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Effects of Substitutions of Gln16 and Asp18 with Phe or Tyr in HP(2-20) on its Structure and Antimicrobial Activity

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Abstract : HP (2-20), a 19-residue peptide derived from the N-terminus of *Helicobacter pylori* Ribosomal Protein L1, has antimicrobial activity but is not cytotoxic to human erythrocytes. Previously, we have synthesized several analogue peptides to investigate the effects of substitutions on the structure and antimicrobial activity. Substitution of Gln^{16} and Asp^{18} with Trp (Anal 3) caused a dramatic increase in bacterial and fungal lytic activities. In this study, analogue peptides were synthesized to investigate the effects of substitution of Gln and Asp with Phe (Anal 6) or Tyr (Anal 7) in HP (2-20) on its structure and antimicrobial activity. Substitution of Gln and Asp with hydrophobic aromatic residues at position 16 and 18 of HP (2-20) caused increase in antibiotic activity without hemolytic effect. Substitution of Gln and Asp with Trp and Try increased antibiotic activity of HP (2-20) twice more compared to substitution with Phe. The tertiary structures of Anal 6 and Anal 7 in SDS micelles has been investigated using NMR spectroscopy. The structures revealed that substitutions of the aromatic residues at C-terminus resulted in longer and well defined alpha-helix and improved their antibacterial activities

INTRODUCTION

Antibiotics has become more important in modern health care system. Antimicrobial peptide is one of the attractive candidates for new mechanism of antibiotics.[1] Antimicrobial

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peptides play important roles in the innate host defense mechanisms of most living organisms, including plants, insects, amphibians, and mammals.[1-3] In addition, they are known to possess potent antibiotic activity against bacteria, fungi, and even certain viruses. HP (2-20), a 19-residue peptide derived from the N-terminus of *Helicobacter pylori* Ribosomal Protein L1, has antimicrobial activity but is not cytotoxic to human erythrocytes.[4] We have synthesized several analogue peptides to investigate the effects of substitutions on the structure and antimicrobial activity. Substitution of Gln^{16} and Asp^{18} with Trp (Anal 3) caused a dramatic increase in bacterial and fungal lytic activities.[5] In this study we investigated the effects of substitution of Gln and Asp with Trp (Anal 3), Phe (Anal 6) or Tyr (Anal 7) in HP (2-20) on its structure and antimicrobial activity. The tertiary structures of Anal 6 and Anal 7 in SDS micelles have been investigated using NMR spectroscopy.

EXPERIMENTALS

Sample preparation

The peptides listed in Table 1 were generated by solid phase synthesis using Fmoc chemistry. Rink amide 4-methyl benzhydrylamine resin (0.55 mmol/g) was used as the support to obtain a C-terminal amidated peptide. The coupling of Fmoc-L-amino acids was performed with N-hydroxybenzotriazole, and dicyclohexylcarbodiimide (DCC). Deprotection and cleavage from the resin were carried out for 2 h at room temperature using a mixture of trifluoroacetic acid, phenol, water, thioanisole, 1,2-ethandithiol, and triisopropylsilane (88:2.5:2.5:2.5:2.5:2.0 [v/v]). The crude peptide was then repeatedly washed with diethylether, dried under vacuum, and purified by reversed-phase preparative high-performance liquid chromatography using a 15-μm Deltapak C_{18} column (19 × 30 cm; Waters, Milford, USA). The purified peptides were hydrolyzed with 6 N HCl at 110°C for 22 h and then dried under vacuum. The residues were dissolved in 0.02 N HCl and amino acid contents were assessed using an amino acid analyzer (Hitachi Model, 8500 A, Tokyo, Japan). The molecular masses of the synthetic peptides were determined using matrix-assisted laser desorption ionization-mass spectrometry.

Table 1. Amino acid sequences of the synthetic antimicrobial peptide HP (2-20) derived from the N-terminus of *H. pylori* ribosomal protein L1 and its analogues.

Peptide	Amino acid sequence	Remarks	Mass
HP (2-20)	AKKVFKRLEKLFSKIQNDK	Native	2403.94
Anal 3	AKKVFKRLEKLFSKI**W**N**W**K	($Q^{16}D^{18}\rightarrow W^{16}W^{18}$)	2450.01
Anal 6	AKKVFKRLEKLFSKI**F**N**F**K	($Q^{16}D^{18}\rightarrow F^{16}F^{18}$)	2371.94
Anal 7	AKKVFKRLEKLFSKI**Y**N**Y**K	($Q^{16}D^{18}\rightarrow Y^{16}Y^{18}$)	2403.94

NMR Spectroscopy

All of the NMR experiments for the sample in SDS micelle were performed at 298K. All the phase sensitive two-dimensional experiments such as DQF-COSY, TOCSY, and NOESY experiments were performed using time-proportional phase incrementation(TPPI) method.[6-10] For these experiments, 400-512 transients with 2K complex data points were collected for each of the increments with a relaxation delay of 1.2-1.5 sec between the successive transients and the data along the t_1 dimension were zerofilled to 1K before 2D-Fourier transformation. TOCSY experiment was performed with mixing times of 80-100 msec, MLEV-17 spin-lock mixing pulse. NOESY experiments were performed with mixing times of 150 and 250 msec. All NMR spectra were recorded on Bruker DPX-400 spectrometer in Konkuk University.

