

Synthesis and Antibiotic Activities of CRAMP, a Cathelin-related Antimicrobial Peptide and Its Fragments

Jong-Myung Ha,[†] Song Yub Shin,[‡] and Shin Won Kang*

[†]Division of New Materials and Biotechnology, Silla University, Pusan 617-736, Korea

[‡]Peptide Engineering Research Unit, Korea Research Institute of Bioscience and Biotechnology,

P.O. Box 115, Yusong, Taejeon 305-600, Korea

Department of Chemistry and Chemistry Institute for Functional Materials, Pusan National University,

Pusan 609-735, Korea

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CRAMP, a 37-amino acid cationic antimicrobial peptide was recently deduced from the cDNA cloned from mouse femoral marrow RNA. In order to investigate the structure-activity relationship and functional region of CRAMP, CRAMP and its 18-mer overlapping peptides were synthesized by the solid phase method. CRAMP showed broad spectrum antibacterial activity against both Gram-positive and Gram-negative bacterial strains (MIC: 3.125-6.25 μ M) but had no hemolytic activity until 50 μ M. CRAMP was found to have a potent anticancer activity (IC₅₀: 12-23 μ M) against two human small cell lung cancer cell lines. Furthermore, CRAMP was found to display faster bactericidal rate in *B. subtilis* rather than *E. coli* in the kinetics of bacterial killing. Among 18-meric overlapping fragment peptides, only CRAMP (16-33) displayed potent antibacterial activity (MIC: 12.5-50 μ M) against several bacteria with no hemolytic activity. Circular dichroism (CD) spectra analysis indicated that CRAMP and its analogues will form the amphipathic α -helical conformation in the cell membranes similar to other antimicrobial peptides, such as cecropins and magainins.

Introduction

During the past decade, the widespread appearance of naturally occurring antimicrobial peptides have been isolated.¹⁻⁵ Their abundance, tissue distribution, and *in vitro* activity suggest an important role in innate immunity and host defense.^{6,7} The role of these antimicrobial peptides in mammals has been described from their expression in neutrophil granules and at sites exposed to multiple microbe such as the skin and gastrointestinal tract.⁸ In general, these antimicrobial peptides can be divided into four major groups according to amino acid composition and secondary structure. One group, which includes cecropins and magainins, exhibits an α -helical structure.⁹⁻¹² A second group contains peptides that form loop structures with one or more disulfide bonds, such as bactenecin and brevinins.¹³ A third group, which includes the defensins, is folded into an antiparallel β -sheet structure, containing three disulfide bonds.¹⁴ The fourth group comprises peptides with a high content of specific amino acid, such as the proline-arginine-rich peptide PR-39 and the tryptophan-rich peptide indolicidin.^{15,16}

One class of antimicrobial peptides, the cathelicidin-derived peptides, contains a highly conserved prepro-region that is homologous to cathelin, a putative cysteine-protease inhibitor originally isolated from pig leukocytes. The cathelicidin-derived peptides contain the C-terminal domain corresponding to the antimicrobial peptides and are highly varied. The antimicrobial peptides belonging to the cathelicidin-derived peptides currently counts over 20 members.¹⁷⁻²⁰ Upon stimulation, the cathelin domain of these peptides was known to be cleaved proteolytically to allow the mature C-

terminal antimicrobial peptide to be released.

In the recent year, the gene, named CRAMP as a member of the cathelicidin gene family, was identified from the nucleotide sequence derived from mouse femoral marrow cells.²¹ This protein is composed of total 172 amino acid residues. It contains a cathelin-like domain in its prepro-region and the antimicrobial portion called the cathelin-related antimicrobial peptide (CRAMP) in its C-terminal region.²¹ CRAMP, a 38-amino acid peptide, was thought to be prepared by a potential processing at dibasic site at position 134-135 of CRAMP protein.²¹

In the present study, in order to investigate the structure-antibiotic activity relationship and the functional region of CRAMP, a series of overlapping 18-mer peptides based on the 38-amino acid sequence of CRAMP were synthesized. Six bacteria strains were used for comparing antibacterial activities of the synthetic peptides. Two different human small cell lung cancer (SCLC) cell lines were used for determining the anticancer activities of the synthetic peptides. The hemolytic activity against the human erythrocytes as cytotoxicity of the synthetic peptides was also examined.²² The secondary structures of the peptides in the aqueous trifluoroethanol (TFE) or sodium dodecyl sulfate (SDS) solution that mimics the environment of cell membranes were estimated by circular dichroism (CD) spectral analysis.

