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ANTIMICROBIAL PEPTIDES FROM THE SKIN OF A KOREAN FROG, *RANA RUGOSA*

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SUMMARY: Six antimicrobial peptides, named gaegurins, were isolated from the skin of a Korean frog, *Rana rugosa*, and their amino acid sequences were determined by automated Edman degradation. All peptides contain two invariant cysteine residues, one at their C-terminus and the second at the seventh position from the C-terminus. The heptapeptides containing these two cysteine residues, which we designate 'Rana boxes', are conserved in the antimicrobial peptides derived from other *Rana* species. Each peptide manifested a broad spectrum of antimicrobial activity against Gram positive and Gram negative bacteria, fungi and protozoa with slightly different specific activities. All gaegurins manifest very little or no hemolytic activity. These properties provide the potential for application of these peptides to effective therapeutic agents for control of pathogenic microorganisms.

Many animals have innate immunity mediated by antibiotic peptides. In many cases, they are released from secretory glands into internal body fluids or onto mucosal epithelia. For example, cecropins, the hemolymph peptides composed of 35-37 amino acids, are synthesized in pupae of the Cecropia moth (*Hyalophora cecropia*) as a response to bacterial infections (1). In mammals, antimicrobial peptides such as defensins are stored primarily in the intracellular granules of phagocytic cells and play a role in non-oxidative killing of engulfed microorganisms (2). A number of peptides from the skin of various amphibians have also been found to have a broad-spectrum of antimicrobial activity (3-9). *Rana rugosa* has been traditionally used for anti-inflammation therapy in Korean medicines since wound-healing activity had been empirically detected in the frog tissues. In this therapy, the frogs are dried, ground and mixed with sessami oil. The resulting jelly-like material is applied to the site of bacterial infection. In the present study, we purified the active 'wound-healing' principles from the frog and determined their primary sequences. In this frog, antimicrobial activity was detected in the skin homogenate or extradermal secretion and this activity was found to be

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; CM, carboxymethyl; HPLC, high performance liquid chromatography; CFU, colony forming unit; LB, Luria-Bertani; YPD, yeast extract-peptone-dextrose; MIC, minimal inhibitory concentration; EDTA, ethylenediaminetetraacetic acid; RBC, red blood cell; GGN, gaegurin; and OD, optical density.

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mediated by more than six different peptides. They have been named gaegurins (from the Korean word, *gaegury*, which means frog).

MATERIALS AND METHODS

Frogs and bacterial strains. Three species of female frogs, *Rana rugosa*, *Rana dybowskii*, and *Rana nigromaculata*, were captured in the wild in the Southern part of the Korean peninsula. Bacterial species used were *Micrococcus luteus* (ATCC4698), *Klebsiella pneumoniae* (ATCC10031), *Shigella dysentariae* (ATCC9752), *Pseudomonas aeruginosa* (ATCC9027), *and Proteus mirabilis* (ATCC25933) obtained from Dr. G. J. Jeong, *Staphylococcus epidermidis* (ATCC12228), *Bacillus subtilis* (KCTC1021), *and Salmonella typhimurium* (ATCC14028) from K. S. Bae, *Serratia marcescens* and *Saccharomyces cerevisiae* from Dr. H. S. Kang, and *Candida albicans* from Dr. B. R. Lee.

Purification of antimicrobial peptides. Crude extracts were prepared by either homogenization or electric shock as described previously (4, 8). In the homogenization method, the skin of frogs was removed surgically and homogenized at 4°C in an extraction buffer, 0.2 M sodium acetate (pH 4.0), 0.2% Triton X-100, and 3 mM phenylmethylsulfonyl fluoride (PMSF). The homogenate was then centrifuged at 20,000x g for 20 min, the supernatant removed and stored at -70°C until used. In the electric shock method, frogs were repeatedly pulsed with electrodes at 40 V for one second and rinsed with distilled water. The washed suspension was lyophilized and resuspended in the extraction buffer. The crude extracts (prepared by either method) were applied to carboxymehtyl (CM)-Sepharose CL-6B (Sigma) equilibriated with 0.2 M sodium acetate (pH 4.0). Antimicrobial activity was eluted with 0.2 M of ammonium acetate (pH5.2). The active fractions were pooled, lyophilized redissolved in water, the sample then loaded onto Sephadex G-50 (Sigma) and eluted with 0.2M ammonium formate (pH 4.0). The fractions containing antimicrobial activity were lyophilized, dissolved in 17% acetonitrile/0.1% trifluoroacetic acid (TFA) and applied to a HPLC C4 column (0.46 cm X 25 cm, Vydac). The peptides were eluted at 0.6 ml/min in a linear gradient (17% to 80%) for 55 min. All fractions were dried under vacuum, dissolved in H_2O and used for the next analyses. Amino acid sequences were determined by the automated Edman degradation method using an Applied Biosystems protein sequencer, Model 473A.

