Characterization of a novel antimicrobial peptide from buffalo casein hydrolysate based on live bacteria adsorption

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ABSTRACT

This study aims to isolate the antimicrobial peptide (AMP) from buffalo casein hydrolyzed by *Dregea sinensis* Hemsl. protease. The AMP was isolated from hydrolysate by live bacteria adsorption, then analyzed using reversed-phase high-performance liquid chromatography, and the fraction with highest antimicrobial activity was identified by liquid chromatography-tandem MS. Further, we characterized the peptide in terms of its peptide sequence, structure, and antimicrobial activity. The results identified the AA sequence of the peptide as YLGYLEQLRLK, which corresponds to residues 106 to 117 of bovine α_{S1} -casein, and we named it BCp12. BCp12 displays α -helical structure, with high hydrophobic moments and net positive charge. BCp12 can inhibit the growth of indicator bacteria, with minimum inhibitory concentration values ranging from 0.8 to 1.6 mg/mL, and can induce low toxicity in mammalian cells. Antimicrobial activity of the BCp12 peptide remained stable under different salt concentrations but was sensitive to trypsin and high temperatures (121°C and above). The results support further research in the application of our newly generated AMP as an antimicrobial agent in the food industry and in food processing facilities.

Key words: buffalo casein, antimicrobial peptide, adsorption, safety, α -helical structure

INTRODUCTION

Antimicrobial resistance is a serious public health threat that results from the selective pressure exerted by antibiotic use and abuse (Farhana et al., 2019). To reduce antimicrobial resistance, alternatives to antibiotics are urgently necessary, particularly in the food industry (Ma et al., 2019). In recent years, researchers have searched for natural sources of antimicrobial agents, including peptides, oligosaccharides, and plant

extracts (Gao et al., 2019). Antimicrobial peptides (AMP) have antiseptic activity and do not develop resistance, so can be used as an alternative to antibiotics (Ciurac et al., 2019).

In higher eukaryotes, AMP are components of the innate immune system to fight pathogens such as viruses, bacteria, fungi, and even cancer cells (Pichu et al., 2009; Peters et al., 2010). By 2017, more than 10,585 AMP have been found and used (Porto et al., 2017). Although synthetic AMP have been generated, natural AMP occur in both prokaryotes and eukaryotes and can be extracted from food proteins by enzymatic hydrolysis (Zasloff, 2002; Pellegrini, 2003). Antimicrobial peptides penetrate the outer membranes of gram-negative bacteria and the cell walls of gram-positive bacteria, creating holes that lead to leakage of intracellular contents (López-Expósito and Recio, 2008). Most AMP have helical, amphipathic, and hydrophobic structures, with positive charge (Bougherra et al., 2017). In general, it is suggested that peptide drugs exhibit lower toxicity and higher specificity and are more easily synthesized and modified than nonpeptide drugs; thus they have great potential for control of bacterial contamination (Lombardi et al., 2019).

Milk proteins are currently the main source of a range of biologically active components that exhibit various physiological activities (Meisel, 1998; Haque et al., 2008). Milk caseins are an important source of AMP, and most AMP are derived from food proteins in milk (Florisa et al., 2003; Sibel Akalin, 2014). Some peptides from milk caseins demonstrating antimicrobial activity, reported in the literature, are released by enzymatic hydrolysis of plant sources such as protease extracted from the latex of *Jacaratia corumbensis* (Arruda et al., 2012) and papain (Abdel-Hamid et al., 2016). New protease-mediated hydrolysis of casein may release new AMP (Arruda et al., 2012). The extract from the stem of *Dregea sinensis* Hemsl. has long been used in making Rubing cheese in Dali, Yunnan Province, China. A cysteine protease with good performance in conversion of milk to curd was identified from *Dregea sinensis* Hemsl. The protease has a molecular weight of 23.803 kDa; its optimal temperature of milk clotting was 80°C, and it

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was stable in the pH range 6.0 to 9.0. It has been used to make cheese and release bioactive peptides from goat milk. (Zhang et al., 2015; Wang et al., 2017; Zhao et al., 2018). Buffalo milk produced in Tengchong, Yunnan Province, China, a regional milk, has been found to be different from that of Holstein cows (Zhang et al., 2012). However, until now no information has been published about the AMP from *Dregea sinensis* Hemsl. protease hydrolyzed from buffalo casein (BC).

Herein we describe the purification and identification of an AMP formed by the hydrolysis of casein from buffalo milk by a self-purified *Dregea sinensis* Hemsl. protease using live bacteria adsorption. Moreover, to better apply the purified peptide in the field of food preservation, its antimicrobial activity, structure, and safety were also studied.

MATERIALS AND METHODS

Materials

Fresh buffalo milk was purchased in Tengchong, Yunnan Province, China. Acetonitrile of gradient grade for liquid chromatography was purchased from Merck KGaA (Darmstadt, Germany). All other chemicals and reagents were of analytical grade and commercially available.

