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A new antifungal peptide from the seeds of *Phytolacca americana*: characterization, amino acid sequence and cDNA cloning

Feng Shao [a], Zhong Hu [b], Yu-Mei Xiong [a], Qin-Zao Huang [b], Chun-Guang Wang [a], Rong-Huan Zhu [a], Da-Cheng Wang [a,*]

[a] *Institute of Biophysics, Chinese Academy of Sciences, Beijing, 100101 China*
[b] *Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, 650204 China*

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Abstract

An antifungal peptide from seeds of *Phytolacca americana*, designated PAFP-s, has been isolated. The peptide is highly basic and consists of 38 residues with three disulfide bridges. Its molecular mass of 3929.0 was determined by mass spectrometry. The complete amino acid sequence was obtained from automated Edman degradation, and cDNA cloning was successfully performed by 3′-RACE. The deduced amino acid sequence of a partial cDNA corresponded to the amino acid sequence from chemical sequencing. PAFP-s exhibited a broad spectrum of antifungal activity, and its activities differed among various fungi. PAFP-s displayed no inhibitory activity towards *Escherichia coli*. PAFP-s shows significant sequence similarities and the same cysteine motif with Mj-AMPs, antimicrobial peptides from seeds of *Mirabilis jalapa* belonging to the knottin-type antimicrobial peptide.

Keywords: Antifungal peptide; Knottin-like peptide; Amino acid sequence; cDNA cloning

1. Introduction

Although plants normally grow on substrates that are extremely rich in microorganisms, infections remain rare events. This is because plants have evolved highly effective defense mechanisms, to restrict the growth of microorganism inside their tissues. A wide array of antimicrobial peptides or proteins, either produced in a constitutive or in an inducible manner, are believed to be involved in such mechanisms. To date, many plant peptides or proteins with antimicrobial activity in vitro have been identified. These peptides or proteins include chitinases [1], chitin-binding proteins (including hevein-like peptides [2–4]), β-1,3-glucanases [5], thionins [6,7], permatins [8], thaumatin-like proteins [9,10], PR-1 type proteins [11], lipid-transfer proteins [12], plant defensins [13] and knottin-like peptides [14]. Among these proteins, thionins, plant defensins, hevein-like and knottin-like peptides are all small (29–54 residues), highly basic ($pI > 10$) and cysteine-rich peptides, but they are highly divergent at the primary structure and exhibit quite different antimicrobial activities. While many kinds of thionins and plant defensins have been identified and studied thoroughly [15,16], hevein and knottin-like peptides, which have a similar antimicrobial spectrum and share the same cysteine motif (C-C-CC-C-C) and cysteine connectivities, have been

* Corresponding author. Fax: +86-10-6487-1293; E-mail: wdc@pewdc.ibp.ac.cn

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investigated less intensively. The only knottin-like antimicrobial peptide reported so far are two highly homologous antimicrobial peptides Mj-AMP1 and Mj-AMP2 (36 and 37 residues, respectively) from *Mirabilis jalapa* L. seeds [14,17].

We have isolated a new antifungal peptide from the seeds of *Phytolacca americana* (Pokeweed), named PAFP-s, which is related to the knottin-like antimicrobial peptides from *M. jalapa*. Although PAFP-s shows 57% sequence homology with Mj-AMPs, there are significant differences in antifungal activity between them. Excepting the strong activities to certain saprophytic fungi, PAFP-s also inhibited some plant pathogens such as *Fusarium oxysporum*, *Fusarium graminearum*, *Alternaria tenuis* and *Pyricularia oryzae*. In the present paper we describe the purification, antifungal activity assays, complete amino acid sequence and also cDNA cloning of PAFP-s. This work will enrich the antimicrobial peptide family and may broaden our insight into the plant defense mechanism.

