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Purification and characterization of antimicrobial peptides from the skin secretion of *Rana dybowskii*

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ARTICLE INFO

Article history:
Received 9 May 2007
Received in revised form
30 June 2007
Accepted 2 July 2007
Published on line 7 July 2007

Keywords:
Peptide
Rana dybowskii
Dybowskin
Amphibian skin
Rana-box

ABSTRACT

Six antimicrobial peptides designated dybowskins were isolated from the skin secretion of *Rana dybowskii*, an edible frog in Korea. Dybowskin-1 (FLIGMTHGLICLISRKC) and dybowskin-2 (FLIGMTQGLICLITRKC) were isoforms differing in only two amino acid residues at the 7th and 14th positions from the N-terminus, and they showed amino acid sequence similarities with ranalexin peptides. Dybowskin-3 (GLFDVVKGVLKGVGKNVAGSLLEQLKCKLSGGC), dybowskin-4 (VWPLGLVICKALKIC), dybowskin-5 (GLFSVVTGVLKAVGKNVAKNVGGSLLEQLKCKISGGC), and dybowskin-6 (FLPLLLAGLPLKLCFLFKKC) differed in both size and sequence, and they were, in terms of amino acid sequence similarities, related to brevinin-2, japonicin-2, esculentin-2, and brevinin-1 peptides, respectively. All the peptides presented in this paper contained *Rana*-box, the cyclic heptapeptide domain, which is conserved in other antimicrobial peptides derived from the genus *Rana*. All the dybowskin peptides showed a broad spectrum of antimicrobial activity against the Gram-positive and Gram-negative bacteria (minimum inhibition concentrations (MIC), 12.5 to >100 μg/ml) and against *Candida albicans* (MIC, 25 to >100 μg/ml). Especially, dybowskin-4 with valine at its N-terminus was the most abundant and showed the strongest antimicrobial activity among all the dybowskin peptides. This result indicates that the dybowskin peptides from *R. dybowskii*, whose main habitats are mountains or forests, have evolved differently from antimicrobial peptides isolated from other Korean frogs, whose habitats are plain fields.

1. Introduction

The emergence of increasing numbers of pathogenic microorganisms which are resistant to the commonly used antibiotics has greatly stimulated searches for novel antimicrobial agents that possess a broad spectrum of antimicrobial activity. Antimicrobial peptides play important roles on innate immunity that many animals have against microorganisms and other pathogens [1]. In case of amphibians, skin glands are rich sources of antimicrobial peptides which are released into skin secretions upon stress or injury [2,27,28].

Frogs from the genus *Rana* comprise an estimated 250 species worldwide, and the analysis of skin secretions and/or skin extracts of different species of ranid frogs has led to the isolation of numerous antimicrobial peptides with virtually no identical peptides with respect to amino acid sequences have been discovered from a single species [4,35]. This molecular diversity is considered to be important in protecting the animal from invasion by a wide array of different microorganisms and providing useful taxonomic and phylogenic markers to clarify the evolutionary history of ranid frogs [4]. These ranid antimicrobial peptides are composed of 11–48

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0196-9781/$ – see front matter
doi:10.1016/j.peptides.2007.07.002

amino acid residues and are characterized by cationic, amphipathic, and helical properties [4,20,34]. Most of these peptides, except for antimicrobial peptides such as the bP peptides from *Rana catesbeiana* [23] and the temporins from a number of *Rana* species [21,27], exhibit a unique pattern of amino acid sequence at their N-terminus, with phenylalanine or glycine as the first amino acid, while their C-terminus contains *Rana*-box, the cyclic heptapeptide domain [25]. The precise role of the *Rana*-box in the antimicrobial activity has not yet been elucidated [12,13,18–20,26]. Peptides from frogs show a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, and fungi. In addition, it has also been reported that some antimicrobial peptides show anti-tumor activity with little toxicity against non-malignant cells [6,14], and that already-existing antimicrobial peptides have been evaluated in order to assess their potential for therapeutic use such as controlling dental cariogenic oral *Streptococci* [15].

