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# Synthesis and Characterization of GGN4 and its Tryptophan Substituted Analogue Peptides

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**Gaegurin 4 (GGN4), a broad-spectrum antibiotic, is a 37-amino acid peptide isolated from the Korean frog, *Rana rugosa*. In this study, we have chemically synthesized and purified GGN4 analogues where the C-terminal portion is truncated and/or substituted with tryptophan. These peptides show significantly different biological activities depending on the location of tryptophan and the number of amino acids truncated from the C-terminal end. While deletion of 9 amino acids from the C-terminal seems to be marginally tolerable in maintaining the antimicrobial activity, further deletion of up to 14 amino acid residues decreases the potency by more than 60-fold towards Gram-positive, and 10-fold towards Gram-negative, bacteria. Surprisingly, the reduced activity of the shorter peptide can be completely restored by a single substitution of aspartic acid 16 to tryptophan 16 (D16W). Also, the truncation seems to decrease the specificity of antibiotic activity more towards Gram-positive than towards Gram-negative bacteria studied. These data suggest a partial role of the C-terminal region in determining the binding specificity and the activity of peptides upon binding to their target cell membranes.**

**Keywords:** Deletion mutant, Gaegurin 4 (GGN4), Hemolysis, Minimum inhibitory concentration (MIC), Tryptophan substitution.

## Introduction

Amphibian skins are known to produce an abundance of biologically active peptides (Boman, 1995; Nicolas and Mor, 1995). For example, biologically active peptides such as bombinin (Csordas and Michl, 1969; Gibson *et al.*, 1991; Simmaco *et al.*, 1991) and magainins (Zasloff, 1987) have been isolated from the species *Bombina* and *Xenopus*, respectively. Many other biologically active peptides have also been isolated from *Ranidae* in particular. For examples, Brevinins (Morikawa *et al.*, 1992), Ranalexin (Clark *et al.*, 1993), Gaegurins (Park *et al*, 1994), Esculentins (Simmaco *et al.*, 1994), and Rugosins (Suzuki *et al.*, 1995) are all antimicrobial peptides from this source.

Gaegurin 4 (GGN4) is one of six antimicrobial peptides found in the Korean frog, *Rana rugosa*, which shows broad antimicrobial activity towards both Gram-negative and Gram-positive bacteria, fungi, and protozoa (Park *et al.*, 1994). GGN4 consists of 37 amino acid residues with a net charge of +4 (6 K & 2 D) and its amphipathic helical structure is maintained by a number of hydrophobic residues (Park *et al.*, 1994). Like other antibiotic peptides derived from *Ranidae*, GGN4 is characterized by the presence of two cysteine residues in positions 1 and 7 counted from the carboxy terminus, which forms a cationic heptapeptide loop between C31 and C37 (CKLAKTC) called the '*Rana* box' (Park *et al.*, 1994). The exact role of this C-terminal loop is not clear, but intensive investigations are underway to understand its functional implications (Lee *et al.*, unpublished results). Among the group of antibiotic peptides from *Ranidae*, GGN4 shows sequence homology to Esculentin 2a from *Rana esculenta* (Simmaco *et al.*, 1994) and Rugosin C from the Japanese *Rana rugosa* (Suzuki *et al.*, 1995), both of which contain the same sequence of 37 amino acids as GGN4. GGN4 also shows relatively high homology to Rugosin A, Rugosin B, Brevinin 2, and Brevinin 2E, but these peptides lack four internal amino acids spanning from residues 15–18 (see Table 1) found in GGN4 and Rugosins.

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**Table 1.** Sequence comparison of Brevinin, Gaegurin 4 and 6, Esculentin, Rugosin A, B and C, and Magainin 2. The boxes represent identical amino acids among the ten peptides compared.

