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Purification and Characterization of an Antibacterial, Antifungal and Non Hemolytic Peptide from *Rana Ridibunda*

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Abstract

Amphibians have a large variety of antimicrobial peptides that serve as natural innate barriers limiting microbial infection or, in some instances, act as an integral component in response to inflammation or microbial infection. A novel peptide with antibacterial effects and without hemolytic activity was purified from skin secretions of *Rana ridibunda* by multisteps cation-exchange FPLC, reversed-phase HPLC and was called Ridibundin 1. Circular dichroism spectra revealed that this peptide strongly prefers to form an amphipathic α-helical structure in the presence of 50% trifluoroethanol. Acting as wide-spectrum microbicides against a variety of bacteria, Ridibundin 1 also shows no hemolytic activity on erythrocyte. These properties reveal its unique characteristics and potential therapeutic application.

Keywords: Antibacterial peptide; Hemolysis; *Rana ridibunda*

1. Introduction

Antimicrobial peptides (AMPs) are essential defense components of both invertebrates and vertebrates. Amphibians have a rich set of chemical arsenals as an integral part of their defense system and regulation of their dermal physiological actions. In response to a variety of stimuli, host defense compounds are secreted from specialized glands on to the dorsal surface or on the gut of the amphibian [1-3]. So far more than two-dozen of peptides have been reported in a single host [4]. They display a large amount of heterogenesity in primary and secondary structures but share common features that seem to underlie their cytotoxic function, such as amphipathy and net positive charge.

Following well-advertised magainins from the skin of *Xenopus laevis*, a number of cationic peptides from various amphibians have been isolated and found to have a broad-spectrum of antimicrobial activity [2]. Dermaceptins, a large family of AMPs expressed in the skin of Phillomedusa tree frogs [5], Bombinins from Bombina species [6-8], Temporin-1Od and temporin-1Ja, isolated from *Rana ornativentris* and *R. japonica*, respectively [9,10]. More recently several potential AMPs have been isolated from amphibian such as japonicin-1 and japonicin-2 from *R. japonica* [9];

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nigrocin 1 and 2, from *R. nigromaculata* [11]; brevinin-20a, from *R. ornaventris* [10]; tigerinin 1, from *R. tigerina* [12]; pseudin-2, from *Pseudis paradoxa* [13]; maximim 1, from *Bombina maxima* [14]; XT-1, from *Xenopus tropicalis* [15], and distinctin, from *Phylomedusa distincta* [16].

With respect to the above-described AMPs, we conducted this project to isolate and characterize those from *Rana ridibunda*.

2. Materials and Methods

2.1. Materials

The strain used for antimicrobial activity was *Klebseilla pneumoniae* ATCC 13883 which was obtained from the Reference laboratory of Bu-Ali Hospital in Tehran (Iran). HPLC grade solvent, TFA and TFE were obtained from Merck Co. (Germany). Chemicals, culture media and related compounds were purchased from Sigma Co. (USA) and Pharmacia Biotech. Uppsala (Sweden).

2.2. Preparation of Skin Secretions

Adult male and female specimens of *Rana ridibunda* were collected from the Gonbad region of Golestan province in north east of Iran and maintained in captivity at the Tarbiat Modarres University. Secretions were obtained from parotoid and dorsal glands through a non-invasive electrical stimulation method [17]. The skin secretions were washed from the animals with 5 ml of 0.01% acetic acid solution. The mixture was centrifuged and the supernatant passed through double stack Amicon filter (cut off 10 kDa) and the utterance solution was collected and finally lyophilized.