Standard assay of antimicrobial activity. *Micrococcus luteus* was used for the standard assay. Cells were grown overnight in Luria-Bertani (LB) broth and inoculated into 5 ml of molten 0.6% LB agar with final 10^7 CFU/ml, which was overlayered on a 150 mm Petri dish containing solidified 2% LB agar. After the top agar hardened, 3-10 μl of samples were dropped onto the surface of the top agar and completely dried before incubating overnight at 37°C. If an applied sample and antimicrobial activity, a clear zone formed on the surface of the top agar representing inhibition of bacterial growth. Minimal inhibitory concentrations were determined by incubating 10^6 CFU/ml of cells in LB broth for bacteria or in yeast extract-peptone-dextrose (YPD) broth for yeasts including variable amount of peptides. Cell growth was quantified by measuring OD_{600} of the culture suspension.

Quantification of the Peptides. Samples to be analyzed were electrophoresed on an 20% acid-urea gel (10) together with the serially diluted lysozyme (Sigma) solutions as standard concentration markers. Gels were stained with Coomassie brilliant blue and the resulting band intensities of the peptides and lysozyme were measured with a densitometer (LKB). The amount of each peptide was extrapolated by comparing the band intensity of antibiotic peptides with those of standard markers.

RESULTS

Purification of Antimicrobial Peptides. In the standard assay of antimicrobial activity employing *Micrococcus luteus*, the skin extract from *Rana rugosa* manifested strong growth inhibitory activity. For purification of the substances that mediate this activity, two methods were employed to prepare

the starting material of column chromatography. One is to homogenize the skin tissue of frogs and the other is to shock the frogs electrically and then wash them with distilled water. The homogenates were centrifugated to remove aggregates and then chromatographed on a CM-Sepharose column. The active fractions from the homogenization technique were further chromatographed on a Sephadex G-50 column. The active fractions contained small peptides whose sizes are in the range between 2,000 and 5,000 daltons (data not shown). The active fractions were pooled and purified further by C4 reverse phase high performance liquid chromatography (HPLC). More than six peaks of antimicrobial peptides were separated by HPLC (Figure 1). Six peptides representing the major antimicrobial activity within frog skins were purified and their amino acid sequences determined (Table I). The antibiotic activity of each sequenced peptide was confirmed by assaying the

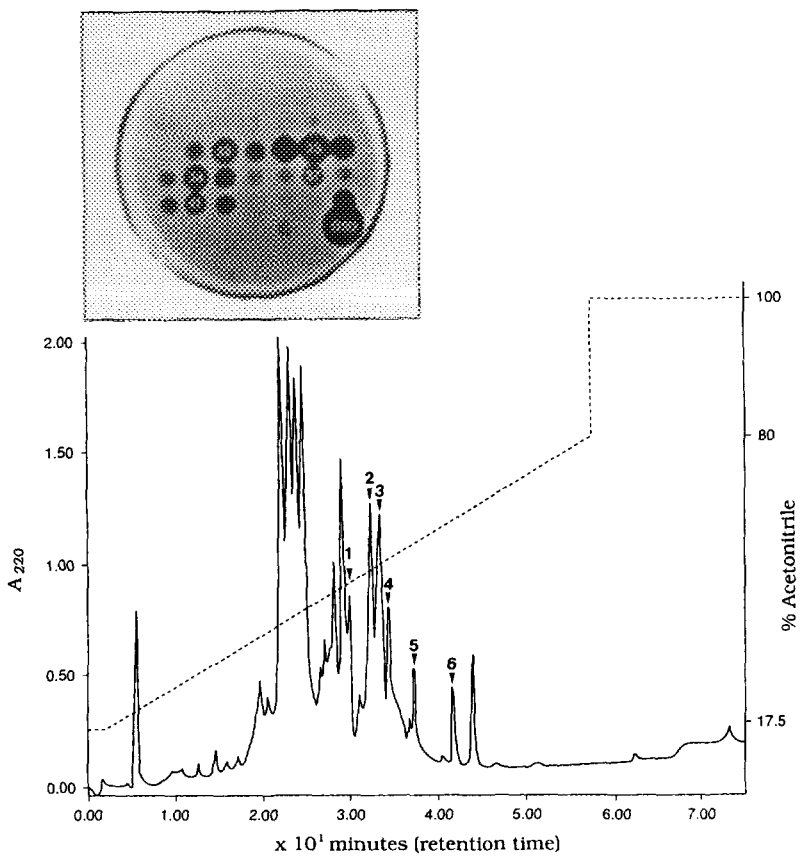

FIGURE 1. Separation of gaegurins by C_4 Vydac high performance liquid chromatography. The number above the peaks designates elution time. Flow rate was 0.5 ml/min and each fraction contains 0.5 ml of eluent. (Inset) Activity assay of eluents with *Micrococcus luteus*. 7μl of eluents dissolved in 70μl of distilled water were applied to each grid. See text for the activity distribution of each gaegurin.

