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# Influence of the Hydrophobic Amino Acids in the N- and C-Terminal Regions of Pleurocidin on Antifungal Activity

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**To investigate the influence of the N- or C-terminal regions of pleurocidin (Ple) peptide on antifungal activity, four analogs partially truncated in the N- or C-terminal regions were designed and synthesized. Circular dichroism (CD) spectroscopy demonstrated that all the analogs maintained an alpha-helical structure. The antifungal susceptibility testing also showed that the analogs exhibited antifungal activities against human fungal pathogens, without hemolytic effects against human erythrocytes. The result further indicated that the analogs had discrepant antifungal activities [Ple>Ple (1–22)>Ple (4–25)>Ple (1–19)>Ple (7–25)] and that N-terminal deletion affected the activities much more than C-terminal deletion. Hydrophobicity [Ple>Ple (1–22)>Ple (4–25)>Ple (1–19)> Ple (7–25)] was thought to have been one of the consistent factors that influenced these activity patterns, rather than the other primary factors like the helicity [Ple>Ple (4–25) >Ple (1–22)>Ple (1–19)>Ple (7–25)] or the net charge [Ple=Ple (4–25)=Ple (7–25)>Ple (1–22)=Ple (1–19)] of the peptides. In conclusion, the hydrophobic amino acids in the N-terminal region of Ple is more crucial for antifungal activity than those in the C-terminal region.**

**Keywords:** Antifungal peptide, hydrophobicity, pleurocidin, truncated analog

Pathogenic fungi possess many complicated mechanisms regarding their resistance to conventional antifungal drugs. In fact, the antifungal resistance has been classified as either of primary, secondary, or third types. The primary (intrinsic) antifungal resistance presents before exposure to antifungal drugs. The secondary (acquired) resistance is that which develops after exposure to antifungal drugs, owing to stable or transient genotypic alterations. The third resistance type can be defined as "clinical resistance" including progression or relapse of an infection by a fungal isolate that seems to be fully susceptible to antifungal drugs [8]. Recently, these resistances have been one of the most critical problems in antifungal therapy; therefore, the suggestion and development of novel therapeutic agents that can overcome this problem have become essential and urgent.

Cationic peptides containing antibacterial and antifungal activities are widely distributed in nature and constituted an essential component of the innate immune system, which is the first line of defense against invasion by pathogenic microorganisms for both vertebrates and invertebrates [17]. As many cationic peptides physically disrupt the cell plasma membrane, causing damage that is hard to fix, they are considered an attractive model of novel therapeutic agents for treating fungal diseases in humans. Among the cationic peptides, pleurocidin (Ple) (GWGSFFKKAAHV GKHVGKAALTHYL-$NH_2$), which was isolated from the skin mucous of the winter flounder *Pleuronectes americanus*, is one of the most well-known α-helical cationic antimicrobial and membrane-active peptides [3]. In this study, four novel analog [Ple (4–25), Ple (7–25), Ple (1–22), and Ple (1–19)], which were partially truncated in the N- or the C-terminal regions of Ple, were designed and their antifungal properties were investigated. Furthermore, the effects of hydrophobic amino acids in the terminal regions on the activity of Ple are suggested.

## Materials and Methods

### Solid-Phase Peptide Synthesis

Peptide synthesis was carried out by Anygen Co. (Gwangju, Korea). The following procedures for peptide synthesis are offered by Anygen Co. The assembly of peptides consisted of a 60-min cycle for each residue at ambient temperature as follows: (i) the 2-chlorotrityl [or 4-methylbenzhydrylamine (MBHA) amide] resin was charged to a reactor and then washed with dichloromethane (DCM) and *N*,*N*-dimethylformamide (DMF) respectively; (ii) a coupling step

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with vigorous was done shaking using a 0.14 mM solution of Fmoc-L-amino acids and Fmoc-L-amino acids pre-activated 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 trifluoroacetic acid (TFA) cocktail solution at ambient temperature [12, 15].

**Analysis and Purification of the Peptide Using HPLC**

Analytical and preparative reverse-phase high-performance liquid chromatography (HPLC) runs were performed with a Shimadzu 20A or 6A gradient system. Data were collected using an SPD-20A 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 $CH_3CN$) over 40 min at flow rates of 1 ml/min 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, respectively.

