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ARTICLE

Screening of antimicrobial peptides from hemolymph extract of tasar silkworm *Antheraea mylitta* against urinary tract and wound infecting multidrug-resistant bacteria

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ABSTRACT Antimicrobial peptides (AMPs) are an evolutionarily conserved component of the innate immune response and they were found among all classes of life forms. In the present study AMPs were extracted from the hemolymph of *Antheraea mylitta* and fractionated by High Performance Liquid Chromatography (HPLC). Antimicrobial activity was tested against three clinically isolated multidrug-resistant (MDR) bacteria, such as urinary tract infecting *Escherichia coli*, wound infecting *Pseudomonas aeruginosa* and *Bacillus pumilus*. Fraction I (comprised of three different peptides of varying mass) did not inhibit the growth of any of these clinical isolates, whereas, fraction III inhibited the growth of *B. pumilus* without affecting the growth of gram-negative isolates. Fraction II exhibited bactericidal effects against *P. aeruginosa* and *E. coli,* whereas, *B. pumilus* was not susceptible. Scanning electron microscopic study revealed that serious structural alterations of cell morphology and disruption of the outer membrane, that facilitates the release of cytoplasmic content through holes and channels in *E. coli*, treated with this isolated peptide. Our results indicate that the peptide from the isolated fraction could be used as potent alternative antimicrobial compounds for the treatment of MDR *E. coli* and *P. aeruginosa* infections.
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KEY WORDS

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antimicrobial peptide
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gram-positive bacteria
hemolymph
multidrug resistance

Introduction

Every living organism on Earth has their own self-defense mechanism of producing a versatile group of soluble factors, which have the immense role in both innate and adaptive immune systems. In higher animals, soluble factors, particularly bioactive peptides are the sole 'attacking weapons' against invading pathogens. More than 2000 AMPs have been isolated from plants and animals, which were identified until today. Their amino acid composition, charge and size allow them to cross the outer barriers in both gram-positive and gram-negative bacteria, fungi and some viruses to form non-specific pore, damage and kill the microorganisms (Bencsik 2013). Many antimicrobial components have been evaluated between 1920 and 1950, but their applicability as therapeutic agents has been considered just in the last 15 years. They represent promising compounds for the treatment of infections to eradicate MDR microbes. In addition to the treatment of infectious diseases, AMPs are used in novel therapeutics development for other diseases, such as atopic dermatitis, Crohn's disease, cystic fibrosis (Zhang et al. 2005) and tumors (Hilchie et al. 2011). Considering its immense importance, pharmaceutical industries in the last five years have exhausted more than 30 billion dollars for the development of this novel drug type (Kang et al. 2014).

Both in developed and developing countries worldwide, MDR bacterial infections are very frequent and their number is increasing day by day, due to improper use of antibiotics and health care deficiencies. In developing countries, nosocomial infections are one of the greatest threats and make expensive to control, due to poor hygienic and environmental conditions. It has been reported, that approximately 150 million people suffer from urinary tract infection (UTI) in each year around the world (Stamm et al. 2001), in which 75% are women between the age group of 16-35 years (Nicolle 2008; Salvatore et al. 2011). UTI is a common bacterial infection, mostly caused by *E. coli* and about 75-95% uncomplicated UTI is mediated by *E. coli.* In India the risk of *E. coli* me-

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diated UTI is 63.44%, followed by other organisms like *Klebsiella* sp. (14.62%), *Pseudomonas* sp. (4.53%), *Proteus* sp. (4.62%) and other bacteria (Maji et al. 2013). Antibiotic resistance in gram-negative bacteria, particularly in *E. coli* is common, due to the spread of strains producing extended-spectrum β-lactamases, such as CTX-M enzymes (Pallett and Hand 2010). *Pseudomonas aeruginosa* is another common causative agent for burns, wounds, airways and urinary tract infection, also causes septicemia. One in ten hospitals, acquired infections are caused by *Pseudomonas* sp. In case of skin infection, its exotoxin A is responsible for hemorrhage and necrosis. The antibiotic resistance mechanism of *P. aeruginosa* has the inherent capacity to produce four-five factors that can facilitate its growth even in mixed antibiotics-treated condition. It has reported that, 75% mortality due to burn, is directly caused by *P. aeruginosa* (Koller et al. 1999; Guggenheim et al. 2011). Due to natural adaptation, bacteria develop many mechanisms to avoid the actions of antibiotics, including ejecting out, efflux, changing the antibiotic attacking site or by modifying/cleaving the antibiotics (Rajaduraipandi et al. 2014; Mukherjee et al. 2007).

