The Influence of the N-Terminal Region of Antimicrobial Peptide Pleurocidin on Fungal Apoptosis

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Introduction

Every living organism produces antimicrobial peptides (AMPs) as a component of the innate immunity against invasion by pathogens [3]. AMPs, composed of less than 100 amino acids, exert a broad spectrum of antimicrobial activity against bacteria, fungi, and viruses, including multidrug-resistant microorganisms [7]. It is generally accepted that most AMPs cause microbial cell membrane disruption with electrostatic or hydrophobic interaction via cationic charge and amphipathicity [21, 30]. In addition, AMPs also inhibit the synthesis of intracellular substances such as DNA, RNA, and proteins [3, 21]. Recently, some AMPs, such as coprisin [12], RsAFP2 [1], dermaseptin S3 (1-16) [18], and pleurocidin (Ple) [4], have been reported to exert an antifungal mechanism via apoptosis induction.

In our previous study, the 25-mer antimicrobial peptide pleurocidin (Ple) had been thought to induce apoptosis in Candida albicans. This study demonstrated that reactive oxygen species (ROS) production was a major cause of Ple-induced apoptosis. Four truncated analogs were synthesized to understand the functional roles in the N- and C-terminal regions of Ple on the apoptosis. Ple, Ple (4-25), Ple (1-22), and Ple (1-19) produced ROS, including hydroxyl radicals, on the order of [Ple > Ple (1-22) > Ple (4-25) > Ple (1-19)], whereas Ple (7-25) did not induce any ROS production. The results suggested that the N-terminal deletion affected the ROS-inducing activities much more than that of the C-terminal deletion, and net hydrophobicity [Ple > Ple (1-22) > Ple (4-25) > Ple (1-19) > Ple (7-25)] was related to ROS generation rather than other primary factors like net charge. Hence, we focused on the N-terminal-truncated peptides, Ple (4-25) and Ple (7-25), and examined other apoptotic features, including mitochondrial membrane depolarization, caspase activation, phosphatidylserine externalization, and DNA and nuclear fragmentation. The results also confirmed the disappearance of apoptotic activity of Ple (7-25) by the truncation of the N-terminal region (1-6) and the specific activity patterns between Ple and analogs. In conclusion, the N-terminal region of Ple played an important role in apoptosis.

Keywords: Pleurocidin, reactive oxygen species, terminal region, Pleuronectes americanus, antimicrobial peptide
antimicrobial activity was greatly decreased by removing the N-terminal region of HP (2-20), suggesting that the N-terminal region was necessary for the antimicrobial activity [19]. On the other hand, LL-31, a truncated peptide missing six amino acids at the C-terminus of LL-37, exhibited a higher killing effect than LL-37 by disrupting the bacterial membrane [11].

In this study, four truncated analogs [Ple (4-25), Ple (7-25), Ple (1-22), and Ple (1-19)] were synthesized and the relationship between Ple-induced apoptosis and the role of each truncated N- or C-terminal regions was investigated.

Materials and Methods

Peptide Characterization

The purity of the peptides was checked by analytical and preparative reverse-phase HPLC runs. The 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% trifluoroacetic acid (TFA) in H2O; B = 0.1% TFA in acetonitrile (CH3CN)) over 40 min at flow rates of 1 and 8 ml/min, using the Shimadzu C18 analytical (5 µm, 0.46 cm × 25 cm) and preparative C18 (10 µm, 2.5 cm × 25 cm) columns. Mass spectrometry was also done.

N-Acetylcycteine (NAC) Assay

For ROS quenching, NAC (Sigma-Aldrich, St. Louis, MO, USA) in solid form was weighed and added to C. albicans (2 × 106 cells/ml) for a final concentration of 5 mM in solution. NAC was added to the culture at the same time as the Ple or H2O2. The concentrations of the quencher used here were determined to minimize the growth inhibition [17]. CFU/ml was monitored after a 2 h exposure to the Ple or H2O2. For the CFU/ml measurements, 100 µl of culture was collected, washed with PBS, and then serially diluted in PBS. Each dilution was plated onto YPD agar, and the plates were incubated at 28°C overnight. The CFU/ml was counted and expressed as a percentage of the survivors using the formula [CFU of sample treated with agent]/(CFU of non-treated control) × 100.

