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Tigerinins: Novel Antimicrobial Peptides from the Indian Frog *Rana tigerina**

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Korrapati Purna Sai‡§, Medicharla Venkata Jagannadham‡, Mariappanadar Vairamani¶, Namburi Prasada Raju¶, Ambure Sharada Devi‡, Ramakrishnan Nagaraj‡‖, and Narasimhaiah Sitaram‡‖

From the ‡Centre for Cellular and Molecular Biology, Uppal Road, Hyderabad, 500 007, India and the ¶Indian Institute of Chemical Technology, Uppal Road, Hyderabad 500 007, India

Four broad-spectrum, 11 and 12 residue, novel antimicrobial peptides have been isolated from the adrenaline-stimulated skin secretions of the Indian frog *Rana tigerina*. Sequences of these peptides have been determined by automated Edman degradation, by mass spectral analysis and confirmed by chemical synthesis. These peptides, which we have named as tigerinins, are characterized by an intramolecular disulfide bridge between two cysteine residues forming a nonapeptide ring. This feature is not found in other amphibian peptides. Conformational analysis indicate that the peptides tend to form β-turn structures. The peptides are cationic and exert their activity by permeabilizing bacterial membranes. Tigerinins represent the smallest, nonhelical, cationic antimicrobial peptides from amphibians.

Antimicrobial peptides constitute a very important component of the innate immune system in organisms across the evolutionary scale (1–8). Amphibians being the first group of organisms forming a connecting link between land and water are forced to adopt and survive in a variety of conditions laden with pathogenic microbes. Thereby, they are endowed with an excellent chemical defense system composed of pharmacological and antimicrobial peptides (9). Bombinins were the first antimicrobial peptides characterized from the skin of *Bombina variegata* in 1969 (10). The discovery of magainins from the skin secretions of *Xenopus laevis* in 1987 (11) triggered extensive search and characterization of antimicrobial peptides from amphibians (12–14). Antimicrobial peptides from genus *Rana* share an interesting structural motif composed of a disulfide-bridged cationic heptapeptide segment at the COOH-terminal end. Peptides with this motif include brevinins and esculentins which are composed of 24 and 46 amino acids, respectively (14). The primary structures of large number of peptides belonging to this family have been determined. Another group of short peptides composed of 13 residues called temporins, which do not contain this COOH-terminal ring, have also been characterized from frogs of genus *Rana* (15). However, considering the large variety of amphibian species in nature, antimicrobial peptides from only a small number of them have been characterized, that too only with respect to primary structure. Also, studies directed toward determining structure-function relationships have been confined to magainins (16) and dermaseptins (17, 18). Hence, characterizing host-defense peptides from other species would be of interest and could conceivably result in the identification of new structural motifs which would be useful in designing peptides for therapeutic applications. *Rana tigerina* is the predominant species of frogs found in India (19). The skin of these frogs have been used traditionally by some tribal communities to heal both open and burn wounds and the antimicrobial components could possibly contribute to the wound healing process (20). In this study, we have described the isolation and characterization of antimicrobial peptides from *R. tigerina*. These peptides are composed of only ~11–12 residues that do not have primary structural homology to any of the known antimicrobial peptides derived from amphibians. They are characterized by a disulfide-bridged loop composed of 9 amino acids. We have named these peptides as tigerinins.

EXPERIMENTAL PROCEDURES

Collection of Skin Secretions

The frogs of the species (*R. tigerina*) were stimulated to release peptides through adrenergic-mediated granular gland secretion by injecting 0.5 ml of 1 mM adrenaline (Loba Chemie) into the dorsal sacs. The secretions were collected from the dorsal surface in ethanol:water (3:1, v/v) and subsequently dried under reduced pressure so as to remove ethanol and redispersed in water acidified with 0.1% trifluoroacetic acid. Solid phase extraction on reverse phase (C_{18}) was carried out with the clarified homogenate which involved the pumping of the extract through eight Sep-Pak C_{18} cartridges (Waters Associates) connected in series at a flow rate of 1 ml/min. Bound material was sequentially eluted with 15, 30, and 60% of acetonitrile in acidified water and freeze dried. These fractions were evaluated for antimicrobial activity.

Purification of the Peptides

The frog skin secretions obtained after partial purification on Sep-Pak cartridges were redissolved in acidified water (HPLC[1] pure) and purified further on an analytical reverse-phase Water's μBondapak C_{18} column equilibrated with 0.1% (v/v) trifluoroacetic acid/water at a flow rate of 1 ml/min. The acetonitrile concentration in the eluting solvent was raised from 0 to 40% in 30 min and from 40% to 100% in 5 min. Fractions were collected according to detection at 210 nm and dried in a vacuum centrifuge and evaluated for antimicrobial activity.

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The peptide sequences of tigerinins reported in this paper has been submitted to the Swiss-Prot Database under Swiss-Prot accession numbers P82651 (tigerinin 1), P82652 (tigerinin 2), P82653 (tigerinin 3), and P82654 (tigerinin 4).

