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Cecropin A–melittin mutant with improved proteolytic stability and enhanced antimicrobial activity against bacteria and fungi associated with gastroenteritis in vitro

Shengyue Ji [a,1], Weili Li [b,1], Lei Zhang [a], Yue Zhang [a], Binyun Cao [a,*]

[a] College of Animal Science and Technology, Northwest A&F University, 22 Xinong Road, Yangling 712100, Shaanxi, PR China
[b] Beijing S&V Science and Technology Co., Ltd., Beijing 102200, Beijing, PR China

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ABSTRACT

Cecropin A–melittin (CAM), a chimeric antimicrobial peptide with potent antimicrobial activity, is threatened by some special extracellular proteases when used to deal with certain drug-resistant pathogenic microbes in the gastrointestinal tract. Thus, a four-tryptophan-substitution mutant (CAM-W) from CAM was developed via the replacement of special amino acid residues to enhance the antimicrobial potency and to improve the proteolytic stability of this agent. The pharmaceutical index of CAM-W was investigated, with a focus on biological potency, cytotoxicity, and proteolytic stability, as well as pH and thermal resistance. CAM-W exhibited potent antimicrobial activity and was approximately 3–12 times higher than that of CAM. CAM-W also exhibited a strong antifungal activity against a series of common pathogenic fungi, in a lower IC50 range between 2.1 mg/L and 3.3 mg/L than that of its reference CAM ranging from 9.8 mg/L to 14.2 mg/L. Besides, CAM-W showed moderate cytotoxicity (IC_{50} > 300 mg/L) in erythrocyte lysis test. In addition, CAM-W overcame challenges under various conditions, including specific temperatures (20, 30, 40, 50, 60, 70, 80, and 90 °C), pH values (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0), and proteases (trypsin, pepsin, human neutrophil elastase, *Pseudomonas aeruginosa* elastase, and *Staphylococcus aureus* V8 protease) that are commonly present in human gastrointestinal tract. These results suggest that the four-tryptophan-substitution can confer CAM-W with a high pharmaceutical index, which is important for CAM-W to become a potential alternative to conventional antibiotics against bacteria and fungi associated with gastroenteritis.

1. Introduction

Gastroenteritis is often clinically observed and has significant economic and social consequences for both health systems and communities because of its high rate of occurrence [1]. The infectious causes of gastroenteritis include a wide array of bacteria, fungi, and viruses. Of these microbes, bacterial and fungal pathogens are important causes. For example, *Salmonella* and other *Enterobacteriaceae*, usually causing gastroenteritis in human, may be extended-spectrum β-lactamase producers. The resulting β-lactamases are usually encoded by plasmids that often carry other resistant genes and easily elicit host strains multidrug-resistant [2]. The re-emergence of gastroenteritis caused by multi-drug-resistant bacteria presents a challenge to clinicians, particularly because of the lack of a pipeline of new antibiotics that are sufficiently active toward these drug-resistant strains.

The development of drug-resistant pathogens also prevents the recommendation of most traditional antibiotics for gastroenteritis treatment. Thus, an alternative way to solve this problem is becoming more attractive. Antimicrobial peptides (AMPs) have recently become a powerful chemotherapeutic alternative to treat drug-resistant pathogenic bacteria regarding their distinct mechanism of action. This mechanism involves outer membrane permeation in bacteria and, thus, the possible reduced likelihood of the emergence of resistance [3–6]. However, bacteria have developed some special extracellular proteases, such as *Pseudomonas aeruginosa* elastase, *Staphylococcus aureus* aureolysin, and *S. aureus* V8. When these proteases presented in the human gastrointestinal tract, they can proteolytically degrade the probably applied AMPs. Moreover, gastrointestinal digestive enzymes, such as pepsin and

* Corresponding author. Fax: +86 29 87092164.
E-mail address: jiziqing2010@163.com (B. Cao).
[1] These authors contributed equally to this work.

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trypsin, inevitably threaten the integrity and activity of AMPs when used to treat gastroenteritis.

