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Isolation and Characterization of Novel Antimicrobial Peptides, Rugosins A, B and C, from the Skin of the Frog, *Rana rugosa*

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SUMMARY: Three antimicrobial peptides were isolated from the skin of *Rana rugosa*. The major component, designated rugosin A, consisted of 33 amino acid residues and had structural homology (45%) with brevinin-2 of *Rana porosa brevipoda*. This peptide strongly inhibited the growth of gram-positive bacteria (e.g. *Staphylococcus aureus* 209P). The second peptide (rugosin B), a minor component,also had 33 amino acid residues, but was less homologous (33%) with brevinin-2. This peptide exhibited a striking antimicrobial activity against both gram-negative (e.g., *Escherichia coli* NIHJ) and gram-positive bacterial species. The third one, named rugosin C, composed of 37 amino acid residues, exhibited an antimicrobial activity against gram-positive bacteria. All three peptides had an intramolecular disulfide bond at the C-terminus.

Studies on antimicrobial peptides are very useful not only for therapeutical applications, but also for understanding the evolutionary aspects of the immune system in animals (1, 2). Cytotoxic and antibiotic peptides were originally found in the skin of the frog *Bombina* (3). Since then, such antimicrobial peptides have been isolated from several anuran species : magainins from *Xenopus laevis* (11), bombinin-like peptides from *Bombina variegata* and *Bombina orientalis* (5, 9), dermaseptin from *Phyllomedusa sauvagii* (7), brevinins from *Rana porosa brevipoda* (8), esculentin from *Rana esculenta* (10). Each of these peptides exhibits a broad spectrum of antibacterial activity and some of them act on both gram-positive and gram-negative bacteria.

Here, we report the primary structures and the antimicrobial activities of three novel antimicrobial peptides from the skin of *Rana rugosa*.

MATERIALS AND METHODS

Preparation and purification of skin peptides. Adult frogs, *Rana rugosa* were collected at a mountainous region in Gunma Prefecture, Japan. The skin was removed from 20 frogs after

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they had been immersed in ice water. The skin was boiled for 10 min and extracted in 0.5 M acetic acid containing 0.02% 2-mercaptoethanol overnight at 4°C. After centrifugation at 10,000 x g for 30 min, the supernatant was collected and lyophilized. The lyophilized material was dissolved in 0.2 M acetic acid was loaded onto 100 x 700 mm column of Sephadex G-25 SF (Pharmacia) and eluted with 0.2 M acetic acid. The fraction exhibiting antimicrobial activity was purified by reverse-phase HPLC on a YMC-Pak S-ODS-5 column (20 x 250 mm) (Yamamura Chemical Lab., Japan) with a linear gradient elution of acetonitrile in 0.1% TFA. Further purification of the fractions containing antimicrobial activity was performed by reverse-phase HPLC on a YMC-Pak S-ODS-5 column (4.6 x 250 mm) with a linear gradient of 50% acetonitrile in 0.1% phosphoric acid. Final purification was carried out on the same HPLC column by elution with a linear gradient of acetonitrile in 0.1% TFA.

Structural analysis. The isolated peptides were first reduced and then carboxymethylated with iodoacetic acid (3). The carboxymethylated peptides were digested with α-chymotrypsin (Type I-S, Sigma) for 1-2 hr at 37°C in 0.1 M $NaHCO_3$ and chymotryptic fragments produced were separated by HPLC on a YMC-Pak ODS-5 column (4.6 x 250 mm) and eluted with a linear gradient of acetonitrile in 0.1% TFA. The amino acid compositions of the carboxymethylated peptides and their chymotryptic fragments were analyzed with a Hitachi amino acid analyzer (Model L-8500) after hydrolysis of the samples in 6 M HCl containing 1% phenol at 110°C for 24 hr in evacuated tubes. The amino acid sequences of the peptides were determined by automated Edman degradation with an Applied Biosystems protein sequence analyzer (Model 477A-120A).

