Research Article

Journal of PeptideScience

Received: 24 April 2011 Revised: 15 June 2011 Accepted: 27 June 2011 Published online in Wiley Online Library: 29 September 2011

(wileyonlinelibrary.com) DOI 10.1002/psc.1409

Identification and characterization of two novel antimicrobial peptides, temporin-Ra and temporin-Rb, from skin secretions of the marsh frog (*Rana ridibunda*)

Ahmad Asoodeh,[a,b]* Hadi Zare Zardini[a] and JamshidKhan Chamani[c]

In this study, two novel antimicrobial peptides from the skin secretions of the marsh frog, *Rana ridibunda*, named temporin-Ra and temporin-Rb, were identified and purified using RP-HPLC. Temporin-Ra and temporin-Rb are composed of 14 and 12 amino acids, respectively. Our results show that these peptides have inhibitory effects on both gram-negative and gram-positive bacteria, especially antibiotic resistant strains prevalent in hospitals, such as *Staphylococcus aureus* and *Streptococcus agalactiae*. The sequences and molecular weights of these peptides were determined using tandem MS. The molecular masses were found to be 1242.5 Da for temporin-Rb and 1585.1 Da for temporin-Ra. Human red blood cells tolerated well exposure to temporin-Ra and temporin-Rb, which, at a concentration of 60 μg/ml, induced 1.3% and 1.1% hemolysis, respectively. MIC values of these peptides are suitable for potent antimicrobial peptides. The low hemolytic effect and wide-spectrum antimicrobial activity suggest a possible therapeutic application of these novel peptides. **Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.**

Keywords: antimicrobial peptides; *Rana ridibunda*; RP-HPLC; MIC; hemolysis

Introduction

Antimicrobial peptides (AMPs) are one of the major components of the innate immune system in all animals and are a very important defense mechanism [1]. In recent years, it has been widely recognized that many organisms use these peptides as a part of their host defense system against invasion by microorganisms [2–5]. These compounds are very small in size and show wide-ranging antimicrobial activity. A common feature among these compounds is their amphipathicity, meaning that about half of their amino acids are hydrophobic. A large number of their remaining amino acids are cationic [6]. Because of the special mechanisms by which these compounds kill microbes, pathogens do not easily develop resistance against these peptides. Frogs are not an exception and secrete some peptides of this type. Several AMPs, such as odorranain-B1 [7], dermaseptin-B2 [8], ranatuerin-2B [9] and brevinin [10–12] isolated from different types of frogs, are reported and exhibit different effects. Anticancer effects of some skin peptides from amphibians have also been demonstrated [10,13–16].

The marsh frog is an amphibian that is confined to parts of Europe, extending to the Baltic and Asia as far as Pakistan. However, few studies have examined the skin secretions of this animal. Only one study has investigated the effect of crude extracts obtained from animal secretions on resistant hospital bacteria *Staphylococcus aureus* [17]. We previously reported the characterization of a new peptide isolated from secretions of the marsh frog found in the northern regions of Iran [13,18]. Therefore, studying skin secretions from frogs found in different parts of Iran may identify peptides with new activity.

Materials and Methods

Chemicals

HPLC grade acetonitrile and TFA, methanol, formaldehyde, acetic acid, sodium chloride, sodium hydroxide, ethanol, TCA, acetone, glycerol and chloridric acid were obtained from Merck and Company Inc. (Whitehouse Station, NJ, USA). Trypticase soy broth (TSB), Mueller Hinton Broth (MHB) and blood agar media cultures were purchased from Himedia Laboratories (Mumbai, India). All other chemicals used, including agarose, methyl green, agar, Triton X-100 (Dow Chemical Company, Midland, MI, USA), Coomassie Brilliant Blue R-250 (Imperial Chemical Industries, London, UK) and bromophenol blue, were of analytical grades.

Collection of Frog Skin Secretions

Between April and June, 20 specimens including male and female marsh frogs were collected from different parts of Khorasan province

* Correspondence to: Ahmad Asoodeh, Department of Chemistry, Ferdowsi University of Mashhad, Mashhad, Iran. E-mail: asoodeh@um.ac.ir

a Department of Chemistry, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran

b Cellular and Molecular Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran

c Department of Biology, Faculty of Sciences, Mashhad Branch, Islamic Azad University, Mashhad, Iran

 Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

in northeast Iran. These frogs were used throughout the cold season when sampling was impossible. The frogs were maintained under normal laboratory conditions at 25 °C. To obtain 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 extracts were collected by washing the frog's dorsal area using 5% acetic acid. The extracts were centrifuged at 10,000 r.p.m. for 15 min, and the supernatant was stored at 4 °C for subsequent analyses.

