

Note

Amino Acid Sequence and Antimicrobial Activity of Chitin-Binding Peptides, *Pp*-AMP 1 and *Pp*-AMP 2, from Japanese Bamboo Shoots (*Phyllostachys pubescens*)

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Two novel chitin-binding peptides, designated *Pp*-AMP 1 and *Pp*-AMP 2, which had antimicrobial activity against pathogenic bacteria and fungi, were purified from Japanese bamboo shoots (*Phyllostachys pubescens*) by a simple procedure based on chitin affinity chromatography. They had the common structural features of the plant defensin family, but they could not be grouped in any type of that family. They showed a high degree of homology to mistletoe toxins.

Key words: antimicrobial peptide (AMP); chitin-binding peptide; plant defensin; thionin; *Phyllostachys pubescens*

Antimicrobial peptides are considered to be important biophylatic substances functioning in self-defense against infection by various harmful pathogens. Plants have various small antimicrobial peptides which are cysteine-rich and highly basic.^{1–3} Since their primary structural features are similar to defensins derived from animals, one group of them is named the plant defensin family.⁴ The plant defensin family was divided into three types by Huang *et al.* according to the number and arrangement of cysteine residues: the knotting type, the hevein type, and the thionin type.⁵ The knotting type has six cysteine residues, and the hevein and thionin types have eight residues. The knotting and hevein types have a continuous sequence of cysteine (–CC–) near the center of the molecule, and the thionin type near the N-terminus.

We found two novel antimicrobial peptides in Japanese bamboo shoots (*Phyllostachys pubescens*), which had common features of the plant defensin family. They bound to chitin in a reversible way. It has been reported that thionin type plant defensin shows affinity against chitin, one of the main cell wall

components of fungi.⁶ This paper deals with their purification by a simple procedure based on chitin affinity chromatography, antimicrobial activity, and structural features. Bamboo shoots were harvested in Kagoshima Prefecture in Japan in March, 2003. Preparation of a chitin column, MALDI-TOF mass spectrometric analysis, pyridylethylation of antimicrobial peptides, analyses of amino acid composition and sequence, and calculation of isoelectric point were done as described previously.⁷ Antimicrobial activity was assayed for the following bacteria and fungi as described previously:⁷ *Erwinia carotovora* MAFF 106567, *Agrobacterium radiobacter* MAFF 520028, *Agrobacterium rhizogenes* MAFF 210265, *Clavibacter michiganensis* MAFF 301044, *Curtobacterium flaccumfaciens* MAFF 301203, *Fusarium oxysporum* IFO 6384, and *Geotrichum Candidum*, isolated from oranges at our university. They were donated by the Laboratory of Phytopathology, Department of Agriculture, Kagoshima University.

Purification at all steps was done at 4 °C. Bamboo shoots (100 g) were homogenized with 10 volumes of 50 mM sodium acetate buffer (pH 5.0). After the homogenate was stirred overnight, it was filtered through gauze and the filtrate was centrifuged at 30,000 g for 20 min. The clear supernatant was concentrated under reduced pressure and saturated with ammonium sulfate. The precipitate was completely dialyzed against 20 mM sodium bicarbonate buffer (pH 8.4), and then put through the chitin column ($\phi 1.5 \times 15$ cm) equilibrated with the same buffer. After loading the same buffer, a chitin-binding fraction was eluted at a flow rate of 0.5 ml/min with 20 mM acetic acid (pH 3.0). SDS-PAGE analysis⁸ showed that most of the proteins in the bamboo shoots passed through the column, and peptides of apparent molecular mass of

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Abbreviations: AMP, antimicrobial peptide; MALDI-TOF MS, matrix assisted laser desorption ionization time-of-flight mass spectrometry; HPLC, high performance liquid chromatography; MeCN, acetonitrile; TFA, trifluoroacetic acid

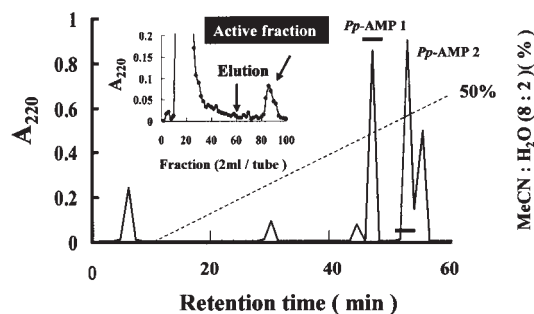


Fig. 1. Separation of *Pp*-AMP 1 and *Pp*-AMP 2 by Reverse-Phase HPLC.

