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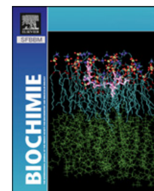

Research paper

Novel families of antimicrobial peptides with multiple functions from skin of Xizang plateau frog, *Nanorana parkeri*

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ABSTRACT

Xizang plateau frog (*Nanorana parkeri*) captured in Lhasa, Tibet, China, solely lives in the subtropical plateau, where there is strong ultraviolet radiation and long duration of sunshine. Considering its harsh living environment, the frog's innate defense against microbes and environmental stress was investigated. In current study, three antimicrobial peptides (AMPs) were purified and characterized from the skin secretion of *N. parkeri*. The coding cDNA sequences were also cloned from the skin cDNA library of *N. parkeri*. By structural characterization, two peptides were identified belonging to Japonicin-1 family, and named as Japonicin-1Npa (FLLFPLMCKIQGKC) and Japonicin-1Npb (FVLPLVMCKILRKC). The third peptide isolated named Parkerin with a unique sequence of GWANTLKNVAGGLCKITGAA did not show similarity to any known amphibian AMPs. Multi-functions of three AMPs were examined (antioxidant, MCD, hemolytic etc). Their solution structures determined by CD and antimicrobial mechanisms investigated by SEM are very well consistent with their functional characters. Current result suggests that these novel multi-functional AMPs could play an important role in defending *N. parkeri* against environmental oxidative stress and pathogenic microorganisms, which may partially reveal the ecological adaptation of these plateau-living amphibians.

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1. Introduction

Over the past several decades, extensive studies have focused on the bioactive compounds present in amphibian skin secretions, especially the Ranidae family [1]. These compounds include a lot of pharmacological peptides with a variety of functions, such as regulatory or hormonal [2,3], and antimicrobial [4,5]. The antimicrobial peptides (AMPs) are an important part of the amphibian innate immune system against the invasion of environmental microorganisms [6]. So far, there have been hundreds of AMPs of different families characterized from ranid frogs, including temporins, brevinins, esculentins, etc. [7]. The structural diversity of AMPs from ranid frogs is quite remarkable with virtually no single peptide from one species identical with another. But most of them share a conserved disulfide-bridged heptapeptide segment at the C-terminal end [7,8], and a common highly conserved N-terminal preproregion, followed by a markedly different C-terminal domain corresponding to the mature AMPs, constantly cationic with relatively hydrophobic nature and α-helical structure [9].

Xizang plateau frog, *Nanorana parkeri*, is mainly found in China and Nepal. Its sole habitat is the eastern and southern Tibet plateau in subtropical zone, where there is strong ultraviolet radiation and long duration of sunshine. Thus, to protect itself from oxidative stress and pathogenic microorganisms from both endogenous and exogenous sources, *N. parkeri* may have developed various mechanisms to cope with these harmful factors. It is rational to hypothesize that skins of *N. parkeri* may have potent free radical scavenging ability combing antimicrobial activity for their survival. In current study, we isolated three novel AMPs from the skin secretion of *N. parkeri*. By structural characterization, they are grouped into Japonicin-1 family firstly found in Japanese brown frog *R. japonica* [10], and named as Japonicin-1Npa and Japonicin-1Npb. The third

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one, named Parkerin, is a brand new amphibian AMP family, and firstly reported here. All three peptides were found to be multi-functional, combining antioxidant (free radical DPPH scavenging), mast cell degranulation and antimicrobial activities.

2. Materials and methods

2.1. Collection of frog skin secretions

Adult specimens of *N. parkeri* ($n = 20$) were captured in Lhasa, Tibet Autonomous Region, China. They were put into a cylinder container and stimulated gently with an electrical device (10 V, with pulse duration of 3 ms). The skin secretions were collected by washing the dorsal region with 0.1 M NaCl containing 0.01 M EDTA. Totally 200 ml solution was collected and centrifuged at 12 000 rpm for 20 min. The supernatant was removed, lyophilized and stored at −20 °C.

2.2. Peptide purification and sequencing

Lyophilized sample (0.8 g, total OD280 nm of 300) was dissolved in 10 ml 0.1 M phosphate buffer saline (PBS), pH 6.0, containing 5 mM EDTA and then applied to a Sephadex G-50 (Superfine, Amersham Biosciences, 2.6 cm × 100 cm) column equilibrated with 0.1 M PBS, pH 6.0. Elution was performed using the same buffer with collecting fractions of 3.0 ml, and monitored at 280 nm. The antimicrobial activity of fractions was screened, and interesting peaks were further purified by C18 reversed phase high performance liquid chromatography (RP-HPLC, Hypersil BDS C18, 30 cm 0.46 cm) column. Complete peptide sequencing was determined by Edman degradation on an Applied Biosystems pulsed liquid-phase sequencer, model 491. Mass fingerprints (MFPs) of purified AMPs were obtained using electrospray ionization, quadrupole orthogonal time-of-flight mass spectrometry (ESI-QTOF-MS Applied Biosystems/MDS Sciex Toronto, Canada) instrument.