2.3. Peptide Purification

The skin extract was dissolved in 50 mM acetate buffer pH 4.8 and chromatographed on a (1×10 cm) SP-Sepharose FF (Pharmacia Biotech. Uppsala Sweden) equilibrated with buffer A (50 mM acetate buffer pH 4.8). The column was eluted with a 10 column volume (CV) gradient of 50% buffer B (50 mM acetate buffer pH 4.8, 1 M NaCl) at a flow rate of 1 ml/min and fractions (2 ml) were collected. Absorbance was measured at 280 nm using a Pharmacia FPLC detector. Fractions named IV, was lyophilized and redissolved in 0.1% (V/V) trifluoroacetic acid (TFA)/water and injected into a (1×25 cm) Vydac 208TP710 C8 reverse-phase HPLC column (Separation group, Hesperia, CA, USA) equilibrated with 20% acetonitrile/water and 0.1% TFA. Elution was achieved with an initial 10-min wash in equilibrated solution and then 20-60% linear gradient of acetonitrile containing 0.085% TFA over 60 min at a flow rate of 2 ml/min. Absorbance was monitored at 214 nm. Selected fractions were further purified by reversed-phase HPLC on a (1×25 cm) VP Nucleosil 300, C18 reverse-phase HPLC column (Macherey-Nagel, GmbH, Co. Germany) with the same solvent system described above with successively lower gradient of acetonitrile at a flow rate of 1.5 ml/min. Finally, the purity of peptide was evaluated with an Aquapore RP-300, 7 micron (0.46×25 cm) column.

2.4. Antimicrobial Assays

The antimicrobial activity was examined during each purification step by radial diffusion assay [18]. *Klebseilla pneumoniae* was grown in 3% (W/V) trypticase soy broth (TSB) at 37°C overnight. To obtain mid-logarithmic phase microorganism, 50 µl of the culture was transferred to 50 ml of fresh TSB broth and incubated for an additional 2.5 h at 37°C. *K. pneumoniae* were centrifuged at 900× g for 10 min at 4°C, washed once with cold 10 mM sodium phosphate buffer (NaPB) pH 7.4 and resuspended in 10 ml of cold NaPB. The cell concentrations were estimated by measuring the optical density at 620 nm, and were based on the relationship of the OD_{620}, $0.2=5\times10^7$ CFU/ml. 2×10^6 *K. pneumoniae* were added to 10 ml of under layer agar broth (10 mM sodium phosphate, 1% (v/v), (TSB), 1% agarose, pH 7.4) and the agar was poured into a Petri-dish. Samples were added directly to 3-mm diameter wells that were made on the solidified under layer agar. After incubation for 3 h at 37°C, the under layer agar was covered with a nutrient-rich top agar overlay and incubated overnight at 37°C. The antimicrobial activity was evaluated by observing the suppression of bacterial growth around the 3-mm diameter wells.

2. 5. Antifungal Activity

In vitro antifungal assays were performed by radial diffusion assay. Sabouraud-2% dextrose broth (pH 5.6 at 25°C; Merck, Darmstadt, Germany) was used as the assay medium. *Candida albicans* freshly grown on slopes of Sabouraud dextrose agar were suspended in physiological saline, and the cell concentrations were estimated by measuring the optical density at 600 nm, and were based on the relationship of the OD_{600}, $0.9 \approx 10^7$ CFU/ml [19]. 10,000 CFU/ml of *C. albicans* were added to 10 ml of under layer agar broth (10 mM sodium phosphate, 1% (v/v), (SBD), 1% agarose, pH 7.4) and the agar was poured into a Petri dish. Samples

were added directly to 3-mm diameter wells that were made on the solidified under layer agar. After incubation for 3 h at 30°C, the under layer agar was covered with a nutrient-rich top agar overlay and incubated overnight at 30°C.

2.6. Hemolysis Assay

Hemolysis activity was assayed as described by Minn *et al.*, 1998, with a slight modification [20]. Three milliliters of freshly prepared sheep erythrocytes was washed with isotonic phosphate-buffered saline, pH 7.4 (PBS), until the color of the supernatant turned clear. The washed erythrocytes were then diluted to a final volume of 20 ml with the same buffer. Peptide sample (20 µl) serially diluted in PBS, were added to 180 µl of the cell suspension in microfuge tubes. Following gentle mixing, the tube were incubated at 37°C for 30 min and then centrifuged at 4000×g for 5 min. 100 µl of supernatant was taken, diluted to 1 ml with PBS, and the absorbance at 567 nm was determined. The relative optical density was compared with that of the cell suspension treated with 0.2% Triton X-100 as 100% hemolysis.