Microbial Strains and Culture

Escherichia coli CICC 10003, *Staphylococcus aureus* CICC 10384, *Listeria monocytogenes* CMCC 54004, *Salmonella typhimurium* CICC 21484 were purchased from the China Center of Industrial Culture Collection (Beijing). *Listeria monocytogenes* CMCC 54004 was obtained from the College of Food Science and Technology, Yunnan Agricultural University (Kunming, Yunnan Province, China). Bacterial strains were conserved at -80°C in glycerol containing nutrient broth and were subcultured twice in Luria-Bertani broth medium under agitation (100 rpm) at 37°C for 24 h before use. The initial concentration of cells was adjusted to 10^6 cfu/mL by dilution in PBS (pH 7.4, containing 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4 , and 1.8 mM KH_2PO_4 , without calcium and magnesium) for subsequent experiments.

Human skin epithelial cells (HaCaT) were provided by the Moringa Institute of Yunnan Agricultural University. Cells were stored with 90% fetal bovine serum and 10% dimethyl sulfoxide at -80°C and cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT) and 1% penicillin streptomycin antibiotic mixture (Gibco, Waltham, MA). All cell lines were cultured at

37°C in a humidified atmosphere at 5% CO_2 and 95% air.

Enzyme Extraction

Dregea sinensis Hemsl. stems (60 g) were cut into small pieces (5 to 10 cm) and mixed with 600 mL of 50 mM phosphate buffer (pH 6.5) containing 2.5% NaCl for 12 h at 4°C . The protease was partially purified by ammonium sulfate precipitation (25–35%). This protease was lyophilized after dialysis. The purity of protease was determined by SDS-PAGE that showed a band. Its activity was 314.378 U/mg.

Preparation of Casein from Buffalo Milk

Skim milk was prepared from whole buffalo milk by centrifugation at $4,000 \times g$ for 20 min at 4°C . Casein from skim buffalo milk was prepared based on a previously described method (Mulvihill, 1992), with slight modification. Skim milk was acidified to 4.6 with citric acid (0.2 M) under continuous stirring at room temperature. After curd degradation for 20 min, the mixture was centrifuged at $4,000 \times g$ for 20 min at 4°C . The precipitated casein was washed 3 times with distilled water. The final precipitates were adjusted to pH 7.0 with NaOH (1 M) and then heated at 80°C for 30 min to inactivate the plasmin, dialyzed against distilled water, and freeze-dried.

Preparation of BC Hydrolysates

Buffalo casein was dissolved in Tris-HCl buffer (50 mM, pH 8.5) at a concentration of 20 mg/mL. *Dregea sinensis* Hemsl. protease was pre-incubated for 10 min and mixed with BC at an enzyme-to-substrate ratio of 1:45 (wt/wt). After 4.5 h of hydrolysis at 54°C , the reaction was stopped by boiling for 15 min and cooling to room temperature in an ice bath. The BC hydrolysate was centrifuged at $10,000 \times g$ for 20 min at 4°C (TLG20M, Changsha Maijiasen Instrument Equipment Co. Ltd., Changsha, China) to remove insoluble material, and the supernatant was adjusted to pH 7.0 with NaOH (1 M), microfiltered (0.22 μm , Millipore, Billerica, MA), and stored at -20°C for further analysis.

Fractionation of BC Hydrolysates by Ultrafiltration

The hydrolysate was fractionated with ultrafiltration membranes with molecular mass cutoff sizes of 10 and 1 kDa. A stirred UF device (UFSC40001, Millipore) with membranes of 76-mm diameter (NMWL, PES, Millipore) was used. The permeate and retentate of each stage of filtration were collected and lyophilized. Frac-

tionation stages of BC hydrolysates by ultrafiltration (BCHU) were marked as follows: BCHU1 (>10 kDa), BCHU2 (<10 kDa), BCHU3 (>1 kDa, <10 kDa), and BCHU4 (<1 kDa). The different fractionations were dissolved in distilled water and filtered using a 0.22- μ m filter. The inhibition rates of *E. coli* and *Staph. aureus* were determined, to select the fractionations with highest inhibition rates.

Separation of AMP by Live *E. coli* Adsorption (BCHU4-LE)

The AMP were screened using the method of Tang et al. (2009), with slight modification. Fifteen mL of cultured *E. coli* were washed 3 times with 0.9% NaCl, followed by centrifugation at $10,000 \times g$ for 10 min to remove culture medium and metabolites. Ten milliliters of BCHU4 (20 mg/mL) were incubated with live *E. coli* at 37°C for 4 h, and the mixed liquid was centrifuged at $10,000 \times g$ for 10 min. The supernatant of BCHU4 after affinity adsorption by live *E. coli* (BCHU4-LE), without AMP, was microfiltered (0.22 μ m) for reversed-phase (RP)-HPLC analysis. The RP-HPLC fingerprint chromatograms of BCHU4 and BCHU4-LE were compared to find the different peaks. The fraction with strongest antimicrobial activity was identified via liquid chromatography-tandem MS (LC-MS/MS).

