Research Article

Journal of PeptideScience

Received: 26 May 2009 Revised: 16 September 2009 Accepted: 16 September 2009 Published online in Wiley Interscience: 30 November 2009

(www.interscience.com) DOI 10.1002/psc.1195

Novel antimicrobial peptides identified from an endoparasitic wasp cDNA library

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We screened an endoparasitic wasp (*Pteromalus puparum*) cDNA library for DNA sequences having antimicrobial activity using a vital dye exclusion assay. Two dozens of clones were isolated that inhibited the growth of host *Escherichia coli* cells due to expression of the cloned genes. Three peptides (PP13, PP102 and PP113) were synthesized chemically based on the amino acid sequences deduced from these clones and assayed for their antimicrobial activity. These peptides have net positive charges and are active against both Gram-negative and -positive bacteria, but are not active against fungi tested. Their hemolytic activity on human red blood cells was measured, and no hemolytic activity was observed after 1-h incubation at a concentration of 62.5 μM or below. A Blast search indicated that the three peptides have not been previously characterized as antimicrobial peptides (AMPs). Salt-dependency studies revealed that the biocidal activity of these peptides against *E. coli* decreased with increasing concentration of NaCl. Transmission electron microscopic (TEM) examination of PP13-treated *E. coli* cells showed extensive damage of cell membranes. The CD spectroscopy studies noted that the enhanced α-helical characteristics of PP13 strongly contribute to its higher antimicrobial properties. These results demonstrate the feasibility to identify novel AMPs by screening the expressional cDNA library.

Keywords: antimicrobial peptide; cDNA library; insect defense; endoparasitic wasp; *P. puparum*

Introduction

Bacterial resistance to existing drugs due to widespread use of antibiotics and other pharmaceutical agents has increased dramatically in the past 20 years and has become an increasingly serious global public health problem [1–3]. To alleviate this problem, a new generation of antibiotics and other antimicrobials should be developed to provide new tools for multitherapy treatments. Antimicrobial peptides (AMPs) have been one of the subjects of focus of the investigation in the past decades in an attempt to develop novel classes of antibiotics. AMPs function through interaction with cell membranes of microbes, and hence it is difficult for microbes to develop resistance via mutations [4–7]. AMPs play an important role in innate immune systems and host defense mechanisms. In the last 20 years, thousands of AMPs have been isolated from a wide range of species, including plants, insects, amphibians and mammals [8–12]. It is commonly accepted that AMPs are mostly relatively of low molecular weight (typically, 20–40 amino acid residues), often membrane-bound, usually cationic and are extremely effective to broad-spectrum pathogens such as the Gram-positive bacteria, Gram-negative bacteria and fungi. Their important advantages are as follows: selectivity, fast killing, broad antimicrobial spectrum and no resistance development [13]. These unique features have encouraged many researchers to devote their efforts to discover new classes of AMPs and their mimics from different kinds of organisms as novel antimicrobial therapeutic over the last two decades [14–16].

Insects lack the adaptive immune system endowed with memory. They rely solely on their multilayered innate immune defenses to fight against a mass of microorganisms of various origins in their environment. The rapid synthesis of various peptides with antimicrobial actions is one of the most important weapons possessed by insects as the first line of defense against invading microbes [17–19]. Numerous AMPs from insect sources produced in the fat body, various epithelia and certain hemocytes [20–22] have been identified, characterized and their coding genes cloned. They are rapidly released into hemolymph to fight against a broad spectrum of microbes, including bacteria, fungi and even certain viruses, playing a central role in the humoral defense response. Since Boman and coworkers isolated two families of antibacterial peptides from immunized pupae of the moth *Hyalophora cecropia* in 1981 [23], more than 200 AMPs have been identified in insects to date (http://www.bbcm.univ.trieste.it/~tossi/amsdb.html). These insects belong to different orders including Lepidoptera, Diptera, Coleoptera, Hymenoptera, Hemiptera, Trichoptera and Ondonata [24]. In Hymenoptera, AMPs have been reported in stinging species such as honeybees, wasps, bumble bees and ants [25–27], but not in endoparasitic wasps, although antibacterial activities have been found in the venom from a solitary endoparasitic wasp, *Pimpla hypochondriaca* (Hyemoptera: Ichneumonidae) [28].

