Characterization of Antimicrobial Peptides from Local Forest Dwelling Ants: In-vitro Screening for Antimicrobial Activity

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ABSTRACT

Antimicrobial peptides (AMPs) recognized as host defense peptide (HDPs), have achieved considerable attention during last two decades as potential components of the instinctive immune system. Amongst the insects overcasted for AMPs the hymenopterans comprising of ants have been identified for possessing potent antimicrobial peptides, those are small linear peptides. An investigation was taken up to screen for antimicrobial peptides collected from ants thriving in Forest Plantation in Hijli of Midnapore District, W.B. Three species of ants, members of the family Formicidae, were collected for in vitro antimicrobial activities of the whole body extract. These were tested by in the 10% of acetic acid solution using the Kirby-Bauer disc diffusion method against test microorganism viz. *Escherichia coli*, *Staphylococcus mutans*, *Vibrio cholerae* and *Candida albicans*. A strong antimicrobial activity was noted in all the extracts against all the microorganisms. The Spectrophotometric scanning was carried out to confirm the presence of peptides. TLC and HPLC were done. The result revealed that all the three species of ants exploited in this study were found to be a promising source of antimicrobial peptides.

Keywords: Antimicrobial peptides, *Escherichia coli*, Formicidae, HPLC, TLC.

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I. INTRODUCTION

Majority of living organisms are susceptible to harmful pathogen ingestion and inhalation which are indeed potential. The endurance of such organisms in a microbe thriving environment depends on a network of host defence system involving a variety of components. Pathogens that penetrate immediately the first line of defence involves the innate immune responses which in turn are followed by acquired immune response involving the activation of T and B cells expression against particular antigens. In the perspective of a distinct approach these acquired immune mechanisms, endogenous peptides, [1] which are constitutively appeared or induced (in a few cases), provides a fast and useful means of defence against the pathogen. Those molecules identified as Antimicrobial Peptides (AMPs) which constitutes primitive immune defence mechanisms and is found in the extensive range of eukaryotic organisms, from human to plants and again to insects. Most of these peptides encoded by genes are mobilized shortly after microbial infections and act rapidly to neutralize a wide range of microbes and usually share several common properties. For instance, most of these abundant groups of antimicrobial peptides are less than 10 KDa, however the overall structure is similar having a net positive charge, hydrophobicity and are membrane active. It is now evident that antimicrobial peptides are primary elements of the innate immunity against microorganisms specially bacteria and fungi in both the animal and the plant kingdom [2]-[4]. Of the remarkable number of

known antimicrobial structures at least 50% were identified in invertebrates and major portion within insects. In 1981, Boman and colleagues isolated and fully characterized the inducible antibacterial peptide from the diapausing pupae of the giant silk moth, *Hylaphora cecropia* [5] Insect cecropins are highly effective against Gram-positive bacteria [6] since the original report of cecropin from Boman, claimed the accessibility of more than 170 antimicrobial peptides/polypeptides in insects. They shared few common features like relatively low molecular weight (below 5 kDa), and are positively charged at physiological pH, while for most of them, amphiphilic α -helices or hairpin-like β -sheets or mixed structures. In the perspective structural features, these peptides can be broadly classified into three categories (i) linear peptides constituting α -helices and devoid of cysteine residues; (ii) cyclic peptides containing cysteine residues and (iii) peptides with an overrepresentation in proline and/or glycine residues.

II. MATERIALS AND METHOD

A. Ant Samples Collection

The worker ants of family Formicidae were collected using a brush and forceps from their natural habitat during day time within 2 KM of Hijli Forest range, of Midnapore Dist, W.B during the year of 2018. Prior to collection a small bud of cotton soaked in ether is inserted in the flask. The collected ants were isolated from nest debris and soil particles and after inactivating them those were transferred to 15 ml falcon tubes & stored at -

80 °C freezer. The frozen ants were sieved again in 1.7 mm sieve for separating the darts.

Fig. 1. Habitat of sample A.

Fig. 2. Habitat of sample B.

Fig. 3. Habitat of sample C.

Fig. 4. Sample A.

Fig. 5. Sample B.

Fig. 6. Sample C.

B. Identification of Ant Sample

The samples were identified up to the genus and species level in Zoology Laboratory, DDE, Vidyasagar University, Midnapore (W.B) by using binocular microscope and based on the literature available [7]-[11]. Species name, authority and spelling had been followed to Catalogue of Ants of the world for the Formicidae [12]. So, finally the samples were identified as Sample A – *Solenopsis geminata*, Sample B – *Solenopsis invicta*, and Sample C – *Camponotus compressus*.