2. Materials and methods

2.1. Isolation and purification of PAFP-s

Fresh ripe berries of pokeweed were rubbed in water to separate the seeds from the fruit juice. Seeds were dried at room temperature. Dried seeds (100 g) were ground and extracted with 0.8 l of distilled water at 4°C for 12 h. After filtration and centrifugation at $10\,000\times g$ for 30 min, the supernatant was passed through a CM-Sephadex C-50 column (4.0×20 cm, Pharmacia) equilibrated with 0.05 mol/l sodium phosphate buffer (pH 7.0). The flow-through solution was discarded. The column was washed with 0.1 mol/l and 0.5 mol/l NaCl in the equilibrating buffer in a stepwise fashion. The fraction eluted with 0.5 mol/l NaCl was dialyzed against distilled water at 4°C and centrifuged at $12\,000\times g$ for 20 min. After the precipitate was removed the supernatant was concentrated by ultrafiltration and applied to a gel filtration column of Sephadex G-50 Fine (2.5×60 cm, Pharmacia), eluted with 0.1 mol/l NaCl. Two fractions G1 and G2 which eluted at 80 ml and 140 ml, respectively, were obtained. The G2 was loaded on a CM-cellulose 52 column (2.5×20 cm, Whatman) equilibrated with 0.05 mol/l sodium phosphate buffer (pH 7.2). The column then was washed with an 800-ml linear gradient of 0.1–0.5 mol/l NaCl in the same buffer. Five peaks were obtained. The fourth fraction (C4) eluted at 0.3 mol/l NaCl showing the highest antifungal activity was pooled and dialyzed against 0.05 mol/l NH_4Ac (pH 6.5) at 4°C for 48 h. Then the protein solution could be further purified by chromatography on cation exchange HPLC on an S-Hyper D10 column (Beckman) eluted with a linear gradient of 0.05–0.2 mol/l NH_4Ac (pH 6.5). The single main peak eluted is PAFP-s. The purity of PAFP-s was checked by chromatography on a SMART Mini-S PC 3.2/3 (Pharmacia) column, eluted with a linear gradient of NaCl in 20 mmol/l MES, pH 6.0.

2.2. Antifungal activity assays

The antifungal potency of the PAFP-s was examined [18] on different species of fungi including *Trichoderma viride*, *Armillaria mellea*, *Morchelia conica*, *Fusarium oxysporum*, *Fusarium graminearum*, *Alternaria tenuis* and *Pyricularia oryzae*. Spores were inoculated onto the center of petri dish plate, containing potato–glucose–agar medium (PGA) of 1 mm in thickness, incubated at 25°C for about 25 h. Aseptic solution of tested sample was prepared in 0.05 mol/l sodium phosphate buffer, pH 6.5. Aliquots of 5 μl of sample solution were dripped onto the spots at a distance of 0.5 cm in front of the growing mycelia. The plates were then incubated at 25°C for 8–24 h. The antifungal activity was shown by the occurrence of visible zone of inhibited mycelia growth. Quantitative estimation of the antifungal activity was carried out by the following procedure. An aseptic solution with the peptide concentration of 5 mg/ml buffered at 0.05 mol/l sodium phosphate (pH 6.5) was prepared; then 40 μl, 20 μl, 10 μl, 5 μl, 2.5 μl and 0 μl, respectively, of the prepared peptide solution were mixed with 0.2 ml of PGA medium and immediately poured onto a small dish of 1.4 cm diameter. Thus a series of agar plates with protein concentration of 1, 0.5, 0.25, 0.125 and 0.065 mg/ml as blank were prepared. A small drop of the mycelia suspension was put onto the center of each dish, and then incubated at 25°C. When the mycelia of the blank grew and arrived at the edge of the plate, the

diameter of mycelia area in each dish was measured. From the average diameter of three repeated determinations, the doses in μg/ml of 50% inhibition of mycelia extension (IC_{50}) were given.

2.3. Mass spectrometry analysis

Mass spectrometry analysis of PAFP-s was performed on a Finnigan LCQ-MS, an instrument that essentially consists of an atmospheric pressure electrospray positive-ion source, attached to a triple-quadrupole mass analyzer. The purified peptide (100 pmol) was dissolved in water/methanol (50:50, v/v) containing 1% (v/v) acetic acid at a peptide concentration of 5 μmol/l, and then applied on the MS instrument.

2.4. Amino acid sequencing

After the purified peptide was reduced and alkylated as the method described by Fullmer [19], automated Edman degradation of the pridylethylated peptide and detection of phenylthiohydantoin derivatives were performed on a pulse liquid automatic sequenator (Applied Biosystems model 491). The amino acid sequence of 38 residues was obtained from the N-terminal sequencing, thereafter the sequencer response dropped abruptly. After treatment of the *s*-carboxymethylated PAFP-s with trypsin, four tryptic peptides were obtained, which were then also subjected to the sequenator and sequenced by the same method described above.

