BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **218,** 408–413 (1996)
Article No. 0071

A Novel Antimicrobial Peptide from *Bufo bufo gargarizans*

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Received November 24, 1995

A potent and structurally novel antimicrobial peptide was isolated and characterized from the stomach tissue of *Bufo bufo gargarizans,* an Asian toad. The 39-amino acid peptide, named buforin I, was purified to homogeneity by heparin-affinity column and reverse-phase HPLC. The amino acid sequence of buforin I was identical in 37 of 39 amino-terminal residues of Xenopus histone H2A. The buforin I showed strong antimicrobial activities *in vitro* against a broad-spectrum of microorganisms and was found to be more potent than magainin 2. In addition, a 21-amino acid peptide, named buforin II, which was derived from buforin I, showed more potent antimicrobial activities than buforin I.

In recent years, a number of antimicrobial peptides, which play one of the most important defense roles against pathogenic microorganisms, have been isolated from various biological sources (1). Melittin was one of the first antimicrobial peptides isolated from the venom of bees (2) and later bombinins were purified from an European frog (3). Steiner and Boman also found that a pupae of cecropia moth produced an antimicrobial peptide named cecropin (4). To date, about 2,000 antimicrobial peptides have been identified (1). These antimicrobial peptides carry net positive charges and most of them have the propensity to form an amphipathic α-helix structure (5,6,7). It was reported, even though the precise action mechanism of the antimicrobial peptides remains to be determined, that they rapidly disturbed the membrane function of pathogenic microorganisms (8,9,10).

Amphibians are rich in antimicrobial molecules, particularly in the antimicrobial peptides. More than 20 different antimicrobial peptides have been isolated from the skins and stomachs of *Xenopus laevis, Bombina,* sp., *Phyllomedusa,* sp., and *Rana* sp (1,5–6,10–11). These molecules are produced and stored in specialized granular glands in dermal structure or gastric mucosa (12). In this study, we report a novel antimicrobial peptide purified from the stomach of *Bufo bufo gargarizans,* an Asian toad, which has been used as a wound-healing agent in traditional Korean medicine.

MATERIALS AND METHODS

Peptide purification. Twenty adult toads (both male and female), captured in the wild in the middle part of the Korean peninsula, were choked by being left for 10 min in a desiccator filled with CO_2 gas. The stomachs were removed (tissue weight, 12.5g) and the toads were subsequently sacrificed. To minimize peptidases activities and maximize solubilization of peptides, the stomachs were homogenized in 120 ml of 1% (v/v) trifluoroacetic acid, 1 M HCl, 5% (v/v) formic acid, 1% (w/v) NaCl and pepstatin A at 1 μg/ml using a homogenizer (Polytron, Sweden) (12). The homogenate was then centrifuged at 20,000 × *g* for 30 min in a Himac SCR20BR (Hitachi, Japan) and the supernatant was collected. The peptides in the supernatant were then subjected to reverse-phase concentration using a Sep-Pak C18 cartridge (Waters associates, Milford, MA) which was activated with 80% acetonitrile containing 0.1% (v/v) trifluoroacetic acid and flushed with 0.1% (v/v) trifluoroacetic acid to remove the excess acetonitrile. After being loaded with the supernatant, the cartridge was washed with 20 ml of 0.1% (v/v) trifluoroacetic acid and the peptides trapped in the Sep-Pak C18 cartridge were eluted with 6 ml of 80% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid. The eluates were then lyophilized and subsequently resuspended in 10 ml of 0.01 M Tris-HCl (pH 7.5) containing 0.01 M NaCl and were loaded onto a 1.0 × 10 cm heparin sepharose column (Pharmacia, Uppsala, Sweden) equilibrated with 0.01 M Tris-HCl (pH 7.5) containing 0.01 M NaCl (13). The peptides were eluted by 0.5 M NaCl and the active fraction pools were further purified by HPLC on a 3.9 × 300 mm Delta Pak C18 column (Waters associates, Milford, MA) with a linear gradient of 0% buffer A to 50% buffer A at 1 ml/min for 1 h (buffer A; acetonitrile containing 0.1% (v/v) trifluoroacetic acid). Each peak was collected and antimicrobial activity of the each peak was tested after dried under the vacuum. The purity of the isolated peptide was assessed by reverse-phase

0006-291X/96 $12.00

HPLC on the same column used for separation of the active peptides under a slower gradient (50% buffer A for 2 h) and a matrix associated laser desorption ionization (MALDI) mass spectroscopy (Kartos Kompact MALDI, England).

