

Original Research Article

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Biophysical Characterization of Chemically Synthesized Antimicrobial Peptide ‘Mastoparan’ and Evaluation of its Activity on *Staphylococcus aureus* Isolated from Mastitic Milk

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ABSTRACT

Keywords

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A well-documented antimicrobial peptide, Mastoparan, initially isolated from the venom of wasp was chemically synthesized using solid phase peptide synthesis methodology. A total of 20 *S. aureus* isolates were isolated from milk samples. Antimicrobial sensitivity of these isolates against 9 (nine) conventional antibiotics, as well as, Mastoparan was studied. Majority of these isolates were resistant to many antibiotics. Mastoparan was observed to exhibit potent antimicrobial activity, even against some antibiotic-resistant isolates. Chloramphenicol was found to be most effective, whereas Ampicillin was least effective against *S. aureus* of milk origin.

Introduction

Antimicrobial peptides (AMPs) are naturally occurring, relatively short (12 to 100 amino acids), positively charged (net charge of +2 to +9), amphiphilic peptide molecules. Antimicrobial peptides are a promising candidate for treatment of diseases caused by pathogenic microbes viz. bacteria, fungi, and viruses (Boman *et al.*, 1995). The key mechanism of action of AMPs is disruption of

bacterial membrane by adopting some cationic and amphipathic structure. This membrane acting mechanism involves physicochemical interaction between the peptide and bacterial membrane, formation of peptide quaternary structure and insertion into bacterial membrane and formation of transmembrane pores which ultimately results in membrane permeabilization. AMPs have been isolated from almost all forms of life, including single-celled microorganisms, insects and other

invertebrates, plants, amphibians, birds, fish, and mammals, including humans (Martin *et al.*, 1995, Wang and Wang, 2004).

In terms of the number of species, insects represent the largest class within the animal kingdom. There is no doubt that insects' resistance invading to pathogens has contributed to their extreme proliferation and diversity. At present, except for the polar regions and the deep marine environment, insects are found in almost all biological niches. More than one million species of insects have been described (Bulet *et al.*, 1999). The cuticle is the first line of insect defense against pathogens and parasites. Nonetheless, a complex process of innate humoral and cellular immune reactions is initiated in both tissues and haemocoel once this barrier has been broken, resulting in the rapid removal of microorganisms. In such conditions, fat bodies and hemocytes of insects produce antimicrobial peptides/polypeptides, and rapidly release into the hemolymph. Insects produce a variety of antimicrobial peptides and are one of the major sources of antimicrobial peptides (Yi *et al.*, 2014). Majority of the antimicrobial peptides identified in invertebrates are predominantly from insects.

Mastoparan is an insect-derived, 14 amino acid residues membrane-active amphipathic peptide. Hydrophobic and basic residues are abundantly present which form amphipathic helical structures, the latter has the capacity to form pores in bacterial membranes (Moreno and Giralt, 2015). Mastoparan was first isolated from the venom of Eusocial wasp (*Polybia paulista*) (Hirai *et al.*, 1979). Since then Mastoparan has been studied extensively for its antimicrobial property against a wide range of Gram-positive and Gram-negative bacteria (Moreno *et al.*, 2015). Milk which is considered the most complete food is also a well-known good medium for the growth of

several food-spoilage, toxin-producing and/or infectious microbes. A number of pathogenic and toxigenic bacteria like *Staphylococcus aureus*, *Escherichia coli* and *Salmonella* have been isolated from raw milk (Lingathurai and Vellathurai, 2010).

In terms of milk and milk products output, the performance of Indian dairy on their international market is rising day by day. However, one of the biggest challenges for this sector is the maintenance of udder health for production of good quality milk. Antimicrobial peptides are considered potent alternatives to existing antibiotics.

Materials and Methods

Peptide sequences and synthesis

In the present study Mastoparan I, a tetradecapeptide having the sequence IDWKKLLDAAKQIL was chemically synthesised by solid phase peptide synthesis (SPPS) methodology developed by Merrifield (1963) using standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on Rink Amide MBHA resin.