2.5. cDNA cloning by 3′-RACE and DNA sequencing

Corresponding to the N-terminal amino acid sequence (AGCIKNG–) of PAFP-s, a primer (5′-CGGAATTC GCC/A GGA/T TGC/T ATA/T AAG/A AAT/C GG-3′) with an additional *Eco*RI restriction site was designed and synthesized. 3′-RACE (Gibco BRL, USA) was performed as recommended by supplier's instruction. At first, 5 μg total RNA isolated from the near-mature seeds of pokeweed was reverse transcripted to cDNA with Super-Script II reverse transcriptase. Then using 0.5 μg of cDNA as template, the first polymerase chain reaction (PCR) was performed with the PAFP-s N-terminal specific primer and the adapter primer (5′-GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TTT T-3′) provided by the 3′-RACE kit. The thermal cycle profile was: 94°C, 1 min; 50°C, 1 min; 72°C, 1 min. Afterwards the second PCR were performed with the first PCR product as template and the following two primers: the PAFP-s N-terminal specific primer and the abridged universal amplification primer (5′-GGC CAC GCG TCG ACT AGT AC-3′). The thermal cycle profile was: 94°C, 40 s; 54°C, 40 s; 72°C, 1 min. Finally the PCR product was directly cloned into the *Sma*I site of M13mp19 vector. The single-strand DNA of positive clones was extracted and sequenced as described in the protocol of T7 sequenase version 2.0 DNA sequencing kit (Amersham) with labeled nucleotide ([α-^{35}S]dATP). All PCR experiments were performed on a Perkin–Elmer GeneAmp PCR System 480.

3. Results and discussion

3.1. Characterization of PAFP-s

Following our purification procedure, i.e., four steps of chromatography on CM-Sephadex C-50, Sephadex G-50, CM-Cellulose 52 and S-Hyper D10 successively, the overall recovery of PAFP-s was approximately 10 mg per 100 g dry seeds. During the

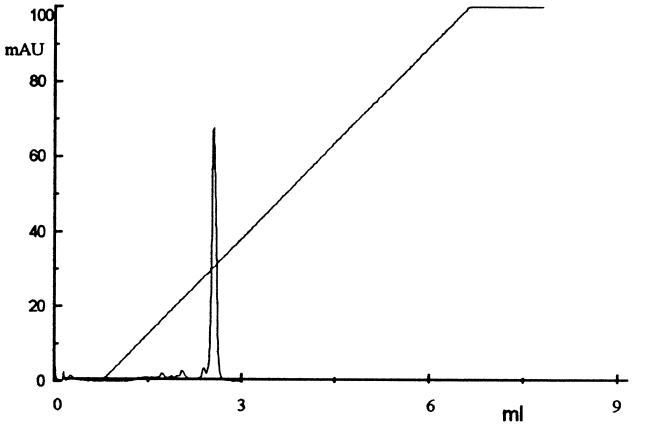

Fig. 1. Purity analysis of PAFP-s on SMART Mini-S. Fifty μg of the purified PAFP-s (dissolved in 100 μl 20 mmol/l MES, pH 6.0) was applied on a Mini-S PC 3.2/3 column (Pharmacia) on the SMART (Pharmacia) system. The column was eluted at a flow rate of 200 μl/min with a linear gradient of 5 ml from 0 to 1 mol/l NaCl in 20 mmol/l MES (pH 6.0). The eluate was monitored for absorbance at 280 nm.

