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HP (2–20) Derived from the Amino Terminal Region of *Helicobacter pylori* Ribosomal Protein L1 exerts its Antifungal Effects by Damaging the Plasma Membranes of *Candida albicans*

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Abstract: The fungicidal effects of the peptide HP (2–20), derived from the *N*-terminal sequence of *Helicobacter pylori* ribosomal protein L1 (RPL1), have been investigated. HP (2–20) displays a strong fungicidal activity against various fungi, without haemolytic activity against human erythrocyte cells, and the fungicidal activity is inhibited by Ca^{2+} and Mg^{2+} ions. In order to investigate the fungicidal mechanism(s) of HP (2–20), the amount of intracellular trehalose was measured in *C. albicans*. It was found that the amounts of intracellular trehalose were decreased when HP (2–20) was used. The action of the peptide against fungal cell membranes was further examined by the potassium-release test; HP (2–20) was found to increase the amount of K^+ released from the cells. Furthermore, HP (2–20) caused significant morphological changes, as shown by scanning electron microscopy, and by testing the membrane disrupting activity using liposomes (phosphatidyl choline/cholesterol; 10 : 1, w/w). Our results suggest that HP (2–20) may exert its antifungal activity by disrupting the structure of cell membranes, via pore formation or direct interaction with the lipid bilayers. Copyright © 2002 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: HP (2–20); antifungal activity; fungicidal mechanism; potassium-release test

INTRODUCTION

It is generally recognized that antimicrobial peptides play an important role in the innate host defence mechanisms against infectious pathogens of living organisms such as plants [1], insects [2–4], amphibians [5] and mammals [6]. These antimicrobial peptides possess a potent antibiotic activity against bacteria, yeasts and even certain enveloped viruses [6–10]. Despite the great diversity in the primary structures of these peptides, most antimicrobial peptides are cationic at physiological pH values, and show the amphipathic characteristic of adopting α-helix and β-sheet structures in hydrophobic environments [11–12].

The mechanisms of action for several antimicrobial peptides have been investigated, including defensin [13–15], cecropin [16–18], magainin [19], protegrin [20–21] and melittin [22]. These studies have shown the major killing mechanism for these

Abbreviations: MBHA resin, 4-methyl benzhydrylamine resin; MIC, minimal inhibitory concentration; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; PBS, phosphate-buffered saline.

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cationic 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 [14].

Although an understanding is essential for the rational development of novel bioactive peptides, the mechanism(s) by which antimicrobial peptides work have not been clearly elucidated. In general, antimicrobial peptides are known to act in two different ways, one by targeting the physiology of the cells, and the other by disrupting the cellular structure of the microorganism. The mechanism of antifungal action has been investigated for several antimicrobial peptides [15]. The major cause for the loss of fungal cell viability is believed to result from the formation of transmembrane channels, increasing membrane permeability, and causing disruption of the microbial cell structure.

Recently, it has been reported that the *N*-terminus of the *H. pylori* ribosomal protein L1 (RPL1) possesses antibacterial activity to which it is self resistant [23–24]. In this study, the antifungal property of this *N*-terminal region was examined. In order to elucidate the antifungal mechanism of the synthetic peptide, HP (2–20), its antifungal effect was investigated on various yeast strains. In order to substantiate the mechanism(s), potassium release tests and measurements of intracellular trehalose were performed on *C. albicans*. Additionally, its effect on the cell morphology of *C. albicans* was explored using a scanning electron microscope.

MATERIALS AND METHODS

Peptide Synthesis

The peptides, HP (2–20) and melittin, were synthesized by Fmoc SPPS [25] 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, 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-MASS.

