/ml and 18 to 109 μg/ml, respectively. There are no synergistic effects between these two peptides (Fig. 2). When calcium ions are added to the PD liquid medium, IC_{50} values rise more than 100-fold (Fig. 2), which shows that 5 mM calcium ion can significantly inhibit the antifungal activity of EAFPs.

Polysaccharide binding assay was carried out. In the experiment EAFPs exhibit a potent affinity with chitin and have no binding affinity with xylan (data not shown).

4. Discussion

The automated Edman degradation in combination with the tandem mass spectrometry and C-terminal ladder sequencing analysis determined the full sequences of antifungal peptides EAFP1 and EAPF2, which both contain 41 residues with the N-terminal blockage by pyroglutamic acid (Fig. 1A). The most striking feature of their structures is 10 cysteines cross-linked to form five disulfide bonds. The connectivities of these disulfide bridges can be reasonably deduced from the sequence alignment between EAFPs and hevein (Fig. 1B). Among 10 cysteines in EAFPs, eight (C1, C3, C4, C5, C6, C7, C8 and C10) are identical to those in hevein with regard to characteristic arrangement and distinctive residues (CS, CG, YC) for the hevein-type peptides (Fig. 1B). Therefore, they should adopt the connectivities (C1–C5, C3–C6, C4–C7, C8–C10) corresponding to those in hevein (C1–C4, C2–C5, C3–C6, C7–C8). Since all 10 cysteines are contained in the disulfide bridges as shown in the results, the additional two cysteines (C2 and C9) in EAFPs must connect with each other. Consequently, it is rational to predict that the connectivities of the 10 cysteines of EAFPs should be C1–C5, C2–C9, C3–C6, C4–C7 and C8–C10, which have been identified

Table 2
Antifungal activities of EAFP1 and EAFP2

Fungus	IC_{50} (μg/ml)			
	EAFP1	EAFP2	EAFP1+EAFP2	Ca^{2+}+EAFP2
A. lycopersici	155	109	ND	ND
F. moniliforme	56	18	ND	ND
F. oxysporum	46	94	ND	ND
C. gossypii	35	56	37	>1000

ND, not detected.

by preliminary X-ray structure analysis at 0.8 Å resolution (Xiang et al., personal communication). This form of connectivities is different from the pattern (C1–C4, C2–C5, C3–C6, C7–C8) in hevein (Fig. 1C). To our knowledge, from a structural point of view, this is a novel type of antifungal peptide identified so far.

The sequence alignment (Fig. 1B) indicated that, as described above, the core of EAFPs is a hevein domain [2] that shows the same cysteine pairing and unique residues as Gly, Ser and Tyr as in hevein [9]. However, the additional disulfide bridge (C2–C9) in EAFPs cross-links the N-terminal with the C-terminal, which should promote certain structural features to the general folding pattern appearing in this type of peptides. The bioassay showed that, except for inhibition to a series of chitin-containing fungi, EAFPs could also be against a chitin-free fungus, *P. infestans*. This result provides a functional evidence, showing the certain peculiarity of EAFP structure. The finding of EAFPs also extends the known families of chitin-binding proteins [2,21] with its unique five-disulfide motif. Certainly, the detailed structural features of EAFPs should be clarified by the three-dimensional structure.

The *Eucommia* antifungal peptide molecules containing 41 residues with five disulfide bonds must be very solid and extremely stable. In fact, they can retain their antifungal activity in boiled water for 30 min (data not shown), and are easily crystallized, in contrast with other small peptides. This solid folding pattern may favor penetration through the fungal cell wall. The bioassay showed that they exhibited evident effects on a relatively broad spectrum against fungi, including a series of pathogens for cotton, wheat, potato, tomato and tobacco. The effective fungi include both chitin-containing and chitin-free fungi. The activity of EAFP1 and EAFP2 is nearly similar, which shows that the micro-heterogeneous substitute at residue 27 (Fig. 1A) does not affect the antifungal activity. The antifungal effects of EAFPs can be strongly antagonized by calcium ion. For all these properties in association with the peculiar primary structure the molecular mechanism of the antifungal activity for EAFPs becomes very interesting.

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