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Identification of an antimicrobial peptide from large freshwater snail (*Lymnaea stagnalis)*: activity against antibiotics resistant *Staphylococcus epidermidis.*

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Abstract

Nowadays, antibiotic resistance in bacteria is a great public health problem of increasing magnitude due to quick evolution through mutation that has generated the urgency to find the effective solutions to address this problem. Aside the conventional antibiotics, antimicrobial peptides are a new class of antimicrobials is known to have the activity against a wide range of bacteria. An antimicrobial peptide was isolated and purified from the *Lymnaea stagnalis,* a fresh water large snail, using ultrafiltration and reversed phase liquid chromatography. The molecular mass of the peptide 2345 Da was determined using MALDI TOF mass spectrometry. This peptide is efficiently prevented the growth of *Staphylococcus epidermidis* that resistant to ampicillin and chloramphenicol antibiotics. The MIC value was 16 µg/mL and specifically damage to bacterial membranes. Hence, this reported peptide revealed an alternative candidate to controlling the *Staphylococcal* infections.

Key words: Antimicrobial peptides, antibiotic resistant, *Lymnaea stagnalis*, *Staphylococcus epidermidis,*

Introduction

The newest challenge in the healthcare setting is multi-drug resistant bacteria. Rapid emergences of drug-resistant to conventional antibiotics are of greatest threats to microbial infection control. It has generated the urgent need to search for new antimicrobial therapeutic molecules to control the microbial infection to cope with the problems associated with public health. In the last few decades, several studies have focused to identify the animal-derived natural antimicrobial compounds to pursue novel strategies for diagnosis and treatment of bacterial diseases [1]. Antimicrobial peptides (AMPs) are the most important and effective antibiotics to combat the increasing emergence of drug resistant bacteria. AMPs have broad-spectrum activity against a wide range of micro-organisms including viruses, Gram-positive and Gram-negative bacteria, protozoa, yeasts and fungi [2]. A large number of antimicrobial peptides have been isolated from amphibians, fish, insects, mammals [3] and different microorganisms [4].

Snail species have developed many distinct types of venom as a survival strategy for feeding and defense. Their venoms contain a diverse mixture of biologically active peptides, mostly small and structurally constrained. The discovery of snail peptides has been greatly accelerated during the past years due to the introduction of sophisticated and effective technical approaches. Many research groups are extraordinarily interested in these small peptides and intend to explore this rich resource. Several peptides have been identified from marine cone snails, including AVC1, effective in treating postsurgical and neuropathic pain, isolated from the Australian Queen Victoria cone, *Conus victoriae* [5]. Most of the bioactive peptides from cone snails are mostly important for interaction with macromolecules involved in neuronal function [6]. But, no studies on the peptides from fresh water large pond snails (*Lymnaea stagnalis)* have been reported as antibacterial agent. In this view, this report describes the isolation and characterization of a novel peptide from *Lymnaea stagnalis* with antibacterial activity against *S. epidermidis* NCIM2493, resistance to ampicillin and chloramphenicol.

Materials and methods

Sample preparation

Fresh water large snails (*Lymnaea stagnalis*) are potent source of protein to many people around

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the world. Snails were purchased from local market of Kharagpur, West Bengal, India. The snails were washed twice to remove residual clay particle. Venom was collected by an external injury at the anterior end where operculum is attached. The collected venom (100 ml) sample was centrifuged twice for 10 mins at 13,000 rpm to precipitate the residual debris. The supernatant was then passed through 3 kDa Biomax polyethersulfone (PES) cut-off membrane using Amicon Stirred Ultrafiltration Cells (MA, USA). The filtrate was then lyophilized and redissolved in 1 ml of 5% (v/v) acetonitrile solution containing 0.01% (v/v) trifluoroacetic acid.

Peptide purification

Resuspended sample was fractionated by reverse phase- HPLC (Agilent 1100 series, USA) with a ZORBAX 300 SB C18 column (4.6 mm x 150 mm, particle size 5 µm). The sterile Milli-Q water with 0.1% TFA (A) and 80% acetonitrile with 0.1% TFA (B) were used as mobile phase. The system was operated at 1 ml min^{-1} flow rate with linear gradient of solvent B (0-60%) for 50 mins and the detection was monitored at 220 nm in a diode array detector. Selected peaks of the HPLC chromatogram were collected using a fraction collector (GILSON, France) coupled with the system. Fractions were concentrated by Speed-Vac and each fraction was re-suspended in same solvent composition where they were eluted and tested for antimicrobial activity against *S. epidermidis.*

