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BREVININ-1 AND -2, UNIQUE ANTIMICROBIAL PEPTIDES FROM THE SKIN OF THE FROG, RANA BREVIPODA PORSA

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SUMMARY: Two unique antimicrobial peptides named brevinin-1 and -2 were isolated from the skin of the frog, Rana brevipoda porsa. Both of the peptides did not have any structural homology with bombinin nor magainin; the frog skin derived-antimicrobial peptides isolated from Bombina and Xenopus, nor even with other known antimicrobial peptides of non-amphibian origin. The minimum inhibitory concentration of brevinin-1 against the growth of St. aureus and E. coli was determined to be 8μg/ml and 34μg/ml while that of brevinin-2 was 8μg/ml and 4μg/ml, respectively, indicating the difference of the two peptides in the antimicrobial selectively on Gram-positive and Gram-negative bacteria.

Occurrence of several kind of antimicrobial peptides has been demonstrated in vertebrate and invertebrate. For example, MCP-1 and -2 were the antimicrobial peptides isolated from rabbit lung macropharge (4) and Bac-5 and -7 were from bovine neutrophil (5,6). Sarcotoxin (12) and defensin (7) are the peptides of insect-origin.

Since frog skin has been known to be rich in extra-secretory glands which contains biogenic amines as well as bioactive or neuroactive peptides (1), bombinin isolated from skin of Bombina bombina (2) and magainin from X. laevis (3) are known as the frog skin-derived antimicrobial peptides.

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Recently, we found active principles, which prevented microbial growth, in the skin of Japanese frog, Rana brevipoda porsa and have characterized these principles as the novel peptides of no precedent for the other known antimicrobial peptides. In this paper, we will report isolation, structural elucidation and antimicrobial activity of the peptides.

MATERIALS AND METHODS

Materials: Rana brevipoda porsa was purchased from SEASCO(Saitama, Japan). Trypsin was purchased from WORTHINGTON, chymotrypsin was from WAKO PURE CHEMICAL INDUSTRIES, LTD. and dialysis tube (Specrta/Por membrane mwco: 1,000, FED FE052640) was from Funakoshi Inc. The bacteria used were Staphylococcus aureus 209P JC-1, Bacillus subtilis ATCC 6633, Escherichia coli NIHJ JC-2, and Candida albicans 7. Magainin-2 was from Peptide Institute Inc.(Osaka Japan). Ves-CP-M and Ves-CP-X were chemically synthesized by solid-phase method. Other chemicals were of guaranteed or protein sequence grade.
Antimicrobial assay: During the purification steps, growth inhibition of St.aureus was employed as a marker. The bacteria were grown in LB culture to an OD_{600} of 0.1. The bacteria broth was diluted to 0.5 % with LB broth containing 1.5 % agar and poured over petri dish. The assay was performed by the methods as described previously (13).
Isolation: Rana brevipoda porsa was anesthetized with diethyl ether for 3 min and was surgically sacrificed. The skin was boiled for 15 min in 1M acetic acid. After cooling, the extract was centrifuged at 12,000 x g for 30 min and the supernatant was dialyzed against 1M acetic acid overnight at 4 ^{o}C to remove low molecular weight substances. Then, the non-dialysale fraction was adsorbed on an SP-sephadex C-50 column (Pharmacia Fine Chemicals, H^{+}-form, 5ml), pre-equilibrated with 1M acetic acid. The column was washed with 15 ml of 1M acetic acid, 2M pyridine and finally 2M pyridine-acetic acid (PH 5.0). After removal of each solvent, each fraction was subjected to antimicrobial assay and the active fraction was applied on an ODS-80TM column (4.6x250mm, TOSOH) for further purification. Elution was carried out with a linear gradient from (A) water/acetonitrile(95/5 v/v) to (B) water/acetonitrile(40/60 v/v), containing 0.05% (v/v)TFA. The flow rate was set at 0.7 ml/min and the column temperature at 40 ^{o}C. Chromatography was monitored by UV absorption at 215 nm.
Structural determination: Reduced and carboxymethylated (cm-) brevinin-1 and -2 (brevinins) were prepared by the standardized method (10). Tryptic and chymotryptic fragments of cm-brevinins were purified by the ODS column. Amino acid composition analysis were performed by post-column derivatization with o-phtalaldehyde (11) after hydrolysis of peptide with 5.7N HCl at 110 ^{o}C for 20 h. Amino acid sequence was determined by Edman degradation using a Protein Sequencer 477A (Applied Biosystems). Molecular weight of brevinins was determined by electrospray MS/MS spectrometry (TSQ700, Finigan MAT). Chemical synthesis of brevinins were performed by a solid phase Peptide Synthesizer 430A (Applied Biosystems).