Although the mode of action of antimicrobial peptides from frogs is not completely understood, studies showed that these peptides are mostly unstructured in water, but have the propensity to form amphipathic α-helices either in a membrane-mimetic solvent or in model micelles [11,27,37]. Thus, the helix-forming potential of peptides are attributable to perturbation of the structure of lipid bilayer, then the peptide-invaded membrane becomes leaky and the cell begins to suffer irreversible damage and dies.

Rana dybowskii is an edible, medium-sized (5–9 cm) brown frog found mainly in wooded regions, broad-leaved and mixed pine-broad-leaved forests, coniferous forests, and valley groves in Korea, Japan, and the Eastern Siberia. In this study, we isolated, purified, and characterized six antimicrobial peptides that were designated dybowskins. Dybowskin-4, in particular, was the most abundant and the most potent antimicrobial peptide isolated from *R. dybowskii*. Dybowskins, therefore, may be used as important taxonomic and phylogenetic markers and their study may provide clues to understand the evolutionary diversification of *Rana* antimicrobial peptides.

2. Materials and methods

2.1. Preparation of skin secretions

R. dybowskii ($n = 40$, males) were purchased from Cheolwon frog farm located north of Seoul, Korea. The frogs were repeatedly pulsed with electrodes at 30 V, 15 mA for 3 s. Skin secretions were washed from the frogs with distilled water containing 0.5 mM phenylmethylsulfonylfluoride (Sigma Chemicals, MO, USA) and then lyophilized.

2.2. Purification of the antimicrobial peptides

The lyophilized skin secretion (57.55 mg) was dissolved in 100 ml of 5% glacial acetic acid (Sigma Chemicals) and partially purified with 4 Sep-Pak C-18 cartridges (Waters Corp., MA, USA) at a flow rate of 2 ml/min. The bound materials were eluted with acetonitrile (ACN)/water/trifluoroacetic acid (TFA, 70.0:29.9:0.1, v/v/v) and lyophilized. Low molecular weight peptides were further separated using Amicon Ultra-15 centrifugal filters (Millipore Corp., MA, USA) and lyophilized. Subsequently, the final product was redissolved in 20% ACN/0.1% TFA, and subjected to a reversed-phase high-performance liquid chromatography (RP-HPLC) by using a semi-preparative C4 column (4.6 mm × 250 mm, Vydac, CA, USA) equilibrated with 20% ACN/0.1% TFA. The peptides were eluted in a linear gradient of 20–60% ACN/0.1% TFA at 4 ml/min for 60 min. The absorbance was measured at 224 nm. The peaks containing antimicrobial activities were pooled and analyzed with MALDI-MS by using a Biflex IV system equipped with a 337 nm nitrogen laser (Bruker, Bremen, Germany). Fractions containing two or more peptides were further chromatographed on an analytical Vydac218TP54 (C-18, 2.1 mm × 250 mm) column.

2.3. Determination of sequences

Amino acid sequences of the purified peptides were determined by the automated Edman degradation method using an automatic protein sequencer (LC 491, Applied Biosystems, CA, USA). Molecular weights of the purified peptides were determined by MALDI-MS. The analyte ions were accelerated at 20 kV under delayed extraction conditions in the positive ionization mode. MALDI-MS spectra were acquired in the 1500–4000 Da range in the reflection mode, and the external standard used for MALDI analysis was a mixture of bradykinin 1–5 (M_r, 572.3070), bradykinin 2–9 (M_r, 903.4603), angiotensin I (M_r, 1295.6774), adrenocorticotropic hormone rat fragment 1-16 (M_r, 1935.9776), adrenocorticotropic hormone fragment 18–39 (M_r, 2464.1909), and parathyroid hormone (M_r, 3285.6694).

2.4. Analysis of physicochemical properties

The mean hydrophobicity was calculated as mean per residue hydrophobicity. The amphipathicity of the peptides was calculated by determining the mean hydrophobic moments (μH) of an idealized α-helix, in which side-chain protrude perpendicular to the helix axis at regular 100 intervals, using a web-based peptide sequence analysis tool (http://www.bbcm.univ.trieste.it/~tossi/HydroCalc/HydroMCalc.html) which is based on Eisenberg's hydrophobic moment formula [8]. In all calculations, the numerical values used are in the consensus scale of Kyte and Doolittle [17]. Net charges were calculated using the sum of charged amino acid resides.