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Pteromalus puparum (Hymenoptera: Pteromalidae) is a gregarious endoparasitic wasp, which parasitizes in the pupae of *Pieris rapae* (cabbageworm). Our previous studies on the interaction between *P. puparum* and *P. rapae* have suggested that the venom of *P. puparum* may contain biological actives [29–31]. In this study, we have characterized several antibacterial peptides from *P. puparum* previously isolated using an expressional cDNA library screening method [32,33]. These peptides are found to be active against Gram-negative and Gram-positive bacteria without noticeable hemolytic activity.

Materials and Methods

Screening of *P. puparum* cDNA Library

Screening of the wasp library has been described elsewhere [32,33]. In brief, a cDNA library was constructed in Lambda ZAP II vector (Stratagene, CA). The library was plated out in a noninductive medium to allow the formation of bacterial colonies and then transferred to an inductive medium to induce the expression of cloned genes. Colonies that had been toxicated by the expressed genes were detected by a vital dye-staining method.

Antibacterial Activity against *E. coli*

A liquid growth inhibition assay was used to determine the antibacterial activities of the stained colonies. Ten-microliter aliquots of cell suspension were inoculated into 5 ml of Luria-Bertani (LB) ampicillin medium (0.5% yeast extract, 1% peptone, 1% NaCl, 100 μg/ml ampicillin) with and without an inducer (1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG)). After 10-h incubation at 37 °C, the absorbance (A_{600}) readings were taken with a spectrometer as an index of cell growth.

Peptide Synthesis

Twenty milligrams of peptides were commercially synthesized by solid-phase methods at the Shanghai Bootech BioScience and Technology Company, using N-9 FMOC chemistry with Fmoc-Ile-Wang resin (STAM, USA) on a 431A Peptide Synthesizer (Applied Biosystems). The peptides were purified by HPLC to 90% purity for use in this study.

MIC Determination

The MIC of the synthetic peptides were determined against Gram-negative bacterium and Gram-positive bacterium according to modified methods described previously [34]. The microorganisms *Staphylococcus aureus* (CVCC1882), *E. coli* (CVCC1570), *Sarcina lutea* (CVCC1600), *Bacillus pumilus* (CVCC709) and *Bacillus subtilis* (CVCC717) were purchased from China Institute of Veterinary Drug Control. The assays were carried out in sterile 96-well titer plates at a final volume of 100 μl. The inocula were preincubated in a nutrient broth (NB, Difco) at 37 °C to a midlog phase (about 12 h), harvested and then adjusted to a concentration of 10^4 colony-forming units (cfu)/ml in NB. Eighty microliters of the bacterium suspensions was incubated with 20 μl of NB containing the peptides at final concentration between 1 and 1000 μM. The control contained all the components except the peptide. The bacterial culture was incubated for 24 h at 37 °C and the absorbance was measured at 600 nm. The MICs were determined, in which 100% inhibition of growth was observed, and the final results were obtained as the average of three independent experiments carried out in duplicate.

To determine if the peptides have activity on fungus, agar diffusion assay was conducted using *Botrytis cinerea* (an important pathogen of many crop diseases) as testing strain. Ten milliliters of PDA (5% potato extract, 0.5% dextrose, 1.7% agar) medium was poured into a 9-cm petri dish. After solidification, a hole with the diameter of 0.6 cm was punched in the center of the plate and 10-μl aliquots of peptides at different concentrations (1, 10, 100, 1000 μM) were added to the hole. Fresh mycelium of *B. cinerea* was inoculated symmetrically on both sides of the hole. The plates were cultured at 28 °C for 24–72 h and observed for fungal growth.

