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Antifungal Mechanism of Antifungal Peptide Derived from Cecropin A(1-8)-Melittin(1-12) Hybrid against *Aspergillus fumigatus*

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Abstract The antifungal mechanism of the antifungal peptide against *Aspergillus fumigatus*, $K^{18,19}$-CA(1-8)-ME(1-12), derived from cecropin A(1-8)-melittin(1-12) was investigated by confocal laser scanning microscopy, cell wall regeneration, ATPase activity inhibition, and released potassium ion. By confocal laser scanning microscopy, $K^{18,19}$-CA(1-8)-ME(1-12) was detected on the surface of *A. fumigatus*, while cecropin A used as a negative control peptide was not detected. The protoplast of *A. fumigatus* treated with $K^{18,19}$-CA(1-8)-ME(1-12) failed to regenerate the fungal cell walls. Compared with cecropin A, the amount of potassium ion released by $K^{18,19}$-CA(1-8)-ME(1-12) was increased. Furthermore, $K^{18,19}$-CA(1-8)-ME(1-12) inhibited the ATPase activity on the plasma membrane. These results suggested that $K^{18,19}$-CA(1-8)-ME (1-12) acts on the plasma membrane of *A. fumigatus* and its antifungal action is due to the ion channel or pore formation on the plasma membrane.

Key words: Cecropin A, melittin, *Aspergillus fumigatus*

A filamentous fungi, *Aspergillus fumigatus*, is known to be the common pathogen which causes both invasive and noninvasive aspergillosis [2, 10], and is especially responsible for several types of respiratory mycosis. Although many investigations have been performed to identify the antibiotic materials either from natural sources or from synthetic compounds, other drug-resistant strains have been isolated continuously. Therefore, constitutive efforts have been succeeded to develop more potent and efficient antibiotic agents against this pathogenic fungi [8].

Among these antibiotics, several antimicrobial peptides such as cecropin A (CA), melittin (ME), and magainin 2 (MA) have been isolated from many organisms [3, 14, 16]. These peptides play a central role in the host defense system and innate immunity against infectious pathogens [4, 9]. Both CA and MA have strong antibacterial activity, but have less or no activity against fungi [13, 14, 16]. ME has a powerful activity against fungi as well as bacteria, while it also shows a strong hemolytic activity [15]. For these reasons, several modified and hybrid peptides were designed to generate potent antibiotic peptides with no hemolytic effect. As part of the efforts, Andreu *et al.* [1] designed the CA-ME hybrid peptides that possess N-terminal basic regions of CA, followed by hydrophobic N-terminal regions of ME, and determined that these hybrid peptides contain increased antimicrobial activities with broader spectrum than CA, while they have no or less hemolytic effect [5].

In our previous study, the CA(1-8)-ME(1-12) hybrid peptide, composed of the N-terminal segments of both CA(1-8) and ME(1-12), has shown powerful antifungal activity with less hemolytic activity [11, 12]. Additionally, to improve the antibiotic activities, CA(1-8)-ME(1-12) analogues were redesigned by amino acid substitution. Among these analogues, $K^{18,19}$-CA(1-8)-ME (1-12) of which Thr residues at position 18 and 19 are substituted with Lys, showed a 2-fold higher antifungal activity against *Trichosporon beigelii* than the CA(1-8)-ME (1-12) hybrid. $K^{18,19}$-CA (1-8)-ME(1-12) showed higher fluorescence intensity in FASCan analysis than CA with low antifungal activity against *Trichosporon beigelii*. This peptide was detected in the intracellular region as well as in the cell membrane of *T. beigelii* in confocal laser scanning microscopy. Therefore, these facts suggested that potent antifungal activity of this peptide against yeast such as *T. beigelii* is due to the pore formation in cell membrane [11].

However, the antifungal mechanism of the antifungal peptide against the filamenteous fungi such as *Aspergillus*

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fumigatus has not been clearly elucidated. Therefore, in this study, the antifungal mechanism for *A. fumigatus* of the antifungal peptide [$K^{18,19}$-CA(1-8)-ME(1-12)] was investigated by confocal laser scanning microscopy, cell wall regeneration, the amount of released potassium ion, and the ATPase activity inhibition on the plasma membrane.

MATERIALS AND METHODS

Fungal Strain and Growth Condition

A. fumigatus (KCTC 6145) was obtained from the Korean Collection for Type Cultures (Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea). It was grown at 28°C in YM medium (1% glucose, 0.3% malt extract, 0.5% peptone' and 0.3% yeast extract).

