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ORIGINAL ARTICLE

Biochemical property and membrane-peptide interactions of de novo antimicrobial peptides designed by helix-forming units

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Abstract Typical peptides composed of Phe, Ile, and Arg residues have not been reported, and the effect of the helix-forming unit (HFU) composed of the tripeptide core on biological activity remains unclear. In this study, multimers of the 3-residue HFU were designed to investigate the structure–function relationships. The in vitro biological activities of the peptides were determined. We used synthetic lipid vesicles and intact bacteria to assess the interactions of the peptides with cell membranes. The well-studied peptide melittin was chosen as a control peptide. The results showed that the antimicrobial and hemolytic activities of the peptides increased with the number of HFUs. HFU3 had optimal cell selectivity as determined by the therapeutic index. HFU3 and HFU4 exhibited strong resistance to salts, pH, and heat. CD spectra revealed that the peptides except HFU2 displayed α-helix-rich secondary structures in the presence of SDS or trifluoroethanol (TFE). The peptides interacted weakly with zwitterionic phospholipids (mimicking mammalian membranes) but strongly with negatively charged phospholipids (mimicking bacterial membranes), which corresponds well with the data for the biological activities. There was a correlation between the cell selectivity of the peptides and their high binding affinity with negatively charged phospholipids. Cell membrane permeability experiments suggest that the peptides targeted the cell membrane, and HFU3 showed higher permeabilization of the inner membrane but lower permeabilization of the outer membrane than melittin. These findings provide the new insights to design antimicrobial peptides with antimicrobial potency by trimers.

Keywords Antimicrobial peptides · Amino acids · Helix · Cell membrane · Residue

Abbreviations

AMP	Antimicrobial peptide
HFU	Helix-forming unit
diSC3(5)	3-3-Dipropylthiadicarbocyanine-iodide
MHC	Minimal hemolytic concentration
CD	Circular dichroism
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
NPN	N-phenyl-1-naphthylamine
Ksv	Stern–Volmer quenching constant
MH	Mueller–Hinton

Introduction

For more than three decades, a large number of peptides with potent antibacterial, antiviral, and antifungal properties have been identified in animals, plants, and bacteria (Sato and Feix 2006). The widespread distribution throughout the animal and plant kingdoms suggests that antimicrobial peptides served a fundamental role in the successful evolution of complex multicellular organs (Zasloff 2002). The diversity and inhomogeneity of amino

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acid sequences make it difficult to design de novo antimicrobial peptides with potent activities (Hancock and Chapple 1999; Matsuzaki 1999). We have designed a series of peptides with systematically incremental hydrophobicity (Ma et al. 2011a, b). Considering the importance of net charge in the potency of antimicrobial peptides, we continued to change the net charge but fix the mean hydrophobicity of the antimicrobial peptides. Therefore, an approach was developed to place basic elements of screened residues on a helical wheel projection to obtain amphipathic helices expected to have antimicrobial activity.

Most naturally occurring antimicrobial peptides are cationic and amphipathic in nature (Jin et al. 2005). Therefore, basic and hydrophobic amino acids play an important role in antimicrobial peptides. As aliphatic and aromatic amino acids, Ile and Phe have the highest hydrophobicity levels, respectively (Eisenberg et al. 1982; Fauchere and Pliska 1983; Tossi et al. 2002). Temporin-SHf, a Phe-rich antimicrobial peptide, has broad-spectrum microbicidal activity against Gram-positive and Gram-negative bacteria and yeasts but does not possess hemolytic activity (Abbassi et al. 2010). Plicatamide and its Phe-rich analogs showed rapid action and potent anti-staphylococcal activity (Tincu et al. 2003). A variant of the peptide indolicidin with five tryptophans replaced by phenylalanines showed antibacterial activities comparable to that of indolicidin. The analog did not exhibit any hemolytic activity (Subbalakshmi et al. 1996). The single tryptophan residue of cecropin had a negligible effect on antimicrobial activity but lessened or eliminated hemolytic activity (Andreu and Merrifield 1985). Therefore, Phe was chosen over Trp as the aromatic amino acid to be used in this study. In addition, the Phe-containing copeptides had broader antimicrobial activity and were more effective than the Leu-containing and Ala-containing series (Zhou et al. 2010). Two aliphatic amino acids, Leu and Val, have been widely studied over the past few years (Ma et al. 2011b). However, very little is known about Ile. In fact, Ile occurred with average frequencies of 7.04 % in 252 α-helical antimicrobial peptides and 6.09 % in 1,628 reported antimicrobial peptides (APD: http://www.aps.unmc.edu/AP/main.php). Mutants of the peptide carnobacteriocin B2 in which Val was replaced by Ile were fully active (Quadri et al. 1997).

As a basic amino acid, the side chain of Arg donates several hydrogen bonds while simultaneously interacting with an aromatic ring, whereas lysine is unable to form hydrogen bonds and interact with the aromatic ring (Aliste et al. 2003; Mitchell et al. 1994). It has been suggested that this difference is responsible for the increased activity of Arg-containing peptides over Lys-substituted peptides. Cation–π interactions make the entry of Arg into the hydrophobic environment inside a lipid bilayer more energetically favorable. Other studies suggest that the antimicrobial activity of peptides containing Arg is higher than those of peptides containing Lys (Pellegrini and Fellenberg 1999; Shafer et al. 1996).