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‖ To whom correspondence should be addressed. Tel.: 91-40-7172241; Fax: 91-40-7171195; E-mail: nraj@ccmb.ap.nic.in or sitaram@ccmb.ap.nic.in.

[1] The abbreviations used are: HPLC, high performance liquid chromatography; MD, molecular dynamics; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; NPN, *N*-phenyl-1-*N*-naphthylamine; ONPG, *o*-nitrophenyl-3-D-galactoside; OM, outer membrane; IM, inner membrane.

Peptide Characterization

HPLC purified active peptide fractions were subjected to amino-terminal sequence analysis using a 473 A Applied Biosystems gas phase sequencer. Cysteine residues were identified by the alkylation of the HPLC purified active peptide fractions redispersed in pyridine buffer (pH 8.3) with iodoacetamide incubated in dark for 1 h at 37 °C. Prior to treatment with iodoacetamide, dithiothreitol was added at a concentration of 10-fold molar excess over expected disulfides and nitrogen was flushed to provide an inert atmosphere throughout the reaction (21). The reaction was terminated by using β−mercaptoethanol at a concentration of 10-fold excess to that of the peptide. The alkylated peptides were desalted, run on reverse phase C_{18} column and were subsequently characterized by protein sequencing and mass spectrometry. Alkylation with iodoacetamide without prior reduction with dithiothreitol was also carried out to ascertain the presence of disulfide bridges.

Mass Spectrometry

The HPLC purified active peptide fractions as well as iodoacetamide-treated fractions were acidified with 0.1% trifluoroacetic acid in water and mixed with α-cyano-4-hydroxycinnamic acid matrix and loaded on a stainless steel target and the molecular weights were determined using matrix-assisted laser desorption/ionization time of flight mass spectrometry (Kratos PC-Kompact MALDI 4VI.1.2).

COOH-terminal Analysis

The status of the carboxyl terminus was investigated by digestion of the natural peptides with carboxypeptidase Y that was pretreated with phenylmethylsulfonyl fluoride to inactivate endopeptidase and amidase activities (22). Aliquots of the enzyme digests were taken at 1- and 2-h time points and were subjected to amino acid analysis (LKB 4151 Alpha Plus Amino acid Analyzer).

Peptide Synthesis

Peptides identified were synthesized manually on amide crowns (Chiron technologies) by the solid phase method using Fmoc chemistry (23). All amino acids were added as Fmoc hydroxy benzotriazole active esters. The peptides were cleaved from the resin by treatment with trifluoroacetic acid/thioanisole/phenol/water/ethanedithiol (16.5:1:1:1:0.5, v/v) overnight at room temperature. The peptides were checked for purity on HPLC using a reverse phase column (Waters μBondapak C_{18}) using a solvent system of 0.1% aqueous trifluoroacetic acid and acetonitrile. The cysteines were deprotected with mercury (II) acetate in the ratio of 2 equivalents for each equivalent of cysteine. Mercuric

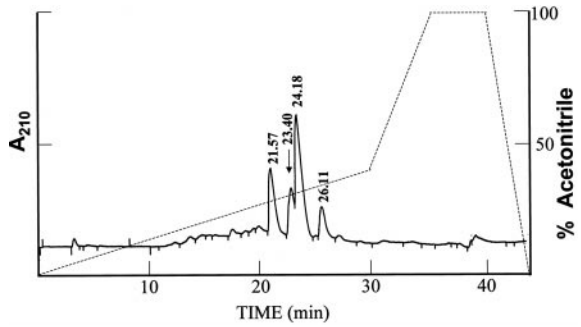

FIG. 1. **Reverse-phase HPLC of skin secretions of *R. tigerina*.** Analysis of adrenergic-mediated skin secretions of *R. tigerina* eluting between 15 and 30% acetonitrile concentration through Sep-Pak cartridges on a C_{18} μBondapak column. Conditions: Solvent A, 0.1% trifluoroacetic acid in water; B, 0.1% trifluoroacetic acid in acetonitrile; gradient of 0−40% B in 30 min and 40% to 100% in 5 min at a flow rate of 1 ml/min. Detection was at 210 nm.

