Biomaterials xxx (2014) 1–13

Contents lists available at ScienceDirect

Biomaterials

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

Insights into the membrane interaction mechanism and antibacterial properties of chensinin-1b

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ARTICLE INFO

Article history:
Received 24 August 2014
Accepted 2 October 2014
Available online xxx

Keywords:
Antimicrobial peptide
Depolarization
Lipopolysaccharide
Cell selectivity
Wound infection

ABSTRACT

Antimicrobial peptides (AMPs) with non-specific membrane disrupting activities are thought to exert their antimicrobial activity as a result of their cationicity, hydrophobicity and α-helical or β-sheet structures. Chensinin-1, a native peptide from skin secretions of *Rana chensinensis*, fails to manifest its desired biological properties because its low hydrophobic nature and an adopted random coil structure in a membrane-mimetic environment. In this study, chensinin-1b was designed by rearranging the amino acid sequence of its hydrophilic/polar residues on one face and its hydrophobic/nonpolar residues on the opposite face according to its helical diagram, and by replacing three Gly residues with three Trp residues. Introduction of Trp residues significantly promoted the binding of the peptide to the bacterial outer membrane and exerted bactericidal activity through cytoplasmic membrane damage. Chensinin-1b demonstrates higher antimicrobial activity and greater cell selectivity than its parent peptide, chensinin-1. The electrostatic interactions between chensinin-1b and lipopolysaccharide (LPS) may have facilitated the uptake of the peptide into Gram-negative cells and be also helpful to disrupt the bacterial cytoplasmic membrane, as evidenced by depolarisation of the membrane potential and leakage of calceins from the liposomes of *Escherichia coli* and *Staphylococcus aureus*. Chensinin-1b was also found to penetrate mouse skin and was also effective *in vivo*, as measured by hydroxyproline levels in a wound infection mouse model, and could therefore act as an anti-infective agent for wound healing.

1. Introduction

Conventional antibiotics have specific intracellular targets. Consequently, bacteria are able to develop compensatory mechanisms that render them resistant, and the infections caused by multi-drug resistant bacteria have become a serious health problem [1–4]. Therefore, the search for new types of antibiotic agents with novel structures and antibacterial mechanisms has become increasingly urgent. Antimicrobial peptides (AMPs), a fundamental component of innate immunity, exhibit broad-spectrum antimicrobial activities, killing both Gram-negative and Gram-positive bacteria, viruses and fungi [5–8]. AMPs initially bind to the negatively charged bacterial cell membrane by electrostatic interactions, after which they insert into the hydrophobic core and perturb its structure, resulting in an increase in bacterial membrane permeability, which induces leakage of cytoplasmic components, and consequently, death of the micro-organism [9]. Evolution of pathogen resistance to AMPs may therefore be a very slow process, resulting from a fundamental change in membrane composition [10,11]. Thus, this membrane active mode might possibly be the most promising way to solve the multi-drug resistance problem. This renders AMPs as potent agents to replace the conventional antibiotics. Although many AMPs differ in their polypeptide chain lengths, amino acid sequence, cationicity, hydrophobicity and amphipathicity, they have common structural features, such as the capacity to adopt an amphipathic α-helical or a β-sheet structure, which are essential for disruption of bacterial membranes [12–14].

We previously identified and reported on a novel type of antimicrobial peptide, chensinin-1, that was isolated and purified from skin secretions of the Chinese brown frog *Rana chensinensis* [15]. Unlike other known amphibian AMPs, chensinin-1 has three histidine residues that may lead to dramatic changes in antimicrobial activity at different pH values [16,17]. Furthermore, the N-terminal sequence (S-A-V) of chensinin-1 differs from that of other relatively short antimicrobial peptides (20–24 residues) from Ranidae [18]. Thus, chensinin-1 is distinctly different from other known

