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Characterization and antimicrobial mechanism of CF-14, a new antimicrobial peptide from the epidermal mucus of catfish

Tingting Li[a], Quanwei Liu[b], Dangfeng Wang[b], Jianrong Li[b,*]

[a] Key Laboratory of Biotechnology and Bioresources Utilization (Dalian Minzu University), Ministry of Education, Dalian Liaoning, 116029, China
[b] College of Food Science and Technology, Bohai University, National & Local Joint Engineering Research Center of Storage, Processing and Safety Control Technology for Fresh Agricultural and Aquatic Products, JinzhouLiaoning, 121013, China

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ABSTRACT

In this study, we identified a novel antibacterial peptide, RIVELTLPRVSVRL-NH_2 (named CF-14), derived from the epidermal mucus of catfish and characterized its antimicrobial activity. Analysis of antimicrobial activity and hemolytic activity of CF-14 revealed broad spectrum, high levels of antimicrobial activity and low toxicity to eukaryotic cells. CF-14 remained stable at pH values ranging from 4.0 to 12.0 and remained bioactive when exposed to high temperature. CD analysis indicated that CF-14 forms a random coil in PBS buffer and an α-helical conformation in the membrane-mimetic 2.5% SDS micelle. Additionally, the antibacterial mechanism of CF-14 against *Shewanella putrefaciens* was investigated. Membrane permeability experiments confirmed that CF-14 could increase cell wall membrane permeability and cause nucleotide leakage. Moreover, observations performed using scanning electron and confocal microscopy indicated that CF-14 could penetrate into the cell membranes of *S. putrefaciens* and accumulate in bacterial cells, but did not break down cell membranes. Further, electrophoresis analysis demonstrated that CF-14 possesses DNA-binding affinity. The results provide a substantial basis for future application of CF-14, a novel cell-penetrating peptide (CPP) derived from catfish.

1. Introduction

China has substantial amounts of aquaculture, but the deterioration of aquatic products caused by microbial contamination is difficult to resolve and is problematic for this valuable industry [1]. *Shewanella putrefaciens* is the main bacterium responsible for aquatic product spoilage, especially during low-temperature storage of seafood products [2,3]. Antibacterial agents can eliminate significant amounts of spoilage-causing microorganisms in foods, but the overuse of antimicrobial agents has led to the worldwide emergence of drug-resistant microorganisms [4]. Therefore, it is critical to develop new antimicrobial strategies for seafood products.

Antimicrobial peptides (AMPs), also known as host defense peptide (HDPs), are important components of the innate immune system. AMPs have been isolated from a variety of plants and animals and typically exhibit relatively broad antimicrobial activity, strong antimicrobial activity against drug-resistant pathogens, and low toxicity towards eukaryotic cells [5,6]. In addition, antibacterial peptides have multiple advantages, including thermal stability, good solubility, low molecular weight, and a lack of resistance [7]. The C-termini of most natural antimicrobial peptides are often amidated, which may be related to their broad-spectrum antibacterial activity, although detailed mechanisms of action for AMPs have not been fully elucidated and appear to be fairly variable [8]. Finally, AMPs can interact with bacteria without using specific receptors, preventing induction of antibiotic resistance [9]. Due to these advantages, AMPs have been considered a potential strategy to inhibit bacteria in an age of drug-resistant bacteria. Up to now, several AMPs have been used in local treatments or clinical trials, suggesting that AMPs have potential application value in food preservation and medicine [10].

Previous studies have shown that antimicrobial peptides can be isolated from a variety of sources [11]. As a result of dwelling in muddy water for many generations, catfish have the ability to defend themselves against many microorganisms. Fish skin mucus is rich in antimicrobial peptides and plays an important role in protecting fish from microbial invasion. Su et al. [12] described the purification and characterization of a novel antimicrobial peptide, pelteobagrin, from the skin mucus of *Pelteobagrus fulvidraco*. Similarly, Park et al. [13] reported that catfish produce the antimicrobial peptide parasin I upon injury to protect against invasion by microorganisms. Despite this, out of the 3019 AMPs in the Antimicrobial Peptide Database (APD) (http://aps.unmc.edu/AP/main.html), only 122 AMPs have been isolated from

* Corresponding author.
E-mail addresses: jwltt@dlnu.edu.cn (T. Li), liuqw11010@163.com (Q. Liu), 15941611651@163.com (D. Wang), lijianrong@bhu.edu.cn (J. Li).

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fish. In our previous study, we identified the novel AMP RIVELTLPR-VSVRL-NH_2 from catfish. In this report, we characterize and describe the antibacterial mechanism of this novel peptide, which we have named CF-14.

2. Materials and methods

2.1. Materials and bacterial strains

S.putrefaciens used in this experiment was previously isolated from contaminated turbots, while *S. aureus* came from lab stocks. Test strains were cultured in Luria-Bertani (LB) broth and shaken at 28 °C, 160 rpm for 12 h. After incubation, the bacterial culture was diluted to 1×10^6 CFU/mL for further experiments. CF-14 peptides with a purity of > 95% and FITC-labeled CF-14 peptide were purchased from Sangon Biotech (Shanghai, China).