Chemical shifts of the samples were measured relative to the methyl resonance of internal 2,2-dimethyl-2-silapentane-5-sulfonic acid(DSS) at 0 ppm. $^3J_{HN\alpha}$ coupling constants were either measured in 1D spectrum or calculated from the separation of absorptive peaks and dispersive peaks in DQF-COSY spectrum. DQF-COSY spectrum was processed to the 4K×2K matrix and used to measure peak-to-peak separations. P.E.COSY experiment was executed to obtain $^3J_{\alpha\beta}$ coupling constants. To identify slowly exchanging amide protons, a series of 1D spectra were acquired after deuterium oxide was added to the sample.

Structure calculation

Structure calculations were carried out using X-PLOR version 3.851.[11] All the NOE intensities are divided into three classes, i.e., strong, medium, and weak with the distance

ranges of 1.8-2.7, 1.8-3.5, and 1.8-5.0 Å, respectively.[12,13] Standard pseudoatom corrections were applied to the non-stereospecifically assigned restraints, and the additional 0.5 Å was added to the upper bounds for NOEs involving methyl protons.[14] Standard distance geometry-dynamical simulated annealing hybrid protocol[15,16] was imployed to generate structures. Center averaging was used to correct distances involving methyl groups and non-stereospecifically assigned methylene. The target function that is minimized during simulated annealing comprises only quadratic harmonic potential terms for covalent geometry, square-well quadratic potentials for the experimental distance and torsion angle restraints, and a quartic van der Waals repulsion term for the nonbonded contacts.

RESULTS and DISCUSSION

We have reported the antimicrobial activities against Gram-positive and -negative bacterial strains of HP (2-20) and its analogues in the previous paper.[5] Here, hydrophobicity was increased by substitution of hydrophilic residues such as Asp^{18} or Gln^{16} with Trp, Phe, or Tyr. As shown in Table 2, the substitution of Gln^{16} and Asp^{18} of HP (2-20) with Trp, Phe, and Tyr resulted in 2-10 times higher antibacterial activity compared to HP (2-20). Anal 3 and Anal 7 has about 2- times higher antimicrobial activities compared to Anal 6 with Phe. The increase of hydrophobicity caused increase of antimicrobial activity

Table 2. Antibacterial activities of HP (2-20) and its analogues. [5]

Microorganism	Peptide (MIC : μM)			
	HP (2-20)	Anal 3	Anal 6	Anal 7
B. subtilis	3.13	1.56	1.56	1.56
S.epiderm idis	6.25	0.78	0.78	3.13
S.aureus	12.5	1.56	3.13	3.13
P.aeruginosa	6.25	3.13	6.25	3.13
E. coli O157	12.5	3.13	6.25	3.13

and the results indicated that Anal 3, Anal 6, and Anal 7 which have high hydrophobicity compared to HP(2-20) displayed approximately 2- to 10-fold more potent than HP (2-20) and aromatic residues at the C-terminus may facilitate the interactions with the membrane.

Using standard sequential assignment strategy, all the proton resonances were assigned. TOCSY and DQF-COSY spectra were used to assign spin systems of most of the amino acid residues. By direct comparison of TOCSY and NOESY spectra, sequence-specific resonance assignments were completed. The sequential NOE connectivities in the NH-NH region of NOESY spectra of Anal 7 in SDS micelle are illustrated in Fig. 1.

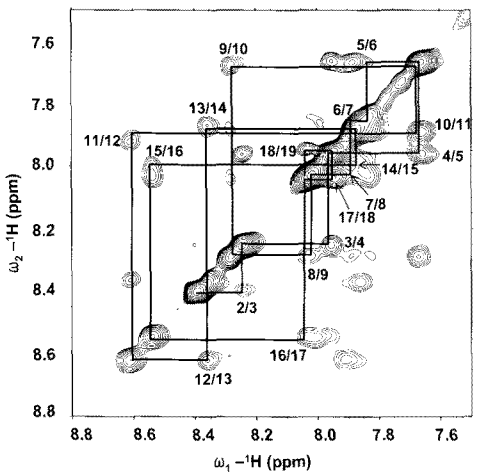

Figure 1. NH-NH region of NOESY spectra with a 250 msec mixing time of Anal 7 in SDS micelles.

Fig. 2 illustrates the summary of the NOE connectivities of Anal 6 and Anal 7 in SDS micelle, which were extracted directly from NOESY spectrum recorded with a mixing time of 250msec. A number of nonsequential NOE connectivities that are characteristics of an α-helix, specifically $d_{\alpha\beta}(i,i+3)$ and $d_{\alpha N}(i,i+3)$, were observed for all of the peptides. The observed value of the $^3J_{HN\alpha}$ coupling constant for the helical region of all of the peptides was generally below 6 Hz.