Peptide Isolation and Purification

To isolate low molecular weight compounds, the supernatant was passed through an ultramembrane with a 10 kDa cutoff. The filtrated solution was concentrated using a 1 kDa ultramembrane and lyophilized. The lyophilized extract was dissolved in the smallest possible volume of distilled water and was purified using a C18 semipreparative RP-HPLC column. The injected amount was 400 μL each time. Elution was performed using solution A (0.1% TFA in water) combined with a 5 to 65% gradient of solution B (0.098% TFA in acetonitrile) over a period of 65 min, at a flow rate of 2 ml/min. According to the absorbance at 220 nm, the fractions were collected and lyophilized. For further purification, each active fraction was purified using the method described previously except that the elution was conducted using a 0.5% per minute increasing gradient of solution B. To evaluate purity, a small amount of each peak was checked on the analytical C18 column using RP-HPLC.

Radial Diffusion Assay

The antimicrobial effects of the collected peptides were investigated with a radial diffusion assay, as described in [19], but with slight modification. One species of gram-positive bacteria (*S. aureus* PTCC 1431) and one species of gram-negative bacteria (*Klebsiella pneumoniae* PTCC 1388) were used for primary assays. An aliquot of bacteria with a titer of 4×10^6 CFU was mixed with 10 ml of medium containing both 0.03% TSB and 1% agarose and was poured into a plate. Holes were then created in the medium using a punch, the peptide sample was loaded into the wells, and the plates were incubated for 3 h at 37 °C. After the 3-h incubation, the secondary medium, enriched with 6% TSB and 1% agarose, was poured into the plate, and the plates were incubated at 37 °C for 18 h. Afterwards, the plate was stained for 24 h using a solution containing the following: 37% formaldehyde, 15 ml; methanol, 27 ml; water, 63 ml; and Coomassie brilliant blue R-250, 2 mg. The plates were destained for approximately 10 min with an aqueous solution of 10% acetic acid and 2% DMSO.

Peptide Sequencing

Peptide sequencing was carried out using MS, in positive ionization mode on a MALDI-TOF/TOF instrument [Ultraflex III, (Bruker Daltonics, Inc., Billerica, MA, USA) at York University, UK]. Purified and lyophilized peptides were reconstituted with 10 μL of 0.1% TFA (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–Aldrich, St. Louis, MO, USA) in 50% aqueous (v : v) acetonitrile containing 0.1% TFA (v : v). Bruker flex analysis (Bruker Daltonics, Inc.) 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-ion and y-ion series were overlaid onto their fragmentation spectra using the Bruker flex analysis software (version 3.3).

Phylogenetic Analysis

Eleven amino acid sequences for peptides from different species were obtained from the antimicrobial peptide database (http://aps.unmc.edu/AP/main.php). These sequences, along with those for temporin-Ra and temporin-Rb of *Rana ridibunda*, were aligned using the program BLAST; the alignment was then adjusted manually. A phylogenetic tree was obtained from the CLC Main Workbench version 5.5 software (CLC bio A/S, Denmark) using the neighbor-joining method. Bootstrap analysis with 100 replications was performed on the phylogenetic tree to estimate the reproducibility of the tree topology.

Determination of MIC

All bacteria were cultivated in MHB at 37 °C for 18 h. Approximately 1 ml of culture was transferred to 9 ml of broth medium and incubated at 37 °C for another 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 0.6–0.01875 mg/ml of peptide were prepared, and 20 μL of the peptide stocks were added to the aforementioned MHB to yield a final concentration of 10^5 CFU/ml in each well. The microplate was incubated at 37 °C for 24 h. After this time, the absorbance at 600 nm for each well was read using an ELISA reader, and the results were compared with the control sample. The MIC was defined as the peptide concentration at which bacterial growth was inhibited after 24 h of incubation at 37 °C [7]. *Escherichia coli* HP101BA 7601c, *K. pneumonia* PTCC 1388, *Salmonella typhimurium* PTCC 1428, *S. aureus* PTCC 1431, *Streptococcus agalactiae* PTCC 1365 and *Streptococcus dysgalactiae* PTCC 1074 were used for MIC determination. Experiments were carried out in triplicate.

Hemolysis Assay

For hemolysis testing, 5 ml of fresh human blood was added to a heparinized tube. After centrifugation at 4500 r.p.m. for 5 min, red blood cells (RBCs) were isolated, washed five times using 4 ml sterile phosphate buffered saline (PBS) and centrifuged at 4500 r.p.m. until the solutions were clear. In the last stage of purification, the RBCs were diluted into 20 ml of PBS buffer. To assess hemolysis, 10 μL of serially diluted peptide samples was added to microfuge tubes containing 190 μL of diluted RBC (10% cells). The samples were incubated for 30 min at 37 °C. The tubes were then centrifuged for 5 min at 4000 r.p.m. A 100 μL aliquot of the supernatant solution was removed and diluted with PBS to a final volume of 1 ml. The absorbance at 567 nm was then measured. Triton X-100 (0.1%), which yields 100% hemolysis, was used as a positive control [20]. The results were compared with the control.

 Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

Results

RP-HPLC and Antimicrobial Assay

In total, 28 major peaks were collected from a semipreparative C18 column, as shown in Figure 1(A). The fractions were numbered sequentially. The results of the AMP assays indicate that peaks R7, R11, R20, R22, R23, R27 and R28 have broad antibacterial effects on gram-positive and gram-negative bacteria (Figure 2).The antimicrobial activities of these peaks are much more than that of the 30 μg of kanamycin used as a control. Consequently, these active peaks were further purified using the same RP-HPLC conditions, except for the use of a gradient of 0.5% eluent B per minute. The purity of R20 and R27 were checked using C18 RP-HPLC, as shown in Figure 1(B) and (C), respectively. The antimicrobial effects of temporin-Ra and temporin-Rb are more than the other peaks.

Peptide Sequencing

Among the peaks obtained, seven that were appropriate in terms of quantity and microbial effects were sequenced. These peaks were the following: R7, R11, R20, R22, R23, R27 and R28. Information about these sequences is summarized in Table 1. Figure 3 (A) and (B) shows the MS/MS spectrometry analyses of the temporin-Rb and temporin-Ra peaks, respectively. MS of peaks R7, R11, R20, R22, R23, R27 and R28 revealed that they have molecular masses of 2480.8, 2668.38, 1585.1, 3322.01, 3319.09, 1242.5 and 2678.38 Da, respectively (Table 1). Our fractions obtained from *R. ridibunda* were compared with the published AMP sequences in the antimicrobial peptide database. As shown in Table 1, results indicated that the sequences of R7, R11, R22, R23 and R28 are identical to brevivin-1Ya, brevinin-ALa, gaegurin-2, brevinin-2Eb and brevinin-1E, respectively, but R20 and R27 show no sequence homology to any AMP in the

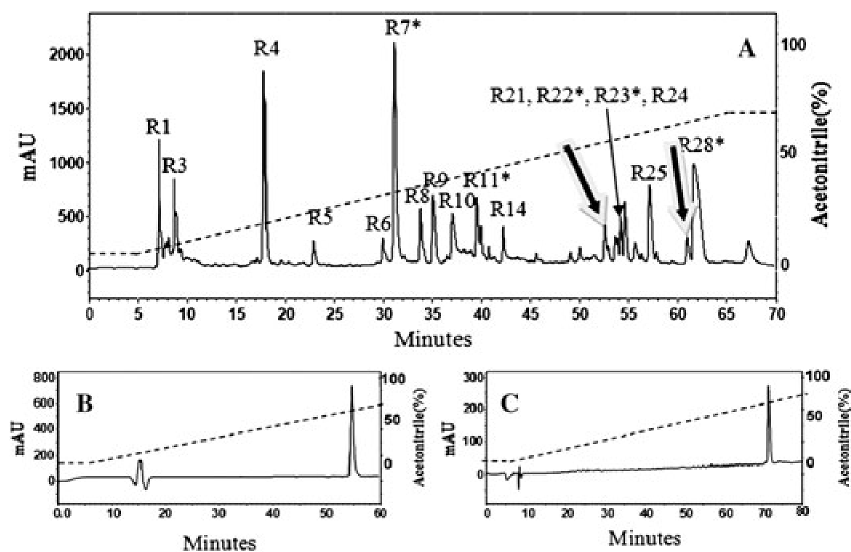

Figure 1. Isolation of peptides from skin secretions of *Rana ridibunda*. A 400 μL aliquot of filtrated extract was loaded onto a semipreparative C18 RP column. The elution was performed with a 1% acetonitrile gradient at a flow rate of 2 ml/min. The absorbance was monitored at 220 nm. In all, 28 peaks were collected (A). The active fractions were further purified using an analytical C18 column by applying a mild slope of the eluent B at a flow rate of 1 ml/min, (B, C). R20 and R27 are indicated by bold arrows in the figure. Asterisk (*) indicates active peaks with appropriate antibacterial effects.

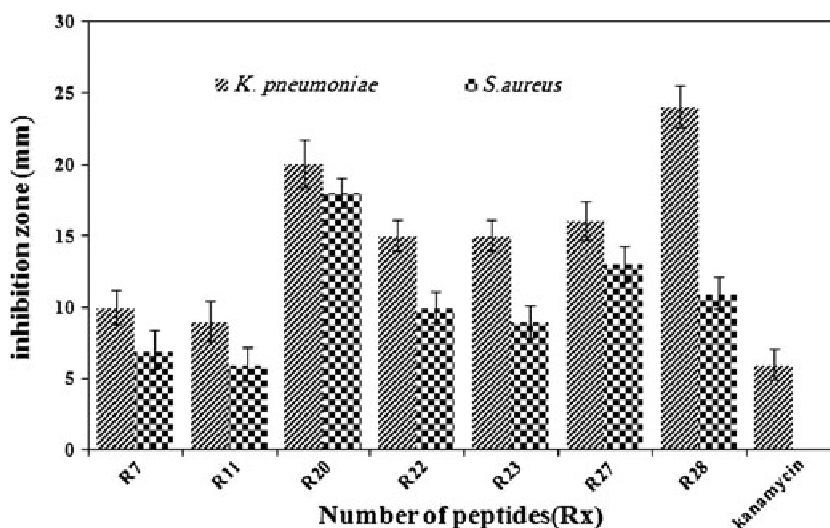

Figure 2. Antimicrobial activity of purified peptides from skin secretions of *Rana ridibunda* against bacteria (*Klebsiella pneumoniae* PTCC 1388 and *Staphylococcus aureus* PTCC 1431 using the RDA method (inhibition zone). *Staphylococcus aureus* was resistant to kanamycin.

 Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

database, suggesting that they are novel AMPs. Thus, the two new AMPs were named temporin-Ra and temporin-Rb on the basis of a systematic nomenclature for AMPs [21]. There are multiple basic amino acids in the sequence of temporin-Ra (net charge +2) and temporin-Rb (net charge +1), as found in other AMPs. Analysis using the ExPASy pI/Mw tool (http://www.

Table 1. Primary structures, molecular weight, homology peptides and pI values of the peptides identified from *Rana ridibunda*

Peptides	Sequence	Mass (Da)	pI	Homology peptide
R7	VIPFVASVAAEMQHVYCAASRKC	2480.8	8.03	Brevivin-1Ya
R11	FLPMLAGLAANFLPKLFCKITKKC	2668.38	9.70	Brevinin-Ala
R22	GIMSIVKDVAKNAAKEAAKGALSTLSCKLAKTC	3322.01	9.51	Gaegurin-2
R23	GILDTLKNLAKTAGKGALQGLVKMASCKLSGQC	3319.09	9.60	Brevinin-2Eb
R28	FLPLLAGLAANFLPKIFCKITRKC	2678.38	9.85	Brevinin-1E
R20	FLKPLFNAALKLLP	1585.1	10.0	None found
R27	FLPVLAGVLSRA	1242.5	9.75	None found

R*x* (R, abbreviation of *Rana*; *x*, number of peak).