Currently, two natural peptides known to exhibit potent antimicrobial activity are cecropin A and melittin. Both peptides are highly potent in lysing bacteria membranes, and the first 13 and last 13 residues of their chimeric peptide CAM (KWKLFKKIEKVGQG IGAVLKVLTTGL-NH_2) are identical to each of the first 13 residues of cecropin A and melittin, respectively. CAM kills bacteria also by disrupting bacterial membranes through mechanisms involving electrostatic interactions with bacterial surfaces. However, CAM possesses a more potent antimicrobial activity against a wide range of clinical pathogens, such as *Escherichia coli* and *P. aeruginosa*, than its parental peptides but with a low hemolytic activity [7].

In the complex environment of the gastrointestinal tract, CAM is inevitably threatened by protease degradation induced by a variety of enzymes that are secreted by both special bacteria and the digestive glands. Previously studies showed that the proteolytic sensitivity of certain AMPs could be decreased by placing tryptophan residue(s) (W) at selected residue positions [8–12]. In this work, a peptide mutation biotechnology was adopted to develop a new and advantageous CAM, and a modified CAM with four-tryptophan-substitution (KWKL***W***KKIEK***W***GQGIGAVLK***W***LTT***W***L-NH2; CAM-W) was designed to possess improved proteolytic stability and enhanced antimicrobial activity against bacteria and fungi associated with gastroenteritis.

2. Materials and methods

2.1. Peptide synthesis

CAM and CAM-W were biosynthesized from *Bacillus subtilis* WB700 using EDDIE autoprotease fusion technology [13,14]. The obtained crude peptide was subjected to a semi-preparative Zorbax 300SB-C8 column (250 × 9.4 mm, 5 μm particle size, 300 Å pore size) (Agilent, Englewood, CO). The column was equilibrated in 0.1% (v/v) trifluoroacetic acid and 10% acetonitrile, and was subsequently developed with a linear 0–60% acetonitrile gradient at a flow rate of 1.0 mL/min. The absorbances at 214, 254, and 280 nm were monitored, and the peaks were pooled and investigated by using an antimicrobial activity assay with *E. coli* as the indicator strain. Purified peptides were checked by using an analytical Zorbax 300SB-C8 column (250 × 4.6 mm, 5 μm, 300 Å) (Agilent, USA) and then freeze-dried in a vacuum freeze dryer (SIM International Group Co., Ltd., USA) at −80 °C for further analysis. The concentration of purified peptide solutions was determined by UV spectrophotometry [15,16]. The molecular weights of the peptides were measured using electrospray ionization mass spectrometry (Agilent, USA).

2.2. Microbial strains

E. coli 25922, *Campylobacter jejuni* ATCC 29428, *Helicobacter pylori* ATCC 43504, *P. aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 14028, *Shigella flexneri* ATCC 12022, *Shigella sonnei* ATCC 11060, *S. aureus* ATCC 25923, and *Streptococcus pyogenes* ATCC 19615 were acquired from American Type Culture Collection (ATCC; Rockville, MD, USA). Ceftriaxone-resistant *S. sonnei*, chloramphenicol-resistant *E. coli*, colistin-resistant *P. aeruginosa*, erythromycin-resistant *S. pyogenes*, fluoroquinolone-resistant *S. flexneri*, methicillin-resistant *S. aureus*, penicillin-resistant *S. typhimurium* and quinolone-resistant *C. jejuni* were kept in our laboratory. *Aspergillus flavus* ATCC 9807, *Aspergillus fumigatus* ATCC 46646, *Aspergillus niger* ATCC 16620, *Candida albicans* ATCC 10231, *Saccharomyces cerevisiae* ATCC 9763, *Trichosporon beigelii* ATCC 28592, and *Trichoderma viridae* ATCC 8678 were also from ATCC.

2.3. Antibacterial activities

The minimum inhibitory concentration (MIC) assay was conducted based on a microtiter broth dilution method as recently described [17], with slight modification. Deionized water, 2 M NaCl, 0.1 M NaOH, deionized water, 70% ethanol, and 10 mM Tris buffer (pH 7.4) were used to pre-equilibrate a volume packed with DEAE-Sephacel (Sigma–Aldrich, Schnelldorf, Germany), to which 100 mL of LB broth or specialized medium in the same Tris buffer was applied twice to prepare the refined medium. The peptide samples were dissolved in Tris buffer to prepare tenfold required serial dilutions. The overnight cultures of test bacteria were washed twice with Tris buffer and then diluted to approximately 5×10^5 CFU/mL in refined medium. The wells of a 96-well microtiter plate were filled with aliquots of 90 μL of bacterial solution. Thereafter, aliquots of 10 μL of above tenfold peptide dilutions were placed into the corresponding wells, yielding serial working concentrations of 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.12, and 0.06 mg/L. The mixtures were incubated at 37 °C for 21 h and determined using a previously described method [18]. Meanwhile, a negative control was set up by adopting an identical reaction system without peptide solution. MIC was defined as the lowest concentration required for inhibiting bacterial growth by more than 90% after overnight incubation.