2.7. Polyacrylamide Gel Electrophoresis and Zymogram Analysis

In order to investigate protease inhibitory capacity of the peptide, Ridibundin 1 at constant concentration of 20 µg/40µl was treated by 5 µg trypsin for 20, 60, and 120 min at 37°C then loaded on a 7.5, 12 and 15% polyacrylamide gel. Tricine-SDS-PAGE experiment was done by the method of Schagger [21] and protein bands were detected by silver nitrate. For zymography, 1 mg/ml gelatin was added to running gel. Ridibundin 1 at constant concentration (20 µg/well) with 0, 4, 6, and 10 µg/well trypsin was loaded into the gel without heating. Protease active bands were detected by the method of Leber [22].

2.8. Circular Dichroism (CD)

CD spectra were measured in either 20 mM NaPB, or 50% (v/v) trifluoroethanol (TFE) in 20 mM NaPB, and were recorded on a JASCO J-715 spectopolarimeter (Japan) using solutions with peptide concentrations of about 0.1 mg/ml. The CD results were expressed as molar ellipticity [θ] ($deg.cm^2d.mol^{-1}$) based on a mean amino acid residue weight (MRW) assuming its average weight 110 [23]. The molar ellipticity was determined as [θ] = (θ. 100 MRW)/(cl), where c is protein concentration in mg/ml, l is the light path length in cm, and θ is the measured ellipticity in degree at wavelength λ. Noise in the data was smoothed using the Jasco J-715 soft ware including the fast Fourier-transform noise reduction routine which allows enhancement of most noisy spectra without distorting their peak shape [23].

3. Results

3.1. Separation and Purification of Small Cationic Peptides

Separations of small cationic peptides from other small non-cationic compounds were archived by SP-Sepharose FF as cation exchange chromatography (Fig. 1a). About 500 mg of crude extract was dissolved in 10 ml of start buffer A (50 mM acetate buffer pH 4.8) and loaded on the column (1×10 cm). Elution of peptides were accomplished with a linear gradient of 0-50% buffer B (50 mM acetate buffer, pH 4.8, 1 M NaCl) at a flow rate of 1 ml/min. Tubes with a volume of 2 ml were collected and grouped according to the absorbance at 280 nm as shown (horizontal bar). The total extract, approximately 32% corresponded to fraction I, 5.5% to fraction II, 44% to fraction III and 18.5% left to fraction IV. Different fractions were lyophilized and desalted for antibacterial activity (data not shown). Fractions II, III and IV showed antibacterial activity against *Klebseilla pneumoniae*. The antibacterial compounds presented in peak IV (Fig. 1a) were purified to homogeneity by the following multisteps procedure.

Fraction IV was subjected to Vydac semi-preparative C_8 reversed-phase column and eluted with a 10 min wash in 20% acetonitrile/water then a linear gradient 20-60% acetonitrile/water over 60 min at flow rate of 2 ml/min (Fig. 1b). Fraction labeled by horizontal bar was injected to Macherey-Nagel semi-preparative C_{18} reversed-phase column with a 30-40% linear gradient by the same solvent described above over 40 min (Fig. 1c). Only peak D from series of peak named A-E in Figure 1c was again injected to semi-preparative C_{18} reversed-phase column and eluted by the same conditions (Data not shown). A pure antibacterial peptide was obtained as judged by analytical C_8 reversed phase chromatography as shown in set of Figure 1c. Finally we named this peptide Ridibundin 1, due to the source named *Rana ridibunda.*

