Journal of Bioscience and Bioengineering
VOL. 122 No. 6, 652–659, 2016

ELSEVIER

www.elsevier.com/locate/jbiosc

Antimicrobial activity against *Porphyromonas gingivalis* and mechanism of action of the cationic octadecapeptide AmyI-1-18 and its amino acid-substituted analogs

Masayuki Taniguchi,[1,2,*] Akihito Ochiai,[1] Kiyoshi Takahashi,[1] Shun-ichi Nakamichi,[1] Takafumi Nomoto,[1] Eiichi Saitoh,[3] Tetsuo Kato,[4] and Takaaki Tanaka[1]

Department of Materials Science and Technology, Graduate School of Science and Technology, Niigata University, Niigata 950-2181, Japan,[1] Center for Transdisciplinary Research, Niigata University, Niigata 950-2181, Japan,[2] Graduate School of Technology, Niigata Institute of Technology, Niigata 945-1195, Japan,[3] and Department of Chemistry, Tokyo Dental College, Tokyo 101-0062, Japan[4]

Received 5 May 2016; accepted 27 May 2016
Available online 28 July 2016

The antimicrobial peptide AmyI-1-18 is a cationic α-helical octadecapeptide derived from α-amylase in rice (*Oryza sativa* L. *japonica*) that contains four cationic amino acid residues (two arginines and two lysines). To enhance the antibacterial activity of AmyI-1-18 against *Porphyromonas gingivalis* (a bacterium associated with periodontal disease), we synthesized 12 analogs bearing substitutions with alanine, leucine, and/or arginine that were designed based on helical wheel projections and investigated their antibacterial properties. The antibacterial properties of four analogs bearing substitution of a single arginine or lysine with alanine were almost similar to those of AmyI-1-18, suggesting that the antibacterial properties depend on the presence of three cationic amino acid residues. Of three single arginine-substituted analogs, AmyI-1-18(G12R) exhibited an antibacterial activity 2.8-fold higher [50% growth-inhibitory concentration (IC_{50}): 4.6 μM] than that of AmyI-1-18 (IC_{50}: 13 μM). Likewise, the antibacterial properties of two single leucine-substituted analogs were significantly enhanced; in particular, AmyI-1-18(N3L) exhibited an antibacterial activity (IC_{50}: 2.5 μM) 5.2-fold higher than that of AmyI-1-18. The hemolytic activity of AmyI-1-18(N3L) against mammalian red blood cells was low (2% at 50 μM). A membrane-depolarization assay using a membrane potential-sensitive fluorescent dye revealed that, similar to AmyI-1-18, the antibacterial activity of AmyI-1-18(N3L) was not dependent on its membrane-disrupting activity. Our results demonstrate that the antibacterial properties of AmyI-1-18 against *P. gingivalis* are significantly improved, without a significant increase in hemolytic activity, by replacing asparagine with leucine at position 3.

[**Key words:** Antibacterial peptide; Substitution of amino acid; *Porphyromonas gingivalis*; Cationic peptide; Hydrophobicity]

Antimicrobial peptides (AMPs) represent promising alternatives to conventional antibiotics because of their potency and different mechanisms of action, which avoid the induction of bacterial resistance. Among AMPs, cationic α-helical AMPs including LL-37 in humans, cecropins and melittin in insects, magainin 2 and buforin 2 in amphibians, and fowlicidins in chickens are attractive alternatives to conventional antibiotics (1–3). For cationic α-helical AMPs to be useful therapeutic agents against human pathogens, they must exhibit a high antimicrobial potency and the ability to distinguish microbial cells from mammalian cells based on their differing membrane lipid compositions. However, it is likely that naturally occurring cationic α-helical AMPs do not possess sufficient antimicrobial activity to kill human pathogens. Furthermore, previous studies have showed that several cationic α-helical AMPs including melittin (4,5) and LL-37 (6,7) exhibit cytotoxic behaviors such as hemolysis. Thus, by replacing amino acids in naturally occurring AMPs, it is necessary to create AMPs that exhibit a high antimicrobial activity and display little or no cytotoxic activity for use as therapeutic agents (8–12).

Structure–function studies on cationic α-helical AMPs indicate that several parameters modulate antimicrobial and cytotoxic activities, including net positive charge, charge distribution, hydrophobicity, amphipathicity, and helical propensity (12–20). Despite the diversity of amino acid sequences in cationic α-helical AMPs, most share a high net positive charge as a result of multiple arginine and lysine residues and hydrophobic amino acids such as tryptophan, phenylalanine, and leucine (11,13,21–23). These cationic properties are crucial for binding to the negatively charged surfaces of microbial lipid membranes. However, the α-helical amphipathic propensity, which depends on the balance between cationic (hydrophilic) and hydrophobic residues, is also a key factor facilitating selective insertion into and/or translocation across microbial cell membranes, ultimately leading to cell death. Therefore, the antimicrobial activities of cationic α-helical AMPs are governed by both the positive charge and hydrophobicity of their residues. Helical wheel projections (http://rzlab.ucr.edu/scripts/wheel/wheel.cgi?sequence) are frequently used to define the structure–antimicrobial activity relationship of cationic α-helical AMPs, as well as to design analogs with improved antimicrobial activity and/or cell selectivity. To date, a large number of AMP

* Corresponding author at: Department of Materials Science and Technology, Graduate School of Science and Technology, Niigata University, Niigata 950-2181, Japan. Tel./fax: +81 25 262 6716.
E-mail address: mtanig@eng.niigata-u.ac.jp (M. Taniguchi).