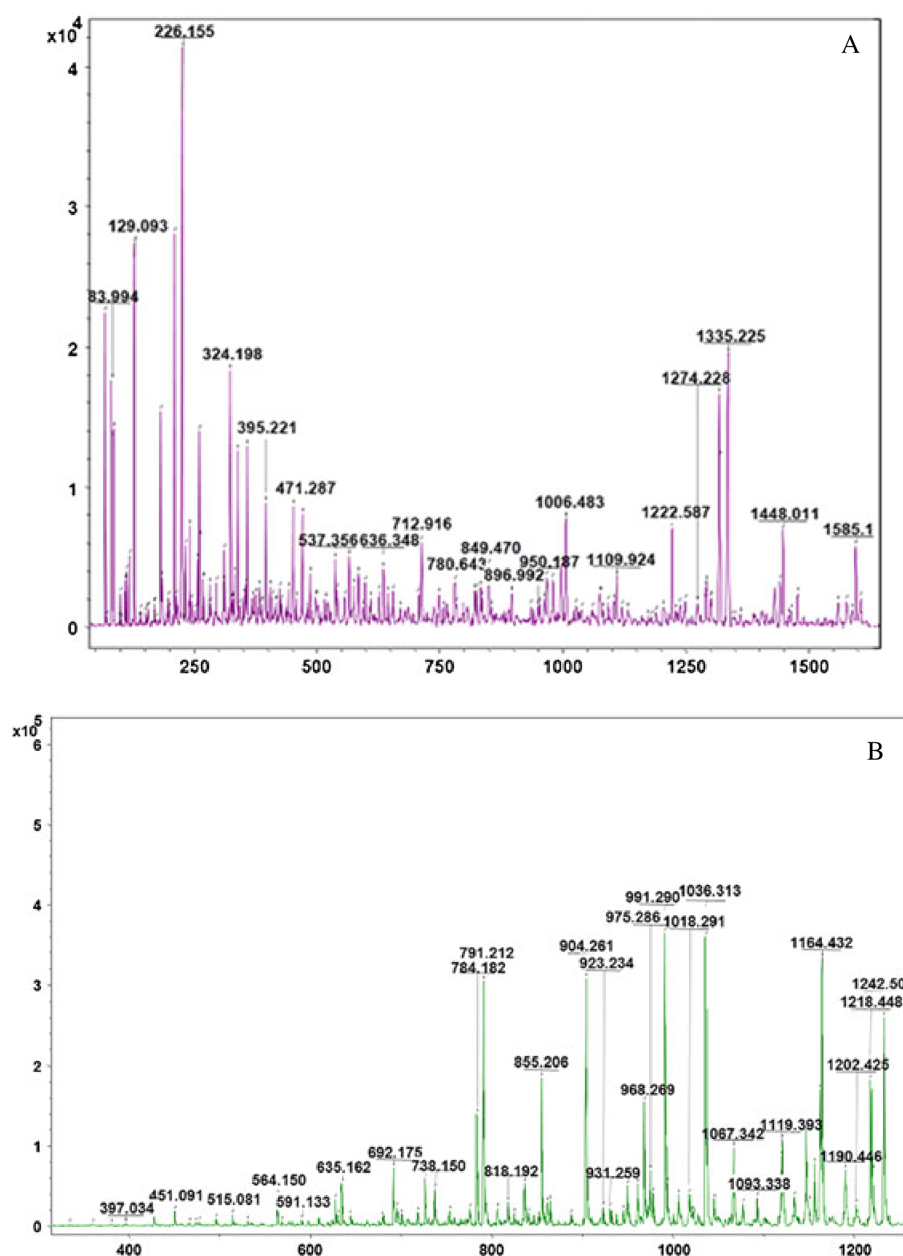

Figure 3. MS/MS spectra of (A) temporin-Ra and (B) temporin-Rb isolated from the skin secretions of *Rana ridibunda*.

 Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

expasy.ch/tools/pi_tool.html) showed that temporin-Ra and temporin-Rb had predicted pI (isoelectric point) values of 10 and 9.75, respectively. Therefore, temporin-Ra and temporin-Rb were selected for further testing, including MIC determination and the hemolysis assay.

Phylogenetic Analysis

Multiple sequence alignments of temporin-Ra and temporin-Rb were carried out along with 11 AMPs from different species. The result is illustrated in Figure 4(A). Temporin-Ra and temporin-Rb from *R. ridibunda* have a high similarity to the temporin-1 AMPs. Phylogenetic analysis of the amino acid sequences was carried out by constructing a phylogenetic tree. On the basis of the length of lines in the phylogenetic tree, the result showed that temporin-Ra and temporin-Rb had the highest similarity to temporin-1bc from *Rana clamitans* and temporin-1Ec from *Rana esculenta*, respectively. Temporin-Ra is most similar (62.5% identity) to temporin-1bc from *R. clamitans*, and temporin-Rb is most similar (61.53% identity) to temporin-1Ec from *R. esculenta* (Figure 4(B)). Despite these similarities, temporin-Ra and temporin-Rb can be considered two novel peptides that belong to the temporin-1 family.

MIC Determination

Temporin-Ra and temporin-Rb exhibited antimicrobial activity against standard and antibiotic-resistant strains (Table 2). Of the tested strains, *E. coli* and *S. dysgalactiae* were the most sensitive to temporin-Ra and temporin-Rb, respectively. The MIC value of temporin-Ra against *E. coli* was 24.2 μg/ml, and the MIC value of temporin-Rb against *S. dysgalactiae* was 21.8 μg/ml. These peptides exhibited antimicrobial activity against resistant hospital bacteria, such as *S. agalactiae* and *S. aureus*. Therefore, temporin-Ra and temporin-Rb may be suitable antimicrobial agents against these bacteria.

