Journal of Colloid and Interface Science 452 (2015) 148–159

Contents lists available at ScienceDirect

Journal of Colloid and Interface Science

www.elsevier.com/locate/jcis

# Probing the role of Proline in the antimicrobial activity and lipopolysaccharide binding of indolicidin

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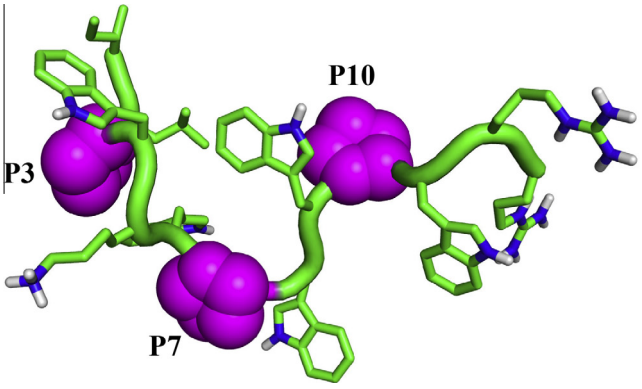
## GRAPHICAL ABSTRACT




| | MIC (μM) |
|---|---|
| IR13 | 4 |
| IR13P3A | 35 |
| IR13P7A | 10 |
| IR13P10A | 15 |
| IR13P3AP7A | 15 |
| IR13P7AP10A | 15 |
| IR13P3P10A | 25 |
| IR13PAAA | 45 |

## ARTICLE INFO

*Article history:*
Received 20 March 2015
Accepted 13 April 2015
Available online 25 April 2015

*Keywords:*
AMP
Indolicidin
LPS
SEM
CD spectroscopy
$^{31}$P NMR spectroscopy
DLS

## ABSTRACT

*Hypothesis:* Indolicidin (ILPWKWPWWPWRR-$NH_2$), an antimicrobial peptide from bovine neutrophils, possesses significant antibacterial activity. An interesting feature of indolicidin is its unusually high content of Tryptophan and Proline residues. While the involvement of Tryptophan has been studied for its hemolytic and antibacterial activity, little is known about the roles played by Proline in these aspects. We herein investigate the structure and biological activities of indolicidin, where Proline at either one or more of the 3rd, 7th, 10th positions has been replaced by Alanine to better understand its structure and biological function.

*Experiments:* Structural aspects of Proline residues of indolicidin and its effect on antimicrobial activity were elucidated by replacing Proline residues with Alanine. Minimum inhibitory concentration (MIC) and scanning electron microscopy (SEM) experiments provide substantial evidence for the importance of Proline residues for antimicrobial activity and cell wall disintegration. Binding affinity of the peptides to Lipopolysaccharide (LPS) was investigated using fluorescence spectroscopy and dynamic light scattering (DLS) in conjunction with $^{31}$PNMR spectroscopy and confirmed the disintegration of LPS layer.

*Findings:* Our study reveals that Proline residues are necessary for interaction of indolicidin with LPS and establishes the significance of the third and tenth Proline residues for its antimicrobial activity. We believe that the presence of so many Proline residues provides the molecule a selective advantage of adopting different conformations varying from a globular, closed conformation to an open extended

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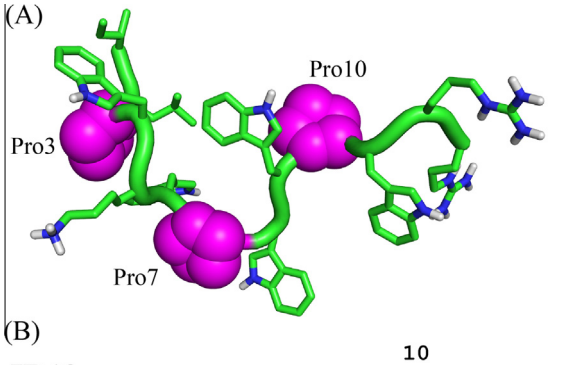
http://dx.doi.org/10.1016/j.jcis.2015.04.031

conformation, and even to a wedge-shaped conformation, which account for the diverse mechanisms of action of indolicidin.



