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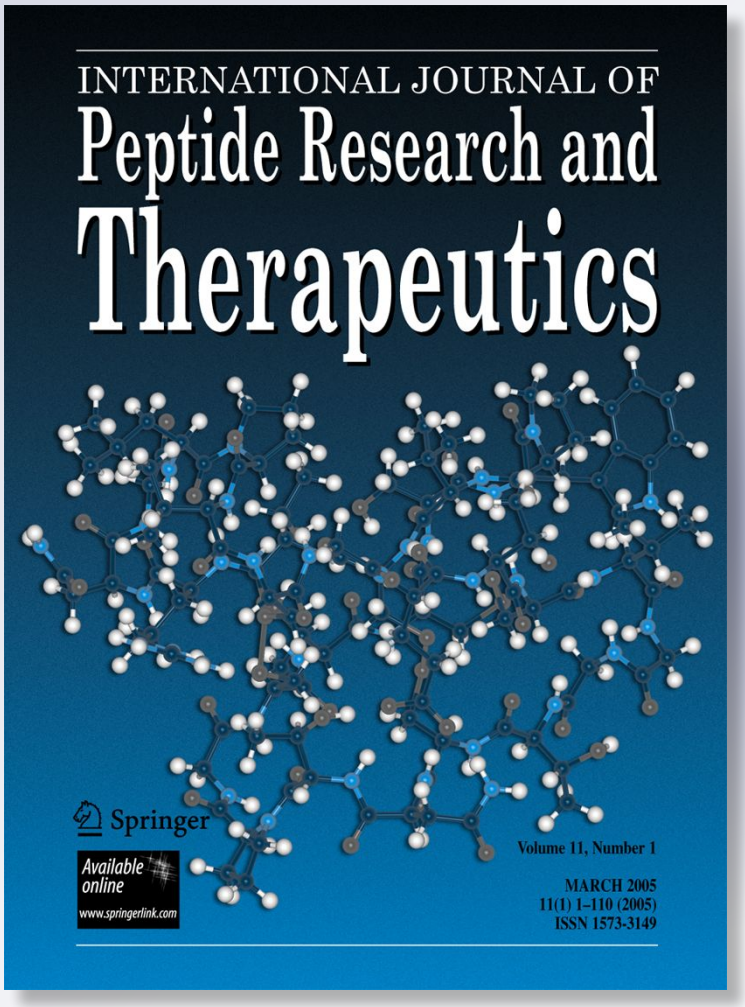

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Identification and Characterization of Novel Antibacterial Peptides from Skin Secretions of *Euphlyctis cyanophlyctis*

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Abstract In this study, we extracted and purified antimicrobial peptides (AMPs) secreted from skin of *Euphlyctis cyanophlyctis* using reverse phase-high performance liquid chromatography. Three AMPs were isolated from skin secretions of this frog and sequenced using tandem mass spectrometry. The purified peptides were named buforin-EC (1875.05 ± 0.5 Da), cyanophlyctin (2347.50 ± 0.5 Da) and temporin-ECa (1013.33 ± 0.5 Da). Multiple alignments and homology search showed that buforin-EC, cyanophlyctin and temporin-ECa had a homology of 71.43, 47.1, and 69.23% to buforin II, brevinin-2EC, and temporin-1CSc, respectively. Antimicrobial tests demonstrated that our peptides have a great antimicrobial effect on both gram-positive and gram-negative bacteria. The results indicated that they have an overall minimum inhibitory concentration (MIC) below 13 μM against *E. coli*. No hemolysis was observed in around of their MIC values. In conclusion, skin secretions of *E. cyanophlyctis* contain a novel class of AMPs with the proper characteristics.

Keywords Antimicrobial peptides · *Euphlyctis cyanophlyctis* · Hemolysis · Minimum inhibitory concentration (MIC) · RP-HPLC

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Introduction

Nowadays, the most important problem dealing with most of the infectious disease is that they are going to become resistant to the most of the conventional antibiotics in many patients. So it is necessary to find novel antibiotics of high potency and lower risk of resistance against them (Kückelhaus et al. 2007). It seems that the natural therapeutic antimicrobial peptides (AMPs) could be considered as a new substituent for antibiotics at the next decades (Gordon et al. 2005; Dashper et al. 2007).

For many years, people in different cultures were using therapeutic effects of amphibian skin to cure any inflammation or lesion and even scorch (Jin et al. 2009; Ostorhazi et al. 2010). The AMPs are found among all classes of life (Wiedow et al. 1998; Vizioli and Salzet 2002; Wessely-Szponder et al. 2010; Shamova et al. 2009). During the past two decades, AMPs from different species of amphibians have been discovered (Barra and Simmaco 1995; Bevins and Zasloff 1990; Giacometti et al. 2005). AMPs are major parts of the immune system which defend the body against bacteria, pathogenic organisms and viruses (Bevins and Zasloff 1990; Albiol Matanic and Castilla 2004; Zhang et al. 2008; Falco et al. 2009). Some studies have disclosed the selective anticancer effects of AMPs on eukaryotic cells (Pilch et al. 2006; Ghavami et al. 2008).

