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Isolation of Antimicrobial Peptides from Skin Secretions of the Oriental Fire-bellied Toad, *Bombina orientalis*

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Antimicrobial peptides are widely distributed in nature and appear to play important roles in the host defense of plants and animals. In this study, we isolated and characterized three antimicrobial peptides from skin secretions of the oriental fire-bellied toad, *Bombina orientalis*. These purified peptides were referred to as P1, P2, and P3 in order of their elution. P1, P2, and P3 have molecular weights of 2569, 2566, and 2370 Da by MALDI-TOF mass spectrometer, respectively. They are heat-stable, amphipathic peptides of 24-27 amino acids without cysteine residues. All three peptides are active against representative gram negative and gram positive bacterial species, and in particular, P1 appears to have distinctive antifungal activity. However, no significant hemolytic activity was found for these peptides.

Many antimicrobial peptides originated from multicellular organisms have been found in skin secretions of frogs. For example, bombinin, a 24-amino acid peptide, was originally isolated from the European toad *Bombina variegata* (Csordas and Michl, 1970), and magainin, a 23-amino acid peptide, from the African clawed frog *Xenopus laevis* (Zasloff, 1987). Recently, esculentin, a 46-amino acid peptide, was isolated from the European frog, *Rana esculenta* (Simmaco et al., 1993), and gaegurins, 35-37 amino acid peptides, from the Korean frog, *Rana rugosa* (Park et al., 1994). These peptides are produced and stored in specialized dermal structures called granular glands, which release their contents onto the external surface of the frog upon adrenergic stimulation or injury (Bevins and Zasloff, 1990)

The antimicrobial peptides can be divided into several classes based on amino acid sequence and secondary structure. The first one consists of small, basic peptides which can be configured as a single, amphipathic α-helix. The N-terminal half of the peptides is rich in charged amino acids and is hydrophilic, whereas the C-terminal half is hydrophobic. The amphipathic nature of the peptides presumably underlies their biological activities which enables them to associate with lipid membranes and disrupt normal membrane functions (Gibson et al., 1991). For example, magainin 2 assumes an amphiphilic helix when bound to acidic phospholipids, forming a pore composed of a dynamic, peptide-lipid supramolecular complex (Matsuzaki et al., 1996). The second class is characterized structurally by two α-helices joined by a hinge region containing glycine and proline residues. This class includes the cecropins, molecules first isolated from insect haemolymph (Boman and Hultmark, 1987) and later from pig intestine (Lee et al., 1989). The third class consists of cationic peptides with three intramolecular disulfide bonds. Members of this class include the defensins, which are found within mammalian neutrophils and macrophages (Lehrer et al., 1991), tracheal epithelium (Diamond et al., 1991), and the Paneth cells of the small intestine of mouse (Ouellette et al., 1989) and human (Jones and Bevins, 1992).

In this paper, we report the isolation and characterization of several antimicrobial peptides from the skin secretions of the oriental fire-bellied toad, *Bombina orientalis.*

Materials and Methods

Preparation of frog skin secretions

Adult frogs, *Bombina orientalis* Boulenger, were collected in the middle eastern region (Dunnae, Kangwon-do) of Korea. The antimicrobial peptide-containing secretions, induced by 5 V electrical shock at the dorsal region of the frog for 5 min, were collected from the surface of the skin by washing with 200 ml of 0.05% acetic acid. The washed solution was then lyophilized and redissolved in 10 ml of Milli Q.

Bacterial strains

Three gram negative strains, three gram positive strains, and two fungi were used in this experiment. The test organisms for antimicrobial activity assays were *E. coli* K12 (ATCC 2223), *E. coli* D21, *Enterobacter cloacae* (ATCC 11438), *Bacillus subtilis* (ATCC 3135), *B. megaterium* Bm11, *B. thuringiensis* (ATCC

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10792), *Candida albicans* (ATCC 10231) and *Aspergillus niger* (ATCC 32656). *E. coli* D21 and *B. megaterium* Bm11, streptomycin resistant mutants, were gifts from Dr J.-Y. Lee, and other organisms, all wild types, were gifts from KCTC, Korea.