As mentioned earlier, Phe, Ile, and Arg residues have the potential to provide peptides with antimicrobial activity. However, helix-forming unit (HFU) composed of the tripeptide core has not been reported, and the effect on biological activity remains unclear. In this study, we synthesized a series of peptides containing between 2 and 5 HFUs (Table 1). The HFU, which is composed of some combination of Phe, Arg, and Ile, is not a standard unit, but rather a string of Phe, Arg, and Ile trimers organized in a way that optimizes an idealized alpha-helix (Fig. 1). We allowed the peptides to interact with synthetic lipid vesicles and whole bacteria (Silvestro et al. 2000). A Trp residue was introduced at position two of the peptides to monitor lipid–peptide interactions by means of tryptophan fluorescence (Song et al. 2004). Furthermore, the C terminus was aminated to increase stability (Liu and Deber 1998).

Materials and methods

Peptide synthesis

The peptides were purchased from GL Biochem Corporation (Shanghai, China). The purity of the peptides (>95 %) was assessed by reverse-phase high-performance liquid chromatography, and the peptides were further subjected to electrospray mass spectrometry to confirm their molecular weight. The peptides were amidated at the C terminus.

Antimicrobial assays

Minimum inhibitory concentration (MIC) testing was performed by a modified version of the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method as described previously (Steinberg et al. 1997). In brief, bacteria were grown overnight in Mueller–Hinton (Difco) broth at 37 °C. Then the cultures were diluted to $\sim 1 \times 10^5$ CFU/ml. Serially diluted peptides in 0.01 % (v/v) acetic acid and 0.2 % (w/v) bovine serum albumin (Sigma) were added to each well of 96-well plates. Each well contained a total volume of 100 µl (50 µl of inoculum and 50 µl of peptide-containing solution). Minimum inhibitory concentrations were determined as the lowest concentration of peptide that prevented visible turbidity by visual inspection after incubation at 37 °C for 20 to 24 h. Independent experiments were carried out three to five times. Uninoculated MH broth was used as a negative control and cultures without added peptides served as the positive control.

Table 1 Amino acid sequence, molecular weight, and net charge of the peptides

Peptide	Sequence	Calculated MW (Da)	Observed MW (Da)	Net charge
HFU2	IWRIFR–NH_2	889.1	889.1	+3
HFU3	IWRIFRRIF–NH_2	1,305.6	1,305.7	+4
HFU4	IWRIFRRIFRIF–NH_2	1,722.1	1,722.2	+5
HFU5	IWRIFRRIFRIFIRF–NH_2	2,138.6	2,138.7	+6

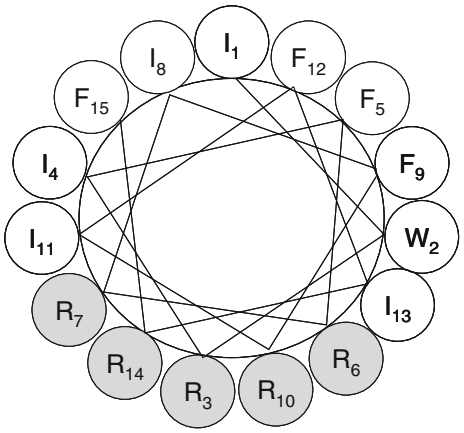

Fig. 1 The sequence template of peptide HFU5. Residues are numbered consecutively from the N terminus to the C terminus, with Arg residues *shaded*. Another four peptides were derived by detracting the HFU. The HFU, which is comprised of some combination of Phe, Arg, and Ile, is not a standard unit, but rather a string of Phe, Arg, and Ile trimers organized in a way that optimizes an idealized alpha-helix

Measurement of hemolytic activity

The hemolytic activity of the peptides was measured as the amount of hemoglobin released by the lysis of human erythrocytes (Stark et al. 2002). Fresh human red blood cells (hRBCs) were obtained by centrifuging whole blood at 1,000*g* for 5 min at 4 °C. The erythrocytes were washed three times with phosphate-buffered saline (PBS) and then resuspended in PBS. The peptides dissolved in PBS (50 µl) were added to the hRBCs solution (50 µl). The suspension was incubated for 1 h at 37 °C. Intact erythrocytes were pelleted by centrifugation at 1,000*g* for 5 min at 4 °C, and the release of hemoglobin was monitored by measuring the absorbance at 492 nm. Peptide concentrations causing 50 % hemolysis were used as the minimal hemolytic concentration (MHC). Zero hemolysis (blank) and 100 % hemolysis were determined by incubating the cells with PBS and 0.1 % Triton X-100, respectively.

The effects of salts on the antimicrobial activity of the peptides

The stability of the peptides was tested in the MIC assay mentioned above with $\sim 5 \times 10^4$ CFU/ml of *E. coli* ATCC 25922. Briefly, inoculum-containing MH broth and peptide-containing solution were added into each well of 96-well plates. Each well contains 50 to 150 mM NaCl, or 1 mM $CaCl_2$ or $MgCl_2$ to evaluate the salt stability of the peptides. The final concentrations of the peptides in 96-well plates ranged from 0.125 to 64 µM. After incubation for 20–24 h at 37 °C, bacterial growth inhibition was determined by inspecting the visible turbidity. The solutions with dilution of bacteria at salts ($CaCl_2$, $MgCl_2$, or NaCl) without peptides were conducted as control.