C. Extraction and Purification of Peptides from the Supernatant of Whole Body Extract of Ant

Whole body extract of ant [13] is done to purify the total peptides present in the ant body. These total peptides will be used to investigate the presence of anti-microbial peptides. 10% acetic acid is prepared (Glacial acetic acid & ultrapure water at 1:9 ratios) and chilled at too. Motor pastel is pre-cooled at -80 °C freezer. The stored samples were taken out from the -80 °C freezer and 2 g of the sample is taken in chilled motor pastel. Liquid Nitrogen is added and immediately crushed to powder. The crushed powdered extract is collected in 50 ml Falcon tube. The powdered extract is suspended in 25 ml of pre-cooled 10% acetic acid solution and vortex vigorously. The homogenate extract is collected in 16 ml centrifuge tube and centrifuged in Sorvall RC 6+ centrifuge (SS34 fixed rotor) at 7000RPM (4 °C) for 25 min, the supernatant is now collected, and others are disposable. The supernatant of the extract is filtered by Whatman 41 filter paper to remove leftover debris after centrifugation. Then the filtrate was filtered in sterile filtration apparatus (0.22 µm filter membrane) by applying vacuum. The filtrate of the 0.22 µm filter membrane is taken upper chamber of the Millipore Amicon ultra filter (10 KD cut up) and centrifuged at 3500RPM (4 °C) for 10 min. The purified protein is collected in a fresh falcon tube and kept in 4 °C freezer.

D. Spectrophotometric Analysis of Purified Peptides

Spectrometric scanning [14] was done at wavelength from 200 nm to 800 nm in Varian Cary 50 split beam

spectrophotometer, to screen the presence of peptides in the purified sample. Absorbance vs wavelength has been plotted with the help of the instrument software.

E. Determination of Concentration of Peptide Sample by Bradford Method

The basic reagents are Bovine Serum Albumin (BSA), Coomassine Brilliant Blue G-250 as dye, Bradford reagent, along with spectrophotometer and Whatman no. 1 filter paper [15]. The maximum absorbance at 470nm when the dye turned into red, when it changes in anionic blue then the absorbance is 595 nm, and when it comes in neutral green the absorption is 650 nm. [16] Two standard solutions of 100 µg/ml and 200 µg/ml, standard protein is prepared using dilution 1mg/ml BSA solution. 20 µl standard solution with sample has taken into 5 micro centrifuge tubes. 100 µl of Bradford reagent is used to each of the protein solution and leftover for 10 mins. Reading was taken in Nanodrop spectrophotometer at 595 nm as per software protocol.

F. Concentrating of the Peptide Samples for Purification and Analysis

The sample is concentrated by freeze drying at -50 °C in Eyela FDU 1200 Freeze dryer for further analysis for antimicrobial activity, HPLC purification, TLC of the sample.

G. Kirby-Bauer Disc Diffusion Sensitivity Test

The disc diffusion method is more suitable for testing isolates are testing for susceptibility to numerous antimicrobial agents. A Mueller Hinton agar plate is equally inoculated with the sample organism and a paper disc had been saturated with a subtlet concentration of antimicrobial agent fixed on the surface of the agar [17]. Diffusion of the antimicrobial agent and the growth of organisms were placed in a circular zone of inhibition through which the antimicrobial agent overruns inhibitory concentration. By using of sterile cotton swab in the previously cultured bacterial sample removed the excess fluid. To obtain uniform growth, the swab streaked at least 3 directions over the Mueller Hinton agar. The plate left for 5 mins to dry out. After that Sample A, B, C were placed by a sterile force for 15 mins. The plates were incubated soon after placing the disc when the diffusion of AMPs and the growth of bacteria initiate at the same time. Need the overnight incubation, the diameter of the zone of growth of the inhibition were measured around each disc. Plates were examined carefully at the zone of inhibition. Disc diffusion method is again repeated after HPLC purification of the peptides, vacuum dried & re-suspended the sample in Phosphate Buffer saline pH 7.4.

H. Bacterial Sample Used Preparation for Testing Kirby-Bauer Disc Diffusion Sensitivity Test

Vibrio cholerae, *Escherichia coli*, *Candida albicans* and *Streptococcus mutans* have been used. Freeze dried bacterial culture were taken out from each vial inside bio-safety cabinet and included in a culture tube containing sterilized Luria Broth medium with inoculation loop. The culture tubes are incubated at 37 °C for 20 h till optimum growth of bacteria observed. For sub-culturing 50 µl of the confluent cell were inoculated in 10 ml of culture media in conical flask containing sterile Luria Broth medium. The flask was again

incubated at 37 °C till the cells become confluent. The culture has been taken out of the incubator and stored at 4 °C.