Earlier reported, that silkworm *Bombyx mori* produces antibacterial protein attacin (~20 kDa) and gloverin (~14 kDa) have abilities to kill *E. coli* (Carlsson et al. 1998). The efficacy of a peptide rich hemolymph extract of *B. mori* has been proved to have growth inhibitory effects on human pathogenic bacteria, such as *S. aureus*, *P. aeruginosa*, *E. coli* and it also suppresses the growth of tumor cells (Moselhy et al. 2012). *Antheraea mylitta*, a tropical tasar silkworm, endemic to India, possess an enormous number of microorganisms, including microsporidia (Hassan and Nath 2014). This made us curious to investigate its hemolymph in search of novel antimicrobial substances. An antifungal protease inhibitor (AmFPI-1) was characterized from *A. mylitta* hemolymph as an effective inhibitor of *Aspergillus oryzae* (Shrivastava et al. 2003). Analysis of fat body Expressed Sequence Tag (EST) library of bacterial challenged *A. mylitta* larvae (Gandhe et al. 2006) achieved, which revealed that *A. mylitta* contains transcripts of various AMPs, such as cecropin, attacin, gloverin and lebocin. No any information is available in the literature about the full AMP profile of *A. mylitta* hemolymph. In the present study, it is further characterized by isolating novel representatives and investigation of their antibacterial activity against MDR gram-positive and gram-negative bacterial isolates.

Materials and Methods

Collection of hemolymph

Eschericia coli DH5α strain was grown in LB (lysogeny broth) medium in absorbance at 600 nm. Bacteria were washed with PBS (phosphate buffer saline) and 10 µl of 10^7 bacterial cell/ml was injected into the haemocoel of *A. mylitta* larvae. After 24 h of post infection, larvae were chilled on ice and hemolymph was collected by cutting appendages in prechilled tubes. The hemocyte free hemolymph was obtained by centrifugation at 500 g for 10 min and then centrifuged further at 13 500 g for 15 min at 4 ºC to remove other cell debris and stored at -20 ºC.

Preparation of hemolymph extracts

Acidic/methanolic extracts of hemolymph were prepared by the method of Schoofs et al. (1990). The hemolymph was diluted 10 times with the extraction solution consisting of methanol:glacial acetic acid:water (90:1:9; v/v/v) and mixed thoroughly. Precipitated proteins were pelleted by centrifugation at 20 000 g for 30 min at 4 ºC. The obtained supernatant was freeze-dried and dissolved in 0.1% (v/v) trifluoroacetic acid (TFA) followed by removal of lipids with n-hexane. This extract was mixed with equal volume of ethyl acetate, centrifuged and finally the aqueous phase, containing peptides, was lyophilized and stored at -20 °C.

Isolation and characterization of clinically isolated bacteria

Bacteria, such as *E. coli* and *P. aeruginosa* were isolated from urine and wound samples at Bankura Sammilani Medical College & Hospital (West Bengal, India). These organisms were isolated and biochemically identified with standard methods described elsewhere (Bhatia and Ichhpujani 2008).