Antimicrobial assays. The antimicrobial activity was examined during each purification step by the ultrasensitive assay using *Bacillus subtilis* described by Lehrer *et al.* (11). *B. subtilis* was grown in 3% (w/v) Trypticase soy broth (TSB) at 37 °C overnight. To obtain mid-logarithmic phase microorganisms, 50 μl of the culture was then transferred to 50 ml of fresh TSB broth and incubated for an additional 2.5 h at 37 °C. 6. *B. subtilis* cells were centrifuged at 900 × *g* for 10 min at 4 °C 6, 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 based on the relationship of OD_{620} $0.2 = 5 \times 10^7$ CFU/ml. 1×10^6 *B. subtilis* cells were added to 6 ml of underlayer agar broth (10 mM sodium phosphate, 1% (v/v) TSB, 1% agarose, pH 6.5) and the agar was poured into a Petri-dish. Samples were added directly to 3-mm-diameter wells that were made on the solidified underlayer agar. After incubation for 3 h at 37 °C, the underlayer agar was covered with a nutrient-rich top agar overlay and incubated overnight at 37°C. The antimicrobial activities were assayed by observing the suppression of bacterial growth around the 3-mm diameter wells. The antimicrobial spectra of peptides were determined by measuring minimal inhibitory concentrations against several Gram-positive and Gram-negative bacteria and fungi.

Molecular weight determination and sequence analysis of antimicrobial peptides. The molecular weights of antimicrobial peptides were determined by MALDI mass spectroscopy. The lyophilized peptides were dissolved in 50% acetonitrile containing 7% (w/v) sinapinic acid and mixed with a Pt probe. After removing the solvent in warm air, the peptides adsorbed to the Pt probe were applied to a vacuum chamber and analyzed. Sequencing was performed by the automated Edman degradation method on an Applied Biosystem gas phase sequencer, Model 447 (Foster city, CA, USA).

Quantification of peptide. The amounts of peptides were determined by amino acid analysis. The lyophilized peptides were hydrolyzed in 6 N HCl for 24 h at 110°C and converted to their phenylthiocarmayl derivatives. Samples were then analyzed using a Pico-tag analysis system on a Beckman 121 MB amino acid analyzer (Fullerton, CA, USA).

Endoproteinase Lys-C cleavage of the antimicrobial peptide. 60 μg of the isolated antimicrobial peptide was dissolved in 0.1 M NH_4HCO_3 buffer (pH 8.3) containing 2 M urea and 1 μg endoproteinase Lys-C (Boehringer Mannheim, Germany), and the mixture was incubated at 37°C for 11. The resulting fragments were directly separated by reverse-phase HPLC under the condition identical to that used for the purification of the toad stomach peptides and the antimicrobial activity of the each fragment was examined.

Peptide synthesis. A 21-amino acid peptide derived from the isolated antimicrobial peptide was synthesized by the solid-phase synthesis method on a Milligen 9050 Pepsynthesizer according to fluoren-9-ylmethoxycarbony (Fmoc)-polypeptide active ester chemistry. The product was purified by reverse-phase HPLC and the homogeneity was assessed by reverse-phase HPLC, amino acid analysis, and MALDI mass spectroscopy.