Previously, we have reported the characterization of new peptides isolated from secretions of the marsh frog (*Rana ridibunda*) found in the northern regions of Iran (Ghavami et al. 2008; Mehrnejad et al. 2008; Asoodeh et al. 2011). The aim of this study is to isolate and characterize AMPs, from skin secretions of *Euphlyctis cyanophlyctis*, founds in different parts of Iran to identify peptides with new activity.

Materials and Methods

Chemicals

Acetonitrile, trifluoroacetic acid HPLC grade, methanol, formaldehyde, acetic acid, and ethanol were obtained commercially from Merck KGaA (Darmstadt, Germany). Trypticase soy broth (TSB), Mueller–Hinton Broth (MHB), blood agar media cultures were purchased from Himedia Laboratories (Mumbai, India). Ultrafiltration membranes with 1 and 10 kDa cut-off were procured from Millipore (Bedford, MA, USA). C_8 and C_{18} semi-preparative columns were purchased from Macherey-Nagel GmbH Co. (Düren, Germany). All other chemicals used, including agarose, Triton X-100, EDTA, Coomassie Brilliant Blue R-250, were of analytical grade.

Sample Preparation

A total of 30 specimens, including female and male of *E. cyanophlyctis*, were collected from the Baluchistan province of Iran and kept at the animal's house of Ferdowsi University of Mashhad. This kind of frog is mostly found in southeast of Iran and areas in Pakistan and India (Duellman 1999). To obtain the frog skin secretions, the animals were washed with distilled water. The dorsal part of the frog was then stimulated several times with a 4–6 V electrical current. The extracted colloid on skin was washed with 5% acetic acid solution. The extracts were centrifuged at 10,000 rpm for 15 min. The extracted sample was passed through an ultrafiltration membrane with cut-off of 10 kDa and the filtered solution was concentrated using a 1 kDa membrane. Samples were dried by freeze-drier to keep them safe and use easily in the subsequent processes of purification and characterization.

RP-HPLC

After the concentration of the sample by ultrafiltration, they used to be fractionated by reverse phase-high performance liquid chromatography (RP-HPLC). The columns that used were C_8 and C_{18} RP-HPLC. For this purpose, 120 mg of the lyophilized ultrafiltrated sample was dissolved in 5 ml of 0.1% (v/v) trifluoroacetic acid (TFA) in water (solution A) and 0.4 ml of the sample was loaded onto a C_8 column (10 × 250 mm) in each run. Elution was performed using the solution A (0.1% TFA in water) combined with a 10–70% increasing gradient of solution B (0.098% TFA in acetonitrile) over a period of 60 min, at a flow rate of 2 ml/min. According to the absorbance at 220 nm, the fractions were collected and lyophilized. To purify the fractions, the reversed phase-HPLC on C_{18} column (10 × 250 mm) was done according to the method, which was described above for C_8 column.

Peptide Sequencing

Among all the fractionated peaks, those which were enough in amounts, were subjected to sequence. Peptide sequencing was carried out using mass spectrometry in positive ionization mode on a MALDI-TOF/TOF instrument (Bruker ultraflex III at York University, UK). Purified and lyophilized peptides were reconstituted with 10 µl of 0.1% trifluoroacetic acid (v:v). A 1 µl aliquot of each peptide solution was applied directly to a ground steel MALDI target plate, followed immediately by an equal volume of a freshly prepared 5 mg/ml solution of 4-hydroxy-α-cyano-cinnamic acid (Sigma) in 50% aqueous (v:v) acetonitrile containing 0.1% trifluoroacetic acid (v:v). Bruker flex Analysis software (version 3.3) was used to perform the spectral processing and peak list generation for both the MS and MS/MS spectra. De novo sequencing was performed by hand, allowing for a maximum mass error of 0.5 Da for any given fragmentation ion. Deduced b- and y-ion series were overlaid onto their fragmentation spectra using the Bruker flex Analysis software (version 3.3).

Phylogenetic Analysis

Fourteen peptides of different species were obtained from the antimicrobial peptide database (http://aps.unmc.edu/AP/main.php). These sequences were aligned with the *cyanophlyctis*-extracted AMPs using Blast program (http://www.ncbi.nlm.nih.gov/BLAST); the alignment was then adjusted manually. A phylogenetic tree was constructed using the CLC main workbench version 5.5 software and the neighbor-joining method. Bootstrap analysis with 100 replications was performed on the phylogenetic tree to estimate the reproducibility of the tree topology.