Antimicrobial activity assay

Antibacterial activity was assayed by measuring clear growth inhibition zones on thin agarose plates seeded with bacteria as described previously (Yoe et al., 1995). A standard curve was made with known amounts of synthetic cecropin A, and 1,000 units were defined as an activity equal to that of 1 μg of cecropin A. Three μl of samples were used for each assay.

Antifungal activity was essentially the same as those described by Schwartz et al. (1988). Spore solutions of 2×10^6 cells were spread on Sabouraud agar medium. Assay samples (20 μl) were applied to 8 mm filter discs and air dried at room temperature before being placed on the seeded assay plates. After incubation for 48 h at 28℃, the size of clear inhibition zones was measured.

Gel electrophoresis

Electrophoresis of native protein was carried out in 12.5% acrylamide containing 5% acetic acid and 5 M urea, using a continuous buffer system (Harwig et al., 1993). No stacking gel was used. Gel was prerun for 1 h at 150 V with reversed polarity (lower chamber-cathode). Both the upper and the lower chambers contained 5% acetic acid. The sample was mixed (2:1) with acid urea gel sample solution (9 M urea in 5% acetic acid containing methyl green as a tracking dye). The sample thus prepared was carefully layered onto the gel. Electrophoresis was carried out at 150 V with the usual polarity of the electrodes reversed so that the cathode was at the bottom.

Gel overlay assay

The electrophoresed gels were rinsed for 15 min in 10 mM sodium phosphate buffer (pH 7.4) and placed on top of a 1 mm-deep agar layer that had been seeded with approximately 4×10^5 bacteria per ml. In addition to bacteria, this agar underlayer contained 0.03% (w/v) trypticase soy broth powder, 1% (w/v) agarose, 10 mM sodium citrate phosphate buffer, and 0.02% Tween 20. Stock citrate phosphate buffer was made by mixing 100 mM citric acid and 100 mM sodium phosphate and diluting this mixture 1:10 into the agar underlayer. The final pH of the agar underlayer was adjusted to 6.5 or 7.4 before the agar was autoclaved, and the bacteria were added after the agar had cooled to 42-43℃, just before being poured. After 3 h of incubation at 37℃ to allow the electrophoresed proteins to diffuse into the bacterial underlayer, the acid-urea polyacrylamide gel was removed, and a nutrient-rich top agar was poured over the agar underlayer to allow the surviving bacteria to grow. After incubation for at least 24 h at 37℃, the presence of a clear zone indicated the locations of antimicrobial peptides.

Purification of skin secretion

The skin secretions (5 ml) were diluted by the addition of 1 ml of 0.1 M ammonium acetate, pH 6.0. The sample was applied to a column (10×1.5 cm) with CM Sepharose CL-6B gel (Pharmacia) equilibrated with the same buffer and eluted with a linear gradient of 0.1-1.0 M ammonium acetate (pH 6.0). The antibacterial activity was determined as above. For further purification, the major peak (corresponding to fractions 95-99, Fig. 1) was pooled, concentrated, and subjected to reverse-phase fast performance liquid chromatography (RP-FPLC) on a PepRPC HR5/5 column (Pharmacia) equilibrated with 0.1% TFA. Elution was performed with a linear gradient of 30-38% acetonitrile in water. Ultraviolet absorption was monitored at 206 nm, and the antibacterial activity was determined with aliquots of the fraction that had been freeze-dried to remove acetonitrile.

Mass spectrometry

The purified P1, P2, and P3 were dissolved in water/acetonitrile (50:50, v/v) containing 0.5% formic acid and analysed on a VG Platform benchtop single quadrupole mass spectrometer (Fisons Instruments/VG BioTech, Altrincham, U.K.) equipped with an electrospray ionization source operating at atmospheric pressure. Mass spectra were recorded in the positive ion mode, and scan range was performed from *m/z* 250 to *m/z* 1,500.