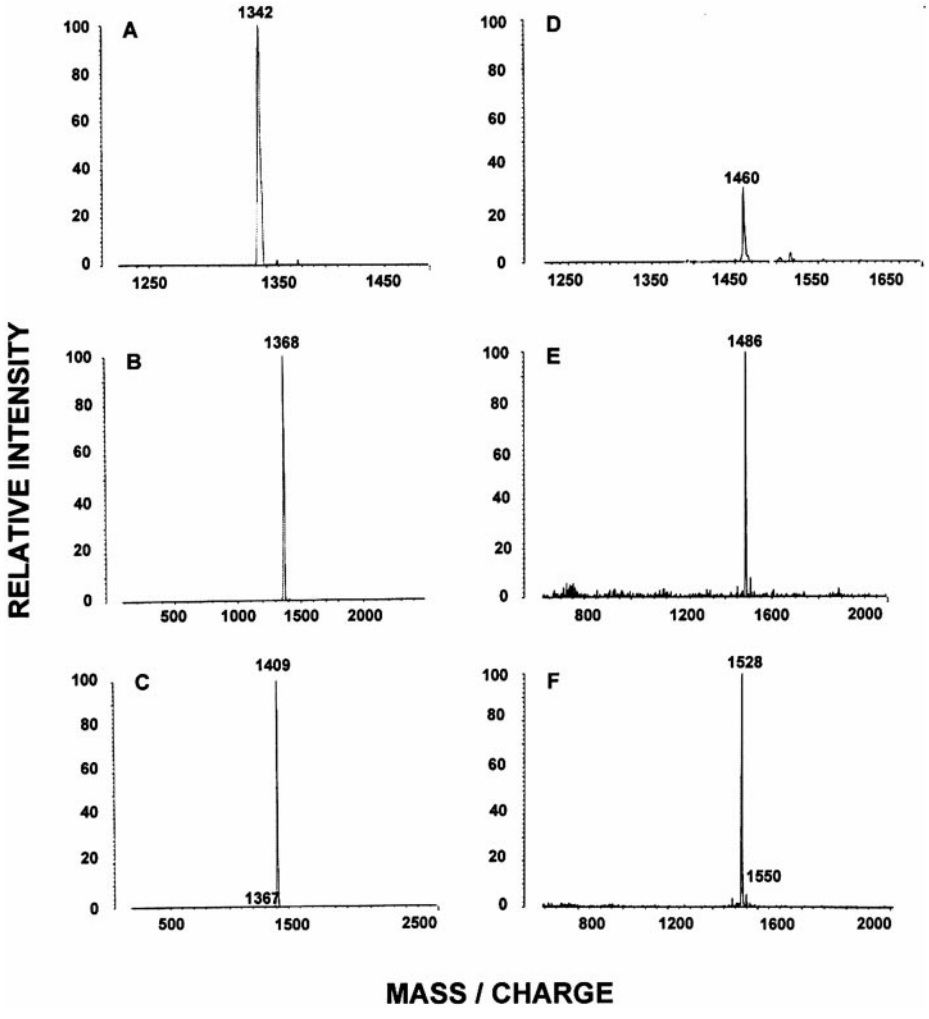

FIG. 2. **Mass spectroscopic analysis of peptides.** *A-C* are the mass spectra of the peaks obtained by HPLC with retention times 21.57, 23.40, and 24.10 min, respectively. *D-F,* correspond to mass spectra of the peptides in *A-C* after treatment with iodoacetamide and purified on HPLC.

sulfide salts formed were precipitated with 20 eq of β-mercaptoethanol (24). The peptides were then desalted and the disulfide bridges were formed with 20% dimethyl sulfoxide (25). The synthesized peptides were run on C_{18} reverse phase HPLC column independently as well as mixing with natural peptide fractions. The synthetic peptides were then used for evaluating antimicrobial activity.

Antimicrobial Activity

Minimal inhibitory concentration of the crude 15, 30, and 60% fractions was monitored by the decrease in turbidity at 600 nm of *Escherichia coli* W160.37 cells grown to logarithmic phase in minimal A medium (10.5 g of KH_2PO_4, 4.5 g of K_2HPO_4, 1 g of $[NH_4]_2SO_4$, 0.5 g of sodium citrate, 0.1 mM magnesium sulfate, 0.1 g of L-arginine, and 1% glucose in 1 liter of water) (26).

For evaluating antimicrobial activity of the purified peptides, liquid culture assays were carried out wherein varying concentrations of the peptides were added to 100 μl of suspension of the organisms diluted from a midlogarithmic phase liquid culture to a concentration of 10^5 cells/ml in sodium phosphate buffer (27, 28). The microbicidal activity was determined by counting the number of viable colony forming units on nutrient agar plates after 2 h of incubation with the individual peptides. The microorganisms used were *Pseudomonas putida, Micrococcus luteus, Bacillus subtilis, Staphylococcus aureus, E. coli,* and *Saccharomyces cerevisiae*. The kinetics of killing was also evaluated for *E. coli* and *S. aureus* by determining the viable cell counts as a function of time.

Outer Membrane Permeability

Outer membrane permeability was assessed by *N*-phenyl-1-*N*-naphthylamine (NPN, Sigma) uptake assay (29). *E. coli* W160-37 cells were grown to late logarithmic phase in bactonutrient broth (Himedia) and the cells obtained were washed twice with 5 mM HEPES buffer (pH 7.4). A 1-ml aliquot of cells so prepared, adjusted to an A_{600} of 0.5 in the same buffer containing 10 μM NPN was taken for each experiment. The excitation monochromator was set at 350 nm and the emission at 420 nm was continuously monitored after the addition of the peptide from an aqueous stock solution.