Antimicrobial Tests

Antimicrobial activity of the RP-HPLC fractions, including the peak numbers of 7, 9, and 16 was tested against *Escherichia coli* (HP101BA 7601c), *Klebsiella pneumonia* (PTCC1388), *Micrococcus luteus* (PTCC1625), and *Staphylococcus aureus* (PTCC1431) bacteria through radial diffusion assay (RDA) (Lehrer et al. 1991). For this purpose, specific numbers of bacteria (4×10^6 CFU) were poured into a 3 ml of cold 10 mM phosphate buffer and mixed with 7 ml of 1% agarose (Sigma-Aldrich) and 0.03% TSB as an underlay culture and poured into the plate. Then peptides were poured into the punched well in the underlay culture and after a 3-h incubation at 37°C, an overlay media culture containing autoclaved 6% TSB and 1% agarose was

poured on the treated microbes gently and kept at 37°C for 24 h. After 3 h, a light-halo was appeared around the punched wells. The antibacterial activity of the test agents was determined by measuring the mean radius of zone of inhibitions in millimeter. Phosphate-buffered saline (PBS) was used as negative control. All results were compared with the standard antibacterial antibiotic (Pattan Teb Company, Tehran, Iran). Each experiment was repeated thrice.

Each type of bacteria have different drug susceptibility. Here, we compared the antimicrobial activities of these peptides with the most prevalent antibiotics. Drug susceptibility was performed using the radial diffusion assay. Erythromycin (15 μg), penicillin (10 μg), and kanamycin (30 μg) discs were used as controls and the results compared with the concentration 15 μg of each peptide.

MIC Determination

The minimum inhibitory concentration (MIC) value is the lowest concentration of an antimicrobial compound at which bacterial growth was inhibited after 24 h of incubation at 37°C (Andrews 2001). To determine MIC values, four types of bacteria, including *E. coli* HP101BA 7601c and *K. pneumonia* PTCC1388 (gram-negative) and *M. luteus* PTCC1625 and *S. aureus* PTCC1431 (gram-positive) were chosen. All kinds of bacteria were cultivated in MHB at 37°C for 18 h. Approximately 1 ml of culture was transferred into 9 ml of broth medium and incubated at 37°C for the next 15 h, cell concentration was then adjusted to obtain final concentration of 10^6 CFU/ml using MHB. To a 96-well microplate, 100 μl of bacterial suspension (1×10^6 CFU/ml) and 80 μl of MHB medium were poured into the microplate. Stock serial dilutions of 0.6 to 0.01875 mg/ml of peptide were prepared and 20 μl of the peptide stock solutions were added to the above MHB to yield a final concentration of 10^5 CFU/ml in each well. The microplate was incubated at 37°C for 24 h. Afterward, the absorbance at 600 nm for each well was recorded using an enzyme-linked immunosorbent assay (ELISA) reader and the results were compared to the control sample. Experiments were done in triplicate.

Hemolysis Assay

The hemolytic activity of peptides was determined as described by Minn et al. (Minn et al. 1988). 2 ml of human red blood cells were washed several times with 5 ml of PBS by centrifugation at 4,000 rpm for 10 min. The washed cells were diluted to a final volume of 40 ml of PBS. A 20 μl of 10× stock serial dilution of peptide samples was added to 180 μl of 5% diluted erythrocytes to make the final peptide concentrations of 3.75 μg/ml to 60.0 μg/ml and the treated cells kept at 37°C for 30 min. 0.1% Triton X-100 was used as a positive control with 100% hemolytic activity. After 30 min, the solution was centrifuged @ 4,000 rpm for 5 min and the supernatant was gently diluted to 1 ml of PBS. The absorbance of the solution was measured at 567 nm. Experiments were done in triplicate.

Results and Discussions

Sample Collection, RP-HPLC, and Mass Spectrometry Analysis

Approximately, 100 ml of crude sample was collected. The crud sample was filtrated using a membrane with cut-off of 10 kDa and then concentrated by a membrane 1 kDa. Nearly, a 15 ml of the filtrated sample with the molecular weight between 1 and 10 kDa was obtained. The concentrated sample then was lyophilized by freeze dryer and kept at 4°C. The lyophilized sample was redissolved in 0.1% (v/v) TFA in water (solution A) and injected into a C_8 column. A total of 23 peaks were collected and named sequentially (Fig. 1a). Only three peaks which were enough amounts of peptide samples were subjected to further analyses. Therefore, these peaks were purified by a C_{18} semi-preparative with the same program which was used for C_8 semi-preparative column (Fig. 2a–c). The purified peptides, including the peak number of 7, 9, and 16 were chosen to sequence using tandem mass spectrometry. The MS–MS spectrum of the peak number of 7, 9, and 16 are shown in Fig. 3. The obtained sequences of the selected peptides are as follows:

a. peak 7: FLNALKNFAKTAGKRLKSLLN (2347.50 ± 0.5 Da)
b. peak 9: RAGLKFPVGRVHRLLR (1875.05 ± 0.5 Da)
c. peak 16: FLPGLLAGLL (1013.33 ± 0.5 Da)

Homology and Phylogeny

Each obtained sequence was searched against all sequence data banks and based on its homologous, each peptide was named. The peaks 7, 9, and 16 were named cyanophlyctin,