Antimicrobial peptide fraction partially purified by chitin affinity chromatography was applied on a Mightysil RP-18 column (particle diameter, 5 μm ; ϕ 4.6 \times 250 mm; Kanto Chemical, Japan) previously equilibrated with 0.1% TFA. *Pp*-AMP 1 and *Pp*-AMP 2 were eluted from the column at a flow rate of 0.8 ml/min using a linear gradient of 0–60% in 60 min with MeCN:H₂O (8:2, v/v) in 0.1% TFA.

about 5 kDa combined to it. Antimicrobial activity was detected only in the eluted fraction. The eluted fraction was subjected to reverse-phase HPLC on a Mightysil RP-18 column, as shown in Fig. 1. After rechromatography on Mightysil RP-18, purified *Pp*-AMP 1 and *Pp*-AMP 2 were obtained in yields of about 0.2 mg and 0.3 mg respectively. Reduced *Pp*-AMP 1 and *Pp*-AMP 2 migrated as single bands with an apparent molecular mass of about 5 kDa on SDS-PAGE. The extinction coefficients of *Pp*-AMP 1 and *Pp*-AMP 2 of 1% aqueous solutions at 280 nm ($E^{1\%}_{280\text{nm}}$) were 7.7 and 7.9 respectively.

The antimicrobial activities of *Pp*-AMP 1 and *Pp*-AMP 2 against several plant pathogenic bacteria and fungi were evaluated as the concentration required for 50% growth inhibition, IC₅₀ (Table 1). The values of IC₅₀ varied from 2 to 25 $\mu\text{g/ml}$. Both *Pp*-AMP 1 and *Pp*-AMP 2 had potent antimicrobial activities, especially against pathogenic fungi.

Pp-AMP 1 and *Pp*-AMP 2 had four and six cysteine residues, respectively, by amino acid composition analysis. Their amino acid sequences are shown in Fig. 2A. *Pp*-AMP 1 and *Pp*-AMP 2 consisted of 44 and 45 amino acid residues respectively. The molecular mass of *Pp*-AMP 1 was 4,693.9 Da, and that of *Pp*-AMP 2 was 4,916.0 Da by MALDI-TOF MS analysis. The molecular mass of *Pp*-AMP 1 was 4,698.1 Da and that of *Pp*-AMP 2 was 4,922.3 Da by amino acid sequence data. The difference in values between the masses obtained by MALDI-TOF MS analysis and those calculated from the amino acid sequence data of *Pp*-AMP 1 and *Pp*-AMP 2, that is 4.2 Da and 6.3 Da respectively, indicate that the number of disulfide bridges on *Pp*-AMP 1 and *Pp*-AMP 2 were two and three, respectively. The isoelectric point of *Pp*-AMP 1 was calculated to be 11.5, and that of *Pp*-AMP 2 to be 11.6.

Pp-AMP 1 and *Pp*-AMP 2 had the common features of plant defensins: they were cysteine-rich and highly basic. Since plant defensins so far isolated contain six or

Table 1. Antimicrobial Activities of *Pp*-AMP 1 and *Pp*-AMP 2

Microorganisms	IC ₅₀ ($\mu\text{g/ml}$)	
	<i>Pp</i> -AMP 1	<i>Pp</i> -AMP 2
Bacteria		
<i>Erwinia carotovora</i>	22	20
<i>Agrobacterium radiobacter</i>	15	13
<i>Agrobacterium rhizogenes</i>	15	15
<i>Clavibacter michiganensis</i>	14	18
<i>Curtobacterium flaccumfaciens</i>	25	20
Fungi		
<i>Fusarium oxysporum</i>	2	2
<i>Geotrichum candidum</i>	2	2

eight cysteine residues, *Pp*-AMP 1 was a unique plant defensin in that it contained four cysteine residues. No antimicrobial peptide containing four cysteine residues has been isolated from plants, except for MBP-1 from maize.⁹⁾ The amino acid sequence of *Pp*-AMP 1 differed completely from that of MBP-1.

Huang *et al.* divided the plant defensin family into three types according to the number and arrangement of cysteine residues described above: knotting type, hevein type, and thionin type. *Pp*-AMP 2 was thought to belong to the knotting type from the number of cysteine residues, but its arrangement was evidently different from that of the knotting type, as shown in Fig. 2B. Additionally, it does not belong to the hevein¹⁰⁾ or thionin type in the number of cysteine residues as shown in Fig. 2C and 2D. *Pp*-AMP 2 as well as *Pp*-AMP 1 showed homology to the thionin type^{11,12)} in the continuous sequence of cysteine and N-terminal amino acid sequences, but did not in the C-terminal region (Fig. 2D). Thus *Pp*-AMP 2 was not grouped into any type of the plant defensin family.