Inner Membrane Permeability

Inner membrane permeability was monitored by the *o*-nitrophenyl-3-D-galactoside (ONPG) influx as described by Lehrer *et al.* (30). In brief, *E. coli* W160-37 cells were grown to late logarithmic phase in bactonutrient broth (Himedia) in the presence of 5×10^{-4} M isopropyl thiogalactoside for inducing the cytoplasmic enzyme β-galactosidase. The culture was then diluted to an A_{600} of 0.03 with 10 mM sodium phosphate buffer (pH 7.4) containing ONPG that serves as a substrate. Aliquots of this were incubated with peptides at 37 °C and the influx of ONPG into the cells was monitored by absorbance measurements at 420 and 550 nm at fixed time intervals. ONPG influx into the cells recorded as $(A_{420} - 1.75 \times A_{550})$ reflects the permeability status of inner

TABLE I
Sequences of antimicrobial peptides from R. tigerina

Am, COOH-terminal amide. All the cysteine residues in tigerinins are linked by intramolecular disulfide bridge.

Peptide	Sequence
Tigerinin 1	FCTMIPIPRCY-Am
Tigerinin 2	RVCFAIPLPICH-Am
Tigerinin 3	RVCYAIPLPICY-Am
Tigerinin 4	RVCYAIPLPIC-Am

TABLE II
Antimicrobial activity of Tigerinins

Microorganisms	MIC			
	Tigerinin 1	Tigerinin 2	Tigerinin 3	Tigerinin 4
	μg/ml			
B. subtilis	30	20	30	NT[a]
S. aureus (ATCC 8530)	30	40	30	50
E. coli (W 160.37)	40	50	40	60
P. putida (NCIM 2102)	40	50	40	60
M. luteus (MT 166)	20	20	30	NT
S. cerevisiae (ATCC 8530)	80	100	80	100

[a] NT, not tested.

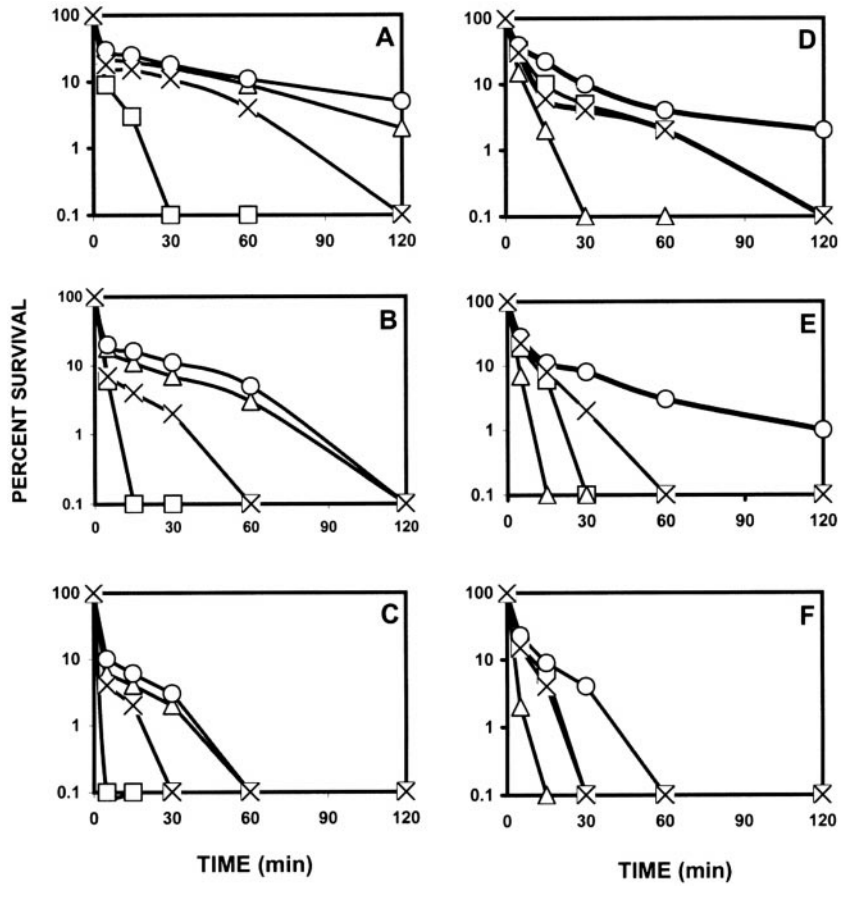

FIG. 3. **Kinetics of killing of bacteria by tigerinins.** *A-C, S. aureus; D-F, E. coli*. Cells in the midlogarithmic phase of growth (10^5 colony forming units) were incubated with different concentrations of tigerinins (*A* and *D,* 10 μg/ml; *B* and *E,* 20 μg/ml, and *C* and *F,* 30 μg/ml) and aliquots were drawn out at different intervals after incubation and were plated on nutrient broth. The number of colonies developed were counted after incubating the plates for 18 h at 37 °C. *Open square,* tigerinin 1; *open triangle,* tigerinin 2; *open circle,* tigerinin 3; *cross,* tigerinin 4. Cells incubated in the absence of any peptide served as controls.

membrane. ONPG influx was monitored also in the presence of divalent cation, calcium.