3.2. Antibacterial, Antifungal and Hemolysis Assay

Antibacterial activity was examined during each purification step by the radial diffusion assay on *Klebseilla pneumoniae*. Figure 2a showed antibacterial activity of isolated peptide against *K. pneumoniae* as an inhibition zone around a well contained 5 µg peptide

compare to 30 µg disk antibiotic Amikacin (AN). Antifungal effect of Ridibundin 1 was surveyed on *Candida albicans* by radial diffusion assay as shown in Figure 2b. At concentration of 3 µg/ml, Ridibundin 1 showed a fungicidal effect compared to 10 µg/well of Amphotericin B as standard antifungal agent. Hemolysis assay of the isolated peptide Ridibundin 1 was tested against sheep erythrocytes based on radial diffusion assay and release of hemoglobin assay that is shown in Figures 2c and 2d, respectively. This peptide had no appreciable (2.5%) hemolytic activity up to 200 µg/ml peptide (Fig. 2d).

3.3. Proteolysis and Zymogram

Tricine-SDS-PAGE and Zymography of Ridibundin 1 in the presence of trypsin showed that Ridibundin 1 not only has no protease inhibitory activity but also is very sensitive to protease digestion (Fig. 3). Ridibundin 1 in the presence of protease was fragmented to very small peptides, which were not detectable by general SDS-PAGE staining. This behavior could be expected from its high cationic residues content, Lys and Arg, which is a general characteristic of antimicrobial peptides.

3.4. CD Spectroscopy

The secondary structure content of Ridibundin 1 was calculated by circular dichroism (CD) in the absence and presence of TFE (Fig. 4). The CD spectrum of Ridibundin 1 in 50 mM NaPB indicated that the content of α-helix, β-sheet, turn and random coils were 9.3%, 19.9%, 38.2% and 32.5%, respectively. Under the hydrophobic condition of 50% TFE solution, the contents of α-helix, β-sheet, turn and random coils changed to 33%, 18.2%, 27% and 21.8%, respectively.

4. Discussion

We have purified cationic antimicrobial peptide Ridibundin 1, from skin of *Rana ridibunda* by mult-isteps FPLC and HPLC chromatography. Our results by radial diffusion assay showed that Ridibundin 1 had antimicrobial activity against gram negative bacteria and *C. albicans* (Fig. 2a, b). Circular dichroism studies showed that Ridibundin 1 has random coil conformation and would change to amphipathic α-helical structure at 50% TFE (Fig. 4). This structure is ideal for interacting with the membrane interface, the transition zone between the hydrophilic (and in the case of bacteria, net negatively charged) head groups and the hydrophobic fatty acyl chains.

