Peptides 30 (2009) 1421–1427

Contents lists available at ScienceDirect

Peptides

journal homepage: www.elsevier.com/locate/peptides

The membrane action mechanism of analogs of the antimicrobial peptide Buforin 2

Gang Hao [a,b], Yong-Hui Shi [a,b], Ya-Li Tang [a,b], Guo-Wei Le [a,b,*]

[a] The State Key Laboratory of Food Science and Technology, JiangNan University, Wuxi, 214122, Jiangsu Province, China
[b] Institute of Food Nutrition and Safety, School of Food Science and Technology, JiangNan University, Wuxi 214122, Jiangsu Province, China

ARTICLE INFO

Article history:
Received 8 March 2009
Received in revised form 15 May 2009
Accepted 15 May 2009
Available online 23 May 2009

Keywords:
Antimicrobial peptide
Buforin 2
Analog
Membrane action
Mechanism

ABSTRACT

Previously, the antimicrobial peptides BF2-A and BF2-B, two analogs of Buforin 2 that was hypothesized to kill bacteria by entering cells and binding nucleic acids, had been designed based on the structure–activity analysis of Buforin 2. In the present study, BF2-A and BF2-B were chemically synthesized and their activities and lipopolysaccharide affinity were assayed. To elucidate the mechanism of action with cytoplasmic membranes, we subsequently examined the membrane permeability of both peptides in detail. Both peptides showed stronger antimicrobial activities against a broad spectrum of microorganisms than their parent peptide. Interestingly, BF2-A did not cause significant membrane permeabilization for influx of ONPG into cells, and hardly caused the leakage of intracellular macromolecules, probably BF2-A slightly disturbed cell membrane causing the K^+ leakage during peptide crossing phospholipids bilayer. Electron micrographs indicated that the cell membrane treated by BF2-A was still intact within 20 min. On the contrary, BF2-B obviously increased the outer and inner membrane permeability, even induced the slight leakage of macromolecules in the cytoplasm. The leakage of cytoplasmic contents was also demonstrated by the electron micrographs. The increase of membrane permeabilization explained why BF2-B displayed better antimicrobial activity and rapid killing kinetics than BF2-A.

1. Introduction

A number of antimicrobial peptides composed of 15–40 amino acids have been discovered in various organisms [14]. Most peptides have been considered to kill bacteria by permeabilizing negatively charged bacterial membranes [3]. In contrast to these membrane-active peptides, other classes of antimicrobial peptides, such as pyrrhocoricin [9] and indolicidin [21], are suggested to have multiple targets including intracellular components other than membranes. A 21-aa peptide, Buforin 2 (BUF2), is isolated from the stomach tissue of an Asian toad, *Bufo bufo gargarizans* and shows much stronger antimicrobial activities against a broad spectrum of microorganisms compared with other cationic polypeptides [17]. Interestingly, BUF2 is proposed to kill bacteria by efficiently crossing cell membrane without inducing severe membrane permeabilization or lipid flip-flop and strongly binding to DNA and RNA [7,8,18]. Indeed, BUF2 shares complete sequence identity with N-terminus of histone H2A that directly interacts with nucleic acids [24].

Recently, a new 17-aa peptide, named BF2-A (RAGLQFPVGRVHRLLRK) and a new 21-aa peptide, named BF2-B (RAGLQFPVGRLLRRLLRRLLR), were designed out based on the structure–activity analysis of BUF2 (TRSSRAGLQFPVGRVHRLLRK). The BF2-A was an N-terminally truncated analog of BUF2 corresponding to residues 5–21. The BF2-B was a hybrid peptide containing the N-terminal residues 5–13 and three repeats of the C-terminal regular α-helical motif RLLR [19]. These two analogs of BUF2 exhibited more powerful antimicrobial activities against a broad spectrum of microorganisms than their parent peptide. Although the previously proposed mechanism was quite intriguing, no one had characterized how both peptides interact with cell membrane on the molecular-level.

In this study, the interaction of BF2-A and BF2-B with phospholipid membrane was investigated in detail by use of liposomes as a model membrane system, and determination of membrane permeability. The results indicated that BF2-A could not cause significant cell membrane permeabilization, however, BF2-B apparently increased cell membrane permeability, and even caused the leakage of intracellular macromolecules.

* Corresponding author at: Institute of Food Nutrition and Safety, School of Food Science and Technology, JiangNan University, 1800 Lihu Road, Wuxi 214122, Jiangsu Province, China. Tel.: +86 510 85917789; fax: +86 510 85917789.
E-mail address: lgw@jiangnan.edu.cn (G.-W. Le).

0196-9781/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2009.05.016

2. Materials and methods

2.1. Peptide synthesis and purification

All peptides were synthesized by the Fmoc (9-fluorenylmethoxycarbonyl)-chemistry in solid phase peptide synthesis by using a model 432A peptide synthesizer [1]. The synthetic peptides were purified by C_{18} reverse-phase HPLC and the purity was confirmed by amino acid analysis and matrix associated laser desorption ionization (MALDI) mass spectroscopy.

2.2. Circular dichroism (CD)

CD experiments were performed by using a Jasco 720 spectropolarimeter (Jasco, Tokyo) to determine the secondary structure of BF2-A/B. The spectra were measured between 190 and 250 nm either in the presence or absence of 50% (v/v) trifluoroethanol in 50 mM NAPB. Five consecutive scans per sample were performed in a 1-mm cell at 25 °C. The helicity of the peptides was determined from the mean residue helicity at 222 nm.