The effects of heat, and enzymes on the antimicrobial activity of the peptides

To test thermal stability and susceptibility to enzymes, the peptides were processed in advance. For heat resistance experiment, the peptides were incubated at 100 °C for 1 h and cooled on ice for 10 min. For the sensitivity of the peptides to proteolytic enzymes, a solution of the peptide was incubated at 37 °C for 1 h with 1 mg/ml final concentration of the following enzymes: trypsin, pepsin, caroid, and proteinase K. After the treatment, the procedures were the same as described above. The peptides were added into the well with final concentrations in the range of 0.125 to 64 µM. Incubation of *E. coli* ATCC 25922 was carried out at 37 °C for 20 to 24 h. Minimum inhibitory concentrations were determined by identifying the concentration of peptide that completely inhibited bacterial growth after approximately 24 h of incubation.

The effects of pH on the antimicrobial activity of the peptides

Mueller–Hinton broth was adjusted with HCl or NaOH to investigate the effects of pH (4.0–10.0) on antibacterial activity. The peptides and bacteria were added into the well and incubated for 2 h at 37 °C. The diluted aliquots were plated on MH agar plates and incubated for another 18–24 h at 37 °C. The residual numbers of CFU were scored. The dilution of bacteria at respective pH values without peptides was always grown in parallel as a control. The MICs was defined as the concentration of peptides at which no growth (i.e., 99.9 % kill) occurred after around 20 h of incubation.

CD analysis

Circular dichroism (CD) spectra of the peptides were measured at 25 °C using a J-720 spectropolarimeter (Jasco, Tokyo, Japan). The peptides with a final concentration of 150 μM were dissolved in 10 mM sodium phosphate buffer, pH 7.4, 50 % TFE, or 25 mM SDS. The solutions were loaded into a 0.1-cm path length rectangular quartz cell, and the spectra were recorded between 190 and 250 nm at 0.1 nm increments. The average mean residue ellipticities were plotted against the wavelength (in nanometers) (Fig. 2).

Preparation of liposomes

Small unilamellar vesicles (SUVs) were prepared for fluorescence spectroscopy as described previously (Lee et al. 2006; Yang et al. 2003). Egg yolk L-α-phosphatidylcholine (PC), egg yolk L-α-phosphatidyl-DL-glycerol (PG), egg yolk L-α-phosphatidylethanolamine (PE), cholesterol, and acrylamide were purchased from Sigma-Aldrich Corporation (St. Louis, MO). Briefly, PE/PG (7:3, w/w) or PC/cholesterol (10:1, w/w) lipids were dissolved in chloroform, dried with a stream of nitrogen, and lyophilized overnight. Dried lipid films were resuspended in 10 mM Tris–HCl buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 0.1 mM EDTA), vigorously vortexed, and sonicated in ice-cold water for 10–20 min using an ultrasonic cleaner until the solutions became clear.

Tryptophan fluorescence

Tryptophan fluorescence spectroscopy was used to evaluate the binding activity of the peptides to lipid vesicles. The tryptophan fluorescence spectra were measured using an F-4500 fluorescence spectrophotometer (Hitachi, Japan). The fluorescence emission spectra were measured in 10 mM Tris–HCl buffer (pH 7.4) as well as in the presence of negatively charged PE/PG (7:3, w/w) SUVs or zwitterionic PC/cholesterol (10:1, w/w) SUVs. The peptide/liposome mixture (molar ratio of 1:50) was incubated at 20 °C for 10 min. The fluorescence was excited at 280 nm and emission was scanned from 300 to 400 nm.

Tryptophan quenching

To examine the relative extent of burial of the tryptophan residues of the peptides into phospholipids, tryptophan fluorescence was quenched with acrylamide, a water-soluble fluorescence quencher. Fluorescence quenching