Peptide synthesis. Rugosins A and B were synthesized with a solid-phase peptide synthesizer (Model 431A, Applied Biosystems). The crude synthetic peptides were purified on a reverse-phase HPLC column (YMC-Pak ODS-5, 20 x 250 mm) and a linear gradient of acetonitrile in 0.1% TFA. The purified peptides were dissolved in water at a concentration of 0.1 mg/ml and the pH of the solution was adjusted to 6.5 with 1 M $NaHCO_3$. Oxidation of the peptides was performed by mixing with air for several hours at 4°C. After lyophilization, the oxidized products were separated on the same HPLC column as mentioned above. The peptide peaks containing rugosins with disufide bonds were identified by HPLC elution time of the corresponding natural peptides and also by relative molar mass determinations using a mass spectrometer (Kompact Maldi III, Kratos Analytical, UK).

Antimicrobial assay. The antimicrobial activity of the peptides was examined by means of an inhibition zone assay on agarose plates seeded with *Staphylococcus aureus* 209P, *Bacillus subtilis* ATCC6633, *Micrococcus luteus* ATCC9341, *Streptococcus pyogenes* COOK, *Escherichia coli* NIHJ, and *Pseudomonas aeruginosa* POA-1. The peptide samples absorbed onto filter papers were placed on thin agarose plates containing bouillon broth (sensitivity disk agar, Nissui, Japan) and about 1 x 10^7 bacterial cells. The inhibition zone around the filter papers was observed after overnight incubation at 35°C. The minimum inhibitory concentration of synthetic peptides was determined by a broth dilution method.

RESULTS

Purification of rugosins A, B and C

The lyophilized material from acidic extracts (dry weight, 1.0 g) dissolved in 0.2 M acetic acid was loaded onto a 100 x 700 mm column of Sephadex G-25 SF (Pharmacia) and eluted with 0.2 M acetic acid. Fractions (600 ml each) were collected at a flow rate of 20 ml/min and lyophilized. An aliquot (0.5 mg) of each lyophilized fraction was assayed for antimicrobial activity. The 3rd fraction was found to exhibit antimicrobial activity against gram-positive bacteria such as *Staphylococcus aureus* 209P, *Bacillus subtilis* ATCC6633, and *Micrococcus luteus* ATCC9341. This fraction (dry weight, 56 mg) was therefore purified by reverse-phase HPLC on a YMC-Pak S-ODS-5 column (20 x 250 mm). Elution was achieved with a linear gradient of acetonitrile in 0.1% TFA. An aliquot (100 µl) of the fractions (10 ml each) was applied for an antimicrobial assay against *Staphylococcus aureus* 209P, and antimicrobial activity was found clearly in the two fractions (60 and 65), as shown in Fig. 1. The fraction containing rugosin B and C (fraction 60) was further purified by reverse-phase HPLC with a linear gradient

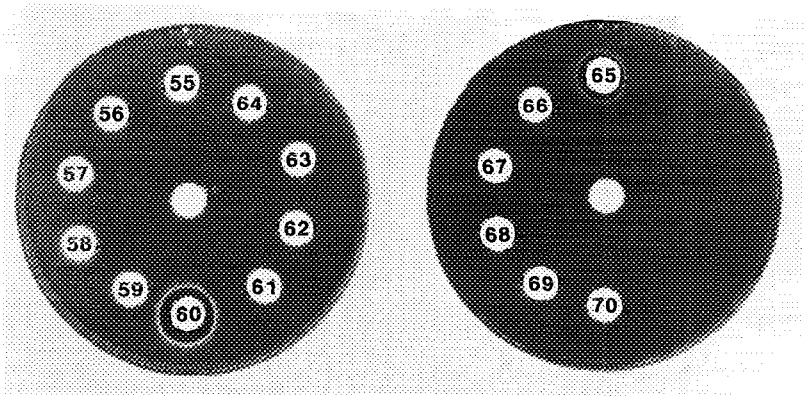

Fig. 1. Antimicrobial activity of reverse-phase HPLC fractions. A 100-μl aliquot of each fraction was concentrated and applied to a sheet of filter paper on a poured lawn of *Staphylococcus aureus* 209P. A black area with a white ring indicates inhibition of bacteria growth. Rugosin B-C (60) and rugosin A (65) fractions exhibited antimicrobial activities. Numbers 55-70 correspond to those of fractions.

of acetonitrile in 0.1% phosphoric acid. The antimicrobial activity against *Staphylococcus aureus* 209P was evident in two fractions (20 and 23) in the chromatogram. Rugosins A, B and C were isolated in single peaks in the final HPLC purification steps on a YMC-Pak S-ODS-5 column (4.6 x 250 mm) and a linear gradient of actonitrile in 0.1% TFA (Fig. 2).

