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Structure-activity analysis and biological studies of chensinin-1b analogues

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ABSTRACT

Chensinin-1b shows a potent and broad-spectrum bactericidal activity and no hemolytic activity and thus is a potential therapeutic agent against bacterial infection. The NMR structure of chensinin-1b consists of a partially α-helical region (residues 8–14) in a membrane-mimic environment that is distinct from other common antimicrobial peptides. However, further analysis of the structural features of chensinin-1b is required to better understand its bactericidal activity. In this study, a series of N- and C-terminally truncated or amino acid-substituted chensinin-1b analogues were synthesized. Next, the bactericidal activity and bacterial membrane effects of the analogues were investigated. The results indicated that the N-terminal residues play a more significant role than the C-terminal residues in the antimicrobial activity of chensinin-1b. The removal of five amino acids from the C-terminus of chensinin-1b did not affect its biological properties, but helix disruption significantly decreased bactericidal activity. The substitution of positively charged residues increased the helicity and antimicrobial activity of the peptide. We also identified a novel analogue [R^4,R^{10}]C1b(3–13) that exhibited similar bactericidal properties with its parent peptide chensinin-1b. Electrostatic interactions between the selected analogues and lipopolysaccharides or cells were detected using isothermal titration calorimetry or zeta potential. The thermodynamic parameters ΔH and ΔS for [R^4,R^{10}]C1b(3–13) were −20.48 kcal mol^{-1} and −0.0408 kcal mol^{-1} deg^{-1}, respectively. Chensinin-1b yielded similar results of −26.36 kcal mol^{-1} and −0.0559 kcal mol^{-1} deg^{-1} for ΔH and ΔS, respectively. These results are consistence with their antimicrobial activities. Lastly, membrane depolarization studies showed that selected analogues exerted bactericidal activity by damaging the cytoplasmic membrane.

Statement of Significance

Antimicrobial peptide chensinin-1b is a candidate for the development of new drugs and a template for the design of synthetic analogues. It mainly exhibits a random coil conformation in membrane environment, and in this manuscript, we characterized the structure of chensinin-1b using NMR spectroscopy, its structure is different than the structures of magainin 2, which has an α-helical conformation and indolicidin, which has a random coil structure. The structural features of chensinin-1b that are required for its potent bactericidal activity were also elucidated. Based on these data, we can fully understand the structure-activity relationship of such peptide and identified a novel analogue with properties that make it an attractive topic for future therapeutic research.

1. Introduction

The use of conventional antibiotics in the past decades has been abused. The majority of these antibiotics have specific intracellular targets; thus, bacteria can develop compensatory mechanisms to resist them. Infections due to multi-drug resistant bacteria have

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become a serious public health issue [1–3]. Therefore, it is extremely important to develop antibiotics with a novel mode of action [4]. From bacteria, insects, vertebrates, and plants, including humans, researchers have discovered an almost inexhaustible source of potential therapeutic agents cationic antimicrobial peptides (AMPs) that are in the gene-encoded. AMPs protect the host, serve as a first line of innate immunity, and exhibit a potent antimicrobial activity against bacteria, fungi and viruses [5,6]. The development of resistance to antimicrobial peptides was illustrated by several *in vitro* experiments; however, the resistance rates are much lower than those for conventional antibiotics [7,8]. The mode of action for AMPs is initially through electrostatic interactions. The majority of AMPs bind to the negatively charged surface of the bacterial cell membrane, insert into the hydrophobic core, and disrupt the bacterial membrane integrity or cooperative permeabilization, which ultimately leads to microbe death [9]. Besides, AMPs display some other biological properties, including endotoxin neutralization, immunomodulating properties, chemokine-like activities, wound repair induction and angiogenesis [10,11]. Altogether, these properties support AMPs as promising candidates for the development of novel antibiotics [12].