2.2. Solid phase peptide synthesis

CF-14 peptides derived from epidermal mucus of catfish were synthesized using solid phase technology and amidation of the C-terminal carboxyl group. The crude peptides were purified to > 95% homogeneity by reversed-phase high performance liquid chromatography (RP-HPLC) on an ODS-SP column (Prominence HPLC, Shimadzu, Japan). The molecular masses of synthesized peptides were determined by mass spectrometry (LCMS-2020, Shimadzu, Japan) and amino acid analysis. These peptides were also produced by Shanghai Sangon Biotech. The confirmed peptides were stored at −80 °C for further study.

2.3. Physicochemical property analysis

The physicochemical properties of the peptide were analyzed by ExPASy (http://www.expasy.ch/) and HeliQuest Computparam form version 3(http://heliquest.ipmc.cnrs.fr/). The potential toxicity of the peptide was predicted by ToxinPred (http://www.imtech.res.in/raghave/toxinpred/). Transmembrane regions of the peptide were predicted on the TMHMM (http://www.cbs.dtu.dk/services/TMHMM/). The minimum energy 3D structure of the peptide was predicted using the Discovery Studio (DS).

2.4. Antimicrobial activity

2.4.1. Agar well diffusion method

The agar well diffusion method [14] was performed to verify the antibacterial activity using some modifications. *S.putrefaciens* and *S. aureus* were used as indicator strains for the antimicrobial activity spectrum of CF-14. First, sterilized Oxford cups were put on a sterile dish, and 15 mL of LB-agar containing $1x10^6$ CFU/mL of indicator strain was slowly added. After the agar solidified, the Oxford cups were removed and 100 μL of 1 mg/mL CF-14 was added to the resulting hole on the dish, with a PBS buffer solution in place of the CF-14 solution as a negative control. Petri dishes were then incubated at 28 °C for another 24 h and the inhibition zones were observed.

2.4.2. Minimal inhibitory concentration

The minimal inhibitory concentration (MIC) of CF-14 against *S. putrefaciens* was determined using the microdilution method to evaluate bactericidal activity [15]. Bacterial cells were collected in exponential phase, washed thrice with PBS buffer, and resuspended at 1×10^6 CFU/mL in fresh LB broth. Next, 50 μL samples were added to 96-well polystyrene plates and 50 μL of prepared CF-14 solutions (diluted to 31.25–500 μg/mL with PBS) were added to plates containing bacterial suspension. The 96-well plates were then incubated at 28 °C for 10 h. Bacterial growth inhibition was determined by measuring the absorbance at 595 nm with a microplate autoreader (Victor X3, PerkinElmer, USA) and compared to growth of cells exposed to 50 μg/mL of ampicillin or PBS buffer alone as positive and negative controls, respectively. The MIC was considered as the CF-14 concentration at which bacterial growth was not significantly different from the positive ampicillin control. The MIC identified represents the average of values obtained in three independent experiments.

2.5. Effects of temperature, pH and proteolytic enzymes on the activity of CF-14

The effects of temperature, pH, and proteolytic enzymes on CF-14 were assessed by determining the antimicrobial activities of CF-14 against *S. putrefaciens* using the agar well diffusion assay after exposure to test conditions [16]. To test temperature sensitivity, CF-14 was incubated at either 40 °C, 60 °C, 80 °C, or 100 °C for 30 min. To examine pH stability, pH was adjusted to 2.0 to 12.0 at increments of 1.0. To test protease sensitivity, CF-14 was incubated with different hydrolytic enzymes including trypsin, pepsin and proteinase K for 30 min at 37 °C, then 80 °C for 10 min to terminate enzyme activity.

2.6. Growth curve assay

S. putrefaciens cells were collected in mid-logarithmic phase, washed thrice with PBS buffer, and suspended at 1×10^6 CFU/Ml in fresh LB broth. 100 μL of resuspended bacteria were incubated in 96-well plates with CF-14 solution at a final concentration of 4MIC, and the same volume of PBS buffer was used as a negative control. OD_{595} was measured every 2 h and up to 12 h at 28 °C.

2.7. Membrane integrity analysis

Membrane permeability of CF-14 was evaluated as described by Chen [17]. *S. putrefaciens* were grown to exponential phase, collected, washed and resuspended in PBS buffer to attain the final density of 1×10^6 CFU/mL. The bacterial suspension was then incubated with CF-14 at a final concentration of 2MIC for either 0, 3, 6, 9, or 12 h, with incubation in the same volume of PBS only as a negative control. After incubation, the mixture was filtered through a 0.22 μm filter to remove bacterial cells followed by measuring the absorbance of each sample at 260 nm (Victor X3, PerkinElmer, USA).

2.8. Hemolytic activity (MHC)

Hemolytic activity was assayed according to previously reported methods [18]. Fresh sheep red blood cells (SRBCs) were washed three times with PBS buffer, centrifugated for 5 min at 8000× *g*, and pellets resuspended (5%) with PBS buffer. 100 μL of CF-14 solution (dissolved and 2-fold serial diluted in PBS) was then added to 100 μL SRBC resuspension. The mixture was then incubated at 28 °C for 30 min, centrifuged at 8000 × *g* for 5 min to remove the cells, and the released hemoglobin quantified by measuring the supernatant absorbance at 595 nm. For absorbance measurements, the positive control was 1% tritionX-100 (A_{triton}) and the negative control was PBS buffer (A_{blank}). The percentage of hemolysis was calculated according to the following equation:

$$\%\text{hemolysis} = 100 \times [(A_{sample}\text{-}A_{blank})/(A_{triton}\text{-}A_{blank})]$$