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http://dx.doi.org/10.1016/j.biomaterials.2014.10.041
0142-9612/

antimicrobial peptides, including the "Rana box"—containing brevinin family of peptides. The 18-amino acid residue peptide has a large net positive charge (+7) at neutral pH, due to the presence of the five Arg and two Lys residues in the sequence, and the charge can increase to +10 under acidic conditions, due to the presence of three His residues. Chensinin-1 has relatively low hydrophobicity (4.289) and low amphipathicity (0.809). Notably, chensinin-1primarily adopts a random-coil structure in a membrane-mimetic environment and may be a novel lead molecule for the design of antimicrobial peptides with possible therapeutic applications [19]. Importantly, chensinin-1 displays potent antimicrobial activity against Gram-positive bacteria, but has no haemolytic activity, and thus has potential as a novel antibiotic. In our previous study [20], a designed chensinin-1 analogue, chesinin-1a, in which its sequence was rearranged by adjusting its hydrophilic/polar residues on one face and its hydrophobic/nonpolar residues on the opposite face, is shown in the helical diagrams in Fig. 1. These changes increased the amphipathicity from 0.809 to 0.930, but resulted in similar antimicrobial activity against Gram-positive bacteria compared to chensinin-1, however, with no antimicrobial activity against Gram-negative bacteria. Leu-substituted chensinin-1 and chensinin-1a analogues were also designed to improve the hydrophobicity of chensinin-1. Although the hydrophobicity of these analogues increased from 4.289 to 8.172, and the alpha-helical content increased from 8% to 66% in membrane-like solutions, their antimicrobial activities against Gram-positive and Gram-negative bacteria were not significantly improved.

Trp residues have been widely reported to exhibit the unique property of being able to interact with the interfacial region of a membrane, facilitating anchoring of the peptide to the bilayer surface [21,22]. This special feature of Trp makes it a very interesting molecule for designing short chain antimicrobial peptides. In this paper, we designed a novel analogue, chensinin-1b, by replacing three Gly residues with Trp on the hydrophobic face of chensinin-1a, as shown in Fig. 1. The antimicrobial and haemolytic activities of chensinin-1b have been investigated. The mechanism of membrane interaction with the peptide has also been determined. Lipopolysaccharide is thought to be the major structural component in the outer membrane of Gram-negative bacteria and forms a protective wall to resist a variety of host defence molecules [23]. To understand why chensinin-1b and its parent peptide chensinin-1 exhibit different antimicrobial activities against Gram-negative bacteria together with the role of LPS in bacterial susceptibility to chensinin-1b, the peptide interaction with LPS was also examined with various biophysical methods [24], including fluorescence spectroscopy, in-situ IR, dynamic light scattering and ITC experiments. The acquired biophysical data suggested that the Trp-substituted analogue chensinin-1b may penetrate the membrane of Gram-negative bacteria. A preliminary evaluation of chensinin-1b treatment on wound healing has also been investigated. This novel antimicrobial peptide, chensinin-1b, may represent a potentially useful antibiotic for wound healing.

2. Materials and methods

2.1. Synthesis and purification of peptides

Chensinin-1b, chensinin-1, FITC-and NBD-chensinin-1b were synthesised using standard Fmoc solid-phase peptide synthesis protocols (GL Biochem Ltd., Shanghai, China). The peptides were purified to near homogeneity (95%) by reverse-phase HPLC using a Vydac 218TP1022 C-18 column (2.2 cm × 25 cm; Separations Group, Hesperis, CA, USA) equilibrated with acetonitrile/water/trifluoroacetic acid. The relative mass of the peptide was obtained using MALDI-TOF MS (Shimadzu, Japan).

2.2. Bacterial strains

The bacterial strains *Escherichia coli* (AS 1.349), *Staphylococcus aureus* (AS 1.72), *Bacillus cereus* (AS 1.126), *Streptococcus lactis* (AS 1.1690), *Pseudomonas aeruginosa* (CGMCC 1.860), *Enterobacteraerogenes* (CGMCC 1.876), *Enterobacter cloacae* (CGMCC 1.58), *Klebsiella pneumonia subsp. pneumonia* (CGMCC 1.176), *Enterococcus faecalis* (CGMCC 1.595) and *Enterococcus faecium* (CGMCC 1.2334) were acquired from the China General Microbiological Culture Collection Centre. The bacterial strains *Staphylococcus epidermidis* (CICC23664) and *Acinetobacter baumannii* (CICC22934) were acquired from the China Center of Industrial Culture Collection.

