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Antifungal effect of CopA3 monomer peptide via membrane-active mechanism and stability to proteolysis of enantiomeric D-CopA3

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ABSTRACT

In our previous study, coprisin, a 43-mer defensin-like peptide, was derived from the dung beetle, *Copris tripartitus*, and a 9-mer CopA3 (monomer), truncated coprisin analog peptide, was designed. However, the antifungal effects of CopA3 are not known yet. In this study, the antifungal activity and mechanism of CopA3 were investigated and to develop a more effective antimicrobial peptide under physiological conditions, the enantiomeric D-CopA3 was designed. L- and D-CopA3 had a similar antifungal activity without chiral selectivity, and their activity was more potent than that of melittin used as a positive control. Furthermore, L- and D-CopA3 did not even show any hemolysis against human erythrocytes. Membrane studies using propidium iodide and bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$], suggested that the antifungal effect of L- and D-CopA3 was due to the membrane-active mechanism, by contrast with coprisin possessing apoptotic mechanism without membrane permeabilization. Finally, the proteolytic resistance and antifungal activity of L- and D-CopA3 against trypsin was analyzed by HPLC and colony count assay. The results showed that only D-CopA3 maintained a potent antifungal activity despite the proteolytic condition. Therefore, this study suggests that D-CopA3 has potential as a novel antimicrobial agent.

1. Introduction

Infectious disease resulting in increased morbidity, mortality, and health-care costs is becoming a serious problem, mainly due to the emergence of antibiotic-resistant pathogens [1]. This clinical problem needs promising candidates for new antibiotics. Antimicrobial peptides produced by every living organism as a component of innate immunity, have received attention because they have many useful biological properties, including broad-spectrum antimicrobial activity, fast action and slow resistance development [2,3]. Although the mechanism of action of antimicrobial peptides has not been shown clearly, it is believed that most antimicrobial peptides kill microbes by membrane permeabilization [4]. The membrane-permeabilizing action significantly hinders the development of resistance to antimicrobial peptides because it is difficult for a microbe to change the lipid composition of its membrane and this is in marked contrast to conventional antibiotics, which target specific molecules such as receptors and enzymes [5].

However, the use of antimicrobial peptides *in vivo* is mainly limited due to the loss of their activity in body fluids because of enzymatic degradation in the presence of proteases [6]. To overcome this limitation, D-amino acid enantiomers are expected to be resistant to proteolytic cleavage and several studies have reported. Synthesized D-enantiomers of naturally occurring membrane-active peptides such as cecropin A, magainin 2 and melittin exhibited antimicrobial activity similar to that of their natural form and were not sensitive to enzymatic degradation [7–9]. These results suggested that D-amino acid peptides would be very attractive candidates as a therapeutic agent.

Recently, we isolated coprisin (VTCDVLSFEAKGIAVNHSACALH-CIALRKKGGSCQNGVCVCRN-NH_2), a natural peptide consisting of 43-amino acids, from Dung beetle *Copris tripartitus* after it had been infected with pathogenic bacteria, and showed that coprisin exhibited antifungal activities by apoptotic mechanisms [10,11]. In addition, we made a synthetic 9-mer analog peptide CopA3 (LLCIALRKK-NH_2) by replacing the histidine residues of CopN5 (LHCIALRKK-NH_2), which is the α-helical region in the natural peptide coprisin, with leucine to increase the hydrophobicity [10,12].

Abbreviations: CH_3CN, acetonitrile; ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Cultures; CLSI, Clinical and Laboratory Standards Institute; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; PBS, phosphate buffered saline; $DiBAC_4(3)$, bis-(1,3-dibutylbarbituric acid) trimethine oxonol; CFUs, colony-forming units; SD, standard deviation.

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The monomer and disulfide dimer of 9-mer CopA3 had antibacterial effects, and the antibiotic activity of CopA3 was higher than that of CopN5 [10,13]. However, the antifungal activity and the mechanism of the CopA3 monomer are not known yet.

Therefore, in this study, the antifungal activity and the mechanism of the CopA3 monomer were investigated. In addition, enantiomeric D-CopA3 was synthesized, and its enzymatic degradation and antifungal activity in the presence of trypsin were investigated.

