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Synthesis and antimicrobial activity of cysteine-free coprisin nonapeptides

Jaeho Lee [a], Daeun Lee [a], Hyemin Choi [b], Ha Hyung Kim [c], Ho Kim [d], Jae Sam Hwang [e], Dong Gun Lee [b], Jae Il Kim [a,*]

[a] School of Life Sciences, Gwangju Institute of Science and Technology, Oryong-dong, Buk-gu, Gwangju 500-712, Republic of Korea
[b] School of Life Sciences and Biotechnology, College of Natural Sciences, Kyungpook National University, Daehak-ro 80, Buk-gu, Daegu 702-701, Republic of Korea
[c] College of Pharmacy, Chung-Ang University, Seoul, Republic of Korea
[d] School of Life Sciences and Biotechnology, College of Natural Science, Daejin University, Pocheon, Gyeonggido, Republic of Korea
[e] Department of Agricultural Biology, Natural Academy of Agricultural Science, RDA, Suwon 441-853, Republic of Korea

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ABSTRACT

Coprisin is a 43-mer defensin-like peptide from the dung beetle, *Copris tripartitus*. CopA3 (LLCIALRKK-NH_2), a 9-mer peptide containing a single free cysteine residue at position 3 of its sequence, was derived from the α-helical region of coprisin and exhibits potent antibacterial and anti-inflammatory activities. The single cysteine implies a tendency for dimerization; however, it remains unknown whether this cysteine residue is indispensible for CopA3's antimicrobial activity. To address this issue, in the present study we synthesized eight cysteine-substituted monomeric CopA3 analogs and two dimeric analogs, CopA3 (Dimer) and CopIK (Dimer), and evaluated their antimicrobial effects against bacteria and fungi, as well as their hemolytic activity toward human erythrocytes. Under physiological conditions, CopA3 (Mono) exhibits a 6/4 (monomer/dimer) molar ratio in HPLC area percent, indicating that its effects on bacterial strains likely reflect a CopA3 (Mono)/CopA3 (Dimer) mixture. We also report the identification of CopW, a new cysteine-free nonapeptide derived from CopA3 that has potent antimicrobial activity with virtually no hemolytic activity. Apparently, the cysteine residue in CopA3 is not essential for its antimicrobial function. Notably, CopW also exhibited significant synergistic activity with ampicillin and showed more potent antifungal activity than either wild-type coprisin or melittin.

1. Introduction

The increasing prevalence of pathogenic bacteria resistant to conventional antibiotics has prompted an intensive search for new antibacterial agents [1,2]. Antimicrobial peptides (AMPs) are attractive in this regard because they mainly kill through global actions at bacterial membranes and do not interact with specific intercellular components, which reduces the likelihood that resistance will develop [3,4]. AMPs are found in a wide variety of organisms including plants, insects, invertebrates and mammals, and are classified into four categories based on their sequence and structure: amphipathic α-helical molecules, extended disordered molecules, cyclic peptide with a single disulfide bond, and cysteine-rich β-sheet molecules [5]. One representative AMP group is the defensin family, members of which contain three or four intramolecular disulfide bonds. Defensins identified to date include sapecin A/B, drosomycin, heliomicin, defensin A and lucifensin [6–10]. Widely distributed in diverse insect species, these molecules exhibit a broad antimicrobial spectrum against bacteria, fungi and enveloped viruses [11].