Hemolytic Assay

The hemolytic activities of the three peptides were determined using freshly prepared human red blood cells. The human red blood cells were prepared from freshly collected human blood by centrifugation at 1500 rpm for 10 min at 4 °C. The cells were washed three times (3000 rpm) with a cold solution of 0.15 M, pH 7.2 phosphate buffered saline (PBS) and diluted to a final concentration of 0.5% in PBS. To each well of a polypropylene microtiter plate, 75 μl of the diluted erythrocytes and 75 μl of peptide solution (250, 125, 62.5, 31.2, 15.6, 7.8, 3.9 and 1.95 μM) were added. The plates were incubated for 1 h at 37 °C, and centrifuged for 10 min at 4000 rpm. Sixty-microliter aliquots of the supernatant were transferred to 96-well plates. Hemolysis was measured by absorbance at 414 nm with an ELISA plate reader. Zero percent and 100% hemolysis were determined in PBS and 0.1% Triton X-100, respectively. The hemolysis percentage was calculated as follows: $[(A_{peptide} - A_{PBS})/(A_{Triton} - A_{PBS})] \times 100$. All hemolysis determinations were performed in duplicate and are the average of three independent determinations using the same stock solution [35].

Salt-dependent Test

E. coli cells were preincubated in LB broth to a midlog phase and diluted to 10^6 colony-forming units (cfu)/ml in M9 medium for use in salt-dependency studies. To determine the effect of salt on antibacterial activity of the peptides, the diluted bacterial culture was aliquoted into 96-well plates and incubated with the peptides at 50% MIC in an M9 medium containing different concentrations of NaCl. The bacterial culture was incubated for 12 h at 37 °C and the absorbance was measured at 600 nm. All assays were performed in triplicate.

Transmission Electron Microscopy (TEM)

E. coli cells were incubated with peptide PP13 at MIC in an LB medium at 37 °C for 2 h and harvested by centrifugation at 1 500 × *g* for 3 min. Negative control was run in the absence of peptide. The pellet was first fixed with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, for 12 h at 4 °C and was washed three times with 0.1 M phosphate buffer and postfixed with 1% (v/v) osmium tetroxide in 0.1 M phosphate buffer for 2 h. After washing three times with the phosphate buffer, the fixed samples were dehydrated through a graded series of ethanol gradients (50, 70, 80, 90, 95 and 100%) for about 15–20 min at each step, and then transferred to absolute acetone for 20 min. Dehydrated samples were suspended in absolute acetone and Spurr resin 1 : 1 (v/v) mixture for 1 h and 1 : 3 mixture for 3 h at room temperature. After suspension in Spurr resin overnight, the

samples were embedded in pure Spurr resin and dried at 70 °C for 9 h. Sections were obtained using a Reichert Mltracut E Microtome and stained with 2% (v/v) uranyl acetate and alkaline lead citrated for 15 min. Sections were observed in TEM of Model JEM-1230 [36].

Circular Dichroism (CD)

The CD spectra were recorded at room temperature on a Jasco J-815 spectropolarimeter (Tokyo, Japan) using a quartz cell of 1 mm path length. Spectra were obtained from 260 to 190 nm by averaging two scans at a scan speed of 10 nm/min. The concentration of the peptides was 10^{-4} M in 10 mM sodium phosphate buffer (pH 7.4). Experiments were also performed in 50% trifluoroethanol (TFE, v/v, Fluka) and 25 mM SDS, Sigma. CD spectra were reported in terms of ellipticity units per mole and the α-helicity was calculated from the mean residue ellipticity at 222 nm [37,38].