| Peptides | | Amino acid sequence |
|---|---|---|
| | | 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 |
| Brevinin | 1E | F L P L L A G L A A N F L P K I F C K I T R K C |
| Gaegurin | 6 | F L P L L A G L A A N F L P T I I C K I S Y K C |
| Gaegurin | 4 | G I L D T L K Q F A K G V G K D L V K G A A Q G V L S T V S C K L A K T C |
| Esculentin | 2a | G I L S L V K G V A K L A G K G L A K E G G K F G L E L I A C K I A K Q C |
| Rugosin | C | G I L D S F K Q F A K G V G K D L I K G A A Q G V L S T M S C K L A K T C |
| Rugosin | B | S L F S L I K A G A K F G • • • • K N L L K Q G A Q Y A A C K V S K E C |
| Rugosin | A | G L L N T F K D W A I S I A • • • • K G A G K G V L T T L S C K L D K S C |
| Brevinin | 2 | G L L D S L K G F A A T A G • • • • K G V L Q S L L S T A S C K L A K T C |
| Brevinin | 2E | G I M D T L K N L A K T A G • • • • K G A L Q S L L N K A S C K L S G Q C |
| Magainin | 2 | G I G K F L H S • A K K F G K A F V G E I M N S 'Rana box' |

In this study, we generated four different C-terminal truncated GGN4 peptides (15, 22, 23, and 28 amino acids in length), where tryptophans were substituted at different sites (L3W, D16W, L17W, V18W, K19W, and T28W; see Table 2). The deletion was carried out sequentially to investigate the effects of the C-terminal truncation on the antimicrobial activity. The tryptophans were introduced to estimate the concentration of peptides accurately by the known extinction coefficient of tryptophan (Gill & von Hippel, 1989). Besides this, it allowed us to estimate site-specific information on the replaced residues in the antimicrobial activity regulation. The utilization of tryptophan as a structural or biological probe (Matsuzaki *et al.*, 1994; Shin *et al.*, 1995; Lim *et al.*, 1997) in peptide research is not uncommon. In this paper, we report the effects of the modifications on the antibiotic activity towards Gram-negative and Gram-positive bacteria. The oligomeric state of GGN4 and its analogue peptides in aqueous solution are also presented.

## Materials and Methods

**Materials** Full-length native GGN4 was purified from the Korean frog, *Rana rugosa*, captured in the wild in the southern part of the Korean peninsula. All other analogue peptides were synthesized as the carboxy form at their C-terminus using a solid-phase peptide synthesizer at the Korea Basic Science Institute (Seoul) or at the Institute for Molecular Biology and Genetics, Seoul National University. Gram-positive (*Micrococcus luteus* KCTC 1056) and Gram-negative (*E. coli* KCTC 2571) bacteria were purchased from the Korean Collection for Type Cultures (KCTC) at Daeduk, Korea. Triton X-100 and heparin were from Sigma (St. Louis, USA). Trifluoroacetic acid (TFA) was purchased from Acros Organics (New Jersey, USA) and acetonitrile and HPLC water were obtained from T. J. Baker (Phillipsburg, USA). LB base reagents were purchased from Difco (Detroit, USA).

**HPLC purification and mass analysis** Synthetic peptides were purified using HPLC on a C18 reversed-phase column (Vydac, Hesperia, USA) with a linear gradient of 17 to 80% acetonitrile in 0.1% TFA at 1.0 ml/min for 20 min. Fractions that demonstrated antimicrobial activity were pooled and dried under vacuum. These peptides were suspended in water and the concentration of the peptides were determined using the molar extinction coefficient of tryptophan at 278 nm as 5500 $M^{-1}cm^{-1}$ (Gill & von Hippel, 1989). The molecular weights of purified peptides were determined by matrix associated laser desorption time-of-flight (MALDI-ToF) mass spectroscopy (Kratos Kompact MALDI 2) at the Korea Basic Science Institute (Daeduk). It was found that the weight of all the peptides investigated fell within 3 Da difference from the calculated mass of the peptides.

**Antimicrobial assays** Antimicrobial activities of GGN4 analogues were determined by measuring the minimal inhibitory concentration (MIC) of *Micrococcus luteus* (Gram-positive, KCTC 1056) and *Escherichia coli* (Gram-negative, KCTC 2571) (Park *et al.*, 1994). They were grown in LB broth at 37°C for 10 h to an $OD_{600}$ of 0.8, representing $10^9$ colony-forming units(CFU)/ml. The bacteria of $10^9$ CFU/ml were diluted 1000 times with LB broth and aliquoted in sterilized 96-well plates to a final volume of 100 $\mu$l. Then, the peptides of known concentration were added to each well. The inhibition of growth was determined by measuring absorbance at 600 nm using an HP 8453 UV/VIS spectrophotometer after an incubation time of 10 h at 37°C.