RESULTS

Isolation and structural elucidation: Fig.1 summarizes the flow chart of purification of the peptides. Since antimicrobial activity was found in the eluate of 2M pyridine-acetic acid (PH5.0) from an SP-sephadex C-50 column, this fraction was applied on an ODS column. As shown in Fig.2, antimicrobial activity resided in the two peaks as indicated by arrows (A and B). Preliminary analysis showed that the active compounds were peptides and we named them brevinin-1 (fractionB) and -2 (fractionA). Amino acid composition of these peptides suggested that brevinins contained cystine in their molecules, so that both peptides were reduced and carboxymethylated prior to enzymatic digestion (chromatograms not shown) and amino acid sequence analysis. Table 1 summarizes respective amino acid compositions of cm-brevinins as well as their tryptic and chymotryptic fragments. On the other hand, the native brevinin-1 and -2 were similarly digested with trypsin or chymotrypsin, and the respective digested fragments were purified by HPLC and sequentially analyzed by the protein sequencer. The proposed sequences are given in Fig.3. As shown in Fig.3,

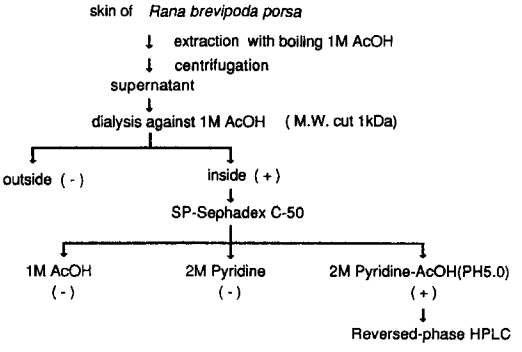

Fig. 1. The purification of brevinin-1 and -2
Skin from 6 frogs was used. All fractions were assayed using St.aureus 209P JC-1. (+); inhibition of growth. (-); not affected.

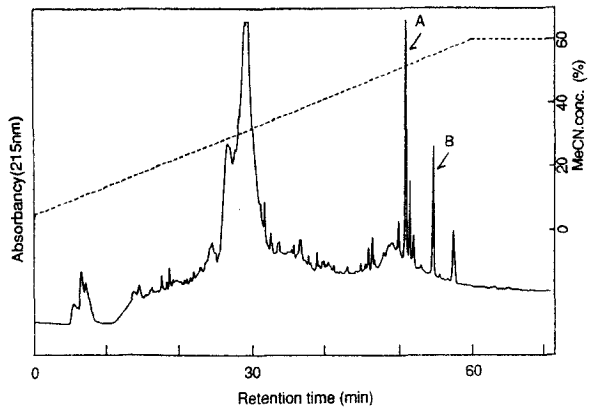

Fig. 2. HPLC chromatogram of 2M pyridine-acetic acid (PH 5.0) fraction of SP-Sephadex C-50 chromatography. The peak fractions indicated by arrows (A and B) possessed antimicrobial activity. A; brevinin-2, B; brevinin-1.