2. Materials and methods

2.1. Solid-phase peptide synthesis

The peptide synthesis was done by Anygen Co. (Gwangju, Korea). Anygen Co. offers the following procedures for peptide synthesis. The assembly of the peptides was achieved with a 60 min cycle for each residue at ambient temperature using the following method: (1) 2-chlorotrityl (or 4-methylbenzhydrylamine amide) resin was charged to a reactor and then washed with DCM and DMF, respectively, and (2) a coupling step with vigorous shaking using a 0.14 mM solution of Fmoc-L-amino acids and Fmoc-L-amino acids preactivated for approximately 60 min with a 0.1 mM solution of 0.5 M HOBt/DIC in DMF. Finally, the peptide was cleaved from the resin using a TFA cocktail solution at ambient temperature [14,15]. Analytical and preparative reverse-phase HPLC runs were performed with Shimadzu 20 A or 6 A gradient systems. Data were collected with an SPD-20 A detector at 230 nm. Chromatographic separations were achieved with a 1%/min linear gradient of buffer B in A (A = 0.1% TFA in H_2O; B = 0.1% TFA in acetonitrile (CH_3CN)) over 40 min at flow rates of 1 and 8 ml/min using Shimadzu C_{18} analytical (5 μm, 0.46 cm × 25 cm) and preparative C_{18} (10 μm, 2.5 cm × 25 cm) columns.

2.2. Fungal strains and antifungal activity assay

Candida albicans (ATCC 90028) and *Candida parapsilosis* (ATCC 22019) were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *Trichosporon beigelii* (KCTC 7707) and *Malassezia furfur* (KCTC 7744) were obtained from the Korean Collection for Type Cultures (KCTC) at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea).

The fungal strains were cultured in YPD broth (Difco) with aeration at 28 °C, and the *M. furfur* was cultured at 32 °C in a modified YM broth (Difco) containing 1% olive oil. The cell suspensions were adjusted to obtain standardized populations by measuring the turbidity with a spectrophotometer (DU530; Beckman, Fullerton, CA, USA). Fungal cells (1×10^6 cells/ml) were inoculated into the broth, and 0.1 ml/well of the mixture were dispensed into microtiter plates. Minimum inhibitory concentrations (MICs) were determined with a 2-fold serial dilution of the test peptides, based on the Clinical and Laboratory Standards Institute (CLSI) method [16] and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay [17]. After 48 h of incubation, the minimal concentration of peptides required to prevent the growth of the microorganisms was determined, and defined as the MIC. The growth was measured with a microtiter ELISA Reader (Molecular Devices Emax, CA, USA) by monitoring the absorption at 580 nm. The MIC values were determined by three independent assays.

2.3. Hemolytic activity assay

A fresh human blood sample was diluted in a phosphate buffered saline (PBS: 35 mM phosphate buffer/150 mM NaCl, pH 7.4) and centrifuged at 2000 rpm for 10 min to remove the plasma and buffy coat, and the supernatant was removed. This washing procedure was repeated three times, and the final concentration of the erythrocytes was 4%. The erythrocyte suspension was transferred into sterilized 96-well plates and incubated with peptides at 37 °C for 1 h. The plate was centrifuged at 1500 rpm for 10 min. An aliquot of the supernatant was taken, and then the hemolytic activity of the peptides was evaluated by determining the release of hemoglobin from a 4% suspension of human erythrocytes at 414 nm with an ELISA reader. Hemolytic levels of zero and 100% were determined in PBS alone and with 0.1% Triton X-100, respectively. The hemolysis percentage was calculated with the following equation: hemolysis (%) = [(Abs_{414nm} in the peptide solution − Abs_{414nm} in PBS)/(Abs_{414nm} in 0.1% Triton X-100 − Abs_{414nm} in PBS)] × 100 [18].