2.4. Antifungal activities

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method was introduced to perform a 50% inhibition concentration (IC_{50}) assay of the peptide against seven common fungi. The fungal strains were cultured at 28 °C in a Yeast Mold medium (1% glucose, 0.5% peptone, 0.3% yeast extract, and 0.3% malt extract). Subsequently, each well of 96-well microtiter plate was seeded with the fungal conidia at a density of 1×10^2 spores in a 90 μL volume with 10 μL tenfold peptide serial dilutions. After incubation of the mixtures at 28 °C for 24 h, 10 μL of 5 mg/mL MTT in PBS (pH 7.4) was added to each well. And then a further incubation was performed at 37 °C for 4 h, followed by the addition of 40 μL of 20 mM HCl containing 20% SDS at 37 °C for 16 h to dissolve the MTT-formazan crystals. The reaction samples were detected at 570 nm with a microtiter ELISA reader (Epoch™, BioTek-® instruments, Inc., Vermont, USA).

2.5. Assessment of stability

Aliquots of 5 mg/L peptide solutions in PBS (pH 7.4) were processed under serial temperatures (20, 30, 40, 50, 60, 70, 80, and 90 °C) for 30 min, under various pH values (pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0) at 37 °C for 30 min, and under different enzymes [trypsin (Sigma Chemical Co., St. Louis, MO), pepsin (Sigma), human neutrophil elastase (HNE; Innovative Research, Novi, MI), *P. aeruginosa* elastase (PE; Innovative Research), and *S. aureus* V8 protease (V8; BioCol GmbH)] with a substrate: enzyme molar ratio of 300:1 at 37 °C for 30 min. Afterwards, aliquots of 100 μL treated peptide solutions were replenished with approximately 5×10^5 CFU/mL of indicator bacteria and processed at 37 °C for 21 h. The same reaction systems with no peptides were performed in parallel as the negative control, and the same reaction at 30 °C, pH 7.0 was employed as the positive control. *E. coli* ATCC 25922 was used as the indicator bacteria. The influence of factors on peptide stability was assessed based on residual activity using the formula below:

$$\text{Inhibition ratio } (\%) = (A_1 - A)/(A_1 - A_2) \times 100\%$$

where A, A_1, and A_2 are the absorbances at OD 600 after treatment of indicator mixtures with different factors, the negative control, and the positive control, respectively.

2.6. Hemolytic activity

The hemolytic activity study was carried out at peptide concentrations of 0.01, 0.02, 0.05, 0.1, 0.2, 0.35, 0.7, 1.5, 3.5, 5.5 and 10.0 mg/L. EDTA-treated human blood was centrifuged at 800×*g* for 10 min. The serum and buffy coat were removed, and erythrocytes were washed twice and diluted in PBS (pH 7.4) to generate a 5% cell suspension for the hemolysis assay. Then, aliquots of 100 μL of erythrocyte solution were plated into 96-well plates, and aliquots of 100 μL serial dilutions of the peptide were added to each well. Subsequently, the mixtures were incubated at 37 °C for 1 h and centrifuged at 150×*g* for 10 min. Thereafter, aliquots of 100 μL supernatant were retransferred to the 96-well plates. The absorbance of liberated hemoglobin was immediately read at 414 nm with a microplate spectrophotometer (Epoch™). Slight spontaneous leakage (<2%) was observed and served as 0% hemolysis, whereas 100% hemolysis was determined in 0.1% Triton X-100 (Sigma–Aldrich, Steinheim, Germany).