Bacteria were also identified by sequencing the 16S rRNA gene. In brief, cells were grown in LB medium and pelleted by centrifugation at 8600 g for 3 min at 4 °C. Pellets were resuspended in 5 ml buffer (1.4 M NaCl, 10 mM EDTA, 0.5%, (w/v) SDS, and 100 mM Tris-HCl, pH 8.0) containing proteinase K and RNase A at a final concentration of 100 µg/ml and incubated at 56 °C for 30 min. Then, it was extracted twice with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) by gentle mixing and centrifuged at 12 400 g for 15 min at 4 °C. From the upper aqueous phase, DNA was precipitated by adding an equal volume of isopropanol and 0.1 volumes of 3 M sodium acetate (pH 5.2). DNA was pelleted by centrifugation at 13 500 g for 20 min at 4 °C, washed thrice with 70% (v/v) ethanol, air dried and finally suspended in 50 µl of TE buffer. Quantity and quality of DNA were determined with a Nanodrop 2000 UV-VIS Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) by checking OD at 260 nm and performing 1% agarose gel electrophoresis, respectively. Polymerase chain reaction (PCR) for the amplification of 16S rRNA was carried out as follows: reaction mixture contained 30 ng of template

DNA, 1 pmol of each primer (27 F: 5′ AGAGTTTGATCMTGGCTCAG 3′; 518 R: 5′ CGTATTACCGCGGCTGCTGG 3′), 1 × PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 2.5 mM $MgCl_2$, 1 unit of Taq DNA polymerase (5 U/µl), 0.2 mM dNTPs (Bioline) and adjusted to the final volume (20 µl) with nuclease free water. PCR was carried out in a GeneAmp 9700 PCR system (Applied Biosystems) for 35 cycles after an initial denaturation for 4 min at 94 ºC. Each PCR cycle comprised of three steps: denaturation at 94 ºC for 1 min, annealing at 58 ºC for 1 min and extension at 72 ºC for 1.5 min with a final extension of 7 min at 72 ºC. A negative control without template DNA was also used. The amplified PCR products were analyzed by gel electrophoresis on 1% agarose gel observed under UV transilluminator and photographed in a gel documentation system (Kodak). Amplicons were sequenced by using Bigdye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), in an automated DNA sequencer (3500 series Genetic Analyzer, Applied Biosystems) based on the manufacturer's instructions. Alongside, a bacterium, *Bacillus* sp. isolated from soil was subjected for molecular identification following the aforementioned technique. The sequences were analyzed by Sequencher software (Gene Codes Corporation, Ann Arbor, MI, USA) and homology were searched in NCBI tool using the BLAST (Basic Local Alignment Search Tool) method.

Antimicrobial assay of hemolymph extract

The bactericidal activity of hemolymph extract of *A. mylitta* was tested against *E. coli*, *P. aeruginosa* and *B. pumilus* isolates grown on Mueller-Hinton agar (HiMedia Laboratories). The antibacterial efficacy of hemolymph extract and commercial antibiotics were tested by the agar disk diffusion method as described by Bauer et al. (1966). The size of the disk was 5 mm in diameter and contained 5 µl of hemolymph extract (2.3 mg/ml) or 20 µg of antibiotics (SRL Diagnostics). All plates were incubated at 37 °C for 24 h and zone of inhibition (mm) was measured.

Peptide purification and assay of antimicrobial activity

The lyophilized hemolymph extract was resuspended in distilled water and 50 µl of it was applied on reversed-phase high-performance liquid chromatography (*RP-HPLC*) (Agilent 1200 Series, Agilent Technologies, USA) ZORBAX-SB-C18 column (5 mm, 250 mm × 4.6 mm). Sterile Milli-Q water (Millipore) (A) and 80% (v/v) acetonitrile (B) containing 0.1% (v/v) TFA were used as mobile phases. Linear gradient of solvent B (5-60%, v/v) was maintained for 50 min at 1 ml/min flow rate. The elution was monitored at 220 nm in a diode array detector, equipped with the RP-HPLC system. Individual peak was collected separately, freeze dried, resuspended in distilled water and tested for antimicrobial activity.

The antibacterial activity of the three major peak fractions (I, II and III) eluted at 3.5, 7.4 and 11.5 min (after loading), finally, were tested again by using the same agar disk diffusion test as described earlier. The concentration of the peptides was monitored using a Nanodrop 2000 UV-VIS Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Thin layer chromatography (TLC)

The purity of peptides was also tested by TLC. Briefly, 5 µl of HPLC-purified peptides were spotted on a pre-coated silica plate (7 cm × 10 cm) along with glycine as standard. The plates were run with a mobile phase solvent of butanol:acetic acid:water (3:1:1, v/v/v) for 1 h at room temperature. After air drying peptides were identified using 2% (w/v in butanol) ninhydrin as a coloring reagent.

