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Antifungal effect and pore-forming action of lactoferricin B like peptide derived from centipede *Scolopendra subspinipes mutilans*

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ABSTRACT

The centipede *Scolopendra subspinipes mutilans* has been a medically important arthropod species by using it as a traditional medicine for the treatment of various diseases. In this study, we derived a novel lactoferricin B like peptide (LBLP) from the whole bodies of adult centipedes, *S. s. mutilans*, and investigated the antifungal effect of LBLP. LBLP exerted an antifungal and fungicidal activity without hemolysis. To investigate the antifungal mechanism of LBLP, a membrane study with propidium iodide was first conducted against *Candida albicans*. The result showed that LBLP caused fungal membrane permeabilization. The assays of the three dimensional flow cytometric contour plot and membrane potential further showed cell shrinkage and membrane depolarization by the membrane damage. Finally, we confirmed the membrane-active mechanism of LBLP by synthesizing model membranes, calcein and FITC-dextran loaded large unilamellar vesicles. These results showed that the antifungal effect of LBLP on membrane was due to the formation of pores with radii between 0.74 nm and 1.4 nm. In conclusion, this study suggests that LBLP exerts a potent antifungal activity by pore formation in the membrane, eventually leading to fungal cell death.

1. Introduction

Antimicrobial peptides (AMPs) are important components of innate immunity, and their distribution is widespread, including in bacteria, fungi, plants, insects, birds, crustaceans, amphibians, and mammals [1]. AMPs are typically relatively short (10–50 amino acids), positively charged (net charge ranging from +2 to +9), and amphiphilic [2]. These characteristics generally allow them to permeabilize bacterial membranes [3]. However, it has been reported that AMPs with antifungal activity had different mechanisms including membrane permeabilization, mitochondrial depletion, and depolymerization of actin cytoskeleton [4]. Chief of all, membrane-permeabilizing action significantly hinders the development of resistance to AMPs 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 usually act by attacking a specific receptor or enzymatic target [5].

Although insecta have been continuously exposed to pathogenic microorganisms, they make up about 80% of all the animals in the kingdom Animalia due to remarkable evolutionary success. Therefore, insects possess potent antimicrobial defense systems and particularly rely on innate immunity rather than adaptive immunity because of energy efficiency. Among components of innate immunity, AMPs play important roles in the innate immune systems of invertebrates such as insects. As a result, a single insect produces approximately 10–15 AMPs upon detection of invading pathogens [6,7]. To date, more than 170 AMPs have been identified in insects [8]. Melittin, the representative insect AMP, is the main toxic component in the venom of the honey bee *Apis mellifera*. It exerts not only potent antimicrobial activity but also hemolytic activity by membrane-disruptive action [9–11].

Centipedes have been thought to produce AMPs because they have only innate immunity [12]. Among them, a big centipede (*Scolopendra subspinipes mutilans*) has been used in traditional medicine for the treatment of various diseases [13]. A few AMPs isolated from the centipede *S. s. mutilans* have been reported, such as scolopendrin I, scolopin 1 and 2 [12,14,15]. Scolopin 1 and 2 exerted cytotoxicity through hemolytic activities against both human and rabbit erythrocytes. In this study, a novel lactoferricin B like peptide (LBLP) was derived from the centipede *S. s. mutilans*, and its antifungal activities and mechanisms were investigated. Among 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

Abbreviations: AMP, Antimicrobial peptides; LBLP, Lactoferricin B like peptide; SmAE, *S. s. mutilans* Assembled EST; CH_3CN, Acetonitrile; ATCC, American Type Culture Collection; KCTC, Korean Collection for Type Cultures; MIC, Minimum inhibitory concentration; PBS, Phosphate buffered saline; CFU, Colony-forming unit; SD, Standard deviation; $DiBAC_4(3)$, Bis-(1,3-dibutylbarbituric acid trimethine oxonol; LUV, Large unilamellar vesicle; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PI, Phosphatidylinositol; FITC, Fluorescein isothiocyanate; FD, FITC-labeled dextran

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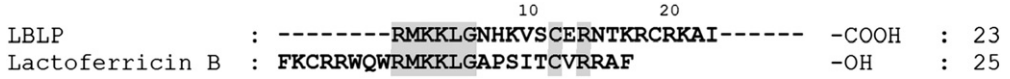

Fig. 1. Sequence alignment of LBLP and lactoferricin B.

50% [16]. Hence, *C. albicans* was selected as a model organism for the experiments of this study.