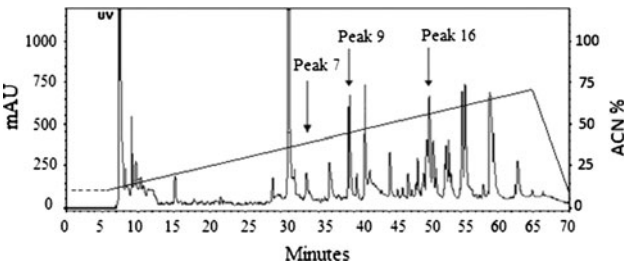

Fig. 1 HPLC chromatogram of peptides derived from skin secretions of *E. cyanophlyctis* using a C8 semi-preparative HPLC column

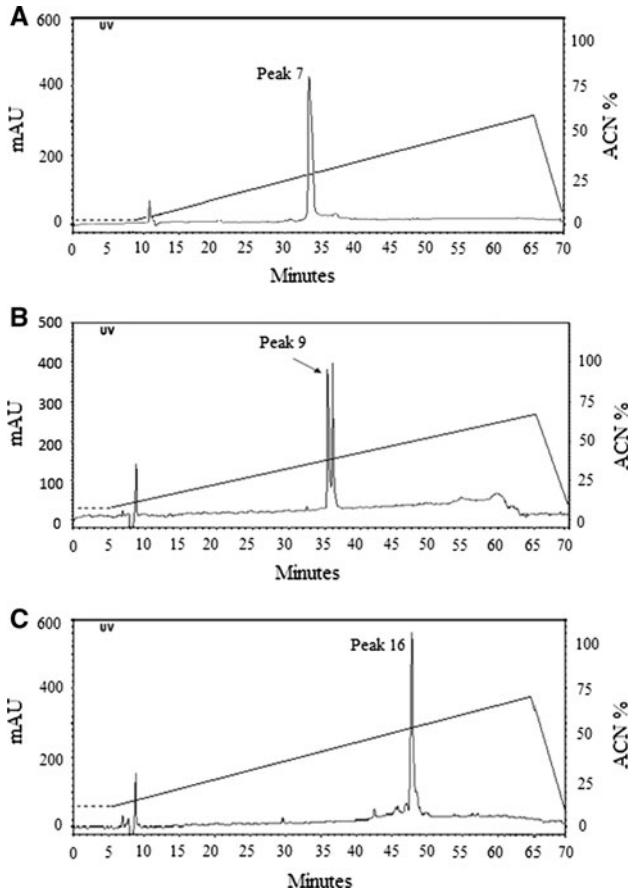

Fig. 2 The HPLC chromatograms of selected peaks, including peak 7(**a**), peak 9 (**b**), and peak 16 (**c**) using a C_{18} semi-preparative column

buforin-EC, and temporin-ECa, respectively. Multiple alignments of the purified peptides with some other similar antimicrobial-extracted peptides from different species are given in Fig. 4. Homology search indicated that the buforin-EC has the most similarity (71.43%) to buforin II (Fig. 4a). The buforin II itself is a partial sequence of buforin I (Yi et al. 1996). The cyanophlyctin has 47.1% similarity to brevinin-2EC from *R. esculenta* and 33% similarity to lycotoxin-I from *Lycosa carolinensis* (Fig 4b). The lycotoxin-I peptide belongs to a species of spider (Simmaco et al. 1994; Yans and Adams 1998). The cyanophlyctin and brevinin-2EC have a clear difference in their amino acid sequences (Fig. 4b). Temporin-ECa isolated from *E. cyanophlyctis* is more similar 69.23% to temporin-1CSc from *Rana muscosa* and 61.54% to temporin-1 M from *Rana cascadae* (Rollins-Smith et al. 2006; Conlon et al. 2007) (Fig. 4c). Temporin-ECa is classified into temporin antimicrobial peptide family with 10 amino acids but with a noticeable difference in amino acid composition. In some other reports, AMPs with even less than nine amino acid residues have been confirmed (Brogden 2005). The instruction of phylogenetic trees for the three peptides, confirmed that these peptides belong to three classes of amphibian AMPs (Fig. 5a–c).

Antimicrobial Activity and MIC Assay

For antimicrobial assay, peptides of different concentrations were poured into the punched wells. After a 24 h-incubation at 37°C, the inhibitory zone around the wells was observed. The inhibition radius of wells was measured in mm as illustrated in Fig. 6. The buforin-EC at concentration of 15 μg similarly acts against both *S. aureus* and *E. coli*, while kanamycin (30 μg) and temporin-ECa (15 μg) showed a higher inhibition zone aginst *S. aureus* than *E. coli*. The results showed that the MIC values for these peptides are in the range of 4.7–12.3 μM (Table 1). All the peptides act against both gram-positive and gram-negative bacteria, but they showed a more antibacterial effects against gram-positive than gram-negative bacteria, which have been tested here. The cyanophlyctin has more antibacterial effects than the two other peptides.

