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Antifungal activity of synthetic peptide derived from halocidin, antimicrobial peptide from the tunicate, *Halocynthia aurantium*

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Abstract **Halocidin is an antimicrobial peptide isolated from the hemocytes of the tunicate. Among the several known synthetic halocidin analogues, di-K19Hc has been previously confirmed to have the most profound antibacterial activity against antibiotic-resistant bacteria. This peptide has been considered to be an effective candidate for the development of a new type of antibiotic. In this study, we have assessed the antifungal activity of di-K19Hc, against a panel of fungi including several strains of *Aspergillus* and *Candida*. As a result, we determined that the MICs of di-K19Hc against six *Candida albicans* and two *Aspergillus* species were below 4 and 16 μg/ml, respectively, thereby indicating that di-K19Hc may be appropriate for the treatment of several fungal diseases. We also conducted an investigation into di-K19Hc's mode of action against *Candida albicans*. Our colony count assay showed that di-K19Hc killed *C. albicans* within 30 s. Di-K19Hc bound to the surface of *C. albicans* via a specific interaction with β-1,3-glucan, which is one of fungal cell wall components. Di-K19Hc also induced the formation of ion channels within the membrane of *C. albicans*, and eventually observed cell death, which was confirmed via measurements of the K^+ released from *C. albicans* cells which had been treated with di-K19Hc, as well as by monitoring of the uptake of propidium iodide into the *C. albicans* cells. This membrane-attacking quality of di-K19Hc was also visualized via confocal laser and scanning electron microscopy.**

Keywords: Antimicrobial peptide; Antifungal activity; Halocidin; Di-K19Hc; Mode of candidacidal action

1. Introduction

In the last two decades, the incidence of human fungal infections has increased dramatically, in parallel with the wide spread of incurable infectious diseases associated with antibiotic-resistant bacteria. Fungal diseases have become a growing threat, especially in immunocompromised patients, for which few or no effective drugs are currently available [1]. Accordingly, a variety of studies have been conducted in an attempt to isolate natural antifungal substances with potential pharmaceutical utility, and to develop and design new synthetic or semi-synthetic drugs [2–4].

Antimicrobial peptides have recently become the focus of considerable interest as a candidate for a new type of antibiotic, due primarily to their potency against pathogenic microbes that are resistant to conventional antibiotics, as well as their broad-spectrum activity [5]. Since the first identification of cecropin [6] and defensin [7] in insect hemolymph and human neutrophils, respectively, more than 880 antimicrobial peptides have been isolated from a wide variety of organisms, including vertebrates, invertebrates, and plants [8]. Tunicates, simple marine protochordates, have recently been reported to harbor several antimicrobial peptides in their hemocytes, including halocidin [9], dicynthaurin [10], clavanins [11], styelins [12], clavaspirin [13], plicatamide [14] and halocyamine [15]. They were also purified and studied in order to characterize the relationship between their structure and activity. Among these compounds, the halocidin detected in *Halocynthia aurantium* appears to exhibit the most potent antibacterial activity. It is a heterodimeric peptide consisting of two α-helical monomers with 18 and 15 amino acid residues, referred to as 18Hc and 15Hc, respectively. From the structure of 18Hc, several synthetic congeners have been designed and evaluated with regard to their antibacterial activities against a variety of antibiotic-resistant bacteria, under diverse conditions [16]. As a result of these trials, it was determined that di-K19Hc, a homodimeric version of a synthetic peptide (K19Hc), formed via the addition of a lysine (K) residue to the N-terminus of 18Hc, exhibited profound antibacterial effects, and was also shown to exhibit much alleviated hemolytic activity against human erythrocytes. Therefore, di-K19Hc was considered to be the most appropriate candidate for the development of a novel peptide antibiotic, although the mechanisms underlying the aforementioned activities remained to be elucidated. In the present study, we tested di-K19Hc with regard to antifungal activities against 13 fungal strains, including two major genera, the *Candida* and *Aspergillus* strains, which are responsible for the majority of fungal pathogenic infections. We also attempted to determine the manner in which di-K19Hc exerts its antifungal effects against *C. albicans*. In this paper, we describe the antifungal activity of di-K19Hc, and the mechanism underlying this effect.

