Original Article

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Antibacterial Activity of Cc-CATH3 Peptide and its N-terminally Truncated Analogues against Gram-positive and Gram-negative Bacteria

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Abstract

Cc-CATH3 is an avian antimicrobial peptide (AMP) with 29 amino acids in length containing a broad-spectrum antibacterial activity. It has been proved that the either N-terminal or C-terminal residues of AMPs is important for antibacterial activity; since the N- or C-termini is the active function that interact with bacterial membranes in an initial step of bactericidal mechanism. The aim of this study was to investigate the antibacterial activity of Cc-CATH3 and its N-terminal truncated analogues against gram-positive and gram-negative bacteria to observe the role of N-terminal of Cc-CATH3 on antibacterial activity. Cc-CATH3 could inhibit gram-positive bacteria *S. aureus* and *B. subtilis* and also gram-negative bacteria *E. coli* and *S. typhimurium* with the MIC values of 1, 8, 2 and 4 μM respectively. The first four-residue N-terminally truncated peptide appeared to be more active since it showed the antibacterial activity against the aforementioned bacteria with MIC values of 0.5, 1, 2 and 2 μM respectively. However, the peptides with eight- or twelve-residue truncation were inactive since no inhibition neither gram-positive nor gram-negative bacteria was observed. The data also indicated that the MBC values of Cc-CATH3 against *S. aureus*, *B. subtilis*, *E. coli* and *S. typhimurium* were 2, 8, 8 and 8 μM respectively while the MBC of the peptide with four-residue truncation were 1, 2, 8 and 8 uM respectively. The results conclude that the N-terminal residues of Cc-CATH3 are important for its antibacterial activity; since the peptide lose the function when it was N-terminally truncated only eight residues.

KEYWORDS: Antibacterial activity, Antimicrobial peptide, Cathelicidin, Truncated peptide

INTRODUCTION

An emergence and rapidly increasing occurrence of antibiotic resistance in bacteria and other microbes has resulted in reduced effectiveness of available conventional drugs and become a major concern for public health[1]. Several promising antimicrobial agents have been discovered, studied and developed to cope with such problem[2]. Among them, antimicrobial peptides (AMPs) are regarded as excellent candidates owing to their higher or equal potencies and broad spectrum antimicrobial activities with less tendency to induce resistance when compared to conventional antibiotic drugs[3].

Various AMPs can be found among all classes of life and play a critical role in innate host defense system. While differing in sequence and structure, AMPs share, to a certain degree, common features such as

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low molecular weight, net positive charge, percent hydrophobicity, and amphipathicity[4-6]. Cathelicidins, which are major part of AMPs, have been identified in several mammals, fish, reptiles and avian. They demonstrated potent and broad antimicrobial activity against a wide range of microorganisms[7-9]. Recently, quail (*Coturnix coturnix*) cathelicidin, Cc-CATH3, has been characterized and proved to be a new strong AMP candidate for therapeutic development due to its high antimicrobial activity but low hemolytic activity[10]. Although some previous studies reported that N-terminal region play important role in antimicrobial property of many AMPs, there has been no report in the case of Cc-CATH3. Therefore it was of interest to investigate the antibacterial activity of Cc-CATH3 and its amino-terminal truncated analogues against gram-positive and gram-negative bacteria to observe the role of its N-termini with respect to antibacterial efficacy.

MATERIALS AND METHODS

Peptides

Cc-CATH3 - a native avian peptide from *Coturnix coturnix* and its three N-terminally truncated analogues (Table 1) were commercially synthesized by China-Peptides Co., Ltd (China) using standard solid-phase synthesis method to the purity of >98% and characterized by Mass Spectrometry. All the lyophilized peptides were stored at -20 ºC and dissolved in DI water to make 320 µM stock solutions prior to use. For antibacterial activity testing of each peptide, these stock solutions were diluted to 80 µM and then performed 2-fold serial dilution to get 7 concentrations ranging between 80-1.25 µM.

Bacterial strains and growth conditions

All the bacteria strains (Table 2) were purchased from the Culture Collection of the Department of Medical Sciences Thailand (DMST) under the Ministry of Public Health, Thailand. They were stored at -80 ºC until use. Subcultures of frozen bacteria were grown in Mueller Hinton (MH) Broth (HiMedia Laboratories Pvt. Ltd, India) overnight at 37 ºC in shaking incubator.