Table I. Amino Acid Sequence Alignment of Gaegurins

Peptide	Amino acid sequence
Family I	* * * * * * * [a]
Gaegurin 1	SLFSLIKAGAKFLGKNLLKQG----ACYAACKASKQC
Gaegurin 2	GIMSIVKDVAKNAAKEAAKGA----LSTLSCKLAKTC
Gaegurin 3	GIMSIVKDVAKTAAKEAAKGA----LSTLSCKLAKTC
Gaegurin 4	GILDTLKQFAKGVGKDLVKGAAQGVLSTVSCKLALTC
Family II	
Gaegurin 5	FLGALFKVASKVLPSVKCAITKKC
Gaegurin 6	FLPLLAGLAANFLPTIICKISYKC

[a] The asterisks over the residues indicate the region of the Rana box motif. See text for explanation.

corresponding synthetic peptide (data not shown). They were named gaegurins (from the word *gaegury* that means frog in Korean).

Antimicrobial activity of gaegurins. The antimicrobial spectrum of each gaegurin was determined by measuring minimal inhibitory concentration (MIC). Table II shows the MIC values of each gaegurin against various bacteria and fungi. *E. coli*, *S. dysentariae*, *S. epidermidis*, *B. subtilis*, *K. pneumoniae*, and *M. luteus* showed relatively high sensitivity to gaegurins. Growth of *Aspergillus*

Table II. Antimicrobial Activity of Gaegurins against Various Bacteria and Yeasts

microorganism	minimal inhibitory concentration[a] (μg/ml)					
	GGN[b] 1	GGN2	GGN3	GGN4	GGN5	GGN6
Micrococcus luteus	5	2.5	2.5	2.5	2.5	2.5
Staphylococcus epidermidis	50	10	10	10	10	10
Bacillus subtilis	50	10	10	10	10	10
Klebsiella pneumoniae	100	25	25	25	50	50
Shigella dysentariae	25	25	25	25	50	50
Pseudomonas putida	50	50	50	100	50	150
Pseudomonas aeruginosa	50	50	50	100	100	150
Escherichia coli	25	75	75	75	50	100
Saccharomyces cerevisiae	>200	150	>200	200	50	50
Candida albicans	>200	150	>200	200	50	50
Salmonella typhimurium	>200	150	150	200	200	>200
Proteus mirabilis	>200	>200	>200	>200	>200	>200
Serratia marcescens	>200	>200	>200	>200	>200	>200

[a] Minimal inhibitory concentrations were determined by incubating 10^6 CFU/ml of cells in Luria-Bertani(for bacteria) or yeast extract-peptone-dextrose (for yeasts) media including the appropriate amounts of gaegurins. Cell growth was quantified by measuring OD_{600} of the culture suspension. [b] GGN, gaegurin.

nidulans, a filamentous fungus, was also inhibited by gaegurins, especially by gaegurins 5 and 6 (data not shown). When gaegurins were added to a culture medium of *Amoeba proteus*, a protozoan, to a final concentration of 25 μg/ml, the cells were ruptured within a few minutes (data not shown). Lower concentration of gaegurins caused contraction of pseudopods and loss of motility. This phenomenon is similar to that observed when synthetic magainin derivatives were treated to a protozoan, *P. caudatum* (11). Although gaegurins exhibit growth inhibitory or cell killing activities, they do not cause hemolysis of human red blood cells (RBC). Addition of gaegurins to human RBC up to 100 μg/ml did not result in significant hemolysis (Table III).