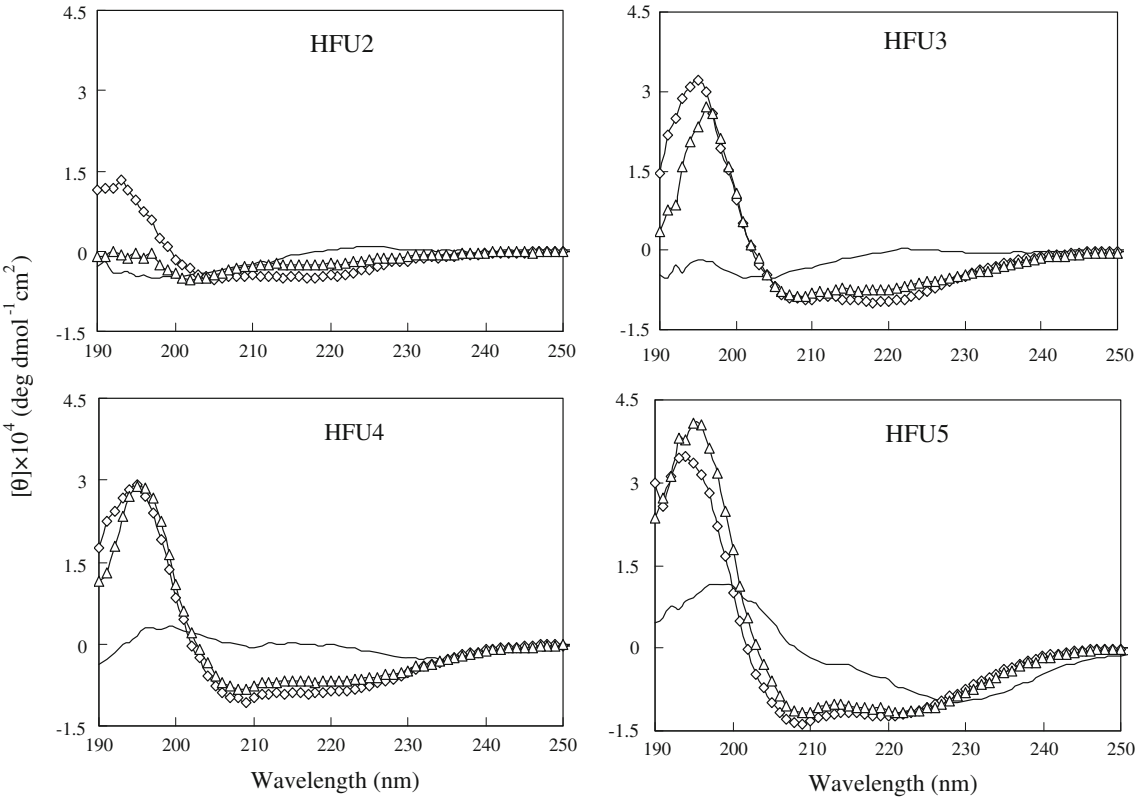

Fig. 2 CD spectra of the peptides. The peptides were dissolved in 10 mM sodium phosphate buffer (*solid line*), pH 7.4, 50 % TFE (*daimonds*), or 25 mM SDS (*squares*). Mean residue ellipticities were plotted against wavelength. Values from three scans were averaged per sample

experiments were measured with an F-4500 fluorescence spectrophotometer (Hitachi, Japan). To reduce absorbance by acrylamide, the fluorescence of Trp was excited at 295 nm instead of 280 nm (Song et al. 2004). A 4-M stock solution of acrylamide was titrated to a final concentration of 0.4 M, and the concentrations of peptides and phospholipid vesicles were 10 and 500 μM, respectively. Quenching of the Trp emission by acrylamide was analyzed using the Stern–Volmer equation: $F_0/F = 1 + K_{SV}(Q)$, where F_0 and F are the fluorescence values of the peptide in the absence and the presence of acrylamide, respectively, K_{SV} represents the Stern–Volmer quenching constant, and Q represents the concentration of acrylamide.

Evaluation of outer membrane permeability

The ability of the peptides to disrupt bacterial outer membrane integrity was evaluated using the fluorescent dye N-phenyl-1-naphthylamine (NPN) as previously described (Loh et al. 1984). Briefly, *E. coli* UB1005 cells were suspended in 5 mM sodium HEPES buffer, pH 7.4, containing 5 mM glucose. N-phenyl-1-naphthylamine was added to log-phase bacteria at a final concentration of 10 μM. The stabilized background fluorescence was recorded (excitation λ 350 nm, emission λ 420 nm). The peptide was added to the quartz cuvette with a final concentration from 2 to 8 μM, and the increase in fluorescence was recorded.

Evaluation of inner membrane permeability

The cytoplasmic membrane depolarization activity of the peptides was measured using *E. coli* and the cyanine $diSC_3(5)$ as previously described (Wu et al. 1999). Briefly, the bacteria were centrifuged and washed with buffer (20 mM glucose, 5 mM HEPES, pH 7.3) and resuspended to an OD_{600} of 0.05 in the same buffer. The cell suspension was incubated with 0.4 μM $diSC_3(5)$ until a stable reduction of fluorescence was achieved (around 1 h). Then KCl was added to a final concentration of 0.1 M to equilibrate K^+ levels. The peptides were added to achieve the concentrations in the range of 0.25 to 8 μM. Changes in fluorescence were recorded using an F-4500 fluorescence spectrophotometer (Hitachi, Japan) with an excitation wavelength of 622 nm and an emission wavelength of 670 nm.

Results

Antimicrobial and hemolytic activities of the peptides

As shown in Table 2, the peptides showed antimicrobial activity against tested bacteria. The peptide HFU4 exhibited the strongest antimicrobial activity, with MICs ranging from 1 to 4 μM. Helix-forming unit 3 and helix-forming unit 5 showed moderate antibacterial activity, with MICs ranging from 4 to 16 μM. The shortest peptide, HFU2, had poor antimicrobial activity. Regarding hemolytic activity, HFU2 displayed no hemolysis, even at 256 μM, and HFU3 caused 50 % hemolysis at 101.4 μM. The peptides caused less hemolysis than the control peptide, melittin. The therapeutic index is calculated by the ratio of MHC (the peptide concentration that causes 50 % hemolysis) to MIC. Larger values in the therapeutic index indicate greater cell selectivity. As shown in Table 2, HFU3 exhibited a greater therapeutic index than melittin and offered an optimal selectivity with a therapeutic index of 21.1.