Drug Susceptibility

Due to possessing sufficient amount and more antibacterial efficiency of the cyanophlyctin peptide, the drug susceptibility of this peptide against *E. coli* and *S. aureus* as a representative of gram-negative and gram-positive bacteria were done. Based on our findings, the cyanophlyctin (10 μg) showed a great antimicrobial effect in comparison with penicillin (10 μg) against *E. coli* and *S. aureus* (Fig. 7). Furthermore, the cyanophlyctin exhibited a more potent effect than erythromycin (15 μg) against *E. coli.* In addition, the peptide showed a similar effect like that of kanamycin (30 μg) against *S. aureus*. Therefore, in many cases, skin secretions peptides act more potent than the conventional antibiotics like kanamycin (30 μg), erythromycin (15 μg), and penicillin (10 μg) in the control of pathogenic agents.

Hemolysis Assay

As shown in Fig. 8, human RBCs resisted hemolysis, while being treated well with cyanophlyctin-extracted peptides at concentrations below 7.5 μg/ml. At higher concentrations of peptides (15 and 30 μg/ml), less than 1% of hemolysis was observed in comparison with Triton X-100 treatment as a positive control. At the concentration of 60 μg/ml, temporin-ECa (59.2 μM), cyanophlyctin (25.5 μM) and buforin-EC (32.0 μM) induce only 2.3, 2.5, and 2.9% human RBC hemolysis, respectively (Fig. 8), which is ignorable for these AMPs. Therefore, no hemolysis was found for these peptides at their MIC values. This observation indicates that the interactions between these AMPs and phospholipids of RBCs are very weak.