Primary structures of rugosins

The isolated peptides, rugosins A, B and C were reduced and carboxymethylated with iodoacetic acid. The primary structures of rugosins A, B and C were determined from the results of the amino acid sequences of the intact peptides and the chymotryptic fragments. The amino acid compositions of the carboxymethylated peptides were analyzed. It was found that the three peptides were rich in lysine and leucine and each peptide contained two cysteine residues. The complete amino acid sequence of rugosin A was determined as follows. The amino acid sequence of carboxymethylated rugosin A was first determined with the sequence analyzer, except for Cys^{27}, Ser^{32} and Cys^{33}. Secondly, the amino acid sequence of its chymotryptic fragment (residues 26-33) was determined. Two carboxymethyl cysteine residues were estimated from the reduced and carboxymethylated rugosin A. However, the carboxymethyl cysteine was not recovered from non-reduced and carboxymethylated rugosin A by amino acid analysis. These data show the lack of free cysteine residues in the molecule. This means the presence of an intramolecular disulfide bond between Cys^{27} and Cys^{33} in rugosin A molecule. Rugosin A therefore consisted of 33 amino acid residues and its relative molar mass was calculated to be 3,438 Da. The second peptide, rugosin B, which also comprised 33 amino acid residues (3,514 Da.) was 27% homologous with the structure of rugosin A. The third peptide, rugosin C, was composed of 37 amino acids (3,814 Da.) and showed 49% sequence homology with rugosin A. The identical amino acid residues were found at seven positions between the structures of the three rugosins. Rugosins B and C also had one disulfide bridge at the COOH-terminus.

Antimicrobial activities of rugosins A, B and C

Using synthetic rugosins A and B, we tested the antimicrobial activity by a dilution method (Table 1). Rugosin A being a main component selectively inhibited the growth of gram-positive

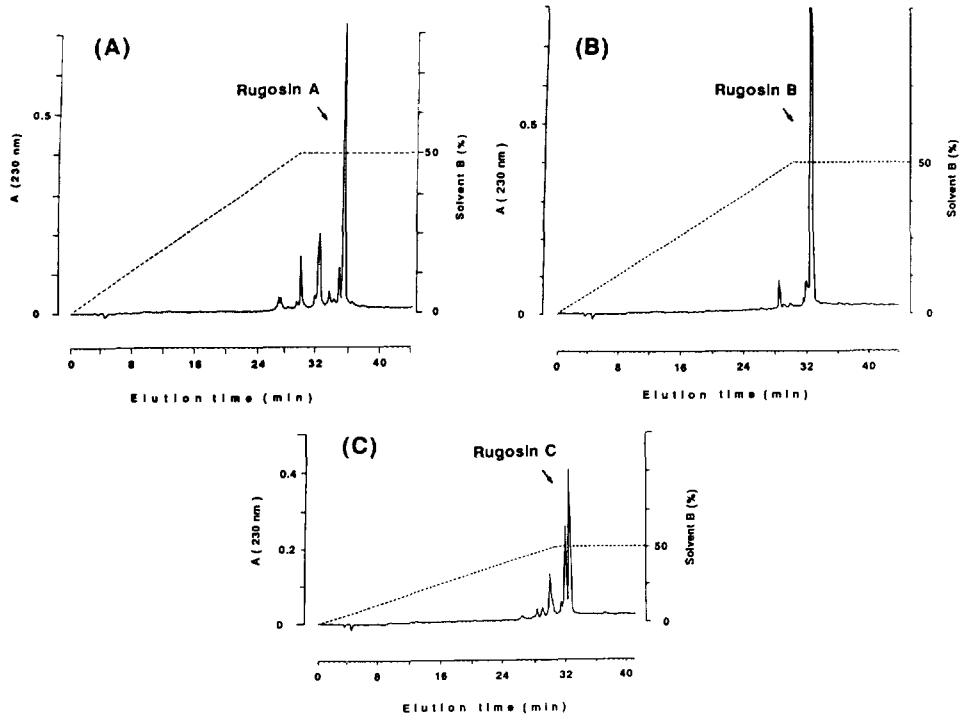

Fig. 2. Reverse-phase HPLC of rugosins A (A), B (B) and C(C). Chromatographic conditions : solvent A, 0.1% TFA. solvent B, acetonitrile in 0.1%TFA. Flow rate, 1 ml/min. Linear gradient from 0 to 50% solvent B for 30 min. HPLC peak containing antimicrobial peptide was shown by an arrow.