Antibacterial assay

The *in vitro* antibacterial activity of Cc-CATH3 and its N-terminally truncated variants were determined as the minimum inhibitory concentration (MIC) by broth microdilution assay using 96-well microtiter plate according to "Broth Microdilution Antibacterial Assay of Peptides" protocol[11] with some modification. An overnight bacteria culture was diluted with MHB to adjust turbidity, and measured the absorbance spectrophotometrically at optical density 600 nm (T70+ UV/VIS spectrometer, PG Instruments Ltd.) The desired absorbance should be in the range of 0.08 - 0.13 (McFarland standard 0.5) which approximately represents bacterial cell number $1x10^8$ CFUs/ml. The bacterial suspension was further diluted in MHB to $5x10^5$ CFUs/ml. 180 µL aliquot of bacterial suspension were transferred to a flat-bottom 96-well microtiter plate per well with an addition of 20 µL of two-fold serially dilute peptides to give final concentrations of 0.125, 0.25, 0.5, 1, 2, 4, and 8 µM in triplicate. After overnight incubation at 37 ºC, the MIC values of each peptide was determined by visual inspection (the lowest peptide concentration that gave no visible bacterial growth) Ampicillin and DI water were used as positive and negative controls respectively. To further determine bactericidal activity of each peptide, bacteria from the wells showing no visible sign of growth were inoculated onto sterile Mueller Hinton (MH) Agar (HiMedia Laboratories Pvt. Ltd, India) plates by streak plate method. The least concentration that showed no bacterial growth after an overnight incubation at 37 ºC was recorded as the MBC value.

RESULTS AND DISCUSSIONS

Physico-chemical properties of AMPs

The amino acid sequences of cationic α-helical antibacterial peptides were collected from the AntiMicrobial Sequence Database (AMSDb). These AMPs were calculated physicochemical properties using a compute pI/MW program. 61 peptides containing required characteristics were recruited from the AMSDb database. The results indicated that the cationic α-helical antibacterial peptides were usually 17-30 residues in length with molecular weight ranging from 1737-3383 Dalton. In addition, the values of pI from these antibacterial peptides were ranging from 6.26-11.47.

Peptides design

The peptides were created based on the previous criteria of physicochemical properties of the AMPs obtained from the AMSDb. Since Cc-CATH3 has not had its structure resolved experimentally, a bioinformatics tool (http://bioinf.cs.ucl.ac.uk/psipred/) was used to theoretically predict its possible secondary structure. The result obtained demonstrated that the first eight amino acids from the N-terminal part of the peptide should be random coil while the rest conformed in helix (Figure 1A). In this study, a series of truncated variants of Cc-CATH3 was created by deleting four amino acids progressively from the amino terminal region to examine the functional significance of its N-terminal region. The amphipathicity of each peptide was also determined using the helical wheel projections (http://blanco.biomol.uci.edu/mpex/) to show the distribution of polar and hydrophobic amino acids (Figure 1B).

Antibacterial activities of Cc-CATH3 and its variants

The antibacterial properties of Cc-CATH3 and its N-terminally truncated variants were evaluated against two gram-positive and two gram-negative bacteria. The MIC values for each peptide are reported in Table 2. According to the table, full-length peptide Cc-CATH3 and Cc-CATH3(5-29) had the ability to inhibit growth of the investigated bacteria with relatively low concentrations. On the other hand, Cc-CATH3(9-29) and Cc-CATH3(13-29) exerted no antibacterial activities at the maximum tested concentration of 32 μM. These findings suggest that the first eight amino terminal residues accounted for nearly 28 percentages of the total amino acids, are indispensable for the antibacterial activity.

When compared the MIC values of Cc-CATH3(1-29) and Cc-CATH3(5-29), Cc-CATH3(5-29) showed marginally higher activity against *S. aureus, B. subtilis,* and *S. typhimurium* but displayed equal activity against *E. coli*. Bactericidal activities were also determined for both peptides (Table 2). Cc-CATH3 and Cc-CATH3(5-29) showed MIC against *S. aureus* at 1 and 0.5 μM respectively and were bactericidal at double MICs. A similar trend of activity was observed in the testing of Cc-CATH3(5-29) against *B. subtilis* (MIC and MBC of 1 and 2 μM) but not in Cc-CATH3. However, the MICs of Cc-CATH3 and Cc-CATH3(5-29) towards *E. coli* and *S. typhimurium* were found to be generally higher. Both peptides were unable to kill these bacteria at two or four times the MICs. Thus, it appeared that both peptides were more potent against gram-positive bacteria both in terms of inhibitory and bactericidal properties. Yet a further study should be conducted with more bacteria strains to confirm this conclusion.