2.9. Circular dichroism analysis

Circular dichroism (CD) spectra of the CF-14 was recorded using a Chirascan system (Chirascan, Applied Photophysics, UK) to investigate the higher order structure of CF-14. All CF-14 samples were maintained at room temperature during analysis. Each sample containing 0.2 mg/mL of CF-14 dissolved in PBS buffer or in PBS with 2.5% SDS. The

sample was loaded into a quartz test tube with a diameter of 1.0 mm. The bandwidth was set to 1.0 nm and the time-per-point was set to 0.5 s. The baseline was subtracted from the resulting scan map and smoothed with Pro-Data Viewer. Three scans per samples were performed over the wavelength range of 190–260 nm (a far-UV region scan).

2.10. Scanning electron microscopy (SEM)

SEM was performed similarly to previously reported methods to examine disruption of membrane integrity by CF-14 [19]. Bacterial cells were collected in mid-logarithmic phase and centrifuged at 8000 × *g* for 10 min. After washing and resuspending the cells in PBS buffer for a final density of 1×10^6 CFU/mL, cells were incubated with a final concentration of 2 MIC CF-14 at 28 °C for either 0, 3, 6, or 12 h. Melittin, which has well known membrane destabilizing activity, was selected as a positive control [20]. After centrifugation, cells were collected and fixed on glass slides using 2.5% (v/v) glutaraldehyde for 4 h, then washed with PBS buffer for 10 min and dehydrated in an ethanol gradient of 50%, 70%, 80%, 90%, and 100% ethanol. Finally, the samples on the glass slide was sprayed with gold (E − 1045, Hitachi, Japan), and the morphology of treated cells observed by scanning electron microscopy (S-4800, Hitachi, Japan).

2.11. Confocal laser scanning microscopy

The attachment site of CF-14 in bacterial cells was determined using confocal laser scanning microscopy according to the method of Park et al. [21] with slight modification. 1×10^6 CFU/mL of tested cells were incubated with 2 MIC of FITC-labeled CF-14 in PBS buffer at 28 °C for 30 min. After incubation, cells were washed with PBS buffer and added to the slide. Cells were then treated briefly with 0.2% NAPB, and the FITC-labeled CF-14observed by using a confocal microscope (SP6, Leica, German) equipped with a 488 nm bandpass filter and 40 × lens.

2.12. DNA binding assay

In order to examine the DNA binding ability of CF-14, a gel retardation assay was performed [22,23]. Genomic DNA was extracted from *S. putrefaciens* using the Ezup column bacterial genomic DNA extraction kit were purchased from Sangon Biotech (Shanghai, China) and the purity of the extracted genomic DNA was evaluated by the optical density ratio of 260 nm–280 nm of the DNA. Next, 1 μL DNA was mixed with 1 μL of CF-14 for final CF-14 concentrations of 160 MIC, 80 MIC, 40 MIC, 20 MIC, 10 MIC, 5 MIC, and 2.5 MIC. DNA was also mixed with PBS buffer only as a negative control. Following, 2 μL of each mixture was loaded onto a 1% agarose gel in Tris-acetate-EDTA (TAE) buffer containing ethidium bromide for DNA visualization. The gel was run at 80 V for 35 min and DNA identified by the fluorescence of ethidium bromide (Ge1Doc XR +, Bio-Rad, USA).

2.13. Statistical analysis

All assays were carried out in triplicates and average values were calculated. Statistical analyses were performed with SPSS 18.0 statistical software. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Validation of synthetic peptides

CF-14 was synthesized by conventional solid-phase synthesis and purified by HPLC on an ODS column. The molecular weight was 1650.04 Da, which is close to the theoretical value. The purity of CF-14 was 97.58% as determined by RP-HPLC, and the molecular masses of synthesized peptides are shown in Fig. 1. These results confirmed successful synthesis of CF-14.

3.2. Physicochemical property analysis of the CF-14

As shown in Table S1, physical and chemical properties of CF-14 were measured and they appeared close to their theoretical values. These values were similar to those of known cationic antimicrobial peptides involved in host innate immunity, suggesting that CF-14 may be an antibacterial agent [24]. The toxicity of CF-14 was then predicted by ToxinPred, indicating that CF-14 was non-toxic, which is important for the potential use of CF-14 in the food industry [25]. TMHMM posterior probabilities for WEBSEQUENCE are given in Fig. S1. The predictive maps showed that CF-14 had no distinct transmembrane helical region, suggesting that CF-14 does not form a transmembrane channel in the membrane and is not inserted into the bacterial cell membrane. This suggests that the target of CF-14 is in the cytoplasm, not membrane, of bacteria [26]. In addition, the predicted 3D structure of CF-14 with minimum energy was identified (Fig. S2) with a proline located in the middle of CF-14. Proline hinges are a known important structural factor for bacterial cell penetration. For example, Park et al. [27] revealed that when the proline hinge in buforin II was replaced with leucine, the bacteriostatic activity of buforin II was significantly reduced. In addition, Otvos et al. [28] revealed that chaperone-assisted protein folding can be inhibited by proline-rich cationic antimicrobial peptides. Thus these predictions of CF-14 properties suggest that CF-14 is an antimicrobial peptide whose mechanism of action does not involve the bacterial membrane.

