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HP(2–9)-Magainin 2(1–12), a Synthetic Hybrid Peptide, Exerts Its Antifungal Effect on *Candida albicans* by Damaging the Plasma Membrane

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Abstract: In our previous study, HP(2–9)-MA(1–12), HP-MA for short, a hybrid peptide incorporating residues 2–9 of *Helicobacter pylori* ribosomal protein L1 (HP) and residues 1–12 of magainin 2 (MA) was shown to have strong antibacterial activity. In this study the antifungal activity of HP-MA was evaluated using various fungi, and it was shown that the activity was increased when compared with the parent peptides. In order to investigate the fungicidal mechanism(s) of HP-MA its action against fungal cell membranes was examined by the potassium-release test, which showed that HP-MA caused an increase in the amount of K^+ released from the cells. Furthermore, HP-MA induced significant morphological changes. These facts suggested that the fungicidal effect of HP-MA involves damaging the fungal cell membranes. CD investigators suggested that the α-helical structure of these peptides plays an important role in their antibiotic effect, but that α-helicity is less directly correlated with the enhanced antibiotic activity of the hybrid. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: HP-(2–9): MA-(1–12); antifungal peptides; fungicidal mechanism(s)

INTRODUCTION

Antimicrobial peptides have been recognized as playing important roles in the innate host defence mechanisms of most living organisms including; plants, insects, amphibians and mammals [1–6]. They are also known to possess potent antibiotic activity against bacteria, fungi and even certain viruses [7–11].

These studies have shown the major killing mechanism for these antimicrobial peptides consists of the formation of transmembrane pores or ion channels, on the cellular membrane, leading to the leakage of essential metabolites, resulting in the disruption of the cell structure [12]. The mechanism of antifungal action has been investigated for several antimicrobial peptides [13]. The major cause for the loss of fungal cell viability is believed to be the result of the formation of transmembrane channels, increasing membrane permeability, and causing disruption of the microbial cell structure.

Helicobacter pylori grown on plates produces cecropin-like antibacterial peptides to which

Abbreviations: MBHA resin, 4-methyl benzhydrylamine resin; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; PBS, phosphate-buffered saline; MIC, minimal inhibitory concentration; HP, *Helicobacter pylori* ribosomal protein L1; MA, magainin 2; HP–MA, HP(2–9)-MA(1–12) hybrid

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H. pylori is resistant. This antibacterial activity has been attributed to fragments from the amino-terminus of ribosomal protein L1 (RPL1) [14,15]. Therefore, in our previous study, hybrid peptides were designed composed of the amino-terminal regions of HP (2–9) and MA (1–12) which had higher antibacterial activity than the parent peptides [16,17].

In this study the antifungal activity of HP-MA was investigated on various yeast strains. In order to substantiate the mechanism(s), potassium release tests were performed on *C. albicans*. Additionally, the effect of HP-MA on the cell morphology of *C. albicans* was explored using a scanning electron microscope.

MATERIALS AND METHODS

Peptide Synthesis

The peptides, HP(2–20), HP-MA and MA, were synthesized by Fmoc SPPS on a Rink amide MBHA resin (0.55 mmol/g) in order to obtain a peptide *C*-terminal amide. Amino acid side-chains were protected with Bu^t (Asp), Trt (Gln), or Boc (Lys) groups. Deprotection and cleavage from the resin were carried out using a mixture of TFA, phenol, water, thioanisole, 1,2-ethanedithiol and triisopropylsilane (88 : 2.5 : 2.5 : 2.5 : 2.5 : 2.0, v/v) for 2 h at room temperature. The crude peptide was then repeatedly washed with Et_2O, dried *in vacuo*, and purified by reverse-phase preparative HPLC on a Waters 15-μm Deltapak C_{18} column (19 × 30 cm). The purity of the peptide was checked by analytical reverse-phase HPLC using an Ultrasphere C_{18} column (Beckman, USA), 4.6 × 25 cm. The purified peptides were hydrolysed with 6 N HCl at 110 °C for 22 h, and then dried *in vacuo*. The residues were dissolved in 0.02 N HCl, and subjected to amino acid analysis (Hitachi Model, 8500 A, Japan) to determine the peptide concentrations. The molecular weights of the synthetic peptide were determined by MALDI-MS.