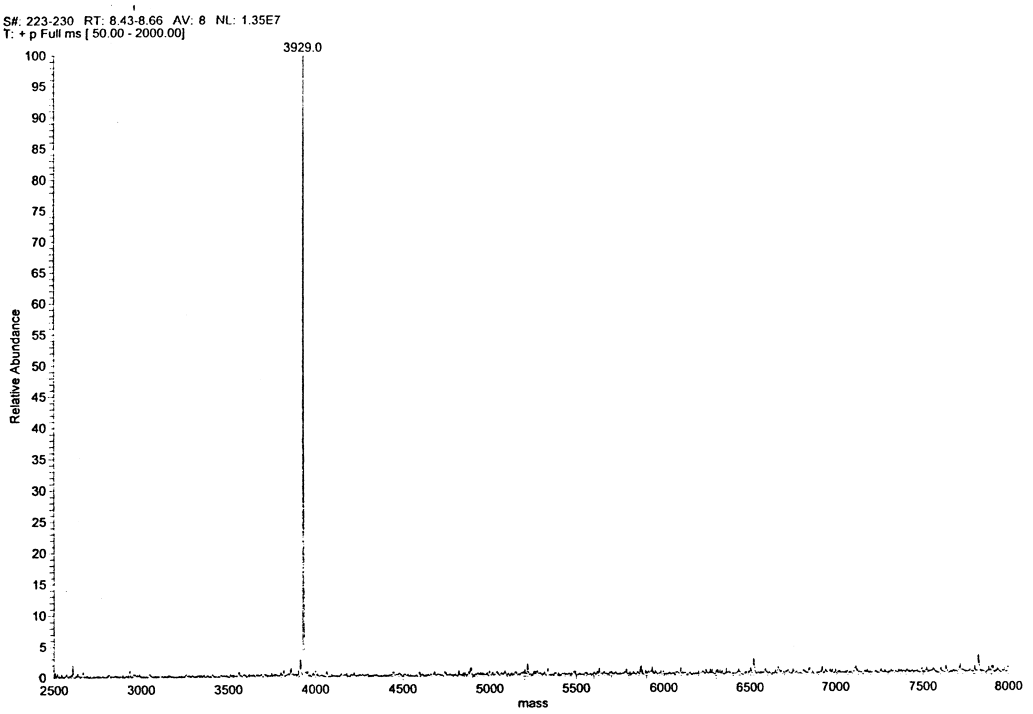

Fig. 2. Mass spectrometry analysis of PAFP-s on Finnigan LCQ-MS. 100 pmol of the purified peptide was dissolved in water/methanol (50:50, v/v) containing 1% (v/v) acetic acid at a peptide concentration of 5 μmol/l. The determined molecular mass by spectrometry is 3929.0.

whole procedure the eluate was monitored for absorbance at 280 nm and the active fraction was traced by the antifungal assay method described above. The purified peptide yielded a single well-resolved peak on the SMART Mini-S chromatography column (Fig. 1) and the purity is about 95%, calculated from the software UNICORN (Pharmacia).

Like other cysteine-rich antifungal peptides, PAFP-s is also highly basic, and its isoelectric point (p*I*) is 10 as determined by isoelectric focusing PAGE (data not shown). The molecular mass obtained from the mass spectrometry analysis is 3929.0 (Fig. 2), and the mass calculated from the 38 residues determined by sequencing (see Fig. 4) is 3935.5, i.e., in excess of 6 Da to that measured by mass spectrometry. This difference of 6 Da is attributable to the arrangement of six cysteine residues into three intramolecular disulfide bridges, a process which eliminates six hydrogen atoms.

Interestingly, although the purity of PAFP-s was high as judged by sequencing, SMART Mini-S chromatography and mass spectrometry, two inseparable peaks were obtained upon HPLC chromatography on C8 Sephasil Peptide 5 μm ST 4.6/250 analytic column (Pharmacia) (data not shown). The two peaks showed the same sequence on the sequenator. Two possible explanations, conformation conversion or aggregation of PAFP-s, could be considered. Given that PAFP-s has a small size and three disulfide bonds, it must have a compact three-dimensional folding. Therefore its conformation is unlikely to be easily changed. On the other hand, PAFP-s is highly soluble (200 mg/ml) and elutes at a relatively low acetonitrile concentration (18%) on reverse-phase HPLC, i.e., this peptide is relatively hydrophilic, notwithstanding that its sequence is rich in hydrophobic residues (see Fig. 4). We presume that PAFP-s molecules may aggregate in polar solvent to hide the hydrophobic residues. One of the two peaks might represent the aggregated form of PAFP-s, while the other may result from dissociation of the aggregate because of acetonitrile solution's hydrophobicity. This phenomenon of aggregation was also found in Mj-AMPs [14].