3.3. Antimicrobial assays

The antimicrobial activity of CF-14 against *S. putrefaciens* and *S. aureus* were tested by the broth microdilution method. CF-14 showed antibacterial activity against *S. putrefaciens* and *S. aureus* (Fig. 2 A), as well as *Escherichia coli* (data not shown) compared to negative controls. When the concentration of CF-14 was 62.5 μg/mL, the antibacterial effect of CF-14 against *S. putrefaciens* was similar to that of 50 μg/mL of ampicillin (Fig. 2 B). Similar results were also found by Cole et al. [29], who isolated a novel antimicrobial peptide from the skin of *Pleuronectes americanus* and demonstrated the broad-spectrum antibacterial activity of that peptide. Our results are also consistent with those of Haney et al. [30], who found that puroindoline A isolated from wheat endosperm protein had strong antibacterial activity against gram-positive bacteria and gram-negative bacteria. Thus, it was confirmed that CF-14 displays potent activity against gram-negative (*S. putrefaciens*) bacteria and gram-positive bacteria (*S. aureus*). Further, the MIC of CF-14 against *S. putrefaciens* was 62.5 μg/mL.

3.4. Effects of temperature, pH, and proteolytic enzymes

CF-14 exhibits strong antimicrobial activity against *S. putrefaciens*; therefore, we sought to identify factors, such as pH, temperature or proteolytic enzymes, that may influence the effectiveness of CF-14 against *S. putrefaciens*. In addition, many food processing procedures involve heating and/or treatment in an acidic or alkaline environment, so the heat and pH stabilities are very important characteristics for application of CF-14 as a food preservative [31]. As shown in Table S2, the antimicrobial activity of CF-14 showed no obvious change after heat treatment for 30 min at either at 40 °C, 60 °C, or 80 °C, and slight inactivation after treatment at 100 °C for 30 min. CF-14 remained stable after 24 h after exposure to a pH values ranging from 4.0 to 12.0, but the antimicrobial activity of CF-14 decreased after exposure to an acidic environment (pH < 4). Finally, CF-14 was susceptible to proteases as no antimicrobial activity was observed after treatment with trypsin, pepsin, or proteinase K. This may be because the CF-14 sequence is too short and easy to be broken down by proteases.

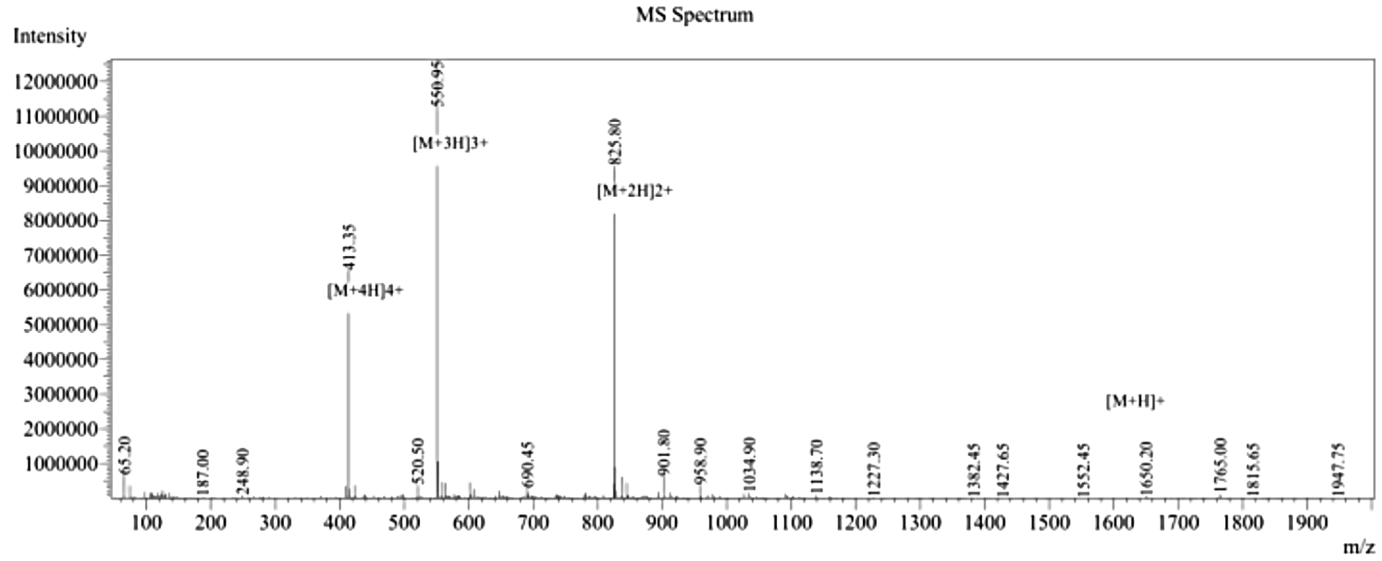
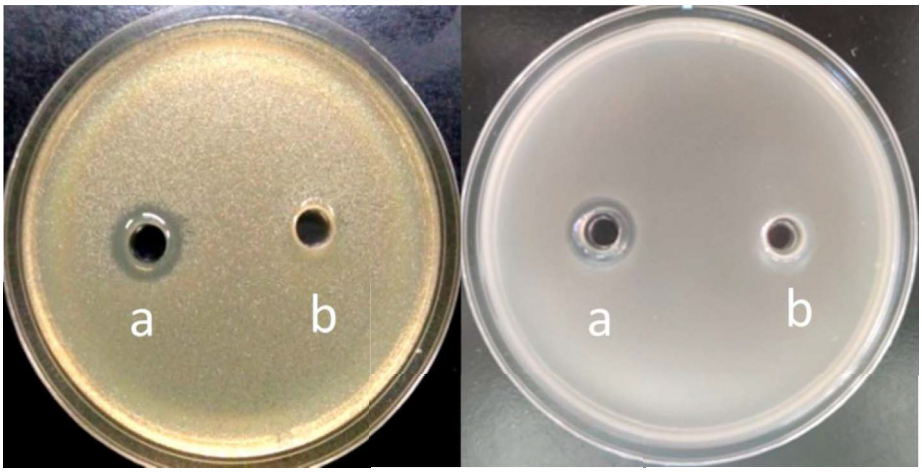