2. Materials and methods

2.1. Peptides

K19Hc and P18, a cecropin-magainin hybrid [17], were synthesized using an automated solid-phase peptide synthesizer (Pioneer Applied Biosystems, Foster, CA), at the Korea Basic Science Institute. The Buforin II [18] used in this study was purchased from Sigma (St. Louis, USA). All of the peptides were then re-purified via C18 reverse-phase (RP) high-pressure liquid chromatography (HPLC). In order to

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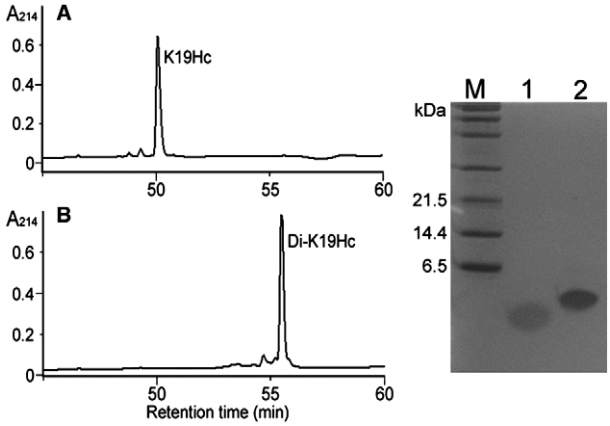

Fig. 1. RP-HPLC profiles and tricine SDS–PAGE analysis for purified K19Hc and di-K19Hc. Each of the HPLC iterations was performed under the same conditions. The HPLC fractions were eluted in various linear gradients of acetonitrile containing 0.1% trifluoroacetic acid (TFA). For the first 10 min after sample loading, the C18 column (Vydac 218TP54: The Separation Group, Hesperia, CA, USA) was washed with 5% acetonitrile at a 0.5 ml/min flow rate. Then the acetonitrile concentration linearly increased by 1%/min, for 60 min. K19Hc was eluted at 45% of acetonitrile (A). Di-K19Hc was eluted at 50% of acetonitrile (B). The right panel shows a Coomassie blue-stained, 16.5% tricine SDS–PAGE gel, containing purified K19Hc and di-K19Hc. Lane M, standard molecular weight markers; lane 1, K19Hc; lane 2, di-K19Hc. Samples were electrophoresed under non-reducing condition. A Model Mini-Protean 3 Cell (Bio-Rad) was used to perform tricine SDS–PAGE [19].

prepare the di-K19Hc, the purified K19Hc was exposed to 0.1 M ammonium bicarbonate (1 mg/ml) for at least 72 h at room temperature. Fig. 1 shows the HPLC profiles of the purification of K19Hc and di-K19Hc. The purity of the peptides was monitored via HPLC profile and tricine SDS–PAGE analysis (right panel in Fig. 1) [19], conducted under non-reducing conditions. The purified peptides were completely dried and stored at −70 °C until used. When necessary, the peptides were dissolved in acidified distilled water (0.01% acetic acid), and the peptide concentrations were then evaluated using the bicinchoninic acid technique (Pierce, Rockford, IL), with bovine serum albumin employed as a standard protein.

2.2. MICs of the peptides against fungi

The minimum inhibitory concentrations (MICs) [20] for di-K19Hc, K19Hc, buforin II, and P18 against 13 fungal strains were determined in accordance with the methods developed by the National Committee on Clinical Laboratory Standards (NCCLS) [21], with some modifications. Two fungal spores (*Pyricularia grisea* KACC40425 and *Fusarium oxysporum* KACC40902) and one yeast (*Geotrichum candidum* KACC40354) were grown in 1/2 Potato Dextrose Broth (Difco, USA), and two other yeast strains (*Cryptococcus neoformans* KCCM50544 and *Trichosporon beigelli* KCTC7707) were cultivated in YM broth (Difco) for 48 h at 30 °C. The *Candida* (*C. albicans* KCTC7121, 7122, 7270, 7728, 7729, 7965) and *Aspergillus* (*A. terreus* KCTC6178, *A. niger* KCTC6461) strains were cultured in Sabouraud Dextrose Broth (SDB: Difco) for 24 and 72 h, respectively, at 30 °C. The SDB was prepared in 10 mM sodium phosphate buffer, at a pH of 7.0. Each of the filamentous or yeast-phase fungal strains was diluted in its corresponding broth, to a final concentration of 2×10^3 spores/ml or cells/ml, respectively. The numbers of spores or yeasts were counted using a hemocytometer. Stock peptide solutions were prepared to a concentration of 640 μg/ml in 0.01% acetic acid, and were then serially diluted twofold, to 10 μg/ml. 100 μl aliquots of the diluted culture media were dispensed into the wells of 96-well polypropylene microtiter plates (Costar 3790, Corning, NY), to which peptide solution (11 μl) was then added. In the experiments conducted to assess the candidacidal activity of peptides in the presence of salt, we used SDB supplemented with up to 200 mM NaCl for the culture medium, which was mixed with the peptide solution. The antifungal activities were evaluated after 48 h of incubation at 30 °C, by observations of visible turbidity in each of the wells in the plates. The MIC values were expressed as intervals ($a - b$), in which a represents the highest tested concentration at which microbes continue to grow, and b is the lowest concentration at which growth inhibition can be detected. These experiments were repeated at least three times, and each was conducted on a different day.