The resistance to salts, enzymes, heat, and pH of the peptides

Helix-forming unit 3 and helix-forming unit 4 are chosen to investigate their resistance to salts, enzymes, heat, and pH. The MICs against *E. coli* after treatment are summarized in Table 3. The MICs of the two peptides were more than 64 μM after treatment with trypsin, caroid, or proteinase K. But pepsin made the peptides retain part of their antimicrobial activity, with a one- to twofold increase for MICs. The antimicrobial activity of the peptides was not affected by 50 or 100 mM NaCl and was slightly decreased when exposed at 150 mM NaCl. After treatment with 1 mM $CaCl_2$ or $MgCl_2$, the peptide HFU3 retained its antimicrobial activity while HFU4 partly lost the antibacterial activity with onefold increase of MICs. The peptides HFU3 and HFU4 exhibited strong thermal stability and antimicrobial activity against *E. coli* remained stable after 1 h of incubation at 100 °C. For the pH stability, colony count showed that the control with dilution of bacteria at respective pH values was grown in parallel. The results revealed that the activity of the peptides was not affected under a wide pH range from 4.0 to 10.0 and the peptides retained their antimicrobial potency.

CD measurements of the peptides

According to the CD spectra, all of the peptides formed random coil structures in sodium phosphate buffer. In the presence of TFE and SDS, the spectra of HFU3, HFU4, and HFU5 displayed showed one positive (∼195 nm) and two negative (∼208 and 220 nm) bands, which represent typically α-helix structures. The secondary structure of the peptides was also estimated from the CD data with the program K2D2 (Perez-Iratxeta and Andrade-Navarro 2008). The predicted results showed that the peptides except HFU2 showed around 35–55 % α-helix in the

Table 2 Antimicrobial and hemolytic activities of the peptides in this study

Peptide	MIC (μM)				GM[a] (μM)	MHC[b] (μM)	TI[c]
	E. coli ATCC 25922	*P. aeruginosa* 27853	*S. aureus* ATCC 29213	*S. epidermidis* ATCC 12228			
HFU2	128	128	128	64	107.6	>256	4.8
HFU3	8	4	4	4	4.8	101.4	21.1
HFU4	4	4	1	2	2.4	19.6	8.2
HFU5	16	16	8	4	9.5	15.0	1.6
Melittin	1	2	1	0.5	1.0	1.9	1.9

[a] The observed geometric mean of the MICs of the peptides against all of the tested bacterial strains

[b] MHC is the minimal hemolytic concentration that caused 50 % hemolysis of human red blood cells (hRBCs)

[c] Therapeutic index is the ratio of the MHC to the geometric mean of the MICs (GM). Larger values indicate greater cell selectivity

Table 3 Effects of salts, pH, enzymes, and heat on antimicrobial activity of the peptides

Treatment	MIC (μM)	
	HFU3	HFU4
Without treatment	8	4
NaCl (50 mM)	8	4
NaCl (100 mM)	8	4
NaCl (150 mM)	16	8
$CaCl_2$ (1 mM)	8	8
$MgCl_2$ (1 mM)	8	8
Pepsin	16	16
Trypsin	>64	>64
Proteinase K	>64	64
Caroid	>64	>64
Heat (100 °C)	8	4
pH 4	8	4
pH 7	8	4
pH 10	8	4

Table 4 α-Helicity of the peptides in various environments

Peptides	Percentage α-helicity[a]		
	Buffer	TFE	SDS
HFU2	1.8	10.5	3.3
HFU3	2.6	38.5	34.6
HFU4	2.1	39.1	33.0
HFU5	3.3	42.6	51.7

The peptides were dissolved in aqueous (10 mM sodium phosphate buffer, pH 7.4) or membrane-mimicking solvent (50 % TFE or 25 mM SDS micelles)

[a] The a-helical content of the peptides was estimated by CD spectroscopy interpreted according to the K2D2 algorithm

presence of TFE or SDS (Table 4). The helical contents of the peptides tend to increase with the extension of chain length. We also used other methods (Deber and Li 1995; Rohl and Baldwin 1998) to estimate the values and similar results were obtained.

Binding of the peptides to model membranes

In Tris–HCl buffer, all of the peptides displayed fluorescence emission maxima around 350 nm, which is typical for Trp in a water-polar environment, suggesting that the tryptophan residues of these peptides are located in a hydrophilic environment. Upon addition of PE/PG or PC/cholesterol vesicles to the solutions containing the peptides, the shift to shorter wavelengths was observed for the fluorescence emission spectra of all the peptides (Table 5). L-α-Phosphatidylethanolamine/L-α-phosphatidyl-DL-glycerol vesicles caused larger blue shifts than PC/cholesterol vesicles, indicating that the Trp side chains of these peptides penetrated into a more hydrophobic environment in negatively charged phospholipids compared with zwitterionic phospholipids. In addition, the GM data of the peptides correlated linearly with the blue shifts in the presence of PE/PG vesicles, with a slope of −21 ($r^2 = 0.98$). Minimum inhibitory concentration values of the two gram-positive strains were purged from the GM values because the *Staphylococcus* membranes have only PG and cardiolipin. These data also fit the MHCs and the blue shifts in the presence of PC/cholesterol vesicles, with a slope of −28 ($r^2 = 0.82$). The lack of hemolytic activity corresponded to a weak ability to penetrate zwitterionic phospholipid membranes, which mimic eukaryotic membranes.