I. Separation of the Peptide Samples by TLC

The freeze dried sample is re-suspended in 20% CAN (Acetonitrile: Water, at 20:80). Mobile phase was prepared by mixing of acetone : butanol : water , at 60:20:20. The TLC plate was dried, Ninhydrin solution sprayed and the plate is heated on hot plate. Ninhydrin (2,2- Dihydroxyindane-1,3-dione) reacts with the free amine [18] of the separated peptides and produced Ruhemann's purple colour.

J. Separation of the Peptide Samples by HPLC

The freeze dried sample is re-suspended in 1ml of 20 aquas acetonitrile and purified in Agilant 1100 gradient HPLC system with Diode Array Detector and Zorobax SB C18, 5 µm, 4.6X, 250 nm reverse phase column was used for separating the sample [19]. The column was previously equilibrated with acetonitrile 50% (CH₃CN) and ultrapure water (Sartorius). 50 µl of the re-suspended sample was injected and eluted stepwise with 10%, 20%, 30%, 40%, & 60% CH₃CN gradient in 0.1% TFA/water. The eluting was detected in diode array detector at 210 nm and 280 nm wavelength. The eluted samples having absorbance at 210 nm were collected in Fraction Collector (Pharmacia). The separated peptides were dried in speed vac concentrator (EYELA) and tested for antimicrobial activity as per Kirby-Bauer's Disc Diffusion sensitivity test (W. Kirby & A. Bauer 1950s).

III. RESULT

A. Analysis of Peptides in Spectrophotometer

Spectrometric scanning was performed peptides obtained from Sample A has absorbance at 239.910 nm & 255.43 nm, for Sample B 239.910 nm, and for Sample C 206.52 nm & 219.94 nm.

Fig. 7. Spectrometric scanning of Sample A.

Fig. 8. Spectrometric scanning of Sample B.

Fig. 9. Spectrometric scanning of Sample C where X-axis indicates Abs and Y-axis indicates Wavelength (nm).

B. Bradford Assay of Peptides

After addition to Bradford reagent samples were incubated for 5 mins & standard curve was plotted by taking the average of 5 successive reading (595 nm) of standard as per instrument software protocol.

Spectrophotometric reading was taken for each of the sample and the sample concentration was examined from the standard curve with the help of software of the instrument. The concentrations of the unknown peptide samples are follows: 1. Sample A- 163 µg/ml, 2. Sample B-169.9 µg/ml, 3. Sample C- 141.3µg/ml.

Fig. 10. Standard curve of BSA standards of Bradford test.

C. Kirby- Bauer Disc Diffusion Sensitivity Test against Bacteria

The purified crude sample is used for testing present of any antimicrobial activity. After incubation for 18 h the culture plates were examined, all the peptide samples antimicrobial activity against *Vibrio cholerae*, *Escherichia coli*, *Candida albicans*, *Streptococcus mutans*.

TABLE 1: DIAMETER OF ZONE OF MICROBIAL GROWTH INHIBITION IS DISC DIFFUSION TEST (10KD PURIFIED SAMPLE)

Strains	Sample A	Sample B	Sample C
<i>Escherichia coli</i>	12 mm	12 mm	16 mm
<i>Vibrio cholerae</i>	11 mm	Nil	15 mm
<i>Candida albicans</i>	14 mm	11 mm	17 mm
<i>Streptococcus mutans</i>	16 mm	12.5 mm	18 mm

Fig. 11. Plate of *E. coli*. Fig. 12. Plate of *V. cholerae*. Fig. 13. Plate of *C. albicans*. Fig. 14. Plate of *S. mutans*.

D. TLC of Purified Peptides

The other part of the sample after freeze drying re-suspended 20% ACN & TLC was done. In TLC separation 3 bands were visualized after staining by Ninhydrin [20] for Sample A, 2 from Sample B, and 3 from Sample C. The Lane profiles were re-confirmed by scanning the TLC plate in densitometry scanner.

Fig. 15. Developed TLC plate (Ninhydrin).

Fig. 16. Densitometer image of TLC plate.

Fig. 17. Densitometric scan of TLC plate Intensity (X-axis) vs Pixel (Y-axis).

E. HPLC Separation of Peptides

Separated peptides in HPLC were detected at 210 nm and peptides were collected at 1.9 min for peptides of Sample A, at 3.9 min for peptides of Sample B, at 3.8 min for peptides of Sample C.

Fig. 18. DAD plot of HPLC separated peptides from sample A.

Fig. 19. DAD plot of HPLC separated peptides obtained from sample B.

Fig. 20. DAD plot of HPLC separated peptides obtained from sample C.