2.3. Colony count analysis for the candidacidal activity of di-K19Hc

100 μl of a predetermined concentration of di-K19Hc was mixed with 1 ml aliquots of yeast-phase *C. albicans* (3×10^7 colony forming units/ml, CFU/ml), in 10 mM sterile sodium phosphate buffer (pH 7.4). The mixtures, in final volumes of 1.1 ml, were then incubated in a shaking incubator at 30 °C, and 100 μl aliquots were removed at predetermined intervals. Each of the aliquots directly or after dilution was plated on SDB agar consisting of 1% agarose and 3% SDB powder in 10 mM sodium phosphate buffer, at neutral pH. The resultant colonies were then counted, after being incubated overnight at 30 °C.

2.4. Potassium efflux

The K^+ efflux from the di-K19Hc-treated *C. albicans* cells was measured with An Istek model 735 pH/ISE meter, fitted with a potassium ion electrode (pHoenix Electrode, USA), as was described in the study of Orlov et al. [22]. In brief, the *C. albicans* KCTC7122 cells were cultured overnight in 50 ml of SDB, at 30 °C. The cells were washed three times with 10 mM sodium phosphate buffer, pH 7.4, and resuspended at a concentration of $\sim 3.5 \times 10^7$ cells/ml in the same buffer. One milliliter aliquots of these *C. albicans* suspensions were then incubated with 100 μl of di-K19Hc (1 mg/ml in 0.01% acetic acid) at 30 °C, for predetermined times. After a 30-s spindown at 12000 rpm, 9 ml of distilled water was added to the supernatant of each of the samples. The total K^+ efflux was then determined, as the concentration of K^+ released from the *C. albicans* after the disruption of the samples via prolonged sonication (3 min at 60% power: Geräte-Type UW 2200, Bandelin Electronic, Berlin, Germany). These experiments were repeated five times, and the mean values were utilized in the construction of the graph. The percent K^+ release was then calculated, according to the following equation: K^+ release (%) = ([K^+] of sample − [K^+] of peptide-free control)/([K^+] of 100% control − [K^+] of peptide-free control) × 100.

2.5. FACScan analysis

The integrity of the yeast membranes after peptide treatment was determined by FACScan analysis, via nuclear staining with propidium iodide (PI; Sigma) as was described by Yeaman et al. [23]. In brief, *C. albicans* KCTC7122 was first harvested after being cultured overnight, and then washed twice with phosphate-buffered saline (PBS, 1.47 mM KH_2PO_4, 10 mM $Na_2HPO_4 \cdot 12H_2O$, 2.7 mM KCl, 137 mM NaCl, pH 7.4). The washed cells (2×10^5 cells) were mixed with peptides at a concentration of 3.12 μg/ml in PBS, then incubated for 10 min at 30 °C. The peptide-treated cells were incubated in PI solution (50 μg/ml final concentration) for 30 min at 4 °C. After incubation, the unbound dye was removed via the excessive washing of cells with PBS. The PI was excited at 488 nm with an argon laser, and the resulting fluorescence emission was collected through a $\geqslant$620 nm long pass filter. Flow cytometry analysis was conducted using a FACScan instrument (Becton–Dickinson, San Jose, CA).

2.6. Assay for binding of di-K19Hc to the component of fungal cell wall

Binding property of di-K19Hc to the surface of *C. albicans* was examined by assessing the effect of fungal cell wall components on the anti-*Candida* activity of di-K19Hc in ultrasensitive radial diffusion assay [9]. 20 μl of 1.25 mg/ml di-K19Hc solution was added to 80 μl of laminarin (β-1,3-glucan polymer; Sigma), pustulan (β-1,6-glucan polymer; Calbiochem, La Jolla, USA) or mannan (mannose polymer; Sigma), each of which was twofold serially diluted in 10 mM sodium phosphate buffer (pH 7.4) ranged in concentration from 10 to 0.312 mg/ml and incubated for 4 h at 37 °C. 5 μl samples were loaded into 3 mm diameter wells that had been punched in underlay gels in which the washed yeast-phase *C. albicans* (4×10^6 colony forming units) were trapped. The underlay agars consisted of 9 mM sodium phosphate, 1 mM sodium citrate buffer, 1% (w/v) agarose (Sigma), and 0.3 mg of tryptic soy broth (TSB: Difco)/ml. After incubation at

37 °C for 3 h, a 10 ml overlay gel of 1% agarose and 6% TSB poured on the underlay gel. After the plates were incubated overnight at 37 °C to allow surviving *C. albicans* cells to form colonies, the diameters of clearing zones indicating antifungal activity were plotted against polysaccharide concentrations. Experiments were performed in triplicate, and mean values were used to make a graph.