**The estimation of hemolysis** The hemolytic activities of the GGN4 analogue peptides were assayed as described previously (Park *et al.*, 1994). Human red blood cells (hRBCs) were collected from a health center at Mokpo National University. Fifteen units of anticoagulant (heparin) were added immediately

**Table 2.** The amino acid sequence of GGN4 and its analogue peptides.

| Peptides | Amino acid sequence |
|---|---|
| | 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 3 4 5 6 7 |
| 1–37 | G I L D T L K Q F A K G V G K D L V K G A A Q G V L S T V S C K L A K T C |
| 1–28 | G I L D T L K Q F A K G V G K D L V K G A A Q G V L S T 'Rana box' |
| 28L3W | G I **W** D T L K Q F A K G V G K D L V K G A A Q G V L S T |
| 28D16W | G I L D T L K Q F A K G V G K **W** L V K G A A Q G V L S T |
| 28L17W | G I L D T L K Q F A K G V G K D **W** V K G A A Q G V L S T |
| 28V18W | G I L D T L K Q F A K G V G K D L **W** K G A A Q G V L S T |
| 28K19W | G I L D T L K Q F A K G V G K D L V **W** G A A Q G V L S T |
| 28T28W | G I L D T L K Q F A K G V G K D L V K G A A Q G V L S **W** |
| 1–23 | G I L D T L K Q F A K G V G K D L V K G A A Q |
| 23L3W | G I **W** D T L K Q F A K G V G K D L V K G A A Q |
| 23D16W | G I L D T L K Q F A K G V G K **W** L V K G A A Q |
| 1–22 | G I L D T L K Q F A K G V G K D L V K G A A |
| 22L3W | G I **W** D T L K Q F A K G V G K D L V K G A A |
| 15L3W | G I **W** D T L K Q F A K G V G K |

to 3 ml of freshly collected hRBCs. hRBCs were then diluted 10 times with 1× PBS and 100 $\mu$l of diluted hRBC was transferred into each well of a 96 multi-well plate. Each peptide was added to hRBCs to give final peptide concentrations of 10 $\mu$g/ml or 100 $\mu$g/ml and the mixture was incubated at 37°C for 30 min. Released hemoglobin was monitored by measuring the absorbance of the supernatant at 414 nm ($A_{414}$) after centrifugation for 15 min at 10000 × *g*. For controls, 0% and 100% hemolysis were determined in 1 × PBS and 0.1% triton X-100, respectively. The percent hemolysis was calculated by the following formula:

$$\% \text{ hemolysis} = 100\times(A_{414} \text{ in the peptide solution} - A_{414} \text{ in PBS})/(A_{414} \text{ in 0.1\% triton X-100} - A_{414} \text{ in PBS})$$

**Analytical ultracentrifugation** The equilibrium sedimentation studies were performed using a Beckman XL-A analytical ultracentrifuge from the National Instrumentation Center for Environmental Management (NICEM) at Suwon, Korea. The experiments were carried out using a 6-hole rotor with a counterbalance at rotor speeds of 30,000 or 40,000 rpm. The peptide concentration was 0. ~0.2 mg/ml in 2.5 mM $KH_2PO_4$, pH 3.3 and in 1 × PBS, pH 7.4. The time required for the attainment of equilibrium at 25°C was within 48 h. The distribution of sample within the cell was determined by measuring the absorbance at 220 or 230 nm. Absorbance was also measured at 330 nm where the peptide has no baseline absorption. The analysis of the ultracentrifugation data was done with the use of the MLAB (mathematical modeling laboratory) program (Knott, 1979).

For data analysis, a mathematical modeling using a non-linear least-squares curve fitting was used and the fitting function was as follows (Kim *et al.*, 1994; Lewis *et al.*, 1994):

$$C(r) = C_b \times \exp(A_p \times M_p \times (r^2 - r_b^2)) + \varepsilon$$
$$A_p = (1-v\rho)\omega^2 / 2RT$$

where C(r) is the total concentration at the radial position r, $C_b$ is the concentration of peptide at the cell bottom, $M_p$ is the molecular weight of peptide, v and $\rho$ are the partial specific volume and the solution density, respectively, $\omega$ is the rotor angular velocity, and $\varepsilon$ is a baseline error term. R is the gas constant and T is the absolute temperature.