tryptic digestion of native brevinin-1 gave a peptide fragment having the sequences corresponding to Val^{12}-Lys^{19} and Lys^{23}-Cys^{24} (T_N). This evidence indicated that Cys^{18} and Cys^{24} are

Table 1. Amino acid composition analysis of brevinin-1, -2, and their enzymatic fragments

	Brevinin-1					Brevinin-2								
	Total	C-1	C-2	T-1	T-2	Total	C-1	C-2	C-3	T-1	T-2	T-3	T-4	T-5
Asx	-	-	-	-	-	1.1(1)	-	-	1.1	-	-	-	-	-
Thr	0.9(1)	-	1.1	-	1.5	2.7(3)	2.0	1.1	-	1.2	-	-	-	1.0
Ser	-	-	-	-	-	3.3(4)	2.0	1.0	1.0	-	-	1.0	0.7	2.4
Glx	-	-	-	-	-	1.1(1)	-	1.2	-	-	-	-	-	0.8
Pro	2.4(2)	1.0	-	0.9	0.8	-	-	-	-	-	-	-	-	-
Gly	1.5(2)	2.1	-	1.1	-	4.0(4)	-	2.6	2.4	-	-	2.2	1.0	1.1
Ala	4.2(4)	2.3	-	2.5	-	4.9(5)	2.2	3.8	-	-	1.0	3.3	-	1.1
Val	1.3(1)	0.7	-	0.9	0.6	0.7(1)	-	0.8	-	-	-	-	-	0.6
Met	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ile	1.8(2)	1.6	1.0	0.9	-	-	-	-	-	-	-	-	-	-
Leu	2.9(3)	2.1	-	1.7	1.2	7.1(7)	1.0	2.9	2.9	-	1.0	-	2.6	2.1
Tyr	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phe	2.1(2)	1.1	-	1.0	0.8	1.0(1)	-	-	1.0	-	-	1.1	-	-
His	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lys	4.0(4)	1.4	2.4	1.0	1.2	3.8(4)	2.2	1.3	1.3	-	1.0	1.1	1.0	1.0
Arg	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cys	2.0(2)	-	-	-	-	2.7(2)	2.1	-	-	1.0	-	-	-	1.2

cm-; carboxy-methylated, Cys; cm-Cys. The numbers in parentheses are from sequencing data.

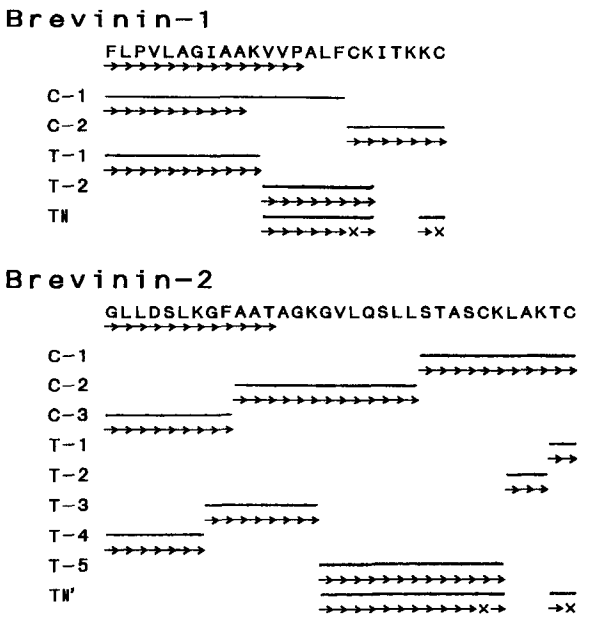

Fig. 3. Amino acid sequence of brevinin-1, -2 and their enzymatic fragments. C-; chymotryptic fragments of cm-brevinin, T-; tryptic fragments of cm-brevinin, T_N; a tryptic fragment of native brevinin-1. T_N'; a tryptic fragment of native brevinin-2. :identified as PTH-amino acid by Edman degradation.

bonded by a disulfide bridge. In the similar way, tryptic fragment of T_N' showed that brevinin-2 has one disulfide bond between Cys^{28} and Cys^{34}. The C-termini of both brevinins were not amidated as identifying by dansyl-Edman analysis (data not shown). The proposed structures were supported by mass spectrometry (brevinin-1, m/e=2528; brevinin-2, m/e=3250).