2.4. Analysis of propidium iodide influx

C. albicans cells (1×10^6 cells/ml YPD) were suspended in PBS, treated with 5.0 μM of the peptides and incubated for 2 h at 28 °C. Cells were harvested by centrifugation and suspended in PBS. Subsequently, the cells were treated with 9 μM propidium iodide and incubated for 5 min at room temperature. The cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) [19,20]. The dot plot is representative of three separate experiments.

2.5. Membrane depolarization assay

C. albicans (1×10^6 cells/ml YPD) were harvested and suspended in PBS. After incubation with 5.0 μM of the peptides for 2 h at 28 °C, the cells were harvested by centrifugation and resuspended in PBS. Subsequently, the cells were treated with 5 μg of bis-(1,3-dibutylbarbituric acid) trimethine oxonol [$DiBAC_4(3)$] (Molecular Probes, Eugene, OR, USA). Flow cytometric analysis was done with a FACSCalibur flow cytometer [21]. The histogram is representative of three separate experiments.

2.6. Proteolytic stability assay

The proteolytic stability of L- and D-CopA3 was assayed with trypsin in a molar ratio of 1:18.7 (trypsin:peptide = 10.7 μM:0.2 mM). Each enantiomeric peptide (5 μg) was mixed with 10 mM sodium phosphate buffer (pH 7.6) containing trypsin (EC 3.4.21.4., Sigma). After incubation for 30 min at 37 °C, the samples were heated for 5 min at 99 °C and cooled for 10 min on ice.

2.6.1. HPLC

The samples were checked by HPLC. Analytical HPLC runs were performed with a Shimadzu C_{18} analytical column (5 μm, 0.46 cm × 25 cm) and a flow rate of 1 ml/min. Data were collected with an SPD-20 A detector at 220 nm. The elution buffers consisted of buffer A (0.1% TFA in H_2O) and buffer B (0.1% TFA in CH_3CN). The elution gradient started ($T = 0$) with 100% buffer A and continued: $T = 5$ min, 100% A; $T = 20$ min, 40% A/60% B; $T = 30$ min, 0% A/100% B; $T = 40$ min, 40% A/60% B, $T = 50$ min, 100% A; $T = 60$ min, 100% A [22].

2.6.2. Colony count assay

C. albicans cells (1×10^6 cells/ml YPD) were incubated with the samples. After overnight incubation, the mixtures were acquired and serially diluted in PBS (pH 7.4). One-hundred microliter aliquots were spread onto YPD agar plates, and then the colony-forming units (CFUs) were counted after incubation for 24 h at 28 °C [23]. The percentage survival was determined relative to the

Table 1
The antifungal activity of L- and D-CopA3.

Fungal strains	MIC (μM)		
	L-CopA3	D-CopA3	Melittin
C. albicans ATCC 90028	5.0	5.0	10.0
C. parapsilosis ATCC 22019	5.0	5.0	10.0
T. beigelii KCTC 7707	10.0	5.0–10.0	10.0
M. furfur KCTC 7744	20.0	20.0	20.0–40.0

Table 2
Hemolytic activity of L- and D-CopA3 against human erythrocytes.

Peptides	% Hemolysis						
	80.0 μM	40.0 μM	20.0 μM	10.0 μM	5.0 μM	2.5 μM	1.3 μM
L-CopA3	0	0	0	0	0	0	0
D-CopA3	0	0	0	0	0	0	0
Melittin	100.0	100.0	100.0	100.0	74.9	38.7	13.5

control treatment. Experiments were performed in triplicate, and the results were expressed as the mean + standard deviation (SD).

3. Results and discussion

3.1. Synthesis of L- and D-CopA3

We focused on the short 9-mer CopA3 which is a functionally improved peptide over the 9-mer CopN5 representing the α-helical region of the natural peptide 43-mer coprisin. According to our previous study, CopA3 has not only cationicity but also an α-helical structure [10,12]. In addition, a short peptide such as CopA3 has been specifically considered as a model for therapeutic agents because it can be produced at low cost and absorbed after oral administration without difficulty [24,25]. Those were the reasons that this short peptide consisting of 9 amino acids, CopA3, was considered as a potential antifungal peptide. Furthermore, in this study, another CopA3 composed of all-D-amino acids was synthesized to investigate the antifungal activity of the D-CopA3 and its potential as a therapeutic agent.