Tryptophan fluorescence quenching by acrylamide

Acrylamide is used as a quenching agent because there are no interactions between the head group of negatively charged phospholipids and acrylamide. The Stern–Volmer quenching constant (K_{SV}) values are presented in Table 5. A decrease in quenching (smaller K_{SV} values) reflects a

Table 5 Tryptophan fluorescence emission maxima of the peptides in Tris buffer, PE/PG (7:3, w/w), or PC/cholesterol (10:1, w/w) vesicles at a molar lipid/peptide ratio of 50:1

Peptide	Fluorescence emission maxima (nm)			K_{SV} (M^{-1})[b]		
	Tris buffer	PE/PG	PC/cholesterol	Tris buffer	PE/PG	PC/cholesterol
HFU2	350	338 (12)[a]	345 (5)	6.5	2.3	3.8
HFU3	350	333 (17)	341 (9)	8.3	1.1	2.4
HFU4	350	332 (18)	339 (11)	16.9	0.8	2.2
HFU5	349	332 (17)	335 (14)	13.1	0.9	1.9
Melittin	351	333 (18)	335 (16)	11.8	0.9	1.7

Smaller K_{SV} values reflect a more protected Trp residue

[a] Blue shift of emission maximum compared with Tris buffer

[b] Stern–Vollmer constants, K_{SV} (M^{-1}), were calculated by the Stern–Vollmer equation: $F_0/F = 1 + K_{SV}(Q)$, where Q is the concentration of the quencher (acrylamide)

more protected Trp residue. The K_{SV} values of the peptides in PE/PG vesicles are larger than those in PC/cholesterol vesicles. Collectively, the Trp residues of the peptides were buried deeper in negatively charged phospholipid vesicles than in zwitterionic phospholipid vesicles.

Outer membrane permeabilization

The ability of the peptides to permeate the outer membrane of *E. coli* was evaluated by the fluorescence-based NPN uptake. N-phenyl-1-naphthylamine is a hydrophobic fluorescent probe that remains quenched in an aqueous environment but fluoresces strongly in a hydrophobic environment. Destabilization of the bacterial outer membrane allows the dye to enter the damaged membrane, where an increase in fluorescence is measured. As shown in Fig. 3, the outer membrane permeabilization of the peptides HFU3, HFU4, and melittin were detected in a dose-dependent manner. Helix-forming unit 3 permeabilized the outer membrane similar with HFU4. However, HFU3 and HFU4 were less effective at disrupting the outer membrane of *E. coli* than melittin. For example, the fluorescence intensity of HFU3 or HFU4 was twofold larger than that of melittin at the concentration of 2 μM.

Inner membrane permeabilization

The cationic dye $diSC_3(5)$ is quenched in the cytoplasmic membrane. When an antimicrobial agent disrupts the transmembrane electrostatic potential, the dye dissociates into the medium and fluoresces strongly. The difference of the cytoplasmic membrane permeability was observed when the peptides were added to the bacteria (Fig. 4). The results showed that that three peptides HFU3, HFU4, and melittin were able to permeabilize the bacterial cell membrane even at the concentrations lower than their MICs. Helix-forming unit 4 was more effective and rapidly

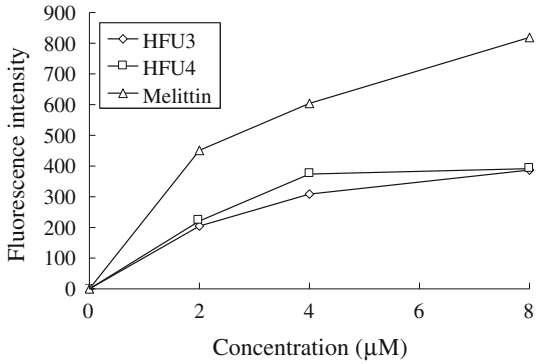

Fig. 3 Uptake of NPN in *E. coli* outer membranes. The ability of peptides to disrupt the outer membrane integrity of bacteria was evaluated with the fluorescent dye N-phenyl-1-naphthylamine (NPN). Changes in fluorescence were measured using an F-4500 fluorescence spectrophotometer at an excitation wavelength of 350 nm and an emission wavelength of 420 nm

at permeabilizing the inner membrane than HFU3 and melittin at the same molar concentrations. The fluorescence intensity rose at first rapidly, then slowly until a certain minimum values were reached. We also tested the inner membrane permeabilization of HFU3 and HFU4 for *S. aureus*. The results showed that HFU3 and HFU4 displayed inner membrane permeabilization in a similar way (data not shown) and HFU4 still displayed the inner membrane permeabilization more effectively and rapidly than HFU3.