RP-HPLC Fingerprint Chromatogram Analysis of BCHU4 and BCHU4-LE

The fingerprint chromatograms of BCHU4 and BCHU4-LE were determined via RP-HPLC (Agilent 1200, Agilent Technologies, Santa Clara, CA) using a C18 column (Agilent XDB-C18; 250 $\times$ 4.6 mm, 5 μ m). The 2 solvent reservoirs contained the following eluents: (A) 0.05% (vol/vol) trifluoroacetic acid (TFA) and (B) 100% acetonitrile. Linear gradients were conducted from 5% to 80% solvent B over solvent A in 30 min with a flow rate of 0.6 mL/min. Elution was monitored by ultraviolet absorbance at 215 nm. Peaks showing reduced peak area were collected, concentrated, and dialyzed against distilled water (with a molecular weight cutoff of 100 Da). Their antimicrobial activity was measured to select the most active fraction.

Peptide Identification via LC-MS/MS

The fraction obtained by RP-HPLC with the highest antimicrobial activity was processed for peptide identification. The LC-MS/MS analysis was performed according to Otte et al. (2007) and Shazly et al. (2017). The sample was dissolved in Nano-HPLC Buffer A and separated by Nano-HPLC (EASY-nLC1200; Thermo

Fisher Scientific, Waltham, MA) liquid phase system. Mobile phase A consisted of 0.1% formic acid in water; phase B consisted of 0.1% formic acid in acetonitrile. The trap column (RP-C18, 100 m $\times$ 20 mm, Thermo Fisher) was balanced with 100% liquid A. Samples were loaded by automatic sampler and adsorbed to the trap column and then separated by analysis column (RP-C18, 75 m $\times$ 150mm, Thermo Fisher) at a flow rate of 300 nL/min. Cleaning between the samples was performed with a blank solvent gradient for 30 min. The samples were separated by capillary HPLC and then analyzed by Q-Exactive MS (Thermo Fisher), which was calibrated with standard correction fluid before use. Analysis time was 60 min, positive ion detection method, spray voltage of 2.5 kV. Ion transport capillary temperature was 320°C, with mother ion scanning in the range of 300–1,600 m/z . Mass spectrometry scanning was performed in the information-dependent analysis (IDA) mode, collecting the 10-piece map (MS2 scan) after each full scan. High-energy collision dissociation (HCD) fracture mode was used, with normalized collision energy (NCE) of 28, dynamic elimination time 30 s. The first mass spectrum (MS¹) has a resolution of 70,000 at 200 m/z , and the second mass spectrum (MS²) has a resolution of 35,000 at 200 m/z . Profile mode was used to collect MS¹, and MS² was collected in centroid mode, to reduce the size of data files. Mass spectra of peptides were exported and compared with the UniProt *Bos taurus* database (<https://www.uniprot.org/proteomes/UP000009136>), using Discoverer 2.3 software (Thermo Fisher). Fragment mass tolerance was ± 10 ppm and 0.02 Da with phosphorylation as dynamic modification; otherwise, default settings were used.

Peptide Synthesis

The identified peptides were chemically synthesized by Guoping Pharmaceutical (Anhui, China) based on AA sequences. The purity of the synthetic peptide was above 98% by HPLC analysis, and its molecular mass was confirmed by MS. Antimicrobial activities of the synthetic peptides will also be determined.

Character Determination of AMP

The hydrophobicity and helical hydrophobic moment of AMP were determined in the AVPdb Database (<http://crdd.osdd.net/servers/avpdb/>), and the AMP Database (APD3: Antimicrobial Peptide Calculator and Predictor, http://aps.unmc.edu/AP/prediction/prediction_main.php). Helical wheel projections were generated with the wheel.pl script via web server (<http://www.bioinformatics.nl/cgi-bin/emboss/pepwheel>).

To investigate the secondary structure under different environments, 0.2 mg/mL peptide solutions were prepared in 10 mM sodium phosphate buffer (pH 7.4), 30 mM SDS (Sigma-Aldrich, St. Louis, MO), or 50% 2,2,2-trifluoroethanol (TFE; Sigma-Aldrich). Circular dichroism (CD) assays were then performed using a JASCO 810 spectropolarimeter (Jasco, Tokyo, Japan). The CD spectra were recorded from 195 nm to 250 nm using a quartz cell with a 1.0-mm path length.

