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Effects of *N*- and *C*-terminal truncation of HP (2-20) from *Helicobacter pylori* ribosomal protein L1 (RPL1) on its anti-microbial activity

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Abstract

HP (2-20) [derived from the *N*-terminal region of *Helicobacter pylori* Ribosomal Protein L1 (RPL1)], a 19-mer peptide, possesses broad-spectrum anti-microbial activity. As the *N*- (residues 2–3) and *C*-terminal (residues 14–20) residues can be deleted without affecting antimicrobial activity, we have now determined the minimum chain length necessary for the retention of antimicrobial activity, and its mode of action. The *N*- (residues 2–3) and *C*-terminal (residues 17–20) truncated fragments [HP (4–16)] induce increased antibiotic activity against several bacterial strains without hemolysis. Flow cytometric analysis, scanning electron microscopy and fluorescence confocal microscopy revealed that HP (4–16) acted rapidly on the plasma membranes of the fungal cells in a salt- and energy-independent manner.

Introduction

Antimicrobial peptides are important components of the non-specific, host defence system and innate immunity of insects (Chesnokova *et al.* 2004), amphibians (Conlon *et al.* 2004), and mammals (Mitsuhara 2001). Among the more potent antibiotics are small bioactive peptides, such as cecropin A, magainin 2, melittin and plant defensin (Park *et al.* 2002a). These peptides have potent activity against bacteria, yeast and even certain enveloped viruses (Boman *et al.* 1991, Hancock & Chapple 1999, Hoffmann & Hetru 1992). Cecropin A is found in the hemolymph of *Hyalophora cecropia* pupae and consists of 37 amino acid residues (Zasloff 1987). Melittin, a 26-residue peptide derived from honeybee venom, shows powerful antibacterial properties but is extremely cytotoxic (e.g. hemolytic) to normal eukaryotic cells (Lee 1998). The bacterium, *Helicobacter pylori,* has been implicated as a pre-disposing factor for gastrointestinal illnesses, such as gastritis and peptic ulcers (Boman 1991). The *N*-terminus from its ribosomal protein L1 (RPL1) possesses antibacterial activity (to which the bacterium is, of course, immune) and this activity has been traced back to HP (2–20), a 19-mer antimicrobial peptide possessing Cecropin-like, *N*-terminal peptides (Katrin *et al.* 1999). In our previous study, in order to elucidate the antimicrobial effects of the *N*- and *C*-terminus of HP (2–20), a series of truncated peptides was synthesized, in which two amino acid residues were deleted stepwise from HP (2–20) (Park *et al.* 2002b). The result suggested that the *N*- (residues 2–3) and *C*-terminal (residues 14–20) residues were not relevant to its antimicrobial activity. Therefore, the aims of the present study were to determine the minimum chain length necessary for the retention of antimicrobial activity, and its mode of action. Two truncated peptides, HP (4–16) and HP (4–14), were synthesized and tested against bacterial and fungal cells, and their cytotoxic activity against human erythrocytes was assessed. Additionally, the resulting peptides were investigated as to their modes of action. The mechanism(s) of action on *Candida*

albicans were examined, both by confocal laser-scanning microscopy and fluorescence-activated flow cytometric analysis. The peptides were also investigated using CD spectra in a cell membrane-mimicking environment, most notably, trifluoethanol or SDS micelles.