2.5. Microorganisms

The bacteria species used were *Micrococcus luteus* (ATCC4698), *Klebsiella pneumoniae* (ATCC10031), *Shigella dysenteriae* (ATCC9752), *Pseudomonas aeruginosa* (ATCC9027), *Proteus mirabilis* (ATCC25933), *Staphylococcus epidermidis* (ATCC12228), and *Bacillus subtilis* (ATCC6633). *Escherichia coli* (KCTC2433) and *Staphylococcus aureus* (KCTC3881) were purchased from Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience and Biotechnology, Korea. *Candida albicans* (ATCC36232) was kindly provided by B.R. Lee.

2.6. Top agar assay

Antimicrobial activity of the peptides was monitored by a standard top agar assay as described previously [25]. Briefly, *M.*

luteus was grown overnight in Luria-Bertani (LB) broth and inoculated into 5 ml of molten 0.6% LB agar at a final concentration of 10^7 CFU/ml, followed by overlaying on a 150-mm petri dish containing solidified 2% LB agar. After the top agar was solidified, 5 μl of the peptide samples were dropped onto the surface of the top agar and completely dried before overnight incubation at 37 °C. The peptide samples forming a clear zone on the surface of the top agar were considered to contain antimicrobial activity.

2.7. Minimal inhibitory concentration

Stock cultures of bacterial strains were grown in Mueller–Hinton (MH) broth, whereas the fungal strain, *C. albicans*, was grown in yeast extract–peptone–dextrose (YPD) broth. Minimal inhibitory concentrations (MICs) were measured by standard microdilution method previously described [25]. Briefly, cells were incubated at a concentration of 10^6 CFU/ml in the media containing appropriate aliquots of the peptides. MIC was defined as the lowest concentration of the peptides inhibiting visible growth after an overnight incubation at 37 °C. Each experiment was performed three times.

2.8. Hemolytic assays

Hemolytic assay was carried out with human red blood cells (hRBCs) as described previously [25]. Hemolysis induced by the peptides was measured by incubating 10% (v/v) suspension of hRBCs in phosphate-buffered saline (PBS) with 100 μg/ml of the peptides at 37 °C for 10 min. After centrifugation at 10,000 × *g* for 10 min, the optical density of the supernatant at 350 nm was measured. The hemolytic activity of the peptides was determined by calculating relative absorbance to that obtained by treatment of 0.1% Triton X-100 (100%). PBS was used as negative control (0%).

2.9. Biochemical synthesis of peptides

Dybowskin peptides were synthesized based on standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry [22]. Briefly, 2-chlorotrityl resin (BeadTech Inc., Seoul, Korea) was used as the support to obtain a carboxylated C-terminal peptide. After synthesis, the peptides were cleaved from the resin by incubating with a mixture of TFA, 1,2-ethanedithiol, and thioanisole (Sigma Chemicals, 90:5:5, v/v/v) for 2 h at room temperature. After removing the resin by filtration through glass wool, the peptides were precipitated with 30 ml of *tert*-butyl methyl ether (Sigma Chemicals). The precipitated peptides were solubilized with 20% ACN and subjected to RP-HPLC purification as described previously [15]. The purity of each synthesized dybowskin peptide was analyzed by both analytical RP-HPLC and mass spectrometry.

3. Results

3.1. Isolation and purification of antimicrobial peptides

From 40 medium-sized (5–9 cm) male *R. dybowskii*, 57.55 mg of crude skin extract was obtained. After the partial purification on Sep-Pak C-18 cartridges, a total of 46.50 mg of the skin extract was obtained from *R. dybowskii*. The subsequent ultrafiltration led to 37.50 mg of small peptides, whose sizes ranged between 1 and 4 kDa. The low molecular weight peptides were resolved by RP-HPLC using a Vydac C-4 column (Fig. 1A). Each fraction was sequentially screened for antimicrobial activity by using the top agar assay, and significant antimicrobial activity against *M. luteus* was detected in six fractions (Fig. 1B). The numbers on the top agar plate correspond to those of the arrowheads in the HPLC profile presented in Fig. 1A. MALDI-MS revealed that fractions 1, 3, and 5 contained two or more peptides. These fractions were subjected to an additional RP-HPLC against analytical Vydac C-18 column by using linear gradients, from 25% to 65% ACN/0.1% TFA, at a flow rate of 2 ml/min for 40 min. Major peaks were pooled and assayed for antimicrobial activity. Finally, six antimicrobial peptides were purified to near homogeneity, as revealed by mass spectroscopy, gel electrophoresis, and the symmetrical peak shapes on the analytical Vydac C-18 column (data not shown). These peptides were designated from dybowskin-1 to dybowskin-6; the final quantities of the pure peptides in dry weight were 2.2, 0.5, 1, 3.0, 1, and 0.3 mg, respectively.