Antimicrobial Activity Assay

Agar Disk Diffusion Assay. The antimicrobial activity of the purified peptide against 4 bacteria strains was tested by the agar disk diffusion method of Fang et al. (2019). Briefly, 150 μ L of bacteria ($\times 10^6$ cfu/mL) were uniformly plated on a Luria-Bertani medium plate. After 20 min, sterile paper disks (6 mm) were placed on the seeded agar plates. We added 8 μ L of synthetic peptide (dissolved in sterile water, pH 6.8) to the sterile paper disks and incubated in a closed humidified container for 3 h at 4°C, for the sample to diffuse into the gel. Tetracycline was used as a positive control for antimicrobial activity, and sterile water served as a negative control. Finally, the plates were incubated at 37°C for 12 h, and the diameter of the bacterial growth inhibition zone was measured.

Minimum Inhibitory Concentration. The MIC is defined as the lowest concentration of an AMP that inhibits the growth of a microorganism following incubation at 37°C for 12 h, as measured at an absorbance of 630 nm (Multiskan MK3, Thermo Fisher). The MIC of the purified peptide was measured in sterile 96-well microplates (costar 3599, Corning Inc., Ewloe, UK) using a modified version of the method of Kobbi et al. (2015). Each well included 190 μ L of fresh Luria-Bertani medium, 50 μ L of peptide solution, and 10 μ L of bacteria ($\times 10^6$ cfu/mL). Sterile water was used as control. The inhibition rate was calculated according to the following equation:

$$\text{Inhibition rate (\%)} = [(A_0 - A_1)/A_0] \times 100,$$

where A_0 and A_1 represent the optical density (OD) values at 630 nm of the control and the sample, respectively.

Stability Assays

The stability of the purified peptide was determined as previously described (Tang et al., 2015). The antimicrobial activity of the purified peptide under different

conditions (heating, salt, and trypsin) was evaluated by measuring OD₆₃₀ of *Staph. aureus* as an indicator bacteria, following the same protocol as for MIC assays. The effect of salt on the recovered antimicrobial activity of the purified peptide by treating *Staph. aureus* with the purified peptide, dissolved in sterile water at a final concentration 0.80 mg/mL (according to the MIC), in the presence of NaCl, KCl, MgCl₂, or CaCl₂ (initial concentrations of 5%, 10%, and 15%, wt/vol). To determine the effect of temperature on the recovered antimicrobial activity of the purified peptide against *Staph. aureus*, the AMP was incubated at different temperatures between 70°C and 121°C for 30 min. To test the effect of trypsin on the antimicrobial activity of the purified peptide against *Staph. aureus*, the purified peptide was incubated with 0.2 mg/mL of trypsin at 37°C for 0 h, 1 h, and 2 h. Sterile water was used as blank, and nontreated peptide served as control. After treatment, the recovered activity of the purified peptide was evaluated as previously described. Recovered activities are calculated as

$$(U) = (A_0 - A_1)/(A_0 - A_2) \times 100,$$

where U is recovered activity and A_0 , A_1 , and A_2 are OD₆₃₀ of the blank, the sample, and the control, respectively.

Hemolysis and Cytotoxicity Assay

The hemolytic activity of the purified peptide was determined as previously described (Ji et al., 2014), with slight modification. Fresh rabbit red blood cells were washed and diluted to 4% with PBS (pH 7.4). The red blood cell suspension was added to 96-well plates (100 μ L/well), mixed with the same volume of serially diluted peptide (4 mg/mL, 2 mg/mL, 1 mg/mL, 0.5 mg/mL), and incubated at 37°C for 1 h with shaking (200 rpm). The plate was centrifuged, and the supernatant was transferred to a new 96-well plate. The absorbance at 414 nm was measured with a Multiskan MK3 reader (Thermo Fisher), with 0.1% Triton X-100 and PBS used as positive and negative controls, respectively. The hemolysis rate was calculated as follows (Han et al., 2016):

$$\text{Hemolysis (\%)} = [(A - A_0)/(A_1 - A_0)] \times 100,$$

where A is OD₄₁₄ nm in the peptide solution, A_0 is OD₄₁₄ in PBS, and A_1 is OD₄₁₄ in 0.1% Triton X-100.

A3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, a technique to measure cell survival, was carried out according to the method of

Table 1. Growth inhibition rates (%) of *Escherichia coli* and *Staphylococcus aureus* by buffalo casein (BC) and by BC hydrolysate by ultrafiltration (BCHU)-derived peptides with different molecular weights (initial peptide concentration: 80 mg/mL); data presented as mean $\pm$ SD¹

Fraction	<i>Escherichia coli</i> CICC 10003	<i>Staphylococcus aureus</i> CICC 10384
BC	-14.317 $\pm$ 2.259 ^a	-17.523 $\pm$ 4.170 ^A
BCHU1 (>10 kDa)	51.304 $\pm$ 2.523 ^b	17.413 $\pm$ 3.856 ^B
BCHU2 (<10 kDa)	80.983 $\pm$ 6.287 ^d	34.190 $\pm$ 1.378 ^C
BCHU3 (>1 kDa, <10 kDa)	71.101 $\pm$ 1.570 ^c	33.748 $\pm$ 2.951 ^C
BCHU4 (<1 kDa)	83.192 $\pm$ 0.707 ^d	46.213 $\pm$ 7.591 ^D

^{a-d, A-D}Means within a row with different superscripts differ ($P < 0.05$).