Another insect defensin, coprisin, was recently isolated from *Copris tripartites* [12]. Its NMR solution structure reveals the presence of the conserved cysteine-stabilized α-helix/*β-sheet* motif frequently seen in the three-dimensional structures of insect defensins [13]; it has an amphipathic α-helical structure from Ala^{19} to Arg^{28} and β-sheets from Gly31 to Gln35 and Val38 to Arg42. Our research group examined the structure–activity relationship of coprisin in an effort to understand its antimicrobial action and to provide insight into the mechanism of action of an insect defense system [12–17]. In addition, we also designed CopA3 (LLCIALRKK-NH_2), a nonapeptide derived from the α-helical region of coprisin, and showed that CopA3 has an effective spectrum of biological properties, including antimicrobial, antifungal, anticancer and anti-inflammatory activities [14–17]. Notably,

Abbreviations: AMP, antimicrobial peptide; ATCC, American Type Culture Collection; CLSI, Clinical and Laboratory Standards Institute; HPLC, high performance liquid chromatography; KCTC, Korean Collection for Type Cultures; LPS, lipopolysaccharide; PBS, phosphate buffered saline.

* Corresponding author.
E-mail address: jikim@gist.ac.kr (J.I. Kim).

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CopA3 possesses just a single cysteine residue in its sequence, which prompted us to wonder whether this cysteine is indispensable for the biological function of CopA3. To address that question, in the present study we chemically synthesized a set of cysteine-related CopA3 analogs that included eight monomeric analogs and two dimeric analogs and tested their antibacterial activity against Gram-negative, Gram-positive and drug-resistant bacteria, as well as their hemolytic activity toward human red blood cells (hRBCs). Our findings indicate that CopW, a cysteine-free nonapeptide derived from CopA3, possesses potent antibacterial and antifungal activities without hemolytic activity, indicating the cysteine residue in CopA3 is not essential for its antimicrobial function.

2. Materials and methods

2.1. Chemical synthesis and oxidative refolding of 9-mer peptides

All peptides were chemically synthesized by Anygen (Gwangju, Republic of Korea) using the solid-phase synthesis, and were then purified by reverse-phase high-performance liquid chromatography (RP-HPLC) on C_{18} columns (20×250 mm; Shim-pack). The purity and molecular masses of the peptides were determined using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Shimadzu, Japan).

2.2. Bacterial strains and antibacterial test

Escherichia coli (KCTC 1682), *Pseudomonas aeruginosa* (KCTC 1637), *Salmonella typhimurium* (KCTC 1926), *Bacillus subtilis* (KCTC 3068), *Staphylococcus epidermidis* (KCTC 1917), and *Staphylococcus aureus* (KCTC 1621) were purchased from the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience & Biotechnology (Daejeon, Republic of Korea). *Enterococcus faecium* (KCCM 12118), *Enterococcus faecalis* (KCCM 29212), and methicillin-resistant *S. aureus* (MRSA) (KCCM 40510) were purchased from the Korean Culture Center of Microorganisms (Seoul, Republic of Korea). Vancomycin-resistant *E. faecalis* (ATCC 51575) and *E. faecium* (ATCC 51559) were purchased from the American Type Culture Collection (Manassas, VA, USA).

Minimal inhibitory concentrations (MICs) were determined using broth microdilution assays. Bacteria were grown to mid-log phase in LB broth and then diluted to 10^6 CFU/ml. Synthetic peptides diluted to final concentrations of 64, 32, 16, 8, 4, 2 and 1 μg/ml were added to the diluted bacteria, after which the mixture was incubated for 20 h at 37 °C. The MIC was defined as the lowest concentration of antibiotic causing complete inhibition of visible growth, as compared to the growth in an antibiotic-free control well. MICs were determined by three independent assays.

2.3. Fungal strains and antifungal susceptibility test

Candida albicans (ATCC 90028) and *Candida parapsilosis* (ATCC 22019) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *Malassezia furfur* (KCTC 7744) and *Trichosporon beigelii* (KCTC 7707) were obtained from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea). Most fungal strains were cultured in YPD broth (Difco) at 28 °C with aeration; *M. furfur* was cultured at 32 °C in modified YM broth (Difco) containing 1% olive oil. Fungal cells at log phase (2×10^6/ml) were inoculated into YPD or YM broth and then dispensed into microtiter plates at 100 μl/well. The MIC values were determined by three independent assays.