3.2. Structural characterization of dybowskin peptides

The amino acid sequences of the purified antimicrobial peptides were determined by automated Edman degradation. The approximate molecular masses determined by MALDI-MS were consistent with the predicted masses (Table 1). Results showed that dybowskin-1 (FLIGMTHGLICLISRKC) and dybowskin-2 (FLIGMTQGLICLITRKC) were isoforms differing in only two amino acid residues at the 7th and 14th positions from the N-terminus. Dybowskin-3 (GLFDVVKGVLKGVGKNVAGSLLEQLKCKLSGGC), dybowskin-4 (VWPLGLVICKALKIC), dybowskin-5 (GLFSVVTGVLKAVGKNVAKNVGGSLLEQLKCKISGGC), and dybowskin-6 (FLPLLLAGLPLKLCFLFKKC) were sequenced and found to contain 33, 15, 37, and 20 amino acid residues, respectively. Cysteine residues were not identified in the underivatized peptides, but the presence of an intramolecular cysteine bridge in all the six peptides was confirmed by MALDI-MS. On an average, the observed peptide masses were 1.2 Da less than the calculated masses of peptides without the disulfide bonds. The synthesized dybowskin peptides were purified to near homogeneity, as assessed by the symmetrical peaks on the analytical Vydac C-18 column. The mass spectroscopy analysis revealed that after oxidation, the molecular masses of the synthetic dybowskin peptides were in accordance with those of native dybowskin peptides (data not shown). The accuracy of the instrument used in this study for mass determination was ±1 Da.

Through the alignment of amino acid sequences using databases provided by UniProtKB/SWISS-PROT at EBI and the antimicrobial peptide database (APD, http://aps.unmc.edu/AP/main.php), all the six dybowskin peptides were found to have significant amino acid sequence similarities with other antimicrobial peptides previously isolated from the *Rana* species. When compared with other *Rana*-originated peptides, dybowskin-1 exhibited 35% amino acid similarity with both ranalexin and ranalexin-1CA, whereas dybowskin-2 displayed