Measurement of ROS Production

Intracellular ROS production was measured with the fluorescent dye dihydrodorhodamine-123 (DHR-123) [22]. C. albicans (ATCC 90028) cells (2 × 106 cells/ml) were treated with 20 µM of Ple or the four analogs or 10 mM of hydrogen peroxide (H2O2) for 2 h at 28°C. After incubation, the cells were washed with phosphate-buffered saline (PBS, pH 7.4), stained with 5 µg/ml of DHR-123, and analyzed using a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Measurement of Hydroxyl Radical (OH•) Accumulation

OH• accumulation was measured with the fluorescent dye 3’-(p-hydroxyphenyl) fluorescein (HPF) [31]. C. albicans (ATCC 90028) cells (2 × 106 cells/ml) were treated with 20 µM of Ple or the four analogs or 10 mM of H2O2 for 2 h at 28°C. After incubation, the cells were washed with PBS, stained with 10 µM of HPF, and analyzed using the FACS Calibur flow cytometer.

Determination of the Mitochondrial Membrane Potential

JC-1 (Molecular Probes) is a mitochondrial membrane potential-sensitive dye. JC-1 was specifically used to confirm the decreases in the membrane potential of mitochondria. C. albicans cells (2 × 106 cells/ml) were treated with 20 µM of Ple or the four analogs or 10 mM of H2O2 for 2 h at 28°C. The treated cells were washed in PBS and then stained with 2 µg/ml JC-1 in the dark at 37°C for 15 min. The cells were analyzed using the FACS Calibur flow cytometer. The ratio of J aggregate (FL2, red fluorescence) to JC-1 monomer (FL1, green fluorescence) intensity was calculated, which shows the change in the mitochondrial membrane potential [8].

Caspase Activity

Activated caspases in C. albicans were measured using the CaspACE FITC-VAD-FMK In Situ Marker (Promega). The cells (2 × 106 cells/ml) were treated with 20 µM of Ple or the four analogs or 10 mM of H2O2 for 2 h at 28°C. The cells were washed twice in PBS and incubated in 1 ml of PBS containing 0.25 µM FITC-VAD-FMK. Caspase activation was analyzed with the FACS Calibur flow cytometer [15].

Analysis of Apoptotic Markers

Annexin V-FITC labeling was done with the following modified method from Madeo et al. [14]. The FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen) was used for the analysis. C. albicans cells were harvested by centrifugation, resuspended in 0.1 M potassium phosphate buffer (PPB, pH 6.0) containing 1 M sorbitol, and digested with lysing enzyme (20 mg/ml). The cells were incubated for 1 h at 28°C and washed with 0.1 M PPB containing 1 M sorbitol. The protoplast cells (2 × 106 cells/ml) were treated with 20 µM of Ple or the four analogs or 10 mM of
H$_2$O$_2$ for 2 h at 28°C, centrifuged, and resuspended in annexin binding buffer. The cells were incubated with 5 µl/ml annexin V-FITC and 1 µl/ml propidium iodide (PI) for 15 min. Flow cytometric analysis was done with the FACSCalibur flow cytometer.

DNA fragmentation in *C. albicans* cells was analyzed by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) method [22]. The cells (2 x 10$^6$ cells/ml) were treated with 20 µM of Ple or the four analogs or 10 mM of H$_2$O$_2$ for 3 h at 28°C, centrifuged, and fixed in 2% paraformaldehyde for 1 h. The cells were washed in PBS, permeabilized in permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) for 2 min on ice, and washed twice in PBS. DNA ends were labeled with an *in situ* cell death detection kit for 1 h at 37°C. The stained cells were observed with a fluorescence microscope (Nikon Eclipse Ti-5; Nikon, Japan).