2. Materials and methods

2.1. Sample collection and treatments

Adult *S. s. mutilans* were purchased from Jirisan industrial insect institute in Jinju, South Korea. For infection, log-phase *E. coli* (2×10^6 colony forming units/centipede) was injected into the body of each centipede.

2.2. Transcriptomic data analysis

2.2.1. RNA preparation and sequencing

Total RNA samples were prepared from the centipedes using the RNeasy Total RNA isolation kit (Qiagen, USA) according to the manufacturer's instructions. Whole body RNA was isolated from *E. coli*-infected *S. s. mutilans* at 18 h after infection. RNA integrity was validated using the Agilent 2100 Bioanalyzer. Beads with Oligo (dT) were used to isolate poly (A) mRNA. After fragmentation buffer was added to produce short mRNA fragments, random hexamer-primers were used to synthesize the first-strand cDNA, after which the second-strand cDNA was synthesized. Short fragments were purified with the QIAquick PCR extraction kit and resolved with EB buffer for end reparation and poly (A) addition. The short fragments were tailed with sequencing adapters. Fragments suitable for PCR amplification were selected as a template with the agarose gel electrophoresis method. Finally, the library was sequenced with the Illumina HiSeq™ 2000 by generating paired-end libraries with an average insert size of 200 bp, following the manufacturer's instructions.

2.2.2. Assembly

The *S. s. mutilans* transcriptome without a reference genome was *de novo* assembled using the Trinity method [17]. Together with the original Trinity assembly result, TGICL software was used to obtain sequences that could not be extended on either end [18]. The resulting sequences, contigs and singletons, were referred to as *S. s. mutilans* Assembled ESTs (SmAEs), sequences of which were translated into amino acid sequences with ESTScan [19]. 51317 SmAEs were assembled.

2.3. In silico prediction of antimicrobial peptides

2.3.1. Screening based on physicochemical properties

With all of the protein-coding genes collected, potential AMPs were predicted based on less than or equal to 100 amino acids in length with EMBOSS PEPSTATS [20]. 52 SmAEs were found.

2.3.2. Novel AMP selection

To predict AMPs based on sequence similarity, BLASTx was used. The sequences obtained were considered as putative AMPs. Among the putative AMPs, we synthesized the following AMP: LBLP (RMKKLGNHKVSCERNTKRCRKAI).

2.4. Solid-phase peptide synthesis

Anygen Co. (Gwangju, Korea) did the peptide synthesis. 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 [21,22].

2.5. Peptide characterization

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. Mass spectrometry was also done with AXIMA CFR MALDI TOF Mass Spectrometer (Kratos/Shimadzu).

2.6. 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). *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).

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 at the log phase (2×10^6/ml) were inoculated into 0.1 ml/wells of YPD or YM broth, and then, dispensed to microtiter plates. Minimum inhibitory concentrations (MICs) were determined with a two-fold serial dilution of the test peptides, based on the Clinical and Laboratory Standards Institute (CLSI) method [23]. The MIC values were determined by three independent assays.

Table 1
Amino acid sequence and physicochemical features of LBLP.

Peptide	Amino acid sequence	Purity	Molecular mass (MW)		Net charge (physiological PH)
			Calculated value	Observed value	
LBLP	RMKKLGNHKVSCERNTKRCRKAI	98.233%	2757.3	2757.6	+8

Table 2
The antifungal activity of LBLP and melittin.

Fungal strains	MIC (μM)	
	LBLP	Melittin
C. albicans ATCC 90028	10.0	5.0
C. parapsilosis ATCC 22019	10.0	2.5–5.0
M. furfur KCTC 7744	10.0–20.0	2.5
T. beigelii KCTC 7707	20.0	5.0

2.7. Hemolytic activity assay

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 phosphate buffered saline (PBS: 35 mM phosphate buffer/150 mM NaCl, pH 7.4) 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 [24].

2.8. Time-kill kinetic analysis

Exponential-phase *C. albicans* cells (2 × 10^6/ml YPD) were incubated with either LBLP or melittin at the MIC. After 2, 4, 6, 8, and 10 h, the samples 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 [25]. The percentage survival was determined relative to the control treatment. Experiments were performed in triplicate, and the results were expressed as the mean ± standard deviation (SD).

2.9. Analysis of propidium iodide influx, cell size, and cell granularity

C. albicans cells in the log phase (2 × 10^6/ml YPD), resuspended in PBS, were treated with 10.0 μM of the peptides and incubated for 4 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) [26,27].