Antifungal Activity Assay

Candida albicans (TIMM 1768) was grown at 28 °C in a YPD medium (dextrose 2%, peptone 1%, yeast extract 0.5%, pH 5.5). The cells were seeded on 96-well plates at a density of 2×10^3 cells per well in 100 μl of the YPD medium. The serially diluted peptides (50, 25, 12.5, 6.25, 3.125, 1.56, 0.78 and 0.39 μM) were added to each well. Synthesized melittin was used as a positive control. After incubation for 24 h at 28 °C, 10 μl 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) solution (5 mg/ml MTT in PBS, pH 7.4) was added to each well, and the plates were incubated for a further 4 h at 37 °C. The turbidity of each well was measured at 570 nm using a microtitre ELISA reader (Molecular Devices Emax, California, USA). All the assays were performed in triplicate.

Haemolysis Assay

The haemolytic activities of the peptides were measured against a 4% human erythrocyte cell suspension at 414 nm. The human erythrocyte cells were washed three times with phosphate-buffered saline (PBS: 35 mM phosphate buffer/0.15M NaCl, pH 7.0). 100 μl of the human erythrocytes suspension, 8% (v/v) in PBS, was added to the 96-well plates, then 100 μl of the peptide solution (HP (2–20) or melittin, used as positive control) was mixed into each well. The mixtures were incubated for 1 h at 37 °C, then centrifuged at 150 × **g** for 5 min. The absorbance of the supernatant was measured at 414 nm. Haemolysis rates of 0 and 100% were determined in PBS and 0.1% Triton-X100, respectively. The percentage of haemolysis was calculated employing the equation: % haemolysis = [($Abs_{414\ nm}$ in the peptide solution − $Abs_{414\ nm}$ in PBS)/($Abs_{414\ nm}$ in 0.1% Triton-X100 − $Abs_{414\ nm}$ in PBS)] × 100.

Ca^{2+} or Mg^{2+} Dose Dependent Test

C. albicans was suspended at a density of 2×10^4 cells per 100 μl of the YPD media in a 96-well

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Table 1 The Amino Acid Sequences of HP (2–20) and Melittin and their Molecular Weights as Determined by MALDI-MASS

Peptide	Sequence	Calculated value	Observed value
HP (2–20)	AKKVFKRLEKLFSKIQNDK-NH_2	2319.3	2320.0
Melittin	GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2	2847.4	2850.6

microtitre plate. HP (2–20), at a final concentration of 25 μM, and calcium chloride and/or magnesium citrate were added, to give final concentrations of 0.1, 0.5, 1 and 3 mM. The mixtures were incubated at 28 °C for 18 h. 10 μl of a MTT solution (5 mg/ml MTT in PBS, pH 7.4) was added to each well, and the plates were incubated at 37 °C for a further 4 h. The turbidity of each well was measured by absorbance at 570 nm using a microtitre ELISA reader (Molecular Devices Emax, California, USA).

Measurement of K^+ Release

The yeast cells (2×10^5) were incubated on 96-microtitre plates, in 100 μl of the YPD media containing peptides (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^+ measured using an inductively coupled plasma mass spectrometer (Fisons, Cheshire, UK).

Determination of Intracellular Trehalose

C. albicans cells, containing 50 μM of HP (2–20), were incubated at 28 °C for 1 h. The negative controls were incubated without peptide, with melittin being used as a positive control. Intracellular trehalose was extracted from 15 mg (dry wt) of fungal cells with 0.5 ml of boiling water, the extracts were reacted with anthrone, and the colour formation was measured at 620 nm, in accordance with the procedure described by Savioja and Miettinen [26]. Glycogen in the trehalose residue was digested with amyloglucosidase to give free glucose which was assayed by the glucose oxidase peroxidase method [27]. The method was validated using glucose as an external standard.

Preparation of Small Unilamellar Vesicles (SUVs)

SUVs were prepared by drying phosphatidyl choline (PC)/cholesterol (10 : 1, w/w) under nitrogen, suspending 100 mM of the film in 50 mM of phosphate buffer at pH 7.5, mixing by vortex and sonicating using the tip of an ultrasonic probe. A drop was deposited on a carbon-coated grid and negatively stained with 2% uranyl acetate. Specimens were examined in a Tecnai 12 (Philips, USA) at an accelerating voltage of 120 kV.