Hemolytic Activity

Hemolytic activities of the peptides were evaluated essentially as described earlier (31), using rat erythrocytes isolated from heparinized blood by centrifugation. The cells were washed three times with 5 mM HEPES buffer containing 150 mM sodium chloride. Aliquots of 1-ml suspension containing 10^7 cells in Eppendorf tubes were incubated with different concentrations of peptides in duplicates at 37 °C for 30 min with gentle mixing. The tubes were then centrifuged and absorbance of the supernatants was measured at 540 nm. The lysis obtained with water was considered as 100%.

Conformational analysis

Circular Dichroism (CD) Studies—CD spectra of the peptides were recorded in 5 mM HEPES buffer (pH 7.4) in a JASCO J-715 spectropolarimeter in 0.1-cm path-length cells at 25 °C. Calibration was carried out with d_{10}-camphor sulfonic acid. CD band intensities are represented as mean residue ellipticity.

Theoretical Studies—The starting structures were generated in extended conformation with $\phi,\psi = -180°$ for nonproline residues and $\phi,\psi = -75°, -180°$ for prolines using BIOPOLYMER module of MSI of version 98. The cysteines were bonded to form a disulfide bridge. These structures were minimized for a short duration to remove bad contacts. The structures were then optimized using a combination of minimizers like Steepest Descent, Conjugate Gradient, and Newton Raphson's methods for 3000 iterations till a final convergence of 0.001 was achieved. The Amber force field "amber.frc" provided in the MSI 98 (Biosym technologies, San Diego) was used for all calculations. The final structures were equilibrated for 10 ps duration before they were subjected to dynamics of 500 ps at a constant temperature of 298 K using a NVT ensemble temperature control method. The velocity-verlet integration method was used in this case. The final structures were once again minimized for 3000 iterations to achieve a convergence of 0.001. The optimization and Molecular Dynamics (MD) studies were conducted by using DISCOVER module provided in MSI 98. The dihedral angles for the final structures were calculated by using STRIDE software program developed by Patrick Argos of EMBL (32). The Procheck Program indicated that all the dihedral angles were in the allowed regions in the Ramachandran map.

RESULTS

Characterization of Peptides—The lyophilized material obtained from the adrenaline-stimulated skin secretions or the skin extracts of *R. tigerina* was initially processed on Sep-Pak cartridges. The fraction that eluted between 15 and 30% acetonitrile contained all the antimicrobial activity against *E. coli*. This fraction was subjected to further fractionation on a μBondapak C_{18} column. A chromatogram obtained is shown in Fig. 1. The peaks eluting at 21.57, 23.40, and 24.10 min exhibited antimicrobial activity against *E. coli*. Mass spectral analysis yielded mass values of 1342, 1368, and 1409, respectively (Fig. 2, *A-C*). Amino acid sequence analysis indicated the sequences as F_TMIPIPR_Y, RV_FAIPLPI_H, and RV_YAIPLPI_Y. To determine whether the gaps in the sequences could arise due to the presence of cysteine residues, the peptides were alkylated with iodoacetamide, purified on HPLC, and subjected to mass spectral analysis. The data shown in Fig. 2, *D-F,* indicate mass increases which correspond to the alkylation of two cysteine residues. When sequencing of the modified peptides were carried out, the alkylated cysteine residues eluted at the blank positions observed for unmodified cysteines. We have named these peptides as tigerinins. Treatment of tigerinins with carboxypeptidase Y did not result in the release of free amino acids under conditions where free amino acids were released from peptides with free COOH-terminal COOH groups, indicating that the COOH-terminal ends of tigerinins 1, 2, and 3 were amidated. The peptides on alkylation with iodoacetamide, without prior reduction with dithiothreitol, did not yield the alkylated derivatives of cysteine indicating that the two cysteine residues were linked by a disulfide bridge. The

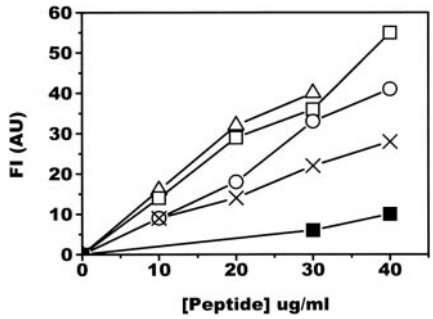

FIG. 4. **Peptide-mediated NPN uptake in *E. coli* W 160.37.** *E. coli* cells were incubated with NPN in the presence of various concentrations of tigerinins. Enhanced uptake was measured by an increase in fluorescence caused by partition of NPN in to hydrophobic interior of the OM. *Open square,* tigerinin 1; *open triangle,* tigerinin 2; *open circle,* tigerinin 3; *cross,* tigerinin 4; *filled square,* linear analog of tigerinin 1, tigerinin 1(cys-Acm).