A survey of the data base for amino acid sequences revealed that the features of primary structures of *Pp*-AMP 1 and *Pp*-AMP 2 closely resembled those of several toxins such as viscotoxin, which have been isolated from mistletoe, a parasitic tree, cultured in various countries (Fig. 2E).^{13–15)} The mistletoe toxins are reported to have a cardiac-contraction-stop action and to be toxic to animal cells, but antimicrobial activity has not been reported. The number and arrangement of cysteine residues in *Pp*-AMP 2 were in complete agreement with those of the mistletoe toxins. *Pp*-AMP 1 differed from these toxins in the number of cysteine residues, but was similar in arrangement of cysteine residues. *Pp*-AMP 1 and *Pp*-AMP 2 had a higher homology, about 70%, to the mistletoe toxins in whole amino acid sequences than to the thionin type (about 40%). The present study shows the diversity of the plant defensin family with regard to the structural features.

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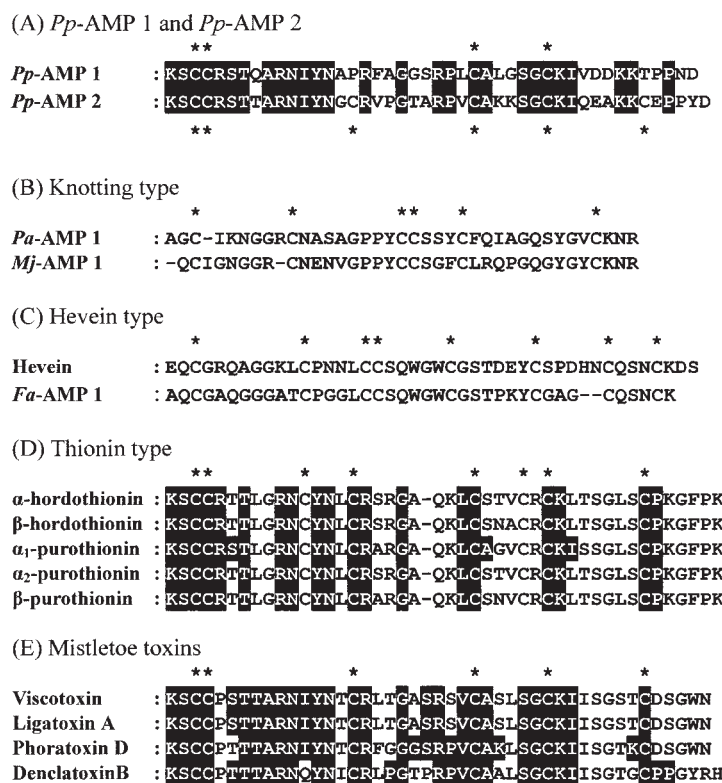


Fig. 2. Sequence Comparison of *Pp*-AMPs with Various Antimicrobial Peptides and Mistletoe Toxins.

The amino acid sequences of *Pp*-AMP 1 and *Pp*-AMP 2 were compared with those of various antimicrobial peptides and mistletoe toxins. Cysteine residues are emphasized by asterisks. Horizontal bars indicate gaps introduced for optimal alignment of the sequences. (A) Amino acid sequences of *Pp*-AMP 1 and *Pp*-AMP 2. The full-length amino acid sequences of these were analyzed with an Applied Biosystems Procise 492 protein sequencer. Identical amino acids are marked in black. (B) Knotting type. This contains six cysteine residues and a continuous sequence of cysteine (–CC–) in the center of the molecules. *Pa*-AMP 1, antimicrobial peptide from pokeweed seed; *Mj*-AMP 1, antimicrobial peptide from nyctaginaceae seed. (C) Hevein type. This contains eight cysteine residues and a continuous sequence of cysteine (–CC–) in the center of the molecules. Hevein, antimicrobial peptide from rubber latex; *Fa*-AMP 1, antimicrobial peptide from buckwheat seed. (D) Thionin type. This contains eight cysteine residues and a continuous sequence of cysteine (–CC–) in the N-terminus region. Common amino acids of *Pp*-AMP 1, *Pp*-AMP 2, and the thionin type are marked in black. (E) Mistletoe toxins. Common amino acids of *Pp*-AMP 1, *Pp*-AMP 2, and the mistletoe toxins are marked in black.

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