3.2. Antifungal and hemolytic activity of L- and D-CopA3

The antifungal effects of L- and D-CopA3 against human pathogenic fungi, such as *C. albicans*, *C. parapsilosis*, *T. beigelii* and *M. furfur* were investigated and described using the MIC. Melittin, as a positive control peptide for comparing the activity of L- and D-CopA3, is derived from the venom of the honey bee *Apis mellifera*. It is the most widely known antimicrobial peptide and possesses potent antimicrobial and hemolytic activity by membrane-disruptive action [26–28]. The result showed that L- and D-CopA3 had antifungal activities, with MIC values in the range of 5.0–20.0 μM, and melittin, with MIC values in the range of 10.0–40.0 μM, also had antifungal activities (Table 1). Interestingly, the antifungal activities of L- and D-CopA3 were more potent than that of melittin, which is the one of the most potent antimicrobial peptides. In addition, L- and D-CopA3 exhibited similar antifungal activities against various fungal strains. It meant that there was no partial chiral selectivity in the antifungal action.

The hemolytic effects of L- and D-CopA3 against human erythrocytes were examined. At any of the tested concentrations, no

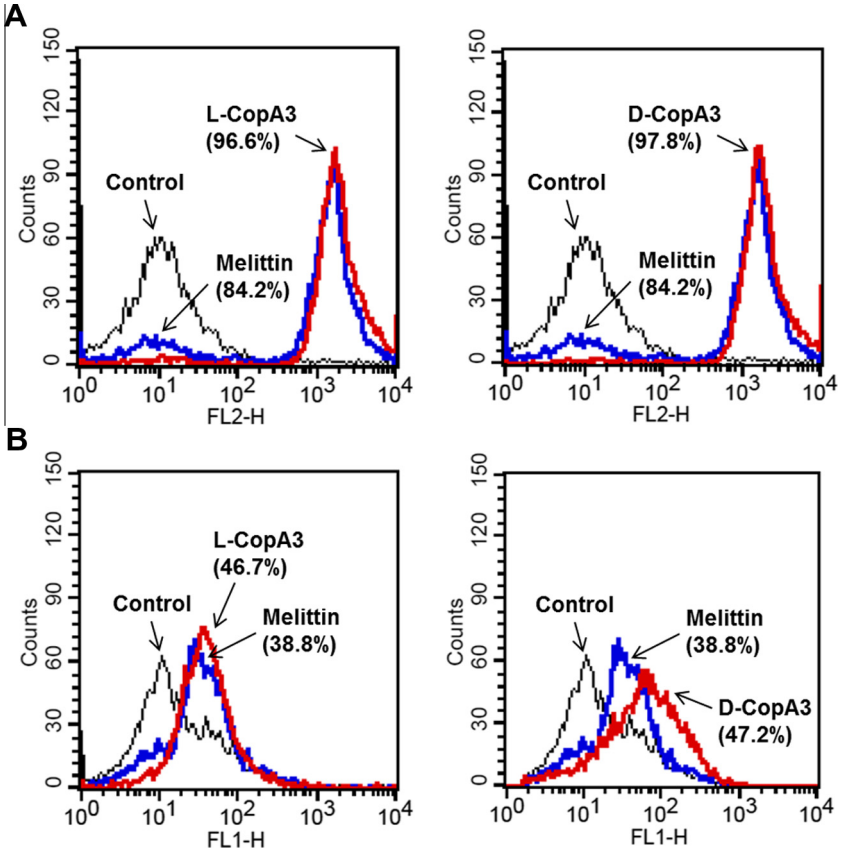

Fig. 1. (A) Flow cytometric analysis of membrane permeabilization detected by propidium iodide influx assay in *C. albicans* (1×10^6 cells/ml). The peptides were treated with 5.0 μM for 2 h at 28 °C. (B) Flow cytometric analysis of $DiBAC_4(3)$ staining in *C. albicans* (1×10^6 cells/ml) after incubation with 5.0 μM of the peptides for 2 h at 28 °C.