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.05.008

analogs exhibiting high antimicrobial activities and displaying few or no cytotoxic behaviors have been successfully designed and created based on helical wheel projections (24–28).

Recently, we identified a novel cationic α-helical octadecapeptide [AmyI-1-18 (175–192 residues)] that contains four cationic amino acids (two lysine and two arginine residues) and eight hydrophobic amino acids (one valine, one isoleucine, four leucine, and two tryptophan residues) (Table 1). In our previous study (29), to elucidate the antimicrobial spectrum and activity of AmyI-1-18, we evaluated its performance against representative human pathogens, including *Candida albicans* (an opportunistic fungal pathogen), *Porphyromonas gingivalis* (a gram-negative bacterium associated with periodontal disease), and *Propionibacterium acnes* (an acne-related gram-positive bacterium). In addition, to improve the antifungal properties of AmyI-1-18 against *C. albicans*, we synthesized 11 analogs bearing substitutions with alanine, leucine, and/or arginine that were designed based on helical wheel projections and investigated their antifungal properties (30). Of the 11 AmyI-1-18 analogs, AmyI-1-18(D15R), in which aspartic acid was replaced with arginine at position 15, exhibited an approximately twofold higher antifungal activity against *C. albicans* than AmyI-1-18 while exhibiting a low hemolytic activity against mammalian red blood cells (RBCs; 4% at 100 μM) similar to that of AmyI-1-18 (30). Interestingly, flow cytometric analysis using propidium iodide suggested that the antifungal properties of AmyI-1-18(D15R) against *C. albicans* were dependent on its membrane-disrupting activity, in contrast to AmyI-1-18 (30).

The antimicrobial and cytotoxic properties of cationic α-helical AMPs targeted to cell membranes are greatly affected by their lipid compositions (31–33). In this study, we selected *P. gingivalis* as a test pathogen, because the lipid composition of Gram-negative bacterial membranes, which are rich in phosphatidylethanol and phosphatidylglycerol, is distinct from that of *C. albicans* membranes, which contain phosphatidylcholine and ergosterol (31–33). To enhance the antibacterial activity of AmyI-1-18 against *P. gingivalis*, we synthesized 12 analogs bearing substitutions with uncharged alanine, cationic arginine, and/or hydrophobic leucine including three analogs bearing substitutions with two amino acids (arginine and/or leucine) designed based on helical wheel structure of AmyI-1-18 (30) and investigated the effects of the substitutions on their antibacterial activity and mode of action. In addition, to select potent AMPs that do not induce cytotoxicity, we investigated the hemolytic activity of arginine- and/or leucine-substituted analogs against mammalian RBCs. Furthermore, to clarify the mode of action of AmyI-1-18 analogs exhibiting higher antibacterial activity against *P. gingivalis*, we investigated their interactions with cell membranes using a membrane-depolarization assay and a membrane potential-sensitive fluorescent dye [3,3′-dipropylthiadicarbocyanine iodide ($diSC_3$-5)] (34–36). Finally, we discussed the effect of arginine and/or leucine substitution on the antibacterial activity against *P. gingivalis* and mechanism of action of AmyI-1-18.

MATERIALS AND METHODS

Materials $diSC_3$-5 and melittin, a component of bee venom known to disrupt cell membranes, were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sterile defibrinated sheep blood was obtained from Cosmo Bio Co., Ltd. (Tokyo, Japan).

Peptides used in this study The amino acid sequences and properties of AmyI-1-18 and its analogs used in this study are summarized in Table 1. Chemically synthesized AmyI-1-18 and its analogs, including alanine-substituted analogs [AmyI-1-18(K4A), AmyI-1-18(R5A), AmyI-1-18(R8A), and AmyI-1-18(K18A)], arginine-substituted analogs [AmyI-1-18(I11R), AmyI-1-18(G12R), and AmyI-1-18(D15R)], leucine-substituted analogs [AmyI-1-18(N3L) and AmyI-1-18(E9L)], and two amino acids-substituted analogs [AmyI-1-18(E9L, G12R), AmyI-1-18(N3L, E9L), and AmyI-1-18(N3L, G12R)], which were designed based on helical wheel projections of AmyI-1-18 (30), were obtained from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan). The synthesized peptides were purified to >95% by reversed-phase high-performance liquid chromatography. The molecular weights of purified AmyI-1-18 and its analogs were confirmed by matrix-assisted laser/desorption ionization–time-of-flight mass spectroscopy.