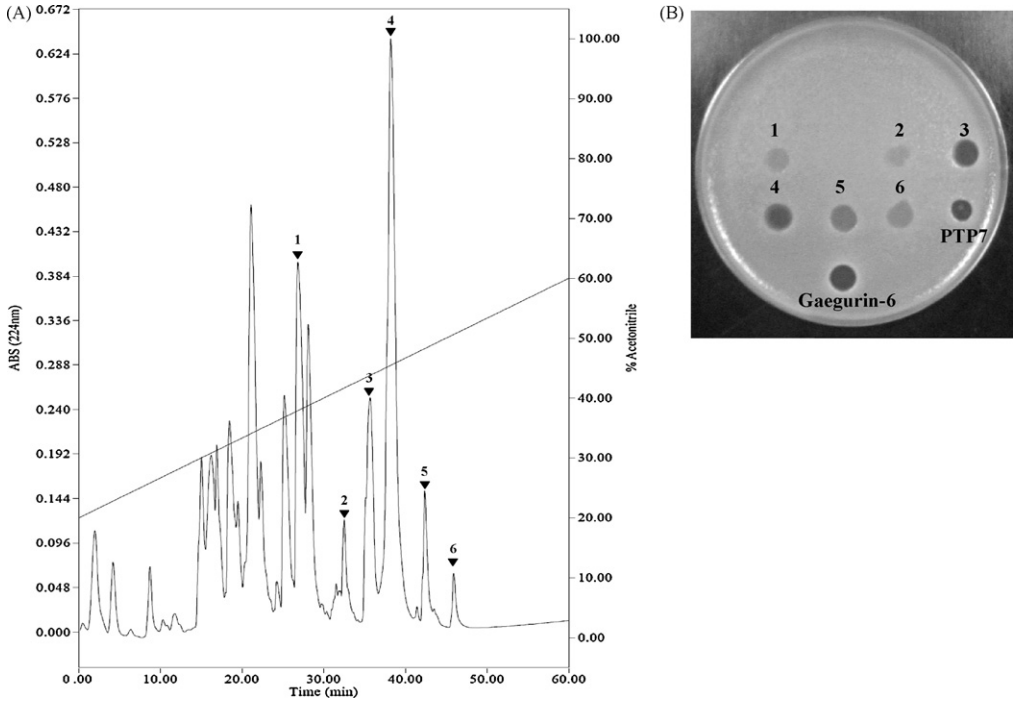

Fig. 1 – Isolation of the antimicrobial peptides by reverse-phase HPLC. (A) Elution profile of a reverse-phase HPLC. Peaks denoted by the numbered arrows indicate the fractions containing antimicrobial activity. The solid line across the profile shows the concentration of ACN in the eluting solvent. (B) Top agar assay showing the growth-inhibitory activity against *Micrococcus luteus*. The numbered spots correspond to the numbers of dybowskin peptides and to the peaks marked in the HPLC profile. Gaegurin-6 [25] and PTP7 [14,15] were used as positive controls for the antimicrobial activity.

41% similarity with the same peptides (Fig. 2A). Fig. 2B shows amino acid sequence similarity between dybowskin-3, containing 33 amino acid residues peptide, and *Rana*-originated brevinin-2 (45%), brevinin-2E (45%), brevinin-2Eb (39%), brevinin-2Ea (36%), gaegurin-2 (39%), gaegurin-3 (39%), and rugosin-A (30%). Dybowskin-4 with 15 amino acid residues was the shortest dybowskin peptide purified and had a unique N-terminus valine residue. As shown in Fig. 2C, its primary structure was similar to those of japonicin-2CHb (33%), japonicin-2CHc (33%), and japonicin-2CHd (26%) that were isolated from *Rana chaochiaoensis* [5]. It should be noted that the mean hydrophobicity (1.66) and hydrophobic moment (1.318) of dybowskin-4 were the highest among all the purified dybowskin peptides (Table 2). Fig. 2D shows the primary structure similarity between the 37 amino acid-long dybowskin-5 and the *Rana* peptides, including esculentin-2a (45%), esculentin-2b (45%), esculentin-2L (40%), esculentin-2B (35%), esculentin-2P (32%), gaegurin-4 (27%), and rugosin-4 (27%). Dybowskin-6 is a 20 amino acid residue peptide which shows 50% amino acid sequence similarity with both gaegurin-6 and brevinin-1E.

All the dybowskin peptides contain *Rana*-box, and the consensus sequence of the dybowskin *Rana*-box is C-[L/K/F]-[I/L/A]-[S/L/F]-[G/K/R]-[K/G/I]-C. The internal sequences of *Rana*-box in the dybowskin peptides also showed a high similarity with those of the antimicrobial peptides listed in Fig. 2.

Table 1 – Primary structures and molecular masses of the antimicrobial peptides isolated from the skin secretion of R. *dybowskii*

Peptide	Amino acid sequence	M_r (obs)[a]	M_r (calc)[a]
Dybowskin-1	FLIGMTHGLI**CLISRKC**	1902.6	1904.0
Dybowskin-2	FLIGMTQGLI**CLITRKC**	1908.0	1909.0
Dybowskin-3	GLFDVVKGVLKGVGKNVAGSLLEQLK**CKLSGGC**	3314.8	3315.8
Dybowskin-4	VWPLGLVI**CKALKIC**	1653.8	1655.0
Dybowskin-5	GLFSVVTGVLKAVGKNVAKNVGGSLLEQLK**CKISGGC**	3671.9	3673.0
Dybowskin-6	FLPLLLAGLPLKL**CFLFKKC**	2275.2	2276.4

[a] The observed molecular masses M_r (obs) and calculated molecular masses M_r (calc) of the antimicrobial peptides are monoisotopic. The bolded letters indicate *Rana*-box.