2.7. Liposome leakages

Two types of liposomes with entrapped glucose were prepared from commercially available phosphatidylethanol (PHE), phosphatidylglycerol (PG), cardiolipin (CA), cholesterol (CH), and 1, 2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (all from Avanti Polar Lipids, AL, USA) with phospholipid compositions resembling those cells of *E. coli* (molar ratio of PHE:PG:CA, 7:2:1; PPC liposome) and erythrocyte (DOPC:CH, 3:2; DC liposome). To prepare the two types of liposomes, PHE:PG:CA, 7:2:1 or DOPC:CH, 3:2 were dissolved in chloroform on flask walls to form dry lipid films. The films were dried overnight under vacuum with a stream of nitrogen at room temperature. Then, 5(6)-carboxyfluorescein (CF; Acros Organics, Geel, Belgium) was added as a leakage marker to 10 mM Tris buffer (pH 7.4, containing 5 mM glucose) at a self-quenching concentration of 100 mM, and the lipids were resuspended in the treated Tris buffer. Liposomes were prepared by using 10 freeze–thaw cycles between liquid nitrogen and then vortex at 62 °C. An Avanti mini-extruder (Avanti Polar Lipids) was used to reduce liposome polydispersity by extrusion through polycarbonate filters with 100 nm pores. Thus, the monolayer liposomes were obtained with approximate 110 nm diameter.

CF leakage from the liposomes to the external environment, which resulted in increased fluorescence intensity and loss of self-quenching, was monitored for liposome integrity [19]. A Shimazu RF-5000 spectrofluorometer (Tokyo, Japan) was used to monitor fluorescence changes by measuring fluorescence intensity at 520 nm with excitation at 490 nm, as induced by 10 serial peptide concentrations ranging from 0.01 mg/L to 5.5 mg/L at a fixed lipid concentration of 10 μM under continuous agitation. Spontaneous leakage at the initial 10 min was detected based on the signal changes in every experiment. The influence of peptide participation on the systems was monitored for 45 min once the induced leakage reached its limits, and 100% liposome leakage was defined as 0.1% Triton X-100 in the system for reaction.

2.8. Circular dichroism spectroscopy analysis

A Jasco 810 spectropolarimeter (Jasco Corporation, Japan) was used in the range from 190 to 250 nm at 50 nm/min at 37 °C to determine the peptide secondary structures in 10 mM PBS (pH 7.4) with or without liposomes at a peptide concentration of 100 mg/L, using a 1 mm-path-length quartz cuvette. The palmitoyl-oleoyl-phosphatidylglycerol (POPG) liposomes (Sigma, USA) was prepared as previously documented [20], with the following slight modification: 10 mM PBS (pH 7.4) dissolved the appropriate amount of POPG to prepare the stock solution of POPG (100 μM). The information of CAM-W and CAM secondary structure was deduced based on a previously reported approach [21].

2.9. Statistics

All experiments were performed three times, and means ± standard deviations were used as the mean values. ANOVA followed by the Student–Newman–Keuls test was employed to determine the significance of CAM-W comparing with CAM in the relative experiments. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Antibacterial and antifungal activity

As shown in Table 1, the results demonstrated that the antimicrobial activity of CAM-W showed three to twelve times higher than that of CAM against a broad spectrum of pathogenic strains, especially against *E. coli* with MIC 0.3 mg/L, approximate one-twelfth lower than MIC 3.7 mg/L of CAM. In addition, like CAM, CAM-W also displayed a more potent antibacterial activity against Gram-negative bacteria, with MICs ranged from 0.3 to 1.0 mg/L, than against Gram-positive bacteria, with MICs ranged from 2.1 to 2.8 mg/L. However, there was no statistically significant difference between the antibacterial activities of CAM-W against each pair of typical and drug-resistant bacteria. As shown in Table 2, CAM-W also exhibited a lower IC_{50} between 2.1 mg/L and 3.3 mg/L than 9.8 mg/L to 14.2 mg/L of CAM against a series of common pathogenic fungi, especially against *A. flavus* with IC_{50} 2.1 mg/L, approximate one-fifth lower than IC_{50} 10.6 mg/L of CAM.

3.2. Liposome leakages

PPC liposomes and DC liposomes were designed as the simulations of bacterial cell membranes and normal animal cell membranes respectively. As shown in Fig. 1, the addition of CAM-W and CAM to either PPC liposomes or DC liposomes all induced

Table 1
MICs of peptides for different bacteria.