**Circular Dichroism (CD) Analysis**

CD spectra of the peptides were recorded using a spectropolarimeter (Jasco J720; Japan). All samples were maintained at 25°C during the analysis. Four scans per sample were performed over a wavelength range of 190–250 nm at 0.1 nm intervals. The spectra were measured in 50% (v/v) TFE (2,2,2-trifluoroethanol) and 30 mM SDS in 10 mM sodium phosphate buffer, pH 7.2, respectively, at 25°C using a 1-mm path-length cell. The peptide concentrations were 50 μM. The mean residue ellipticity, [θ], is given in deg·cm$^2$·dmol$^{-1}$: $[\theta]=[\theta]_{obs}$ (MRW/10lc), where $[\theta]_{obs}$ is the ellipticity measured in millidegrees, MRW is the mean residue molecular weight of the peptide, c is the concentration of the sample in mg/ml, and l is the optical path-length of the cell in cm [9, 11].

**Antifungal Susceptibility Testing**

*Aspergillus flavus* (KCTC 1375) and *Malassezia furfur* (KCTC 7744) were obtained from the Korean Collection for Type Cultures (KCTC) of the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Korea). *Candida albicans* (ATCC 90028) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). Amphotericin B, fluconazole, and itraconazole were purchased from Sigma-Aldrich Co. and used as positive control antifungal compounds. Fungal cells were cultured in YPD broth (Difco) with aeration at 28°C. *M. furfur* was cultured at 32°C in YM broth (Difco) containing 1% olive oil. Fungal cells ($2\times10^4$ cells/ml) were inoculated into either YPD or YM broth. Thereafter, 0.1 ml/well of the mixture was dispensed into microtiter plates. The minimum inhibitory concentration (MIC) was determined by means of a serial 2-fold dilution of the peptides or compounds, following the microdilution method and MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay. After 48 h of incubation at either 28°C or 32°C, the minimal peptide or compound concentration to prevent the growth of a given test organism was determined and defined as MIC. Growth was assayed with a microtiter ELISA Reader by monitoring absorption at 580 nm. MIC values were determined by three independent assays [7, 10].

**Hemolysis Assay**

The hemolytic activity of the peptides was evaluated by determining the release of hemoglobin from an 8% suspension of fresh human erythrocytes at 414 nm with an ELISA plate reader. The percentage of hemolysis was calculated by using following equation: % hemolysis = [($Abs_{414\ nm}$ in the peptide solution – $Abs_{414\ nm}$ in PBS)/($Abs_{414\ nm}$ in 0.1% Triton X-100 – $Abs_{414\ nm}$ in PBS)] × 100 [16].

## RESULTS

### Secondary Structures of the Analogs of Ple

To examine the alteration or the maintenance of the secondary structures of the analog peptides, CD spectroscopy was