Morphological Changes induced in *C. albicans* by HP (2–20) and Melittin

C. albicans cells were incubated at 28 °C for 4 h with 12.5 μM of HP (2–20) or melittin. Negative controls were run with neither HP (2–20) nor melittin. 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 (Tokyo, Japan).

RESULTS AND DISCUSSION

A peptide corresponding to HP-(2–20)-amide was chemically synthesized, purified to homogeneity and

Table 2 Antifungal Activities of HP (2–20) Compared with Melittin

Peptide	MIC (μM)		
	C. albicans	*T. beigelii*	*S. cerevisiae*
HP (2–20)	25	12.5–25	25
Melittin	3.125	3.125–6.25	3.125

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subjected to MALDI mass spectroscopic analysis, and its antifungal mechanism studied (Table 1).

HP (2–20) was shown to inhibit the growth of *E. coli* and *B. megaterium* [23]. Melittin, a honeybee venom toxin, was also reported to possess potent antimicrobial activity over a broader spectrum [28]. However, the antifungal activity and mechanism(s) of action of HP (2–20) are not thoroughly understood. Therefore, the antifungal MIC of HP (2–20) against various fungal cells was determined as MIC using the MTT assay [29] (Table 2). The haemolytic percentage was measured using human erythrocyte cells at various peptide concentrations. HP (2–20) exhibited no haemolytic activity, while melittin, used

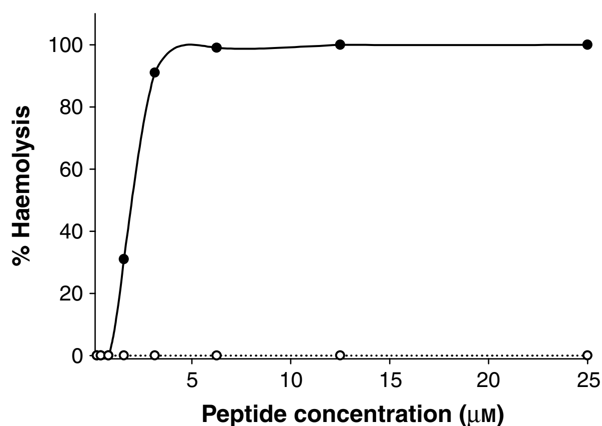

Figure 1 Haemolytic activities of the *H. pylori* derived antimicrobial peptide, HP (2–20). The HP (2–20) treated (○), the melittin treated, used as a positive control (●).

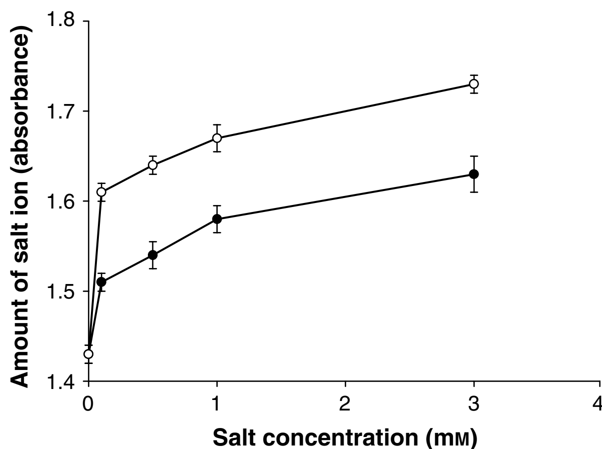

Figure 2 The antifungal effect of HP (2–20) against *C. albicans*, in the presence of calcium and magnesium ions. HP (2–20) was added to a final concentration of 25 μM and calcium chloride and/or magnesium citrate added to a give a final concentration of 0.1, 0.5, 1 and 3 mM. The turbidity of each well was measured at 570 nm using a microtitre ELISA reader. The cells were incubated and treated with Ca^{2+} ions (●) and Mg^{2+} ions (○), respectively.