Material and methods

Peptide synthesis

Peptides were synthesized by the solid phase method using Fmoc (9-fluorenyl-methoxycarbonyl)-chemistry (Merrifield 1986). Arg (pmc)-Wang-Resin was used as the support to obtain a *C*-terminal free peptide. The coupling of Fmoc-amino acids was performed with *N*-hydroxybenzotriazole and dicyclohexylcarbodiimide. Amino acid side chains were protected with *tert*-butyl (Asp and Thr), trityl (Gln), *tert*-butyloxycarbonyl (Lys and Trp), and pmc (Arg). Deprotection and cleavage from the resin were carried out using a mixture of trifluoroacetic acid/phenol/water/thioanisole/1,2-ethandithiol/triisopropylsilane (82.5:5:5:5:2.5:2, by vol.) for 3 h at 25 °C. The crude peptide was repeatedly washed with diethylether and dried in a vacuum. The crude peptides were purified by a reversed-phase preparative HPLC on a Waters 15 μm Deltapak C_{18} column (19 × 30 cm). The purified peptides were hydrolyzed with 6 M HCl at 110 °C for 22 h, and then dried in a vacuum. The residues were dissolved in 0.02 M HCl and subjected to an amino acid analyzer (Hitachi Model, 8500 A, Japan). Peptide concentrations were determined by amino acid analysis. The molecular masses of the peptides were confirmed with a MALDI (matrix-assisted laser desorption/ionization) mass spectrometer.

Microbial strains

Streptococcus aureus (KCTC 1621), *Bacillus subtilis* (KCTC 1918), *Pseudomonas aeruginosa* (KCTC 1637), *Salmonella typhymurium* (KCTC 1926), *Escherichia coli* (KCTC 1682), *Candida albicans* (KCTC 7270), *Trichosporon beigelii* (KCTC 7707) and *Saccharomyces cerevisiae* (KCTC 7296) were obtained from the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience & Biotechnology (KRIBB), Taejon, Korea.

Antibacterial assay

Bacteria were grown to mid-growth phase in medium (g l^{-1}) (10 Bacto-tryptone/5 yeast extract/10 NaCl, pH 7). The peptide was added containing up to 100 μM in 1% Bactopeptone. The tested organism (final bacterial suspension: 5×10^3 colony formation units (c.f.u.) ml^{-1}) was suspended in growth medium (100 μl), and then mixed with 100 μl of the test peptide solution in a microtiter plate well, with three replicates for each test solution. Microbial growth was determined from the optical density at 620 nm after 10 h incubation at 37 °C.

Antifungal activity assay

Yeasts were grown at 28 °C in an YPD medium (dextrose 2%, peptone 1%, yeast extract 0.5%, pH 5.5). The cells were seeded into 96-well plates at 2×10^3 cells per well in 100 μl YPD medium. The serially diluted peptides (50–0.39 μM) were added to each well. Melittin was used as a positive control. After 24 h at 28 °C, 10 μl 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) (5 mg MTT ml^{-1} PBS, pH 7.4) was added to each well, and the plates were incubated for a further 4 h at 37 °C. The absorbancy of each well was measured at 570 nm using a microtiter ELISA reader (Molecular Devices Emax, CA). All the assays were performed in triplicate.

Hemolysis assay

The hemolytic activity of the peptides was evaluated by determining the released hemoglobin of 8% (v/v) suspensions of fresh human erythrocytes at 414 nm (Blondelle & Houghten 1992). Human red blood cells were centrifuged and washed three times with phosphate-buffered saline (35 mM phosphate buffer/0.15 M NaCl, pH 7). Human red blood cells suspended in 8% (v/v) PBS were plated into 96-well plates, and then 100 μl of the peptide solution HP (2–20), its truncated were added to each well. Melittin was used as a positive control.

The plates were incubated for 1 h at 37 °C and centrifuged at 150 g for 10 min. One hundred microliter aliquots of the supernatant were transferred to the 96-well plates. Hemolysis was measured by absorbance at 414 nm with an ELISA

plate reader (Molecular Devices Emax, Sunnyvale, CA). Zero percent and 100% hemolysis were determined in PBS and 0.1% Triton X-100, respectively. The hemolysis percentage was calculated using the following equation: % hemolysis = [(A_{414} in the peptide solution − A_{414} in PBS)/(A_{414} in 0.1% Triton-X 100 −A_{414} in PBS)] × 100.