RESULTS

Purification of toad stomach antimicrobial peptides. Toad stomachs were homogenized in an acidic medium designed to maximize solubilization of peptides. Tricine-SDS-PAGE analysis results showed that the extracts were mostly composed of peptides of which molecular weights below 14,000 (Fig. 1, lane A). The acid extracts were fractionated on an heparin-affinity column. The

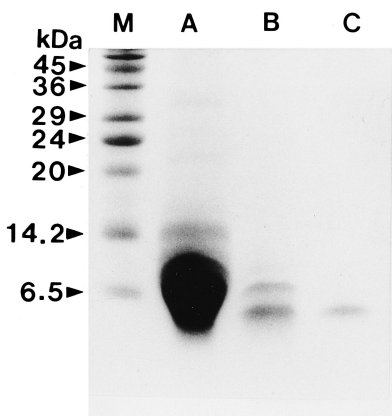

FIG. 1. Tricine–SDS–PAGE of toad stomach peptides. Lanes M, A, B, and C show the peptide size marker, toad stomach crude extracts, active fraction after heparin chromatography, buforin I isolated from reverse-phase HPLC, respectively.

active fraction was recovered by a step elution while removing a large number of non-antimicrobial peptides (Fig. 1, lane B). The active fraction was concentrated and further purified by reverse-phase HPLC (Fig. 2). Among several peaks, one which showed the strongest antimicrobial activity was pooled and lyophilized. The purified antimicrobial peptide was confirmed to be homogeneous by reverse-phase HPLC (Fig. 2), tricine-SDS-PAGE (Fig. 1, lane C) and MALDI mass spectra and was named as buforin I (Fig. 3). The total amount of purified buforin I recovered was 200 μg per g wet tissue of toad stomach. A 21-amino acid peptide, which also showed a strong antimicrobial activity, was obtained from 39-amino buforin I after treatment with endoproteinase Lys-C and was named as buforin II. The purified buforin I and buforin II were used for further chemical and biological analyses.

Primary structure determination. To determine the precise molecular weights, both buforin I and buforin II were subjected to MALDI mass spectroscopy from which the actual molecular weights were confirmed to be 4309 and 2432, respectively (Fig. 3). After amino acid analysis of buforin I and buforin II, the peptides were subjected to amino acid sequence analysis by an automated gas-phase amino acid sequencer. The complete sequences of buforin I and buforin II were Ala-Gly-Arg-Gly-Lys-Gln-Gly-Gly-Lys-Val-Arg-Ala-Lys-Ala-Lys-Thr-Arg-Ser-Ser-Arg-Ala-Gly-Leu-Gln-Phe-Pro-Val-Gly-Arg-Val-His-Arg-Leu-Leu-Arg-Lys-Gly-Asn-Tyr and Thr-Arg-Ser-Ser-Arg-Ala-Gly-Leu-Gln-Phe-Pro-Val-Gly-Arg-Val-His-Arg-Leu-Leu-Arg-Lys, respectively. The molecular weights, which were observed by the mass spectroscopy, were in agreement with the molecular weights calculated from the complete sequences of each peptide.

Antimicrobial activity of the isolated peptides and synthetic peptide. Both buforin I and buforin II showed strong antimicrobial activities. At the concentration of 30 μg/ml, *B. subtilis* lost viability