bacteria, especially against *Staphylococcus aureus* 209P. On the other hand, rugosin B strongly inhibited both gram-positive and gram-negative bacteria. The growth of *Escherichia coli* NIHJ was inhibited in the presence of 12.5 μg/ml rugosin B. Natural rugosin C exhibited antimicrobial activity against gram-positive bacteria, such as *Staphylococcus aureus* 209P in the inhibition zone assay.

Table 1. Antimicrobial activity of synthetic rugosins

	Minimum inhibitory concentration (μg/ml)	
	rugosin A	rugosin B
Staphylococcus aureus 209P	6.25	6.25
Bacillus subtilis ATCC6633	12.5	6.25
Micrococcus luteus ATCC9341	25	1.56
Streptococcus pyogenes COOK	50	12.5
Escherichia coli NIHJ	100	12.5
Pseudomonas aeruginosa PAO-1	> 100	100

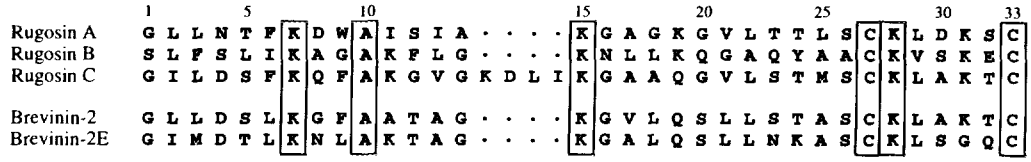

Fig. 3. Structural comparison of rugosins, brevinin-2 and brevinin-2E. Identical amino acid positions are boxed. The presence of an intramolecular disulfide bond at COOH terminus is characteristic of all antimicrobial peptides shown in the figure.

DISCUSSION

Up to date, the presence of six antimicrobial peptides was reported in three different frog families : *Xenopus laevis* (Pipidae) (6, 11). *Bombina variegata* and *Bombina orientalis* (Discoglossidae) (5, 9), *Rana porosa brevipoda* and *Rana esculenta* (Ranidae) (8, 10). The amino acid sequences of these known antimicrobial peptides had strong structural similarities in the same genus, though they are very different from the peptides of different family origin. In the present study, three novel antimicrobial peptides, named rugosins A, B and C, were isolated from *Rana rugosa*. The amino acid sequences of these rugosins showed similarities to those of brevinin-2 (8) and brevinin-2E (10) displaying antimicrobial activities against gram-positive and gram-negative bacterial species (Fig. 3). Rugosin A was similar to brevinin-2 with 45% structural homology and brevinin-2E with 39% homology. However, unlike brevinin-2 and brevinin-2E, rugosin A showed strong antimicrobial activity selectively against only gram-positive bacteria. On the other hand, rugosin B inhibited the growth of gram-positive and gram-negative bacteria and it was 33% homologous to brevinin-2 and brevinin-2E. The third peptide, rugosin C exhibited antimicrobial activity against gram-positive bacteria and the number of amino acid residues was different from those of brevinin-1 (8), brevinin-1E and esculentin (10). The amino acid sequence of rugosin C contained four extra amino acid residues at a midregion of the molecule when compared with those of rugosins A and B (Fig. 3). Between the structures of rugosins, brevinin-2 and brevinin-2E, there were six identical positions (Fig. 3, boxed). It is interesting that the unique C-terminal structure of rugosins, -CKXXXXC, is identical to that of all antimicrobial peptides found in the *Rana* family (brevinin-1, brevinin-1E, brevinin-2, brevinin-2E and esculentin). However, no similar C-terminal structure has been found in frogs of different families (*Xenopus laevis*, *Bombina variegata*, *Bombina orientalis* or *Phyllomedusa sauvagii*). This sequence may be characteristic of antimicrobial peptides from *Rana* skin. It is not yet clear which structure of the native sequence of these regosins is essential for the antimicrobial activity. The relationship between the structure and the antimicrobial activity of rugosins should be clarified in a further study.

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