Salt- and energy-dependency test

To analyse the membrane integrity after peptide treatment, *C. albicans* (2×10^5 cells in YPD media) were harvested at mid-growth phase. Peptides were added to give 6.25 μM at 28 °C for 4 h, and NaCl, magnesium citrate and 0.05% NaN_3 (as a respiration inhibitor) were added for the salt- and energy-dependency test. Permeabilization of the cell membrane was detected by incubation of the peptide-treated cells in propidium iodide (50 μg ml^{-1} final concentration) at 4 °C for 30 min, followed by removal of unbound dye via excessive washing with PBS. The fluorescence of the PI was monitored in the FL2-H channel. Flow cytometric analysis was performed with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

Confocal laser-scanning microscopy (CLSM)

Intracellular localization of the fluorescein isothiocyanate (FITC)-labeled HP (4–16) in *C. albicans* was analyzed by confocal laser scanning microscopy. *C. albicans* cells were inoculated into 3 ml yeast medium and incubated at 28 °C for 12 h. FITC-HP (4–16) was added to 100 μl cell suspension at 6.25 μM, and the cells were incubated at 28 °C for 15 min. Intracellular localization of FITC-HP (4–16) was examined by a Leica TCS 4D, connected to an Olympus IX 70 upright microscope (Olympus, Japan).

Scanning electron microscopy (SEM)

Mid-growth phase *C. albicans* were resuspended at 10^8 c.f.u. ml^{-1} in sodium-phosphate buffer, pH 7.4, supplemented with 100 mM NaCl (buffer A), and incubated at 28 °C with HP (4–16). After 30 min the cells were fixed with an equal volume of 5% (w/v) glutaraldehyde in 0.2 M sodium-cacodylate buffer, pH 7.4. After fixation for 2 h at 4 °C, the samples were filtered on Isopore filters (0.2 μM pore size, Millipore, Bedford, MA) and extensively washed with 0.1 M cacodylate buffer, at pH 7.4. The filters were then treated with 1% (w/v) osmium tetroxide, washed with 5% (w/v) sucrose in cacodylate buffer and subsequently dehydrated with a graded ethanol series. After lyophilization and gold coating, the samples were examined on a Hithachi S-2400 instrument.

Circular dichroism analysis

CD spectra were recorded at 25 °C on a Jasco 715 spectropolarimeter (Jasco, MD) equipped with a temperature control unit. A 0.1 cm path-length quartz cell was used with a 12.5 mM protein 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 in the 250–190 nm range. All CD spectra are reported in terms of mean residue ellipticity, $[\theta]_{MRW}$, in deg cm^2 $dmol^{-1}$. The α-helical content was determined from the mean residue ellipticities at 222 nm, as indicated in Equation (1) (Maeng *et al.* 2001).

$$\%\text{Helix} = ([\theta]_{obs} \times 100)/\{[\theta]_{helix} \times (1 - 2.57/l)\}, \quad (1)$$

where $[\theta]_{obs}$ is the mean-residue ellipticity observed experimentally at 222 nm, $[\theta]_{helix}$ is the ellipticity of a peptide of infinite length with 100% helix population, taken to be −39,500 deg cm^2 $dmol^{-1}$, and l is the peptide length or, more precisely, the number of peptide bonds.

Results and discussion

Peptides corresponding to HP (2–20), HPC2 (2–16), HP (4–16) and HP (4–14) were chemically synthesized, purified to homogeneity and subjected to MALDI mass spectroscopic analysis, and the antimicrobial activity and mechanisms operant in these peptides were assessed (Table 1).

The HP (2–20) peptide had antibacterial activity (Boman *et al.* 1989). In our previous study, it also exhibited activity against some fungi but had no hemolytic effect against human erythrocyte cells, at up to 12.5 μM (Lee *et al.* 2002). In order to elucidate the antimicrobial effects of the *N*- and *C*-termini we synthesized a truncated peptide series, in which two amino acid residues were deleted from HP (2–20) (Park *et al.* 2002b). These results suggested that the residues (2–20),

Table 1. Amino acid sequence and molecular masses determined by MALDI-MS of HP (2–20) truncated peptides.