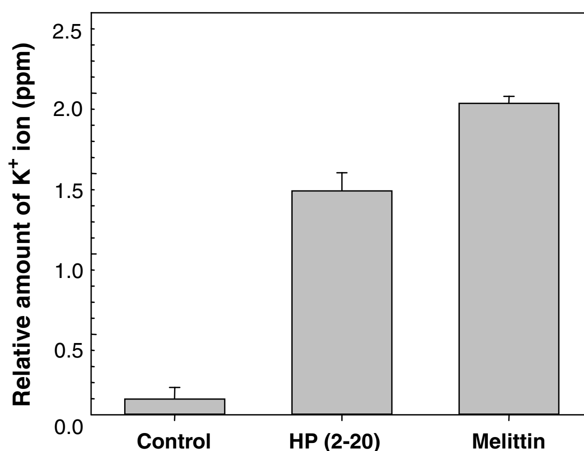

Figure 3 The concentration of potassium ion released from *C. albicans* by the HP (2–20). *C. albicans* (2×10^5 cells per well) were incubated, at 28 °C, on flat-bottom microtitre 96-well microtitre plates in 100 μl of YPD media, containing 10 μM of the peptides. After incubation, the cell suspensions were measured for levels of released potassium ions. No peptide treated (●), cells incubated with HP (2–20) treated sample (○), and cells incubated with melittin, (positive control) (▼).

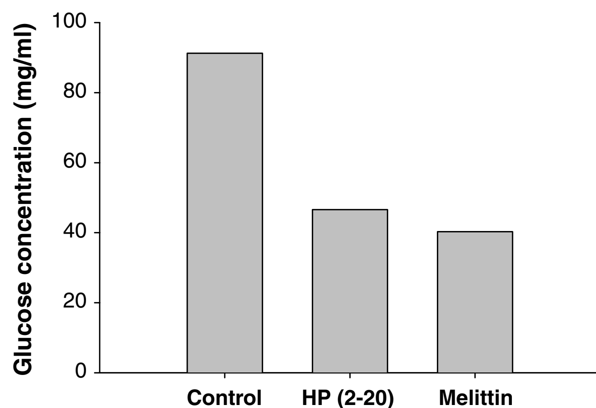

Figure 4 Trehalose assay after addition of peptides, HP (2–20) and melittin. Subcultured *C. albicans* cells (20 μM), with peptides, were incubated at 28 °C for 2 h. Peptide-treated cells were washed with distilled water and dried under vacuum. To determine the glucose in the trehalose residue, samples were submerged in boiling water and 2 μl of amyloglycosidase added. After incubation, anthrone reagent was added, and the mixture was incubated at 90 °C for 15 min. The glucose was determined at a wavelength of 620 nm.

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as a positive control, showed high haemolytic activity (Figure 1).

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 obtain further information on whether HP (2–20) causes damage to the plasma membrane or affects cell physiology, the cells were incubated with HP (2–20) and salts (calcium chloride and magnesium citrate). Recently, it has been proposed that high salt concentrations inhibit the activity of antimicrobial peptides [30]. In this study, the fungicidal activity of HP (2–20), against *C. albicans*, was markedly reduced at high salt concentrations (Figure 2). The initial binding of the antimicrobial peptide, HP (2–20), is thought to depend on a membrane interaction that is vulnerable to increases in salt concentration, and is followed by the formation of ion channels [30]. In this report, it was shown that HP (2–20) peptide possesses a potent fungicidal activity that heavily depends on ionic strength.

The amount of K^+ released from *C. albicans* by HP (2–20) was compared with the action of melittin as a positive control. HP (2–20) and melittin both released more K^+ than the negative control (Figure 3). This increase in the amount of released K^+ after peptide treatment provides further evidence that HP (2–20) 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 (2–20) 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