Capillary electrophoresis

Peptide purity was tested by capillary electrophoresis. Samples were injected under vacuum into a model HP^{3D} capillary electrophoresis system (Hewlett Packard) equipped with a capillary (50 μm × 56 cm). Separation from anode to cathode was carried out in 0.1 M phosphate buffer, pH 2.5 with a voltage of 20 kV at 25℃. Capillary effluent was detected by its ultraviolet absorption at 214 nm.

Amino acid analysis

For general amino acid analysis, purified P1, P2, and P3 were vacuum-dried, hydrolyzed in 6 N HCl at 110℃ for 24 h, and subjected to high performance liquid chromatography (510 solvent delivery pump, 712 WISP automated sample processor, picotag (TM) column, 990 photodiode array detector; Millipore). Samples were oxidized with performic acid in advance of acid hydrolysis for cysteine and hydrolyzed with 4 M methane sulfonic acid for tryptophan.

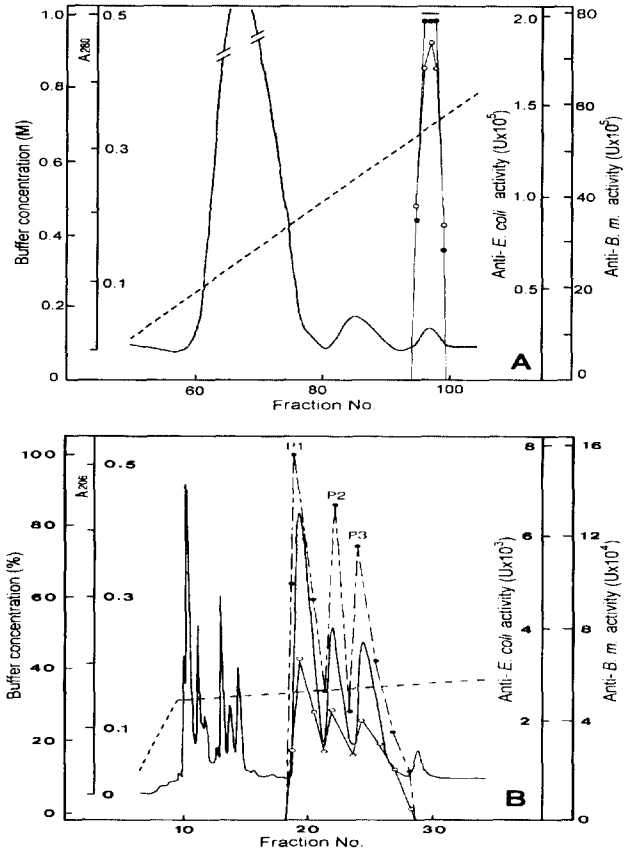

Fig. 1. Isolation of antimicrobial peptides from *B. orientalis*. A, Elution profile of CM-Sepharose cation exchange chromatography of skin secretions. The skin secretions were applied to a column (10×1.5 cm) with CM-Sepharose CL-6B gel equilibrated with ammonium acetate buffer (pH 6.0) and eluted with a linear gradient of 0.1-1.0 M of the same buffer. (—), absorbance at 280 nm; (---), ammonium acetate concentration; (-○-), anti-*E. coli* activity; (-●-), anti-*B. megaterium* activity. B, Elution profile of RP-FPLC with a PepRPC HR5/5 column equilibrated with 0.1% TFA of active fractions from CM-Sepharose column. Elution was performed with a linear gradient of 30-38% acetonitrile in water. (—), absorbance at 206 nm; (---), ammonium acetate concentration. The bar indicates the active peak with anti-*E. coli* and anti-*B. megaterium* activity.