Results and Discussion

Screening of Wasp cDNA Library for Antimicrobial DNA Sequences

In an effort to identify AMPs in wasps, we followed a strategy that was different from traditional biochemistry approaches, where individual peptides/proteins are isolated from various living cells or tissues, purified and assayed for their antimicrobial activity. Instead, we constructed a cDNA expression library using mRNA from the wasp, and screened the cDNA library for DNA sequences having antimicrobial activity. Expression of cDNA library in *E. coli* cells results in killing or impairing of the host cells if the expressed products are toxic to the cells. Reversely, identifying cells that have been toxicated due to expression of the cDNA leads to isolation of the cDNA potentially coding for an antimicrobial protein or peptide. We used a vital dye-staining method to identify *E. coli* colonies derived from the cDNA library that had been toxicated, and isolated several dozens of clones that may contain antimicrobial genes [32]. Three clones were selected for further investigation in this study. The growth of the *E. coli* cells containing the clones reduced from 33.6 to 77.8% when the expression of the cloned genes were induced by IPTG; meanwhile, no significant change was observed on the growth rate for *E. coli* cells harboring a clone that was unstained (Table 1). Plating of the cells from the selected clones gave rise to smaller and blue colonies after culture on an IPTG-supplemented medium when staining with the vital dye (Figure 1). The results indicated that the cDNA sequences in these colonies may code for products that are inhibitory or lethal to the host cells.

Table 1. Comparison of cell growth in noninductive and inductive medium[a]

Colony ID	Noninductive	Inductive	Growth reduction (%)
Control	1.079	1.013	6.11
PP13	0.916	0.495	46.0
PP102	0.642	0.175	77.8
PP113	0.633	0.184	70.9

[a] 10-µl aliquots of *E. coli* cells were inoculated in 5 ml of LB medium (0.5% yeast extract, 1% peptone, 1% NaCl, 100 µg/ml ampicillin) with and without inducer (1 mM IPTG). The A_{600} was measured after 10-h incubation as an index of cell growth.

DNA and Peptide Analysis

Sequence analysis of the cDNA inserts showed that the three clones (PP13, GenBank accession no. **EF444540.1**; PP102, GenBank accession **EF444542.1** and PP113, GenBank accession **EF444543.1**) are fusions between fragment of the β-glucosidase on the vector and short cDNA segments. Once translated, these cDNA segments can code for peptides consisting of 22 to 43 amino acids (Table 2). These peptides are all cationic with net charge between three and six, similar to a class of peptides discovered to have a broad spectrum of antimicrobial activity [11]. They have high isoelectric points (IP) ranging from 10 to 12, and have similarly high ratio of hydrophobic amino acid residues (Table 2). At the *C*-terminal, PP113 is rich with glutamine and arginine, while PP102 has a serine-rich *N*-terminal. Arginine rich peptides have been previously reported to have antimicrobial activity [39,40].

A search of public databases indicated that the peptides have no homolog to previously characterized protein or peptide sequences, indicating that they are novel peptides. At the genomic level, *P. puparum* is a minimally characterized species. It is therefore expected that these peptide sequences will not appear during the Blast search. Furthermore, as these peptides are deduced from the

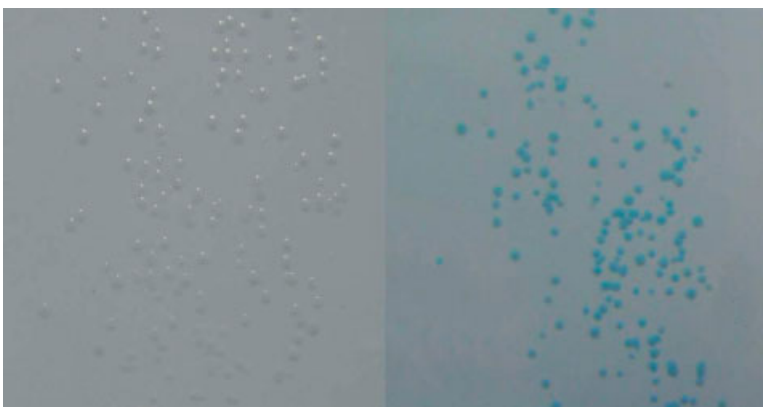

Figure 1. IPTG-dependent cell-staining reaction to vital dye. *E. coli* cells of isolated clones from the cDNA library screening were spread onto membrane filters, and grown on a noninductive medium to form visible colonies. The filters were further cultured on medium without (left) and with (right) 1 mM inducer IPTG for additional 3–5 h at 37 °C and stained with trypan. Culture in IPTG medium induced expression of cloned genes and thus resulted in increased cell membrane permeability as visualized by dye staining.