After completion of stepwise addition of amino acids, the peptide was cleaved from the resin, and precipitated with chilled diethyl ether (dry) and peptide powder obtained was stored at room temperature for further use as method described by Shrivastava, 2006 with some modification.

***In silico* physico-chemical analysis**

Physico-chemical properties *viz.* molecular weight, net charge, hydrophobic ratio, aliphatic index, GRAVY hydrophobic moment and theoretical PI were predicted using online software (Mendes *et al.*, 2005). Three-dimensional secondary structure conformation and helical wheel projection of

the synthesized peptide was predicted using online software PEP-FOLD3 and Heliquist respectively.

Purification of synthesized peptide

The synthesized peptide was purified by reversed-phase semi-preparative HPLC (Shimadzu, Japan) on a C18 column (Shim-pack GIST C18 5 μ m, 250 X 14 mm) using an appropriate 95–5% water/ acetonitrile gradient in the presence of trifluoroacetic acid (TFA).

Antimicrobial assays

Minimum inhibitory concentration (MIC) of AMP Mastoparan against *Staphylococcus aureus* (ATCC 29213) was determined by broth microdilution method following Clinical Laboratory Standard Institute guidelines (CLSI, 2016) with slight modification. Briefly, bacterial culture was grown in Mueller-Hinton broth (MHB) for 12-18 hours till mid-log phase (0.4-0.6 OD₆₀₀). Test inoculums of $\sim 1 \times 10^6$ CFU.mL⁻¹ were prepared in fresh MHB. In a 96-well microtiter plate, 50 μ L of the bacterial suspension and 50 μ L of serially diluted Mastoparan was added. After overnight incubation at 37°C, MIC was determined as the lowest concentration of the peptide that resulted in no bacterial growth similar to the negative control with pure broth without bacteria.

Minimum bactericidal concentration (MBC) of Mastoparan against *S. aureus* (ATCC 29213) was determined by spreading 10 μ L of serially diluted samples from each well of the MIC plate on to fresh MHA plates and incubating overnight. MBC was indicated by the concentration of Mastoparan that completely inhibited bacterial growth.

Culture media and *S. aureus* (ATCC 29213) used in the study were procured from HiMedia Pvt Limited, India.

Isolation of bacteria from milk samples

S. aureus was isolated from a total of 30 milk samples collected from cases of mastitis in cattle. Immediately after collection, the samples were grown in enrichment medium, Brain Heart Infusion broth, at 37°C overnight in shaking. Post-incubation a loop-full of broth culture was streaked onto selective mannitol salt agar (MSA) plate and incubated overnight. Individual colony, showing characteristic colony morphology on selective agar was picked and sub-cultured for antibiogram studies.

Antibiogram study of field isolates

The antimicrobial sensitivity of the field isolates of *S. aureus* against a selected range of conventional antibiotics was performed by disc diffusion test. Antibiotic discs of standard concentration were procured from HiMedia Pvt Limited, India. Overnight broth culture of the isolates was taken and spread onto fresh MHA plates with a sterile spreader. Antibiotic discs were then placed, and plates were incubated at 37°C overnight. Post-incubation, zone of inhibition of bacterial growth around the antibiotic disc on the agar plates was measured using an antibiotic sensitivity scale. Sensitivity or resistance of the bacterium to a particular antibiotic was interpreted according to area of bacterial growth inhibition as per CLSI guidelines (CLSI, 2016).

Antibacterial activity of the synthesized AMP Mastoparan on *S. aureus* isolates was studied by broth microdilution method following CLSI guidelines. The study was conducted using a fixed concentration of 1MIC (as determined with standard *S. aureus*). Similar steps were followed as in case of MIC determination experiment. Growth or inhibition of bacteria was determined by measuring the absorbance at 600nm at 0th and 24th hours using a microplate reader (SkanIt

MultiSkanoGo, Thermo Scientific, USA). Each bacterium was tested in duplicate wells for each concentration of Mastoparan, and the tests were repeated twice on separate days.