Results and Discussion

Purification of antimicrobial peptides from skin secretions

The antimicrobial peptides from skin secretions were isolated by cation-exchange chromatography and RP-FPLC. The skin secretions were applied to a CM-Sepharose column under conditions as described (Fig. 1), and aliquots of the eluted fractions were assayed against *E. coli* and *B. megaterium*. The major peak (corresponding to fractions 95-99, Fig. 1A) was pooled, concentrated, and further purified by RP-FPLC. Fractions displaying the highest antimicrobial activity against both *E. coli* and *B. megaterium* were further purified by RP-FPLC. As shown in Fig. 1B, antimicrobial activity resided in the three peaks indicated as P1, P2, and P3.

To confirm the purity of single peptide, the isolated three peaks, P1, P2, and P3 were tested. Fig. 2 shows

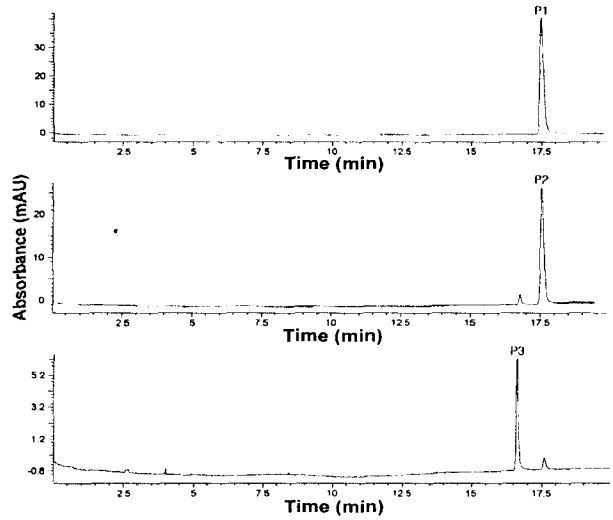

Fig. 2. Capillary electrophoresis of P1, P2, and P3 from *B. orientalis*.

that each peak was well separated by capillary electrophoresis system.

Electrophoresis, molecular weight, and amino acid composition

The purified P1, P2, and P3 were analyzed by acid-urea PAGE and the gel was stained for protein, or overlaid with *E. coli*. P1 and P2 gave a single band with similar but slightly different mobilities (Fig. 3A). Next, we examined whether it had antimicrobial activity. After acidic electrophoresis of the samples and 3 μg synthetic magainin 1, the gel was overlaid with

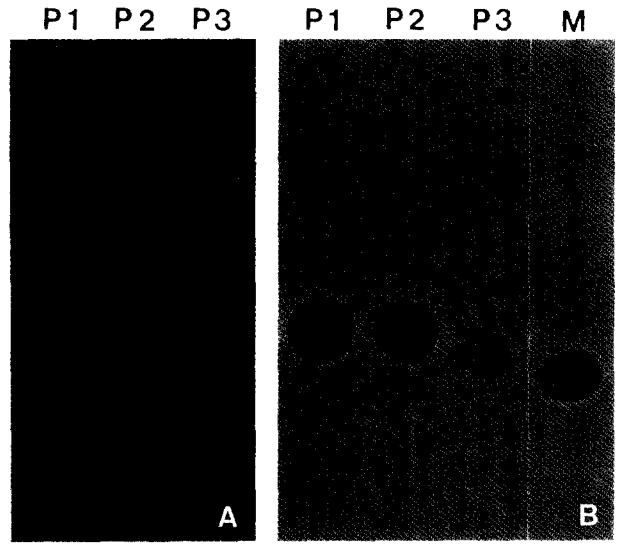

Fig. 3. Acid-urea PAGE of P1 (4 μg), P2 (4 μg), and P3 (4 μg) secreted from skin of *B. orientalis*. A, Gel was stained for protein with Coomassie brilliant blue. B, Gel was overlaid with viable *E. coli* D21 to detect antibacterial activity. M, Synthetic magainin 1 (3 μg).