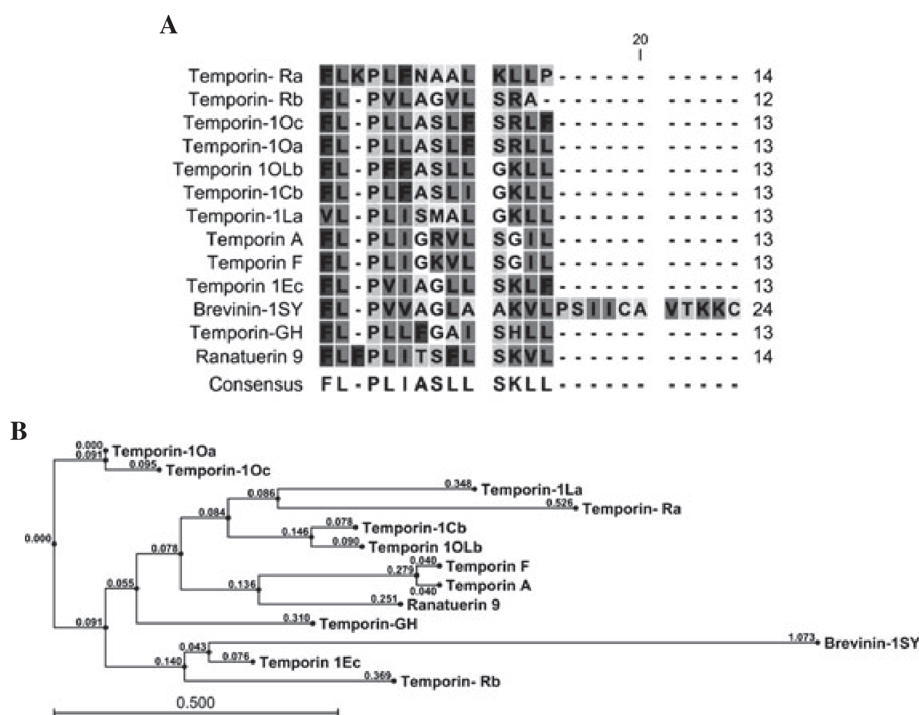

Figure 4. Alignment and phylogenetic tree of temporin-Ra and temporin-Rb. (A) Alignment of amino acid sequences of temporin-Ra and temporin-Rb with the sequences of other antimicrobial peptides. The alignment was carried out with CLC Main Workbench version 5.5 software. (B) Phylogenetic tree of temporin-Ra and temporin-Rb. Amino acid sequences of the 11 reference peptides obtained from the antimicrobial peptide database were incorporated into the tree using the neighbor-joining method. The name of each sequence is typed at the end of the corresponding branch. Reliability of the tree was assessed by bootstrap analysis with 100 replications. The substitutions per amino acid position are typed above each branch.

Table 2. MIC values of peptides from skin secretions of *Rana ridibunda* (temporin-Ra and temporin-Rb)

	MIC(μg/ml)					
Peptides	*Escherichia coli*	*Streptococcus dysgalactiae*	*Streptococcus agalactiae*	*Staphylococcus aureus*	*Staphylococcus typhimurium*	*Klebsiella pneumoniae*
Temporin-Ra	24.2 ± 1.2	33.2 ± 2.4	24.7 ± 1.2	35.2 ± 1	ND	31.2 ± 1.1
Temporin-Rb	34.5 ± 1.4	21.8 ± 1.3	30.5 ± 1.1	42.1 ± 1	31.8 ± 1.5	35.1 ± 2.1

ND, nondetected activity.

 Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

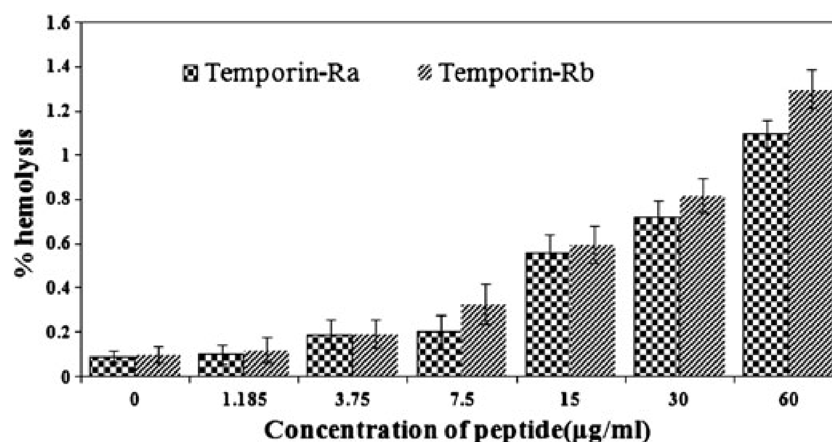

Figure 5. Hemolytic activities of the antimicrobial peptides isolated from the skin secretions of *Rana ridibunda* (temporin-Ra and temporin-Rb).

Hemolysis Assay

As shown in Figure 5, human RBCs tolerated well treatment with temporin-Ra and temporin-Rb peptides. At high concentrations of peptide, only small amounts of hemolysis are observed in comparison with Triton X-100 treatment. At a concentration of 60 μg/ml, temporin-Ra and temporin-Rb induce only 1.3% and 1.1% human RBC hemolysis, respectively (Figures 5). Therefore, the purified peptides have no hemolytic effect near their MIC values. This observation indicates that the interactions between our peptides and phospholipids of RBCs are very weak.

Discussion

Today, research in the field of AMPs is active, and within this field, amphibians are highly regarded because of their unique chemical defense system [2,14,22,23]. Large numbers of AMPs with different structures and functions from the skin secretions of amphibians have been reported [6,8,10]. In this study, peptides with antimicrobial activity against various gram-negative and gram-positive bacteria, particularly types prevalent in hospitals, such as *S. aureus* and *S. agalactiae*, were purified from the skins of native marsh frogs found in Iran. According to their chromatogram patterns, in which high retention times are observed, temporin-Ra and temporin-Rb are highly hydrophobic (Figure 1(A)), as reported by other authors [24]. These hydrophobic and cationic properties are hallmarks observed in nearly all AMPs. Although most AMPs are 10–50 amino acids in length, the new peptides temporin-Ra and temporin-Rb have 14 and 12 amino acids, respectively [25,26]. Unlike some AMPs, temporin-Ra and temporin-Rb have no disulfide bridges. Our results show that peptides isolated from *R. ridibunda* have a potent antibacterial effect against various bacteria, and their antimicrobial activity is much more than current antibiotics such as kanamycin (Figure 2 and Table 2). The net charge and the number of amino acids in temporin-Ra and temporin-Rb are similar to those of other reported temporins [27]. It has been reported that some temporins such as temporins B and D have a great hemolytic effect, whereas other temporins such as temporin-Ra and temporin-Rb as well as temporin-1AUa and temporin-1Va show low percentage of hemolysis [28,31,32]. On the other hand, temporins show a low antibacterial activity, whereas temporin-Ra and temporin-Rb have appropriate inhibitory effects at low concentrations compared with other temporins [29–31]. In addition to our peptides, other AMPs belonging to the same family as brevinin-2, such as gaegurin-2, gaegurin-3 from *Rana rugosa* [33,34] and nigrocin-1 from *Rana nigromaculata* [35] were reported to have no hemolytic activity. Consequently, temporin-Ra and temporin-Rb belong to a superfamily of nonhemolytic AMPs that were isolated from different *Rana* species. Considering their promising properties, these two small AMPs isolated from *R. ridibunda* may find a potential application in the treatment of pathogenic diseases.