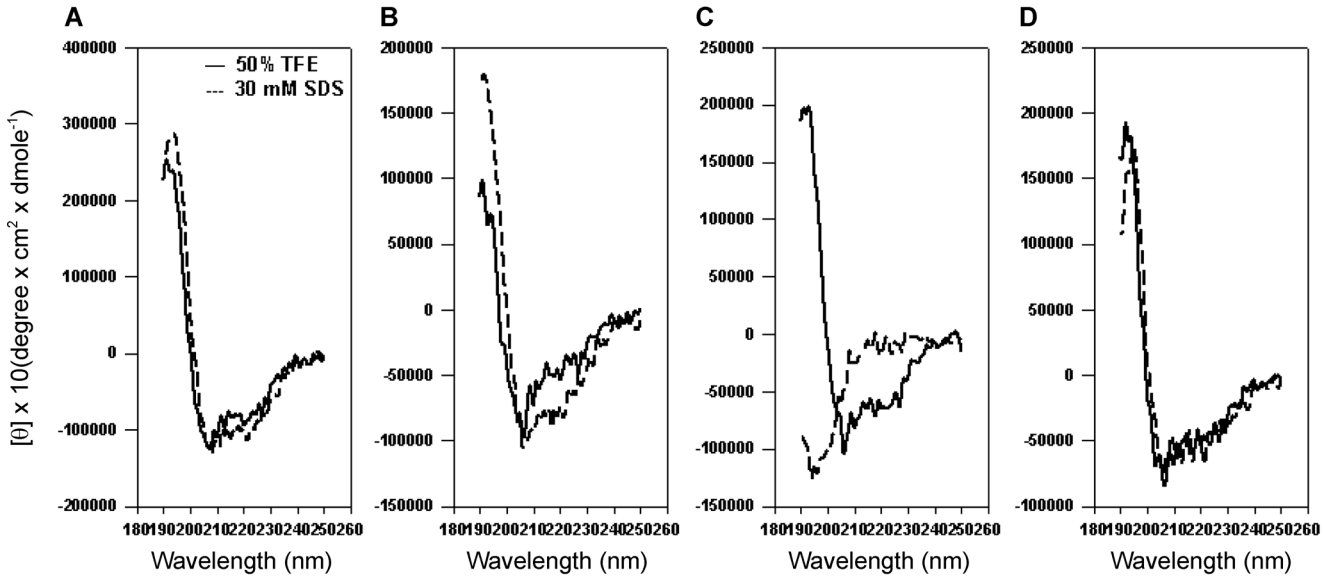


**Fig. 1.** CD spectra of Ple and its analogs.
**A**. Ple (4–25). **B**. Ple (7–25). **C**. Ple (1–22). **D**. Ple (1–19). —, 50% TFE; --, 30 mM SDS.

**Table 1.** Amino acid sequence and physicochemical features of Ple and its analogs.

| Peptide | Amino acid sequence | Molecular mass (MW) | | % alpha helicity | Net charge | Retention time (min) |
|---|---|---|---|---|---|---|
| | | Calculated value | Observed value | | | |
| Ple | GWGSFFKKAAHVGKHVGKAALTHYL-$NH_2$ | 2,710.1 | 2,710.5 | 100 | +7 | 16.967 |
| Ple (4–25) | SFFKKAAHVGKHVGKAALTHYL-$NH_2$ | 2,409.8 | 2,409.4 | 60.6 | +7 | 15.742 |
| Ple (7–25) | KKAAHVGKHVGKAALTHYL-$NH_2$ | 2,028.4 | 2,028.6 | 25.5 | +7 | 14.458 |
| Ple (1–22) | GWGSFFKKAAHVGKHVGKAALT-$NH_2$ | 2,296.6 | 2,296.3 | 45.0 | +6 | 16.150 |
| Ple (1–19) | GWGSFFKKAAHVGKHVGKA-$NH_2$ | 2,011.3 | 2,011.3 | 33.7 | +6 | 15.567 |

first performed in membrane-like environments. The result showed that the analogs were specifically characterized by exhibiting double-negative maximum ellipticity in the 50% TFE, suggesting that they maintained an α-helical structure like the template peptide, Ple (Fig. 1) [9]. It meant that the partial deletion of the terminal residues of Ple did not affect the secondary structure of the analogs.

**Antifungal and Hemolytic Activities of the Analogs of Ple**

Subsequently, to understand the biological properties of the analogs, the antifungal susceptibility testing and hemolysis assays were conducted against human fungal pathogens and human erythrocytes, respectively. In the present study, melittin, which is derived from the venom of the honey bee *Apis mellifera* [5], was used as a positive-control peptide (Table 1). Additionally, conventional antibiotics, such as amphotericin B, fluconazole, and itraconazole, were employed to compare their antifungal activities with the activities of the peptides. Amphotericin B, an amphipathic polyene macrolide, is a representative antibiotic derived from *Streptomyces nodosus* [6] and its primary target is known to be cell membrane ergosterol [13]. Fluconazole is a well-known azole-derived antifungal agent. Itraconazole is also an effective azole antifungal agent, inhibiting ergosterol synthesis through an interaction with C-I4 alpha demethylase [2]. However, the resistance of fungi to polyenes can be induced by a reduction in the amount of plasma membrane ergosterol, to which polyenes bind. Moreover, high-level azole resistance in several *Candida* species correlates with overexpression in the plasma membrane of proteins pumping the drug out of the cell, thereafter reducing intracellular azole concentrations to levels at which Erg11p is not inhibited [1, 13, 14, 18].