2.7. Western blotting analysis

Acid urea–PAGE and Western blotting analyses were conducted in accordance with the method described by Panyutich and Ganz [24]. In the Western blotting experiments, we used an antiserum that had previously been generated via an injection of 18Hc into a rabbit [25]. In brief, the samples were electrophoresed on acid urea–PAGE gels and transferred to nitrocellulose membranes in Tris–glycine buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) at 100 V for 1 h. After transference, the membranes were equilibrated in Tris–buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5; TBS) for 10 min and then incubated for 30 min in blocking solution (3% skim milk in TBS). After a brief washing in TBS containing 0.05% Tween-20 (TTBS), the membrane was incubated for 1 h in a primary antibody solution (antiserum diluted 100-fold with blocking solution). The membrane was washed twice in citrate-buffered saline (20 mM citrate, 500 mM NaCl, pH 5.5) containing 0.05% Tween-20, for 5 min per washing, and then incubated for 1 h in blocking solution containing a 3000-fold diluted secondary antibody (GAR-HRP conjugated IgG; Bio-Rad, Richmond, CA). Finally, the membrane was washed twice in TTBS, once in TBS, and finally submerged in HRP color development solution (50 ml of TBS containing 30 μl of H_2O_2 mixed with 0.3% of chloronaphthol in 10 ml of ice-cold methanol).

2.8. Confocal laser scanning microscopy

At the inception of the work to observe the interaction between *C. albicans* and peptide via confocal laser scanning microscopy, we were going to use FITC-labeled di-K19Hc. Prior to this microscopic experiment, we first compared the antifungal activity of the FITC-labeled di-K19Hc against *C. albicans* with that of the non-labeled di-K19Hc via comparative colony count assays. As a result, FITC-labeled di-K19Hc was determined to have no anti-*Candida* activity (data not shown). Therefore we concluded that FITC affected the structure of di-K19Hc; consequently FITC-labeled material was not appropriate for use in this experiment. As the antiserum to 18Hc was determined to cross-react with di-K19Hc in the Western blotting analysis (Fig. 6B), the antiserum to 18Hc was employed in order to observe the distribution of di-K19Hc on peptide-treated *C. albicans* KCTC7122, via confocal laser scanning microscopy. One milliliter aliquots of *C. albicans* cells (2×10^8 cells/ml) were washed three times with PBS, and then incubated for 1 min with 100 μl of 1 mg/ml di-K19Hc at 37 °C. After a brief centrifugation, the pelleted cells were fixed for 30 min in phosphate-buffered 2.5% paraformaldehyde. The fixed cells were then rinsed three times with PBS, incubated for 30 min in blocking solution (PBS containing 3% gelatin) and washed extensively with PBS, after which they were incubated for 1 h in antiserum diluted (1:100) with PBS, containing 1% gelatin. After washing the cells three times for 10 min each with PBS, the cells were incubated for 1 h with FITC-conjugated goat anti-rabbit antibody (Alexa Flour 488 Goat Anti-Rabbit, Invitrogen. USA) diluted to 1:300 in PBS containing 1% gelatin. After an additional washing in PBS, the di-K19Hc on *C. albicans* was visualized and localized using Confocal Laser Scanning Microscope FV500 (Olympus, Japan).

2.9. Scanning electron microscope (SEM)

The *C. albicans* KCTC7122 was resuspended in PBS, to a final concentration of 10^8 cells/ml. 200 μl aliquots of *C. albicans* suspension were incubated with 22 μl of 250 μg/ml di-K19Hc. For a control, the same amount of *C. albicans* suspension was mixed with 22 μl of 0.01% acetic acid and incubated for 5 min at 30 °C. The di-K19Hc-treated or untreated *C. albicans* samples were then fixed in 0.05 M sodium cacodylate buffer (2% glutaraldehyde, 2% paraformaldehyde, pH 7.2) for 4 h. After fixation, the samples were centrifuged at 2000 rpm and rinsed three times for 20 min each with sodium cacodylate buffer. The samples were then dehydrated through a graded ethanol series (50%, 70%, 90%, 95%, 100% ×2), for 30 in each. The samples were freeze-dried for 8 h, and coated with a light coat of gold palladium. The specimens were then viewed with an S-2400 scanning electron microscope (Hitachi, Japan).

3. Results

3.1. Peptides

The primary amino acid sequences of the synthetic halocidin congeners used in this study, di-K19Hc and K19Hc, are shown in Table 1. Table 1 also provides the sequences of P18 and buforin II, used as the standard peptides in this study.

3.2. Antifungal activity for di-K19Hc

Table 2 shows the established MIC ranges for di-K19Hc and the three other peptides (K19Hc, buforin II and P18) against the four filamentous and eight yeast-phase fungi, including the *Aspergillus* and *Candida* strains. Di-K19Hc was determined to be more than two times stronger than its monomer (K19Hc) against most of the fungal or yeast strains, a finding which was consistent with the results reported in a previous study on the antibacterial activities of halocidin congeners [16]. Interestingly, although the three standard peptides exerted no detectable effects against the two *Aspergillus* strains, even at the highest concentration (64 μg/ml) utilized in the assay, di-K19Hc exhibited anti-*Aspergillus* effects with an MIC of less than 16 μg/ml. The di-K19Hc also exhibited more potent activity against *C. albicans* species than against any of the other

Table 1
Sequences of the antimicrobial peptides used in this study

Peptide	Amino acid sequence	Reference
Di-K19Hc	KWLNA LLHHG LNCAK GVLA* KWLNA LLHHG LNCAK GVLA*	12
K19Hc	KWLNA LLHHG LNCAK GVLA*	12
P18	KWKLF KKIPK FLHLA KKF*	20
Buforin II	TRSSR AGLQF PVGRV HRLLR K	25

Asterisks (*) signify C-terminal amidation. Two cysteine residues connected by a disulfide linkage in the structure of di-K19Hc are shown in the box.