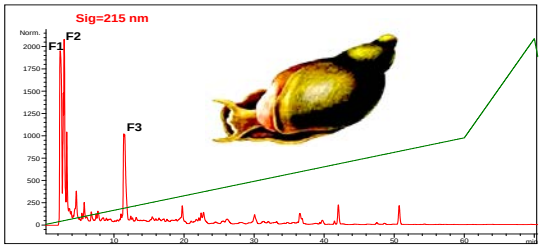

Figure 1. Reversed-phase HPLC chromatogram profile of < 3 kDa fraction from Lymnaea stagnalis venom. Inset representing the picture of Lymnaea stagnalis.

Antimicrobial assay

S. epidermidis NCIM2493, strain was purchased from National Chemical Laboratory, Pune, India. Strain was cultured from a -70°C stock when required and grown to mid-logarithmic phase in brain heart infusion medium (HiMedia Laboratories) at 37°C with shaking. Mueller-Hinton agar (MHA) medium was used for inhibition zone assay by disk diffusion method. The zone of inhibition with peptide and respective antibiotics was determined following the Kirby-Bauer disk suceptibility test. All disks (5 mm diameter) contained 20 µg of the respective antibiotics and purified peptides.

MIC value of the fraction 3 was determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines. The concentrations of peptide used for the assay ranged from 1.0 to 64 µg/mL. MIC value was determined by the microtitre plate dilution method, where no visible growth is observed. A range of peptide concentrations were added to sterile 96-well microtitre plates and the volume was made up to 250 µL/ well with MHB. Culture of the strain was added to the mixtures in such a way that the total inoculum load was ca. 10^5 cells/ well. Finally, microtitre plates were incubated at 37 °C for 18 h. Bacterial growth was measured by the optical density at 600 nm using a Multiskan Spectrum spectrophotometer (model 1500; Thermo Scientific, Nyon, Switzerland) and the MIC value of the peptide was determined by comparing the cell densities with wells where no peptide was added as positive control and no bacteria with peptide was used as negative control to maintain the sterility. Minimum Bactericidal Concentration (MBC) was determined by colony forming unit (CFU) of each concentration following dilution plating method. Determination of all values were carried out in triplicate and repeated at least four times for accuracy.

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MALDI-TOF mass spectrometry

The molecular mass of the HPLC purified fr-3 was determined by Voyager DE Pro^{TM} mass spectrometer equipped with 337 nm N_2 laser (Applied Biosystem, USA). The lyophilized dried peptide was directly applied (non reduced form) and separately reduced with dithiothreitol (DTT) in 50 mM NH_4HCO_3 at 37 °C for 2 h and followed by acidification with 1% TFA. Two micro liter of peptide solution was mixed with 24 µl of CHCA (a-cyano-4-hydroxycinnamic acid) 10 mg ml^{-1} which was used as matrix. Then, 1.0 µl sample was spotted onto the 100 well stainless steel MALDI plate and allowed to air dried prior to the MALDI analysis [1]. The spectra were recorded in the linear, positive mode ion mode with an accelerating voltage 20 kV and average 100 laser shots with a grid voltage of 90%. Sequence of the peptide was determined with tandem mass spectrometry (MS/MS) following Gauri et al. 2011 [7].

Molecular modeling

Initially, the input sequence of the peptide was run with HHpred (HHsearch 1.6.0.0) in order to find the best template for homology detection and structure prediction by HMM-HMM comparison. This structure was then placed with water molecules and a partial simulation for energy minimization was done with the GROMOS96 implementation of Swiss-PdbViewer, using 2000 steps from Steepest Descent in order to remove possible stereochemical disturbances. The final model was visualized using Pymol [7].

Results and Discussion

Peptides less than 3 kDa were purified from fresh water snail venom by reversed phase-HPLC after ultra filtration member technique. Fig. 1 shows HPLC chromatogram of isolated venom. Three major peaks were detected in HPLC chromatogram and their antibacterial activity with equal concentration was tested against *S. epidermidis* by disk diffusion method. Only, fraction 3 showed the activity against *S. epidermidis (*Fig.2*)*. The antibiotic susceptibility assay showed that the strain was resistant to ampicillin and chloramphenicol [7]. The MIC value of the peptide was 16 µg/mL.

