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Antimicrobial properties of derivatives of the cationic tryptophan-rich hexapeptide PAF26

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Abstract

Short antimicrobial peptides represent an alternative to fight pathogen infections. PAF26 is a hexapeptide identified previously by a combinatorial approach against the fungus *Penicillium digitatum* and shows antimicrobial properties towards certain phytopathogenic fungi. In this work, PAF26 was used as lead compound and its properties were compared with two series of derivatives, obtained by either systematic alanine substitution or N-terminal amino acid addition. The alanine scan approach underlined the optimized sequence of PAF26 in terms of potency and permeation capability, and also the higher contribution of the cationic residues to these properties. The N-terminal addition of amino acids resulted in new heptapeptides with variations in their antimicrobial characteristics, and very low cytolysis to human red blood cells. Positive (Arg or Lys) and aromatic (Phe or Trp) residue addition increased broad spectrum activity of PAF26. Noteworthy, addition of selected residues had specific effects on the properties of derivatives of PAF26.

Keywords: Tryptophan-rich cationic antimicrobial peptide; AMP; Antifungal peptide; Phytopathogenic fungi; *P. digitatum*; *B. cinerea*; *M. grisea*; *F. oxysporum*

Antimicrobial peptides (AMP) are important components of an evolutionarily ancient mechanism of immunity, found in a wide range of organisms [1]. AMP differ in length, sequence, and structure, but generally are amphipathic and a great number have positive charge and are refered as cationic antimicrobial peptides (CAMP). In many examples, these peptides are effective against microorganisms resistant to antibiotics or fungicides. In addition, AMP are unlikely to cause rapid emergence of resistance [2]. These facts and their short length, fast and efficient action against microbes, and low toxicity to mammal cells have made them potential candidates as peptide drugs.

Rational design of AMP is an attractive approach to the improvement of antimicrobial properties. Agriculture could also greatly benefit from this emerging research area, with the identification, design, and selection of peptides targeted to specific plant protection problems [3–6]. Soluble combinatorial libraries (SCL) represent an extensive source of molecular diversity for the *de novo* identification of lead AMP with new properties [7]. SCL have been used to identify novel peptides towards phytopathogenic fungi such as 66–10 hexapeptide (Ac-frlkfh-NH_2) [8] and its derivative heptapeptide 77–3 (Ac-frlkfhf-NH_2), which has activity against fungal strains of *Fusarium sambucinum* that are resistant to the fungicide thiabendazole (TBZ) [5]. In a previous work, we have used a synthetic D-hexapeptide library in a positional scanning format to identify AMP against selected phytopathogenic fungi that cause postharvest decay in fruits, such as *Penicillium digitatum* [6]. One of these peptides is PAF26 (Table 1), which showed strong activity against certain filamentous fungi and lower toxicity to *Escherichia coli* and *Saccharomyces cerevisiae* [6].

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Table 1
Amino acid sequences of peptides

Peptide	Sequence[a]
PAF26	Ac–rkkwfw–NH_2
PAF34	Ac–rk**wl**fw–NH_2
PAF26.r1a	Ac–**a**kkwfw–NH_2
PAF26.k2a	Ac–r**a**kwfw–NH_2
PAF26.k3a	Ac–rk**a**wfw–NH_2
PAF26.w4a	Ac–rkk**a**fw–NH_2
PAF26.f5a	Ac–rkkw**a**w–NH_2
PAF26.w6a	Ac–rkkwf**a**–NH_2
PAF38	Ac–**r**rkkwfw–NH_2
PAF39	Ac–**k**rkkwfw–NH_2
PAF40	Ac–**h**rkkwfw–NH_2
PAF41	Ac–**f**rkkwfw–NH_2
PAF42	Ac–**w**rkkwfw–NH_2
PAF43	Ac–**y**rkkwfw–NH_2
PAF44	Ac–**l**rkkwfw–NH_2
PAF45	Ac–**t**rkkwfw–NH_2
PAF46	Ac–**q**rkkwfw–NH_2
PAF47	Ac–**a**rkkwfw–NH_2

[a] The D-amino acids are shown in lower case. Residues distinct from PAF26 are in bold.

PAF26 is a tryptophan-rich CAMP with sequence similarities to other AMP [9–13]. It shares some properties with similar peptides, as absence of hemolytic activity [12,14]. PAF26 is active against strains resistant to fungicides and performed better than TBZ in experimental fruit decay tests [15]. Additionally, we have also demonstrated that PAF26 belongs to the class of AMP endowed with cell-penetrating properties [16,17], being capable to specifically interact with and locate inside target fungal cells [14]. PAF26 and similar peptides synthesized with either D- or L-enantiomers do not differ substantially in antimicrobial potency [9,11,14], which makes biotechnological production feasible.