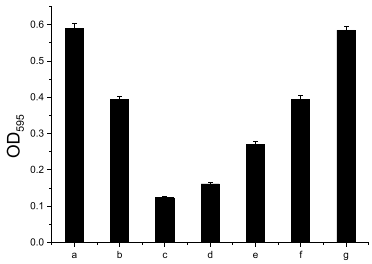
Fig. 1. Mass spectrum of the synthetic peptide CF-14.

3.5. Membrane permeability

The nucleotide leakage of bacterial cells treated with CF-14 was significantly higher compared to the control group (Fig. S3), indicating that CF-14 can cause membrane permeabilization in *S. putrefaciens*. A similar process was observed by Yali et al. [32], who noted that MDpep9 significantly destroys the bacterial cell membrane then enters the cell. Similarly, Li et al. [33] showed that the treatment of *C. albicans* with APP for 30 min led to clear leakage of nucleic acids. Our results thus confirm that CF-14 has the ability to enhance the membrane



(A)



(B)

Fig. 2. (A) The antimicrobial activity test of CF-14 on *S. putrefaciens* and *S. aureus*. (a) 1 mg/mL CF-14; (b) PBS buffer solution.
(B) Minimum inhibitory concentration of CF-14 against *S. putrefaciens*. (a) PBS buffer solution; (b) 50 μg/mL of ampicillin; (c-g) CF-14 concentration was 500, 250, 125, 62.5 and 31.25 μg/mL, respectively.

permeability of *S. putrefaciens*.

3.6. Antibacterial curve

Growth curves of *S. putrefaciens* in the absence or presence of 4 MIC of CF-14 were obtained by OD_{595} measurements and are shown in Fig. S4. *S. putrefaciens* can rapidly grow in the absence of CF-14, with OD_{595} reaching 0.7 within 12 h. However, when 4 MIC of CF-14 was added to the culture, *S. putrefaciens* growth was minimal. The results confirmed that CF-14 treatment can prevent *S. putrefaciens* growth.

3.7. Hemolytic activities

The hemolytic activity of CF-14 was evaluated using sheep red blood cells (Fig. S5). Results revealed hemolysis of less than 1% at 4 MIC, indicating low hemolytic activity of CF-14, and thus confirming its weak toxicity towards mammals. In addition, hemolysis by CF-14 occurred at far above its MIC, further supporting its low toxicity and its potential use as an antimicrobial agent and food preservative. The hemolytic activity of CF-14 follows the same trend as hemolytic activity of other previously identified antimicrobial peptides. For example, the cationic antimicrobial peptide Apep 10isolated by Tang et al. [34] showed no cytotoxic effects against mouse erythrocytes at the observed MIC and Duval et al. [35] showed only slight hemolysis for the antimicrobial peptide K4 when used at 160 μg/mL, above all MICs measured for the K4 peptide. In general, antibacterial peptides are minimally hemolytic and can be used in practical food production.

3.8. CD measurement of CF-14

CD spectroscopy was used to investigate the secondary structure of CF-14 in PBS buffer with 2.5% SDS (a membrane-mimetic environment). As shown in Fig. 3, the CD spectra exhibited low α-helical content in PBS only. However, in PBS with 2.5% SDS, the CD spectra for CF-14 showed double-negative bands at 208 nm and 222 nm, indicating that CF-14 formed an α-helical structure under membrane-mimetic conditions.

These results, including the impact of SDS on structure, are similar to those reported by Li et al. [23], who showed that while the peptide P7 showed a random coil structure in PBS buffer, P7 showed an α-helical structure under membrane-simulating conditions. In addition, Cerovský et al. [36] demonstrated that some of the analogs of PMM were present in an SDS-containing environment, the peptide structure also becomes more α-helical. In addition, it has been shown that having α-helical character in the secondary structure of antimicrobial peptides is key for antimicrobial activity [37].

3.9. SEM analysis

SEM was used to further investigate the antimicrobial effects of CF-14(Fig. 4). *S. putrefaciens* cells were treated with 2 MIC of CF-14 for 3 h, 6 h and 12 h. The cells without CF-14 treatment showed smooth and intact cell surfaces (Fig. 4a and b). After 3 h of treatment, the antimicrobial peptides adsorbed around the bacteria, likely due to electrostatic effects, and no significant changes were observed in the cell membrane structure (Fig. 4c). After treatment with CF-14 for 6 h, increased wrinkling and pore formation was observed on the cell membrane (Fig. 4d). Finally, cells collapsed after 12 h of treatment, indicating that bacterial death (Fig. 4e). In comparison, melittin-treated cells showed tight peptide binding to the cell membrane after 6 h of treatment (Fig. 4g) and complete lysis after 12 h of treatment (Fig. 4h).