Results and Discussion

Chemical synthesis and physico-chemical properties of Mastoparan

The antimicrobial peptide Mastoparan was chemically synthesized as C-terminal amides with free N-terminal. Dry peptide powder, obtained after cleavage, and precipitation with chilled diethyl ether, was dissolved in a minimum volume of sterile HPLC grade water and stored at -80°C. It was observed that being a chemically synthesized C-terminal amidated peptide Mastoparan carried a net positive charge +2. Its predicted molecular weight was 1655.01 gram/mol, and has aliphatic index and hydrophobic moment of 153.57 and 0.511, respectively. Mastoparan exhibited a three dimensional secondary structure as per PEP-FOLD3 structure simulation. Helical wheel

projection of Mastoparan indicated three positively charged lysine (K) residues on one side of the helix with two consecutive K-residues separated from a single K-residue by a negatively charged (D), a non-polar (I) and a polar uncharged (Q) residue. This helical arrangement maybe the drive force for peptide-membrane interaction.

Online predicted Physico-chemical properties, three-dimensional conformation and helical wheel projection of Mastoparan are presented in Table 1 and Figure 1.

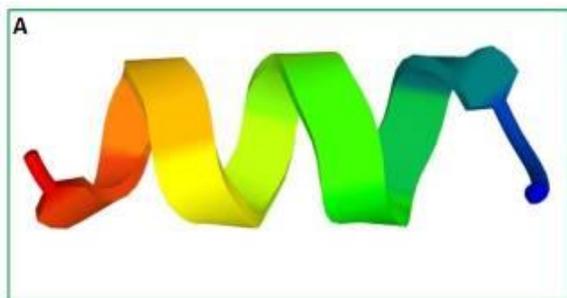
RP-HPLC purification of synthesized peptide

The synthesized AMP was desalted using RP C-18 analytical column. The pure fraction was collected for the desired peak at specific retention time. RP-HPLC data showed that Mastoparan exhibited a retention time of 22.386 minutes in RP C-18 analytical column. RP-HPLC chromatogram of Mastoparan is shown in Figure 2.

Table.1 Physico-chemical properties of Mastoparan

M. Wt. g/mol	Net Charge	Hydrophobic ratio (%)	Aliphatic index	GRAVY	Hydrophobic moment (μH)	Thrtcl. pI
1655.01	+2	57	153.57	0.064	0.511	8.50