Scanning electron microscopic analysis of the antimicrobial activity of the isolated peptide on E. coli

Mid-logarithmic phase *E. coli* culture was washed with PBS and finally resuspended in PBS (10^6 cell/ml). Fifty microliter of this cell suspension was incubated with 0.125 µg of HPLC-purified peptide and incubated for 1.5 h at 37 ºC. Cells were then fixed with 4% glutaraldehyde at 4 ºC for 4 h, then washed with PBS and dehydrated with graded ethanol (40-100%), incubating 10 min at each alcohol concentration. Finally, bacterial pellet was resuspended with 100% alcohol, smeared on a coverslip, coated with gold and changes in bacterial cell morphology were studied by using an atmospheric scanning electron microscope (ASEM, ZEISS).

Results

Characterization of various bacterial isolates and effect of hemolymph extract

Clinical isolates of *E. coli* and *P. aeruginosa* were identified by standard biochemical procedures. Both the strains as well as *Bacillus* sp. were subsequently identified by 16S rDNA sequencing. Obtained sequences were searched in the Genbank database: the results of the homology search are presented in Table 1. Based on this, bacterial isolates were proved to be *E. coli*, *P. aeruginosa* and *B. pumilus* as it shows 76%, 83% and 91% nucleotide sequence identities, respectively, with the available sequences of the same organisms in the database. Hemolymph extract exhibited antimicrobial activity against all tested pathogenic bacterial isolates. *Escherichia coli* was resistant to cephalosporine and ampicillin (20 µg), but sensi-

Table 1. 16 S rDNA sequence homology of isolated bacteria.

Name of clinical isolates	Sequence accession number in the database	Identities shown
E. coli	AY996985.1	271/358 (76%)
P. aeruginosa	KX109926.1	281/337 (83%)
B. pumilus	JQ282821.1	448/495 (91%)

Table 2. Effect of *A. mylitta* hemolymph extract (11.5 µg) and ampicillin (20 µg) on *E. coli*, *P. aeruginosa* and *B. pumilus*.

Name of pathogen	Zone of inhibition including disk (mm)	
	Hemolymph extract	Ampicillin
E. coli	18±0.70	0
P. aeruginosa	11±0.35	0
B. pumilus	12±0.70	22±0.50

tive to hemolymph extract (11.5 µg). Similarly, *P. aeruginosa* showed resistance to cephalosporine, kanamycine, chloramphenicol and ampicillin (20 µg), but sensitive to hemolymph extract (11.5 µg). *B. pumilus* was proved to be sensitive to both hemolymph extract (11.5 µg) and ampicillin (20 µg). The zone of inhibition of bacterial growth by hemolymph extract was measured and depicted in Table 2.

Purification of antimicrobial peptide by RP-HPLC

Peptides, which present in the hemolymph extract were separated by RP-HPLC using a C18 column and three major peaks (I, II, and III) were collected (Fig. 1). Antimicrobial potencies of these fractions were evaluated and presented in Table 3. Fraction I did not show any inhibitory activity against the tested bacteria. Fraction II exhibited strong inhibitory action on *E. coli* (zone of inhibition is 9.0±0.35 mm) and *P. aeruginosa* (zone of inhibition is 6.5±0.40 mm). It is interesting to note that fraction III exhibited antimicrobial effect on the gram-positive *B. pumilus* giving an inhibition zone of 7.5±0.30 mm, but not onto the gram-negative isolates.