Nuclear condensation and fragmentation were analyzed by 4',6-diamidino-2-phenylindole (DAPI) staining [14]. *C. albicans* cells (2 x 10$^6$ cells/ml) were treated with 20 µM of Ple or the four analogs or 10 mM of H$_2$O$_2$ for 3 h at 28°C. For nuclear staining, the cells were washed with PBS and incubated with DAPI (1 µg/ml) in the dark for 20 min. The cells were then examined by fluorescence microscopy.

### Results

**Effect of Ple-Induced ROS on the Apoptosis and Characterization of Ple and Analogs**

To investigate whether ROS production by Ple and H$_2$O$_2$ influences apoptotic cell death, the *C. albicans* cells exposed to Ple or H$_2$O$_2$ were additionally treated with a ROS scavenger, NAC (5 mM). NAC did not have a toxic effect on the *C. albicans* cell culture (Fig. 1). The viability of the cells exposed to Ple and H$_2$O$_2$ for 2 h without NAC treatment was 44.4% and 2.3%, respectively, whereas the survival of the cells treated with NAC before exposure to Ple and H$_2$O$_2$ was significantly increased to 85.1% and 55.1%, respectively (Fig. 1).

The four analog peptides were synthesized by partially truncating amino acids in the N- or C-terminal regions of Ple (Table 1). To investigate the effect of the terminal regions on apoptosis, Ple (4-25) and Ple (7-25) were synthesized by cutting GWG (1-3) and GWGSFF (1-6) in the N-terminal region, respectively. Ple (1-22) and Ple (1-19) were synthesized by deletion of HYL (23-25) and ALTHYL (20-25) in the C-terminal region of Ple (Table 1). The net charges of Ple (4-25) and Ple (7-25) are +7, like that of Ple, whereas the net charges of Ple (1-22) and Ple (1-19) are +6 (Table 1).

Hydrophobicity was calculated by using the Eisenberg-Weiss scale and came up with the conclusion that Ple had the value of 0.026, Ple (4-25) had the value of -0.0536, Ple (7-25) had the value of -0.092, Ple (1-22) had the value of -0.032, and Ple (1-19) had the value of 0.056 (Table 1).

**ROS Production by Ple and Analogs in *C. albicans***

To compare the content of ROS production by Ple and the four analogs, DHR-123 was used. DHR-123 is an oxidation-sensitive lipophilic agent that enters a cell and

### Table 1. Amino acid sequences and physicochemical features of Ple and the four analogs

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Amino acid sequence</th>
<th>Molecular weight (MW)</th>
<th>Net charge</th>
<th>Retention time (min)</th>
<th>Hydrophobicity (Eisenberg-Weiss)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ple</td>
<td>GWGSFKKAHHVGKHVGKAALTHYL-NH$_2$</td>
<td>2,710.1 2,710.5</td>
<td>+7</td>
<td>16.967</td>
<td>-0.026</td>
</tr>
<tr>
<td>Ple (4-25)</td>
<td>SFFKKAHHVGKHVGKAALTHYL-NH$_2$</td>
<td>2,409.8 2,409.4</td>
<td>+7</td>
<td>15.742</td>
<td>-0.0536</td>
</tr>
<tr>
<td>Ple (7-25)</td>
<td>KKAHHVGKHVGKAALTHYL-NH$_2$</td>
<td>2,028.4 2,028.6</td>
<td>+7</td>
<td>14.458</td>
<td>-0.092</td>
</tr>
<tr>
<td>Ple (1-22)</td>
<td>GWGSFKKAHHVGKHVGKAALT-NH$_2$</td>
<td>2,296.6 2,296.3</td>
<td>+6</td>
<td>16.150</td>
<td>-0.032</td>
</tr>
<tr>
<td>Ple (1-19)</td>
<td>GWGSFKKAHHVGKHVGKA-NH$_2$</td>
<td>2,011.3 2,011.3</td>
<td>+6</td>
<td>15.567</td>
<td>-0.056</td>
</tr>
</tbody>
</table>
fluoresces when oxidized by intracellular ROS to positively charged rhodamine 123 derivatives [23]. In *C. albicans* cells treated with Ple, an increase in fluorescence intensity was observed (Fig. 2), indicating intracellular ROS production of 52.6% compared with untreated cells, but the fluorescence intensities of the four analogs were less than that of Ple. Ple (1-22), Ple (4-25), and Ple (1-19) induced intracellular ROS production of 46.9%, 43.6%, and 37.9%, respectively. However, Ple (7-25) did not induce ROS production at all (Fig. 2).