In our previous works, we isolated and characterized an 18-amino acid peptide, chensinin-1 (SAVGRHGRRFGLRKHRKH), from the skin secretions of the Chinese brown frog *Rana chensinensis* [13,14]. Chensinin-1 contains three histidine residues that is different with other known amphibian antimicrobial peptides. Histidine normally acts as a proton shuttle that can vary the antimicrobial activity of the peptide by altering pH values. Thus, chensinin-1 has a net positive charge (+7) at a neutral pH because of the existence of two Lys and five Arg residues in its sequence. Under acidic conditions, the net charge can increase to +10. Furthermore, the N-terminus residues (SAV) of chensinin-1 are unlike other relatively short antimicrobial peptides (20–24 residues) isolated from *Ranidae*. Thus, chensinin-1 is distinct from other reported antimicrobial peptides, including the 'Rana box' that contains the brevinin peptide family. Chensinin-1 shows a relatively low amphipathicity and hydrophobicity, and it primarily forms a random coil conformation in a membrane-mimetic environment. Thus, this peptide could be a novel lead compound for the design of antimicrobial peptides with therapeutic applications. Chensinin-1 shows moderate antimicrobial activity against Gram-positive bacteria but no antimicrobial activity against Gram-negative bacteria. Therefore, we rearranged the sequence of chensinin-1 by adjusting its hydrophobic/nonpolar residues on one face and its hydrophilic/polar residues on the opposite face. The novel analogue chensinin-1a showed increased amphipathicity but did not show antimicrobial activity against Gram-negative bacteria [15]. Moreover, when a Gly residue in the sequence was substituted with a Leu residue, the hydrophobicity was enhanced. However, the peptide showed no activity against Gram-negative bacteria. Therefore, Trp residues were introduced to replace Gly residues in chensinin-1a and the new peptide chensinin-1b (C1b) was synthesized. This substitution resulted in potent antimicrobial activities against both Gram-positive and -negative bacteria. Chensinin-1b produced a significant improvement in wound healing in mice when compared with mupirocin ointment [16]. Chensinin-1b exhibits a mostly random coil conformation in a membrane environment, and binds with the outer and cytoplasmic membranes through electrostatic interactions. Next, the peptide accumulates on the surface of the lipid core. Once the threshold concentration is reached, pores are formed and the bacteria are killed. Chensinin-1b is a promising candidate for the development of new antibacterial drugs because of its specific mechanism of membrane disruption. Therefore, it is of utmost importance to understand the structure-activity relationship of peptides with random coil conformations at the molecular level. Based on NMR data for chensinin-1b, a series of structurally altered chensinin-1b analogues were designed and synthesized, and their antimicrobial activities and secondary structures were also examined. Furthermore, the action of select chensinin-1b analogues on the membrane permeation of intact bacteria was investigated by determining the membrane depolarization. The interactions between chensinin-1b analogues and lipopolysaccharide (LPS, mimics the outer membrane of Gram-negative bacteria) were also studied to understand the bactericidal action of the analogues.

2. Materials and methods

2.1. Peptide preparation

All peptides were synthesized by standard Fmoc chemical protocols by GL Biochem Ltd. in Shanghai, China. The synthetic peptides were purified to approximately 95% homogeneity by reverse-phase HPLC on a C-18 column (Vydac 218TP1022; 2.2 cm × 25 cm; Separations Group, Hesperis, CA, USA) equilibrated with acetonitrile/trifluoroacetic/water acid. The peptides were characterized using matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF MS; Shimadzu, Japan).

2.2. Bacterial strains

Seven bacterial strains were selected and obtained from the China General Microbiological Culture Collection Center, including five Gram-positive bacteria and two negative bacteria: *Staphylococcus aureus* (AS 1.72), *Escherichia coli* (AS 1.349), *Bacillus cereus* (*AS 1.126*), *Streptococcus lactis* (AS 1.1690), *Pseudomonas aeruginosa* (CGMCC 1.860), *Enterococcus faecium* (CGMCC 1.2334), and *Enterococcus faecalis* (CGMCC 1.595).

2.3. Antimicrobial activity assay

The antimicrobial activities of the peptides against selected bacteria were determined using the standard broth micro-dilution method [17]. Briefly, each peptide with an initial concentration of 200 μM was serially diluted to 1.56 μM for use. Next, the peptide solution was added to the wells of a 96-well plate (50 mL/well). After inoculation (50 μL/well, 10^6 CFU mL^{-1}), the cultures were incubated for 18–24 h at 37 °C. The absorbance at 600 nm was detected using a microtiter plate reader. The MIC was defined as the lowest peptide concentration that completely inhibited bacterial growth. Parallel incubations in the presence of gentamicin sulfate were performed to verify the accuracy of the assay.

2.4. Hemolysis assay

The hemolytic activity of the peptides was determined using a previously reported method [18]. Briefly, aliquots of 2×10^7 human erythrocytes were washed three times with 0.9% (w/v) NaCl and incubated with diluted peptides at 37 °C for 3 h. Next, the mixtures were centrifuged for 5 min at 900*g*, and the absorbance of each resuspended pellet was detected at 545 nm. An erythrocyte suspension incubated in water served as the positive control (100% hemolysis), and an erythrocyte suspension incubated in 0.9% (w/v) NaCl (0% hemolysis) served as the negative control. The HC_{50} was determined by the mean peptide concentration from three independent experiments that induced 50% hemolysis of the human erythrocytes.

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