These findings suggest that CF-14 caused minor damage to the cell membrane of *S. putrefaciens* and that CF-14 induced pore formation altered morphology of *S. putrefaciens* rather than lysis. Such membrane changes caused by CF-14 were similar to results reported for other antimicrobial peptides. Li et al. [23] showed that although the cell penetrating peptide P7 can form pores in the *E.coli* cell surface, it cannot lyse cells. Pei et al. [38] also found that the antimicrobial peptide plantaricinSLG1 can damage bacterial cell membranes and cause leakage of cytoplasmic components. Our results suggest CF-14 may be not a membrane-acting peptide, in contrast to other peptides such as melittin.

3.10. Internalization of FITC-labeled CF-14 in S. putrefaciens cells

To investigate CF-14 localization in *S. putrefaciens*, cells were incubated with 2 MIC FITC-labeled CF-14 for 30 min then FITC-labelled CF-14 localization in bacterial cells examined by confocal laser scanning microscopy. As shown in Fig. 5, FITC-labeled CF-14 was located at the cell membrane, implying that CF-14 can penetrate the cell

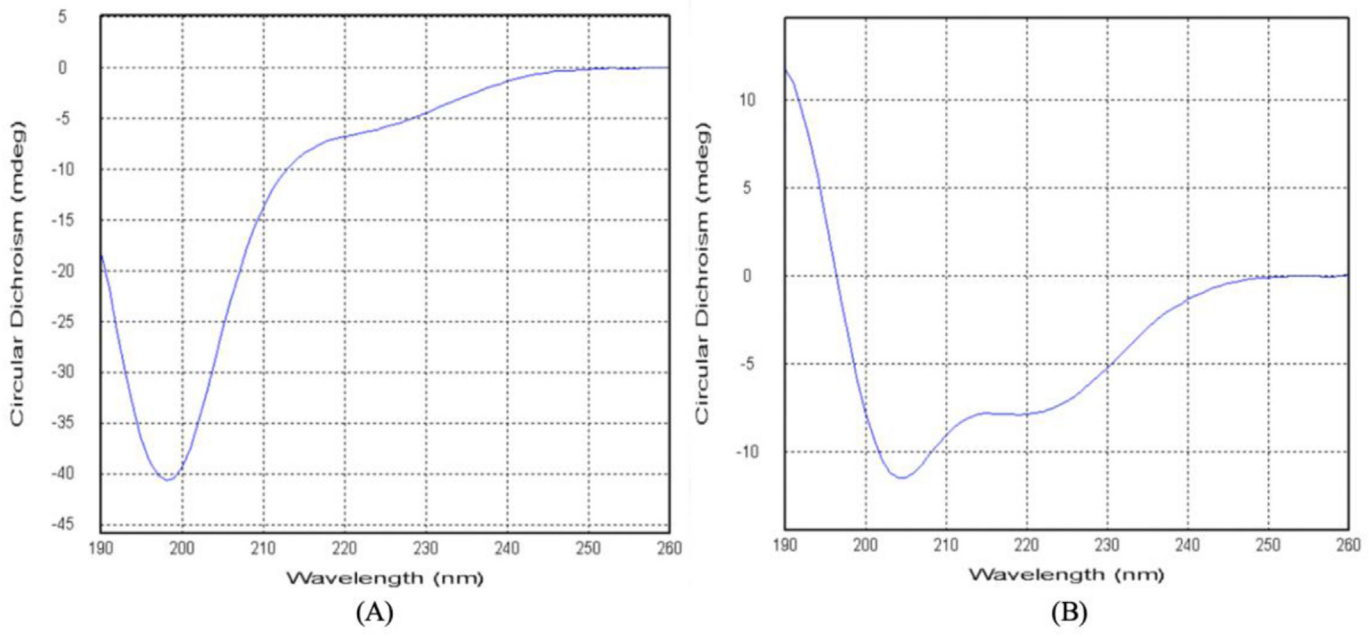


Fig. 3. CD spectra of the CF-14 (0.2 mg/ml) were measured in PBS buffer (A) and in 2.5% SDS buffer (B).