Strains	MIC (mg/L)	
	CAM-W	CAM
E. coli ATCC 15224	0.3 ± 0.12	3.7 ± 0.21
Fluoroquinolone-resistant *E. coli*	0.3 ± 0.14	3.6 ± 0.23
C. jejuni ATCC 43051	0.6 ± 0.16	4.0 ± 0.15
Macrolide-resistant *C. jejuni*	0.7 ± 0.14	4.4 ± 0.20
H. pylori ATCC 43526	0.8 ± 0.15	4.6 ± 0.19
P. aeruginosa ATCC 90271	0.6 ± 0.10	4.4 ± 0.22
Carbapenem-resistant *P. aeruginosa*	0.6 ± 0.11	3.8 ± 0.20
S. aureus ATCC 29213	2.5 ± 0.12	7.3 ± 0.24
Oxacillin-resistant *S. aureus*	2.8 ± 0.14	8.6 ± 0.23
S. pyogenes ATCC 10389	2.1 ± 0.13	7.5 ± 0.20
Tetracycline-resistant *S. pyogenes*	2.3 ± 0.13	7.7 ± 0.19
S. typhimurium ATCC 13311	0.7 ± 0.13	4.9 ± 0.18
Cefotaxime-resistant *S. typhimurium*	0.8 ± 0.10	4.4 ± 0.22
S. flexneri ATCC 25875	0.8 ± 0.12	4.2 ± 0.20
Ciprofloxacin-resistant *S. flexneri*	1.0 ± 0.13	4.7 ± 0.21
S. sonnei ATCC 25931	0.8 ± 0.10	4.5 ± 0.20
Ampicillin-resistant *S. sonnei*	0.9 ± 0.11	4.7 ± 0.23

Table 2
IC_{50} of peptides for different fungi.

Fungi	IC_{50} (mg/L)	
	CAM-W	CAM
A. flavus ATCC 46283	2.1 ± 0.32	10.6 ± 0.99
A. fumigatus ATCC 16424	2.8 ± 0.29	10.5 ± 0.63
A. niger ATCC 20611	2.5 ± 0.29	9.0 ± 0.61
C. albicans ATCC 32354	2.7 ± 0.26	11.3 ± 0.82
S. cerevisiae ATCC 24860	3.7 ± 0.37	14.2 ± 0.68
T. viride ATCC 36316	3.3 ± 0.29	13.2 ± 0.88

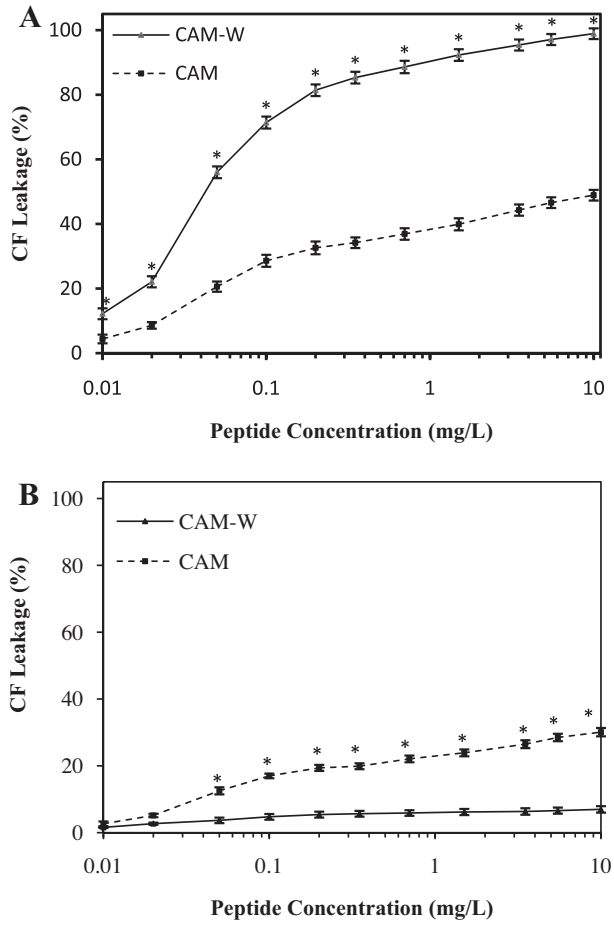

Fig. 1. CF leakage from liposomes PPC (PE:PG:CL, 7:2:1) (A) and DC (DOPC:CH, 3:2) (B), resembling *E. coli* and erythrocyte membranes respectively, induced by CAM-W and its reference CAM. The liberated CF fluorescence was measured at $\lambda_{excitation}$ = 490 nm and $\lambda_{emission}$ = 520 nm. The data points show the means, and error bars show the standard deviations of three independently generated and almost identical triplicate sets of data. *Suggests that the value is statistically and significantly higher than that of its parental peptide ($P < 0.05$).