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Table 2. Amino acid sequences, secondary structures and physical–chemical properties of the three peptides

Peptide	Amino acid sequence	Net charge[a]	Hydrophobic ratio[a] (%)	IP[a]
PP13	GAARKSIRLHRLYTWKATIYTR	+6	78	12
PP102	GSCSCSGTISPYGLRTCRATKTKPSHPTTKETHPQTLPT	+4	69	10
PP113	GKWGWIYITILFADVGGFKSSRHPEERRVQERRFKRITRGPD	+5	57	11

[a] Net charge, hydrophobic ratio and IP were calculated from Innovagen peptide property calculator at http://www.innovagen.se/custom-peptide-synthesis/peptide-property-calculator/peptide-property-calculator.asp.

short cDNA sequences fused to the vector, it is not clear if the peptides are part of native proteins, or artificial products produced as a result of cDNA library construction. Further investigation will be required to determine if these peptides are derived from the native wasp proteins and present within the wasp cells naturally. Nevertheless, their antimicrobial activity is worth further investigation as novel AMPs.

Antimicrobial Activity

The clones were identified due to the toxicity of their expressed products inside the host *E. coli* cells. It is therefore interesting to know if these peptides will exhibit antimicrobial activity when applied from outside the cells, as other AMPs do. They were synthesized chemically and tested against both Gram-negative and -positive bacteria (Table 3). The three peptides exhibited various potencies of antibacterial activities against both Gram-negative and Gram-positive bacteria, with MIC ranging from 8.0 μM for PP13 against *S. lutea* to 166.7 μM for PP102 against *E. coli*. PP13 showed a potent antimicrobial activity against all the bacteria tested. PP102 and PP113 demonstrated a strong antimicrobial activity against Gram-positive bacteria, but were not active against Gram-negative bacteria such as *E. coli*. All the peptides were inactive to the fungus *B. cinerea* (data not shown) in agar diffusion assay. These results clearly demonstrate that the three peptides have *in vitro* antibacterial activity, and are likely responsible for the *E.coli* inhibitory effect initially observed in the cDNA library screening.

Hemolytic Assay

Some AMPs exhibit hemolytic activities [41]. To assess the cytotoxicity of the three peptides against mammalian cells, the percentage of hemolysis was measured against human red blood cells at various concentrations (0–250 μM). After 1 h of coincubation, a low level of hemolytic activity was observed at peptide concentrations higher than 125 μM. No hemolytic activity was observed at 62.5 μM (Table 4) or below – a concentration above MIC in most of the bacteria tested. The results indicate that these peptides are relatively safe to mammalian cells.

Table 3. The minimal inhibitory concentration of the peptides against Gram-negative and -positive bacteria

Peptide	Minimal inhibitory concentration (μM)				
	E. coli	*B. subtilis*	*S. aureus*	*S. lutea*	*B. pumilu*
PP13	16.7	13.3	23.3	8.0	9.0
PP102	n.d.	25	13.3	63.3	23.3
PP113	73.3	23.3	13.0	16.7	23.3

The bacteria were preincubated to the midlogarithmic phase in nutrient broth (NB, Difco) and adjusted to 10^4 colony-forming units (cfu)/ml. 80 μl of bacterial suspension was incubated with 20 μl of NB containing different concentration synthetic peptides. The absorbance was measured at 600 nm after 24-h incubation at 37° as an indicator of cell growth. The results were determined as the mean value of three independent experiments carried out in duplicate. n.d. means not detected.