Table 2
Minimum inhibitory concentration (MIC, μg/ml) for antifungal activity of di-K19Hc and control peptides

	Di-K19Hc	K19Hc	P18	Buforin II
Filamentous fungi				
P. grisea[a] KACC40425	4–8	4–8	4–8	8–16
F. oxysporum KACC40902	4–8	8–16	4–8	8–16
A. terreus[b] KCTC6178	8–16	>64	>64	>64
A.niger KCTC6461	8–16	>64	>64	>64
Yeast-phase fungi				
G. candidum KACC40354	8–16	16–32	8–16	16–32
Cr. neoformans[c] KCCM50544	16–32	16–32	32–64	32–64
T. beigelli KCTC7707	2–4	4–8	2–4	16–32
C. albicans KCTC7121	1–2	8–16	<1	32–64
C. albicans KCTC7270	2–4	16–32	8–16	>64
C. albicans KCTC7728	2–4	16–32	4–8	>64
C. albicans KCTC7729	2–4	8–16	2–4	16–32
C. albicans KCTC7965	2–4	16–32	32–64	>64

[a]KACC, Korean Agricultural Culture Collection.
[b]KCTC, Korean Collection for Type Cultures.
[c]KCCM, Korea Culture Center of Microorganisms.

tested fungi, and retained its anti-*Candida* activities, even at elevated concentrations of salt (Table 3). Overall, our antifungal assays showed that di-K19Hc was superior to other peptides, with regard to both antifungal activity and spectrum width.

3.3. Colony count assay for the candidacidal activity of di-K19Hc

We performed a colony count assay in order to determine the ability of di-K19Hc to kill *C. albicans*, and also to determine the time course associated with this activity. With regard to di-K19Hc exposure, the loss of viability of *C. albicans* occurred dose-dependently. 40 μg/ml of di-K19Hc effected a reduction in the number of viable *C. albicans* cells by more than 4 log 10 after 10 min of incubation, and slightly affected fungal viability during further incubation (Fig. 2, long-term). Therefore, we attempted to assess the candidacidal effects of di-K19Hc within a 10-min incubation period. Short-term analysis showed that the minimum number of viable colonies was achieved after 30 s of incubation, and reached a plateau thereafter (Fig. 2, short-term), thereby illustrating the marked rapidity with which di-K19Hc killed the *C. albicans* cells: its candidacidal activity was completed within 30 s.

3.4. Effects of di-K19Hc on the integrity of the C. albicans membrane

As many antimicrobial peptides have been determined to exert their effects via the induction of permeability changes in the membranes of susceptible microbes, we attempted to characterize the effects of di-K19Hc on the integrity of *C. albicans* membranes, by monitoring the permeability of the membranes to intracellular K^+, and by recording the uptake of PI, a DNA-staining fluorescent probe, into the *C. albicans* cells. Fig. 3 shows the content of K^+ efflux from *C. albicans* treated with di-K19Hc for the predetermined incubation time. An estimated 88% ± 2% ($n = 5$) of the total intracellular K^+ was released after 10 min of incubation in every one of these experiments, and the percentage of K^+ efflux remained constant during further incubation (Fig. 3, long-term). In addition, our short-term test showed that the amount of released K^+ began to increase rapidly at 30 s after the initiation of peptide treatment (Fig. 3, short-term). The time course associated with the K^+ efflux from the *C. albicans* treated with di-K19Hc exhibited a pattern consistent with the results obtained from the colony count assay for the candidacidal activity of di-K19Hc. Therefore, according to these results, we concluded that di-K19Hc induced an immediate and massive efflux of K^+ from *C. albicans*. Fig. 4 shows the results of the intracellular PI measurements, which were analyzed via flow cytometry. The majority of the *C. albicans* cells were labeled fluorescently after 10 min of incubation with 3.12 μg/ml di-K19Hc, thereby indicating that di-K19Hc induced the influx of PI into the cells.

Table 3
Effect of salt on candidacidal activity of di-K19Hc

Peptides	*Candida albicans* KCTC71 22			
	NaCl:	0 mM	100 mM	200 mM
Di-K19Hc		2–4	4–8	8–16
K19Hc		8–16	16–32	32–64
P18		1–2	8–16	16–32
Buforin II		32–64	>64	>64

The MIC values are shown in μg/ml.