**OH· Accumulation by Ple and Analogs in *C. albicans***

To investigate the accumulation of OH· among the produced ROS, HPF was used for selectively detecting OH·. HPF, a cell-permeable reagent, is immediately oxidized by OH·. Upon oxidation, HPF is converted to the highly fluorescent molecule, fluorescein [25]. In *C. albicans* cells exposed to Ple, the fluorescent intensity was increased 50.3% compared with untreated cells (Fig. 3). As shown in Fig. 3, the cells treated with Ple (1-22), Ple (4-25), and Ple (1-22), Ple (4-25), and Ple (1-19) induced intracellular ROS production of 46.9%, 43.6%, and 37.9%, respectively. However, Ple (7-25) did not induce ROS production at all (Fig. 2).

**Fig. 2.** Intracellular ROS production was detected by flow cytometry using DHR-123. The increase in fluorescence intensity indicates an increase in ROS. (A) Cells without any treatment, (B) cells treated with H<sub>2</sub>O<sub>2</sub>, (C) cells treated with Ple, (D) cells treated with Ple (1-22), (E) cells treated with Ple (4-25), (F) cells treated with Ple (1-19), and (G) cells treated with Ple (7-25).

**Fig. 3.** Flow cytometric analysis of hydroxyl radical generation. The hydroxyl radical generation was monitored by HPF, which reacts selectively with highly reactive oxygen species. (A) A histogram of HPF-stained cells. (B) Data are given as the mean ± SD (a) H<sub>2</sub>O<sub>2</sub>, (b) Ple, (c) Ple (1-22), (d) Ple (4-25), (e) Ple (1-19), and (f) Ple (7-25).
19) produced reactive $\text{OH}^-$ of 44.5%, 41.4%, and 0.4%, respectively. Like the result of ROS production, Ple (7-25) did not produce any reactive $\text{OH}^-$. **Depolarization of Mitochondrial Membrane by Ple and Analogs in *C. albicans***

We focused on the N-terminal-truncated peptides, because Ple (7-25) did not produce any ROS by truncating the N-terminal region (1-6) of Ple. The loss of mitochondrial membrane potential, a hallmark for apoptosis, was observed using JC-1. JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria. In non-apoptotic cells, JC-1 accumulates as J-aggregates in the mitochondria, which appear red. In apoptotic cells, JC-1 exists in a monomeric form and stains the cytosol green [28]. The FL2/FL1 value of JC-1 fluorescence intensity by Ple treatment decreased significantly (4.2), indicating the depolarization of mitochondrial membrane. The FL2/FL1 value in the presence of Ple (4-25) and Ple (7-25) was slightly decreased to 45.5 and 51.3, respectively, compared with that of untreated cells (61.0) (Fig. 4).

**Caspase Activation by Ple and Analogs in *C. albicans***

Caspase activity was monitored using FITC-VAD-FMK. The fluorescein isothiocyanate (FITC)-labeled caspase inhibitor VAD-FMK, which is a cell-permeable fluorescent marker, binds irreversibly to the activated caspases in apoptotic cells [15]. In flow cytometric analysis, *C. albicans* cells exposed to Ple and Ple (4-25) displayed increased fluorescence intensity of 33.8% and 22.4%, respectively, indicating the activation of intracellular caspase. However, only 3.5% of the cells treated with Ple (7-25) showed caspase activation (Fig. 5).

**Phosphatidylserine Externalization by Ple and Analogs in *C. albicans***

Phosphatidylserine exposure serves as a sensitive marker for early stages of yeast apoptosis. It can be detected with FITC-labeled annexin V, which binds to phosphatidylserine with high affinity in the presence of Ca$^{2+}$, and then fluoresces [14]. In this study, phosphatidylserine exposure in the fungal plasma membrane was examined using annexin V-FITC and propidium iodide (PI) co-staining, a representative method for detecting early apoptosis. The result showed that the cell populations in the lower-right quadrant, which means the percentage of early apoptotic cells (Annexin V-positive and PI-negative), increased 15.4% in the presence of Ple (Fig. 6). As shown in Fig. 6, 4.1% and 0.8% of the cells treated with Ple (4-25) and Ple (7-25), respectively, were annexin V-positive and PI-negative.