2.10. Membrane depolarization assay

The log-phased cells of *C. albicans* (2 × 10^6/ml YPD) were harvested and suspended in PBS. After incubation with 10.0 μM of the peptides for 4 h at 28 °C, the cells were harvested by centrifugation and resuspended in PBS. Subsequently, the cells were treated with 50 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 [28]. The histogram is representative of three separate experiments.

Table 3
Hemolytic activity of LBLP and melittin 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
LBLP	0	0	0	0	0	0	0
Melittin	99.0	91.1	70.2	54.3	23.8	13.3	1.9

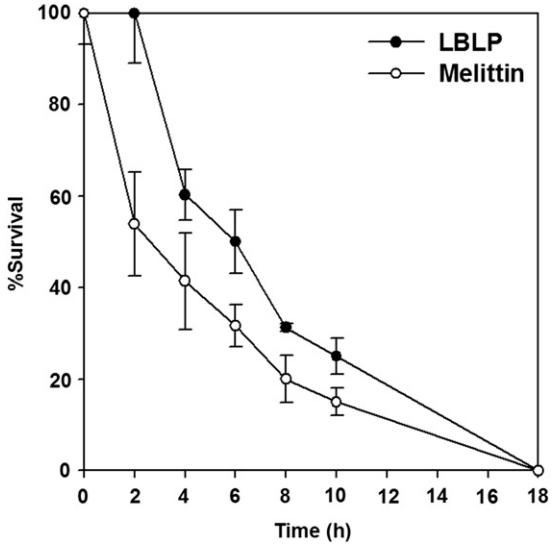

Fig. 2. Time-kill kinetics of 10.0 μM of LBLP and melittin in *C. albicans*.

2.11. Calcein leakage measurement

Large unilamellar vesicles (LUVs) encapsulating calcein, composed of phosphatidylcholine (PC)/phosphatidylethanolamine (PE)/phosphatidylinositol (PI)/ergosterol (5:4:1:2, *w*/*w*/*w*/*w*), were prepared by vortexing the dried lipids in a dye buffer solution (70 mM calcein, 10 mM Tris, 150 mM NaCl, and 0.1 mM EDTA [pH 7.4]). The suspension was freeze–thawed in liquid nitrogen for 11 cycles and extruded through polycarbonate filters (two stacked 200 nm pore size filters) with a LiposoFast extruder (Avestin Inc., Ottawa, Canada). Calcein-entrapped LUVs were separated from free calcein by gel filtration chromatography on a Sephadex G-50 column. The leakage of calcein from the LUVs was monitored at 25 °C by measuring the fluorescence intensity at an excitation wavelength of 490 nm and an emission wavelength of 520 nm, with a spectrofluorophotometer (Shimadzu RF-5301PC, Shimadzu, Kyoto, Japan). To determine 100% dye release, 30 μl of 10% Triton X-100 was added to the vesicles. The percentage of dye leakage caused by the peptides was calculated as follows: dye leakage (%) = 100 × (F − F_0) / (F_t − F_0), where F represents the fluorescence intensity achieved after addition of the peptides, and F_0 and F_t represent the

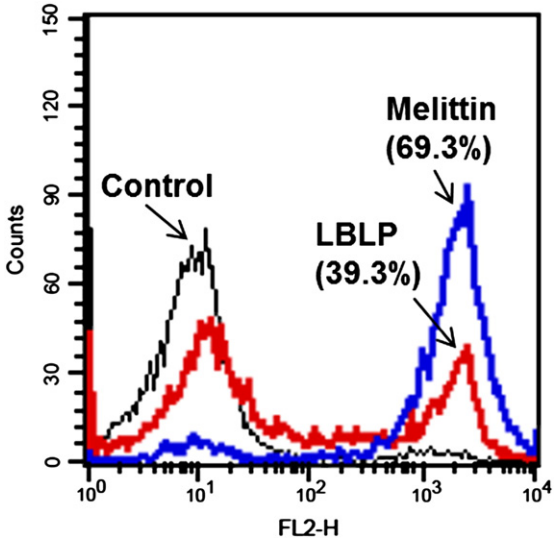

Fig. 3. Flow cytometric analysis of membrane permeabilization detected by propidium iodide influx assay in *C. albicans* (2 × 10^6/ml). The peptides were treated at 10.0 μM.