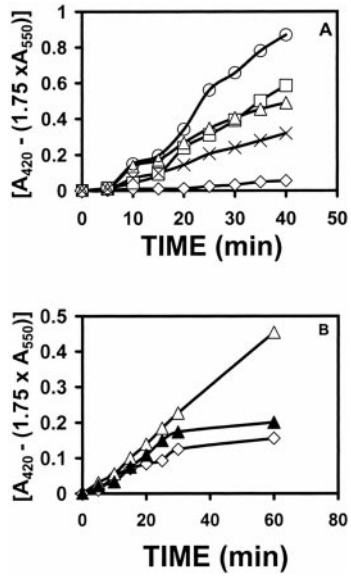

FIG. 5. **Effect of tigerinins on the influx of ONPG in *E. coli* W 160.37.** *E. coli* cells in the logarithimic phase of growth were diluted to a A_{600} of 0.03 in phosphate buffer containing ONPG and incubated at 37 °C with the peptides (40 μg/ml). The absorption at 420 and 550 nm were recorded at various time points. The value of $(A_{420} - 1.75 \times A_{550})$ was taken to denote ONPG influx. *Panel A: open square,* tigerinin 1; *open triangle,* tigerinin 2; *open circle,* tigerinin 3; *cross,* tigerinin 4; *diamond,* control without any peptide. *Panel B: open triangle,* tigerinin 2; *filled triangle,* tigerinin 2 in the presence of 600 μM Ca^{2+}; *diamond*, control without peptide.

sequences of the peptides were further confirmed by chemical synthesis. The synthetic peptides coeluted with their natural counterparts on HPLC and exhibited identical mass values. Reproducible HPLC profiles were obtained with different batches of skin secretions. When the skin extracts were processed using similar protocols, along with the other three peptides an additional peptide, which coeluted with tigerinin 3, with a mass of 1247 was obtained. On treatment with iodoacetamide, this peptide could be separated from tigerinin 3. On the basis of sequence and mass spectral analysis, the primary structure of this peptide is RVCYAIPLPIC-amide. The primary structures of the peptides are summarized in Table I.

Antimicrobial Activity—The minimal inhibitory concentrations of the natural tigerinins are shown in Table II. All the peptides exhibit activities against Gram-positive and Gram-negative bacteria as well as yeast with minimal inhibitory concentrations varying between 30 and 100 μg/ml. The bactericidal activity of tigerinins was investigated by studying the

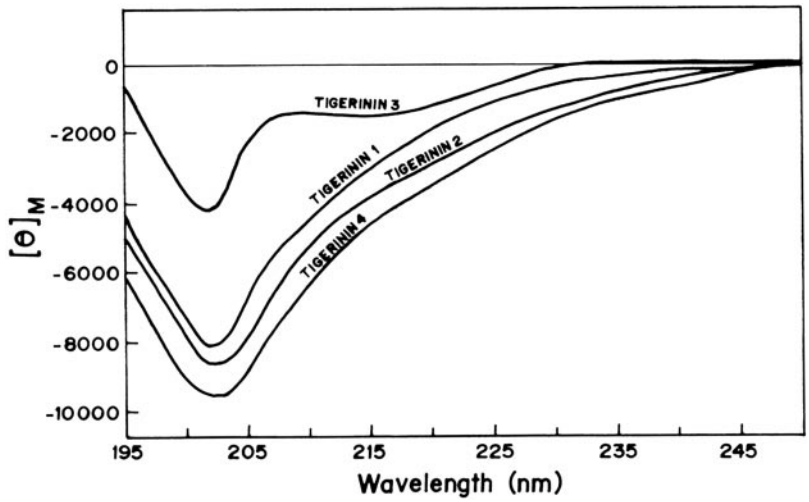

FIG. 6. **Circular dichroism spectra of tigerinins 1, 2, 3, and 4 in 5 mM HEPES buffer (pH 7.4).** Peptide concentration = 60 μM.

kinetics of killing of two representative organisms, *E. coli* and *S. aureus*. The results are shown in Fig. 3. In the first 5 min of incubation itself, between 60 and 80% of cells are killed in the case of all the peptides, even at the lowest concentration of 10 μg/ml. Complete killing is seen between 30 and 120 min. While tigerinin 1 is the most active peptide for *S. aureus*, tigerinin 2 is the most active on *E. coli*. All the peptides are bactericidal and killing is rapid.

The rapid killing of microbial cells by cationic peptides is generally mediated by membrane permeabilization (1–8) and hence the ability of tigerinins to permeabilize the bacterial membranes was examined. The extent to which *E. coli* OM becomes permeable to NPN in the presence of tigerinins is shown in Fig. 4. Tigerinin 1 is most effective in permeabilizing the OM of *E. coli* and tigerinin 2 and 3 are marginally less active with 50% permeabilizing concentrations (PC_{50}) of 12, 15, and 22 μg/ml, respectively. Tigerinin 4 also possesses considerable OM permeabilizing ability. However, the linear analog of tigerinin 1 (*i.e. S*-acetamido protected derivative) did not exhibit antimicrobial activity and also did not permeabilize the OM of *E. coli*. Hence, the S-S bridge appears to be essential for activity.