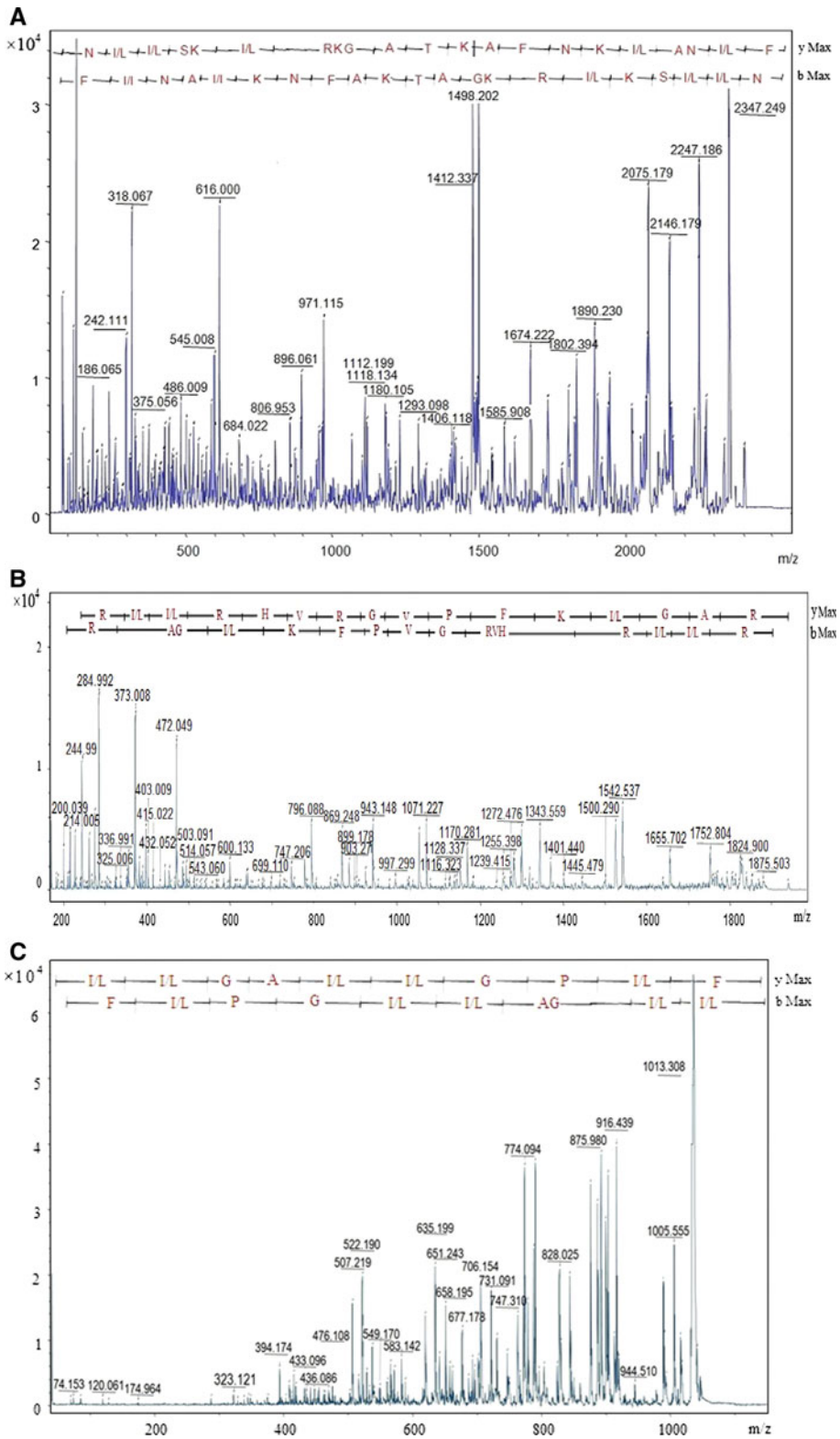

Fig. 3 Mass–mass spectrometry analysis of the peak 7 (**a**), 9 (**b**), and 16 (**c**) isolated from skin secretions of *E. cyanophlyctis*

Discussion

Here, we identified three novel AMPs from *E. cyanophlyctis*. According to their chromatogram patterns, three peptides were eluted based on hydrophobicities of their amino acids. The lowest and highest retention times are observed for cyanophlyctin and temporin-ECa, respectively. To our knowledge, the most AMPs are 10–50

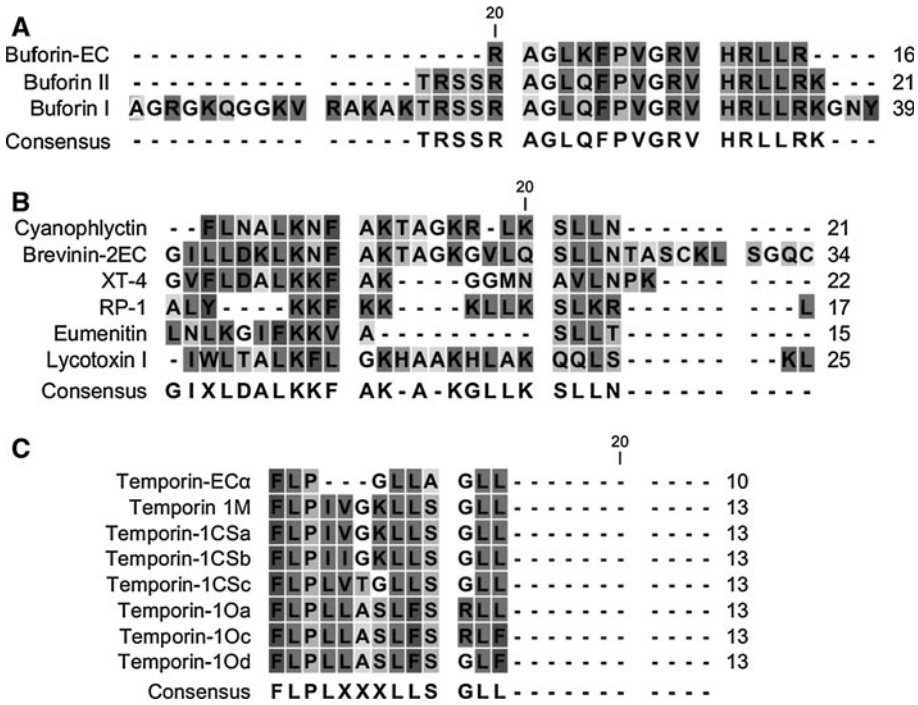

Fig. 4 Multiple alignments of the *cyanophlyctis*-extracted peptides. **a** Buforin-EC, **b** cyanophlyctin, and **c** temporin-ECa. The name of the each peptide was typed at the end of the corresponding branch. Reliability of the tree was assessed by bootstrap analysis with 100 replications. The substitutions per nucleotide position, typed above of the each branch. The figures show that the buforin-EC, cyanophlyctin and temporin-ECa, have the most similarity to the buforin II (**a**), brevinin 2-EC (**b**), and and temporin-CSc (**c**), respectively