## Results

**Chemical synthesis and physicochemical properties of analogue peptides** In Fig. 1, an example of HPLC purification and mass-spectroscopic analysis of 28L3W peptide is shown. The purity of each peptide after HPLC purification was greater than 99%, with the expected molecular masses as confirmed by MALDI mass spectrometry. All the purified peptides were as highly soluble (5 ~ 10 mg/ml) as the native full-length GGN4 which has a solubility of about 10 mg/ml in water, indicating that the C-terminal deletion and the hydrophobic tryptophan substitution have no profound effects on the solubility. All of the purified peptides were subjected to analytical ultracentrifugation at two pHs (pH 3.3 and pH 7.4) to characterize the oligomeric state of the peptides in aqueous solution. In Fig. 2, an example of the equilibrium sedimentation data for 28L3W peptide in 2.5 mM $KH_2PO_4$, pH 3.3, fitted to monomer, dimer and, decamer models is shown. For the analysis, a molecular mass of 2888.5 Da for 28L3W peptide calculated from the amino acid sequence and a density of 1.010 g/cm$^3$ at 25°C were used. The root-means-square error for the peptide to the monomeric fit analysis was about $8.50 \times 10^{-3}$, demonstrating the goodness of the fit. The quality of the fit to the monomeric model suggests that it is appropriate to conclude that

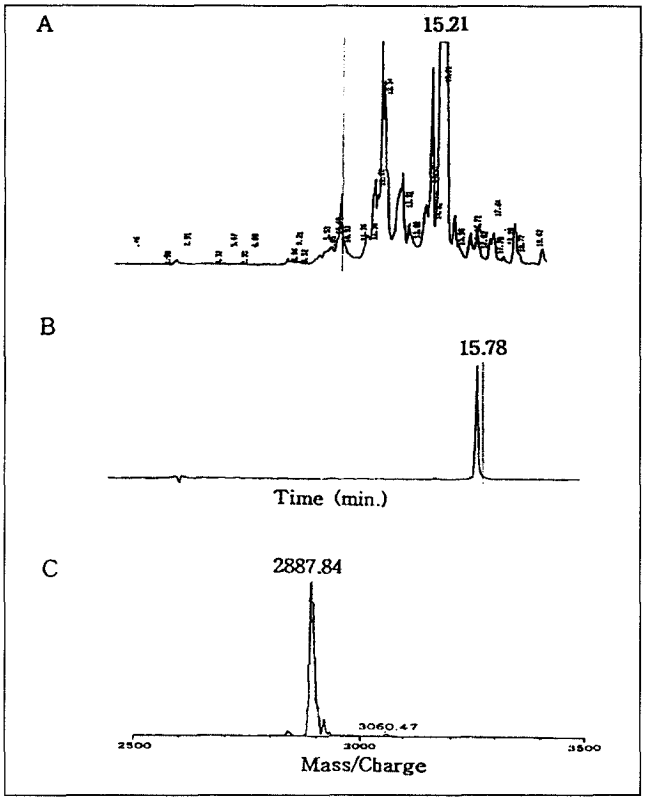


**Fig. 1.** HPLC and MALDI mass spectroscopic analysis of 28L3W peptide. A, B. HPLC profile of unpurified and HPLC purified 28L3W peptide, respectively. C. MALDI mass spectroscopic analysis result of 28L3W peptide. The calculated mass of 28L3W is 2888.5 and the experimental mass is 2887.8 Da.