2.3. Antimicrobial assay

The minimum inhibitory concentration (MIC) of chensinin-1b was determined using a standard micro-dilution method [25]. Briefly, the initial concentration of the peptide was 200 μM and was serially diluted to 1.56 μM for use. 50 μL of peptide solution were added to the wells of a 96-well microtiter plate. Then, 50 μL of an inoculum (10^6 CFU mL^{-1}) from a log-phase bacterial culture were added. The absorbance at 600 nm was recorded using a microtiter plate reader after the cultures were incubated for 18–24 h at 37 °C. The MIC was defined as the lowest peptide concentration that completely inhibited bacterial growth. To assure the accuracy of the assay, parallel incubations in the presence of gentamicin sulphate were also performed.

2.4. Haemolysis assay

The haemolytic activity of chensinin-1b was determined as previously described [26]. Briefly, 2×10^7 human erythrocytes were washed three times with 0.9% (w/v) NaCl and then incubated with diluted chensinin-1b in 0.9% (w/v) NaCl for 3 h at 37 °C. After centrifugation, the absorbance at 545 nm of each resuspended pellet was measured. The positive control was an erythrocyte suspension incubated in water (100% haemolysis), and the negative control was an erythrocyte suspension incubated in 0.9% (w/v) NaCl (0% haemolysis). The HC_{50} was defined as the mean peptide concentration from three independent experiments that caused haemolysis of 50% of the human erythrocytes.

2.5. Morphological changes of chensinin-1b-treated bacteria detected by scanning electron microscopy

E. coli cells were suspended at 1×10^8 CFU/mL in PBS buffer (pH 7.2) and incubated in separate suspensions with 3.91 and 7.82 μM chensinin-1b for 1 h at 37 °C. Then, the bacteria were pelleted by centrifugation at 3000 × *g* for 5 min and

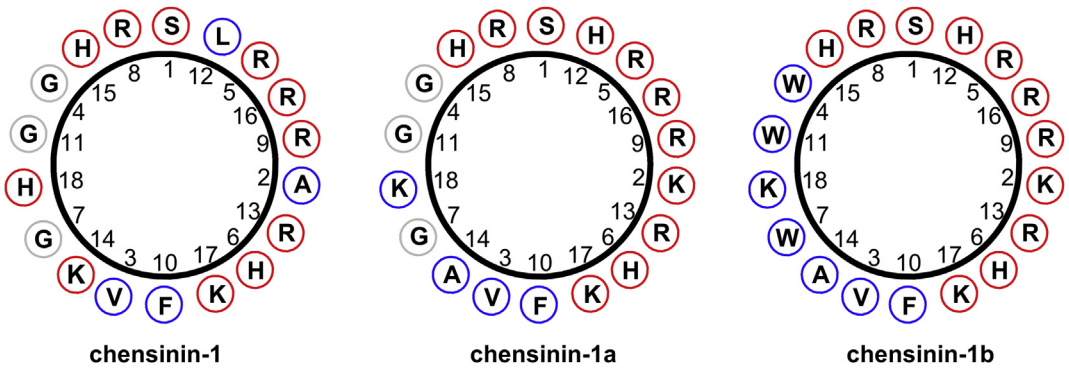

Fig. 1. Helical diagrams for chensinin-1 and its analogues, chensinin-1a and chensinin-1b (created by Antheprot 4.3 and redrawn using ChemDraw). Red circles: hydrophilic residues; blue circles: hydrophobic residues; and grey circles: other residues. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Please cite this article in press as: Sun Y, et al., Insights into the membrane interaction mechanism and antibacterial properties of chensinin-1b, Biomaterials (2014), http://dx.doi.org/10.1016/j.biomaterials.2014.10.041