¹CICC = China Center of Industrial Culture Collection (Beijing).

Mosmann (1983). Briefly, human skin epithelial cells (HaCaT) cultures (3×10^5 cells/mL) were prepared in 96-well plates, and 200 μ L of serial dilutions (0.8 to 1.6 mg/mL) of either purified peptide or 20 mg/mL SDS (positive control) were added. Fresh Eagle's minimum essential medium (E-MEM) was used as negative control. Plates were incubated for 24 h at 37°C in a humidified 5% CO₂ atmosphere. After, the medium was replaced by 50 μ L of MTT solution (1 mg/mL; USB Corporation, Cleveland, OH) prepared in E-MEM medium, and the plates were incubated for 4 h at 37°C. The MTT solution was removed without disturbing the cells, and 100 μ L of dimethyl sulfoxide was added to each well to dissolve formazan crystals. After gently shaking the plates for 10 min, the absorbance was read at 540 nm (Multiskan MK3, Thermo Fisher). The percentage of viable cells was calculated as A_T/A_C , where A_T and A_C are the absorbances of treated and control cells, respectively.

Statistical Analysis

All experiments were performed in triplicate. Data are presented as mean $\pm$ standard deviation. Statistical significance ($P < 0.05$; Duncan's test) was determined using SPSS version 22.0 software (IBM Corp., Armonk, NY). Graphs were made using GraphPad Prism 8.0.1 (GraphPad Software Inc., La Jolla, CA) and Adobe Illustrator CS6 (Adobe Inc., San Jose, CA).

RESULTS AND DISCUSSION

Inhibition Rates of BC Hydrolysates

The BC hydrolysates were ultrafiltered and incubated with *E. coli* and *Staph. aureus*, to determine their growth inhibitory capacity. The bacterial growth inhibition rates of ultrafiltration components (BCHU) with different molecular weights are shown in Table 1. Compared with BCHU, BC did not show antimicrobial activity against the indicator bacteria. Conversely,

BCHU exhibited antimicrobial activity to varying degrees. Among the different fractions, BCHU4 (<1 kDa) showed significantly higher inhibitory effect against *E. coli* and *Staph. aureus* than the others. Therefore, we chose this fraction (BCHU4) for further AMP isolation by live *E. coli* affinity adsorption. Similar results were reported previously for camel casein and *Apostichopus japonicas* protein hydrolysates, from which AMP with molecular weight <1 kDa were isolated during ultrafiltration (Meng et al., 2016; Almi-Sebbane et al., 2018). Previous research has revealed that peptides with low molecular weight could exhibit effective antimicrobial activity (Al Saiqali et al., 2018).

Selective Separation of AMP by Live *E. coli* Adsorption

In addition to the active peptides, the total hydrolysate contains a large number of peptides without antimicrobial activity (Bougherra et al., 2017). The method of live *E. coli* affinity adsorption was used to selectively absorb the AMP contained in the hydrolysate, which avoided cumbersome separation methods (e.g., ion exchange, gel filtration, liquid chromatography). In this study, the major steps for separation of AMP from BC hydrolysate are shown in Figure 1. Seven steps form the core of this approach: (1) obtaining the component (BCHU4) with antimicrobial activity; (2) culturing and washing *E. coli*; (3) affinity adsorption; (4) collecting the supernatant (BCHU4-LE) by centrifugation; (5) analyzing the differential peaks of BCHU4 and BCHU4-LE via RP-HPLC fingerprint; (6) collecting the fraction with the strongest antimicrobial activity; and (7) identifying the fraction via LC-MS/MS.

Purification and Identification of AMP

The RP-HPLC fingerprints of BCHU4 and BCHU4-LE are shown in Figure 2A, which shows 3 peaks (**F1**, **F2**, and **F3**) with decreasing areas after incubation with live *E. coli*. The adsorption of live *E. coli* to

BCHU4 suggested that these fractions might contain AMP, which could be interacting with the live *E. coli*. Three fractions were manually collected, and their antimicrobial activities were tested. We found that F3 was the most effective against *Staph. aureus*. The purified F3 fraction was subjected to LC-MS/MS to determine its molecular weight and AA sequence. Four peptides (YLGYLEQLRLK, TKVIPYVRYL, AYFYPEL, and PFPIIV) were identified from F3 and chemically synthesized (Supplemental Table S1, <https://doi.org/10.3168/jds.2020-18577>). We measured the antimicrobial activity of these 4 peptides respectively and found that the activity of peptide YLGYLEQLRLK was significantly higher than the others (Table 2). Thus, peptide YLGYLEQLRLK was selected as the target AMP in this study. Peptide YLGYLEQLRLK corresponded to the AA residues 106 to 117 of bovine α_{S1} -casein. We found no AMP matching it in the NCBI (<https://www.ncbi.nlm.nih.gov/protein/?term=>) or APD databases, suggesting the peptide is novel; we thus named it BCp12.