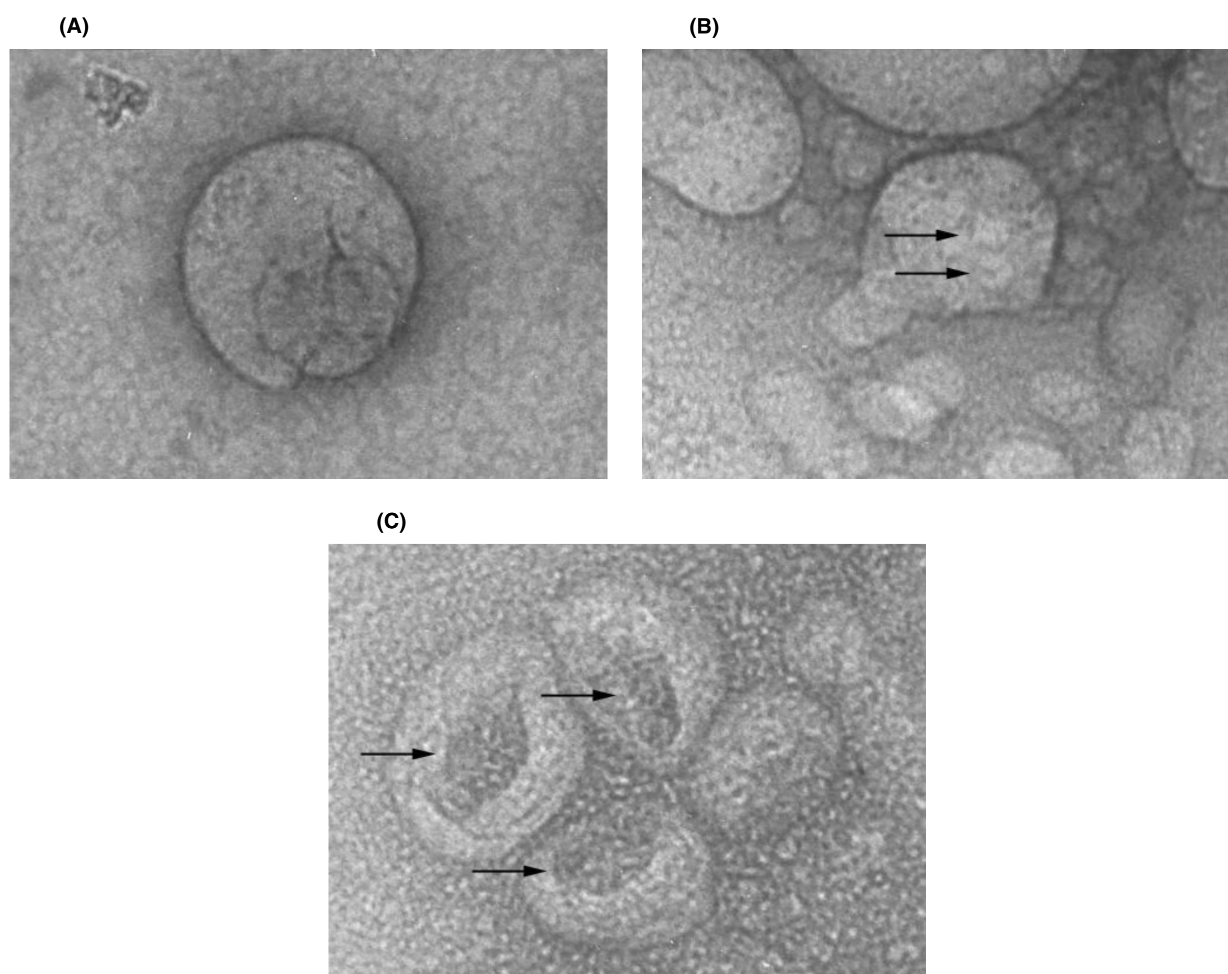

Figure 5 Electron micrographs of negatively stained SUV composed of PC/cholesterol (10 : 1, w/w) in the absence (A), or in the presence (B), of HP (2–20), or melittin (C). The scale bar represents 100 nm.

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well as dissipating the electrical potential of the membrane.