Characterization of peptides by thin layer chromatography

RP-HPLC purified fractions I, II and III were further separated by thin layer chromatography with glycine as a migration control. A distinct and isolated spots of peptides were observed in TLC plates. The chromatogram also revealed that fractions II and III are homogeneous and contain a single

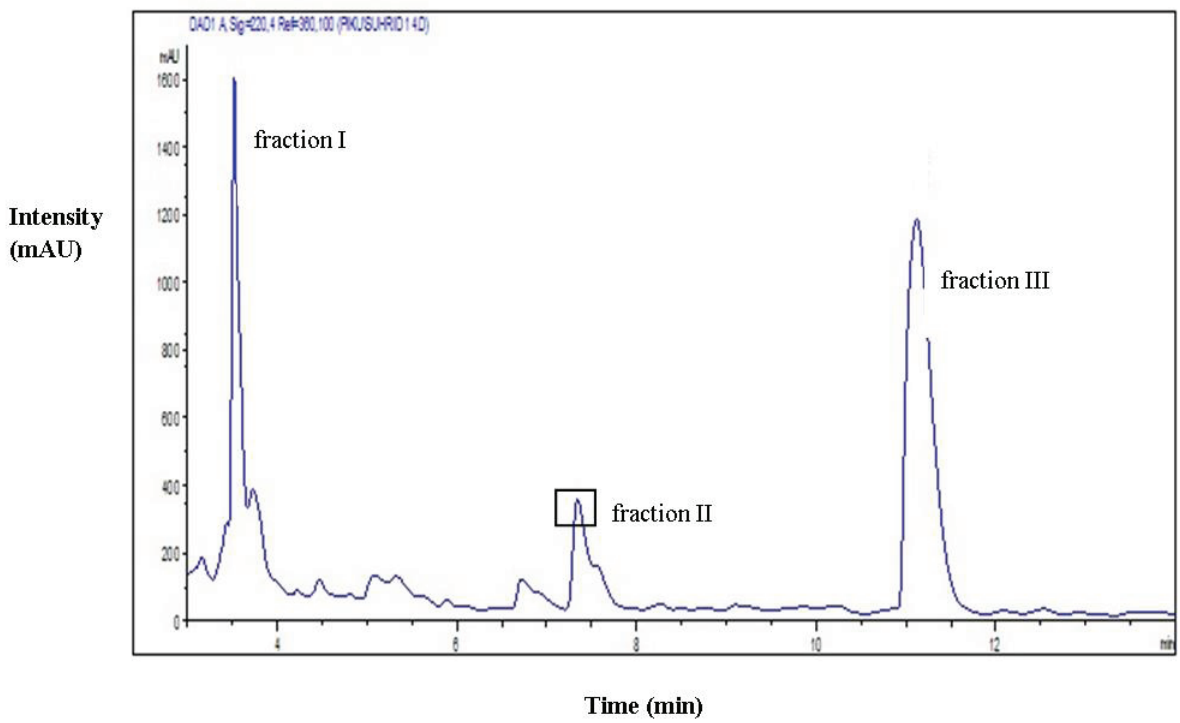

Figure 1. Reversed-phase HPLC chromatogram of *A. mylitta* hemolymph extract. Rectangle indicates fraction II, which showed antibacterial effects.

Table 3. Analysis of antibacterial activity of HPLC purified fractions I, II, III and ampicillin against *E. coli*, *P. aeruginosa* and *B. pumilus.*

Peptide fractions	Zone of inhibition (mm) against tested organisms		
	E. coli	*P. aureginosa*	*B. pumilus*
I	0.0±0.00	0.0±0.00	0.0±0.00
II	9.0±0.35	6.5±0.40	0.0±0.00
III	0.0±0.00	0.0±0.00	7.5±0.30
Ampicillin	0.0±0.00	0.0±0.00	22.0±0.50

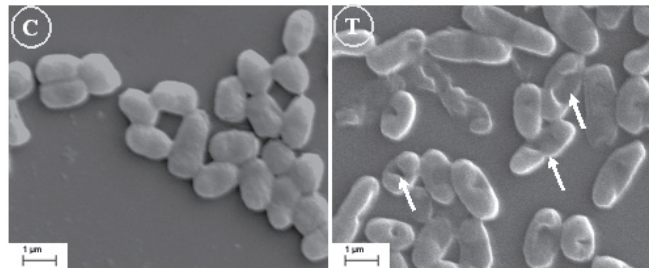

Figure 2. Scanning electron microscopy of untreated (C) and peptide treated (T) *E. coli*. Distortion of cell wall and pore formation was indicated by white arrows.

type of peptide, whereas, fraction I contains three different peptides.