Peptide Synthesis and Biotinylation

CA, ME, and $K^{18,19}$-CA(1-8)-ME(1-12) were synthesized by the solid method using Fmoc-chemistry [13]. Biotinamidocaproate N-hydroxysuccinimide ester (Sigma, St. Louis, U.S.A.) was used to prepare the biotinylated peptides of CA and $K^{18,19}$-CA(1-8)-ME(1-12) as described in our previous report [11, 12]. Prior to the final clevage-deprotection step of the protected peptide-resin, the biotinylation of the peptides was carried out using biotinamidocaproate N-hydroxysuccinimide ester and HOBt (N-hydroxybenzotriazole)

Confocal Laser Scanning Microscopy (CLSM)

The intracellular distributions of the analogue peptide, $K^{18,19}$-CA(1-8)-ME(1-12), and CA were analyzed by CLSM. Cells were treated with the biotinylated peptides (10/ml) of CA or $K^{18,19}$-CA(1-8)-ME(1-12). After incubation at 30°C for 10 min, the unbounded peptides were removed by washing with PBS and cells were incubated with streptavidin-FITC conjugates (Becton Dickinson, San Jose, U.S.A.). Visualization and localization of the labeled peptides were performed by Leica TCS 4D connected to a Leica DAS upright microscope (Leica Lasertech GmbH, Heidelberg, Germany).

Protoplast Preparation and Cell Wall Regeneration

For the *A. fumigatus* protoplast preparation, the spores (3×10^7/ml) were digested with 10 mM phosphate buffer (pH 6.0) containing 0.8 M NaCl, 5 mg/ml of Novozyme 234 (Sigma, St. Louis, U.S.A.) and 5 mg/ml of cellulase (Sigma, St. Louis, U.S.A.) for 5 h at 30°C by gentle agitation. After incubation, the reaction mixtures were filtered through 3G3 glass filter. The obtained protoplasts were gently centrifuged at 700×g and then resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing 0.8 M NaCl, and 10 mM $CaCl_2$. To examine the cell wall regeneration depending on the antifungal peptides, the peptide solutions (10 μg/ml) were added to the protoplasts. After incubation for 3 h at 30°C, the protoplasts treated with peptides were transferred into the YM soft-agar solutions containing 0.8 M NaCl and 0.5% agar, and then spreaded on agar plates in YM medium containing 0.8 M NaCl and 2% agar. The plates were incubated at 30°C for 4 days, and then the regenerated colonies were counted.

Measurement of the Released Potassium ion

A. fumigatus spores (2×10^4/ml) were inoculated on the culture tubes containing 100 μl YM media-peptide (5 μg/ml) mixture and the tubes were incubated at 30°C for 30 h. After incubation, the cell suspensions were centrifuged at 10,000 rpm for 10 min and the supernatants were collected for the next step. The released amounts of potassium ion were measured by Inductively Coupled Plasma Mass Spectrometer (FISONS, Cheshire, U.K.).

Plasma Membrane Preparation and ATPase Activity Assay

For the plasma membrane preparation, the cell walls of *A. fumigatus* spores (5×10^9) were removed as the same method described in protoplast preparation. The obtained protoplasts were homogenized using a Telfon homogenizer. Cell debris and unbroken cells were removed by centrifugation at 1,000×g for 10 min, and then the supernatants were ultracentrifuged at 100,000×g for 1 h. The pelleted plasma membrane fractions were resuspended in 10 mM phosphate buffer, pH 6.0. The amounts of membrane proteins in plasma membrane preparations were determined according to the method described in Bio-Rads protein assay. The ATPase activity was measured by the liberation of inorganic phosphate in 0.5 ml of reaction mixture contained 5 mM NaATP (Sigma, St. Louis, U.S.A.), 5 mM $MgCl_2$, 5 mM phosphoenolpyruvate, 10 unit pyruvate kinase (Sigma, St. Louis, U.S.A.), 0.5 mM EDTA, Oligomycin (10 μg/ml) (to inhibit residual mitochondrial ATPase), and 10 mM Tris/PIPES buffer, pH 6.7 [6]. The reaction was started by the addition of 5 μg of plasma membrane preparation and serially diluted CA-ME analogue peptide [$K^{18,19}$-CA(1-8)-ME(1-12)]. After incubation at 30°C for 10 min, the reaction was stopped by the addition of 100 μl 50% trichloroacetic acid. The tubes were then centrifuged at 2,000×g for 5 min and the amount of inorganic phosphate in 300 μl of the supernatant was measured by the methods described in Dryer *et al.* [7].