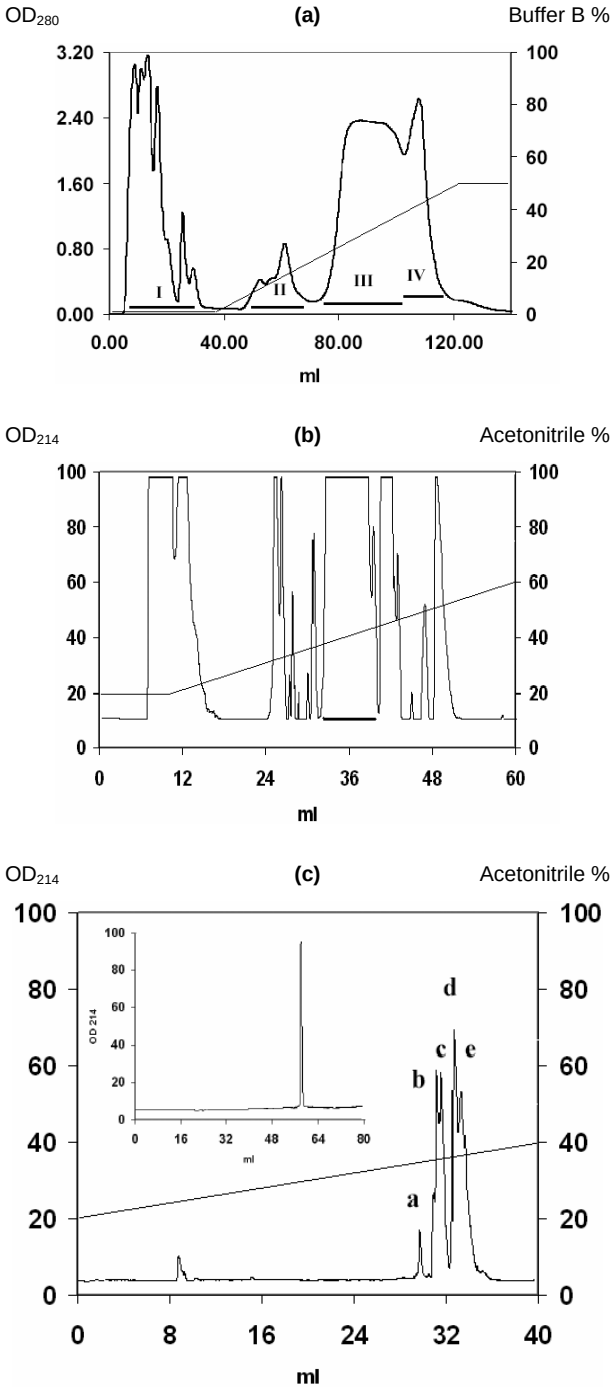

Figure 1. (**a**) Cation exchange of skin extract on a SP-Sepharose FF column. Only fraction IV was further characterized by HPLC. (**b**) Fraction IV (40 mg) was separated in a C8 reverse- phase semi- preparative column. The component labeled with a horizontal bar was subjected to further purification. (**c**) Horizontal bar labeled fractions of Figure 1b were separated in a C_{18} reverse-phase semi preparative column. The inset shows the HPLC profile of the pure component (fraction D of Fig. 1c) using an analytical C_8 reverse-phase column.