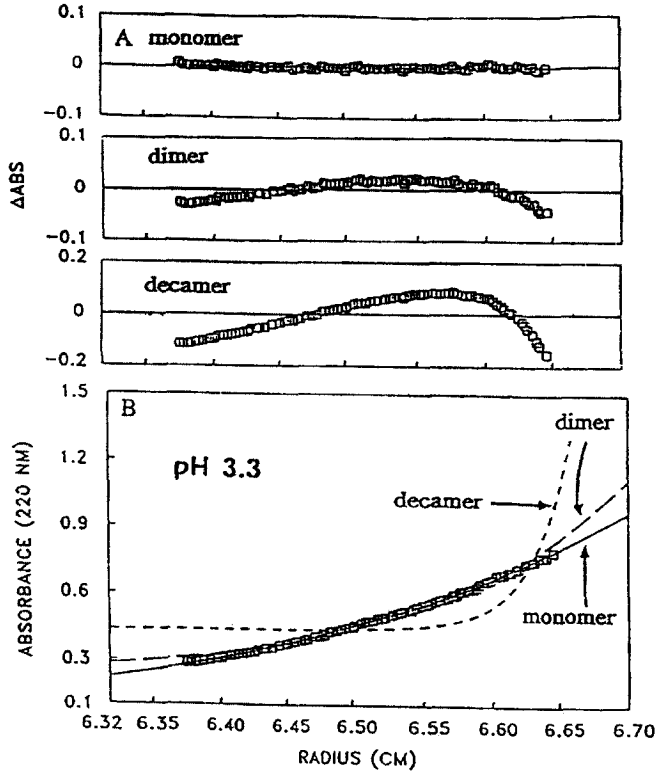


**Fig. 2.** A. Distributions of the residuals of the 28L3W peptide fitted to monomer (top), dimer (middle), and decamer (bottom) as a function of radial position. B. Distributions of the absorbance of the 28L3W peptide at 220 nm in 2.5 mM potassium phosphate buffer, pH 3.3, at ultracentrifugal equilibrium at 30000 rpm at 25°C. The fitting solid line is for a thermodynamically ideal monomer fitted to the data. The long dotted line is a fitting line for the dimer and the short dotted line is a fitting line for the decamer.

28L3W exists as a monomer in the conditions studied, and that the thermodynamic ideality of the solutes is demonstrated. The plots of distribution of residuals to the given models are shown in the upper section (A), and the actual fit to the given models is shown in the lower section (B). These results indicate that the investigated peptide exists as a monomer in the given conditions at pH 3.3. In Fig. 3, the monomeric nature of 28L3W measured at pH 7.4 (in 1× PBS) is also shown (data for the other peptides are not shown due to the similar patterns of the plots). Therefore, it is clear that the C-terminal truncation and/or introduction of tryptophans seems to have no effects on the aggregation state of peptides in the conditions studied and that the peptides are monomers in aqueous solution. Detailed characterization of the aggregation state and the secondary structure of peptides in membrane mimicking solvents and artificial membrane are currently under way.

**Effects of C-terminal deletion on antimicrobial activity**

To evaluate the structural features responsible for the antimicrobial activity of GGN4, the effects of C-terminal truncation was tested against Gram-positive (*M. luteus*) and Gram-negative (*E. coli*) bacteria. For Gram-positive bacteria, the C-terminal truncation caused non-trivial changes in the antimicrobial activity. For example, the shortening of GGN4 peptide to 28 amino acids, 1–28, resulted in a 7-fold decrease in activity. However, further deletion of the chain length to residues 1–23, 1–22, and 1–15 yielded peptides derivatives more than 60-fold less active than the native GGN4, thus almost neutralizing the bacteriocidal activity. In an extreme case, the shortest one (15L3W) failed to kill both Gram-positive and Gram-negative bacteria even at more than a 400 μg/ml concentration. From previous results (Park *et al.*, 1994), it has been shown that GGN4 is more potent towards Gram-positive than towards Gram-negative bacteria. This specificity towards Gram-positive bacteria is also true for all the derivatives investigated (Table 3). However, reducing the peptide length seems to decrease the Gram-positive specificity as judged from the decrease in minimal inhibitory concentration (MIC) ratios for the two bacteria (Table 3). For example, the activity of 1–28 dropped 3-fold