2.3. Antimicrobial assay

Antimicrobial activity was expressed as the minimal inhibitory concentration (MIC), which was defined as the lowest concentration of peptides that completely inhibited microbial growth [4]. Single colonies of bacteria were inoculated in nutrition broth and cultured overnight at 37 °C. An aliquot of each was transferred to 10 mL of fresh culture medium and incubated for additional 3–5 h at 37 °C to obtain mid-logarithmic-phase organisms. Then, 50 μL of a set of twofold serial dilutions of peptides in 1% bactopeptone was added to 100 μL of bacterial suspension (1.0×10^6 CFU/mL) in 96-well microtiter plates and the plates were incubated at 37 °C for 12 h. After incubation, microbial growth was determined by the increase in the turbidity of each well measured at 630 nm using a microplate reader.

2.4. Hemolytic activity

Hemolytic activity of the peptides was tested against human red blood cells (hRBCs) [23]. Human red blood cells were centrifuged for 10 min at 3000 rpm, washed three times with saline (0.85% NaCl) and re-suspended in saline. The peptide solutions (serial twofold dilutions in saline) were then added to 100 μL hRBCs to give a final volume of 200 μL and a final erythrocyte concentration of 4% (v/v), which were incubated for 1 h at 37 °C and centrifuged at 3000 rpm for 5 min. Aliquots (100 μL) of supernatant were transferred to 96-well plates and hemoglobin release was measured using an ELISA plate reader by absorbance at 414 nm. Zero and 100% hemolysis were determined in saline and 0.1% Triton X-100, respectively. The hemolysis percentage was calculated using the following equation: % hemolysis = [($Abs_{414\ nm}$ in the peptide solution − $Abs_{414\ nm}$ in saline)/($Abs_{414\ nm}$ in 0.1% Triton X-100 − Abs_{414nm} in saline)] × 100.

2.5. LPS binding affinity

The lipopolysaccharide (LPS) affinity of peptides was determined by endotoxin neutralization of peptides using quantitative chromogenic tachypleus amebocyte lysate (TAL) endpoint assay. Briefly, after the addition of 0.1 mL TAL in test tubes, 10 μL of control standard endotoxin and 90 μL of peptide solution of various concentrations dissolved in TAL reagent water were added. Then an aliquot of 0.1 mL of chromogenic substrate was added into test tubes after incubation at 37 °C for 45 min. After reaction of intermixture at 37 °C for 6 min, 1.5 mL of stopping reagent was added and mixed by vortex. The endotoxin concentration was monitored by reading absorbance at 545 nm on a spectrophotometer. The endotoxin neutralization rate at absorbance, Abs, was calculated by the following equation: % endotoxin neutralization = $100 \times (Abs_{control} - Abs)/Abs_{control}$. The $Abs_{control}$ denotes the absorbance of reaction solution without antimicrobial peptides.

2.6. Calcein release from large unilamellar liposomes

Calcein-encapsulated large unilamellar vesicles (LUV) composed of EYPC/EYPG (4:1, w/w) were prepared by the reversed-phase ether evaporation method using 10 mM calcein [22]. The initially formed vesicles were extruded through Nuleopore filter (0.6 μm). To remove free calcein dye, the vesicles were passed through a Bio-Gel A 0.5m (Bio-Rad, USA) column (1.5 cm × 30 cm) using phosphate buffered saline, pH 7.4, as the eluting buffer. An aliquot of 100 μL of the separated LUV fraction, after appropriate dilution to a final concentration of phospholipids of 20 mg/mL, was mixed with 100 μL of peptide solution on 96-well microtiter plate at 25 °C. The leakage of calcein from the LUV was monitored by measuring fluorescence intensity at 520 nm excited at 490 nm on an Anthos Zenyth 3100 spectrofluorometer. The apparent percent release value at fluorescence intensity, F, was calculated by the following equation: % of apparent leakage = $100 \times (F - F_0)/(F_t - F_0)$. F_t denotes the fluorescence intensity corresponding to 100% leakage after the addition of 5 μL of 10% Triton X-100. F_0 represents the fluorescence of the intact vesicle.

2.7. Outer and inner membrane permeability

In order to determine if there was an OM permeability-increasing activity, the hydrophobic antibiotics erythromycin and rifampin were tested in association with both peptides in a synergistic growth inhibition assay by using checkboard dilutions [25]. The washed logarithmic-phase *E. coli* ATCC25922 cells, re-suspended in fresh nutrition broth medium (2×10^6 CFU/mL), were incubated on the 96-well plates with antimicrobial peptides and antibiotics of various concentration at 37 °C for 10 h. Decrease of absorbance was monitored at 630 nm using a microplate reader.

Determination of the inner membrane permeability was performed measuring the β-galactosidase activity of *E. coli* ML-35p at 37 °C using ONPG as substrate [20,27]. Aliquots of 800 μL of a suspension of logarithmic-phase bacteria (10^8 CFU/mL) in 10 mM Na-phosphate buffer (pH 7.4), with 1% TSB and 100 μL of 10 mg/mL ONPG were added to a 37 °C preheated cuvette. After 15 min of incubation, 100 μL of the polypeptide sample solution of various concentrations was added. The inner membrane permeability was monitored by measuring the rate of *o*-nitrophenol production at 405 nm by using a microplate reader. Furthermore, the bacterial suspension was centrifuged and β-galactosidase activity in the supernatant was measured using ONPG. As a positive control for the enzymes, a reaction mixture was sonicated to disrupt the bacteria.

2.8. Measurement of the released potassium ion

E. coli ATCC25922 cells (2×10^6 CFU/mL) were incubated in test tubes in 1 mL of deionized water containing peptides (5 μg/mL) and the test tubes were incubated at 37 °C for various time. Following incubation, the cell suspensions were centrifuged at 10,000 rpm for 10 min and the supernatants were diluted at 100-fold [11]. The amounts of released K^+ were measured by atomic absorption spectrometer (VARIAN, USA).