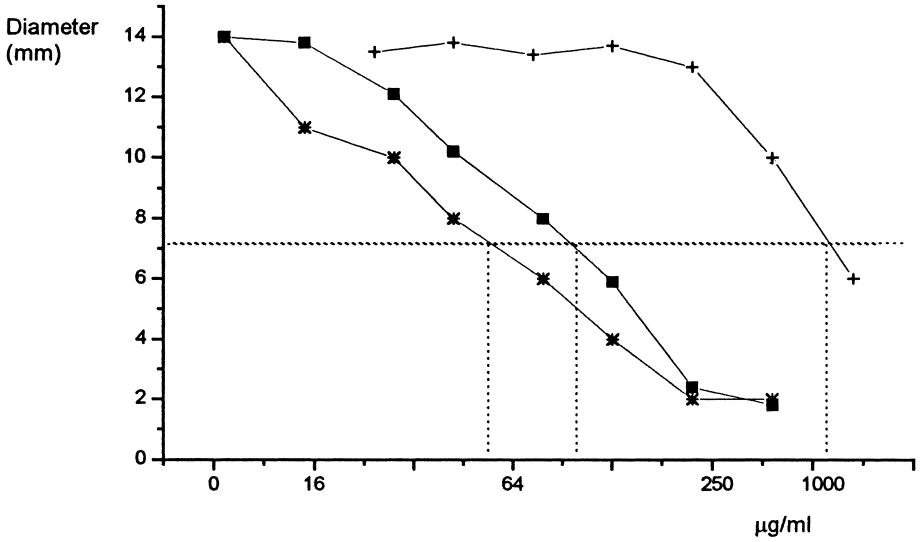

Fig. 3. Inhibition of fungi by PAFP-s and IC_{50} determination. The ordinate shows the diameter (mm) of the growing mycelia zone on the plates containing the peptide at different concentration (μg/ml) shown on the abscissa. The IC_{50} doses of PAFP-s to *T. viride* (*), *F. oxysporum* (■) and *P. oryzae* (+) were 48 μg/ml, 100 μg/ml and 900 μg/ml, respectively.

3.2. Bioassays

PAFP-s is thermostable, and its antifungal activity was not affected even by heat treatment at up to 95°C for 30 min. PAFP-s caused a visible inhibition of the growth of *T. viride* at an agar-based medium at a minimal dose of 4 μg/ml of the peptide in the medium. PAFP-s can also inhibit the growth of a mushroom *M. conica*, *F. oxysporum* (a pathogen of cotton) and *P. oryzae* (a pathogen of rice) at 40 μg/ml. The spectrum of antifungal activity of PAFP-s seems to be rather wide. The strongest antifungal activity was found towards the saprophytic fungi such as *T. viride*, *A. mellea* and *M. conica* with IC_{50} of 48 μg/ml. To some plant pathogens such as *F. oxysporum*, *F. graminearum* and *A. tenuis*, PAFP-s also exhibited inhibitory activities with an IC_{50} of about 100 μg/ml. The antifungal activity to *P. oryzae* was weaker with an IC_{50} of more than 500 μg/ml. The IC_{50} determinations are shown in Fig. 3. Besides this, in a liquid culture medium, PAFP-s at 500 μg/l was found not to inhibit the multiplication of bacteria *E. coli* [20], which is the same as Mj-AMPs. Since the method used to quantify the antifungal activity is