Fungal Strains and Antifungal Activity Assay

Saccharomyces cerevisiae (KCTC 7296) and *Trichosporon beigelii* (KCTC 7707) were obtained from the Korean Collection for Type Cultures (KCTC), at the Korea Research Institute of Bioscience and Biotechnology (KRIBB), Taejon, Korea. *Candida albicans* (TIMM 1768) was obtained from the Center for Academic Societies, Osaka, Japan. The fungal strains were grown at 28 °C in YPD (dextrose 2%, peptone 1%, yeast extract 0.5%, pH 5.5) medium for 3 h. The fungal cells were seeded on the well of a flat-bottom 96 well microtitre plate (Greiner, Nurtingen, Germany) containing YPD media at a density of 2×10^3 cells counted by haemocytometer (100 μl per well). The serially diluted peptide solutions (50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 μM) of HP (2–20), HP-MA or MA used as a positive control, were added to each well, and the cell suspension was incubated at 28 °C for 24 h. 10 μl of a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution [5 mg/ml MTT in phosphate-buffered saline (PBS), pH 7.4] was added to each well, and the plates were incubated at 37 °C for 4 h. The turbidity of each well was measured by absorbance at 570 nm using a microtitre ELISA reader (Molecular Devices Emax, California, USA). All assays were performed in triplicate [18].

Measurement of K^+ Release

The yeast cells (2×10^5) were incubated on 96-microtitre plates, in 100 μl of the YPD media containing peptide (10 μM), at 28 °C. After incubation, the cell suspensions were centrifuged at 2000 × ***g*** for 5 min and the resultant supernatants retained for the next step. For the measurement of released K^+, the supernatant was diluted 100 fold and the released K^+ was measured using an inductively coupled plasma mass spectrometer (FIsons, Cheshire, UK).

Morphological Changes Induced in *C. albicans* by HP-MA Hybrid Peptide

C. albicans cells were incubated at 28 °C for 4 h with 12.5 μM of HP-MA. Negative controls were run with no peptide. The cells were fixed with equal volumes of 4% glutaraldehyde and 1% paraformaldehyde in 0.05 M cacodylate buffer (pH 7.2). After fixation for 3 h at 4 °C, the samples were centrifuged at 150 × ***g*** and washed twice with the same buffer. The samples were dehydrated with a graded ethanol series (50%, 70%, 90%, 95%, 100% EtOH). After lyophilization and gold coating, the samples were examined on a Hitachi S-2400 scanning electron microscope (Tokyo, Japan).

Circular Dichroism (CD) Spectroscopy

CD spectra were recorded at 25 °C on a Jasco 715 spectropolarimeter (Jasco, MD, USA) equipped with

a temperature control unit. A 0.1 cm pathlength quartz cell was used for 40 mM of the peptide solution. At least five scans were averaged for each sample and the averaged blank spectra were subtracted. Each spectrum was obtained by averaging five scans area 240–190 nm. All CD spectra are reported in units of mean residue ellipticity, $[\theta]_{MRW}$, in deg·cm^{2}dmol^{-1}.

RESULTS AND DISCUSSION

HP-MA has been chemically synthesized, purified to homogeneity and subjected to MALDI-MS analysis, and its antifungal mechanism has been studied (Table 1).

HP-(2–20) has been shown to inhibit the growth of *E. coli* and *B. megaterium* [14]. MA, a cationic 23-amino acid antimicrobial peptide exhibits strong antibacterial activity [17]. However, the antifungal activity and mechanism(s) of action of HP-MA are not thoroughly understood. Therefore, the antifungal activity of HP-MA, against various fungal cells, was determined as MIC using the MTT assay [18]. The result demonstrated that HP-MA displayed approximately 8-fold greater antifungal activity than the HP (2–20) and MA against microorganisms tested (Table 2).

In order to visualize the fungicidal effect, the pathogenic fungus, *C. albicans* was treated with the peptide and spread on the YPD agar plate. As shown in Figure 1, the peptide inhibited the growth of *C. albicans*.

The measurement of spore germination, or growth, is commonly used in antifungal activity studies, but it is difficult to determine the exact nature of antifungal mechanism(s) with this method. In order to establish whether HP-MA causes damage to the plasma membrane or affects cell physiology, the cells were incubated with HP-MA. The amount of K^+ released from *C. albicans* by HP-MA was compared with the action of MA as a positive control. HP-MA and MA both released more K^+ than the negative control (Figure 2). This increase in the amount of released K^+ after peptide treatment provides further evidence that HP-MA acts on the plasma membrane, either by specific disruption of the ion channels, or by nonspecific pore formation. As for the mechanism by which HP-MA breaks down the membrane permeability barrier, it is possible that the peptide perturbs the membrane lipid bilayers, causing the leakage of certain cellular components, as well as dissipating the electrical potential of the membrane.