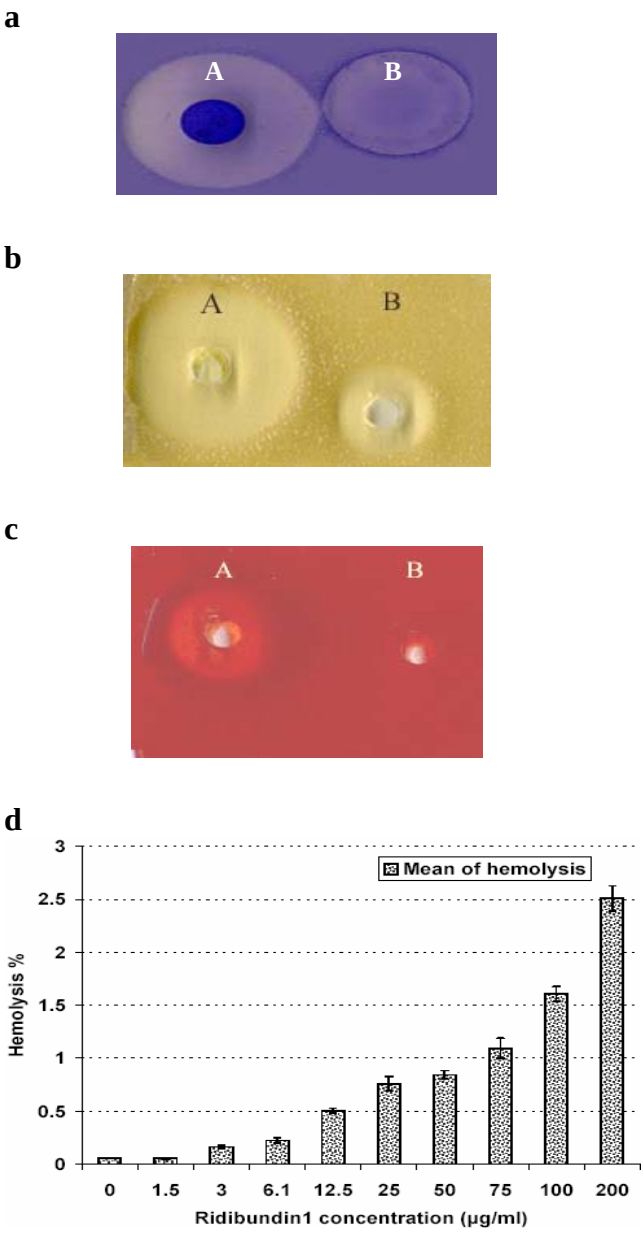

Figure 2. Antimicrobial activities against *Klebseilla pneumoniae*, antifungal effect against *Candida .albicans* and hemolytic effect of Ridibundin 1 on sheep erythrocytes. (**a**) A: Inhibition zone created by one disk 30 µg of amikacin (AN), B: shows suppression of bacterial growth around the 3-mm diameter well contains 5 µg/ml Ridibundin 1 peptide. (**b**) A: Inhibition zone created by 10 µg of Amphotericin B as positive control of antifungal agent. B: shows suppression of fungi growth around the 3-mm diameter well contains 3 µg/ml Ridibundin 1 peptide. (**c**) Shows hemolytic effect of 5 µl of 0.2% triton X100 on sheep erythrocytes blood agar plate (A), in comparison with non hemolytic effect of Ridibundin 1 at concentration of 10 µg/ml (B). (**d**) Only 2.5% hemolysis was observed upon 200 µg/ml of Ridibundin 1. Negative control was BPS and positive control was 0.2% triton X100.