Experimental Section

Peptide synthesis. The peptides was synthesized manually by the solid-phase strategy.²³ The α -Amino group of amino acids were protected by 9-fluorenylmethoxycarbo-

nyl (Fmoc) group. Fmoc-Glu(*t*Bu)-polyethyleneglycolpoly-styrene-Resin (0.17 mmol/g; PerSeptive Biosystem, Framingham, MA) was used as the starting support. The side-chain protecting group of amino acids were as follows: Arg, 2,2,5,7-pentamethylchroman-O-sulfonyl (Pmc); Asn and Gln, trityl (Trt); Ser and Glu, *t*-butyl (*t*Bu); Lys, *t*-butyloxy-carbonyl (Boc). All coupling stages were performed with 4-fold excess of protected amino acid derivatives with an equimolar mixture of DIPC1 (N,N'-diisopropylcarbodiimide)/HOBt (1-hydroxybenzotriazole) as the coupling agent. The coupling cycle was performed according to the following steps: 1. DMF wash; 2. 20% (v/v) piperidine/DMF (3 min); 3. 20% piperidine/DMF (17 min); 4. DMF wash (4×); 5. Coupling (3 hrs); 6. DMF wash (4×). After coupling the last amino acid, the Fmoc-group was removed with 20% piperidine/DMF, and protected peptide-resins were cleaved and deprotected with a mixture of TFA-based reagent (92.5% TFA, 2.5% 1,2-ethanedithiol, 2.5% H₂O and 2.5% triisopropylsilane, v/v) for 3 hr at room temperature, and then precipitated with diethylether, and dried in vacuum. The crude peptides were purified by a reversed-phase preparative HPLC (Waters) on a Delta-Pak C₁₈ column, 3.9 × 15 cm. Purity of the purified peptides was checked by the analytical reversed-phase HPLC (Waters) on a Delta-Pak C₁₈ column, 3.9 × 50 cm. The purified peptides were hydrolyzed with 6 N-HCl containing 2% phenol at 110 °C for 22 h, and then dried in a vacuum. The residues were dissolved in 0.02 N HCl and subjected to amino acid analysis (Hitachi Model, 8500 A, Japan).

Hemolytic activity assay. The hemolytic activity of the peptides was determined using human erythrocytes. Fresh human erythrocytes were rinsed three times with PBS (35 mM phosphate buffer, 150 mM NaCl, pH 7.0), centrifuged for 15 min at 900 × g, and resuspended in PBS. One hundred μL of the suspension (0.4% in PBS, v/v) were plated in 96-well plates (Nunc, F96 microtiter plates), and then 100 μL of the peptide solution dissolved in PBS was added (the peptide final concentration: 100 μg/mL). The plates were incubated for 1 hr at 37 °C, and then centrifuged at 1000 × g for 5 min. One hundred μL aliquots of the supernatant were transferred to 96-well microtiter plates (Nunc). Release of hemoglobin was monitored by measuring the absorbance at 414 nm with an ELISA plate reader (Molecular Devices *E*_{max}, Sunnyvale, California). Zero percent hemolysis and 100% hemolysis were determined in PBS and 0.1% Triton-X 100, respectively. The hemolysis percentage was calculated by the following formula:

$$\% \text{ hemolysis} = \left[\frac{\text{Abs}_{414 \text{ nm}} \text{ in the peptide solution} - \text{Abs}_{414 \text{ nm}} \text{ in PBS}}{\text{Abs}_{414 \text{ nm}} \text{ in 0.1\% Triton-X 100} - \text{Abs}_{414 \text{ nm}} \text{ in PBS}} \right] \times 100$$

Antimicrobial activity assay. *Escherichia coli* (KCTC 1682), *Salmonella typhimurium* (KCTC 1926), *Pseudomonas aeruginosa* (KCTC 1637), *Bacillus subtilis* (KCTC 1918), *Streptococcus pyogenes* (KCTC 3096) and *Staphylococcus aureus* (KCTC 1621) were supplied from the Korea Collection for Type Cultures (KCTC). The bacterial strains

were grown in Luria-Bertani medium (LB, 10 g of bactotryptone, 5 g of yeast extract and 10 g of NaCl per litre) at 37 °C overnight and diluted in a basal medium of 1% bacto-peptone (Difco) to 1 : 200 [*i.e.* final bacterial suspension containing 2-4 × 10⁶ colony formation units (CFU) / mL]. The peptide solution were prepared from 200 μg/mL stock solutions to give a range of 100-1.56 μg/mL in 1% bacto-peptone media. One hundred μL of peptide stock solution was added to the 100 μL of bacterial suspension, and then incubated at 37 °C overnight. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of peptide at which there was no change in optical density (OD) at 620 nm after 18 hr.