RESULTS AND DISCUSSION

Synthesis of $K^{18,19}$-CA(1-8)-ME(1-12) and Its Biotinylated Peptide

The purity of synthetic CA, ME, and $K^{18,19}$-CA(1-8)-ME (1-12) was confirmed by the profile of analytical reversed-phase HPLC (data not shown). The correct amino acid

composition of synthetic CA, ME, and $K^{18,19}$-CA(1-8)-ME (1-12) was confirmed by amino acid analysis of their 6N-HCl hydrolysate (data not shown). Biotinamidocaproate N-hydroxysuccinimide ester as the biotin-reagent was used to prepare the biotinylated peptides of CA and $K^{18,19}$-CA(1-8)-ME(1-12) [11, 12]. Minimal inhibitory concentation (MIC) of $K^{18,19}$-CA(1-8)-ME(1-12) against *A. fumigatus* determined by the MTT assay [11] described in our previous study was 2.5 μg/ml (data not shown).

Interaction of $K^{18,19}$-CA(1-8)-ME(1-12) on the Cell Surface of *A. fumigatus*

In order to investigate where the antifungal peptide, $K^{18,19}$-CA(1-8)-ME(1-12), interacts with the filamentous fungi, *A. fumigatus* mycelium was incubated both with the biotinylated-$K^{18,19}$-CA(1-8)-ME(1-12) and CA (negative control peptide) with no or less antifungal activity. The distribution and the localization of the peptides were determined by confocal laser scanning microscopy after sequential incubation with streptavidin-FITC conjugates. If the antifungal action of the peptides was due to ion channel or pore formation, the fluorescence signal should be observed in the cell membrane. As shown in Fig. 1, $K^{18,19}$-CA(1-8)-ME(1-12) was distributed through the mycelium surface, while signals in CA were not detected. These facts suggest that $K^{18,19}$-CA(1-8)-ME(1-12) interacts directly on the mycelium surface and the antifungal activity by this peptide is due to ion channel or pore formation in cell membranes of *A. fumigatus*.

Influence of $K^{18,19}$-CA(1-8)-ME(1-12) on the Plasma Membrane of *A. fumigatus*

The above results indicated that $K^{18,19}$-CA(1-8)-ME(1-12) acts directly on the mycelium surface, but could not define whether the action sites were the cell wall or the cell membrane. In order to define the action site more accurately, the *A. fumigatus* protoplasts were prepared and the effects of $K^{18,19}$-CA(1-8)-ME(1-12) on the plasma membrane were examined. The prepared protoplasts were incubated either with $K^{18,19}$-CA(1-8)-ME(1-12) or CA. After incubation, plasma membrane damage was determined by calculating the frequency of regeneration, which indicates the regeneration of cell wall. As we expected, the protoplasts treated with $K^{18,19}$-CA(1-8)-ME(1-12) did not produce any colony (Table 2), which indicates the cell membrane was selectively disrupted or, at least, the cell wall regeneration functions were collapsed. On the contrary, the frequencies of regeneration value in the negative control (no peptide) and in CA-treated protoplasts were

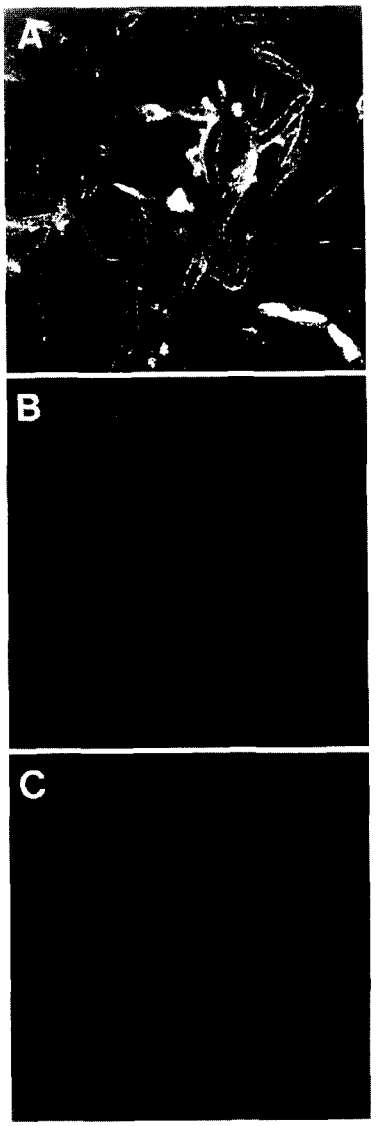

Fig. 1. Distribution of $K^{18,19}$-CA(1-8)-ME(1-12) in *Aspergillus fumigatus* as visualized by confocal laser scanning microscopy. A: *A. fumigatus* cells were incubated with biotinylated $K^{18,19}$-CA(1-8)-ME(1-12). B: *A. fumigatus* cells were incubated with biotinylated CA. C: *A. fumigatus* cells were incubated without peptide treatment.