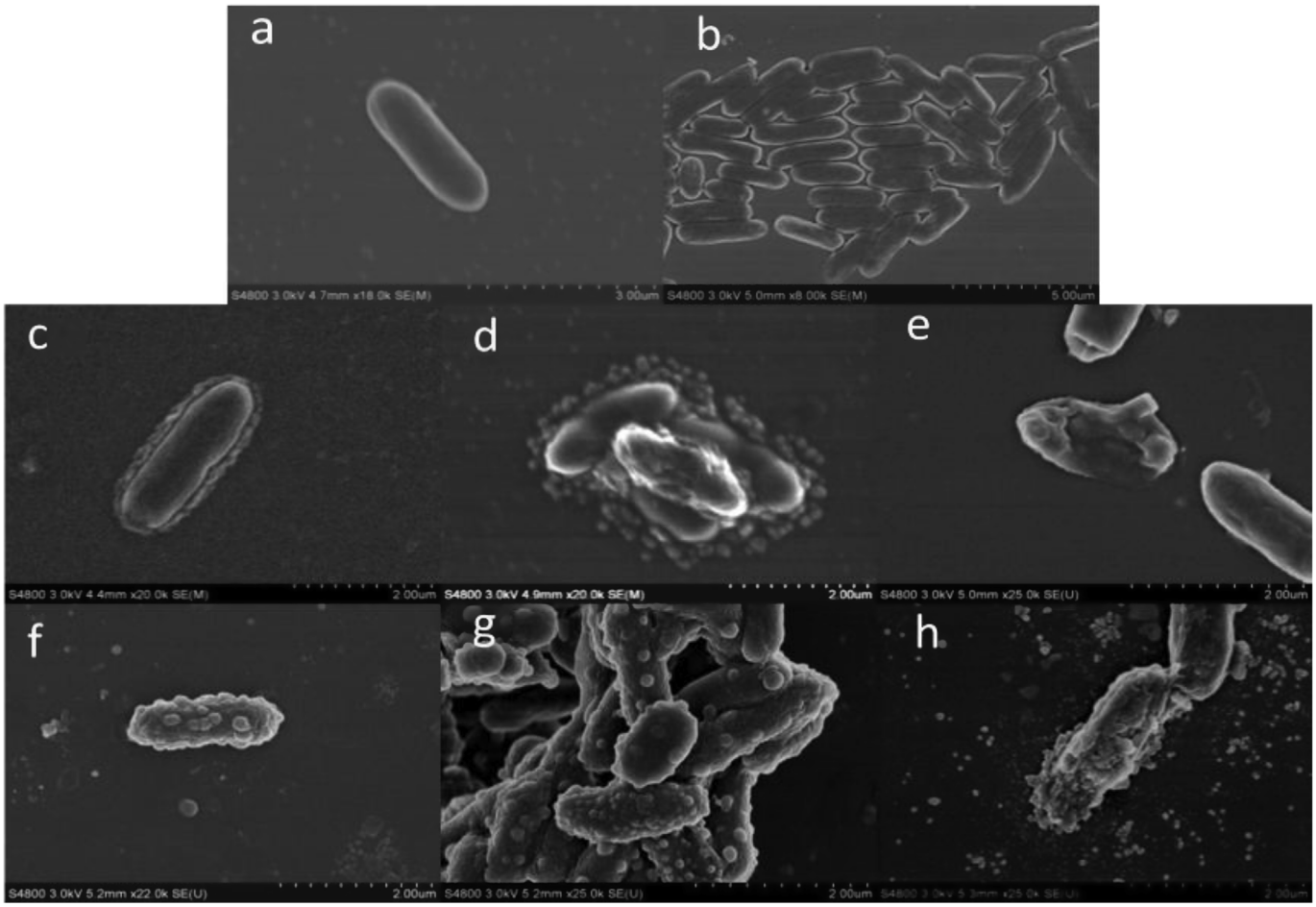


Fig. 4. Scanning electron microscope photographs of *S. putrefaciens*. Scanning electron microscope photographs of *S. putrefaciens* treated by CF-14.Morphology changes of *S. putrefaciens* cells treated by CF-14 (2 MIC). (a-b) Control; (c-e) CF-14 treatment for 3 h, 6 h and 12 h; (f-h) Melittin treatment for 3 h, 6 h and 12 h.

membranes of *S. putrefaciens*. After treatment with FITC-labeled CF-14 for 60 min, the cells were filled with CF-14, although cells showed no changes in membrane morphology even after 3 h of treatment (Fig. 4c).

Previous studies have shown that in addition to affecting cell membrane morphology, antimicrobial peptides often have binding sites within cells [39]. For example, Malanovic & Lohner [40] indicated that some antimicrobial peptides inhibit protein synthesis and reducing the activity of the enzyme in the bacterial cell. Li et al. [33] examined *C. albicans* incubated with FITC-APP for 30 min by using confocal laser scanning microscopy and showed that FITC-APP penetrated the cell membrane and accumulated in the cytoplasm.

3.11. DNA-binding activity

Previous studies have shown that antimicrobial peptides may bind to genomic DNA in cells [23]. Therefore, we indirectly measured the DNA binding by the CF-14 by monitoring the effect of increasing CF-14 concentration on the migration of *S. putrefaciens* DNA in a 1% agarose gel (Fig. 6). DNA without treatment showed a bright band in the gel electrophoresis (Fig. 6a) while a CF-14 concentration of 10–160 MIC caused CF-14 to bind strongly with DNA (Fig. 6b–f), slowing the DNA migration rate and forming a long tail in the gel. In addition, very high concentrations of CF-14 prevented any DNA migration through the gel.

These results are similar to those observed for buforinII and APP, which kill bacteria by penetrating cells and binding to DNA [27,33]. Our results indicate that CF-14 also binds to DNA and that the binding degree of the antimicrobial peptide to DNA increases with the increasing CF-14.