![Fig. 4. Depolarization of the mitochondrial membrane was detected by JC-1 and measured by flow cytometry. The fluorescence of JC-1 was expressed as the ratio of FL2/FL1. (A) Cells without any treatment, (B) cells treated with H$_2$O$_2$, (C) cells treated with Ple, (D) cells treated with Ple (4-25), and (E) cells treated with Ple (7-25).](image-url)
Fig. 5. The activation of caspases in *C. albicans* was detected by FITC-VAD-FMK staining. (A) Cells without any treatment, (B) cells treated with H$_2$O$_2$, (C) cells treated with Ple, (D) cells treated with Ple (4-25), and (E) cells treated with Ple (7-25).

Fig. 6. Phosphatidylserine externalization from the inner leaflet of the plasma membrane to the outer leaflet, which is observed at an early stage of apoptosis, as shown by annexin V-FITC and PI co-staining. (A) Cells without any treatment, (B) cells treated with H$_2$O$_2$, (C) cells treated with Ple, (D) cells treated with Ple (4-25), and (E) cells treated with Ple (7-25).

**DNA Fragmentation and Nuclear Condensation by Ple and Analogs in *C. albicans***

DNA fragmentation and nuclear condensation are considered the representative phenomena in late-stage apoptotic cells [27]. As cleaved DNA during apoptosis exposes more abundant free 3'-OH termini, it can be detected by TUNEL assay, which labels modified nucleotides catalyzed by terminal deoxynucleotidyl transferase.
The modified nucleotides are secondarily labeled with a marker [22]. The TUNEL method is a fast and sensitive way to visualize the DNA fragmentation in individual cells with fluorescence microscopy [14]. In the *C. albicans* cells exposed to Ple and Ple (4-25), the fragmented DNA showing intensive TUNEL fluorescence, mainly located around the edge of the cells (Fig. 7A). However, DNA fragmentation did not happen in the cells treated with Ple (7-25) (Fig. 7A).

The chromatin condensation and fragmentation can be visualized by fluorescence microscopy after DAPI staining. DAPI passes through the intact cell membrane and binds to the minor groove of A-T-rich sequences of DNA with very high affinity [13]. DAPI staining of the *C. albicans* cells treated with Ple and Ple (4-25) showed concentrated and split fluorescence compared with that of untreated cells, indicating nuclear condensation and fragmentation (Fig. 7B). However, Ple (7-25) did not induce any nuclear morphological changes, like the result of TUNEL assay (Fig. 7B).

**Discussion**

In a previous study, it was reported that intracellular ROS production by Ple could trigger apoptosis in *C. albicans* [4]. The effect of ROS production on apoptotic cell death remains poorly understood. In this study, the cause of apoptosis was investigated using a ROS scavenger, NAC. The changes in viability of *C. albicans* treated with Ple alone and in combination with NAC were observed. NAC remarkably increased the survival of the cells by the free scavenging of ROS, which are commonly harmful to the cells. The result confirmed that intracellular ROS production was a major cause of the apoptosis in *C. albicans*, although ROS-independent apoptosis was slightly induced, and the ROS production played an important role as early signal mediators of apoptosis.

Ple (4-25), Ple (7-25), Ple (1-22), and Ple (1-19), which are N-terminal or C-terminal truncated peptides, were synthesized to investigate the role of the N- and C-terminal regions in the apoptosis. The four analogs had physicochemical patterns, including net charge and hydrophobicity, which are factors that affect the antimicrobial activity of AMPs [29]; net charge [Ple = Ple (4-25) = Ple (7-25) > Ple (1-22) = Ple (1-19)] and hydrophobicity [Ple > Ple (1-22) > Ple (4-25) > Ple (1-19) > Ple (7-25)].