Table 4. Hemolytic activity of the three peptides on human red blood cells

Peptides	Percentage hemolysis (μM)							
	250	125	62.5	31.2	15.6	7.80	3.90	1.95
PP13	8.2	5.9	0	0	0	0	0	0
PP102	10.6	8.5	0	0	0	0	0	0
PP113	9.4	6.8	0	0	0	0	0	0
PBS	0	0	0	0	0	0	0	0
Triton X-100	100	100	100	100	100	100	100	100

The hemolytic activities of the three peptides were determined using human red blood cells. The cells were incubated with peptide for 1 h at 37 °C and measured by absorbance at 414 nm with an ELISA plate reader. Zero percent and 100% hemolysis were determined in PBS and 0.1% Triton X-100, respectively. The hemolysis percentage was calculated as follows: $[(A_{peptide} - A_{PBS})/(A_{Triton} - A_{PBS})] \times 100$. All hemolysis determinations were performed in duplicate and are the average of three independent determinations using the same stock solution.

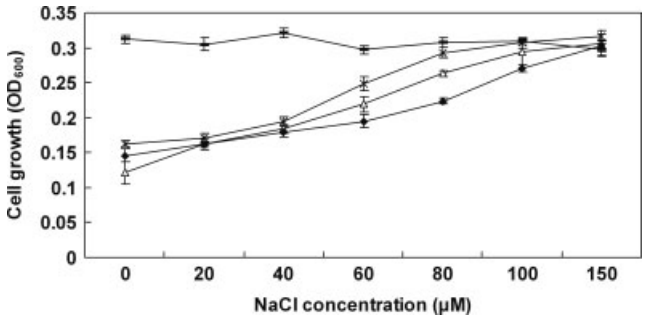

Figure 2. Effects of NaCl on antimicrobial activity of peptides against *E. coli* cells. Midlog phase bacteria (2 × 106 cfu/ml) were incubated with peptides at their 50% MIC in M9 medium containing different concentrations of NaCl. The bacterial culture was incubated for 12 h at 37 °C and the absorbance was measured at 600 nm. BSA (–), PP13(♦), PP102(×), PP113(△).

Salt dependency

Most AMPs have a common undesirable property that their antimicrobial activity is salt dependent. That is, the bactericidal

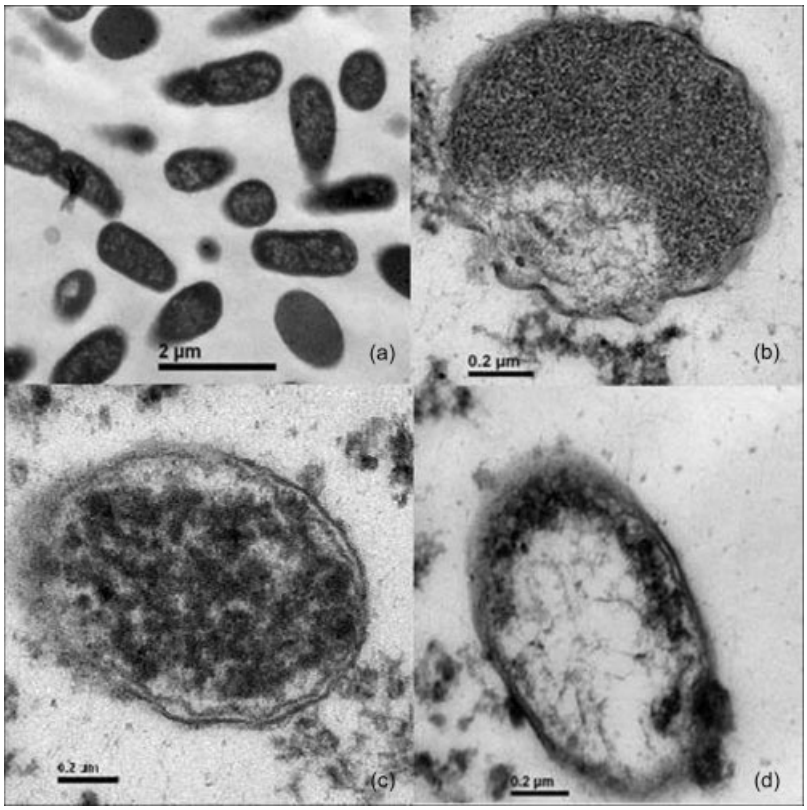

Figure 3. Morphological changes in *E. coli* caused by PP13 visualized by TEM. (a) *E. coli* control, (b, c, d) bacterial cells incubated with PP13 at MIC.