Acknowledgement

This work was funded by grant number 87020301 from the Iran National Science Foundation (INSF).

References

1 VanCompernolle SE, Taylor RJ, Oswald-Richter K, Jiang J, Youree BE, Bowie JH, Tyler MJ, Conlon JM, Wade D, Aiken C et al. Antimicrobial peptides from amphibian skin potently inhibit human immunodeficiency virus infection and transfer of virus from dendritic cells to T cells. *J. Virol.* 2005; **79**: 11598–11606.
2 Boman HG. Antibacterial peptides. Key components needed in immunity. *Cell* 1991; **65**: 205–207.
3 Gabay JE. Ubiquitous natural antibiotics. *Science* 1994; **264**: 373–374.
4 McPhee JB, Hancock REW. Function and therapeutic potential of host defence peptides*J. Pept. Sci.* 2005; **11**: 677–687.
5 McGillivary G, Ray WC, Bevins CL, Munson RS, Bakaletz LO. A member of the cathelicidin family of antimicrobial peptides is produced in the upper airway of the chinchilla and its mRNA expression is altered by common viral and bacterial co-pathogens of otitis media. *Mol. Immunol.* 2007; **44**: 2446–2458.
6 Nicolas P, Mor A. Peptides as weapons against microorganisms in the chemical defense system of vertebrates. *Annu. Rev. Microbiol.* 1995; **49**: 277–304.
7 Wang H, Lu Y, Zhang XQ, Hu YH, Yu HN, Liu JZ, Sun JS: The novel antimicrobial peptides from skin of Chinese broad-folded frog, *Hylarana latouchii* (Anura:Ranidae). *Peptides* 2009; **30**: 273–282.
8 Tennessen JA, Blouin MS. Selection for antimicrobial peptide diversity in frogs leads to gene duplication and low allelic variation. *J. Mol. Evol.* 2007; **65**: 605–615.
9 Mor A, Hani K, Nicolas P. The vertebrate peptide antibiotics Dermaseptins have overlapping structural features but target specific micro-organisms. *J. Biol. Chem.* 1994; **269**: 31635–31641.
10 Li JX, Xu XQ, Xu CH, Zhou WP, Zhang KY, Yu HN, Zhang YP, Zheng YT, Rees HH, Lai R et al. Anti-infection peptidomics of amphibian skin. *Mol. Cell. Proteomics* 2007; **6**: 882–894.
11 Reshmy V, Preeji V, Parvin A, Santhoshkumar K, George S. Three novel antimicrobial peptides from the skin of the Indian bronzed frog *Hylarana temporalis* (Anura: Ranidae). *J. Pept. Sci.* 2011; **17**: 342–347.

 Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.