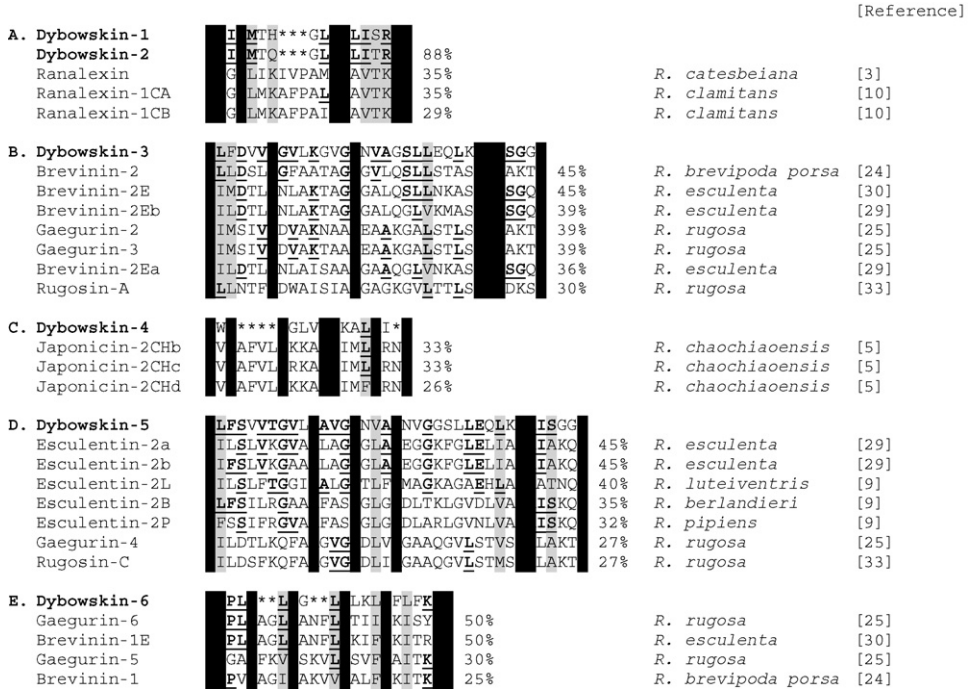

Fig. 2 – Comparison of sequence similarities between the antimicrobial peptides isolated from the skin secretion of *Rana dybowskii* and the other *Rana*-originated peptides from *Rana brevipoda porsa*, *Rana esculenta*, *Rana berlandieri*, *Rana pipiens*, *Rana luteiventris*, *Rana rugosa*, *Rana catesbeiana*, *Rana clamitans*, and *Rana chaochiaoensis*. (A) Group 1: dybowskin-1 and dybowskin-2, (B) Group 2: dybowskin-3, (C) Group 3: dybowskin-4, (D) Group 4: dybowskin-5, and (E) Group 5: dybowskin-6. Percent amino acid sequence identities are shown on the right. The residues shaded in black denote the conserved domains in the peptides, whereas the residues shaded in grey represent the conserved substitutions of similar amino acid residues. Bold and underlined residues represent identical amino acids shared by dybowskin peptides in each family. *, the deleted residues.

3.3. *Antimicrobial and hemolysis assays*

The antimicrobial spectrum of each peptide was determined by measuring the MICs. Table 3 shows the MIC value of each peptide against the Gram-positive bacteria, the Gram-negative bacteria, and the fungus. All the dybowskins showed a strong antimicrobial activity against *M. luteus* (MIC, 1.56–12.5 μg/ml) indicating that *M. luteus* is highly sensitive to the peptide antibiotics. Among the dybowskin peptides, dybowskin-4 with valine at its N-terminus, exhibited the strongest antimicrobial activity against all the tested bacteria. Dybowskin-2 to dybowskin-5 (MIC, 15–60 μg/ml) showed a moderate antimicrobial activity against *S. dysenteriae*, *E. coli*, and *K. pneumoniae*, while dybowskin-1 and dybowskin-6 were not active against the same strains (MIC, >100 μg/ml). *P. aeruginosa* and *P. mirabilis*, which have been known to secrete metalloproteases, were insensitive to all the tested peptides (MIC, >100 μg/ml). Antifungal activity of the dybowskin peptides against *C. albicans* was also tested; the MIC values were found to range from 25 to >100 μg/ml. The strongest antifungal activity was also shown by dybowskin-4 (MIC, 25 μg/ml). The pattern of antimicrobial activities of synthetic dybowskin peptides, as revealed by the MIC values, was similar to that of native dybowskin peptides (data not shown). The cytotoxicity of each peptide was measured by hemolytic activity. As shown in Table 3, dybowskin-1, dybowskin-2, dybowskin-3 and