activities are often strongly reduced or ablated in the presence of physiological concentrations of ions such as Na^+ and Mg^{2+} [42]. The effects of NaCl on the antibacterial activities of the three peptides were studied and are shown in Figure 2. Like many other AMPs [7], the bactericidal activity of the three peptides against *E. coli* decreased with increasing concentration of NaCl. PP102 lost its activity against *E. coli* at 80 mM. At 100 mM, the activity of PP113 decreased considerably. The activity of PP13 against *E. coli* was almost completely lost in the presence of 150 mM NaCl (Figure 2). This salt-dependent bactericidal activity might be explained by assuming that the high ionic strength weakened the initial electrostatic interactions between the three peptides and bacterial targets [7]. The higher net positive charge in PP13 as compared to other peptides may have a role in modulating antimicrobial activity in the presence of NaCl [36].

Transmission Electron Microscopy

The significant advantage of AMPs resides in the common mechanism of their action, which is remarkably different from that of conventional antibiotics. It is generally accepted that the inhibition effect of AMPs on pathogens relies the most on their interaction with membranes of microorganisms, often resulting in pore formation and/or cell lysis [22]. To investigate the effect of the newly identified peptides on cell membrane, we examined the membrane integrity of *E. coli* cells after treatment with peptide PP13 at MIC. Transmission electron microphotographs of the PP13-treated cells shown in Figure 3 indicate that PP13 treatment caused remarkable cell structural changes and extensive damage of cell membranes. Compared to the untreated *E. coli* cells (a), PP13-treated cells show abnormal morphologies, such as cell rapture and leakage of cell contents. Although detailed time course of the events in cell damage remains unknown, the observations are in line with previous microscopical studies of AMP's effect on cells [43], and indicate that the bacterial cell membrane is a target of PP13.

Conformational Study by CD

To investigate the secondary structure of the linear antibacterial peptides in lipid membranes, which are assumed to correlate well with their antimicrobial activity, we used TFE/H_2O mixture and SDS, which are composed of an aliphatic tail and a negatively charged head group to mimic the amphiphilic environment of the lipid membrane. CD experiments on the three peptides were performed in 100 μM phosphate buffer (pH 7.4), as well as in 50% TFE and 25 mM SDS. In Figure 4A, the CD spectra of the three peptides in phosphate buffer are shown. All the peptides adopted a random conformation as a major structure in the phosphate buffer.

Compared to the phosphate buffer, TFE favors the formation of an α-helical structure. As shown in Figure 4B, the CD spectra of PP13 exhibited a positive band at 193 nm and two negative bands at 207 and 217 nm, which indicate that PP13 must adopt a well-defined α-helical structure in the presence of 50% TFE. Under the same condition, the CD spectra of PP113 showed double minimum bands at 206 and 219 nm, indicating that they both adopt helical structures in the presence of 50% TFE. However, the CD spectra of PP102 showed a slight shift. According to the α-helicity calculated from the molar ellipticity at 222 nm, the percentage of helical content of PP102 was 53.5%. All the peptides had considerable α-helicity in the presence of 50% TFE.