Kinetics of bacterial killing. The kinetics of bacterial killing of the peptides was evaluated using *E. coli* and *B. subtilis*. Log-phase bacteria (6 × 10⁵ CFU/mL) were incubated with 3.0 μM peptide in LB broth. Aliquots were removed at fixed time intervals, appropriately diluted, plated on LB broth agar plate, and then the colony-forming units were counted after 16 hr incubation at 37 °C.

Anticancer activity assay. Human SCLC cell lines (NCI-H126 and NCI-H148) were purchased from the Cell Bank of Seoul National University, Korea. The cells were grown in a RPMI-medium supplemented with heat-inactivated 10% fetal bovine serum (FBS), 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate. The cells suspended RPMI 1640 medium with 10% FBS were plated in 96-well plates (Nunc, F96 microtiter plates) at a density of 2.0 × 10⁴ cells/well. After incubating the plates overnight at 37 °C in 5 % CO₂ atmosphere, 20 μL of the serially diluted-peptides were added in each wells and then incubated for 3 days. Twenty μL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) solution (5 mg/mL, MTT in PBS) was added in each well and then the plates were incubated for 4 hrs at 37 °C. Thirty μL of 20% sodium dodecyl sulfate (SDS) solution containing 0.02 M HCl was added in each wells, and then incubated 3 hrs at 37 °C to the dark blue crystals (MTT-formazan product). Absorbance was monitored at 570 nm on ELISA plate reader.

Circular dichroism (CD) analysis. CD spectra of peptides were recorded using a spectropolarimeter (Jasco J720, Japan). All samples were maintained at 25 °C during analysis. Four scans per sample performed over wavelength range 190-250 nm at 0.1 nm intervals. The spectra were measured in 0% and 50% (vol/vol.) TFE and 30 mM SDS in 10 mM sodium phosphate buffer, pH 7.2, respectively at 25 °C using a 1 mm pathlength cell. The peptide concentrations were 100 μg/mL. The mean residue ellipticity, $[\theta]$, is given in deg · cm² · dmol⁻¹; $[\theta] = [\theta]_{\text{obs}} (\text{MRW}/10/c)$, where $[\theta]_{\text{obs}}$ is the ellipticity measured in millidegree, MRW is the mean residue molecular weight of the peptide, *c* is the concentration of the sample in mg/mL, and *l* is the optical path length of the cell in cm. The percent helicity of the peptides were calculated with the following equation:²⁴

$$\% \text{ helicity} = 100 \left(\frac{[\theta]_{222} - [\theta]_{222}^0}{[\theta]_{222}^{100}} \right)$$

where $[\theta]_{222}$ is the experimentally observed mean residue

Table 1. Amino acid sequence, net charge and retention time of native CRAMP and its 18 meric-fragment peptides with overlapping sequence synthesized in this study

Peptides	Amino acid sequence	Net charge	RT (min) ^a
CRAMP	H-ISRLAGLLRKGGEKIGEKI.KKIGQKIKNFFQKLVQPPE-OH	+7	17.5
CRAMP (1-18)	H-ISRLAGLLRKGGEKIGEK-OH	+3	11.3
CRAMP (6-23)	H-GLI.RKGGGEKIGEKI.KKIG-OH	+4	10.2
CRAMP (11-28)	H-GGEKIGEKI.KKIGQKIKN-OH	+4	7.5
CRAMP (16-33)	H-GEKIKKIGQKIKNFFQKLV-OH	+5	13.6
CRAMP (21-38)	H-KIGQKIKNFFQKLVQPPE-OH	+3	12.5

^aThe peptides were eluted with a linear gradient (1.5°/min) of 10-60% (by vol.) acetonitrile containing 0.1 % trifluoroacetic acid at a flow rate of 1 mL/min (Column: Waters Novapak-C18, 3.9 × 150 mm, 5 μ, 300 Å). The effluent was monitored at 220 nm.