the release of entrapped CF; the percentage of CF efflux was CAM-W and CAM concentration dependent. However, the CAM-W/PPC liposomes demonstrated a statistically significant CF efflux in comparison with CAM/PPC liposomes (Fig. 1A). The leakage rate of the former ranged from 56.0% to 98.9% corresponding to the CAM-W concentration between 0.05 and 10.0 mg/L; that of the latter was from 20.6% to 48.9%. Meantime, CAM-W/DC and CAM/DC liposomes induced an opposite effect on CF efflux ratio compared with the above result (Fig. 1B). Wherein, the former generated a statistically significant CF efflux in contrast to the latter, 2.1–4.1% versus 6.8–12.0% with peptide concentration ranging from 1.0 to 10.0 mg/L.

3.3. Hemolytic activity

As illustrated in Fig. 2, the hemolytic activity of CAM-W and CAM against human erythrocytes was determined as the peptide concentration ranging from 0.01 to 10.0 mg/L. Significantly, peptide CAM-W showed a negligible hemolysis (<5%) against human red blood cells after incubation at 37 °C for 1 h even at the extremely high peptide concentration of 10.0 mg/L. At the same time, peptide CAM exhibited a statistically significant enlargement in erythrocyte hemolysis, for which the value is approximately 12% at CAM concentration of 10.0 mg/L. In addition, IC_{50} > 300 mg/L was determined for both CAM-W and CAM against human red blood cells.

3.4. Thermal, pH and proteolytic stability

The result demonstrated that heat-treating at temperatures ranging from 20 °C to 50 °C for 30 min exerted a no more than 10% damage effect on the antibacterial potency of CAM-W. Other than that, especially at temperatures ranging from 50 to 90 °C, CAM-W presented a more statistically significant thermal stability than that of CAM (Fig. 3A). After treatment at pH values 9.0 and between 2.0 and 5.0, CAM-W exhibited a statistical significance of pH stability in contrast to its reference CAM (Fig. 3B). Compared with CAM, the four-tryptophan-substitution CAM-W maintained a statistically significant proteolytic stability, with 82.4%, 80.2%, 84.2%, 95.1%, and 86.9% antibacterial activity preserved after treatment with trypsin, pepsin, HNE, PE, and V8 respectively (Fig. 3C).

3.5. Circular dichroism spectrum analysis

The secondary structure of CAM-W and its reference CAM were analyzed using circular dichroism spectra. As shown in Fig. 4A, the data illustrated that both CAM-W and CAM with a concentration of 100 mg/L in POPG liposome solution all displayed a distinct double-negative-peak at 207 and 222 nm, however the minimal peaks of CAM-W were significantly lower than that of CAM. Meanwhile, as shown in Fig. 4B, the double-negative-peak was not clearly

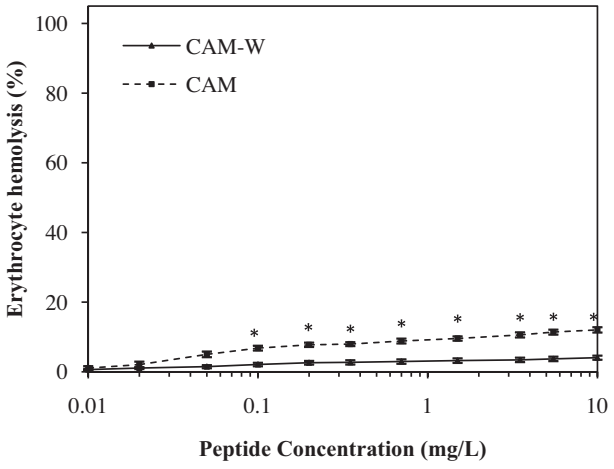

Fig. 2. Erythrocyte hemolysis induced by CAM-W and its reference CAM. The data points show the means, and error bars show the standard deviations of three independently generated and almost identical triplicate sets of data. *Suggests that the value is statistically and significantly higher than that of its parental peptide ($P < 0.05$).