4. Conclusions

In summary, we characterized a novel 14-residue antimicrobial peptide called CF-14 encoded by the sequence RIVELTLPRVSVRL-NH_2. Synthetic CF-14 showed a wide range of antibacterial activities and little toxicity to red blood cells. In particular, our study showed that the CF-14 is a promising novel antibiotic candidate and has a significant inhibitory effect on *S. putrefaciens*, which is a specific spoilage organism during aerobic refrigeration of aquatic products and a key factor in determining the quality of aquatic products during storage and processing. CF-14 showed good bioactivity over a wide range of temperatures and pH values. In terms of mechanism of action, the interaction between CF-14 and the bacterial cell membrane appears to the first step while the primary target of CF-14 may be and intracellular components such as DNA, rather than the bacterial membrane itself. Furthermore, electrophoretic analysis indicated that CF-14 possessed DNA binding affinity. Such properties make CF-14 very promising for applications such as seafood preservation in the food industry. However, further studies will be needed to determine the exact antimicrobial mechanism of CF-14.

Acknowledgments

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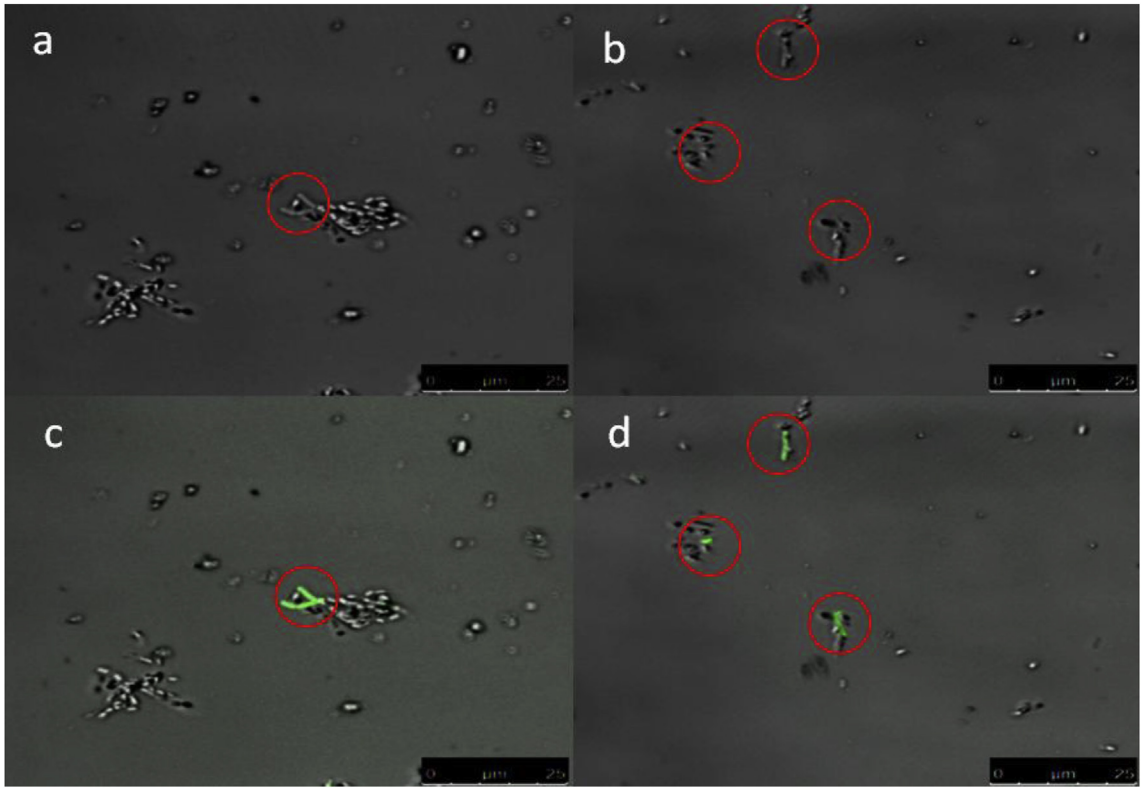

Fig. 5. Confocal laser-scaning microscopy bright field and fluorescence image merged image of localization of FITC-labeled peptides in *S. putrefaciens*. *S. putrefaciens* cells were treated with biotinylated peptides (2MIC) at 28 °C for 30min and visualized with streptavidin-FITC. (a-b) Bright field; (c-d) Fluorescence image merged image. Bars represent 25 μm.

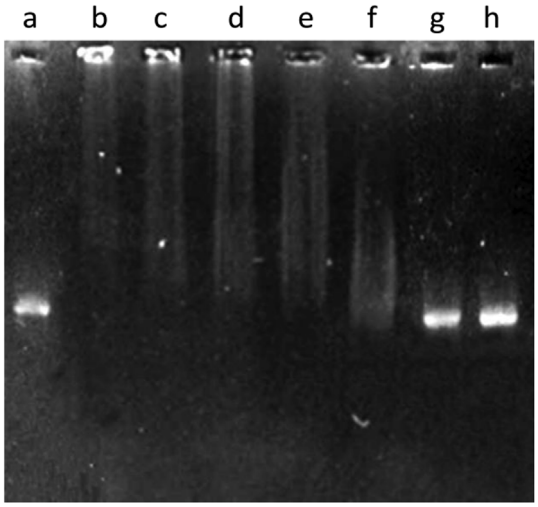

Fig. 6. Gel retardation assay of DNA-peptide binding showed the result of the gel retardation analysis. (a) Control, (b-h) CF-14 concentration was 160 MIC, 80 MIC, 40 MIC, 20 MIC, 10 MIC, 5MIC, 2.5 MIC.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.07.015.

Declarations of conflicts of interest

None.

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