Table 2 Antifungal Activity of HP-MA Hybrid Peptide

Peptide	Fungal strain MIC (μM)		
	S. cerevisiae	*T. beigelii*	*C. albicans*
HP(2–20)	25	12.5–25	12.5
HP-MA	3.12–6.25	3.12–6.25	3.12
MA	6.25	6.25	6.25

The morphological changes induced in *C. albicans* by HP-MA were examined by scanning electron microscopy. Untreated cells had a normal, smooth cell surfaces, whereas HP-MA treated cells had large holes in their surfaces (Figure 3). These results provide additional evidence that HP-MA probably acts on the plasma membrane, by forming pores, causing the leakage of ions and other materials from the cells.

The circular dichroism (CD) spectra of the hybrid peptides were measured in phosphate buffer with or without the α-helix including solvents TFE or SDS, which are composed of an aliphatic tail and a negatively charged head group, this mimicking the lipid membrane. As shown in Table 3, HP-MA resulted in a marked decrease in α-helical content in 50% TFE and 30 mM SDS solutions, but HP and MA had a higher α-helical content in cell membrane-mimicking environments such as TFE and SDS micelles, where it had a lower antimicrobial

Table 1 Amino Acid Sequence and Molecular Masses Determined by MALDI-MS of HP-MA

Peptide	Amino acid sequence	Calculated value	Observed value
HP(2–20)	AKKVFKRLEKLFSKIQNDK-NH_2	2318.38	2318.88
HP-MA	AKKVFKRLGIGKFLHSAKKF-NH_2	2301.43	2303.20
MA	GIGKFLHSAKKFGKAFVGEIMNS-NH_2	2464.34	2465.28

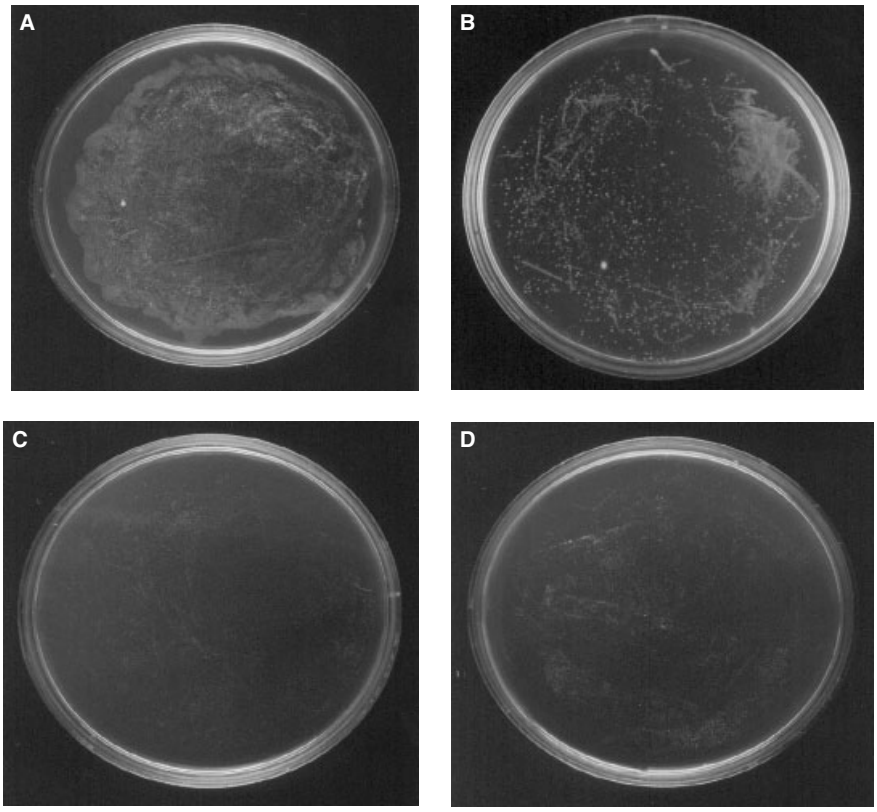

Figure 1 Effect of HP-MA on *C. albicans* colony formation. Yeast cells were suspended to a density of approx. 2×10^3/ml in YPD medium and peptide was added to a final concentration of 3.12 μM. The reaction mixture was spread on YPD agar plate after incubation at 28 °C for 3 h. The plate was then incubated for 18 h at 28 °C. A, No peptide treatment; B, Cells treated with HP (2–20); C, HP-MA; D, Magainin 2.