Scanning electron microscopy

The bactericidal activity of the isolated peptides on the *E. coli* was studied by scanning electron microscopy (SEM). Figure 2 clearly demonstrates that alteration of cell morphology with depression and shrinkage of cell wall due to leakage of cytosol, indicating peptide (fraction II) mediated disruption of outer layers. In contrast, PBS treated cells showed compact, smooth and intact outer layers.

Discussion

It is well-documented that insect hemolymph contains many antimicrobial substances, like melanin, immune proteins and AMPs to protect against invading pathogens as they do not have any adaptive immune system. Many AMPs like cecropin (Steiner 1982), attacin (Hultmark et al. 1983), defensin (Bulet et al. 1991) and gloverin (Kaneko et al. 2007) were identified in different order of insects, including Lepidoptera (Gandhe et al. 2006). Ravichandran et al. (2010) have reported that the crab hemolymph extract can kill *Salmonella typhi* and *Klebsiella* species. Similar report also noted in case of hemolymph extracts of diplopod, chilopod species (Xylander 1990), crab (Veeruraj 2008; Rethna and Chandran 2014), spider (Yigit and Benli 2008), shrimp (Kumaravel et al. 2010) and Mollusca (Vini et al. 2013).

Rapid emergence of MDR bacteria is a burning threat to human health worldwide; it raises the urge to develop some alternative compound, which would be effective against MDR pathogens. Here, for the first time we reported the antimicrobial activity of hemolymph extract of *A. mylitta* on MDR *E. coli* and *P. aeruginosa*. In addition, *B. pumilus* a food poisoning and diarrhea causing bacteria also included in this study. Moreover, it also infects skin and caused cutaneous ulcer, very similar to *B. anthracis* (Tena et al. 2007). MDR strains, as well as antibiotic sensitive *B. pumilus* were inhibited by crude hemolymph extract of *A. mylitta*. The zone of inhibition in all the cases of RP-HPLC isolated peptides were small, because the amount of purified peptides used in the experiment was very low in amount (125 ng). The antibacterial activity of fraction II is very similar to the AMP Api137 (Berthold et al. 2013) and oncovin (Knappe et al. 2010), which also shows antimicrobial effects on pathogenic *E. coli* and *P. aeruginosa*.

Observation of peptide fraction II treated *E. coli* under SEM revealed massive structural deformation and depression in the cell wall caused by the disruption of the outer membrane, which eventually leads to release of the cytoplasmic contents by opening membrane surface. Similar type of observation of the cell surface and leakage of cellular materials has also been made in case of *Xanthomonas oryzae* pv. *oryzae* and *Shigella* sp. treated with mellitin and venom of European honey bee (Shi et al. 2016) and spider *Agelena labyrinthica* (Yigit and Benli 2008). Polymerization of antimicrobial peptide in the bacterial membrane (Zangger et al. 2008) is the initial step of its permeation. It was found, that peptides rich in glycine residues remained untidy in aqueous environments and gradually inclined to order itself in contact with bacterial membrane upon polymerization (Nguyen et al. 2008). A synthetic analog of keratin derived AMP-KDAMP was found to bind and penetrate the bacterial cell wall by upsetting membrane structure for its bactericidal activity against various human pathogens, like *P. aeruginosa, E. coli, Streptococcus pyogenes* and *S. aureus*. Therefore, it seems that the killing of *E. coli* and *P. aeruginosa* by our isolated peptide fraction II, possibly follow similar pattern as observed in case of KDAMPs and mellitin. In conclusion, hemolymph extract of Indian tasar silkworm *A. mylitta* contains effective antimicrobial peptides acts against clinically isolated MDR pathogenic bacteria and therefore, could be exploited as potent alternative therapeutics.

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