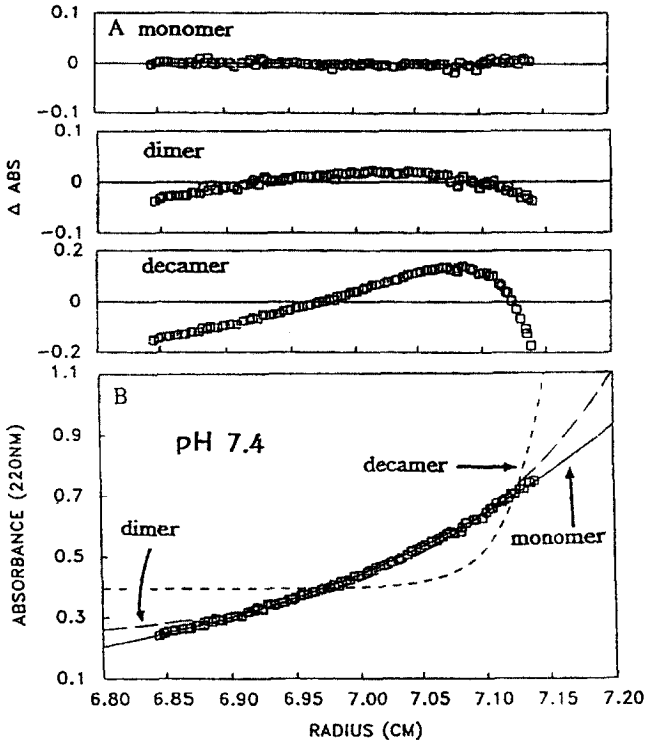


**Fig. 3.** A. Distributions of the residuals of the 28L3W peptide fitted to monomer (top), dimer (middle), and decamer (bottom) as a function of radial positions. B. Distributions of the absorbance of the 28L3W peptide at 220 nm in 1× PBS bufffer, pH 7.4 at ultracentrifugal equilibrium at 30000 rpm at 25°C. The fitting solid line is for a thermodynamically ideal monomer fitted to the data. The long dotted line is a fitting line for the dimer and the short dotted line is a fitting line for the decamer.

against Gram-negative bacteria but dropped 7-fold against Gram-positive bacteria as compared to the full-length GGN4, indicating the more deleterious effects towards the Gram-positive bacteria.

**Effects of tryptophan substitutions on antimicrobial activity** The effect of tryptophan substitution at different positions on the activity was also investigated. For Gram-positive bacteria, tryptophan substitution at the N- or C-terminus (28L3W & 28T28W) caused a minor increase in activity (10 μg/ml of MIC) as compared to the unsubstituted 1-28 (17.5 μg/ml of MIC). This slight increase of activity was also observed in other terminally-substituted tryptophan analogues (23L3W & 22L3W). In contrast, tryptophan substitutions at L17, V18, and K19 residues located in the middle of the native GGN4 sequence significantly decreased the activity to give MICs of 75, 50, and 37.5 μg/ml, respectively, suggesting the sensitivity of the peptide to those particular substitutions. While all the derivatives showed lower antibiotic activities than the full-length native GGN4, one exception was observed. That is, when a negatively charged D16 was

**Table 3.** The antimicrobial activity of GGN4 and its analogue peptides.

| Peptides | MIC (μg/ml) *M. luteus* (+)[b] | | *E. coli* (−)[c] | | Ratio[d] |
|---|---|---|---|---|---|
| 1–37[a] | 2.5 | (x1) | 75.0 | (x1) | 30.0 |
| 1–28 | 17.5 | (x7) | 225.0 | (x3) | 12.9 |
| 28L3W | 10.0 | (x4) | 75.0 | (x1) | 7.5 |
| 28D16W | 2.5 | (x1) | 37.5 | (x0.5) | 15.0 |
| 28L17W | 75.0 | (x30) | 300.0 | (x4) | 4.0 |
| 28V18W | 50.0 | (x20) | 300.0 | (x4) | 6.0 |
| 28K19W | 37.5 | (x15) | 225.0 | (x3) | 6.0 |
| 28T28W | 10.0 | (x4) | 150.0 | (x2) | 15.0 |
| 1–23 | >150.0 | (>60) | >400.0 | (>10) | N/D |
| 23L3W | 150.0 | (x60) | >400.0 | (x6) | N/D |
| 23D16W | 2.5 | (x1) | 75.0 | (x1) | 30.0 |
| 1–22 | >150.0 | (>60) | >400.0 | (x6) | N/D |
| 22L3W | 150.0 | (x60) | 375.0 | (x5) | 2.5 |
| 15L3W | >150.0 | (>60) | >400.0 | (>15) | N/D |

[a]Park *et al.* (1994)
[b]Gram-positive bacteria; values in parentheses indicate the fold decrease in activity of peptide
[c]Gram-negative bacteria; values in parentheses indicate the fold decrease in activity of peptide
[d]MIC Ratio (*E. coli* MIC / *M. luteus* MIC)

substituted for a neutral W16 residue, there was a substantial increase in the activity. For instance, the MIC of 28D16W and the shorter 23D16W to Gram-positive bacteria is 2.5 μg/ml, the same potency as the full-length GGN4. In particular, the 60-fold enhancement of the activity for the shorter 23D16W peptide (MIC of 2.5 μg/ml) as compared to the unsubstituted 1–23 (MIC of 150 μg/ml) is remarkable considering that the effect is a consequence of a single amino acid substitution. For Gram-negative bacteria, the substitution of tryptophan also caused similar effects on the activity as in the Gram-positive bacteria. In particular, D16 to W16 substitution (D16W) also dramatically improved the potency of the peptide, even higher (35.7 μg/ml) than the activity of the full-length native GGN4 (75 μg/ml).