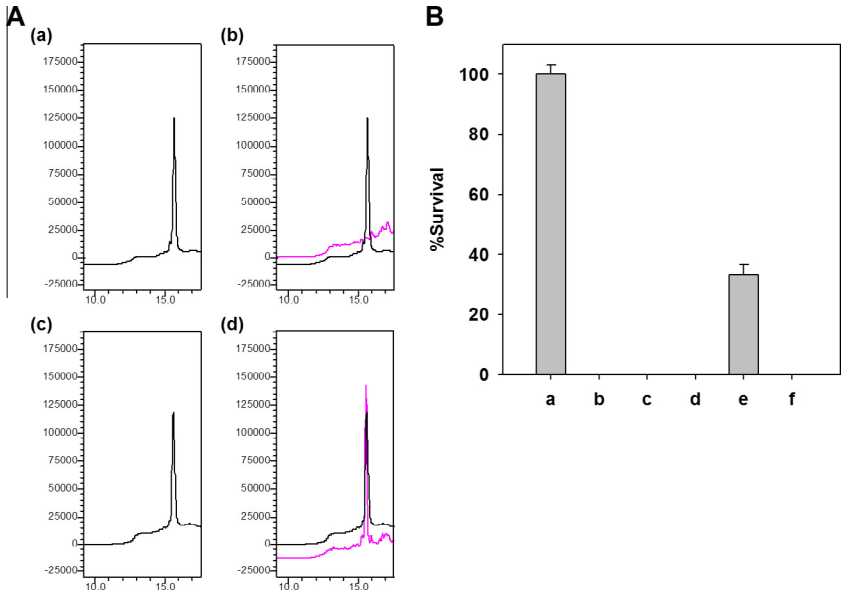

Fig. 2. L-CopA3 (5 μg) and D-CopA3 (5 μg) were treated with 10.7 μM of trypsin. Incubation conditions were for 30 min at 37 °C. (A) Proteolytic resistance of D-CopA3. (a) only L-CopA3, (b) L-CopA3 treated with trypsin, (c) only D-CopA3 and (d) D-CopA3 treated with trypsin. (b) and (d) Were an overlay with (a) and (c), respectively, for comparison with the peptide itself. The elution buffers consisted of buffer A (0.1% TFA in H_2O) and buffer B (0.1% TFA in CH_3CN). Elution gradient started ($T = 0$) with 100% buffer A and continued: $T = 5$ min, 100% A; $T = 20$ min, 40% A/60% B; $T = 30$ min, 0% A/100% B; $T = 40$ min, 40% A/60% B, $T = 50$ min, 100% A; $T = 60$ min, 100% A. (B) The viability was determined by counting CFUs after overnight incubation with samples and expressed as the percentage of survivals. The data represents the mean + SD for three independent experiments. (a) Control, (b) only melittin, (c) only L-CopA3, (d) only D-CopA3, (e) L-CopA3 treated with trypsin and (f) D-CopA3 treated with trypsin.

hemolytic activity were found in the hemolytic assay of L- and D-CopA3. However, melittin caused 100.0% hemolysis at its MIC value (10.0 μM) against *C. albicans*, *C. parapsilosis*, and *T. beigelii* (Table 2). The results of the antifungal and hemolytic activity suggested that L- and D-CopA3 had selective toxicity toward fungal cells without hemolysis, and potential as a novel antifungal agent for treating fungal diseases. Among the tested fungal strains, *C. albicans* is the fourth most common cause of hospital-acquired infectious disease and the primary cause of systemic candidiasis, with mortality rates approaching 50% [29]. Hence, *C. albicans* was selected as a model organism for the experiments of this study.

3.3. Membrane-active mechanism of L- and D-CopA3

Most α-helical antifungal antimicrobial peptides exert their activity by membrane permeabilization [30,4]. Hence, the changes in the integrity of fungal membranes were first observed using 2 dyes, propidium iodide and $DiBAC_4(3)$. The membrane-impermeant dye, propidium iodide, only enters cells with a compromised membrane, after which the fluorescence of the dye is enhanced by 20- to 30-fold due to its intercalation into double-stranded nucleic acids [31]. $DiBAC_4(3)$, is an anionic lipophilic dye and sensitive to changes in membrane potential. It enters only depolarized cells and then binds reversibly to the hydrophobic core of the lipid membrane, resulting in an increased fluorescence [32].