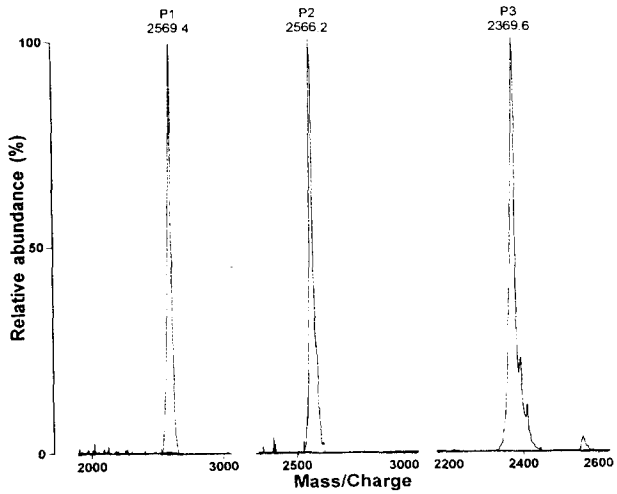

Fig. 4. MALDI-TOF mass spectrum of P1, P2, and P3 from *B. orientalis*.

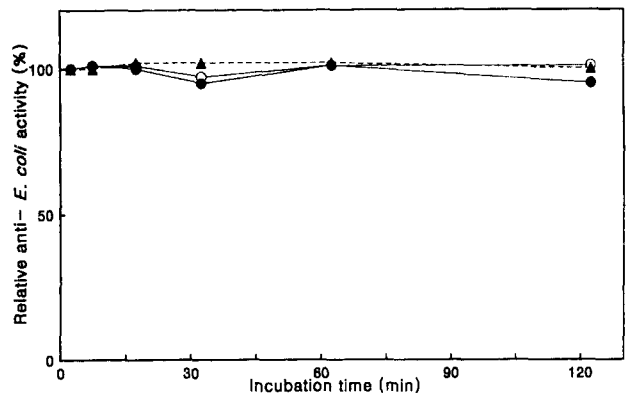

Fig. 5. Effect of temperature on antibacterial activity of P1 (○), P2 (●), and P3 (▲) from *B. orientalis*. The samples were incubated at 100℃. Each time, 3 μl aliquots were withdrawn and assayed against *E. coli* K12, using the inhibition zone assay.

viable *E. coli* to detect antimicrobial activity (Lehrer et al., 1991). Fig. 3B shows that one spot inhibiting growth of bacteria was found in each of the purified P1, P2, and P3. The mobility of these peptides were almost similar to that of synthetic magainin 1.

The molecular weight of each peptide was estimated to be about 2.5 kDa by tricine SDS-PAGE (data not shown). To clarify the molecular weight precisely, we used a MALDI-TOF mass spectrometer. The molecular weights of P1, P2, and P3 are 2569.4, 2566.2, and 2369.6 Da, respectively (Fig. 4), which is similar to that of bombinin-like peptides (BLPs) (Gibson et al., 1991) and corresponds to the value calculated by amino acid analysis.

The amino acid compositions of all three peptides were analyzed by HPLC (Table 1). Amphipathic nature with net positive charge is observed in all three peptides. It was known that positive charge undergoes electrostatic attraction with negative charge on the surface of the bacteria (Gabay, 1994). Total amino acid residues of P1 and P2 are 27 and 26, respectively without cysteine residues, and in the case of P3, 24 amino acid residues. Gibson et al. (1991) reported that three bombinin-like peptides isolated from the skin of *B. orientalis* are amphipathic, cationic peptides of 25-27 amino acids in length. These results suggest that a family of closely related peptides, differing from each other by only one or a few amino acids, are present in the skin of *B. orientalis*. Therefore, these peptides are related but not identical to BLPs.