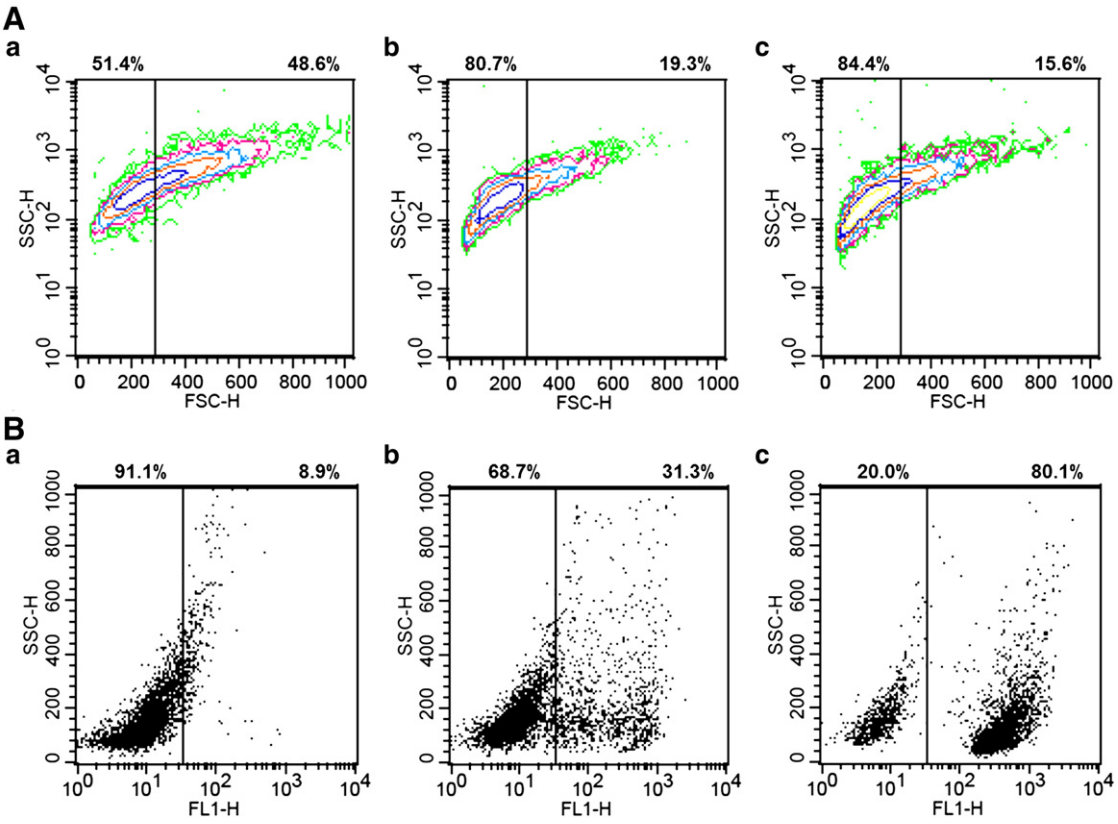

Fig. 4. (A) Flow cytometric analysis of *C. albicans* (2×10^6/ml) treated with 10.0 μM of the peptides. FSC is an indicator of cell size and SSC (log) is an indicator of cell granularity. (a) Control, (b) LBLP, and (c) melittin. (B) Flow cytometric analysis of $DiBAC_4(3)$ staining in *C. albicans* (2×10^6/ml) after incubation with 10.0 μM of the peptides. (a) Control, (b) 10.0 μM of LBLP, and (c) 10.0 μM of melittin.

fluorescence intensities without any compound and with Triton X-100, respectively [29]. The data represent the mean ± SD for three independent experiments.

2.12. Preparation of dextran-loaded liposomes and leakage assay

Fluorescein isothiocyanate (FITC)-labeled dextrans (FD4 and FD10) were used to evaluate the membrane-active mechanism of LBLP. LUVs containing FD were prepared with the reverse-phase evaporation method [30]. To prepare liposomes containing FD, buffer I (1 ml, 50 mM potassium phosphate, pH 7.4, with 0.1 mM EDTA), containing 2 mg/ml of FD, was sonicated (JAC 2010, KODO, Korea) for 30 min with 20 mg/ml of lipid [PC/PE/PI/ergosterol (5:4:1:2, *w*/*w*/*w*/*w*)] solution in chloroform on ice. Chloroform was removed with a rotary vacuum evaporator for 2 h at 25 °C, resulting first in the formation of a viscous gel and then, a liposome suspension. Buffer I (2 ml) was added and the suspension was evaporated further for the removal of eventual traces of chloroform. The liposome suspension was sonicated, centrifuged, and washed for several cycles at 13,000 rpm for 30 min to remove the unentrapped-FD. For the assay, a suspension