The purity of synthesized BCp12 is shown in Figure 2B, illustrating a single peak (purity above 98%). The BCp12 from BCHU4 (<1 kDa) had a molecular weight 1,508.82 Da. Because UF membrane is not an absolute filter membrane, its molecular weight cutoff efficiency will have a range (about 800–1000 Da). However, membrane retention efficiency may also be affected by UF pressure. As Almi-Sebbane et al. (2018) reported, tandem MS analysis of the most active fraction, that

with the lowest molecular mass (<1 kDa), showed a molecular mass between 1 and 2 kDa, which suggested that UF separation does not rely upon a strict molecular mass threshold. That is, the separation between peptides crossing the UF membrane or not is also influenced by peptide conformation, as well as by potential interactions between peptides leading to the formation of complexes with higher molecular mass than individual peptides.

Previous reports have also shown that some peptides probably derived from α_{S1} -casein exhibit bactericidal activity in vitro against various pathogenic bacteria, such as *Staph. aureus* (Gobbetti et al., 2004; López-Expósito et al., 2006). Our results indicate that live *E. coli* adsorption can efficiently separate AMP from complex casein hydrolysate, in a simpler way than classic purification methods.

Structure Prediction and Characterization of BCp12

Helical wheel modeling was used to predict the hydrophobic and hydrophilic regions in BCp12, having determined 66% hydrophobic ratio and 2 positive charges for BCp12. The peptide was shown to be a hydrophobic and cationic AMP (Figure 3A). To determine the secondary structure of peptides in membrane-mimicking environments, CD spectra were obtained from peptides dissolved at a final concentration of 0.2 mg/mL in the following solutions: 10 mM sodium phosphate buffer, pH 7.2, which mimics the aqueous environment; 30 mM

Figure 1. Flowchart of purification of antimicrobial peptides from buffalo casein hydrolysate by live *Escherichia coli* affinity adsorption. RP-HPLC = reversed-phase HPLC; LC-MS/MS = liquid chromatography-tandem MS. BCH = buffalo casein hydrolysate; BCHU4 = buffalo casein hydrolysate ultrafiltration (molecular weight < 1 kDa); BCHU4-LE = supernatant of BCHU4 after affinity adsorption by live *E. coli*.

Figure 2. Purification and identification of antimicrobial peptides. (A) Liquid phase diagram: a = buffalo casein hydrolysate by ultrafiltration (BCHU4, before adsorption), b = BCHU4-LE (the supernatant of BCHU4 after affinity adsorption by live *Escherichia coli*). F1, F2, and F3 = difference peaks 1, 2, and 3. (B) Reversed-phase-HPLC (Agilent 1200; Agilent Technologies, Santa Clara, CA) profile of novel peptide BCp12. Column type: Agilent XDB-C18; 250 × 4.6 mm, 5 μm. The profile was eluted at a flow rate of 1.0 mL/min; inject volume 10 μL. (C) MS spectrum (LC-2010A, Shimadzu Inertsil, Tokyo, Japan; column type: Shimadzu Inertsil ODS-SP, 4.6 × 250 mm × 5 μm, Shimadzu Inertsil) of BCp12.

Figure 3. Structural characteristics of novel peptide BCp12. (A) Helical wheel projection of BCp12. The hydrophobic residues are represented in yellow, hydrophilic residues in circles, negatively charged residues in red, positively charged residues in blue, and particular polar residue in gray. (B) Circular dichroism spectra of BCp12 in PBS, in SDS, and in trifluoroethanol (TFE).

SDS, which mimics the negatively charged prokaryotic membrane environment; and 50% TFE, which mimics the hydrophobic environment of microbial membranes (Kim et al., 2018). The CD analysis (Figure 3B) showed that BCp12 in 30 mM SDS and 50% TFE solution had negative ellipticities at about 208 nm and 222 nm, respectively, indicating that the secondary structures of BCp12 were mainly of α -helical conformation, suggesting that the peptides adopted an α -helical conformation in membrane-mimicking environments.