Peptides	Amino acid sequence	Calculated values	Observed values
HP (2–20)	AKKVFKRLEKLFSKIQNDK-NH_2	2318.4	2318
HPN1 (4–20)	KVFKRLEKLFSKIQNDK-NH_2	2119.3	2120
HPN2 (6–20)	FKRLEKLFSKIQNDK-NH_2	1892.1	1893
HPN3 (8–20)	RLEKLFSKIQNDK-NH_2	1617.9	1618
HPC1 (2–18)	AKKVFKRLEKLFSKIQN-NH_2	2075.3	2076
HPC2 (2–16)	AKKVFKRLEKLFSKI-NH_2	1833.2	1834
HPC3 (2–14)	AKKVFKRLEKLFS-NH_2	1592.0	1592
HP (4–16)	KVFKRLEKLFSKI-NH_2	1635.2	1634.6
HP (4–14)	KVFKRLEKLFS-NH_2	1393.9	1394
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	2847.4	2850.6

Table 2. Antimicrobial activity of HP (4–16) peptide.

	MIC (μM)				
	HP (2–20)	HPC2 (2–16)	HP (4–16)	HP (4–14)	Melittin
Strep. aureus	12.5	6.25	6.25–3.13	12.5	0.39
B. subtilis	6.25	6.25	3.13	6.25	1.56–0.78
P. aeruginosa	3.13	3.13	1.56	6.25–3.13	3.13–1.56
Salm. typhimurium	0.78	0.78	0.78	6.25	0.19
E. coli	6.25	6.25	3.13	6.25	1.56
C. albicans	12.5	6.25	6.25–3.13	25	6.25–12.5
T. beigelii	3.13	3.13	3.13	6.25	1.56
Sacch. cerevisiae	25	12.5	12.5	25	6.25–12.5

(4–20), (2–16), and (2–14) exhibit broad-spectrum antimicrobial effects against bacteria and fungi, while displaying no cytotoxic effect on human erythrocyte cells. Therefore, the *N*-terminal (residues 2–3) and *C*-terminal (residues 15–20) were not relevant to the antimicrobial activity exhibited by HP (2–20). In the present study, in order to obtain shorter peptides, while retaining the antimicrobial activity of the HP (2–20) peptide, we synthesized two truncated peptides from the *N*- and *C*-termini of HP (2–20).

Activity against bacterial and fungal cells was increased to a greater degree by removing the *N*- (2–3) and *C*-terminal (17–20) amino acid residues of HP (2–20) [HP (4–16)], than HPC2 (2–16) (Table 2). This result demonstrated that HP (4–16) displayed approximately 2- to 4-fold greater antimicrobial activity in *S. aureus* and *C. albicans* than did HP (2–20) or HPC2 (2–16) (Table 2).

In order to assess the cytotoxicity of the peptides against mammalian cells, hemolysis of human erythrocytes was measured. The synthetic peptides used in this study evidenced no hemolytic activity, while melittin exhibited pronounced hemolytic activity (data not shown). This result demonstrated that HP (4–16) exerts remarkable antimicrobial activity but no hemolytic activity.

Although the overall antibiotic mechanisms of the amphipathic antibacterial peptides with α-helical structures have not yet been clearly elucidated, disruption of the cell structure via pore formation (Lee *et al.* 1997) or ion channel generation seems to be the most likely mechanism (Bechinger *et al.* 1993). In order to investigate whether the antibiotic effect of HP (4–16) is predicated on damage to the plasma membrane or by altering cell physiology, the cells were incubated with HP (4–16) and propidium iodide (PI) (Lee