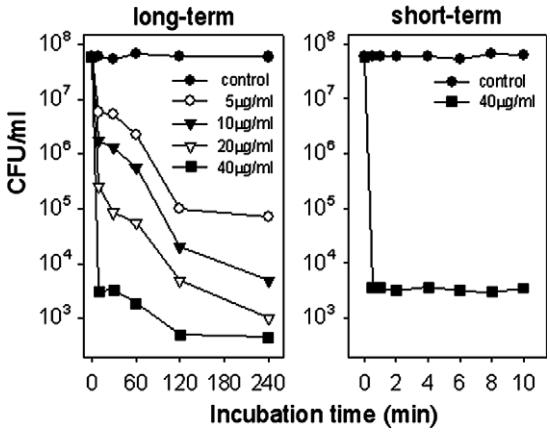

Fig. 2. Colony count assay for the candidacidal activity of di-K19Hc. In the long-term and short-term analyses, we treated the *C. albicans* cells with di-K19Hc for the predetermined times, and spread them on SDB agar. As a control, we added an equivalent volume of 0.01% acetic acid containing no peptide to the control tubes. After being cultured overnight, the viable colonies in each sample were counted. The mean values obtained from three individual experiments were then used in the construction of a graph. The error bars were too narrow for the display.

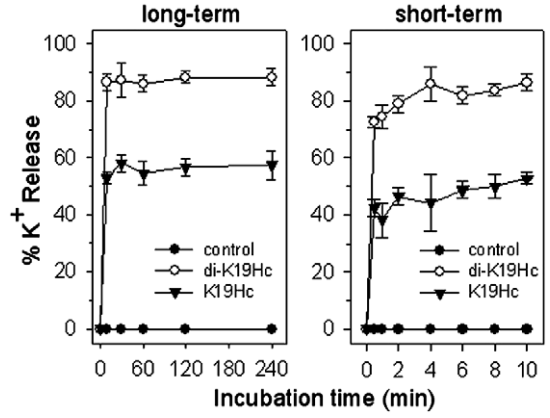

Fig. 3. Potassium efflux assay. The *C. albicans* cells were treated with either di-K19Hc or K19Hc for the predetermined times, and the relative amounts of K^+ released from the cells were measured. Each of the symbols represents a mean value from five replicate experiments. The error bars show the standard deviations.

3.5. Specific binding of di-K19Hc to the component of fungal cell wall

In order to investigate if di-K19Hc specifically binds to fungal cell surface, effects of several polysaccharides composing of fungal cell wall on the anti-*Candida* activity of di-K19Hc were examined (Fig. 5). The constant amount of di-K19Hc was incubated at different concentrations of laminarin (β-1,3-glucan), mannan or pustulan (β-1,6-glucan), and then the mixture was tested for anti-*Candida* activity in agar diffusion assay. As a result, its activity was significantly reduced as the amount of laminarin increased in the mixture. By contrast, neither mannan nor β-1,6-glucan affected the anti-*Candida* activity of di-K19Hc. These results indicate that laminarin prevented

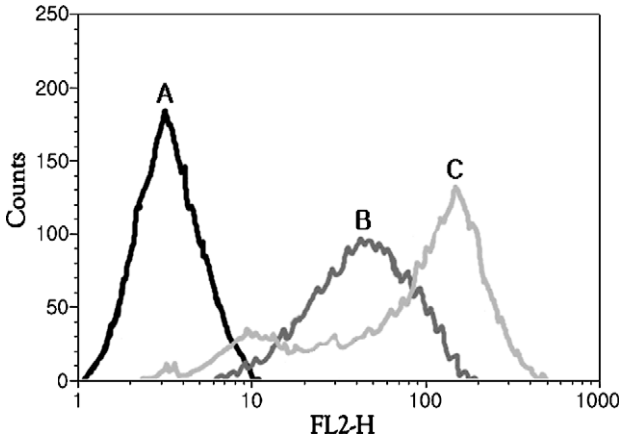

Fig. 4. Flow cytometric analysis. *C. albicans* cells were treated with di-K19Hc and stained with PI. Cellular fluorescence was then analyzed via FACScan flow cytometry. The increments of the log fluorescence signal represent the uptake of PI by the yeast cells. Cells were treated with no peptide (A), K19Hc (B), and di-K19Hc (C).

di-K19Hc from interacting with the cell surface of *C. albicans*, resulting in the abrogation the di-K19Hc-induced antifungal activity in a dose-dependent manner. Therefore it was concluded that di-K19Hc bound to *C. albicans* cells via a specific interaction with β-1,3-glucan, thereby exerted its antifungal effects.