2.4. Hemolytic activity

Fresh hRBCs were washed through three cycles of rinsing in phosphate-buffered saline (PBS; 35 mM phosphate containing 10 mM NaCl, pH 7.4). Aliquots (100 μl) of 8% (v/v) hRBCs in PBS were dispensed into 96-well plates, after which 100 μl of the peptide solution were added to give a final hRBC concentration of 4% (v/v). The plates were then incubated for 1 h at 37 °C before centrifugation at 1000*g* for 10 min. Aliquots (100 μl) of the resultant supernatant were then transferred to new 96-well plates, and hemolysis was monitored at 414 nm using an ELISA plate reader (Molecular Devices, Sunnyvale, CA, USA). Controls for 0% and 100% hemolysis were determined in PBS and 0.1% Triton X-100 solution, respectively. The percent hemolysis was calculated using the following formula: $\%\ \text{hemolysis} = [(\text{Abs}_{414\text{nm}}\ \text{in the peptide solution} - \text{Abs}_{414\text{nm}}\ \text{in PBS})/(\text{Abs}_{414\text{nm}}\ \text{in}\ 0.1\%\ \text{Triton X-100} - \text{Abs}_{414\text{nm}}\ \text{in PBS})] \times 100$.

2.5. Peptide-induced permeabilization for E. coli ML35

E. coli strain ML35, a lactose permease-deficient strain with cytoplasmic β-galactosidase activity, was used to monitor permeation of two chromogenic substrates. The lactose analogue 2-nitrophenyl β-D-pyranoside (ONPG) and nitrocefin are chromogenic reporter molecules that cannot cross the inner or outer membranes under normal conditions. Upon membrane disruption, however, nitrocefin and ONPG diffuse into the bacterial periplasmic and cytoplasmic regions, where they are hydrolyzed by β-lactamase and β-galactosidase, respectively, causing a color change. Thus cleavage of nitrocefin and ONPG can be monitored by measuring the absorbance at 450 and 490 nm, respectively, using a SpectraMax plate spectrophotometer (Molecular Devices, Sunnyvale, CA). For the assay, a single colony of ML35 was inoculated into 5 ml of LB media. After growth overnight at 37 °C, the bacteria were washed in PBS containing 3% LB and diluted to an OD_{600} of 0.2 (1×10^8 CFU/ml). Aliquots of this suspension (100 μl) were added to all the wells of sterilized 96-well plates. In duplicate wells, bacteria were exposed to the test peptides in the presence of 2.5 mM ONPG and 30 μM nitrocefin for 1 h at 37 °C, during which hydrolysis of ONPG and nitrocefin was monitored as described above. Readings were taken every 2 min.

2.6. Killing kinetics

Exponential phase *S. aureus* and *E. coli* were washed three times with PBS and resuspended to 10^6 CFU/ml. CopW was then added to the cells to final concentrations of 16 or 32 μg/ml and 0.5×10^6 CFU/ml. This mixture was incubated for 2 h at 37 °C, after which 100-μl aliquots were collected at timed intervals. Each aliquot was diluted 1000-fold with PBS, and 100 μl of each diluent was spread on an LB agar plate and incubated for 16 h. Cell viability was then assessed by counting the resultant colonies on the plates.

2.7. Measurement of NO in conditioned culture medium

Nitric oxide (NO) levels in the culture supernatants were measured using the Griess reaction. Cells from the RAW264.7 mouse macrophage line were pre-incubated overnight and then plated at a density of 5×10^5 cells/well in 12-well plates. To stimulate the cells, LPS (1 μg/ml) from *E. coli* O111:B4 (Sigma) in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (Invitrogen, Carlsbad, CA, USA) was added to each well with or without CopW. After 24 h, the culture media were collected and mixed with the same volume of Griess reagent [1% sulfanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride, 2% phosphoric acid]. NO production was then quantified by comparing

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