2.3. Screening of cDNA encoding AMPs

The dorsal skin of frog was removed and cut into small pieces, which then were quickly frozen with liquid nitrogen and grinded to powder. mRNA was isolated using a mRNA isolation kit (Dynal Biotec., UK), and cDNA library was constructed using a Creator™ SMART™ cDNA Library Construction Kit (Clontech). According to the conserved signal peptide domain of previously characterized AMPs from ranid frogs, a sense oligonucleotide primer (5′-CCCCAT GTTCACCTTGAAG-3′) was designed and coupled with 3′ antisense primer (5′-ATTCTACAGGCCGAGGCGGCCGACATG-3′) supplied by the Kit to screen the cDNA Library. The PCR procedure was: 5 min of denaturation at 94 °C; 30 cycles: denaturation at 94 °C for 30 s, primer annealing at 56 °C for 30 s, extension at 72 °C for 1 min. The PCR product was purified by gel electrophoresis, cloned into pGEM-T vector (Promega Corporation).

2.4. Circular dichroism (CD) spectroscopy

To investigate the secondary structure of the peptides, CD spectroscopy was performed using a Jasco J-715 spectro-photometer. Samples of 0.5 mg/ml were prepared in three different solvents: water, 50% (v/v) sodium dodecyl sulfate (SDS)–water, 50% (v/v) trifluoroethanol (TFE)–water, then added in a quartz optical cell with a path length of 0.5 mm at 25 °C. The spectra were averaged over three consecutive scans, followed by subtraction of the CD signal of the solvent.

2.5. Antimicrobial assay and bacteria killing kinetics

Standard and clinical-isolated drug-resistant strains of bacteria and fungus used in assays were listed in Table 1. The assay was conducted as described in our previous paper [4]. Minimal inhibitory concentration (MIC) was determined in 96-well microtiter plate by a standard dilution method. Bacteria were incubated in Mueller–Hinton broth (MH) at 37 °C to exponential phase of growth and diluted with fresh MH broth to 10^6 CFU/ml. 50 μl of serial dilutions of the peptides in MH were prepared in 96-well microtiter plates and mixed with 50 μl of bacteria inoculum. Plates were incubated at 37 °C for 18 h and the minimal concentration at which no visible growth occurred was recorded.

The bactericidal effects of three peptides against *Staphylococcus aureus* were tested at 1, 5 and 10 times of their corresponding MICs, ampicillin as the positive control. Fresh overnight colonies were added to a total of 20 ml MH, and incubated at 37 °C for 1 h. Then, 1 ml broth from each flask was replaced with the test solution. The colony counting was performed at different intervals of time [11].

2.6. Scanning electron microscopy (SEM)

S. aureus (ATCC2592) was incubated with the AMPs (1×, 10× MIC) at 37 °C for 30 min. Aliquots of the cultures were fixed with 6% glutaraldehyde solution for 4 h. Then the bacteria were centrifuged (300 × g for 10 min) and washed with 0.1 M PBS, pH 7.2. The pellets were then fixed in 1% osmium tetroxide in 0.1 M PBS, pH 7.2 for 1 h. Cells were rinsed with same buffer and dehydrated in a graded series of ethanol, then, were frozen in liquid nitrogen cooled tert-butyl alcohol and vacuum dried overnight. The samples were mounted onto aluminum stubs. After sputter-coating with gold, they were analyzed by Hitachi S-4800 SEM.

Table 1
Antimicrobial activity of Japonicin-1Npa, Japonicin-1Npb and Parkerin.

Microorganism	MIC (μg/ml)[a]				
	Npa	Npb	Par	Amp	Str
Gram-positive					
Staphylococcus aureus ATCC2592	37.5	9.38	37.5	4.69	>100
Nocardia asteroides (IS)	ND	9.38	ND	0.29	>100
Enterococcus faecalis (IS)	ND	ND	ND	–	–
Enterococcus faecium (IS)	ND	ND	37.5	>100	>100
Gram-negative					
Escherichia coli ATCC25922	>100	>100	>100	>100	>100
Serratia marcescens (IS)	>100	>100	>100	2.35	>100
Pseudomonas aeruginosa (IS)	ND	ND	ND	>100	>100
Klebsiella oxytoca (IS)	ND	ND	ND	>100	>100
Xanthomonas maltophilia (IS)	ND	ND	ND	>100	>100
Aeromonas sobria (IS)	ND	ND	ND	>100	>100
Acinetobacter baumannii (IS)	ND	ND	37.5	>100	>100
Salmonella lignieres (IS)	ND	ND	ND	–	–
Proteus mirabilis (IS)	ND	ND	ND	–	–
Klebsiella pneumoniae (IS)	>100	>100	>100	–	–
Fungus					
Candida tropicalis (IS)	ND	ND	ND	>100	>100
Candida albicans ATCC2002	ND	ND	75	4.69	37.5
Cryptococcus neoformans (IS)	ND	ND	ND	–	–

MIC: minimal inhibitory concentration. These concentrations represent mean values of three independent experiments performed in duplicates. Npa: Japonicin-1Npa, Npb: Japonicin-1Npa, Par: Parkerin, Amp: ampicillin, Str: Streptomycin Sulfate. ND: no detectable activity in a dose up to 100 μg/ml; >100: have detectable antimicrobial activity in inhibition zone assay, but not totally inhibit cell growth in liquid medium in a dose up to 100 μg/ml; –: no assay, IS: clinically isolated strain.

[a] These concentrations represent mean values of three independent experiments performed in duplicates.

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