Table 2. Amino acid composition of the synthetic peptides determined by amino acid analysis

Amino acids	CRAMP	CRAMP (1-18)	CRAMP (6-23)	CRAMP (11-28)	CRAMP (16-33)	CRAMP (21-38)
Asp	1.1 (1)	–	–	0.9 (1)	0.9 (1)	0.9 (1)
Ser	0.6 (1)	0.6 (1)	–	–	–	–
Glu	5.9 (6)	1.9 (2)	1.9 (2)	2.8 (3)	2.7 (3)	3.9 (4)
Pro	2.0 (2)	–	–	–	–	1.6 (2)
Gly	4.9 (5)	3.9 (4)	4.8 (5)	3.8 (4)	1.9 (2)	0.9 (1)
Ala	1.0 (1)	1.0 (1)	–	–	–	–
Val	1.1 (1)	–	–	–	–	1.0 (1)
Ile	4.0 (4)	1.9 (2)	1.9 (2)	2.7 (3)	1.9 (2)	1.9 (2)
Leu	5.0 (5)	3.0 (3)	3.0 (3)	1.0 (1)	2.0 (2)	1.0 (1)
Phe	2.2 (2)	–	–	–	2.1 (2)	1.8 (2)
Lys	8.0 (8)	3.0 (3)	4.7 (5)	5.9 (6)	6.3 (6)	3.8 (4)
Arg	1.6 (2)	1.9 (2)	0.9 (1)	–	–	–

Acid hydrolysis of the synthetic peptides was done with 6N-HCl in the presence of 2% phenol at 110 °C for 24 hr. Amino acid analysis was performed by amino acid analyzer (Hitachi Model. 8500 A, Japan). Numbers in the parenthesis as the theoretical values.

ellipticity at 222 nm. Values for $[\theta]_{222}^{100}$ and $[\theta]_{222}^{0}$ corresponding to 0% and 100% helical contents at 222 nm, are estimated to be -2,000 and -30,000 deg · cm²/dmol, respectively.²⁴

Results and Discussion

CRAMP and its six overlapping 18-meric peptides derived from the primary sequence of 38 amino acid residues were synthesized by the solid phase method²⁵ using Fmoc-chemistry (Table 1). The homogeneity of the synthetic peptides was confirmed by the profile of analytical HPLC on C₁₈ column (data not shown). The correct amino acid composition and molecular weight of the synthetic peptides were also confirmed by amino acid analysis (Table 2) and matrix-assisted laser desorption ionization (MALDI) mass spectra (Table 3). The antibacterial activities of the peptides were determined by measuring minimal inhibitory concentration (MIC) of the growth against Gram-negative (*E. coli*, *S. typhimurium*, and *P. aeruginosa*) and Gram-positive bacteria (*B. subtilis*, *S. pyogenes*, and *S. aureus*), respectively (Table 4). Each peptides were tested in triplicate in a two-fold dilution series ranging from 50 μM to 0.78 μM. Native CRAMP showed potent antibacterial activity against both Gram-positive and Gram-negative bacteria (MIC: 3.125-6.25 μM). Furthermore, in order to examine the kinetics of bacterial

Table 3. Molecular weights of the peptides determined by MALDI-MS

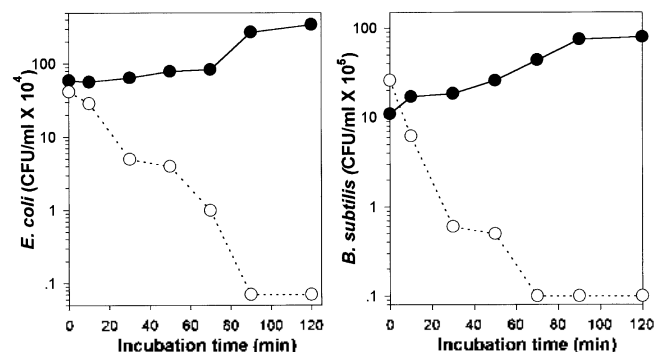
Peptides	Observed value	Calculated value
CRAMP	4292.67	4291.81
CRAMP (1-18)	1924.45	1925.59
CRAMP (6-23)	1924.20	1924.64
CRAMP (11-28)	1969.0	1968.65
CRAMP (16-33)	2148.12	2147.92
CRAMP (21-38)	2141.63	2142.96

killing of native CRAMP, a time course experiment was performed in the LB broth agar at 3.0 μM peptide using the *E. coli* and *B. subtilis* (Figure 1). Native CRAMP inactivated about 90% of *E. coli* within 30 min and about 80% of *B. subtilis* within 10 min (Figure 1). This result indicated that CRAMP display similar MIC values in *E. coli* and *B. subtilis*, but has faster bactericidal rate in *B. subtilis* than *E. coli*.