## 1. Introduction

Despite a large number of natural or designed antibiotics available against life threatening diseases, the emergence of antibiotic resistant bacterial species has been a major challenge toward treating infections [1]. The increasing emergence of these multi-drug resistant (MDR) bacterial strains has initiated vital structure/function studies of membrane-perturbing cationic antimicrobial peptides [2]. These host defense antimicrobial peptides (AMPs) have appeared as a prospective novel group of antibacterial agents because of their evolutionarily preserved role in innate immunity [3,4]. Most cationic AMPs are thought to be important in natural defense against a wide range of invading pathogens, including bacteria, viruses and fungi, by disrupting the integrity of membrane structures [5]. Due to their amphipathic features, they can interact with and invade into cytosolic membranes of microorganisms by one or several mechanisms, e.g., barrel stave, toroidal pore, or carpeting which leads to cell lysis [6,7]. Therefore, detailed studies of structures and interactions of AMPs with different standard membranes by means of various biophysical and biochemical methods along with high resolution solid and solution state NMR is crucial for a better understanding of AMP mechanisms and structure/activity correlations [8–11]. It has been shown that the negatively charged Lipopolysaccharide (LPS) layer of the outer membrane of Gram-negative bacteria prevents translocation of AMPs [5]. In order to achieve accessibility to plasma membrane, AMPs should overcome the LPS permeability barrier first [12,13]. LPS molecule consists of conserved lipid A (glucosamine residues with fatty acid chain), core oligosaccharide unit composed of 10–15 sugar residues, and the O-antigen part (polysaccharide chain composed of consecutive units of 1–8 sugar residues) [14]. The disruption of the outer membrane by the AMP occurs by a "self-promoted" uptake mechanism that involves an electrostatic interaction between phosphate groups of LPS and cationic residues of peptides after displacing bivalent cations. However, LPS or endotoxins may induce the innate immune system causing fatal septic shock syndrome in humans [15,16]. Thus, several AMPs having LPS binding and neutralizing capability would afford a preparatory stage for the development of anti-bacterial drugs [17].




**Scheme 1.** (A) Three dimensional solution structure of IR13 in DPC (1G89.pdb). All proline residues are marked by pink color. (B) Amino acid sequence of IR13 and its mutants. Proline residues of 3rd, 7th and 10th position were sequentially mutated to Alanine i.e., IR13P3A, IR13P7A and IR13P10A. Similarly, for double mutants, two Proline residues were mutated to two Alanine residues such as IR13P3P7A, IR13P3P10A and IR13P7P10A. Finally, all three Proline residues were mutated to Alanine and referred as IR13AAA.

In spite of their antimicrobial activity, these peptides are generally hemolytic and cytotoxic to host cells. Therefore, structure–activity relationship studies of AMPs in negatively charged LPS phospholipids are essential to comprehend their methods of action and to propose effective antibiotic peptides in the future [14,18,19]. This would help in the development of newly engineered analogs that would conserve the elevated antimicrobial strength of the parent peptide without an undesired activity toward the host. The Tryptophan and Proline-rich 13 residue cationic antimicrobial peptide indolicidin (ILPWKWPWWPWRR-$NH_2$) (hereafter denoted as IR13) (Scheme 1) from the Cathelicidin family, is widely known for its potential antimicrobial activity against various Gram-positive and Gram-negative pathogens [20]. IR13 is isolated from cytoplasmic granules of bovine neutrophils. The peptide adopts a disordered structure in aqueous solutions and a wedge-shaped conformation in biological membrane mimicking micelles such as those of DPC and SDS [21]. Five interspersed Tryptophan residues along with three Proline residues in the central core of the sequence of IR13 (Scheme 1) provide a fascinating structural feature which tunes its bioactivity related to its antimicrobial and hemolytic functions [21]. The favorable salt bridge, electrostatic and hydrogen bonding interactions as well as Tryptophan modulated hydrophobic interactions with bacterial lipid membrane provide its foremost function. The mode of action of IR13 has been described in literature as membrane permeabilization [22]. Recent studies demonstrate that after passing through the outer membrane, IR13 interacts with bacterial DNA, and the PWWP motif has immense importance in DNA binding [23]. This in turn causes a complete inhibition of bacterial replication and transcription which leads to cell lysis [24]. However, the structural details for this mechanism are not completely explored and require further and detailed investigation. The implication of Proline residues of IR13 on its antimicrobial activity is poorly understood. It has been hypothesized that Proline plays a major role in structure activity relationship of AMPs due to its restricted stereochemistry and conformation [25]. Furthermore, Proline residues of IR13 have a major contribution in LPS binding. In this study, we have elucidated LPS binding phenomenon of IR13 and its Proline analogs using various biochemical and biophysical techniques and revealed the role of each Proline residue in antimicrobial activity, bacterial cell lysis, and LPS binding.

## 2. Materials and methods

### 2.1. Reagents

The parent Indolicidin peptide (IR13) was purchased from GL Biochem (Shanghai, China). The Proline analogs of Indolicidin were