12 Liu X, Liu R, Wei L, Yang H, Zhang K, Liu J, Lai R. Two novel antimicrobial peptides from skin secretions of the frog, *Rana nigrovittata*. *J. Pept. Sci.* 2011; **17**: 68–72.
13 Ghavami S, Asoodeh A, Klonisch T, Halayko AJ, Kadkhoda K, Kroczak TJ, Gibson SB, Booy EP, Naderi-Manesh H, Los M. Brevinin-2R(1) semi-selectively kills cancer cells by a distinct mechanism, which involves the lysosomal-mitochondrial death pathway. *J. Cell. Mol. Med.* 2008; **12**: 1005–1022.
14 Conlon JM, Abraham B, Galadari S, Knoop FC, Sonnevend A, Pal T. Antimicrobial and cytolytic properties of the frog skin peptide, kassinatuerin-1 and its L- and D-lysine-substituted derivatives. *Peptides* 2005; **26**: 2104–2110.
15 Hoskin DW, Ramamoorthy A. Studies on anticancer activities of antimicrobial peptides. *Bba-Biomembranes.* 2008; **1778**: 357–375.
16 Rozek T, Bowie JH, Wallace JC, Tyler MJ. The antibiotic and anticancer active aurein peptides from the Australian Bell Frogs *Litoria aurea* and *Litoria raniformis*. Part 2. Sequence determination using electrospray mass spectrometry. *Rapid Commun Mass Sp.* 2000; **14**: 2002–2011.
17 Abbasi MA, Mosaffa N, Taheri S, Molla jaafari Kh. Effect of Antibacterial skin secretion of "*Rana ridibanda*" frog on methycillin resistant Staph aureus. *Yakhteh Medical Journal.* 2007; **9**: 208–211.
18 Mehrnejad F, Naderi-Manesh H, Ranjbar B, Maroufi B, Asoodeh A, Doustdar F. PCR-based gene synthesis, molecular cloning, high level expression, purification, and characterization of novel antimicrobial peptide, Brevinin-2R, in *Escherichia coli*. *Appl Biochem Biotech.* 2008; **149**: 109–118.
19 Lehrer RI, Rosenman M, Harwig SSSL, Jackson R, Eisenhauer P. Ultrasensitive assays for endogenous antimicrobial polypeptides. *J. Immunol. Methods* 1991; **137**: 167–173.
20 Kim SS, Shim MS, Chung J, Lim DY, Lee BJ. Purification and characterization of antimicrobial peptides from the skin secretion of *Rana dybowskii*. *Peptides* 2007; **28**: 1532–1539.
21 Conlon JM. Reflections on a systematic nomenclature for antimicrobial peptides from the skins of frogs of the family Ranidae. *Peptides* 2008; **29**: 1815–1819.
22 Lai R, Zheng YT, Shen JH, Liu GJ, Liu H, Lee WH, Tang SZ, Zhang Y. Antimicrobial peptides from skin secretions of Chinese red belly toad *Bombina maxima*. *Peptides* 2002; **23**: 427–435.
23 Lu Y, Li JX, Yu HN, Xu XQ, Liang JG, Tian YQ, Ma DY, Lin GQ, Huang GQ, Lai R. Two families of antimicrobial peptides with multiple functions from skin of rufous-spotted torrent frog, *Amolops loloensis*. *Peptides* 2006; **27**: 3085–3091.
24 Kim S, Kim SS, Lee BJ. Correlation between the activities of α-helical antimicrobial peptides and hydrophobicities represented as RP HPLC retention times. *Peptides* 2005; **26**: 2050–2056.
25 Zairi A, Tangy F, Bouassida K, Hani K. Dermaseptins and magainins: antimicrobial peptides from frogs' skin—new sources for a promising spermicides microbicides—a mini review. *J. Biomed. Biotechnol.* 2009; **2009**: 452567.
26 Boman HG. Antibacterial peptides. Basic facts and emerging concepts. *J. Intern. Med.* 2003; **254**: 197–215.
27 Mangoni ML. Temporins, anti-infective peptides with expanding properties. *Cell. Mol. Life Sci.* 2006; **63**: 1060–1069.
28 Mangoni ML, Rinaldi AC, Di Giulio A, Mignogna G, Bozzi A, Barra D, Simmaco M. Structure–function relationships of temporins, small antimicrobialpeptides from amphibian skin. *Eur. J. Biochem.* 2000; **267**: 1447–1454.
29 Zhou J, McClean S, Thompson A, Zhang Y, Shaw C, Rao P, Bjourson AJ. Purification and characterization of novel antimicrobial peptides from the skin secretion of *Hylarana guentheri*. *Peptides* 2006; **27**(12): 3077–3084.
30 Halverson T, Basir YJ, Knoop FC, Conlon JM. Purification and characterization of antimicrobial peptides from the skin of the North American green frog *Rana clamitans**. *Peptides* 2000; **21**(4): 469–476.
31 Conlon JM, Sonnevend A, Davidson C, Demandt A, Jouenne T. Host-defense peptides isolated from the skin secretions of the Northern red-legged frog *Rana aurora aurora*. *Dev. Comp. Immunol.* 2005; **29**(1): 83–90.
32 Conlon JM, Abraham B, Sonnevend A, Jouenne T, Cosette P, Leprince J, Vaudry H, Bevier CR. Purification and characterization of antimicrobial peptides from the skin secretions of the carpenter frog *Rana virgatipes* (Ranidae, Aquarana). *Regul Peptides* 2005; **131**: 38–45.
33 Park JM, Jung JE, Lee BJ. Antimicrobial peptide from the skin of a Korean frog, *Rana rugosa* (Vol 205, Pg 948, 1994). *Biochem Bioph Res Co.* 1995; **209**: 775.
34 Suzuki. Isolation and characterization of novel antimicrobial peptides rugorsins A,B and C from the skin of the frog, *Rana rugosa*. *Biochem. Biophys. Res. Commun.* 1995; **212**: 249–254.
35 Park S, Park SH, Ahn HC, Kim S, Kim SS, Lee BJ, Lee BJ. Structural study of novel antimicrobial peptides, nigrocins, isolated from *Rana nigromaculata*. *FEBS Lett.* 2001; **507**: 95–100.

 Copyright © 2011 European Peptide Society and John Wiley & Sons, Ltd.