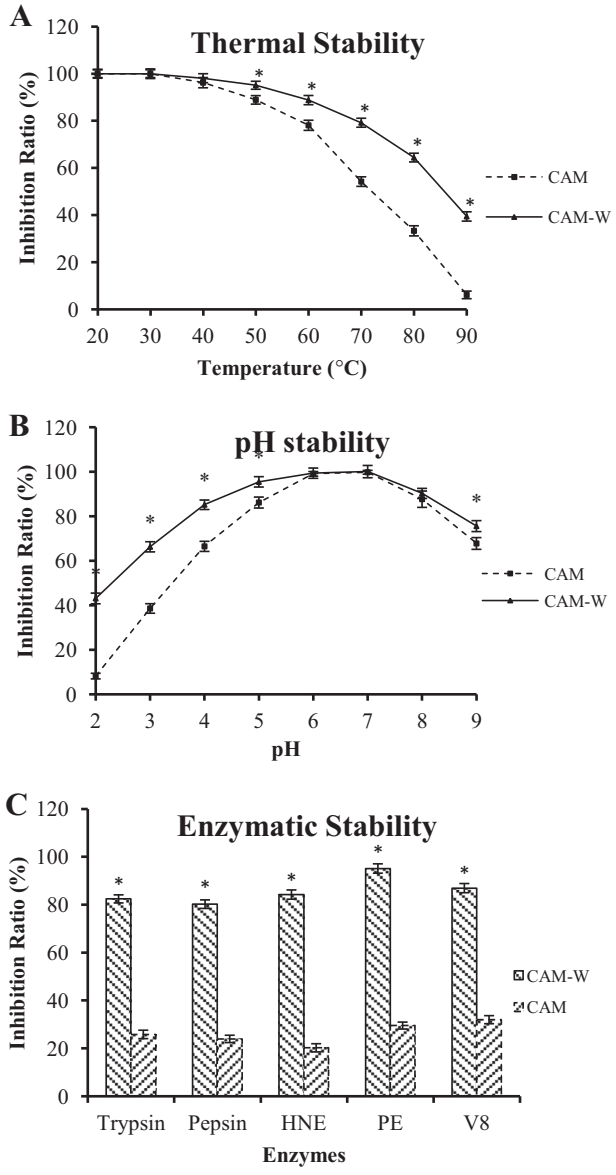

Fig. 3. Influence of different temperatures (A), pH values (B), and enzymes (C) on CAM-W and CAM. The proteases used were trypsin, pepsin, human neutrophil elastase (HNE), *P. aeruginosa* elastase (PE), and *S. aureus* V8 protease (V8). Data points show the means, and error bars show the standard deviations of three independently generated and almost identical triplicate sets of data. *Suggests that the value is statistically significantly higher than that of CAM ($P < 0.05$).

observed either from CAM-W or from its reference CAM in PBS solution.

4. Discussion

CAM-W was studied in terms of its antibacterial and antifungal activity, combined with cytotoxicity and the liposome lytic property. The growth of a large spectrum of pathogenic bacteria and fungi was statistically significantly inhibited by CAM-W in comparison with CAM, and some of these microorganisms, such as *A. flavus*, producer of aflatoxins and capable of causing severe gastroenteritis [22,23], often colonize the gastrointestinal tract and serve as the major etiological agents of gastroenteritis [24]. Compared with CAM, antibacterial and antifungal activity of CAM-W was significantly improved probably as a result of the reduced susceptibility to the proteolytic enzymes secreted by these bacteria. This