Sequence analysis of gaegurins. According to the sizes and amino acid sequences, they can be grouped into two families. Members in one family exhibit a unique pattern of sequence arrangement, which distinguishes them from those in the other family. Family I includes 33- or 37-residue peptides designated gaegurins 1, 2, 3 and 4, which are characterized by the conserved lysines periodically appearing four times at fixed positions in each peptide (Table I). Family II contains two homologous 24-residue peptides, gaegurins 5 and 6. Amphipathic nature with net positive charge is observed in all six peptides. In addition, All peptides in both families share the highly conserved heptapeptide module at their carboxy termini. Two cysteine residues that punctuate both ends of these heptapeptide modules are invariant within all six peptides and are likely engaged in intramolecular disulfide bonds. However, we cannot eliminate the possibility that these cysteines may be involved in intermolecular disulfide bridges, which consolidate the plausible chnnel structure on the lipid bilayer. The heptapeptide module (C-K-[V/I/L]-[A/S/T]-K-[K/T/Q]-C) appears without exception in the antimicrobial peptides from other species in the genus *Rana* (7-9). This heptapeptide module is, therefore, designated 'Rana box'. According to the analyses by Garnier's prediction method (12), gaegurins 1, 2, 3 and 4 have a high propensity for alpha-helix formation. If the periodically recurring lysine residues and nearby amino acids within these gaegurins are projected on the Edmundson wheel

Table III. Hemolytic Activity of Gaegurins and Melittin

concentration (μg/ml)	% hemolysis of human red blood cells [a]						
	GGN[b] 1	GGN2	GGN3	GGN4	GGN5	GGN6	Melittin
0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.1	0.04	0.19	0.11	0.50	0.19	0.39	9.63
1.0	0.14	0.16	0.12	0.74	0.31	0.62	37.67
10.0	0.41	0.31	0.35	0.78	0.78	0.78	78.55
100.0	0.59	0.82	1.20	1.67	1.01	0.86	98.92

[a] Hemolyses induced by peptides were determined by incubating 10% (v/v) suspension of human red blood cells in phosphate-buffered saline with the appropriate amount of gaegurins or melittin at 37℃ for 10min. After centrifugation at 10,000x*g* for 10min, OD_{350} of the supernatant was measured. The relative optical density compared to that of the suspension treated with 0.1% Triton X-100 defined % hemolysis. [b] GGN, gaegurin.

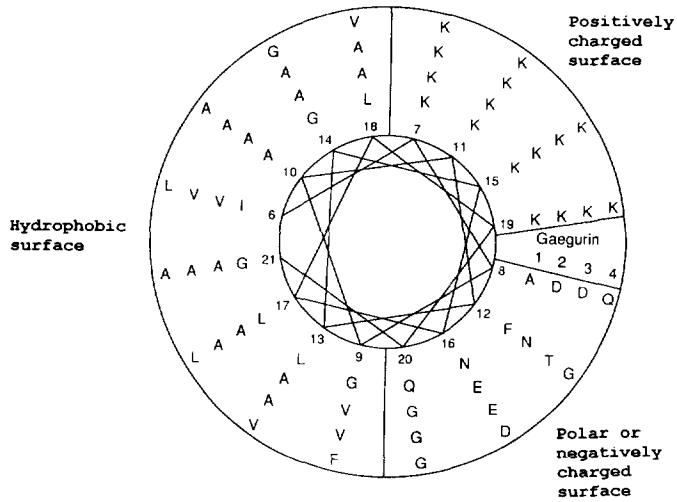

FIGURE 2. Edmundson wheel diagram of gaegurins 1, 2, 3, and 4.

diagram, amino acids with similar biochemical properties are clustered onto an arc in the narrow angle, thus arrayed at the helical surface facing one direction (Figure 2). The clustering of lysine residues and the consequential formation of a local, positively charged surface are especially notable. Likewise, another sector can display polar or negatively charged residues and the remainder of the surface comes to protrude hydrophobic amino acids. When several bundles of this helix are laid in parallel, a channel-like structure may be constructed with the hydrophobic surface outwardly facing membrane lipids and the polar surfaces arrayed inwardly, where ions are allowed to pass through. Further investigation is required to determine whether this hypothetical channel is to be formed from gaegurins.

DISCUSSION

Six different peptides with broad-spectrum antimicrobial activities were purified from the *R. rugosa* skin. They are small peptides with amino acid residues ranging from 24 to 37. The detailed mechanism of how gaegurins inhibit growth of microorganisms is not yet understood. Nevertheless, some explanations of the possible mechanism may be offered. In our studies, gaegurins induced cytolysis of the protozoan, *Amoeba proteus*. The fact that the amoebas are killed by bursting suggests gaegurins may work on the membrane of the target cell by forming an ion channel, like other peptide antibiotics. In addition, preformed electrochemical gradient was removed by adding gaegurins to an artificial membrane (data not shown). However, it is not yet determined how an ion-channel is formed and whether ion channel formation is the only action mechanism of gaegurins. As described in the Results, gaegurins actually retain the potential to adopt conformations expected of ionophores or ion

channels. The strong antimicrobial activity and nonhemolytic property of gaegurins suggest that they may be good candidates for the control of pathogenic bacteria, fungi and protozoa.

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