Table 2 – Structural parameters of the dybowskin peptides

Peptide	Hi[a]	Hμ[b]	Net charge	Number of amino acids
Dybowskin-1	1.218	0.936	2	17
Dybowskin-2	1.206	0.944	2	17
Dybowskin-3	0.47	0.674	3	33
Dybowskin-4	1.66	1.318	2	15
Dybowskin-5	0.559	0.416	4	37
Dybowskin-6	1.515	0.69	3	20

[a] Mean hydrophobicity of each peptide calculated using the Kyte and Doolittle scale [17].
[b] Mean hydrophobic moment of each peptide calculated using the Kyte and Doolittle scale [17].

Table 3 – Antimicrobial and hemolytic activities of the antimicrobial peptides isolated from the skin secretion of *R. dybowskii*

Peptide	Minimal inhibitory concentration (μg/ml)										Percent hemolysis
	M. luteus	*Staphylococcus aureus*	*Bacillus subtilis*	*Staphylococcus epidermidis*	*Shigella dysenteriae*	*Escherichia coli*	*Klebsiella pneumoniae*	*Proteus mirabilis*	*Pseudomonas aeruginosa*	*Candida albicans*	
Dybowskin-1	12.5 (6.6)[a]	25 (13.1)	50 (26.3)	60 (31.5)	>100 (>52.5)	>100 (>52.5)	>100 (>52.5)	>100 (>52.5)	>100 (>52.5)	>100 (>52.5)	0.99
Dybowskin-2	6.25 (3.3)	15 (7.9)	25 (13.1)	25 (13.1)	50 (26.2)	60 (31.4)	60 (31.4)	>100 (>52.4)	>100 (>52.4)	100 (52.4)	10.99
Dybowskin-3	6.25 (1.9)	30 (9.0)	50 (15.1)	60 (18.1)	60 (18.1)	15 (4.5)	15 (4.5)	>100 (>30.2)	>100 (>30.2)	100 (30.2)	1.86
Dybowskin-4	1.56 (0.9)	3.13 (1.9)	6.25 (3.8)	6.25 (3.8)	30 (18.1)	15 (9.1)	15 (9.1)	>100 (>60.4)	>100 (>60.4)	25 (15.1)	47.58
Dybowskin-5	6.25 (1.8)	25 (6.8)	50 (13.6)	50 (13.6)	50 (13.6)	60 (16.8)	50 (13.6)	>100 (>27.2)	>100 (>27.2)	50 (13.6)	17.84
Dybowskin-6	6.25 (2.7)	12.5 (5.5)	50 (22.0)	50 (22.0)	>100 (>44.0)	>100 (>44.0)	>100 (>44.0)	>100 (>44.0)	>100 (>44.0)	50 (22.0)	59.39

[a] μM concentrations are in brackets.

dybowskin-5 induced low or moderate hemolysis, whereas dybowskin-4 and dybowskin-6 induced relatively greater hemolysis.