3.6. Microscopic observation

We used a rabbit antiserum raised against 18Hc and FITC-labeled secondary antibody in order to observe the interaction occurring between *C. albicans* and di-K19Hc, via confocal laser scanning microscopy. In order to examine the target sites of di-K19Hc in *C. albicans*, we have treated the cells with di-K19Hc, with the antiserum and the FITC-labeled secondary antibody sequentially, after which the samples were visualized under confocal microscopy. The FITC-labeled secondary antibody was determined to have accumulated on the cell surface (Fig. 6D), thereby indicating that di-K19Hc acts on the membranes of the fungal cells, and induces the alteration of the cell membrane. We also evaluated, via scanning electron microscopy, the morphological changes in the *C. albicans* cells induced by di-K19Hc treatment. Whereas the untreated cells exhibited normal, smooth surfaces (Fig. 7A), the cell walls of the *C. albicans* that had been incubated with di-K19Hc revealed a series of dramatic alterations, including surface roughening and disruption, within 5 min of exposure (Fig. 7B and C). We considered this to be a strong indication that the membrane had been modified substantially by di-K19Hc, and surmised that this might constitute a critical step in the compound's candidacidal effects.

4. Discussion

For the last forty years, over 100 natural peptides have been determined to exhibit activities against pathogenic fungi. These compounds have been isolated from a wide variety of organisms, including bacteria, fungi, plants, invertebrates, and vertebrates [26]. However, the majority of these compounds have not been used in clinical trials, due to defects in their antifungal activities, or in the breadth of their spectra. The search for such antifungal peptides continues, in the hopes of locating an effective candidate for the development of a new type of antibiotic [27,28]. We recently began to study the antimicrobial activity of halocidin, again in the hope that it could be developed into a novel therapeutic agent, which might prove useful in the treatment of several incurable infectious diseases. Previ-

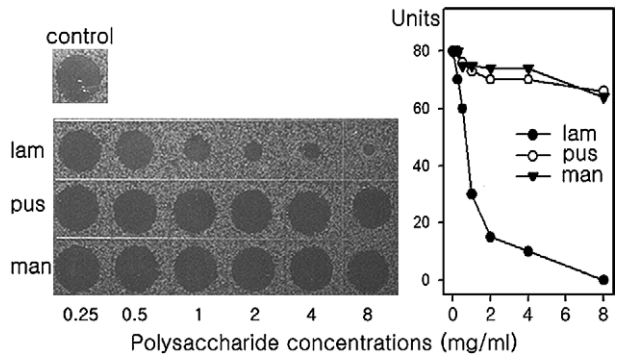

Fig. 5. Specific binding of di-K19Hc to laminarin. Binding/radial diffusion assay was performed by mixing various amounts of pustulan, laminarin or mannan with 1.25 μg of di-K19Hc in a final volume of 5 μl. The mixtures of di-K19Hc with laminarin, pustulan or mannan were introduced into the wells of radial diffusion assay plates seeded with *C. albicans* ACTC7121, which was confirmed as a highly susceptible yeast to di-K19Hc. The left panel shows photo of gel in radial diffusion assay. Numbers represent the polysaccharide concentrations (mg/ml) of the mixture loaded in each well. lam, laminarin; pus, pustulan; man, mannan. 5 μl of 250 μg/ml di-K19Hc was used for polysaccharides-free control. In the right panel, the anti-*Candida* activities of di-K19Hc in the mixture were graphed against concentration of polysaccharides. Diameters of clearing zone have been expressed in units (1 mm = 10 U). The error bars were too small to be expressed.

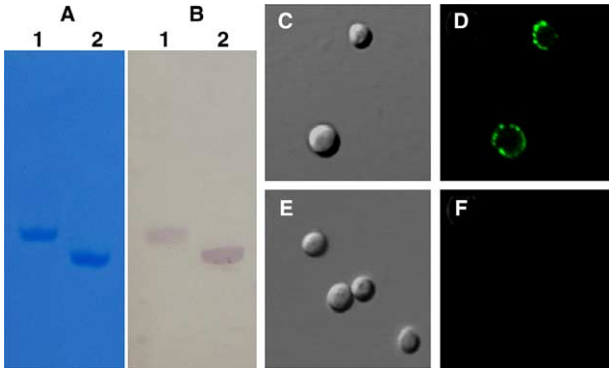

Fig. 6. Western blot analysis and Confocal laser scanning microscopy. Western blotting analysis indicated that the antiserum to 18Hc recognized di-K19Hc, the primary sequence of which is identical to 18Hc in terms of its 18/19 residues. (A) An acid urea–PAGE gel stained with Coomassie's blue. (B) Western blot analysis conducted with a duplicate gel of (A). Lane 1, 18Hc; lane 2, di-K19Hc. *C. albicans* cells were incubated with di-K19Hc, then treated with antiserum to 18Hc and FITC-labeled secondary antibody. The cells were then visualized via confocal laser scanning microscopy. For the control experiment, *C. albicans* cells which had not been treated with di-K19Hc were incubated with antiserum to 18Hc, and treated with FITC-labeled secondary antibody. (C) Phase-contrast image of *C. albicans* treated with di-K19Hc. (D) Fluorescent image of *C. albicans* treated with di-K19Hc. (E) Phase-contrast image of control sample. (F) Fluorescent image of control sample.