Based on the proposed amino acid sequences, we chemically synthesized the peptides. The synthetic products possessed the same elution profiles on reversed-phase HPLC, and the same antimicrobial activity as the native brevinins (data will be published elsewhere).

Antimicrobial activity: Table 2 shows the antimicrobial spectra examined by the agar dilution methods. Brevinin-1 showed growth inhibition against Gram-positive bacteria; St.aureus

Table 2. Antimicrobial activity of brevinin and other related bioactive peptides

peptides	origin	structure	MIC(μg/ml) *B.sub.*	*St.aur.*	*E.coli*	*Can.alb.*
brevinin 1	frog	**FLPVLAGIAAKVVPALFCKITKKC**-OH	2	8	34	>130
brevinin 2	frog	**GLLDSLKGFAATAGKGVLQSLLSTASCKLAKTC**-OH	2	8	4	>130
Pipinin	frog	**FLPIIAGVAAKVFPKIFCAISKKC**-x	—	—	—	—
Ves-CP-M	wasp	**FLPIIGKLLSGLL**-NH_2	25	—	>50	—
Ves-CP-X	wasp	**FLPIIAKLLGGLL**-NH_2	25	—	>50	—
Magainin 2	frog	**GIGKFLHSAKKFGKAFVGEIMNS**-OH	100	50	100	>100
Bombinin	frog	**GIGALSAKGALKGLAKGLAQHFAN**-NH_2	—	—	—	—

B.sub ; *Bacillus subtilis* ATCC 6633, *St.aur* ; *Staphyrococcus aureus* 209P JC-1,
E.coli; Escherichia NIHJ JC-2, *Can.alb* ; *Candida albicans* 7.
-; not determined.

209P and B.subtilis, but relatively weak against Gram-negative bacteria; E.coli NIHJ. On the other hand, brevinin-2 inhibited the growth both Gram-positive and Gram-negative bacteria in the similar concentration. However, brevinin-1 and -2 did not affected for Can.albicans even at 130μg/ml.

DISCUSSION

In the present study, we purified two unique antimicrobial peptides from the skin of Rana brevipoda porsa and named them brevinin-1, and -2. Although, bombinin and magainin have been known as antimicrobial peptides isolated from the skin of frog, Bombina bombina and X.laevis (2,3), brevinin-1 and -2 are structurally different from bombinin or magainin. Thus, brevinins are the new class of antimicrobial peptides. However brevinin-1 has structural homology with pipinin (Table 2) which was previously isolated from the skin of the frog Rana pipiens by its histamine releasing activity from the rat peritoneal mast cell (8). It is noteworthy that the N-terminal sequential

motif of brevinin-1, (i.e., Phe-Leu-Pro-X-X'-, X, X';hydrophobic amino acid) is similar to those of Ves-CP-M and Ves-CP-X known as wasp venom-derived chemotactic peptides (9).

As regards antimicrobial activity, it was interesting that brevinin-2 inhibited the growth of both Gram-positive and Gram-negative bacteria while brevinin-1 was not potent on the growth of E.coli (about 8 times weaker than brevinin-2 in Minimal Inhibitory Concentration (MIC) value). Magainin-2, whose MIC for E.coli D31 has been reported to be 5μg/ml (3), was less potent than brevinin-1 and -2 in the present study. Ves-CP-M and Ves-CP-X, which have the sequence homology in the N-terminal with brevinin-1, actually inhibited the bacteria growth but their MIC were higher than that of brevinin-1, suggesting that the complete molecule of brevinin-1 were required for the effective antimicrobial activity. The structure-activity relationship is now under study by using chemically synthesized brevinins.

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