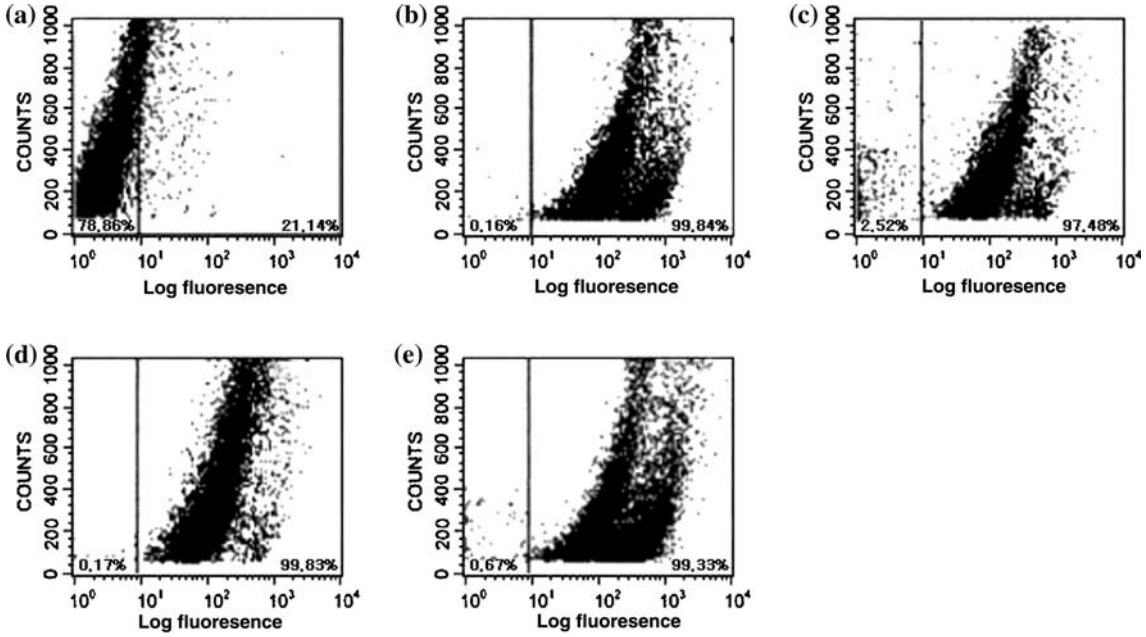

Fig. 1. The effect of salt- and sodium azide (NaN_3) on HP (4–16). Mid-growth phase *C. albicans* cells were treated with 6.25 μM HP (4–16). The cell population showing fluorescence was analysed using a FACScalibur flow cytometer. The increments of the log fluorescence signal represent PI uptake by peptides. a–e shows the NaCl-treated cells with (a) no peptide and no salt, (b) no salts, (c) 150 mM NaCl, (d) 20 mM $MgCl_2$, and (e) NaN_3-treated cells.

et al. 1997). Detection of internal PI in single cells was analyzed by FACScalibur flow cytometric analysis. The results showed that while most untreated, normal cells showed no signal with respect to PI fluorescence activity, cells treated with HP (4–16), at 6.25 μM for 30 min on ice, showed a total shift of the peak to the right (Figure 1b). Figure 1 shows a plot of forward light scatter (horizontal axis) against propidium iodide fluorescence (vertical axis), in which the dots indicate individual cells.

In order to evaluate whether or not salts influence the fungicidal activity of the synthetic peptides, the fungicidal effects of HP (4–16) were investigated using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA). The fungicidal activity of HP (4–16) against *C. albicans,* was not affected in any way by the tested salts, which included $CaCl_2$ and magnesium citrate (Figures 1c and d).

Cells were incubated with the HP (4–16) in the absence or presence of NaN_3 as a respiratory inhibitor. The *C. albicans* cells showing PI uptake were analyzed via flow cytometer. As shown in Figure 1e, the activity of HP (4–16) was unaffected by NaN_3, suggesting that its effect was mediated by a cellular function which does not require energy consumption.