Properties and antimicrobial spectra

Three gram negative strains, three gram positive strains, and two fungi were used to test the antimicrobial activity of P1, P2, and P3. P1 showed a relatively high antimicrobial activity against the *Escherichia*

Table 1. Amino acid analysis of P1, P2, and P3 from *B. orientalis*

Amino acid	P1		P2		P3	
	Molecular percent	Number of residues	Molecular percent	Number of residues	Molecular percent	Number of residues
Aspartate[a]	2.16	1	2.53	1	0.36	0
Glutamate[b]	3.69	1	4.92	1	3.41	1
Serine	10.85	3	11.18	3	8.30	2
Glycine	19.53	6	19.88	5	20.49	5
Histidine	3.31	1	3.05	1	3.79	1
Arginine	0.57	0	1.27	0	0.52	0
Threonine	0.53	0	1.12	0	0.27	0
Alanine	24.02	6	19.33	5	25.36	6
Proline	0.00	0	1.23	0	0.00	0
Tyrosine	0.55	0	2.33	1	1.25	0
Valine	0.37	0	1.05	0	0.37	0
Methionine	0.46	0	0.83	0	0.27	0
Cysteine	0.09	0	0.22	0	0.06	0
Isoleucine	6.95	2	6.46	2	7.25	2
Leucine	14.70	4	14.05	4	15.39	4
Phenylalanine	3.53	1	3.11	1	3.49	1
Trptophan	0.35	0	0.81	0	0.61	0
Lysine	8.34	2	6.63	2	8.81	2
Total residues		27		26		24

[a]Aspartate and [b]glutamate mean sum of asparagine and aspartic acid and glutamine and glutamic acid, respectively.

Table 2. Antimicrobial spectra of P1, P2 and P3 from *B. orientalis*

Microorganism	Antimicrobial activity (diameter of inhibition zone, mm)		
	P1	P2	P3
Escherichia coli K12	7.7	7.1	4.6
E. coli D21	7.6	7.5	4.4
Enterobacter cloacae	3.0[a]	3.0[a]	3.0[a]
Bacillus megaterium Bm11	14.5	12.5	11.9
B. subtilis	6.3	5.5	5.9
B. thuringiensis	5.3	5.8	5.2
Candida albicans	12.2	8.0[b]	8.0[b]
C. tropicalis	8.0[b]	8.0[b]	8.0[b]

*Antibacterial activity was evaluated by measuring as inhibition zones on thin agar plates with about 8×10^4 viable cells in 6 ml of a rich medium. The diameter of the inhibition zone is proportional to the logarithm of the concentration of the inhibiting agent. The volume applied to each well was 3 μl and the concentration was μg/μl. The inhibition zone around the wells was recorded after overnight incubation at 37℃. The diameter of the well was 3 mm. For antifungal activity, an 8 mm filter-paper disc was used (Schwartz et al., 1988). [a,b]The diameters of the well were 3 and 8 mm, respectively and no inhibition zone was detected.

strains, *Bacillus* strains, and one fungal strain tested. *Enterobacter cloacae* and *Candida tropicalis,* however, do not show any sensitivity to all three peptides (Table 2). Moreover, although these three peptides show antimicrobial activity, they do not show significant hemolytic activity against human red blood cells (data not shown). This result is consistent with studies reported previously for BLPs from *B. orientalis* (Gibson et al., 1991), magainin 1 and 2 from *Xenopus laevis* (Soravia et al., 1988), and gaegurins from *Rana rugosa* (Park et al., 1994). The lack of hemolytic activity is in marked contrast to the reported hemolytic activity for bombinin from *B. variegata* (Csordas and Michl, 1970).

The isolated three peptides were tested for their heat stability. All three peptides retained almost full activity after 120 min of incubation at 100℃ (Fig. 5). Thus, it can be concluded that the peptides show a selectivity for prokaryotic over eukaryotic membranes and are quite stable in heat.

Acknowledgements

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