Discussion

Many studies have reported biological activities of antimicrobial peptides containing repeated elements (Deslouches et al. 2005; Liu et al. 2007; Niidome et al. 2005). It was reported that antibacterial activity decreased with an increase in the chain length of the peptides, whereas the opposite trend was found for hemolytic activity (Niidome

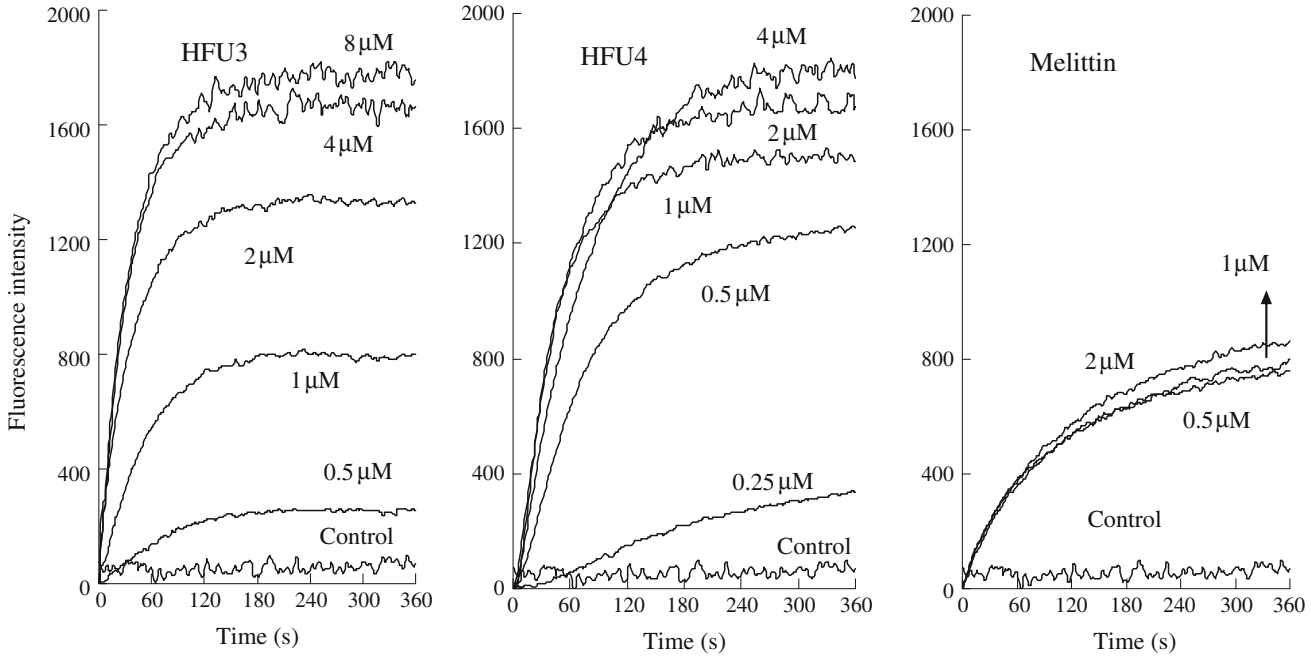

Fig. 4 Cytoplasmic membrane depolarization of *E. coli* by the peptides. The cytoplasmic membrane permeabilization of the peptides was measured using the cyanine $diSC_3(5)$. Changes in fluorescence were recorded with an F-4500 fluorescence spectrophotometer at an excitation wavelength of 622 nm and an emission wavelength of 670 nm

et al. 2005). However, in another study, the antimicrobial activity of the peptides increased with chain length, as did the hemolysis of red blood cells (Liu et al. 2007). In this study, the data can be fit to a power law between the hemolytic activity (MHC) and chain length (n) of the peptides ($\log(\text{MHC}) = \log(b) + a \log(n)$, where a and b are constants, $n = 6$, 9, 12, or 15), with a slope of -4 ($r^2 = 0.97$) (Fig. 5). Considering the intramolecular and intermolecular self-aggregation of longer peptides, we assume that the reduced activity of the longest chain is an artifact. Therefore, log (MHC) was replaced by log (GM) to obtain a linear correlation with a slope of -6 ($r^2 = 0.96$, $n = 6$, 9, or 12). Antimicrobial potency and toxicity to mammalian cells are inclined to increase simultaneously but not synchronously. This suggests that an appropriate chain length will lead to optimal biological activity (HFU3 was found to have optimal selectivity). However, the observed behavior of the peptides may be based on the charge of the molecule because the average net charge of all peptides in the APD is +3.82. This is close to the charge of the HFU3 peptide. Circular dichroism spectroscopy is widely employed to analyze the secondary structure of proteins or peptides because it is extremely sensitive to conformational changes. The peptides displayed α-helix-rich

