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Inhibitory effects of a novel antimicrobial peptide from kefir against *Escherichia coli*

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ABSTRACT

Antimicrobial peptide F1, a novel antimicrobial peptide from Tibetan kefir, have shown strong antimicrobial activity against several bacteria and fungi. We identified the amino acid sequence and studied the antimicrobial mechanism of peptide F1 against *Escherichia coli*. Our results showed that antimicrobial peptide F1 contained 18 amino acids (Thr-DAP-Asn-Thr-PEA-His-Pro-Asn-Thr-His-Leu-Ile- PEA-CySH-Val-Asn-PEA-Tau), which increased the outer and inner membrane permeability of *E. coli*, and the leakage of the cytoplasmic β-galactosidase and potassium ions was detected in the process. Morphologies of *E. coli* were observed by confocal laser scanning microscopy and transmission electron microscopy, which visually showed that antimicrobial peptide F1 could penetrate and accumulate into cell causing disruption of cell membrane functions. Furthermore, we elucidated the DNA binding ability of antimicrobial peptide F1 by agarose gel retardation and atomic force microscopy. Our findings indicated that antimicrobial peptide F1 has multiple targets in the killing of *E. coli*.

1. Introduction

Food safety is an important worldwide issue for both food industry and public health. The growing concern on the use of synthetic preservatives, the discovery of foodborne pathogens with resistance to classical antimicrobial agents (Teuber, 1999), and the increased consumer demand for natural and minimally processed foods have created many technological challenges in the food industry. The novel bio-preservatives, such as bacteriocins, have been considered as one of the most promising candidates to combat these obstacles (Cleveland, Montville, Nes, & Chikindas, 2001).

Bacteriocins are a group of antimicrobial peptides that are produced by a variety of bacteria, and they can kill and/or inhibit the growth of other bacteria (Bendjeddou, Fons, Strocker, & Sadoun, 2012; Gálvez, Abriouel, López, & Omar, 2007; Martinez et al., 2015; Milioni et al., 2015; Sonsa-Ard, Rodtong, Chikindas, & Yongsawatdigul, 2015). In addition, bacteriocins usually possess a strong and broad-spectrum of antimicrobial activity and microorganismal eradication with minimum resistance development. The bacteriocins have been isolated from a variety of bacteria that predominantly consist of lactic acid bacteria (Anastasiadou, Papagianni, Filiousis, Ambrosiadis, & Koidis, 2008; Kruger et al., 2013; Riley & Wertz, 2002). Nisin was the first bacteriocin approved for food use, which was produced by *Lactococcuslactis* subsp. *lactis* and has been used in more than 40 countries for over 50 years.

In our previous research, we found that antimicrobial peptide F1, a novel bacteriocin produced by *Lactobacillus paracasei* subsp. *Tolerans* FX-6 isolated from Tibetan kefir, has a wide antimicrobial spectrum and stability to heat, pH and protease (Miao et al., 2014). Elucidating the mechanism of antimicrobial peptide F1 is particularly important for enhancing its application in food preservation. In our current study, we chose *Escherichia coli* ATCC 25922, a major gram-negative foodborne pathogen, as a target strain to determine the antimicrobial mechanism of antimicrobial peptide F1.

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2. Materials and methods

2.1. Materials

E. coli ATCC 25922 was stored in the microbial culture laboratory in South China Agricultural University, College of Food Science (Guangzhou, China). *E. coli* was cultured in Luria–Bertani broth, and the overnight *E. coli* cultures (10^8 CFU/mL) were used in subsequent experiments. Fluorescein isothiocyanate (FITC), o-nitrophenyl-β-D-galactopyranoside (ONPG) and erythromycin were all purchased from Sigma–Aldrich (Shanghai, China).

2.2. Purification and identification peptide

Antimicrobial peptide F1 was purified from the cell-free supernatant of 72-h fermented pure milk by *L. paracasei* subsp. *tolerans* FX-6 according to a three-step purification procedure. First, cell-free supernatant was separated by a reversed-phase (RP) C18 column (20 mm × 450 mm, 10 μm, Macherey Nagel, France). Then, the active fraction was further purified by a RP Shim-pack PRC-ODS(K) column (30 mm × 250 mm, 15 μm, Shimadzu). Lastly, the antimicrobial peptide F1 was obtained by a RP ECOSIL C18 column (4.6 mm × 250 mm, 5 μm, Lubex, Japan). *E. coli* was used as an indicator strain during the process of purification. The full purification method of antimicrobial peptide F1 was explained in our previous paper (Miao et al., 2014).

The molecular mass of antimicrobial peptide F1 was determined in our previous work using an ABI 4800 MALDI-TOF-MS (Shanghai Applied Protein Technology Co. Ltd, Shanghai, China) (Miao et al., 2014). The amino acid sequence was performed on an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Electron, Bremen, Germany) equipped with an ESI ion source and coupled to a Surveyor solvent delivery pump and a Surveyor autosampler (College of Veterinary Medicine, South China Agricultural University, China).

2.3. Growth curve assay

After overnight incubation at 37 °C in Luria–Bertani broth, the culture of *E. coli* was diluted to an optical density (OD) of 0.2 at 600 nm and 50 mL of dilution was placed in a 100 mL conical flask. Antimicrobial peptide F1 was added to a final concentration of 1 MIC. The culture without antimicrobial peptide F1 was used as negative control. The cultures were grown for 20 h and the OD at 600 nm was recorded at different time intervals (0, 1, 2, 3, 4, 6, 8, 10, 12, 14, 16, 18 and 20 h). All the testing points were measured in triplicate.