Determination of antimicrobial activity *P. gingivalis* ATCC 33277 was used as a test microorganism for estimating the antimicrobial activity of AmyI-1-18 and its analogs. *P. gingivalis* was precultivated overnight at 37°C in test tubes containing modified Gifu anaerobic medium (GAM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) under strictly anaerobic conditions. The cells obtained were further cultivated under the same culture conditions to obtain mid-logarithmic growth-phase cells. The antimicrobial activity of AmyI-1-18 and its analogs was measured in 96-well plates under anaerobic conditions in the presence of a deoxygenating reagent (AnaeroPack A03; Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan) as reported previously (29,30,34–36). In brief, aliquots (20 μL) of *P. gingivalis* cell suspension at a concentration of 5×10^5 colony forming units (CFU)/mL were added to wells containing modified GAM (80 μL) with each peptide at different concentrations (or water as a control). The concentrations of AmyI-1-18 and its analogs were varied by serial dilution. After 48–60 h of incubation, we determined the viable cell concentration using the BacTiter-Glo Reagent (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. The viable cell concentration was expressed in relative light units (RLUs), which are calculated based on the level of ATP released from intact cells. The growth ratio was calculated using the following equation:

$$\text{Growth ratio (\%)} = \{(L_P - L_0)/(L_C - L_0)\} \times 100 \quad (1)$$

where L_P and L_C represent the RLUs of the samples treated with and without the peptide, respectively, and L_0 represents the RLU of the medium alone. The 50% growth-inhibitory concentration (IC_{50}) of each peptide was determined using a plot of the growth ratio versus the log-transformed peptide concentration. The results are expressed as the mean ± standard deviation (SD) of three to five individual experiments.

TABLE 1. The amino acid sequences and properties of peptides used in this study.

No.	Peptide	Sequence[a]	Molecular weight (Da)		α-helix content[a] (%)	MH[b]	Net charge	pI (−)
			Measured	Calculated				
1	AmyI-1-18(parent peptide)	HLNKRVQRELIGWLDWLK	2341.99	2341.75	61.1	11.3	+2	9.99
2	AmyI-1-18(K4A)[c]	HLN**A**RVQRELIGWLDWLK	2247.24	2247.65	61.1	11.3	+1	8.75
3	AmyI-1-18(R5A)[c]	HLNK**A**VQRELIGWLDWLK	2219.35	2219.64	72.2	11.5	+1	8.60
4	AmyI-1-18(R8A)[c]	HLNKRVQ**A**ELIGWLDWLK	2219.49	2219.64	55.6	11.5	+1	8.60
5	AmyI-1-18(K18A)[c]	HLNKRVQRELIGWLDWL**A**	2247.45	2247.65	55.6	11.3	+1	8.75
6	AmyI-1-18(I11R)[c]	HLNKRVQREL**R**GWLDWLK	2347.58	2347.78	72.2	10.2	+3	10.90
7	AmyI-1-18(G12R)[c]	HLNKRVQRELI**R**WLDWLK	2403.67	2403.88	66.7	11.4	+3	10.90
8	AmyI-1-18(D15R)[c]	HLNKRVQRELIGWL**R**WLK	2345.43	2345.85	61.1	11.3	+4	11.72
9	AmyI-1-18(N3L)[c]	HL**L**KRVQRELIGWLDWLK	2303.16	2303.80	83.3	12.7	+2	9.99
10	AmyI-1-18(E9L)[c]	HLNKRVQR**L**LIGWLDWLK	2288.60	2288.79	72.2	12.5	+3	11.00
11	AmyI-1-18(E9L, G12R)	HLNKRVQR**L**LI**R**WLDWLK	2387.65	2387.93	72.2	12.5	+4	11.72
12	AmyI-1-18(N3L, E9L)	HL**L**KRVQR**L**LIGWLDWLK	2287.79	2287.65	83.3	13.8	+3	11.00
13	AmyI-1-18(N3L, G12R)	HL**L**KRVQRELI**R**WLDWLK	2402.58	2402.94	77.8	12.7	+3	10.90

[a] α-helix content and α-helical region (indicated by an underline) were estimated using MLRC secondary prediction available at the NPS website (https://npsa-prabi.ibcp.fr).
[b] The mean hydrophobicity (MH) values of the peptides were calculated using the hydrophobicity indices of amino acid residues reported by Shang et al. (37). Replaced amino acids are indicated by bold letters.
[c] The single amino acid-substituted analogs (Nos. 2–10) were used for examining the antifungal activity against *C. albicans* in our previous study (30).