Although physicochemical properties of AMPs are considered to be, to some extent, responsible for their antibacterial activities, it should be noted that a relationship between the antibacterial activity of each peptide and its physicochemical properties; including length, molecular weight (MW), isoelectric point (PI), charge, % hydrophobic was not observed in this study (Table 1).

In addition, AMPs with amphipathic character are generally able to associate with and permeabilize lipid membranes which likely leads to the killing of certain microorganisms[12]. However, it is not apparent that each peptide possesses strong amphipathicity given that both hydrophobic residues were not mainly occupied one side of the sphere and polar residues the opposite (Figure 1B). Therefore it could be assumed that potencies of these peptides are irrespective of their amphipathicities.

Table 1. Amino acid sequences and physicochemical properties of Cc-CATH3 and its N-terminally truncated variants.

Peptides	Sequences	Length	MW	PI	Charge	% HB
Cc-CATH3 (1-29)	RVRRFWPLVPVAI NTVAAGINLYKAI RRK	29	3379.1	12.6	7	55.17
Cc-CATH3 (5-29)	FWPLVPVAINTVA AGINLYKAIRRK	25	2811.4	11.5	4	60
Cc-CATH3 (9-29)	VPVAINTVAAGIN LYKAIRRK	21	2267.7	11.5	4	57.14
Cc-CATH3 (13-29)	INTVAAGINLYKAI RRK	17	1901.3	11.5	4	52.94

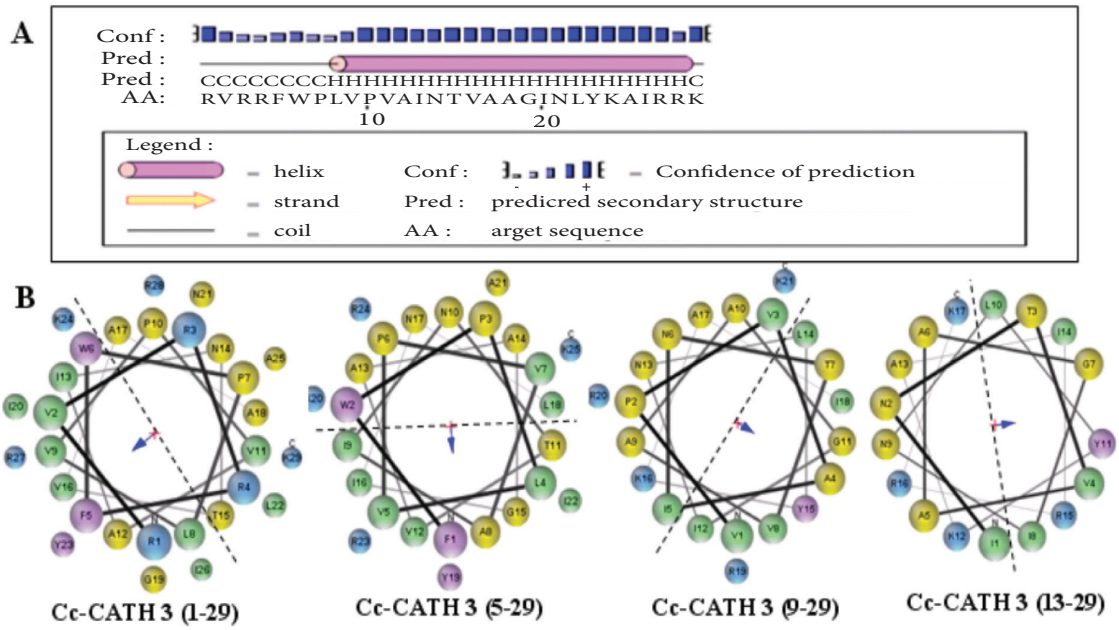

Figure 1. A) Secondary structure prediction of full length Cc-CATH3 and B) Helical wheel projections of (from left to right) Cc-CATH3(1-29), Cc-CATH3(5-29), Cc-CATH3(9-29), and Cc-CATH3(13-29). The arrow is a vector indicating the direction the hydrophobic moment, pointing towards the hydrophobic face of peptides. The dashed line separates the interface between membrane and water.

Table 2. Antibacterial activity of Cc-CATH3 and its N-terminally truncated variants against gram-positive and gram-negative bacteria. Mid-log phase bacteria in MHB were incubated overnight with 2-fold serial dilutions of peptides. The minimum inhibitory concentrations (MIC) of individual peptides against bacteria were determined as the peptide concentration that gave no visible growth after overnight incubation. The minimum bactericidal concentration (MBC) was assessed by streak the suspension from the MIC well and above onto agar plates. The least concentration showing no bacterial growth after incubation is considered MBC.