In this work, we have used PAF26 as a lead in an optimization strategy to design two sets of peptides with single residue variations. The purpose was to analyze the effect of such variations in the antimicrobial properties of the resulting peptides. First, alanine substitution analogues addressed the influence of each residue on PAF26 antimicrobial properties. Second, we designed and compared novel heptapeptides obtained by addition of different N-terminal residues to PAF26 in terms of (i) spectrum of activity, (ii) specificity, (iii) microbicidal properties, and (iv) cytolysis of human red blood cells.

Materials and methods

Microorganisms. We used microorganisms that included fungal isolates of agricultural relevance (three distinct species of *Penicillium*, and *Alternaria* sp., *Fusarium oxysporum*, *Botrytis cinerea* and *Magnaporthe grisea*) as well as fungal (*Aspergillus nidulans*), yeast (*S. cerevisiae*) and bacterial (*E. coli* and *Bacillus subtillis*) model strains (see Supplemental Table 4). Fungi were cultured on potato dextrose agar (PDA) (Difco-BD Diagnostics, Sparks, MD) plates at 24 °C with the exception of *M. grisea*, which was maintained on rice flour medium. Conidia were collected and adjusted to the appropriate concentration. *S. cerevisiae* was grown in YPD (1% yeast extract, 1.5% peptone, 2% dextrose) at 30 °C and bacteria were grown in Luria–Bertani (LB) medium at 37 °C.

Peptides. Peptides used in this work (Table 1 and Supplemental Table 3) were purchased at >90% purity (GenScript Corporation, Piscataway, NJ). Peptides were acetylated at the N-terminus (Ac) and amidated at the C-terminus (NH_2). Stocks were prepared at 1 mM in 5 mM 3-(*N*-morpholino)-propanesulfonic acid, pH 7, buffer and stored at −20 °C. Peptide concentrations were determined by absorbance at 280 nm.

Growth inhibition assays. The antimicrobial activities of the peptides were determined using a microtiter plate assay [6,18]. Growth was quantified as optical density (OD) at 492 nm. Potato dextrose broth (PDB) (Difco-BD Diagnostics) diluted one twentieth (5% PDB) was used as growth medium for fungi, and YPD diluted one tenth (10% YPD) for yeast, in both cases containing 0.003% (w/v) chloramphenicol. In antibacterial assays, the medium was LB diluted one tenth (10% LB). Three replicates were prepared for each treatment.

The minimum inhibitory concentration (MIC) of a peptide for a given microorganism was the lowest peptide concentration that showed no growth at the end of the experiment. The IC_{50} of a peptide was the concentration required to obtain 50% inhibition of growth, and the value in each experiment was estimated by adjustment of the experimental data (SigmaPlot v 8.02, SPSS Inc., Chicago, IL). Statistical analyses were carried out with the software package StatGraphics Plus 4.0 (Manugistics Inc., Rockville, MD).

Membrane permeation assays. Membrane permeation was determined with the probe Sytox Green (SG) (Molecular Probes-Invitrogen Corp., Carlsbad, CA) and fluorometric measurement with a microplate reader (Fluoroskan Ascent FL, Labsystems, Finland) at an excitation of 485 nm and emission of 538 nm wavelengths [14]. Three replicates were prepared for each treatment. The FC_{50} of a peptide was defined as the concentration inducing 50% of the maximum fluorescence emission, and the values were calculated by adjustment of the experimental data as above.

Fungicidal and bactericidal activity assays. Assessment of peptide microbicidal activity was conducted as follows. In the case of *P. digitatum*, 2.5×10^4 conidia/ml were incubated with peptides in 5% PDB at 24 °C. After 1 day of incubation, 50 µl samples were spread onto peptide-free PDA plates to monitor colony forming units. *S. cerevisiae* and *E. coli* (5.0×10^5 CFU/ml) were incubated for 1 day with peptides in either 10% YPD at 30 °C or 10% LB at 37 °C, respectively, and 2.5 µl drops of samples were placed onto YPD or LB peptide-free plates. The lethal concentration (LC) of a peptide was defined as the lowest peptide concentration at which no growth or <1% of CFU was recovered after peptide treatment.

Hemolytic activity assay. The cytolytic activity of the peptides on human red blood cells was determined as release of hemoglobin monitored by absorbance at 415 nm [14]. Peptides were used at final concentrations of 1, 10 or 100 µM. Zero percent hemolysis and 100% hemolysis controls were determined in PBS and 0.1% Triton X-100, respectively.

Results and discussion

Antimicrobial properties of a series of alanine substitution analogues of PAF26

We designed a set of six Ala substitution analogues of the cationic tryptophan-rich hexapeptide PAF26 (PAF26.r1a to PAF26.w6a, Table 1 and Supplemental Table 3). Distinct antimicrobial properties were determined and the results are summarized as IC_{50}, MIC and LC towards *P. digitatum* (Table 2). We observed lower activity for all the analogues, although the decrease was higher in the peptides with substitution of the positively charged residues (PAF26.r1a, .k2a, and .k3a), which approximately