2.4. Outer membrane permeabilization assay

Erythromycin has a weak effect on gram-negative bacteria and penetrates poorly with the intact enterobacterial outer membrane, but it can easily traverse the damaged outer membrane (Vaara & Porro, 1996). The efficacy of antimicrobial peptide F1 in enhancing the outer membrane permeability was determined in *E. coli* with four experimental groups. *E. coli* (10^8 CFU/mL) in all groups was incubated in Luria–Bertani broth. Before the test, erythromycin or antibacterial peptide F1 were dissolved in sterile water. In the first group, antimicrobial peptide F1 (the final concentration 0.5 MIC, namely 31.25 μg/mL) was added with different concentrations of erythromycin (the final concentration 0.4 μg/mL, 0.6 μg/mL, 0.8 μg/mL, 1 μg/mL, and 2 μg/mL, respectively). In the second group, the erythromycin in different concentrations (the final concentration 0.4 μg/mL, 0.6 μg/mL, 0.8 μg/mL, 1 μg/mL, and 2 μg/mL, respectively) was added. In the third group, antimicrobial peptide F1 (the final concentration 0.5 MIC, namely 31.25 μg/mL) was added. The fourth group was the negative control group, without erythromycin and antimicrobial peptide F1 being added, and the same volume of sterile water was added instead. All the groups were incubated at 37 °C for 10 h. The bacterial growth was measured by a microplate reader (Multiskan MK3, Thermo, USA) at 630 nm. All assays were carried out in triplicate.

2.5. Inner membrane permeabilization assay

Inner membrane permeabilization assay was performed by measuring the release of cytoplasmic β-galactosidase activity from *E. coli* in a culture medium using ONPG as the substrate. It was determined by the previous methods with slight modifications (Marri, Dallai, & Marchini, 1996; Tsuji et al., 2001). After overnight incubation at 37 °C in Luria–Bertani broth, *E. coli* cultures were centrifuged at 3000 × g, and the harvested cells were induced in M9 lactose medium (1.28 g Na_2HPO_4, 0.3 g KH_2PO_4, 0.05 g NaCl, 0.1 g NH_4Cl, 0.05 g $MgSO_4$, 0.001 g $CaCl_2$, 0.5 g lactose and dissolved in 100 mL double-distilled water) at 37 °C for 8 h. Then the cells were centrifuged at 3000 × g for 1 min. The centrifuged cells were washed twice in sterile saline and resuspended to an optical density (OD) of 0.2 at 600 nm by the assay buffer (0.8 g NaCl, 0.02 g KCl, 0.29 g Na_2HPO_4, 0.024 g KH_2PO_4, 0.025 g $MgSO_4$, 0.39 g β-mercaptoethanol and dissolved in 100 mL double-distilled water). In addition, ONPG and antimicrobial peptide F1 were added to a final concentration of 0.1 mg/L and 1 MIC (62.5 μg/mL), respectively. The resuspended cell solution was incubated at 37 °C. The production of o-nitrophenol over time was measured using a microplate reader (Multiskan MK3, Thermo, USA) at 420 nm.

2.6. Potassium ion release assay

The cell membrane integrity was assessed by measuring potassium ions released from *E. coli* treated with antimicrobial peptide F1. The exponential phase *E. coli* cells in Luria–Bertani broth were centrifuged, washed and resuspended in 0.9% sterile saline (10^8 CFU/mL). The cell suspensions were incubated with antimicrobial peptide F1 (final concentration 1 MIC) at 37 °C. At multiple time intervals (30, 60, 90, 120 and 150 min) the cell suspensions were centrifuged at 10,000 rpm for 10 min then the supernatants were measured using atomic absorption spectrometer (S7-AA-7000, Shimadzu, Japan) (Hao, Shi, Tang, & Le, 2009). A control was carried out with 0.9% sterile saline. All assays were carried out in triplicate.

2.7. Confocal laser scanning microscopy

Antimicrobial peptide F1 was labeled with fluorescein isothiocyanate (FITC) as previously reported with some modifications (Helmerhorst et al., 1999). In summary, 1 mg/mL of FITC dissolved by Me_2SO was added to 2 mg/mL of antimicrobial peptide F1 in 50 mM potassium phosphate buffer (final pH 7.6), and the final concentration of FITC was 25 mg/mL. The mixture of FITC and antimicrobial peptide F1 was incubated for 16 h in the dark at 4 °C. Then 50 mM NH_4Cl was added to inactivate the residual FITC. The mixed solution was placed in a dialysis bag with a molecular mass cutoff of 500 Da and dialyzed for 24 h in 0.01 mol/L phosphate buffer. The FITC-conjugated antimicrobial peptide F1 was finally obtained by freeze-drying the mixed solution in the dialysis bag. The exponential phase *E. coli* cell suspension (10^8 CFU/mL) was mixed with a 2 mg/mL stock solution of FITC-conjugated antimicrobial peptide F1 to a final concentration equal to 2MIC (125 μg/mL). Samples were kept in the dark for 1 h at 37 °C. At multiple time intervals (10, 30, and 60 min) the cells were washed with the PBS buffer three times and observed using an LSM 710 Zeiss confocal laser-scanning microscope (Zeiss, Germany).