Bacteria	Cc-CATH3 (1-29)		Cc-CATH3 (5-29)		Cc-CATH3 (9-29)	Cc-CATH3 (13-29)
	MIC (μM)	MBC (μM)	MIC (μM)	MBC (μM)	MIC (μM)	MIC (μM)
Gram +						
S. aureus (DMST 6512)	1	2	0.5	1	>32	>32
B. subtilis (DMST 15896)	8	>8	1	2	>32	>32
Gram -						
E. coli (DMST 4212)	2	>8	2	>8	>32	>32
S. typhimurium (DMST 562)	4	>8	2	8	>32	>32

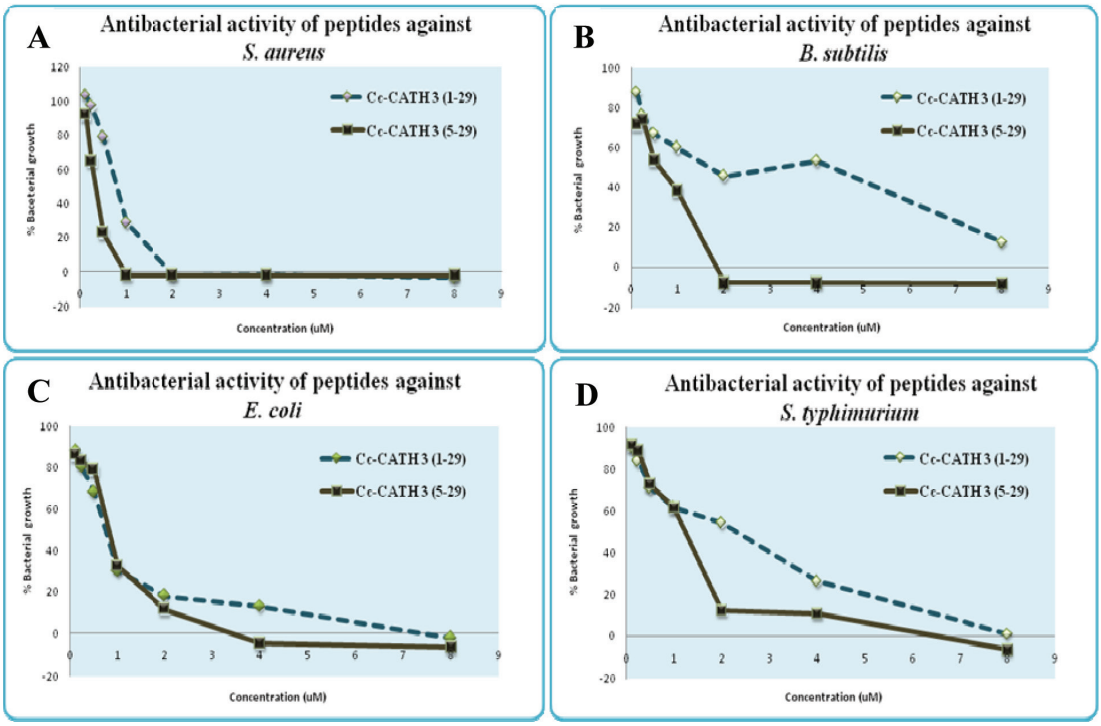

Figure 2. Antibacterial activities of Cc-CATH3(1-29)(♦) and Cc-CATH3(5-29)(■) peptides against (A) *S. aureus* (B) *B. subtilis* (C) *E. coli* and (D) *S. typhimurium.*

CONCLUSIONS

Full length Cc-CATH3 exhibits broad spectrum antibacterial activity at a low concentration. Our study proves that the peptide with four-residue truncation is active, with higher or equal potency than that of the native peptide. On the other hand, the peptides with eight- and twelve-truncation show no antibacterial activities even at concentration of up to 32 μM. Our results provide clear evidence that the amino terminal region of Cc-CATH3(1-29) is very essential for its antibacterial activity. The absence of only eight residues from amino-terminal region leads to the loss of its antibacterial property. In addition, it is worth to note that the tested gram positive bacteria appear to be more susceptible to these two active peptides than gram negative bacteria. It could be explained by the difference in the cell surface morphology; however, a further investigation with more bacteria strains is required.

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