The results showed that a significant propidium iodide influx was induced in *C. albicans* when treated with L-CopA3 (96.6%), D-CopA3 (97.8%) and melittin (84.2%), respectively (Fig. 1A), thereby indicating that L- and D-CopA3 caused membrane permeabilization. Changes in membrane potential were also detected in the $DiBAC_4(3)$ staining assay. In *C. albicans* cells exposed to L-CopA3, D-CopA3 and melittin, the $DiBAC_4(3)$ fluorescence intensity increased 46.7%, 47.2% and 38.8%, respectively (Fig. 1B), indicating that L- and D-CopA3 depolarized the membrane potential. Interestingly, these results suggested that L- and D-CopA3 exerted its antifungal activity via a membrane-active action in contrast to the natural peptide coprisin that has antifungal activity via apoptosis induction without any membrane permeabilization [11]. Furthermore, like the result of MIC test, it is important to note that the activity of L- and D-CopA3 on the fungal membrane was greater than that of melittin. In addition, the activity of D-CopA3 on the membrane was similar to that of L-CopA3, indicating that membrane-active mechanism of CopA3 did not involve a stereoselective interaction with a chiral enzyme or receptor in the fungal membrane.

3.4. Proteolytic resistance and therapeutic potential of D-CopA3

Peptide stability *in vivo* is a major obstacle to the systematic use of antimicrobial peptides because of the proteolytic action of human proteases [33]. In this sense, D-peptides have several advantages: (i) they are resistant to proteases, which can dramatically increase half-life, (ii) short D-peptides can be absorbed systemically when taken orally, whereas L-peptides must be injected to avoid digestion [34]. To examine the proteolytic resistance of L- and D-CopA3, the susceptibility of peptides to trypsin, which is a digestive enzyme component of pancreatic juice, was investigated with HPLC. Trypsin, a serine protease, cleaves peptide chains mainly at the carboxyl side of arginine (R) or lysine (K). The results showed that L-CopA3 was sensitive to degradation by trypsin due to L-arginine (R^7) and L-lysine (K^8) residues, while D-CopA3 remained completely intact even after 30 min of incubation with trypsin (Fig. 2A). The result suggested that D-CopA3 might exert remarkable antifungal activity under exposure to trypsin.

To investigate the changes in the antifungal activity of L- and D-CopA3 under proteolytic condition, a colony count assay was performed. The result showed that after overnight incubation, no visible colonies were seen on the plate when treated with L-CopA3, D-CopA3 and melittin. As we expected, no colonies grew on the plate exposed to D-CopA3 incubated with trypsin, like the result of D-CopA3 alone, due to the proteolytic resistance of D-CopA3 toward trypsin. However, 33.3% of the colonies were counted on the plate incubated with 7-mer L-CopA3 segment that was believed to be missing the C-terminal 2-amino acids (R^7, K^8) by proteolysis with

trypsin (Fig. 2B). This result showed that L-CopA3 had impaired activity although 7-mer L-CopA3 segment exhibited a weak antifungal activity, while D-CopA3 exerted remarkable antifungal activity under proteolytic condition. Therefore, D-CopA3 might be a potential candidate for clinical use.

In conclusion, 9-mer monomer CopA3 exerted a more potent antifungal activity than that of melittin, without hemolysis against human erythrocytes. The antifungal effect of CopA3 was due to the membrane permeabilization in the fungal membrane. Furthermore, D-CopA3 showed more potent antifungal activity under proteolytic conditions than that of L-CopA3 because of its stability against proteolysis. The design or discovery of antimicrobial peptides that can overcome the inhibitory effects of physiological conditions is an important research field. Therefore, D-CopA3 could be a novel antibiotic by itself or a template for designing new therapeutic agents with proteolytic resistance toward proteases in body fluids.

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