The amount of intracellular trehalose from *C. albicans* was measured in the presence and absence of HP (2–20). As expected, the intracellular trehalose of the HP (2–20)-treated cells was lower than that of the negative controls (Figure 4). These results were comparable to that of melittin and suggest both HP (2–20) and melittin may act in a similar way.

In order to confirm the ability of HP (2–20) to disrupt microbial plasma membranes, experiments were performed with liposomes. Artificial small unilamellar vesicles (SUVs) (PC/cholesterol; 10 : 1, w/w) and neutral vesicles of phosphatidylcholine (PC) were used as model membrane systems. The antimicrobial activity of melittin is based on the formation of transmembrane channels. Pores were formed from SUVs, after treatment with HP (2–20) or melittin (Figure 5), suggesting the peptides may cause perturbation of the lipid components of the plasma membranes. It is conceivable the effect of the peptide has the following stages: (i) binding to the membrane; (ii) membrane destabilization, and (iii) stabilization of the peptide–lipid complex.

The morphological changes induced in *C. albicans* by HP (2–20) and melittin were examined by scanning electron microscopy. Untreated cells had a normal, smooth cell surface (Figure 6), whereas HP (2–20) and melittin treated cells had large holes in their surfaces, and cell lysis was also observed (Figure 6). These results provide additional evidence that HP (2–20) probably acts on the plasma