To determine whether tigerinins are capable of permeabilizing the inner (cytoplasmic) membrane (IM) of *E. coli*, the influx of the chromogenic substrate of the cytoplasmic enzyme β-galactosidase, ONPG in the absence and presence of the peptides was monitored. The data is presented in Fig. 5*A*. It is evident that tigerinins permeabilize the IM of *E. coli* effectively. As tigerinin 2 is the most effective, among tigerinins, in permeabilizing the IM of *E. coli*, the effect of stabilizing the OM with Ca^{2+} (33) on its IM permeabilizing ability was also investigated. The results are shown in Fig. 5*B*. Ca^{2+}, at a concentration of 600 μM, almost completely inhibited IM permeabilization by tigerinin. Tigerinins are thus unable to permeabilize a stabilized OM, suggesting that OM permeabilization is an essential and critical step for its activity.

Conformation of Peptides—The CD spectra of tigerinins 1–4 in buffer are shown in Fig. 6. All the peptides show a minimum ~204 nm with crossover at wavelengths <195 nm. The spectra indicate a population of unordered and β-turn conformations (34). The conformations of tigerinins 1, 2, and 3 were analyzed by theoretical methods involving energy minimization and Molecular Dynamics Simulations. Comparison of the structures are shown in Fig. 7. The dihedral angles of the structures obtained after 500 ps Molecular Dynamics Simulations for both the peptides with those documented for standard β-turn types (35) indicate that although β-turn structures are observed for tigerinin 1, 2, and 3, they are not of type I, II, or III and can be categorized as type IV.

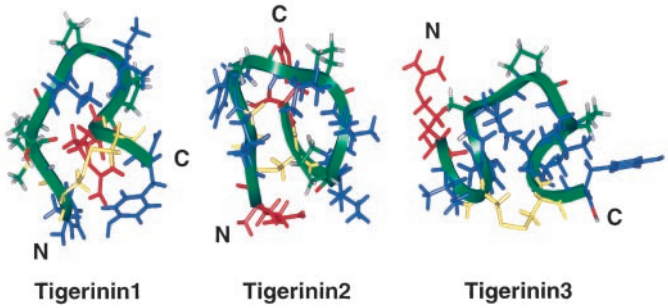

FIG. 7. **Structures of tigerinins after molecular dynamics simulations.** Side chains of cationic residues are indicated in *red* and hydrophobic residues in *blue*. Backbone structure is depicted as *green* ribbons. Cysteine side chains are in *yellow*.

DISCUSSION

We have described the purification and characterization of two novel 12-residue peptides and a 11-residue peptide, with broad-spectrum antimicrobial activity, from the skin secretions of *R. tigerina*. The susceptible microorganisms include Gram-positive and Gram-negative bacteria as well as yeast with minimal inhibitory concentrations in the range of 30 to 100 μg/ml. These three peptides exhibit high homology among themselves, with COOH-terminal amidation and a disulfide bridge between two cysteine residues to form a nonapeptide ring but are not related to any described previously antimicrobial peptides from amphibians. Extensive homology searches from the protein data banks did not yield any other peptide homologous to these peptides. These three peptides were also obtained from the skin extracts of *R. tigerina*. An additional peptide of 11 residues which lacked the COOH-terminal Tyr in tigerinin 3, but marginally less active than tigerinin 3 on all the organisms tested, was also obtained from the skin extracts. None of the peptides exhibited any hemolytic activity up to a concentration of 200 μg/ml. Tigerinins with 11 and 12 residues are the smallest antimicrobial peptides characterized from amphibians and are different from temporins which are linear peptides with 13 residues (15).

A large number of antimicrobial peptides have been characterized from skin tissue of amphibians (10–14). In the genus *Rana* itself peptides belonging to eight families based on their structural similarities, have been described. These are brevenin 1 and 2, esculentin 1 and 2, ranatuerins 1 and 2, ranalexin, and temporins (15, 36–41). Of these, the first seven families of peptides are all characterized by a highly basic, heptapeptide

TABLE III

Representative primary structures of antimicrobial peptides from amphibian skin

Am-amide. Cationic residues are in bold, hydrophobic residues are in bold italics. All the cysteine residues in cysteine containing rana peptides and tigerinins are linked by intramolecular disulfide bridge.