Antimicrobial Activity of BCp12

Antimicrobial activity of BCp12 was determined against the gram-positive bacteria *Staph. aureus* CICC 10384 and *L. monocytogenes* CMCC 54004, as well as the gram-negative bacteria *E. coli* CICC 10003 and *Salmonella typhimurium* CICC 21484. As shown in Table 3, BCp12 was active against both gram-positive and gram-negative bacteria, with MIC values ranging from 0.8 to 1.6 mg/mL. In addition, the MIC of BCp12 against gram-positive bacteria was lower, indicating that the gram-positive bacteria used in this experi-

ment were more susceptible to BCp12 than the gram-negative bacteria were. The diversity of bacterial cell structure may lead to differences in the antimicrobial activity of BCp12. Compared with previous reports, the BCp12 peptide showed similar MIC values against gram-negative bacteria (Hayes et al., 2006).

An agar disk diffusion test was performed to evaluate the antimicrobial activity of BCp12 (Figure 4A). The values of the diameter of the inhibition zone showed that BCp12 was able to inhibit *Staph. aureus* growth. The diameter of the BCp12 inhibition zone was about 11.500 mm. Tetracycline, a broad-spectrum antibiotic, was used as positive control (15.333 ± 0.577 mm).

The growth of *Staph. aureus* under different BCp12 concentrations (0, 0.5, or 1.0 MIC) was studied to correlate the observed bacterial density at 630 nm with the antimicrobial efficacy of the BCp12. As shown in Figure 4B, in the absence of BCp12 (the control), *Staph. aureus* showed a normal growth curve, with lag, exponential, stationary, and declining growth phases within 24 h. Upon incubation with 0.5 MIC, we observed that BCp12 barely affected the growth of *Staph. aureus*, but it did postpone the late exponential phase of the growth curve. When bacteria were treated with BCp12 at 1.0 MIC, we observed that the growth of *Staph. aureus* was significantly inhibited within 24 h.

BCp12 Sensitivity to Trypsin, Temperature, and Salt Concentrations

The trypsin sensitivity of BCp12 is shown in Figure 5A. We detected no significant change in the antimicrobial activity of BCp12 treated for 0 h. But after 1 and 2 h of trypsin treatment, activity of BCp12 was reduced significantly ($P < 0.05$) compared with the control.

Table 2. Growth inhibition rates of *Staphylococcus aureus* by F3 (difference peak 3 in Figure 2A) and by 4 chemically synthesized peptides at the same concentration; data presented as mean $\pm$ SD

Sequence	Inhibition rate (%)
F3	87.284 ± 1.670^c
YLGYLEQLRLK	89.944 ± 1.210^d
TKVIPYVRYL	53.198 ± 2.664^b
AYFYPEL	25.840 ± 1.304^a
PFPIIV	52.412 ± 1.590^b

^{a-d}Means within a row with different superscripts differ ($P < 0.05$).

Table 3. Minimum inhibitory concentration (MIC) of novel peptide BCp12, an antimicrobial peptide from buffalo casein hydrolyzed by *Dregea sinensis* Hems. protease

Item	Bacterial strain	MIC of BCp12 (mg/mL)
Gram-positive bacteria	<i>Staphylococcus aureus</i> CICC 10384	0.8
	<i>Listeria monocytogenes</i> CMCC 54004	1.0
Gram-negative bacteria	<i>Escherichia coli</i> CICC 10003	1.6
	<i>Salmonella typhimurium</i> CICC 21484	1.4

Only 74.679% activity of BCp12 remained after 1 h of treatment, and 68.109% activity of BCp12 remained after 2 h of treatment. To maintain the antimicrobial activity of BCp12, we suggested that trypsin should be avoided in the actual application environment of BCp12. If BCp12 is used in vivo, some techniques may help protect it from trypsin damage. Most AMP are sensitive to in vivo enzymes such as trypsin; a potential solution is microencapsulation, through which peptide drugs are encapsulated, preventing degradation, and allowing the drug to release in a sustained and controlled manner (Zhang, 2011).

As depicted in Figure 5B, a temperature increase from 70°C to 115°C yielded no significant effect ($P > 0.05$) of the antimicrobial activity of BCp12 against *Staph. aureus*; BCp12 retained more than 95% of its activity after being heated from 70°C to 115°C for 30 min. Our results indicate that BCp12 is stable at temperatures below 115°C, suggesting its potential application as a food preservative in thermal processing. But when incubated at 121°C for 30 min, BCp12 activity was significantly decreased ($P < 0.05$) to 40% of its original activity. Not all AMP can retain their original antimicrobial activity after high-temperature treatment. Similar results were obtained for lysozyme-derived peptides, produced by limited protease hydro-

lysis of hen egg lysozyme, which lost about 57% of their original antimicrobial activity after treatment at 121°C for 15 min (Abdou et al., 2007).