**The estimation of hemolysis** Peptide cytotoxicity was tested against human erythrocytes and the results are summarized in Table 4. From the results, it is clear that all the peptides investigated barely lysed the erythrocytes either at 10 μg/ml or 100 μg/ml peptide concentrations. At 10 μg/ml, the maximum lytic percentage was only 0.3% and, even at 100 μg/ml, less than 1% lysis was observed. Considering that the hemolytic percentage of the full-length native GGN4 is 1.67% at 100 μg/ml, the C-terminal modifications seem to decrease the overall hemolytic activity. The deletion of this region seems to obscure the

**Table 4.** The percent hemolysis of GGN4 and its analogue peptides

| | GGN4[a] | 1–23 | 28L3W | 28T28W | 28L17W | 1–28 | 28V18W | 28K19W | 28D16W | 15L3W | 23L3W | 23D16W |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 10 μg/ml | 0.78% | 0% | 0% | 0% | 0% | 0% | 0.02% | 0% | 0% | 0.04% | 0.30% | 0.02% |
| 100 μg/ml | 1.67% | 0% | 0.07% | 0.19% | 0.73% | 0.09% | 0.50% | 0% | 0.69% | 0.07% | 0.40% | 0.38% |

[a]Park *et al.* (1994)

cell-specific recognition process to reduce the lytic action on RBCs. In indolicidin (Subbalakshmi *et al.*, 1996), it was reported that tryptophan substitution increased hemolysis. However, tryptophan substitutions in GGN4 analogues caused different results depending on the locations of the substitutions (Table 4). Considering that the bee-venom-originated peptide melittin (Park *et al.*, 1994) or mastoparan derivatives (Park *et al.*, 1997) display high lytic percentages of 10 to 100% at 100 μg/ml, the hemolysis level of the GGN4 derivatives seems to be negligible.

## Discussions

In this study, we characterized C-terminal deleted and tryptophan-substituted analogues of GGN4, an antibiotic peptide from the Korean frog, *Rana rugosa*. Using equilibrium sedimentation analysis, we found that GGN4 and its derivative peptides exist as random-coiled (CD data not shown) monomers in aqueous solution. Therefore, this data suggests that there is no effect on the aggregation state of the peptides due to the deletions or tryptophan substitutions. However, the possibility of changing their oligomeric state at different pH or salt conditions still remains as was shown from other antimicrobial peptides (Javadpour and Barkley, 1997). Since there is no difference in the oligomeric state, the differences in antimicrobial activity shown in Table 2B seems to arise from the peptide conformation changes in the lipid environments. Side-chain orientation, charge, length, or oligomeric state of peptides probably change radically upon interacting with the target cell membrane. Our ongoing spectral analysis supports this view (Kim S.-J., unpublished results). To understand the structural and mechanistic aspects of the killing action more closely, detailed physicochemical analysis on GGN4 and its derivative peptides is necessary.

To estimate the minimal length of GGN4 peptide that contains antimicrobial activity, we first investigated the C-terminal truncation effects. Based on our analysis, the minimum length of peptide with a high antibiotic potency is a 23 amino acid, since the shorter peptide with residues 1–15 showed essentially no activity. From other amphipathic peptide studies, it was shown that at least 18 residues are required to be able to provide an α-helix capable of spanning the hydrocarbon moiety of the lipid bilayer (Lear *et al.*, 1988). Therefore, mini GGN4 with its membrane-spanning potency is likely to have residues in a range from 18 to 23 residues. Some amphipathic peptides shorter than 18 residues (McLean *et al.*, 1991; Blondelle & Houghteen, 1992) were shown to kill certain micro-organisms and/or bind to membranes to form ion-channels. However, in these cases, oligomeric bundles of head-to-tail dimers were proposed to explain the channel-forming and killing activity (Agawa *et al.*, 1991). However, because the 1–15 peptide has essentially no antibiotic activity, the possibility of the shorter GGN4 analogues acting in a head-to-tail conformation to form oligomeric bundles in a phospholipid environment is unlikely.