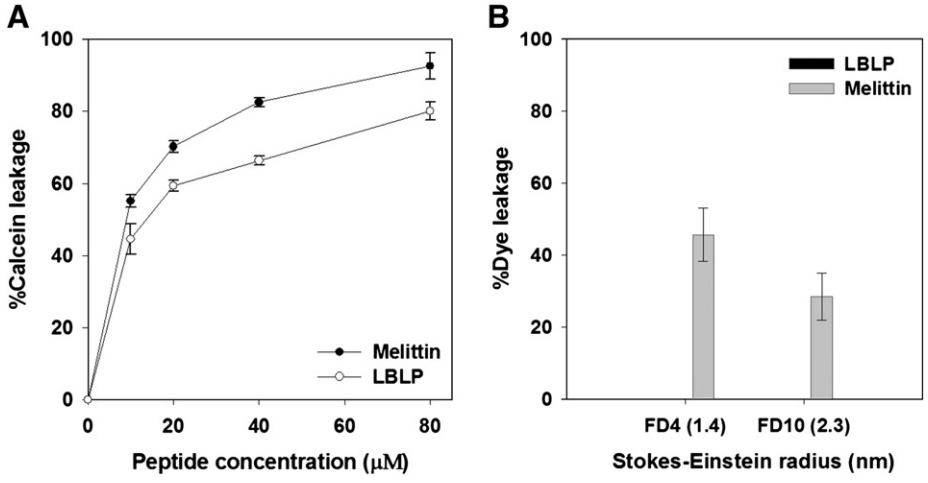

Fig. 5. (A) Percentage of calcein leakage from the LUVs [PC:PE:PI:ergosterol = 5:4:1:2 (*w*/*w*/*w*/*w*)] was measured after treatment with the peptides for 10 min at various concentrations (10.0, 20.0, 40.0, and 80.0 μM). (B) Percentage of FITC-dextrans (FDs) leakage induced by 10.0 μM of the peptides from PC/PE/PI/ergosterol (5:4:1:2, *w*/*w*/*w*/*w*) liposomes.

of FD-loaded liposomes was treated with 10 μM of the peptides. A molar ratio of peptide:lipid was 1:50. The mixture (1 ml, final volume) was stirred for 10 min in the dark and then centrifuged at 13,000 rpm for 20 min. The supernatant was recovered and its fluorescence intensity was recorded at an excitation wavelength of 494 nm and an emission wavelength of 520 nm with a spectrofluorophotometer. The maximum fluorescence intensity was determined by adding 20 μl of 10% Triton X-100 to a liposome suspension. The percentage of FD leakage was calculated the same way as the percentage of calcein leakage. The data represent the mean ± SD for three independent experiments.

3. Results and discussion

3.1. A novel AMP candidate

In order to find novel AMPs, a sequential BLASTx search was used and a peptide with 34.4% sequence similarity to lactoferricin B was found (Fig. 1). The selected LBLP was synthesized and characterized. The purity and observed mass of LBLP were 98.233% and 2757.6 Da (Calculated mass = 2757.3 Da), respectively. The net charge in physiological PH of LBLP was +8 (Table 1).

3.2. Antifungal and hemolytic activity of LBLP

In this study, the antifungal effects and mechanisms of a novel AMP, LBLP, derived from the centipede *S. s. mutilans* were investigated. Melittin was used as a positive control peptide for comparing the potency of LBLP. First, the antifungal activities of LBLP against human pathogenic fungi, such as *C. albicans*, *C. parapsilosis*, *M. furfur*, and *T. beigelii*, were examined. As shown in Table 2, LBLP had antifungal activities, with MIC values in the range of 10.0–20.0 μM. Melittin, with MIC values in the range of 2.5–5.0 μM, was more potent than that of LBLP. In the hemolysis assay, LBLP did not cause hemolysis at any concentration, while melittin caused 23.8% hemolysis at its MIC value (5.0 μM) against *C. albicans*. At the highest concentration (80.0 μM), melittin even showed 99.0% hemolysis (Table 3). These results suggested that LBLP had remarkable potential to be considered as a novel AMP for treating fungal diseases with selective toxicity toward fungal cells over human erythrocytes.