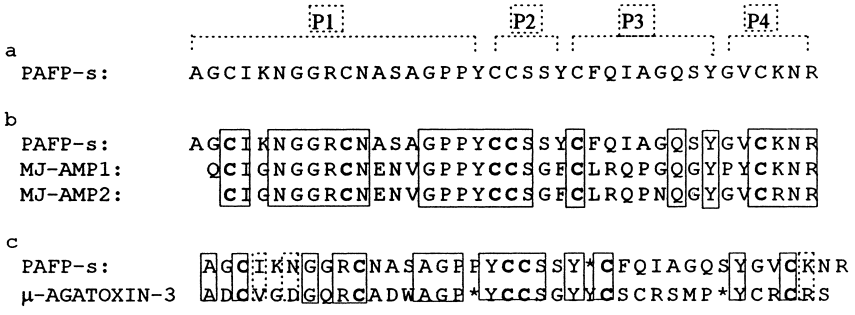

Fig. 4. Amino acid sequence of PAFP-s and comparison with related peptides. (a) The amino acid sequence of PAFP-s was determined by automated Edman degradation. The four tryptic peptides (P1, P2, P3, P4) can make up the whole sequence. (The sequence has been deposited at the SWISS-PROT database with the accession number P81418). (b) Sequence comparison of PAFP-s and Mj-AMPs [14] shows 57% homology and the same cysteine motif. The identical residues are boxed. (c) Alignment of the amino acid sequence of PAFP-s and μ-agatoxin 3 [17], a neurotoxin from spider venom. Sixteen identical residues are boxed with a solid line and three conserved changes are boxed with a dotted line.

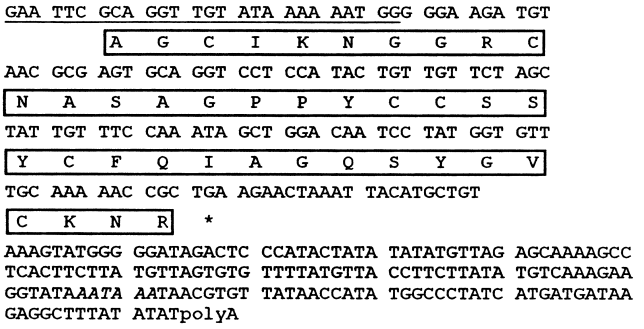

Fig. 5. The nucleotide sequence and deduced amino acid sequence of the partial cDNA encoding PAFP-s. The sequence was determined from 3′-RACE products and from cDNA amplified by reverse transcription–PCR. The primer is underlined. Asterisks show the termination codon. The deduced amino acid sequence corresponding to mature PAFP-s is boxed.

different from that of Mj-AMPs, we cannot compare the activities between them. However, we still can see that they exhibit a relatively broad antifungal spectrum and high degree of antifungal selectivity against different species of fungi.

3.3. Amino acid sequence and cDNA sequence

The sequence of 38 residues obtained by N-terminal sequencing of PAFP-s is shown in Fig. 4a. Since only four tryptic peptides (peptides 1–4, Fig. 4a) were obtained after trypsin treatment and also since they can be assembled into a sequence in consistency with the N-terminal sequencing, the sequence of 38 residues must be the complete sequence of PAFP-s. The accuracy of the sequence was also confirmed by the cDNA sequence (see below) and by mass spectrometry (see above). The first residue of the sequence is not methionine; therefore PAFP-s is probably synthesized as part of a larger precursor.

3′-RACE produced one major DNA fragment about 300 bp. Unfortunately, it is only a partial cDNA missing some information on the N-terminal part of the PAFP-s precursor. Whether PAFP-s is also synthesized as a pre-protein like Mj-AMPs awaits further investigation. Sequencing of this cDNA showed that it encoded a peptide of 38 residues (Fig. 5). The deduced amino acid sequence was identical with that determined by protein sequencing. This partial cDNA clone could be used for a mutational analysis of PAFP-s, which will allow the study of the structure–function relationship of PAFP-s.

3.4. Sequence comparison

Although PAFP-s, thionins, and defensins are all highly basic, small-sized, contain three or four disulfide bridges, and have similar biological activities [6,15], there is no sequence homology among them. Therefore PAFP-s could not be classified into these two classes of antimicrobial peptides. Sequence similarities between PAFP-s and known proteins were searched (June 1998) using the NCBI BLAST electronic mail servers. The result shows 57% sequence identity and the same cysteine motif between PAFP-s and Mj-AMPs (Fig. 4b). Thus PAFP-s should be considered as a new member of the knottin-like antimicrobial peptides. Interestingly, there are also significant similarities between PAFP-s and μ-agatoxin [21], a class of neurotoxic peptides from spider venom. Sequence alignment (Fig. 4c) of PAFP-s and μ-agatoxin 3, allowing three gaps, showed 16 identical residues and three conserved changes in the first 36 residues. Information on the three-dimensional structure may be helpful in elucidating the molecular mechanism of antifungal activity of the knottin-type antimicrobial peptides. However, to date there are no reports on the three-dimensional structure of knottin-type antimicrobial peptides. The results reported here provide a sound base to perform NMR structural analysis for PAFP-s, and this is already in progress in our laboratory.

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