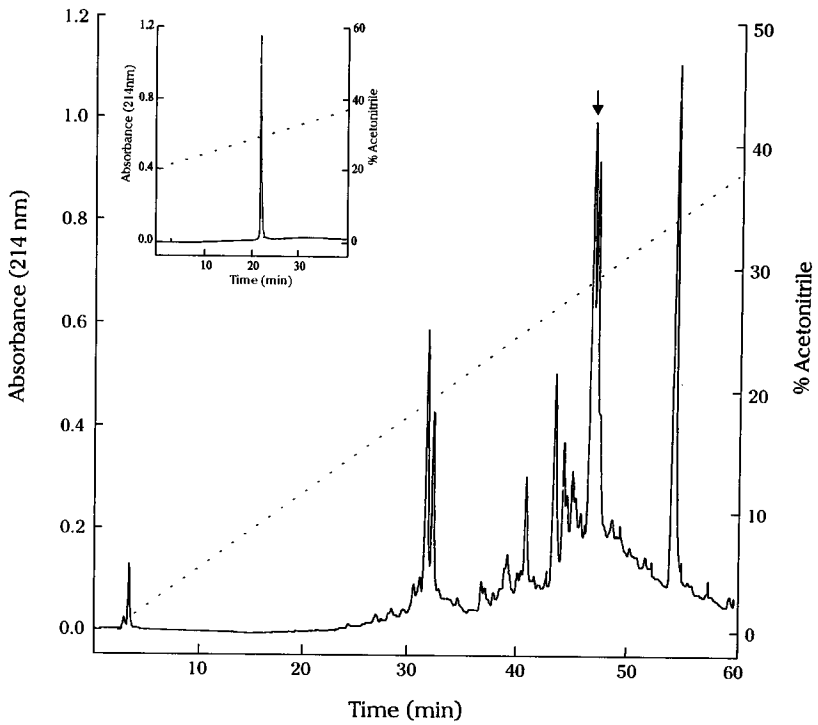

FIG. 2. Purification of buforin I. The active fraction after heparin chromatography was loaded on a Delta-pak (Waters, C18 3.9×300 mm) column and elution was achieved with a linear gradient of acetonitrile in aqueous trifluoroacetic acid (80% acetonitrile/0.1% TFA). The elution position of buforin I is indicated by an arrow. The left-hand inset represents the HPLC profile of the isolated buforin I with a slow gradient.

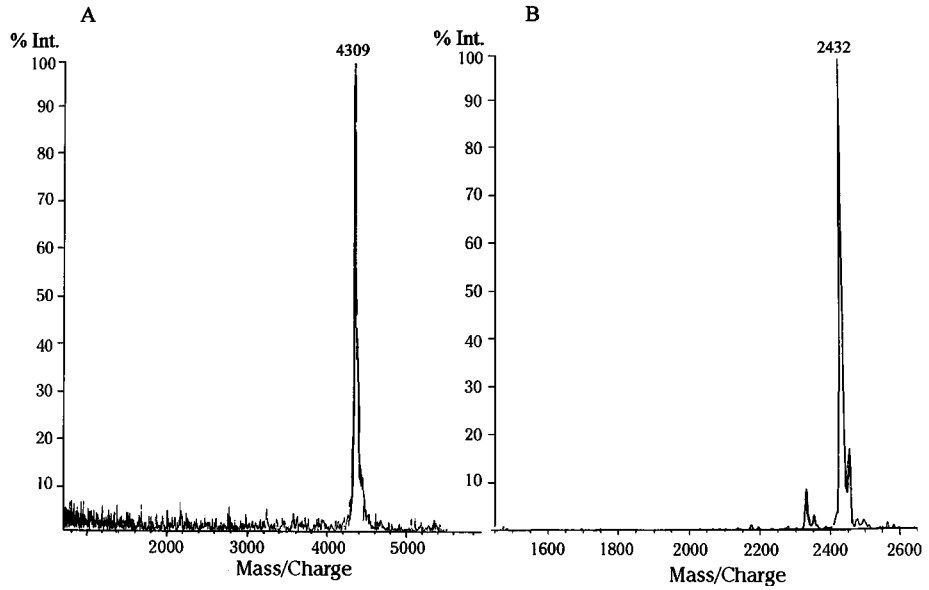

FIG. 3. Mass spectroscopic analysis of buforins. Masses for buforin I ($MH^+ = 4309$) (A) and buforin II ($MH^+ = 2432$) (B) were determined by MALDI mass spectrometer.

irreversibly within 5 min (data not shown). The antimicrobial spectrum of buforin I and buforin II were determined by measuring minimal inhibitory concentrations against several Gram-positive and Gram-negative bacteria and fungi. As shown in Table 1, both buforin I and buforin II displayed strong antimicrobial activities against a broad spectrum of bacteria including, *B. subtilis*, *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus pneumoniae*, *Escherichia coli*, *Serratia* sp., *Pseudomonas putida*, and *Salmonella typhimurium*. Furthermore, *Candida albicans*, *Saccharomyces cerevisiae* and *Cryptococcus neoformance* were also killed. And compared to magainin 2

TABLE 1
Antimicrobial Activities of Buforin I, Buforin II, and Magainin 2

Microorganism	Minimal inhibitory concentrations* (μg/ml)		
	Buforin I	Buforin II	Magainin 2
Gram positive			
Bacillus subtilis	4	2	50
Staphylococcus aureus	4	4	50
Streptococcus mutans	8	2	100
Streptococcus pneumoniae	4	4	50
Pseudomonas putida	4	2	50
Gram negative			
Escherichia coli	8	4	100
Salmonella typhimurium	4	1	25
Serratia sp.	8	4	50
Fungi			
Candida albicans	4	1	25
Cryptococcus neoformans	4	1	12
Saccharomyces cerevisiae	4	1	25

* Minimal inhibitory concentrations were determined by serial dilutions of antimicrobial peptides that inhibit the growth of microorganisms around 3-mm diameter wells. See Materials and Methods for details.