In order to examine the target sites of HP (4–16) in *C. albicans*, the peptide was labeled with FITC and visualized by confocal microscopy. FITC had no effect on the antimicrobial activity of HP (4–16). The FITC-labeled HP (4–16) penetrated the cell membrane and accumulated in the plasma membrane of the cell immediately after addition to the cells (data not shown). This result suggests that the major target site of HP (4–16) is the plasma membrane of the microorganisms and the antibiotic activities of these synthetic peptides, in terms of perturbation of the lipid membrane, also correlate with the antimicrobial activities of these peptides.

The morphological changes induced in *C. albicans* by HP (4–16) were visualized by scanning electron microscopy. Untreated cells had normal, smooth cell surfaces, whereas HP (4–16)-treated cells had large holes in their surfaces (Figure 2). These results provide additional evidence that HP (4–16) probably acts on the plasma membrane, namely, by pore formation.

The circular dichroism (CD) spectra of the peptides were measured in phosphate buffer with trifluoethanol (TFE) or SDS. As shown in Figure 3, HP

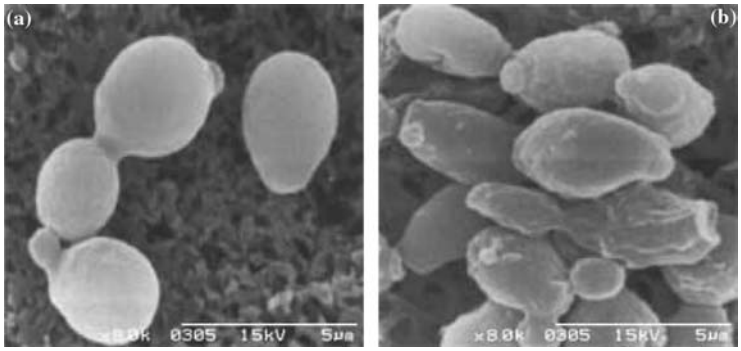

Fig. 2. Scanning electron micrographs of untreated *C. albicans* (a), and after treatment for 30 min at 30 °C with peptides.

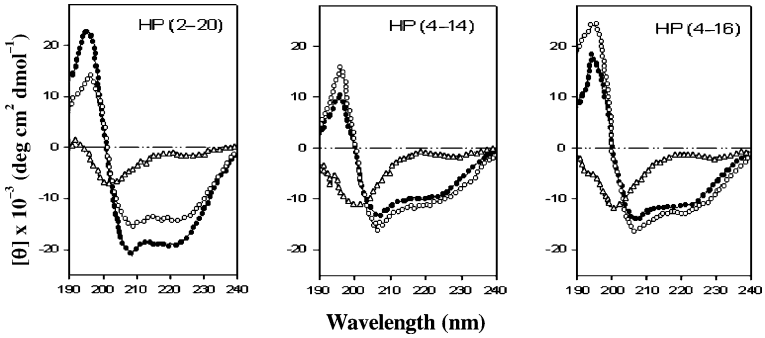

Fig. 3. CD spectra of peptides in alone (△), in 30 mM SDS (○), and in 50% (v/v) trifluoethanol (TFE) (●) and 10 mM sodium phosphate buffer, pH 7.2.

(4–16) showed a remarkable decrease in α-helical content in 50% TFE but HP (2–20) had a higher α-helical content in cell membrane-mimicking environments such as TFE, in which it exhibited less pronounced antimicrobial activity than did HP (4–16). These results suggest that the α-helical content is not directly connected to enhanced antibiotic activity, although it may play an important role in killing microbial cells in the synthetic HP (4–16) peptide.

Conclusions

Truncation of the *N*- and *C*-terminal amino acid sequence of the perfect amphipathic peptide, HP (2–20), resulted in a significant increase in antimicrobial activity. Our results indicate that the HP (4–16) peptide, composed of 4–16 residues of the *H. pylori* ribosomal protein L1, damages the plasma membranes of microbial cells and thereby exerting profound antimicrobial effects. We believe that these observations also indicate that the shortened peptide, HP (4–16), which is also active against bacterial and pathogenic fungal cells, may have great potential as a lead compound in the development of novel antibiotic drugs.

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