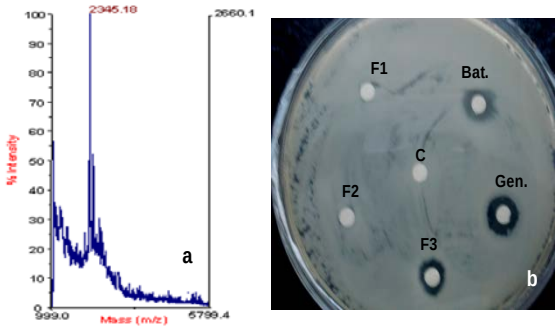

Figure 2. MALDI TOF MS analysis of fraction 3 (a). Antibacterial activity of major fractions against S. epidermidis. Bat, bacitracin; Gen, gentamycin; C, control; F1, Fraction 1; F2, Fraction 2; F3, Fraction 3.

MALDI TOF-MS analysis of Fr.3 showed that the molecular mass of peptide was 2345 Da (Fig .2). Tandem mass spectrometry (MS/MS) provides a means for fragmenting mass selected precursor peptide ions and measuring the mass to charge (m/z) ratio of any product daughter ions. The process usually produces two principal classes of fragment ions, b and y type ions respectively [1]. Manual interpretation of peptide spectra for the purpose of protein identification, a process usually referred to as *de novo* sequencing which is informative about the primary sequence of the peptide. After de novo sequencing the successive m/z values have permitted to assign the sequence as AGCCGSYAAACHCPCKGRSTICGQ. The amino acids residues were compared to AMP database (http://aps.unmc.edu/AP/main.html) in order to understand the homology with other antimicrobial peptides. *Bb*-AMP4 showed a hydrophobic ratio of 45% and a boman index of 0.69 kcal/mol. Molecular modeling of the peptide finally revealed a reliable model (Fig. 3).

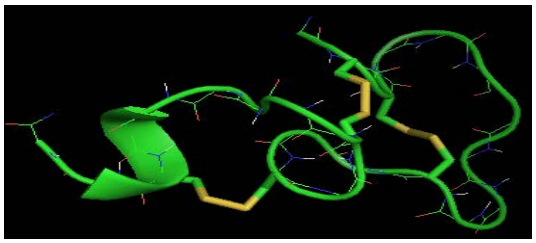

Fig.3. Cartoon representation of the purified peptide. A homology was determined using HHpred (HHsearch 1.6.0.0) and structure was constructed with Modeller 9.4. Golden colour indicating the disulfide bond.

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Several hundreds of antimicrobial peptides have been isolated and identified from a variety of organisms. Their mode of action includes disrupting membranes, interfering with metabolism, and targeting cytoplasmic components have been well studied. In this study, one novel antimicrobial peptide with 1676 Da molecular weight showed activity against *S. epidermidis* NCIM2493, resistance to ampicillin and chloramphenicol. *Staphylococcus epidermidis* is the most common species responsible for infection causing significant morbidity, mortality, and incurring healthcare costs worldwide, one of the most prevalent cutaneous resident bacteria and resistant to many antibiotics. Several studies have been identified *S. epidermidis* as a common opportunistic pathogen of human skin and easily colonize on the indwelling catheters surface, prosthetic joints, cerebrospinal fluid shunts and other medical devices by the production of adhesin like extracellular polysaccharide [8].

Sequence identity analysis showed that the isolated peptide has a 38% homology to an antimicrobial peptide, Varv peptide F (AP01031). Molecular modeling of the peptide revealed that glycine residues were present in the centre of the peptide that conferring greater flexibility to this structure and also rich with aromatic residues, indicating a possible membrane interaction by hydrophobic attraction. Interestingly, the peptide is cysteine rich and belongs to the conotoxin superfamily. Cysteine rich AMPs isolated from different origin showed broad spectrum antimicrobial activity [9]. Hydrophobicity or net charge and chain flexibility is important factor of AMP to improve the antibacterial activity [10]. The peptide showed positive charge (+2) and a significant rate of hydrophobicity suggesting that the main cause for antibacterial activity. Ionic interaction probably is the initial attraction between AMPs and target cell, which occur through an electrostatic bonding between cationic peptide and negatively charged components present on the outer bacterial envelope.

Structural constraints contributed by disulfide pairing are essential for many antibacterial proteins such as β-defensins, tachylepsins and protegrins. This is the first report of an antimicrobial peptide from fresh water large snails against human pathogen *S. epidermidis* resistance to ampicillin and chloramphenicol. The activity can be improved by modifying the peptide by substitution or deletion of residues. Thus it may be a promising candidate as clinically useful antimicrobial drug for treatment of antibiotic resistant *Staphylococcal* infections.

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