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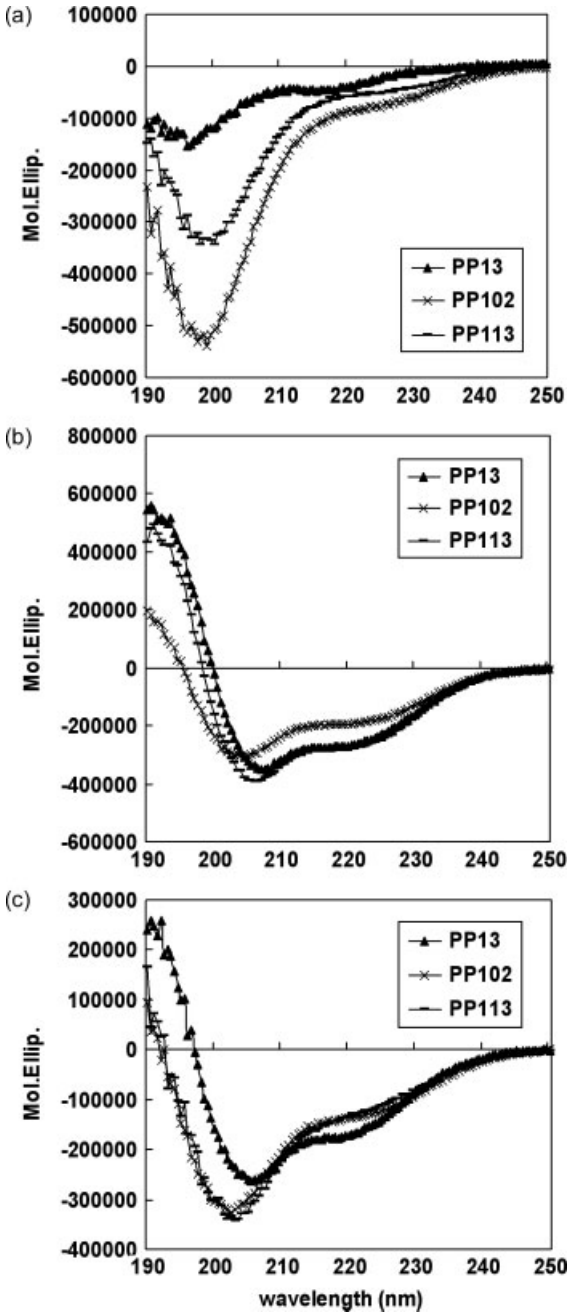

Figure 4. Circular dichroism of peptides in phosphate buffer, TFE and SDS. CD spectra were measured at the concentration of sample (100 μM) in 10 mM sodium phosphate buffer (pH 7.4) (A) including (B) TFE/H2O (50/50, v/v) or (C) 25 mM SDS.

The effect of SDS on the conformation of peptides was also investigated. As shown in Figure 4C, all the peptides had a slightly different secondary structure. The CD spectrum of PP13 measured in the presence of SDS was similar to that of PP13 measured in the presence of TFE; PP13 adopted an α-helical structure in the presence of either TFE or SDS micelles. PP102 and PP113 adopted a random conformation as a major structure in the presence of SDS micelles. The different conformation that the same peptide adopted in phosphate buffer (pH 7.4), 50% TFE and 25 mM SDS might depend on the individual helix propensity of the residues and on the environment.

Conclusions

Our screening of an expressional cDNA library from *P. puparum* resulted in the isolation of a number of cDNA clones, whose sequences code for products that are toxic to host *E.coli* cells. Three clones that can be translated to novel peptides were investigated in the present study. With the synthetic peptides, we have demonstrated that these peptides can inhibit the growth of both Gram-negative and -positive bacteria, with different potency. They likely attack bacterial cell membranes to cause cell death, as observed in TEM studies. The potency of the peptides decreased as the concentration of salts increased, which is often observed in other AMPs. CD spectroscopy indicated that the α-helicity of the

peptides increased in membrane-mimic environments. Their low hemolytic activity would allow the use of these peptides as novel antibiotics in drug development.

Acknowledgements

This work was supported by program for 973 Program (National Basic Research and Development Program of China, Grant No. 2006CB102005), NSFC(National Nature Science Foundation of China, Grant No. 30971959), Zhejiang Provincial Natural Science Foundation of China (Grant No. Z3090191), and the Innovation Research Team Program of the Ministry of Education of China (Grant No. IRT0535).

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