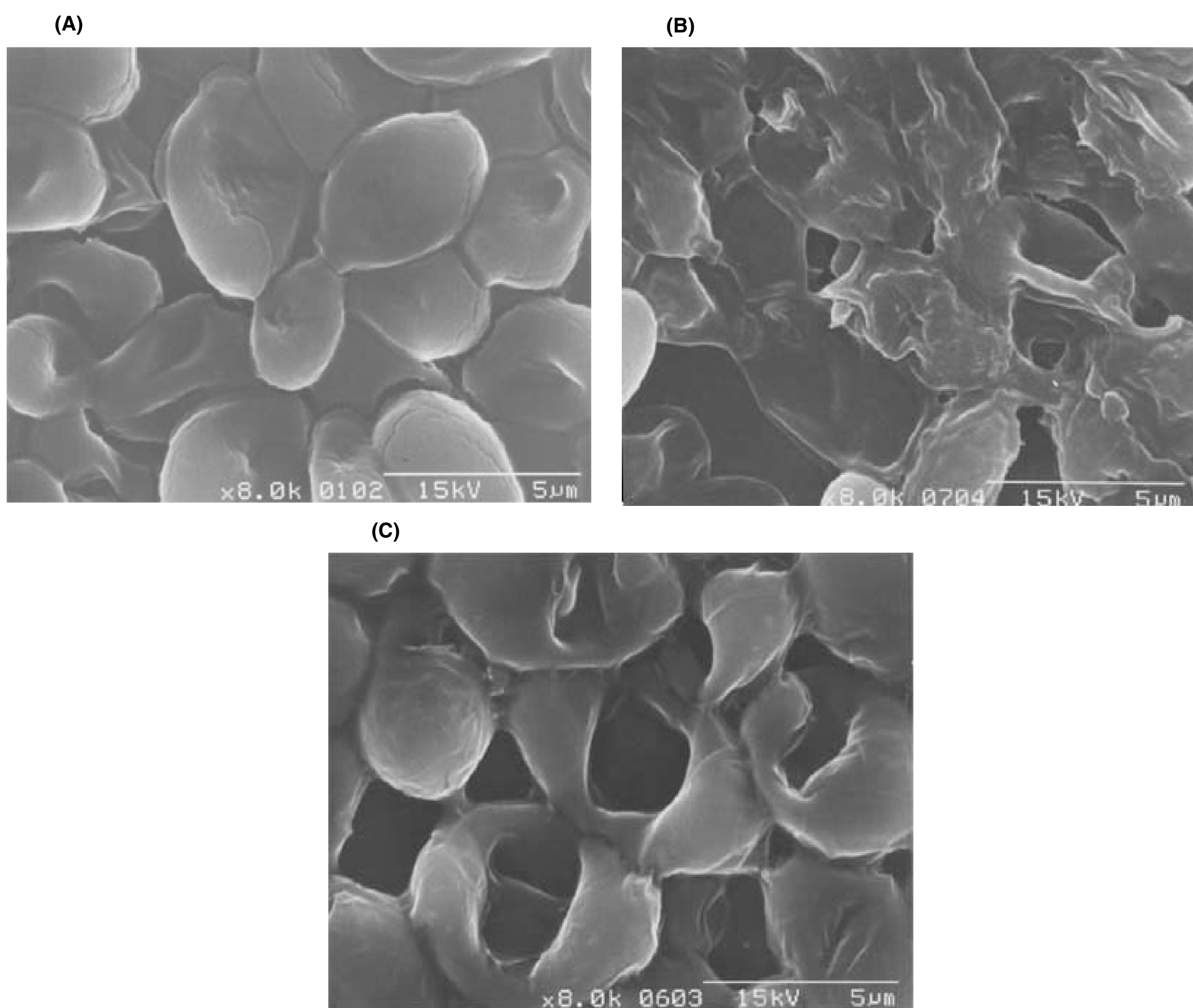

Figure 6 Scanning electron micrographs of untreated (A), and after treatment of *C. albicans* for 4 h at 28 °C with; 12.5 μM melittin, (B) and 12.5 μM HP (2–20), (C).

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membrane, by forming pores, causing the leakage of ions and other materials from the cells.

CONCLUSION

The synthetic peptide amide HP (2–20) corresponding to an *N*-terminal region of *H. pylori* ribosomal protein L1 damages the plasma membranes of *C. albicans* and thereby exerts potent antifungal effects similar to those of melittin, but, in contrast to melittin, without haemolytic activity. We believe that these observations give HP (2–20) which is also active against *T. begelii* and *S. cerevisiae* potential as a lead compound for the development of novel antifungal drugs.