Fig.1(A) Three-dimensional secondary structure conformation



(B) helical wheel projection of Mastoparan

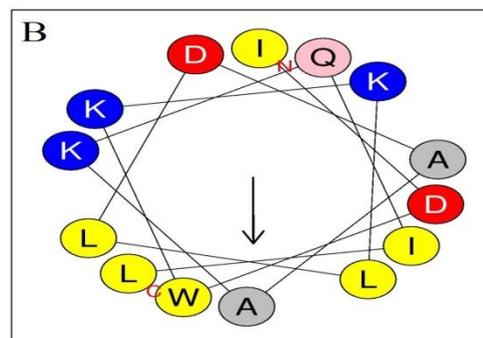


Fig.2 RP-HPLC chromatogram of antimicrobial peptide Mastoparan

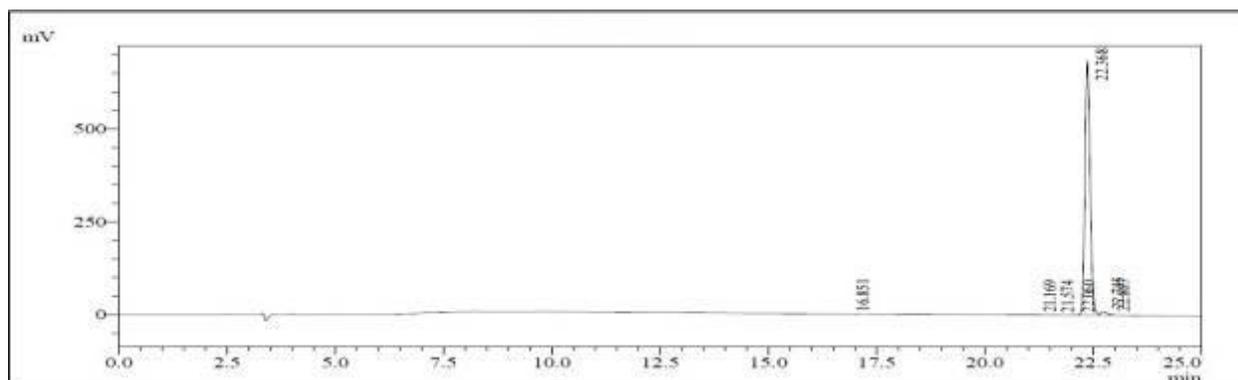
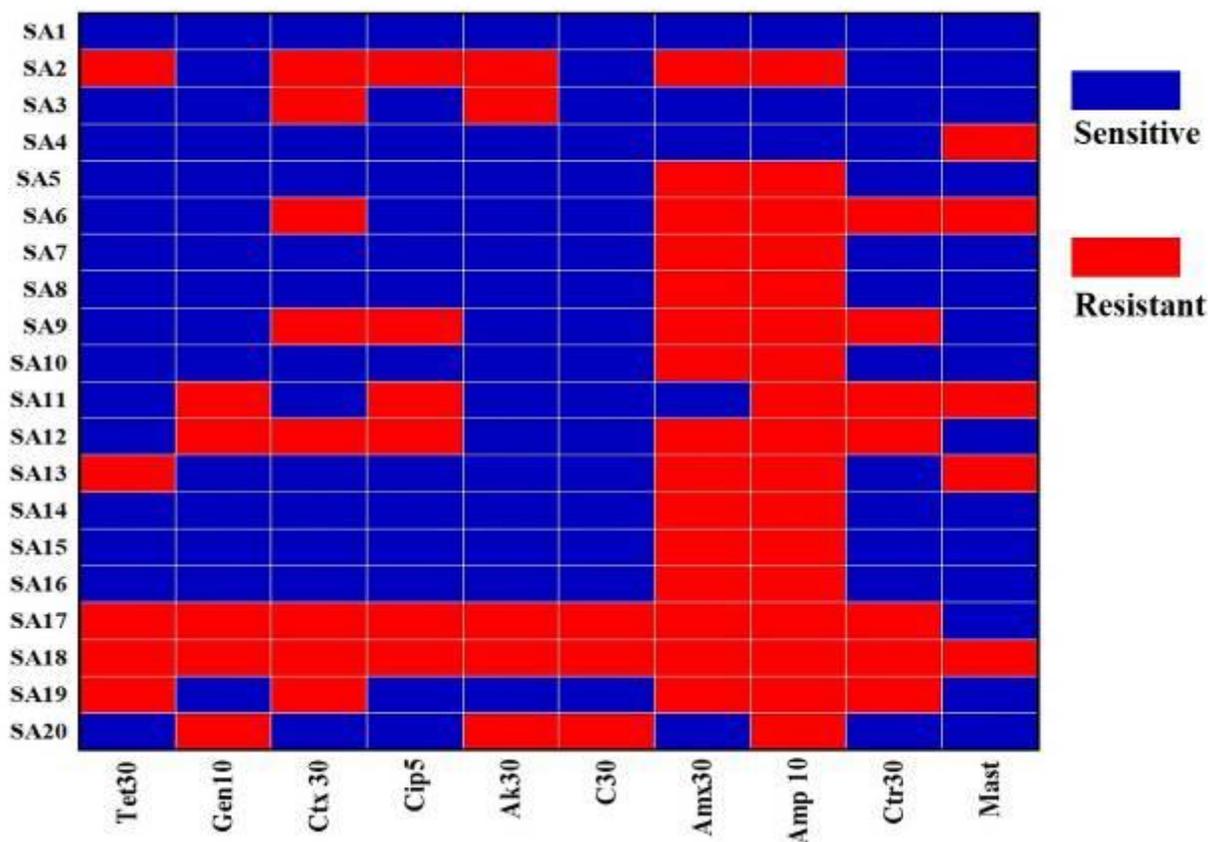