3.3. Fungicidal activity of LBLP

LBLP exhibited lower MIC against the *Candida* species than that of the other strains tested (Table 2). Time-killing assay was conducted to assess whether LBLP showed fungicidal or fungistatic activity for *C. albicans*. The result showed that the activity of LBLP resulted in killing activity as seen with melittin (Fig. 2). This result indicated that LBLP exerted candidacidal activities.

3.4. Membrane damage by LBLP

The effect of LBLP on the integrity of fungal membranes was first analyzed by monitoring the influx of propidium iodide. Propidium iodide only enters membrane-compromised cells and then, intercalates between bases with guanine and cytosine pairs or with a stoichiometry of one dye per 4-5 base pairs [31]. If cell membranes were damaged by LBLP, propidium iodide would diffuse into the cells and bind to the nucleic acids, exhibiting an increased fluorescence intensity. When treated with LBLP and melittin, 39.3% and 69.3% of the *C. albicans* cells exhibited propidium iodide fluorescence, respectively, thereby indicating that LBLP caused membrane permeabilization (Fig. 3).

3.5. Cell shrinkage and membrane depolarization by LBLP

Damage to the cytoplasmic membrane of the cell by AMP causes the loss of the structural and functional integrity of the membrane [32]. The morphological changes of the cell and function of the membrane are measurable by flow cytometry. The morphological changes in peptide-treated *C. albicans* cells were examined. FSC is an indicator of cell size and SSC (log) is an indicator of granularity. The result showed that in the cells treated with LBLP and melittin, 29.3% and 33.0% of cell population showed decreased FSC values based on main population of control, respectively, which indicates cell shrinkage (Fig. 4A).

To further examine whether LBLP can affect the functions of the fungal membrane, the changes in membrane potential were detected with the dye $DiBAC_4(3)$. $DiBAC_4(3)$ is an anionic lipophilic dye sensitive to membrane potential and enters only depolarized cells according to the Nernst equation, where it binds reversibly to lipid-rich intracellular components [33]. Thus, when the membrane potential decreases, $DiBAC_4(3)$ can penetrate and bind to the hydrophobic core of the lipid membrane, resulting in an increased fluorescent signal. In cells exposed to LBLP and melittin, the fluorescence intensity increased 22.4% and 71.2%, respectively, compared to the untreated cells, indicating the depolarization of the membrane potential (Fig. 4B). These data strongly suggested that the fungal membrane was a major target of LBLP. Therefore, we confirmed that LBLP exerted its antifungal activity by means of a membrane-active mechanism.

3.6. LBLP-induced pore formation in the fungal membrane

Propidium iodide is also used to detect pore formation [34], and from the propidium iodide influx by LBLP, we hypothesized that the membrane-active mechanism of LBLP might be related to pore formation. To investigate the membrane-active mechanism of LBLP and the extent of membrane damage induced by the peptides, the leakage content of different dyes with various molecular weights from artificial liposomes was measured, which included calcein (average molecular weight = 623 Da, Stokes–Einstein radius = 0.74 nm) [35], FD4 (average molecular weight = 4 kDa, Stokes–Einstein radius = 1.4 nm) and FD10 (average molecular weight = 10 kDa, Stokes–Einstein radius = 2.3 nm) [36]. The LUV resembled the outer leaflets of the plasma membrane of *C. albicans* composed of PC/PE/PI/ergosterol (5:4:1:2, *w*/*w*/*w*/*w*) [37]. The results showed that LBLP induced dose-dependent calcein leakage at all the tested concentrations. The 10.0 μM of LBLP and Melittin caused 44.5% and 55.1% calcein leakage from the LUVs, respectively (Fig. 5A). In the FD leakage assay, melittin induced the release of FD4 and FD10, suggesting the size of the pore formed by melittin was larger than 2.3 nm. However, LBLP did not cause the release of the FDs from the liposomes at all (Fig. 5B). This result means that LBLP induced formation of pores with radii between 0.74 nm and 1.4 nm. Taken together, these results showed that LBLP formed pores resulting in an increased permeability of the fungal membrane. Hence, the fungal cells experienced membrane depolarization which causes a loss of ions, and then LBLP eventually led to fungal cell death and cell shrinkage.

In conclusion, LBLP, a novel AMP, exerted remarkable antifungal activities, especially against *Candida* species, without hemolysis. The antifungal effects through the pore-forming action of LBLP ultimately resulted in fungal cell death.

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