Name	Source	Sequence	Net charge at pH 7
Magainin 2	*X. laevis*	G***I***G***KFL***H**S**A**KK***F***G**KA*FV*GE***I***MNS	+3
Dermaseptin S1	*Phylomedusa sauvagil*	A***L***W**K**TM***LKKL***GTMA***L***H**AG**K**AA***L***GAAADT***I***SQGTQ	+3
Brevinin-1	*Rana brevipoda*	F***L***P***VL***AG***I***AA***KVV***PA***LF***C***KI***T**KK**C**	+4
Esculentin-1	*Rana esculenta*	G***IF***S***KL***G**RKK***I***K**N***LLI***SG***LK***N***V***G***K***E***V***GMD***VV***RTG***I***DAGO**KI**K)GEC	+6
Gaegurin 5	*Rana rugosa*	***FL***GA***LF******K******V***AS**K**V***L***PS***V***K**CA***ImT*****KK**C	+5
Ranalexin	*Rana catesbiana*	***FF***GG***LIKIV***PAM***I***P**K***I***F**C**K***I***T**RK**C**	+5
Ranateurin 4	*R. temporaria*	***FLPFI***AR***L***AA**K**V***F***PS***II***C)S***V***T**KK**C**	+4
Temporin A	*R. temporaria*	***FL***P***LI***G**R**V***L***SG***IL***-Am	+2
Tigerinin 1	*R. tigerina*	***F***CTM***I***P***I***P**R**CY-Am	+2
Tigerinin 2	*R. tigerina*	**R*V***C***F***A***I***P***L***P***I***C**H**-Am	+2
Tigerinin 3	*R. tigerina*	**R*V***CYA***I***P***L***P***I***C**Y-Am	+2

loop linked by a disulfide bridge at the COOH-terminal end, but with a highly variable sequence and length at the NH_2-terminal end. However, the presence of the disulfide bridge does not appear to be critical either for activity or for structure in the case of *Rana* peptides identified so far (42–45). The primary structures of tigerinins are compared with other amphibian peptides in Table III which evidently indicates no sequence homology. It is thus unlikely that tigerinins are derived from similar class of peptides from *R. tigerina*. All the amphibian antimicrobial peptides, including the *Rana* peptides, known thus far, are known to adopt helical structure (15, 36–41). Conformational analysis by CD and theoretical methods suggest β-turn conformations for tigerinins and these represent the first examples of nonhelical amphibian antimicrobial peptides. A majority of cationic antimicrobial peptides (1, 6, 12, 13), including amphibian skin peptides like magainin (16) and dermaseptins (17, 18), are known to exert their antimicrobial activity by permeabilizing the cytoplasmic membrane. Despite being structurally distinct from other amphibian antimicrobial peptides, tigerinins also are capable of permeabilizing bacterial membranes. While it is conceivable that tigerinins also exert their antimicrobial activity by permeabilizing the microbial membranes, other mechanisms cannot be ruled out. Indolicidin, a 13-residue Trp-rich antimicrobial peptide from bovine neutrophils, which is known to permeabilize the outer and cytoplasmic membranes of *E. coli* also preferentially inhibits synthesis of DNA leading to filamentation of cells (46). This later mechanism appears to contribute to its antimicrobial activity. Recently, many other cationic antimicrobial peptides have been proposed to kill bacteria by alternate mechanisms (47). Like all other amphibian antimicrobial peptides (10–14), tigerinins are also cationic (Table III). All of them carry a charge of +2 with 1 Arg residue and amidated COOH-terminal end. This low cationicity appears to be sufficient for its biological activity. Earlier we have shown that SPF, a 13-residue synthetic peptide corresponding to the most hydrophobic region of bovine seminal plasmin, with a charge of +1, has both antimicrobial and hemolytic activity (31). Apart from brevinins, another antimicrobial peptide, with a single disulfide bond, that is well characterized is thanatin from the hemipteran insect *Podisus maculiventus*. Thanatin is a 21-residue peptide with 50% homology to brevinins (48). However, in contrast to a heptapeptide loop at the COOH-terminal end of brevenins, thanatin has an eight-membered ring. Despite the homology, the secondary structure and mechanism of action differs from that of brevenin. Thanatin appears to assume a β-turn structure stabilized by a disulfide bond and is suggested to exert its action by a nonpore forming mechanism.

The only other short antibacterial peptide with a similar nonapeptide disulfide linkage is bactenecin which has been isolated from bovine neutrophils (49). However, bactenecin has 4 arginine residues as compared with one in tigerinins and therefore considerably more cationic. Reduction of the single disulfide bond in bactenecin was observed to result in a drastic change in the antibacterial spectrum (50). The reduced bactenecin showed high selectivity for Gram-positive bacteria with little activity against Gram-negative bacteria whereas native bactenecin was more active against Gram-negative bacteria. In tigerinin, absence of disulfide bridge resulted in loss of antibacterial activity indicating the importance of disulfide bridge for activity. It is very unlikely that tigerinins are related to bactenecins.

Thus, this paper describes a family of short and nonhelical antimicrobial peptides in amphibians. As short peptides have the obvious advantage of easy chemical synthesis, the new structural motif observed in tigerinins would be easily amenable for the synthesis of analogs with improved activity and conceivably useful against multidrug-resistant microbes. We are currently investigating other pharmacological activities of peptides from the skin secretions of *Rana tigerina*.

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