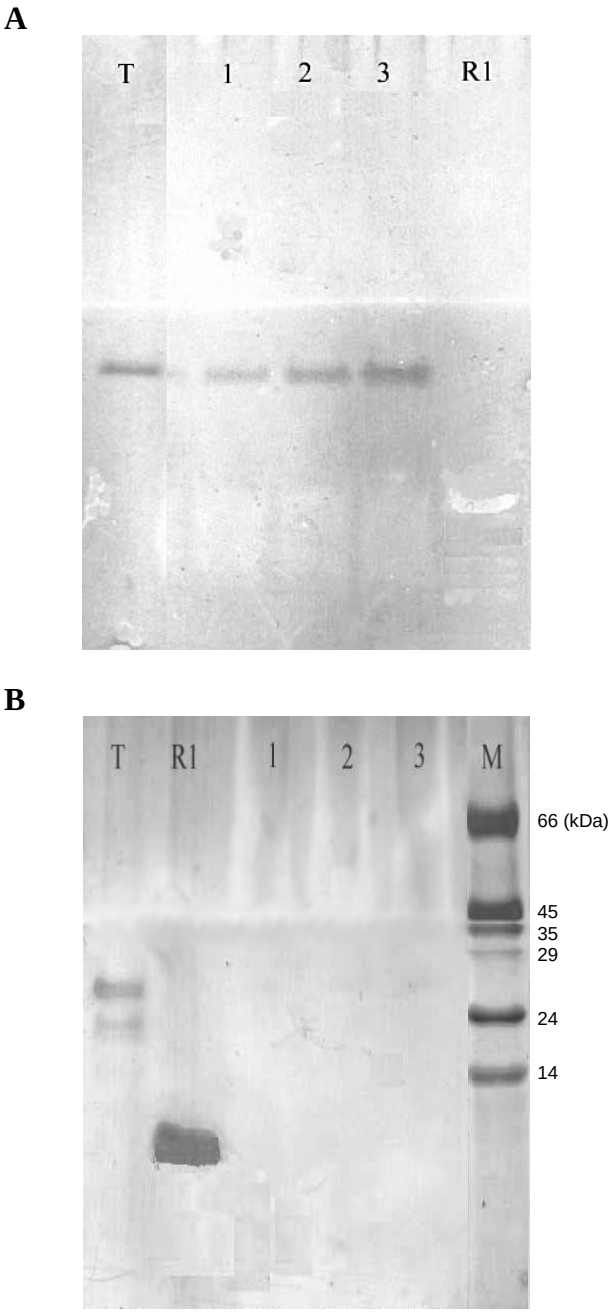

Figure 3. Tricine-SDS-PAGE and zymogram of Ridibundin 1 in absence and presence of trypsin. **A**) Zymogram of Ridibundin 1 in presence of different concentrations of trypsin. Lane T, was trypsin control, lane 1, 2, 3 were Ridibundin 1 that was treated by concentrations of 4, 6 and 10 µg/well gel of trypsin respectively, lane R1 was Ridibundin 1 control. **B)** Tricine-SDS-PAGE of Ridibundin 1 that was treated by trypsin and was incubated for different times. Lane T was control of trypsin; R1 was Control of Ridibundin 1; lanes of 1, 2, and 3 were Ridibundin 1 that were incubated with trypsin for the times of 20, 60 and 120 min, respectively; Lane M was size markers.

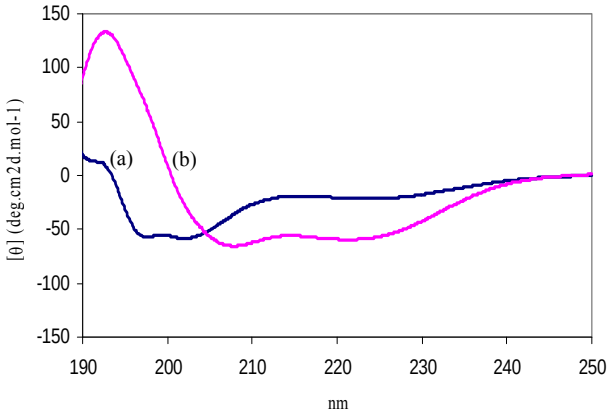

Figure 4. CD spectra of Ridibundin1 in 50 mM NaPB (a) or in the presence of 50% TFE (b).

A hallmark of most antimicrobial peptides is a membrane-permeabilizing activity upon initial interaction with the target membrane. Antimicrobial peptides disintegrate the cell membrane in a detergent-like manner. Antimicrobial activity and low level of hemolytic activity of Ridibundin 1 on sheep erythrocytes could be due to the differences in membrane compositions of its targets. It was suggested that the difference between bacteria and eukaryotic cells exists in the electrostatic properties of their surfaces [24,25]. Bacteria have negatively charged surface structures, such as lipopolysaccharides or lipoteichoic acids, and their membranes contain negatively charged phospholipid, such as phosphatidylglycerol. On the other hand, eukaryotic cells such as erythrocytes contain zwitterionic phosphatidylcholine. Therefore, the antimicrobial peptides with positively charged amino acid residues might selectively bind to the outer leaflet of the bacterial membrane rather than the eukaryotic membrane *via* the electrostatic interaction [26,27]. The low level of hemolytic activity of Ridibundin 1 supports the fact that, it must be small in size or have a low level of hydrophobicity. The low level of hydrophobicity seems to be a key factor for the differentiation of mammalian cells from bacteria cells because high level hydrophobic interactions have been reported to be necessary for the lysis of the erythrocyte membrane [26,27]. There are other cases too such as indolicidin, a very hydrophobic peptide, consisting of 13 amino acid residues, that has been reported to have hemolytic activity as well as antimicrobial activity [26].

The mechanism of antifungal action has been investigated for several antimicrobial peptides. The major cause of the loss of fungal cell viability is believed to be the result of the formation of transmembrane channels, which increase membrane permeability and cause disruption of the microbial cell structure. This is also believed to be generally applicable to the action mechanism(s) of antifungal peptides [28-30]. In contrast, some other antifungal peptides such as IBAMPs, derived from the seeds of *Impatiens balsamina,* do not cause pore formation in the plasma membrane, and therefore, have a different antibiotic mechanism, which involves DNA synthesis inhibition by interaction with a target protein [31]. By now, we don't know the antifungal mechanism of Ridibundin 1. Our results suggest that Ridibundin 1 has a potent antimicrobial activity but no hemolytic activity, which could make it useful as a topical antimicrobial agent, and a model peptide for studying the relationships between structure and antimicrobial activity. This needs further investigation to elucidate the general cytotoxic mechanism of Ridibundin 1 peptide.

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