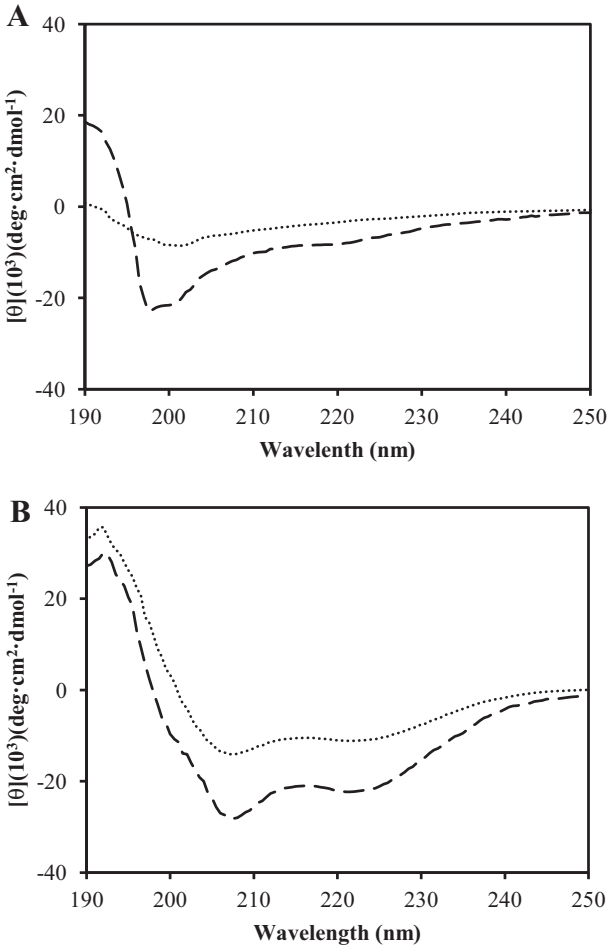

Fig. 4. Circular dichroism spectra of CAM (dotted line) and CAM-W (dashed line) in sodium phosphate buffer (A) and in liposome solution (B). Both CAM and CAM-W were determined with a concentration of 100 mg/L.

result is in agreement with previous report showing high activity of CSA-13, another AMP, against protease-positive *Porphyromonas* [25].

In addition, the findings showed that CAM-W not only had a more potent antimicrobial activity than CAM for inhibiting bacterial strains and fungal cells tested associated with gastrointestinal infections but also had a negligible adverse effect on normal cell proliferation. Liposome leakage analyses also confirmed this action mode and demonstrated that CAM-W bound to negatively charged PPC liposomes, simulations of bacterial cell membranes, and resulted in their remarkable leakage while generating a comparatively lesser or negligible leakage than CAM did for DC liposomes, simulating the membranes of the normal animal cells. Such modified activity emphasized the importance of improving the pharmaceutical index of CAM-W for treating bacterial- and fungal-mediated gastroenteritis.

As a potential alternative to conventional antibiotics, the biochemical stability of CAM-W under simulated in vivo conditions, such as under specific pH, temperatures, and protease-rich circumstances, was investigated. The mean pH values in the gastric juice, proximal small intestine, terminal ileum, and caecum are 1.0–2.5, 6.6, 7.5, and 6.4, respectively [26]. This pH distribution enables CAM-W to exert potent biological actions in these tissues, except in the stomach. In the normal temperature range of human body, no significant difference in antimicrobial activity existed between heat-treating CAM-W and CAM against indicator strains; the residual antimicrobial activity of both agents retained above 90%.

However, the statistically significant enhancement of thermal stability of CAM-W compared with CAM presented when temperature was between 50 and 90 °C, suggesting advantage of capable of withstanding post-treatment process with high temperature even up to 90 °C. Sensitivity to proteolytic degradation is another challenge for application of CAM-W in treating bacterial- and fungal-mediated gastroenteritis [27]. The statistically significantly improved tolerability of CAM-W challenged by a number of bacterial proteolytic enzymes and gastrointestinal digestive enzymes, such as PE, V8 protease, pepsin, and trypsin, suggested this novel agent has a potential application in human medicine for gastroenteritis. However, considering all experiments were performed in vitro, the potential clinical use of CAM-W still requires further investigation.

The result of circular dichroism spectrum analysis demonstrated that CAM-W was more helical than its reference CAM in liposome solution, even though the latter adopted a low α-helical conformation in aqueous but possessed the potential to form α-helices in partially organic solvent as reported [28]. Furthermore, in addition with neural network prediction (http://www.predictprotein.org), secondary structure analysis indicated that CAM-W adopted a typical α-helical conformation with a helical and loop content of 88.46% and 11.54% but lack of β-sheets; the helical and loop content of CAM is 76.92% and 23.08%, which is coincident with the previous report [28]. The total results suggested that two α-helical conformations with a higher helical content than that of CAM combined with a flexible hinge part in between was the feature of CAM-W. For that both of CAM-W and CAM carrying five positive charges, this structure feature of CAM-W possibly acted an important role in its antibacterial activity and biological stability.

Acknowledgments

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