The cells were treated with Ple and the four analogs, and the level of ROS production, which is the major cause of Ple-induced apoptosis, was examined first. Ple induced a significant increase in intracellular ROS, whereas ROS production by the four analogs was not greater than that of Ple on the order of [Ple > Ple (1-22) > Ple (4-25) > Ple (1-19) > Ple (7-25)]. Specifically, Ple (7-25) had no effect on ROS production when truncating GWGSFF (1-6). Furthermore,
OH⁻ accumulation by Ple and its analogs was assessed. Since OH⁻ is known to be a common mediator of apoptosis [24], and a previous study reported that OH⁻ occupied a significant part of ROS produced by Ple [4], it was important to detect OH⁻ production during apoptosis. The result showed the same pattern [Ple > Ple (1-22) > Ple (4-25) > Ple (1-19) > Ple (7-25)] as the ROS production. Ple (7-25) still did not induce OH⁻ accumulation. These results suggested that the N-terminal region of Ple was more crucial for apoptotic activity than the C-terminal region of the peptide, and the hydrophobicity scale of Ple and the four analogs [Ple > Ple (1-22) > Ple (4-25) > Ple (1-19) > Ple (7-25)] seemed to correlate with the apoptotic activity, rather than other factor such as net charge. Therefore, the remaining experiments were done using Ple, Ple (4-25), and Ple (7-25) to understand the relationship between the apoptotic activity and the N-terminal region of Ple.

ROS production can initiate mitochondrial de-energization, finally resulting in the loss of the mitochondrial membrane potential [6]. The dysfunction of the mitochondrial membrane potential promotes the release of pro-apoptotic factors such as cytochrome c, which is able to trigger caspase activation [2, 20]. Changes in the mitochondrial membrane potential and caspase activation by Ple and Ple (4-25) and Ple (7-25) were observed. After the C. albicans cells were treated with Ple, significant depolarization of the mitochondrial membrane and activation of caspases were induced; however, the effects of the two analogs were not more potent than that of Ple. In addition, specific patterns such as [Ple > Ple (4-25) > Ple (7-25)] and the disappearance of apoptotic activity in Ple (7-25) by the truncation of the N-terminal region (1-6) were observed. The results provided further evidence on the importance of the N-terminal region of Ple in apoptosis.

Yeast cells undergo apoptosis, showing characteristic apoptotic makers such as externalization of phosphatidylserine to the outer leaflet of the plasma membrane, DNA fragmentation, and chromatin condensation and fragmentation [14]. In the early phases of apoptosis, phosphatidylserine is translocated from the inner leaflet of the plasma membrane to the outer leaflet [14], and it is measured with the FITC-annexin V and PI double-staining method. Because phosphatidylserine translocation also occurs during necrosis, the FITC-annexin V and PI double-staining method is widely used to discriminate between apoptotic and necrotic cells [9]. In the C. albicans cells treated with Ple and the two analogs, phosphatidylserine externalization was induced; however, the plasma membrane permeability changed little in the specific patterns of activity [Ple > Ple (4-25) > Ple (7-25)]. Phosphatidylserine exposure generally precedes DNA fragmentation and nuclear condensation and fragmentation, which are late-stage apoptotic markers. In TUNEL and DAPI staining, Ple (7-25) did not induce any nuclear and DNA damage, and the specific patterns of the activity [Ple > Ple (4-25) > Ple (7-25)] remained the same. The results showed that the GWGSFF (1-6) in the N-terminal region of Ple played an important role in apoptosis in C. albicans. Furthermore, the apoptotic pattern between Ple and the four analogs was related to the net hydrophobicity rather than to the net charge. It may mean that hydrophobic amino acids, like tryptophan (W) or phenylalanine (F), in the N-terminal regions could have influenced the apoptosis.

In conclusion, the cause of apoptosis induced by Ple was confirmed, and the influence of the N- and C-terminal regions on apoptosis was investigated by synthesizing four truncated analogs. ROS production by Ple was an important cause of apoptosis in C. albicans, and the N-terminal region of Ple also played a key role.

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References