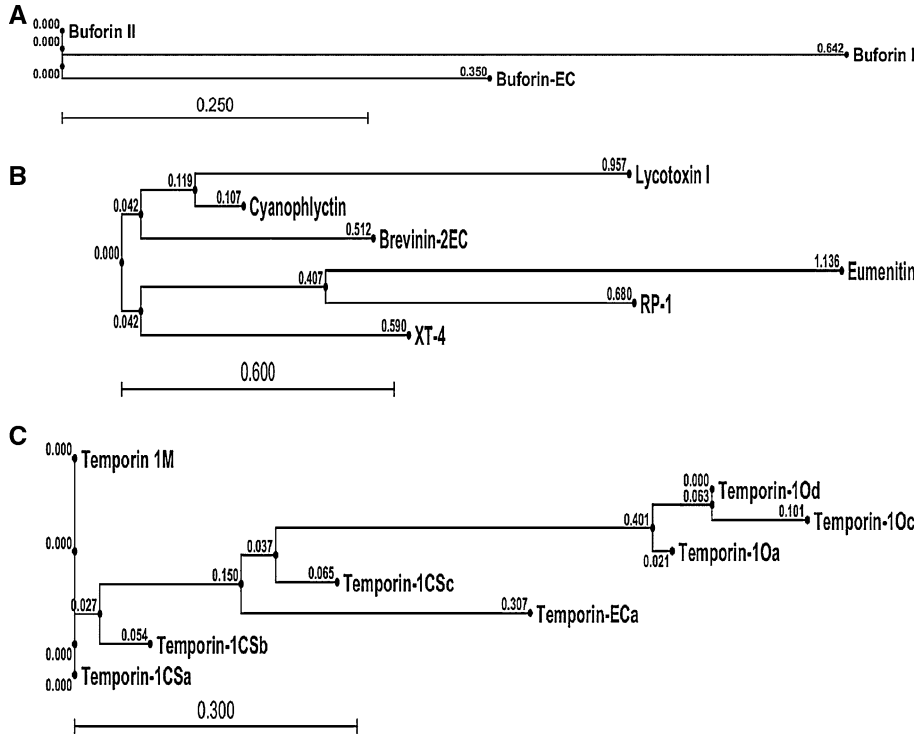

Fig. 5 The phylogenetic trees of the *cyanophlyctis*-extracted peptides. **a** Buforin-EC, **b** cyanophlyctin, and **c** temporin-ECa

amino acids in length, the new peptides cyanophlyctin, buforin-EC, and temporin-ECa have 21, 16, and 10 amino acids, respectively. Unlike some AMPs, these peptides have no disulfide bridges on their structures.

Buforin II is a histon H2A derived antimicrobial peptide isolated from the stomach tissue of the Asian toad *Bufo bufo garagrizans* (Park et al. 2000). It has been demonstrated that a proline hinge situated at amino acid position 11 of buforin II was responsible for the cell-penetrating ability of this peptide (Park et al. 2000). Due to the highest similarity (71.4%) of buforin-EC to buforin II, the position the proline 7 of buforin-EC would likely have a similar function as that of the hinge position of buforin II. However, this needs to be studied further to assess this conjecture. Proline residues are

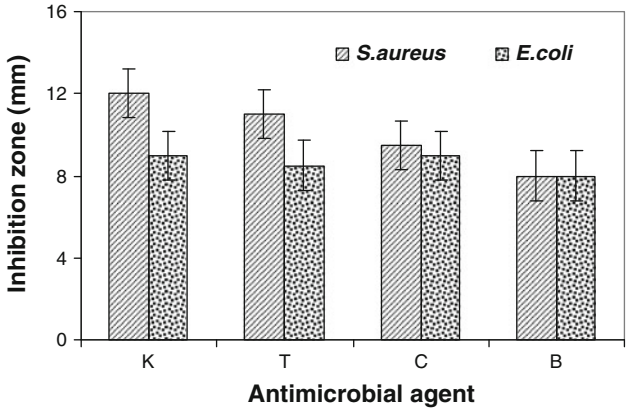

Fig. 6 Inhibition zone of *cyanophlyctis* isolated peptides against *S. aureus* PTCC1431 and *E. coli.* PTCC1431. *K* shows control (kanamycin 30 μg) and *T*, *C*, and *B* show temporin-ECa, cyanophlyctin, and buforin-EC (15 μg for each), respectively. Each inhibition zone radius was measured thrice

Table 1 MIC of *cyanophlyctis*-extracted antimicrobial peptides

Bacteria	MIC (μM)		
	Buforin-EC	Cyanophlyctin	Temporin-ECa
E. coli (HP101BA 7601c)	9.6	6.4	12.3
K. pneumonia (PTCC1388)	10.6	7.3	9.8
M. luteus (PTCC1625)	6.0	4.7	8.3
S. aureus (PTCC1431)	8.1	5.3	10.6

Minimal inhibitory concentration (MIC) is defined as the lowest concentration at which no growth was detectable. Each data point represents the mean of three independent assays (the standard errors were less than 5% of the means)

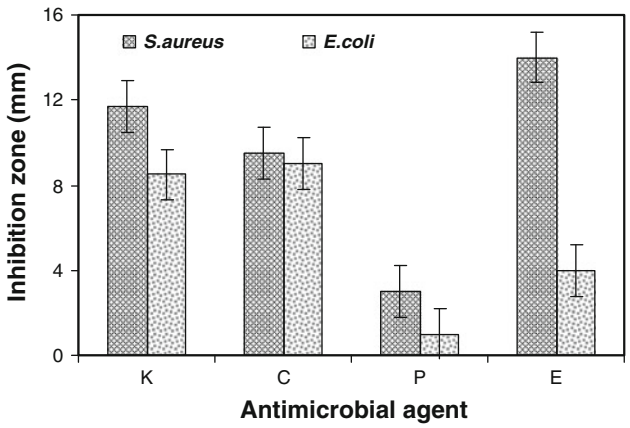

Fig. 7 Drug susceptibility (DS) of cyanophlyctin. **a** DS for *S. aureus* as gram-positive and **b** drug susceptibility of *E. coli* as gram-negative. *K* kanamycin 30 μg, *C* cyanophlyctin 15 μg, *E* erythromycin 15 μg, *P* penicillin 10 μg

prevalent in several AMPs such as peptides in the family of proline-rich antimicrobial peptides (PRAPs) (Otvos 2002). It had been reported that proline substitution in the buforin