Acknowledgements

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REFERENCES

1. Barra D, Simmaco M. Amphibian skin: a promising resource for antimicrobial peptides. *TIBTECH* 1995; **13**: 205–209.
2. Lee DK, Kim BS, Kim D-H, Kim S, Chun JH, Han DM, Lee BL, Lee Y. Expression of an insect antifungal protein of *Tenebrio molitor. Escherichia coli. Mol. Cells* 1995; **5**: 429–435.
3. Dempsey CE. The actions of melittin on membranes. *Biochem. Biophys. Acta* 1990; **1031**: 143–161.
4. Thevissen K, Ghazi A, De Samblanx GW, Brownlee C, Osborn RW, Broekaert WF. Fungal membrane responses induced by plant defensins and thionins. *J. Biol. Chem.* 1996; **271**: 15 018–15 025.
5. Broekaert W, Terras F, Cammue BPA, Osborne R. Plant defensins: novel antimicrobial peptides as components of the host defense system. *Plant Physiol.* 1995; **108**: 1353–1358.
6. Mangoni ME, Aumelas A, Chamet P, Roumestand C, Chiche L, Despaux E, Grassy G, Calas B, Chavanieu A. Change in membrane permeability induced by protegrin 1: implication of disulfide bridges for pore formation. *FEBS Lett.* 1996; **383**: 93–98.
7. Hoffmann JA. Innate immunity of insects. *Curr. Opin. Immunol.* 1995; **7**: 4–10.
8. Lehrer R, Lichtenstein AK, Ganz T. Defensins: antimicrobial and cytotoxic peptides of mammalian cells. *Annu. Rev. Immunol.* 1993; **11**: 105–128.
9. Hoffmann JA, Hetru C. Insect defensins: inducible antibacterial peptides. *Immunol. Today* 1992; **13**: 411–415.
10. Ganz T, Lehrer RI. Defensins. *Curr. Opin. Immunol.* 1994; **6**: 584–589.
11. McCarthy PJ, Troke PF, Gull K. Mechanism of action of nikkomycin and the peptide transport system of *Candida albicans. J. Gen. Microbiol.* 1985; **131**: 775–780.
12. Mclain N, Ascanio R, Baker C, Strohaver RA, Dolan JW. Undeclenic acid inhibits morphogenesis of *Candida albicans. Antimicrob. Agents Chemother.* 2000; **44**: 2873–2875.
13. De Lucca AJ, Walsh TJ. Antifungal peptides: novel therapeutic compounds against emerging pathogens. *Antimicrob. Agents Chemother.* 1999; **43**: 1–11.
14. Mor A, Nguyen VH, Delfour A, Migliore-Samour D, Nicolas P. Isolation, amino acid sequence of dermaseptin, a novel antimicrobial peptide of amphibian skin. *Biochemistry* 1991; **30**: 8824–8830.
15. Kim D-H, Lee DG, Kim KL, Lee Y. Internalization of tenecin 3 by a fungal cellular process is essential for its fungicidal effect on *Candida albicans. Eur. J. Biochem.* 2001; **268**: 4449–4458.
16. Christensen B, Fink J, Merrifield RB, Mauzerall D. Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes. *Proc. Natl Acad. Sci. USA* 1988; **85**: 5072–5076.
17. Pouny Y, Rapaport D, Mor A, Nicolas P, Shai Y. Interaction of antimicrobial dermaseptin and its fluorescently labeled analogues with phospholipid membranes. *Biochemistry* 1992; **31**: 12 416–12 423.
18. Szoka F, Papahadjopoulos D. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl Acad. Sci. USA* 1978; **75**: 4194–4198.
19. Hung HW. Action of antimicrobial peptides: two-state model. *Biochemistry* 2000; **29**: 8347–8352.
20. Stintzi A, Heitz T, Prasad V, Wiedemann-Merdinoglu S, Kauffmann S, Geoffroy P, Legrand M, Fritig B. Plant 'pathogenesis-related' proteins and their role in defense against pathogens. *Biochemie* 1993; **75**: 687–706.
21. Sawistowska-Schroder ET, Kerridge D, Perry H. Echinocandin inhibition of (1,3)-beta-D-glucan synthase from *Candida albicans*. *FEBS Lett.* 1984; **173**: 134–138.
22. Steiner H, Hultmark HD, Engstrom A, Bennich H, Boman HG. Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature* 1981; **292**: 246–248.
23. Putsep K, Branden CI, Boman HG, Normarks S. Antibacterial peptide from *H. pylori. Nature* 1999; **398**: 671–672.

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24. Putsep K, Normarks S, Boman HG. The origin of cecropins; implications from synthetic peptides derived from ribosomal protein L1. *FEBS Lett.* 1999; **451**: 249–252.
25. Merrifield RB. Solid phase synthesis. *Science* 1986; **232**: 341–347.
26. Savioja T, Miettinen JK. On trehalose metabolism in yeast. *Acta Chem. Scand.* 1966; **20**: 2451–2455.
27. Zimmermann FK, Eaton NR. Genetics of induction and catabolite repression of Maltese synthesis in *Saccharomyces cerevisiae*. *Mol. Gener. Genet.* 1974; **134**: 261–272.
28. Lee DG, Park J-H, Shin SY, Lee SG, Lee MK, Kim KL, Hahm K-S. Design of novel analogue peptides with potent fungicidal but low hemolytic activity based on the cecropin A-melittin hybrid structure. *Biochem. Mol. Biol. Int.* 1997; **3**: 489–498.
29. Jahn B, Martin E, Stueben A, Bhakdi S. Susceptibility testing of *Candida albicans* and *Aspergillus* species by a simple microtiter menadione- augmented 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide assay. *J. Clin. Microbiol.* 1995; **33**: 661–667.
30. Shin SY, Yang S-T, Park EJ, Eom SH, Song WK, Kim Y, Hahm K-S, Kim JI. Salt resistance and synergistic effect with vancomycin of α-helical antimicrobacterial peptide P18. *Biochem. Biophys. Res. Commun.* 2002; **290**: 558–562.

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