The result showed that all the peptides and compounds contained significant antifungal activities (Table 2). However, the truncated analogs exhibited less potent activities, containing different potencies from one another, with MIC values in the 10 to 80 μM range, compared with the template peptide, Ple. Melittin and conventional antibiotics also had potent antifungal activities, with MIC values in the 1.25 to 10 μM range. With regard to the hemolysis assay, the analogs did not induce hemolysis at all the concentrations, whereas Ple caused about 7% hemolysis at the highest concentration (Table 3). These results indicated that the truncated analogs may be applied as novel therapeutic models for treating fungal diseases in humans, without cytotoxic effects.

**Table 2.** Antifungal activity of Ple and its analogs.

| | MIC (μM) | | |
|---|---|---|---|
| | *A. flavus* | *C. albicans* | *M. furfur* |
| Ple | 5 | 5 | 5 |
| Ple (4–25) | 20 | 10–20 | 20 |
| Ple (7–25) | 40 | 40 | 40-80 |
| Ple (1–22) | 10 | 10 | 10 |
| Ple (1–19) | 20–40 | 40 | 40 |
| Melittin | 1.25 | 1.25 | 2.5 |
| Amphotericin B | 2.5 | 2.5 | 5 |
| Fluconazole | 10 | 5 | 10 |
| Itraconazole | 10 | 5 | 10 |

## DISCUSSION

Several strategies have been suggested to generate highly active antimicrobial peptides containing low hemolytic activity. The primary approach has involved the analysis of sequences of naturally occurring antimicrobial peptides in order to select sequence regions that may contribute to its activity, and thereafter to synthesize novel analog peptides

**Table 3.** Hemolytic activity of Ple and its analogs.

| Peptide | % Hemolysis (μM) | | | | | |
|---|---|---|---|---|---|---|
| | 100 | 50 | 25 | 12.5 | 6.25 | 3.13 |
| Ple | 7 | 0 | 0 | 0 | 0 | 0 |
| Ple (4–25) | 0 | 0 | 0 | 0 | 0 | 0 |
| Ple (7–25) | 0 | 0 | 0 | 0 | 0 | 0 |
| Ple (1–22) | 0 | 0 | 0 | 0 | 0 | 0 |
| Ple (1–19) | 0 | 0 | 0 | 0 | 0 | 0 |
| Melittin | 100 | 100 | 100 | 98 | 98 | 97 |

[4]. Meanwhile, with respect to the antimicrobial activity of the peptide, Dathe and Wieprecht [4] focused on the role of the structural parameters like helicity, hydrophobicity, hydrophobic moment, and the size of the polar/apolar helix domain or peptide charge for both binding and permeabilizing efficiencies.

As mentioned, the antifungal and hemolytic activities of the truncated analogs of Ple were examined, and the results showed that the analogs contained remarkable activities without cytotoxicity against human erythrocytes. Interestingly, the analogs had discrepant hydrophobicity [Ple>Ple (1–22)>Ple (4–25)>Ple (1–19)>Ple (7–25)] and the pattern of their antifungal activities [Ple>Ple (1–22)>Ple (4–25)>Ple (1–19)>Ple (7–25)] was nearly consistent with their hydrophobicity. We also considered that other significant factors could influence the activities; however, the helicity [Ple>Ple (4–25)>Ple (1–22)>Ple (1–19)>Ple (7–25)] or the net charge [Ple=Ple (4–25)=Ple (7–25)>Ple (1–22)=Ple (1–19)] were not shown to affect the activity patterns. It also meant that the deletion of the hydrophobic amino acids, like tryptophan (W) or phenylalanine (F), in the N-terminal regions influenced the activities more than the deletion of leucine (L) in the C-terminal regions.

In conclusion, although the exact factor(s) related to the antifungal activities of Ple must be further clarified, we have shown that the hydrophobicity of the analog peptides was predominately connected to their activities, and specifically, the hydrophobic amino acids in the N-terminal regions of the Ple were more important for the activities than those in the C-terminal regions.

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