4. Discussion

The present study has led to the isolation six antimicrobial peptides, designated dybowskins, from the skin secretion of *R. dybowskii*. Dybowskin-1 had two amino acid residues different from dybowskin-2: the former contained histidine[7] and serine[14], whereas the latter possessed glutamine[7] and threonine[14]. The substitution ($His^7 \rightarrow Gln^7$) can be easily accomplished by mutation of a single base in the nucleotide sequence at the wobble position (CAU/CAC → CAA/CAG), whereas a single base substitution at the second nucleotide position in the codon is required for the replacement of serine with threonine (ACU/ACC → AGU/AGC). As shown in Fig. 1A, the amount of dybowskin-1 purified by RP-HPLC was significantly greater than that of dybowskin-2. On the contrary, dybowskin-2 exhibited approximately 2-fold and 11-fold higher antimicrobial and hemolytic activities, respectively, than dybowskin-1. As shown in Table 2, the differences in the structural parameters such as hydrophobicity and hydrophobic moment were insignificant; however, when the helicity was predicted by, NNPREDICT, a web-based secondary structure program, the glutamine residue at the 7th position was found to induce a stable helix formation [16]. It has been reported that helicity is important in modulating the activity and selectivity of antimicrobial peptides [7,36,37]. Therefore, it can be speculated that dybowskin-2 is generated from dybowskin-1 to increase its specific activity by increasing the helical content. Dybowskin-3 was found to exhibit amino acid sequence similarities with brevinin-2-like peptides, and as shown in Fig. 2B, it contained seven highly conserved amino acid residues (Gly^1, Lys^7, Lys^{15}, Cys^{27}, Lys^{28}, Leu^{29}, and Cys^{33}). This suggests that the conserved residues within *Rana*-box, internal lysine residues, and the N-terminal glycine may be important factors governing the antimicrobial activity of this family. The structurally unique dybowskin-4 is the shortest antimicrobial peptides isolated from the genus *Rana* that contains valine as its N-terminal residue and the C-terminal *Rana*-box. According to the HPLC profile presented in Fig. 1A and the final dry weight, dybowskin-4 was the most abundant, and it exhibited the strongest specific antimicrobial activity among all the dybowskins. As shown in Table 2, dybowskin-4 exhibited the highest mean hydrophobicity and hydrophobic moment. Such a strong hydrophobic property may lead to indiscriminant membrane perturbation that would in turn explain its high hemolytic activity (47.58%). In addition, dybowskin-4 exhibited a potent antifungal activity against *C. albicans* with a MIC of 25 μg/ml. Based on its strong antifungal activity and natural abundance, dybowskin-4 can be used to develop an antifungal agent. Dybowskin-5 showed amino acid sequence similarities with esculentin-2-like peptides listed in Fig. 2D. Dybowskin-5 showed seven amino acid residue homology (Gly^1, Lys^{11}, Lys^{15}, Lys^{19}, Cys^{31}, Lys^{32}, and Cys^{37}) with seven different peptides listed in Fig. 2D. Such amino acid sequence conservation pattern is comparable to that of dybowskin-3, which has two cysteine residues at the

ends of *Rana*-box, reoccurring internal lysine residues, and the N-terminal glycine residue. Dybowskin-6, the last dybowskin peptide eluted, exhibited amino acid sequence similarities with brevinin-1-like peptides as shown in Fig. 2E. The peptides listed in Fig. 2E contain proline residue(s). NMR spectroscopy has shown that this residue produces a stable kink in the molecule which was suggested to be important with respect to a peptide's antimicrobial activity [31,32]. Therefore, it appears that the mode of antimicrobial activity might differ between dybowskin-6 and other dybowskin peptides.

A diverse array of antimicrobial peptides has been isolated from the skins of *Rana* frogs. Although the *Rana*-originated antimicrobial peptides show a significant amount of variability and diversity, highly conserved regions still exist within the amino acid sequences that are speculated to govern the secondary structure as well as the antimicrobial activity. In this study, six antimicrobial peptides exhibiting different biological activities were isolated, purified, and characterized from *R. dybowskii*. Based on their primary structures presented in Fig. 2, these peptides could be categorized into five groups indicating that *R. dybowskii* contains one of the most highly diversified array of antimicrobial peptides among the *Rana* species. Therefore, dybowskin peptides can be used as the genetic tools for deducing the evolutionary relationship and understanding the phylogenetic interrelationships between the *Rana* species, especially in regions wherein several species coexist and produce hybrids.

Acknowledgments

This study was supported by a grant from the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2005-005 J16 001) as well as in part by Peptipharm Inc. S.S.K., M.S.S., and D.Y.L. were supported by the BK21 Research Fellowship from the Ministry of Education and Human Resources Development.

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