As shown in Table 4, among six overlapping peptides, CRAMP (16-33) [GEKLLKIGQKIKNFFQKL] displayed substantial antibacterial activity (MIC: 12.5-50 μM) within the peptide concentration of 50 μM against five bacteria strains except *S. aureus*. On the other hand, the other fragments was almost inactive. CRAMP (16-33) displays 4-16-fold low lytic activity against six bacterial cells than native CRAMP. This result is owed to the fact that native CRAMP

Table 4. Antibacterial activity of CRAMP and its overlapping peptides

Peptides	Gram-negative bacteria (MIC: μM)			Gram-positive bacteria (MIC: μM)		
	<i>E. coli</i>	<i>S. typhimurium</i>	<i>P. aeruginosa</i>	<i>B. subtilis</i>	<i>S. pyogenes</i>	<i>S. aureus</i>
CRAMP	3.125	3.125	3.125	3.125	6.25	3.125
CRAMP (1-18)	> 50	> 50	> 50	> 50	> 50	> 50
CRAMP (6-23)	> 50	> 50	> 50	> 50	> 50	> 50
CRAMP (11-28)	> 50	> 50	> 50	> 50	> 50	> 50
CRAMP (16-33)	50	12.5	12.5	50	50	> 50
CRAMP (21-38)	> 50	> 50	> 50	> 50	> 50	> 50

**Figure 1.** Kinetics of killing *E. coli* and *B. subtilis* by CRAMP. Bacteria, either untreated (●) or treated with 3.0 μM CRAMP (○) were diluted at the indicated time intervals, and then plated on LB broth agar. The colony forming units were calculated by counting the plates after 16 hr incubation at 37 °C.

has higher positive overall charge and hydrophobicity than CRAMP (16-33) (Table 1). Enhancement of the positive net charge and hydrophobicity in antimicrobial peptides results in a higher antibiotic activity has been reported.^{25,26} The hydrophobicity of the peptides was measured by retention time on C-18 reversed-phase HPLC. Hydrophobic interaction of the individual peptides with the lipid group of the C-18 of stationary phase can be considerable to be comparable to the interaction of the peptides with the lipid bilayer of cell membranes.^{27,28}

The anticancer activities of the synthetic peptides were determined by 50% growth inhibitory concentration (IC_{50}) against human small cell lung cancer (SCLC) cell lines. The IC_{50} values of the peptides were obtained from the concentration-growth inhibition response curves against human SCLC cell lines (graph not shown). As shown in Table 5, native CRAMP exhibited effective anticancer activity against human SCLC cell lines (IC_{50} : 13-20 μM). However, no anticancer activity was observed in all overlapping peptide fragments within the peptide concentration of 100 μM . CRAMP (16-33) showed antibacterial activity at 12.5-50 μM , while it did not display even at 100 μM . This effect may be considered to be caused by the difference in the membrane structure and lipid composition between eukaryotic cancer cells and prokaryotic bacterial cells. As a measure of cytotoxicity, the hemolysis of human erythrocytes induced by the peptides was measured (Table 5). Native CRAMP showed no hemolytic activity at the peptide concentration of 50 μM but was slightly hemolytic at 100 μM (2.4% hemoly-

Table 5. Anticancer and hemolytic activity of CRAMP and its fragments

Peptides	SCLC cells (IC_{50} : μM)		% Hemolysis (100 μM) (Human erythrocytes)
	NCI-H126	NCI-H148	
CRAMP	20	13	2.4
CRAMP (1-18)	> 100	> 100	0
CRAMP (6-23)	> 100	> 100	0
CRAMP (11-28)	> 100	> 100	0
CRAMP (16-33)	> 100	> 100	0
CRAMP (21-38)	> 100	> 100	0

sis). However, all five 18-meric peptide fragments of CRAMP did not exhibit any hemolytic activity against human erythrocytes until the peptide concentration of 100 μM (Table 5).