Fig.3 Heat map showing antibiogram of *S. aureus* isolates. The synthetic AMP Mastoparan was found to be active against most of the isolates some of which were resistant to many conventional antibiotics



Antimicrobial assays

Antimicrobial activity of AMP Mastoparan was screened against *S. aureus* using a range of AMP concentrations viz. 5 to 50 $\mu\text{mol L}^{-1}$.

Mastoparan was found to exhibit potent antimicrobial activity against Gram-positive bacteria *S. aureus*. Minimum inhibitory concentration (MIC) of Mastoparan against *S. aureus* was found to be 25 $\mu\text{mol L}^{-1}$. At this

concentration, no increase in OD600 was observed after overnight incubation at 37°C.

Minimum bactericidal concentration (MBC) of Mastoparan against *S. aureus* (ATCC 29213) was determined by sub-culturing the broth used for MIC determination (after 24 hours) on to fresh agar plates. MBC, the lowest concentration of the peptide that results in killing at least 99.9% of bacteria, was expressed as x MIC (times MIC). MBC of AMP Mastoparan was found to be twice the MIC concentration (2 X MIC) *i.e.* 50 µmol L⁻¹. No bacterial growth was observed on the plates where bacterial cultures exposed to 50 µmol L⁻¹ concentration of Mastoparan were spread.

Isolation and Antibiogram Study of Field Isolates

A total of 20 antibiotic-resistant isolates of *S. aureus*, were isolated from milk samples. Antibiotic sensitivity test (ABST) was done for these isolates against a battery of standard antibiotics (*viz.* tetracycline, gentamicin, cotrimoxazole, ciprofloxacin, amikacin, chloramphenicol, amoxicillin, ampicillin and ceftriaxone) by disk diffusion method as per Clinical and Laboratory Standards Institute guidelines (CLSI, 2016).

These isolates were also screened for their sensitivity towards synthetic AMP Mastoparan. The antibiogram of these isolates is summarized in Figure 3. It was observed that out of the 20 field isolates of *S. aureus* two isolates, (SA17 & SA18) were resistant to all the 9 conventional antibiotics. Only two isolates (SA1 & SA4) were sensitive to all the 9 antibiotics, while other isolates were resistant to at least two or more antibiotics. Interestingly, only 5 isolates (SA4, 6, 11, 13 & 18) were not inhibited by Mastoparan at 1MIC concentration, rest all isolates got inhibited with Mastoparan.

It was also observed that chloramphenicol was the most active antibiotic against field isolates of *S. aureus*. While, ampicillin was the least effective antibiotic followed by amoxicillin, cotrimoxazole, ciprofloxacin, ceftriaxone, gentamicin, amikacin and tetracycline. It was also observed that the synthetic AMP Mastoparan was active against 15 isolates of *S. aureus* out of which one isolate (SA17) was resistant to all the 9 antibiotics tested.

Milk is considered the most complete food globally, however, it may also be a potential source of various pathogenic microbes. Nonetheless, there have been various reports of economic losses and spread of food-borne diseases due to microbial contamination of milk or milk product. In addition to that, bacteria isolated from milk have been reported to harbour drug-resistant microorganisms imposing severe public health hazards. In this context, antimicrobial peptides are considered a potent alternative to existing antibiotics. Mastoparan is one of such potent antimicrobial peptide for combating drug-resistant bacteria. However, further studies on the spectrum of activity, safety and stability of this antimicrobial peptide needs to be evaluated in detail.

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