Total of 50 structures were generated by hybrid distance geometry-dynamical simulated annealing algorithm, and 10 structures having lowest energies were selected for further analysis. All of structures satisfies the experimental NOEs well within 0.02 Å. All

structures display good covalent geometry and small NMR constraint violations. Figure 4 shows the superposition of the 10 lowest energy structures of Anal 6 and Anal 7

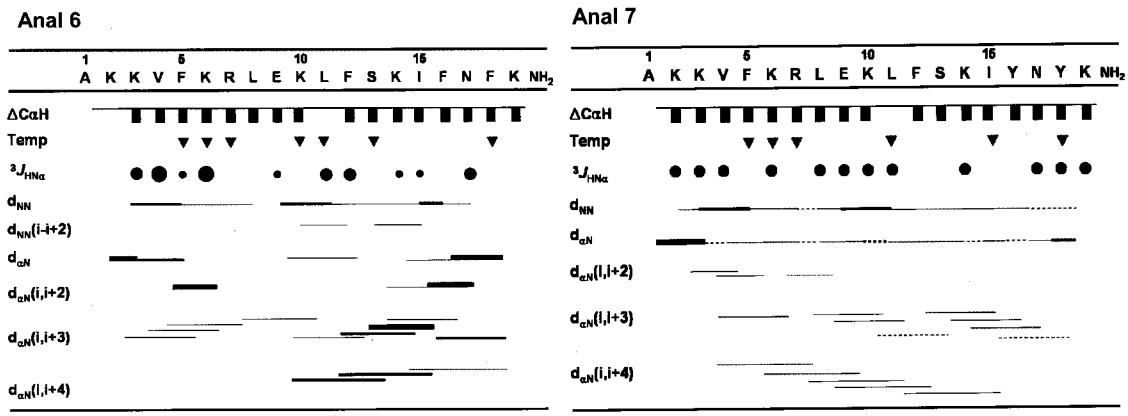

Figure 2. Summary of the NOE connectivities, $J_{HN\alpha}$ coupling constants ($J_{HN\alpha}$ < 6 Hz), and CαH chemical shift indices for Anal 6 and Anal 7 in SDS micelles. The line thickness for the NOEs reflects the intensity of the NOE connectivities.

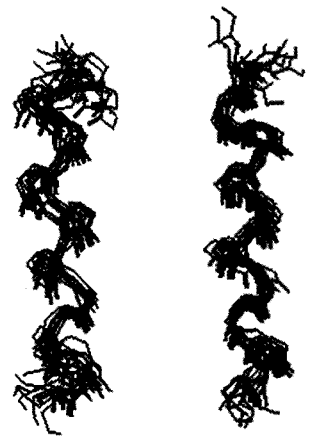

Figure 3. The superpositions of the 10 lowest energy structures calculated from the NMR data for (A) Anal 6, and (B) Anal 7.

over the backbone atoms in 150 mM SDS micelles. None of the structures have violations over 0.5 Å from the NOE distance restraints or 3 degrees from dihedral angle restraints, and all the structures exhibit good covalent geometry. When we superimposed the 20 lowest energy structures of Anal 6 and Anal 7 over the backbone atoms (from Val^4 to Gln^{18}), their root mean squared deviations from the mean structures were 0.892±0.259 Å and 0.676±0.210 Å for the backbone atoms (N, Cα, C′, O) and 1.818±0.366 Å and 1.506±0.243 Å for all heavy atoms, respectively.

HP (2-20) peptides have a stable amphiphilic helix from Val^4 to Gln^{18}.[5] Because of the increase in hydrophobicity caused by substitution of Trp, Phe, and Tyr at the C-terminus, Anal 3, Anal 6, and Anal 7 have longer amphiphilic helical structures from Val^4 to Gln^{18} than HP (2-20). HP (2-20) do not have aromatic residues at the C-terminus. As predicted by NOE connectivities, $^3J_{HN\alpha}$ coupling constants, and chemical shift index values shown in Figures 3, increase of the hydrophobicity by the addition of aromatic residues at the C-terminus stabilizes the overall α-helical structures in Anal 3, Anal 6, and Anal 7 and results in longer amphiphilic helical structures and higher antimicrobial activity.

It is well known that aromatic rings in the peptides play important roles in interactions with membrane. Trp residues, in particular, are known to have important roles in the interactions between peptides and biological membranes.[17-18] The partial insertion of aromatic rings of Trp, Phe, or Tyr residues into the membrane, as well as the electrostatic interactions between the positively charged Lys residues and the anionic phospholipid head groups, mediate the primary binding of Anal 3, Anal 6, and Anal 7 to the cell membrane. From the results of antimicrobial activities of these, it can be concluded that phenyl ring in Phe residue in Anal 6 is less effectively inserted into the membrane compared to those of Trp and Tyr residues in Anal 3 and Anal 7.

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