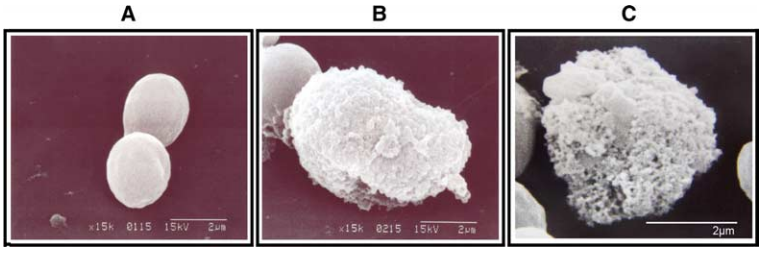

Fig. 7. Scanning electron microscopy. Scanning electron micrographs revealed that the surface of the untreated *C. albicans* cells were smooth (A) and the surfaces of the di-K19Hc-treated cells were ruffled, and became rugged (B,C).

ously, a diverse set of synthetic halocidin congeners were designed and tested with regard to their antibacterial activities [16]. As a result, di-K19Hc emerged as the most promising candidate for the development of a new antibiotic against antibiotic-resistant bacteria. As in the case of many other peptides, including cecropin [29], magainin II [30], and protegrin [31], all of which exhibited antifungal and antibacterial effects, di-K19Hc was also anticipated to exhibit antifungal properties. Hence, in this study, we have assessed the activity of di-K19Hc against a panel of fungi, in a series of assays that were conducted in accordance with NCCLS procedures. Our MIC data (Tables 2 and 3) indicated that di-K19Hc retained an antifungal activity that might be suitable for the treatment of fungal diseases. Accordingly, it has become clear that di-K19Hc was an active peptide possessing both antifungal and antibacterial efficacy, against a wide range of pathogenic microbes. As marine animals live in a habitat characterized by high salinity, it has been often postulated that antimicrobial peptides originating from marine sources may maintain their activities in conditions of high salinity. Actually, certain antimicrobial peptides, such as styelins from the tunicate [12] and oncorhyncin III from rainbow trout [32], have been shown to exert their effects in the presence of 300 and 547 mM NaCl, respectively. Considering the results obtained from the previous [16] and the present studies, it has become evident that di-K19Hc was also capable of exerting its antimicrobial activity under elevated salt concentration conditions, although the structural motifs of di-K19Hc required for its salt-resistant activity remain to be elucidated.

In order to elucidate the mechanisms underlying di-K19Hc antifungal effects, we attempted to determine the manner in which di-K19Hc actually carries out the destruction of *C. albicans*. We performed colony count assays in order to determine the time required for the compound to kill *C. albicans*. With regard to the results reported in studies of the time-dependent bacteria-killing kinetics of antimicrobial peptides, it has generally been reported that these compounds exert their antibacterial effects within minutes of exposure to bacteria [33–35]. This rapid killing process has been most plausibly attributed to the action of these compounds on the bacterial membranes [36,37]. Our study also showed that di-K19Hc was capable of killing *C. albicans* within 30 s (Fig. 2). This result led us to hypothesize that di-K19Hc might act principally on the membrane of *C. albicans* cells, inducing cell lysis. In accordance with our hypothesis, the treatment of *C. albicans* cells with di-K19Hc induced a major efflux of intracellular K^+ (Fig. 3) or a major influx of PI (Fig. 4), demonstrating that di-K19Hc executed its anti-*candida* effects via binding to the surface of the cells, followed by the formation of membrane pores. Interestingly, our results indicated that the antifungal activity of the K19Hc dimer (di-K19Hc) was over two times as strong as that of the monomer against all tested fungi, except for the *P. grisea* species. As reported in other studies involving the disulfide-dimerized analogues of magainin II [38,39] and melittin [40], it has been surmised that di-K19Hc may induce the formation of larger pores in a fungal membrane with a longer lifetime than is associated with K19Hc, thereby resulting in more profound antifungal activity. In addition, it was verified that binding of di-K19Hc to the fungal surface occurred via specific interaction with β-1,3-glucan, which is one of components of the fungal cell wall (Fig. 5). Also, it was strongly supported by the observations made under confocal (Fig. 6D) and scanning electron microscopy (Fig. 7). Taken together, the antifungal activity of di-K19Hc was concluded to result from the disintegration of the cell membrane rather than from any effect on the physiology of the cells.

Because some of antimicrobial peptides physically attack the microbial membranes, thereby killing even microbes that are equipped with antibiotic-resistant mechanisms, they have been the focus of increasing interest, and are being heralded as excellent candidates in the search for new types of antibiotics [41,42]. Among these compounds, di-K19Hc appears to be one of the most promising as a possible therapeutic agent for infections caused by pathogenic fungi or bacteria that are resistant to currently available drugs.

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