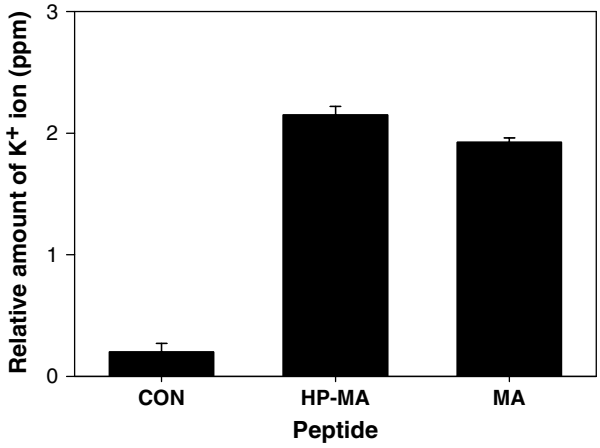

Figure 2 Amount of potassium ion released by HP-MA from *C. albicans*. *C. albicans* (2×10^5 cells per well) were incubated on flat-bottom microtitre 96-well microtitre plates in 100 μl of YPD media containing peptides (5 μM) and the plates were incubated at 28 °C at a various time course. After incubation, the cell suspensions were measured of released potassium ions.

activity than the HP-MA hybrid peptide. These results suggest that the α-helical content is not directly correlated with the enhanced antibiotic activity even though it may play an important role in killing fungal cells in synthetic HP-MA hybrid peptide.

In summary, the above results show that HP-MA, incorporating residues 2–9 of *H. pylori* ribosomal protein L1 (HP) and residues 1–12 of MA, damages the plasma membranes of *C. albicans* and thereby exerts potent antifungal effects similar to those of MA. We believe that these observations give HP-MA, which is also active against pathogenic fungal cells, a potential as a lead compound for the development of novel antifungal drugs.

CONCLUSION

HP(2–9)-MA(1–12), a synthetic hybrid peptide incorporating residues 2–9 of *Helicobacter pylori* ribosomal protein L1 (HP) and residues 1–12 of magainin 2

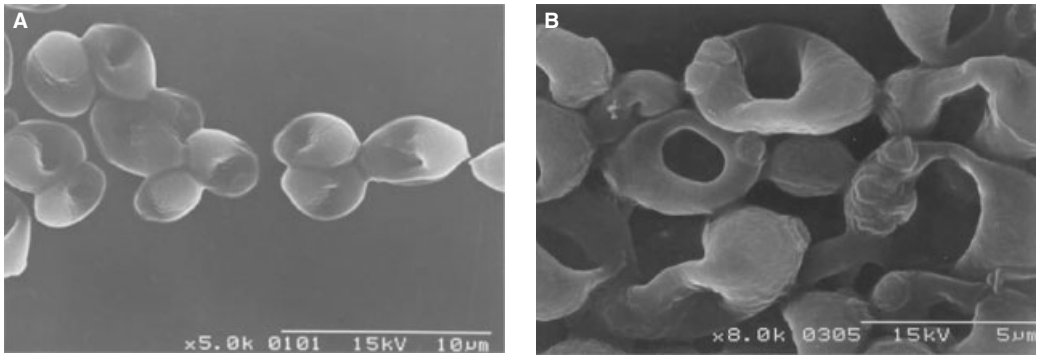

Figure 3 Scanning electron micrographs of (A) untreated, and (B) after treatment for 4 h at 28 °C with 12.5 μM HP-MA, of *C. albicans*.

Table 3 Percent α-Helicity of HP-MA

Peptide	Phosphate buffer	50% TFE	30 mM SDS
HP(2–20)	16.21	79.33	48.96
HP-MA	4.20	10.97	1.36
MA	3.02	70.69	81.94

(MA), damages the plasma membranes of *C. albicans* and thereby exerts potent antifungal effects. It may be useful as a template or a lead compound for development of novel therapeutic agents.

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