TABLE 2
Comparison of Amino Acid Sequences of Buforin I, Buforin II and N-terminal Xenopus Histone H2A

Peptide	Amino acid sequence
N-terminal of Xenopus histone H2A	S G R G K Q G G K T R A K A K T R S S R A G L Q F P V G R V H R L L R K G N Y A E R V -
Buforin I	A G R G K Q G G K V R A K A K T R S S R A G L Q F P V G R V H R L L R K G N Y
Buforin II	T R S S R A G L Q F P V G R V H R L L R K

Homologous residues between N-terminal of Xenopus histone H2A and buforin I are boxed and residues of buforin II in the buforin I are underlined.

purified from *Xenopus laevis,* buforin I and buforin II were approximately 10 times more potent against a broad range of microorganisms. Especially, buforin II was more active against fungi than buforin I. The synthetic buforin II was also as active as the one derived directly from buforin I.

DISCUSSION

The present study described the purification and characterization of an antimicrobial peptide from the stomach of Asian toad which show strong antimicrobial activities against a wide range of microorganisms including Gram-positive and Gram-negative bacteria and fungi. It seems that not all of the 39 amino acids of the buforin I is needed for the antimicrobial activity because the peptide fragment of 21 amino acids (buforin II) produced from the 39-amino acid buforin I after treatment with endoproteinase Lys-C was also as active as the buforin I. There was no difference in antimicrobial activities between natural buforin II and synthetic buforin II. Both buforin I and buforin II share a motif of amphipathic structure based on an Edmundson wheel diagram.

In the sequence comparison, it was found that buforin I was strongly homologous to N-terminal of Xenopus histone H2A (Table 2). Histones are nuclear proteins which are composed of core histones (H2A, H2B, H3, H4) forming nucleosome with 146 bp chromosomal DNA and H1 histone linker. The antimicrobial properties of histones have long been recognized even though their antimicrobial activities were not strong (14). A structural motif of histone, which strongly binds to DNA, may be used as an antimicrobial peptide to bind to cell membrane. Recent studies suggested that one main biological action of antimicrobial peptides such as defensin (15), bactenecin (16) was due to lipid and cell membrane bindings. And it is also known that several phospholipid binding proteins (bovine lung annexins and human serum lipoproteins) and peptide (tachyplesin I) can bind to DNA (17). One biologically active compound has often more than two kinds of macromolecule binding activities. Hence, it can be suggested that the isolated peptides adopted the histone-like structure to bind strongly to lipid or cell membrane which is necessary for antimicrobial activity. Recently, three antimicrobial proteins homologous to histone H1 and H2B were found from murine macrophages (18). But our result is the first one to find an antimicrobial peptide which is homologous to N-terminal of histone H2A. How was the histone H2A converted to 39-amino acid antimicrobial peptide? It seems that histone H2A, which is plentiful especially in dividing cells, is used as a defense protein after being cleaved by a protease. The replication dependent histone mRNA increases 30–50 fold during S-phase (19). It is likely that a limited number of histone H2A might be targeted to nucleus and the remaining histone H2A might be cleaved at a specific site in the cytosol resulting in the 39-amino acid antimicrobial peptide. To find an correct explanation for the homology of buforin I with N-terminal of histone H2A, more research is needed. Now we are working on finding a protease which cleaves histone H2A to produce buforin I. The relationship between histone H2A and antimicrobial peptide buforin I is under study.

ACKNOWLEDGMENTS

This work was partially supported by the grants from the Ministry of Education, Korea, KOSEF (Korea Science and Engineering Foundation) and RCNBMA (Research Center for New Bio-Materials in Agriculture).

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