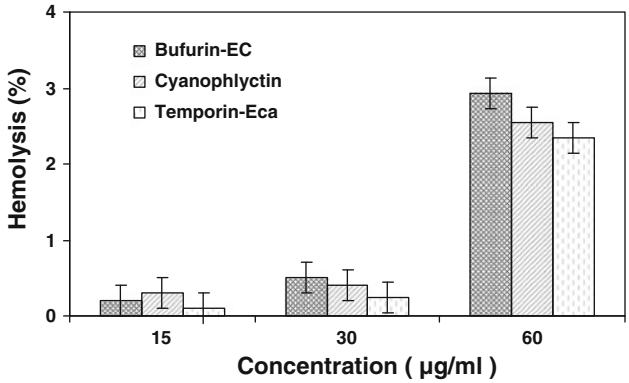

Fig. 8 The effect of isolated peptides, including buforin-EC, cyanophlyctin, and temporin-ECa on hemolysis of human red blood cells. For three isolated peptides, no hemolytic effects were observed at the concentration below 15 μg/ml

II (BF2) analog P11A and P11L mutations decrease the antimicrobial activity of BF2 (Lee et al. 2004; Xie et al. 2011).

Our results revealed that cyanophlyctin has a 47.7 similarity to brevinin-2EC from *R. esculenta*. Brevinins are families of related AMPs, which have been isolated from the skin secretions of various Rana species (Morikawa et al. 1992; Park et al. 1994; Conlon et al. 2004). Brevinin-2EC isolated from skin secretions of *R. saharica* exhibited antimicrobial and insulin releasing activities (Marenah et al. 2006). The isolated cyanophlyctin has a similarity of 32.3% to brevinin-2R from *R. ridibunda* (Ghavami et al. 2008). Instead of a low percentage identity of the two peptides, both peptides had been antibacterial as well as no hemolytic activities at their MICs values.

Our findings indicated that all isolated peptides have overall MICs below 13 μM against tested species (Table 1). In this study, it was shown that the AMPs isolated from skin secretions of *E. cyanophlyctis*, had the least cytotoxicity against human red blood cells in vitro. Some conventional antibiotics showed a few hemolytic activities. Anantharaman et al. (2010) reported a 2.1% hemolysis for kanamycin in its MIC value.

In this study, the temporin-ECa was the smallest isolated peptide, which isolated from *E. cyanophlyctis* lacks the basic amino acid residue that is usually present in members of the temporin family, is completely devoid of hemolytic activity but is still capable of inhibiting the growth of the gram-positive and gram-negative bacteria. Temporin-ECa showed more antibacterial activity against gram-positive bacteria (*S. aureus*) than gram-negative bacteria (*E. coli*) as it confirmed by previously reported results of temporin-1CSc (Conlon et al. 2007). To understand the mechanism of antibacterial activity of temporin-ECa, it is necessory to be investigated, the effect of its synthetic peptide and analogs on different bacteria. Although, more characterized

temporins with antimicrobial activity contain a C-terminally alpha-amidated residue (Mangoni et al. 2000). The C- terminally free acid form is 1 mass unit greater from amidated form. The isolated temporin-ECa exhibited to be a free amino acid at its C-terminal, as it was previously observed for temorin-1M from *R. muscosa* (Rollins-Smith et al. 2006) as well as temporin-Ra and temporin-Rb from *R. ridibunda* (Asoodeh et al. 2011). It has been reported that some temporins showed low percentage of hemolysis, for example temporin H ($HC_{50} = 100$ μM) from *R. temporaria*, temporin-1AUa ($HC_{50} > 300$ μM) from *R. aurora aurora*, temporin-1Va ($HC_{50} = 120$ μM) from *R. virgatipes* and temporin-1CSc ($HC_{50} > 300$ μM) from *R. cascadae*, all have been exhibited a low level of the hemolytic effect against human erythrocytes (Mangoni et al. 2000; Conlon et al. 2005, 2007).

Concluding Remarks

In our study, we identified three AMPs named buforin-EC, cyanophlyctin, and temporin-ECa from skin secretions of *E. cyanophlyctis* using the conventional methods, including HPLC and tandem mass spectrometry. The isolated peptides exhibited valuable properties like a great antimicrobial activity against some prevalent gram-positive and gram-negative bacteria as well as a low level hemolytic effect on human red blood cells. This study suggests that the isolated peptides or their analogs may have potential for drug development. Furthermore, the isolated peptides may show additional functions such as insulin releasing and antioxidant properties that need to elucidate in future studies.

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