The deletion of peptide from the C-terminal end reduced the microbicidal activity for both Gram-negative and Gram-positive bacteria. If we consider that shortening of small portions of peptides does not always decrease the antibiotic activity, as exemplified by dermaseptin (Mor *et al.*, 1994), the degree of the activity loss due to GGN4 truncation is non-trivial. However, considering the invariant presence of the 'Rana box' among all the *Ranidae* (Table 1), the activity decrease for the 1–28 peptide is less drastic than our expectations. Because the 1–23 peptide displayed more than a 60-fold decrease in antimicrobial activity compared to a 7-fold decrease for 1–28, it can be speculated that residues 24 to 28 (24-GVLST-28) are also important to maintain the bacteriocidal activiy in certain conditions.

The removal of the Rana box and its vicinity also seems to have some impact on the specificity of the peptide binding to bacteria. For example, as compared to the MIC value ratios (Table 3) of 30-fold (75/2.5 = 30) for the full-length GGN4, the ratio for 1–28 is about 12.9 (225/17.5). The trend is also shown in tryptophan-substituted shorter peptides, reducing to 2.5 for 22L3W. Thus, as the peptide gets shorter, the MIC ratio for both bacteria becomes smaller, demonstrating the gradual decrease in the specificity of GGN4 towards the Gram-positive bacteria. This aspect is also reflected in the cytotoxicity analysis showing much reduced hemolysis for the truncated peptides as compared to the full-length GGN4 (Table 4).

The antibiotic activity of the tryptophan substituted peptides are quite different from each other depending on

the locations of the tryptophan residues. While introductions at the N- or C-terminus caused a slight increase in the activity, substitutions at the middle caused significant loss of activity as compared to the unsubstituted 1–28 peptide, with the exception of D16W. The terminally substituted (L3W & T28W) tryptophan residue seems to enhance the activity through favorable hydrophobic interactions between the tryptophan residue and hydrophobic portions of the membrane. Contrary to this, substitutions at L17, V18, or K19 substantially decreased the activity as compared to the unsubstituted 1–28 peptide. Because of the absence of the four residues (15-KDLV-18) in other Rana family members, except Rugosin C and Escluentin 2a (in Table 1 dotted region), it was expected to have a minimal effect on the activity by those substitutions. However, the decrease in the activity suggests the importance of the residues for optimal activity. Due to the insufficient biophysical data on gaegurins at the present time, the structural information of GGN4 and its analogue peptides in membranes is not available. However, from the amphipathic character of GGN4 (Park *et al.*, 1994) and its analogue peptides, it is expected that these peptides form multimeric pores or oligomers in hydrophobic environments, as was shown for other amphipathic peptides (Matsuzaki *et al.*, 1994; He *et al.*, 1995; Perez-Paya *et al.*, 1995). From the Edmundson wheel diagram of gaegurins (Park *et al.*, 1994), the location of D16 is at the negatively-charged surface and those of L17 and V18 are at the hydrophobic surface on the diagram. Our ongoing analytical ultracentrifugation (Kim S.-J., unpublished results) and electrochemical analyses (Kim *et al.*, 1998) support this view of GGN4's channel-forming capacity in hydrophobic environments. Considering this and the importance of leucine and valine in coiled-coil interactions to form multimers (Harbury *et al.*, 1993), the substitution of these residues to the less efficient tryptophan in the interaction is expected to reduce the antibiotic activity.

It is of great interest that the single substitution of D16 to W16 (23D16W & 28D16W) can completely restore the activity loss caused by the C-terminal truncations. From Table 1, the location of D16 is shown to be at the non-essential region where an extra four amino acid residues (15–18) are found in Esculentin 2a, Rugosin C, and GGN4. The removal of unconserved negatively-charged aspartic acid probably enhances the membrane binding capacity by reducing the unfavorable charge-charge repulsion between the peptide and phospholipid membrane. Considering that designing cost-efficient peptides with enhanced activity is one of our goals, it is encouraging to utilize the D16W peptides as leading compounds to design better peptides. From this perspective, Rugosin C (Susuki *et al*, 1995) from Japanese *Rana rugosa* also contains D16 and the possibility of utilizing the same residue to enhance the activity of the peptide is also predictable.

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