Table 1. Amino acid sequences of the peptides.

Peptides	Sequences
CA	KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH_2
ME	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2
CA(1-8)-ME(1-12)	KWKLFKKIGIGAVLKVLTTG-NH_2
$K^{18,19}$-CA(1-8)-ME(1-12)	KWKLFKKIGIGAVLKVL<u>KK</u>G-NH_2

The underlined amino acid residues indicate the substituted ones from CA(1-8)-ME(1-12).
The superscript numbers indicate the positions in CA(1-8)-ME(1-12).

Table 2. Effects of the peptides on the regeneration of protoplast of *A. fumigatus*.

Peptidesm	Frequency of regeneration (F. R.[b])
Control[a]	85.3
CA	79.3
$K^{18,19}$-CA(1-8)-ME(1-12)	3.4

[a]Control indicates no peptide treatment.
[b]Frequency of regeneration (F.R.) values were calculated by the following equation: F.R. (%)=[(No. of colonies on plate)/(No. of protoplasts)]×100.

85.3% and 79.3%, respectively (Table 2). These results suggest that the antifungal activity of $K^{18,19}$-CA(1-8)-ME (1-12) is due to the interaction between the peptide and plasma membrane instead of the cell wall.

Potassium Ion Released by $K^{18,19}$-CA(1-8)-ME(1-12)

In order to characterize the antifungal action of $K^{18,19}$-CA(1-8)-ME(1-12) on *A. fumigatus*, the amount of potassium released by the incubation of $K^{18,19}$-CA(1-8)-ME(1-12) and CA with *A. fumigatus* were examined. Compared with the negative control (no peptide) and CA, the amount of potassium released by $K^{18,19}$-CA(1-8)-ME (1-12) were increased (Table 3). This result suggests that $K^{18,19}$-CA(1-8)-ME(1-12) acts on the plasma membrane of *A. fumigatus* either by channel or pore formation.

Table 3. Amount of the released potassium ion by incubating *A. fumigatus* with the peptides.

Peptides	Potassium ion (ppm)
Control[a]	74.9
CA	82.6
$K^{18,19}$- CA(1-8)-ME(1-12)	102.8

[a]Control indicates no peptide treatment.

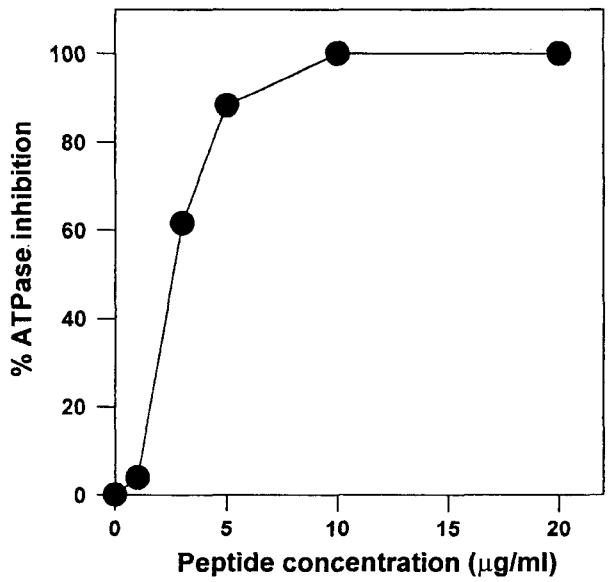

Fig. 2. The curve of plasma membrane ATPase activity inhibition of *A. fumigatus* by $K^{18,19}$-CA(1-8)-ME(1-12).

Inhibition of ATPase Activity on the Plasma Membrane of *A. fumigatus*

In order to further elucidate that $K^{18,19}$-CA(1-8)-ME(1-12) acts on the plasma membrane of *A. fumigatus*, the inhibition of ATP activity on the plasma membranes of *A. fumigatus* by $K^{18,19}$-CA(1-8)-ME(1-12) was investigated. Oligomycin was used for the inhibition of mitochondrial ATPase activity of *A. fumigatus*. As shown in Fig. 2, $K^{18,19}$-CA(1-8)-ME(1-12) inhibited the ATPase activity in a dose-dependent manner and gave complete inhibition at 10 μg/ml. Based on the results in this investigation, the possible action site of $K^{18,19}$-CA(1-8)-ME(1-12) might be the plasma membrane rather than cell wall of *A. fumigatus*. The inhibition of membrane ATPase activity might be due to the disruption of plasma membrane instead of direct interaction with ATPase. The precise relationship between antifungal peptide and membrane ATPase activity inhibition will be elucidated in a further study.

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