Salt environments have a negative effect on AMP (Kim et al., 2018). When AMP interact with bacteria, they first bind to the bacterial cell membrane through electrostatic attraction (Brogden, 2005). The ionic strength in the environment affects the surface charge of the AMP, thus affecting the binding of the AMP to bacterial cells. Therefore, we studied the salt stability of BCp12. Figure 5C shows recovered antimicrobial activities of Bcp12 treated with different salts. The antimicrobial effect did not change significantly ($P > 0.05$) in the presence of a concentration of 5% NaCl, 5% CaCl₂, and 10% CaCl₂. However, antimicrobial effectiveness was significantly reduced ($P < 0.001$) in the presence of concentrations of 15% NaCl, 10% KCl, 15% KCl, 15% MgCl₂, and 15% CaCl₂. The addition of high-concentration ions weakened the electrostatic interaction between AMP and bacteria and reduced AMP binding (Bellamy et al., 1993).

Hemolytic and Cytotoxic Activities of BCp12

Although BCp12 is sensitive to trypsin, as described above, it is still expected to be used in vivo. Therefore,

Figure 4. Antimicrobial activity of novel peptide BCp12. (A) Agar disk diffusion test. 1 = sterile water; 2 = casein (20 mg/mL); 3 = tetracycline (40 µg/mL); 4 = BCp12 (4 mg/mL). (B) Effect of BCp12 on growth of *Staphylococcus aureus*. OD₆₃₀ = optical density at a wavelength of 630 nm; MIC = minimum inhibitory concentration.

it is essential to investigate the toxicity of BCp12. As shown in Figure 6A, its toxicity to red blood cells was examined. The hemolysis rates of BCp12 were 7.033, 4.572, 1.854, and 0.519%, at the respective initial concentrations of 4.0, 2.0, 1.0, and 0.5 mg/mL. According to previous reports, a hemolysis rate of more than 5% indicates hemolysis (Che et al., 2008). We found that BCp12 at a concentration of 4 mg/mL showed a hemolysis rate of 7.033% ($P < 0.001$ compared with negative control). Our positive control, 0.1% Triton X-100, caused complete hemolysis, whereas the negative control, PBS (pH 7.4), showed no hemolysis (Figure 6B).

Cytotoxicity of BCp12 was assessed via MTT assay in HaCaT cells (Figure 6C). In past studies, SDS has shown high cytotoxicity (Vaucher et al., 2010), revealed

by cell survival rates of 0.148% at 20 mg/mL SDS. Cell survival rates in BCp12 were 25.211, 95.129, 98.953, and 101.371% at the respective concentrations of 1.6, 1.2, 1.0, and 0.8 mg/mL. Compared with the negative control, BCp12 caused significant damage ($P < 0.001$) at high concentrations (1.6 mg/mL). As shown in Figure 6D, HaCaT cells in the positive control (20 mg/mL SDS) were completely ruptured, but in the 1.2 mg/mL BCp12 group, cells grew well. In the previous study, Kim et al. (2018) evaluated the toxic effects of AMP on HaCaT cells, which served as a prerequisite to observing the antimicrobial effect of AMP in *Pseudomonas aeruginosa*-infected mice in vivo. Our result could provide the basis to evaluate the efficacy of BCp12 in vivo, as with *Staph. aureus*-infected skin diseases.

Figure 5. Recovered antimicrobial activity of novel peptide BCp12 against *Staphylococcus aureus* after treatment. (A) Recovered antimicrobial activity of BCp12 after incubation by trypsin. (B) Recovered antimicrobial activity of BCp12 after heating at 70, 80, 90, 100, 115, and 121°C for 30 min. (C) Recovered antimicrobial activity of BCp12 in the presence of increasing concentrations of NaCl, KCl, MgCl₂, and CaCl₂. All data presented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control.

Figure 6. Hemolytic activity and cytotoxicity of novel peptide BCp12. (A) Hemolytic activity of BCp12 against red blood cells. (B) Image of hemolysis test: PC = positive control, NC = negative control, BCp12 = BCp12 at a concentration of 4 mg/mL. (C) Cytotoxicity of BCp12, determined by examining its effect on the survival rate of HaCaT cells using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. (D) Experimental image of HaCaT cells. All data presented as mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control.

CONCLUSIONS

In this study, we successfully isolated a novel AMP from buffalo casein hydrolysate. The peptide derived from α_{S1} -casein, named BCp12, exhibits amphipathic α -helical structures, with high hydrophobic moments and net positive charge. We determined that BCp12 exhibited potent antimicrobial activity and high stability against salts and temperatures up to 121°C. Furthermore, BCp12 induced low hemolytic and cytotoxic effects on mammalian cells at concentrations less than 4.0 mg/mL and 1.6 mg/mL, respectively. Overall, BCp12 is an effective inhibitor against bacterial

pathogens, with potential applications in the food and medical fields, such as use as a preservative for dairy products and as an antibiotic substitute for treating *Staph. aureus*-infected skin diseases.

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