F. Kirby- Bauer Disc Diffusion Sensitivity Test against Bacteria with the HPLC Purified Sample

After incubation for 18 hrs the culture plates were examined, all the peptide samples have antimicrobial activity of the HPLC purified sample against *Escherichia coli*, *Candida albicans*, *Vibrio cholera*, *Streptococcus mutans*.

TABLE 2: DIAMETER OF ZONE OF MICROBIAL GROWTH INHIBITION IS DISC DIFFUSION TEST (HPLC PURIFIED SAMPLE)

Strains	Sample A	Sample B	Sample C
<i>Escherichia coli</i>	30 mm	30 mm	25 mm
<i>Vibrio cholerae</i>	12 mm	30 mm	25 mm
<i>Candida albicans</i>	18 mm	nil	nil
<i>Streptococcus mutans</i>	25 mm	20 mm	23 mm

Fig. 21. Plate of *S. mutans*.

Fig. 22. Plate of *E. coli*.

Fig. 23. Plate of *C. albicans*.

Fig. 24. Plate of *V. cholerae*, treated with HPLC fraction.

IV. DISCUSSION

A. Spectrophotometric Scanning of Purified Peptides

Though peptides having their max absorbance at 210 nm [14] but there is a slight shift of absorbance between 206.52 nm to 239.910 nm due to presence of more than one peptides in 10 KD cutup sample.

B. Bradford Assay

Coomassine Brilliant Blue binds with the arginine residue of the all the peptides and gave max absorbance at 595 nm.

C. Kirby-Bauer Disc Diffusion Sensitivity Test for Antimicrobial Peptides

In this test, the growth of *Escherichia coli*, *Candida albicans*, *Streptococcus mutans* are inhibited by peptides obtained from sample A, B, C. The growth of *Vibrio cholerae* is resisted by peptides of sample A & C only. The growth of the above mentioned bacterial strains are resisted due to presence of AMPs in the purified samples. As the samples were freeze dried before the test to remove residual amino acid used for extraction, so there are no chances of bacterial growth inhibition by amino acid. *Streptococcus mutans* is very sensitive to the AMPs of sample A, B, C. *Vibrio cholerae* is moderately sensitive to AMPs of sample A, strongly sensitive to sample C and not sensitive to sample B. *Escherichia coli* is very sensitive to the AMPs of sample A & C. And moderately sensitive to AMPs of sample B. *Candida albicans* is very sensitive to the AMPs of sample A, B, C. The purified fraction of HPLC shows strong antimicrobial activity against *Escherichia coli* and *Streptococcus mutans*. In *Candida albicans* peptide obtained from HPLC purification of sample has slightly inhibited in the growth at all. In *Vibrio cholera* peptides of sample A has very little effect of inhibition and peptides of sample B & C has slightly inhibited the growth.

D. TLC of Sample

As there is a shift of spectrophotometric absorbance from 210 nm, TLC was done to check to different peptides in the samples. Developed TLC plates have clearly shown separated distinct peptide bands. Peptides obtained from sample A

contains mixture of 3 peptides, A1, A2, A3. Peptides obtained from the sample B contain mixture of 2 peptides, B1, B2. Peptides obtained from sample C contain mixture of 3 peptides, C1, C2, and C3. The bands were reconfirmed by densitometry scanning and it also gave 3 peaks for peptides of sample A, 2 peaks for sample B, and 3 for sample C.

E. HPLC Purification of the Peptides

The samples were further separated in HPLC and detected in 210 nm by changing the polarity of peptides by applying a gradient of acetonitrile. Though the graph profile of sample A is shows 3 peptides in which 1 only separated in optimum quantity for further study. Only 1 peptide of sample B and more than 3 peptides show in sample C in which 1 only separated optimally.

V. CONCLUSION

Ants are well known for potential sources of antimicrobial peptides, so far, several antimicrobial peptides are isolated from several genera of ants. By purifying and chemical characterizing of these peptides from these ants, many unknown peptides are received which have antimicrobial activity on pathogenic microbes. By Kirby-Bauer's disc diffusion sensitivity test it is clearly stated that the sensitivity of bacteria to the ant's AMPs. *Streptococcus mutans* noted very strongly sensitive to all of three testing AMPs. The purified fraction of HPLC shows strong antimicrobial activity against *Escherichia coli* and *Streptococcus mutans*. These peptides can be artificially synthesized and can be used as therapeutic drugs. In recent years, AMPs attracted global attention as potential outbreak for therapeutic applications in animal and human health and agriculture. This had led to a flurry of research on insects to identify candidate AMPs in further process. Hence, the future prospect would be to identify more of such novel antimicrobial peptides and develop novel recombinant protocols to obtain greater yield of peptides at a lower cost.

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