A secondary structure analysis predicted that CRAMP and its peptide fragments will take an amphipathic α -helical structure similar to FALL-39,²⁹ and non-cathelin-related antibacterial peptides, such as cecropins and magainins. The CD spectra of CRAMP and its peptide fragments revealed a random coil conformation in phosphate buffer, pH 7.2 with no α -helical content (Figure 2, and Table 6). All peptides form the α -helical structure in the presence of a α -helix-pro-

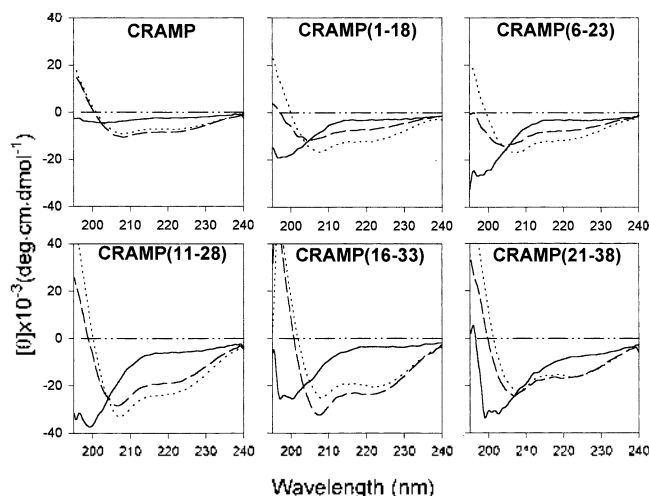
**Figure 2.** Circular dichroism spectra of CRAMP and its fragments. CD spectra were recorded at 50 μM peptide in 10 mM sodium phosphate buffer, pH 7.2 (—), 50% TFE/10 mM sodium phosphate buffer, pH 7.2 (---) and 30 mM SDS/10 mM sodium phosphate buffer, pH 7.2 (· · ·).

Table 6. Percent α -Helicity of CRAMP and its fragments in various solution

Peptides	Buffer		50% TFE		30 mM SDS	
	$[\theta]_{222}$	α -helix (%)	$[\theta]_{222}$	α -helix (%)	$[\theta]_{222}$	α -helix (%)
CRAMP	-2,290	1.0	-8,260	20.9	-7,160	17.2
CRAMP (1-18)	-3,000	3.3	-6,840	16.1	-11,930	33.1
CRAMP (6-23)	-2,980	3.3	-7,560	18.5	-11,310	31.0
CRAMP (11-28)	-5,860	12.9	-18,500	55.0	-22,890	69.6
CRAMP (16-33)	-3,530	5.1	-23,530	71.8	-20,520	61.7
CRAMP (21-38)	-7,800	19.3	-16,490	48.3	-16,270	47.6

moting solvent, 50% TFE or 30 mM SDS micelles that mimics the environment of cell membranes (Figure 2. and Table 6). CRAMP (16-33) with potent antibacterial activity showed a high α -helix contents of 61.7% in 30 mM SDS micelles. Nevertheless, CRAMP (11-28) displayed somewhat higher α -helix contents than CRAMP (16-33) in 30 mM SDS micelles than CRAMP (16-33), but showed no antibacterial activity until 50 μ M. This result suggests that the α -helicity of the peptide may not be a key factor in killing bacterial cells but the characteristics of the α -helix itself may be important. CRAMP (16-33) has more potent antibacterial activity than other fragments is owed to the fact that CRAMP (16-33) has higher positive overall charge and hydrophobicity than other fragments (Table 1).

In summary, in the present study, we identified that CRAMP has effective antibiotic activities against both cancer and bacterial cells, but was inactive against human erythrocytes. CRAMP displayed faster bactericidal rate in *B. subtilis* than *E. coli*. We also found that the synthetic peptide composed of 18 amino acid residues of CRAMP (16-33) is the functional region in antibiotic activity and displayed a potent antibacterial activity against Gram-positive and Gram-negative bacteria with no hemolytic activity at a high peptide concentration of 100 μ M.

In order to design the short peptides having more improved antibiotic activity than parental CRAMP with no undesirable hemolytic effect, the study of the structure-activity relationships based on CRAMP (16-33), the functional region of CRAMP, in more detail will be necessary.

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