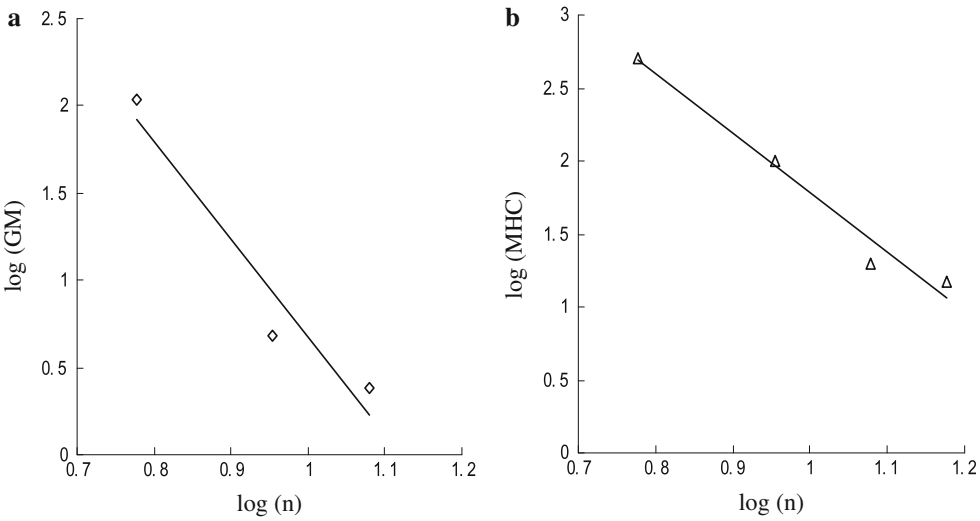

Fig. 5 Correlation between biological activities and chain length of the peptides. **a** The data can be fit to a power law between the antimicrobial activity (GM) and chain length (n) of the peptides ($\log(\text{MHC}) = \log(b) + a \log(n)$, where a and b are constants and $n = 6$, 9, or 12), with a slope of -6 ($r^2 = 0.96$). **b** Log (GM) is replaced by log (MHC) to obtain a linear correlation with a slope of -4 ($r^2 = 0.97$, $n = 6$, 9, 12, or 15)

structures in the presence of membrane-mimicking environments, which agreed with our hypothesis. Biochemical stability is important for peptides that are to be used in industrial manufacturing. For example, the screened peptide HFU3 showed high resistance to heat. Heat stability is potentially a useful characteristic because many procedures such as food or feed processing involve a heating step.

To verify whether the different activities against bacteria and blood cells are associated with selectivity for negatively charged bacterial phospholipids, tryptophan fluorescence blue shift and tryptophan quenching experiments were carried out to compare the membrane-binding affinities of the peptides. Prokaryotic and eukaryotic membranes are organized in different ways (Matsuzaki 1999; Song et al. 2004). The outermost leaflet of the membranes of bacteria is composed principally of lipids with negatively charged phospholipid headgroups. However, the outer leaflet of the eukaryotic membranes is heavily populated by lipids with zwitterionic headgroups while most of the lipids with negatively charged headgroups are located in the inner leaflet (Shai 2002). Cationicity is important for the initial electrostatic attraction of antimicrobial peptides to negatively charged phospholipid membranes of bacteria and other microorganisms (Yeaman and Yount 2003). In the present study, the peptides integrated more effectively into PE/PG vesicles (a phospholipid composition typical of negatively charged bacterial membranes) than into PC/cholesterol vesicles (a phospholipid composition used to mimic the outside of neutral eukaryotic membranes), which was in accordance with the observed data for the biological activity of the peptides. Collectively, the bacterial cell selectivity of the peptides was due to their high binding affinity to the negatively charged phospholipids in bacteria.

After the interaction of the peptides with model membranes, we further investigated the permeabilization of the peptides with whole bacteria. The NPN uptake assay showed the permeabilizing effects of peptides on the bacterial outer membrane, and the $diSC_3(5)$ release assay showed the ability of the peptides to disrupt the membrane potential across the inner cytoplasmic membrane. Melittin was more effective than HFU3 and HFU4 in outer membrane permeabilization while the cyanine dye fluorescence intensity is higher in the inner membrane when HFU3 and HFU4 was assayed than observed for melittin at the same molar concentrations. However, MIC against *E. coli* for melittin is eight times lower than for HFU3 and four times for HFU 4. At their respective MICs, HFU3 showed similar outer membrane permeabilization with melittin while the fluorescence intensity of HFU3 and HFU4 was significantly greater than that of melittin in the inner membrane permeabilization. Different antimicrobial activity between HFU3 and HFU4 may be correlated with the inner membrane permeabilization of the peptides. These results suggest that the difference in antibacterial activity between HFU3 and melittin is probably due to difficulties in permeabilizing the bacterial outer membrane, and not the inner membrane.

The mechanism behind the observed activity of the peptides is not clear. Membrane disruption is believed to occur either via a detergent-like carpet mechanism (Shai 2002) or through the formation of discrete pores (Huang 2000). Although the membrane permeabilization assay reflects in part the movement of peptides across the outer membrane to access the inner membrane (Falla and Hancock 1997), it seems reasonable to assume that the peptides attack bacteria via membrane disruption rather than by end-to-end pore assembly because of their short lengths (Liu et al. 2007). In the carpet-like model (Huang 2000; Sato and Feix 2006), it is not necessary for peptides to adopt an amphipathic α-helical structure and insert into the hydrophobic core of the membrane.

In summary, we successfully designed antimicrobial peptides by assembling HFUs composed of Ile, Arg, and Phe residues. It was shown that biological activity generally increased with the chain length of the peptides, and HFU3 exhibited the optimal selectivity for bacteria over blood cells. The peptides infiltrated more deeply into negatively charged phospholipids than zwitterionic phospholipids.

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