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Isolation and characterization of haemolymph from insects

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Abstract

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Antimicrobial peptides play a crucial role in insect immunity against invading pathogens. AMPs of biological, especially from eukaryotic cells are explore morein recent days as pathogens are developing resistance against habitual antibiotics. The hemolymph of insect is a dynamic substance, which has many significant functions. Exploring more on these Antimicrobial peptides may serve as a new biotechnological protein based antibiotics. In this present study, hemolymph of immune-challenged moth *Eupterote mollifera* larvae was isolated and characterized. The Antimicrobial activity of hemolymph was tested against Gram-positive, Gram-negative bacteria and filamentous fungi. The extra cellular protein/peptide was concentrated by AMICON (MWCO-3kDa) ultra-centrifugal filters. The protein concentration was estimated by Bradford dye binding assay and then the haemolytic and haemagglutination activity of the in human RBC cells. Finally, the molecular weights of the protein/peptide were identified by using Tricine SDS-PAGE. Thus the expected antimicrobial protein/peptide, need to be isolated and cloned for large scale antibiotic production.

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Introduction

Insects (Class: Insecta/Hexapoda) are the largest class of Arthropods, characterized by segmented bodies, jointed legs, and exoskeletons. Their bodies are divided into three regions: the head (with sensory organs), the thorax (with legs and wings), and the abdomen (housing digestive and reproductive organs). Insects play vital ecological roles, including decomposition, pollination, and serving as predators. They also provide

commercially valuable products like honey, silk, and wax but can cause agricultural damage.

Haemolymph: This fluid in the circulatory system of arthropods, including insects, transports nutrients and proteins but does not primarily function in oxygen transport like vertebrate blood. Haemolymph contains hemocytes, contributing to the insect's immune system, and antimicrobial peptides (AMPs), which are a major component of innate immunity. AMPs are promising

alternatives to antibiotics due to their broad-spectrum activity and low resistance development.

Study Focus: The research emphasizes the characterization of haemolymph from *Eupterote mollifera* (a pest of *Moringa oleifera*), investigating its antimicrobial peptides for potential antibiotic development. The study includes isolating and identifying these peptides, analyzing their genetic sequences, and exploring their application through gene cloning for cost-effective large-scale antibiotic production. The objective of the study is isolation of haemolymph protein from insect.

Materials and methods

Source for haemolymph: Haemolymph was extracted from *Eupterotemollifera* moths (Family: Eupterotidae) via insect abdomen puncture.

Sterilization process: Water: Triple distilled and autoclaved at 121°C for 1 hour.

Laminar Air Flow Chamber: Cleaned with 70% ethanol and UV sterilized for 15–20 minutes.

Lab Wares: Washed, air-dried, wrapped in aluminum foil, and heated in a hot air oven at 185°C.

Rearing of moth larvae

Sample collection: Larvae collected from *Moringa oleifera* fields in Tamil Nadu, India.

Natural diet feeding: Larvae reared on fresh *Moringa oleifera* leaves. Containers cleaned daily to prevent death.

Immune challenge: Final instar larvae were injected with *E. coli* D31, maintained at 30°C, and haemolymph collected after 48 hours.

Haemolymph collection

Haemolymph was collected into chilled tubes containing anti-coagulant (sodium citrate buffer, pH 4.5).

Hemocyte-free haemolymph preparation

Centrifuged at 200 × g and 20,000 × g sequentially to

remove hemocytes and debris. Stored at -20°C.

Ultracentrifugation

Samples processed using ultracentrifugal filters (MWCO-3kDa), yielding >3kDa retentate and <3kDa filtrate.

Protein estimation

Performed using Bradford assay, measuring absorbance at 595 nm.

Antimicrobial activity

Antibacterial activity: Colony Counting Assay: Evaluated bacterial growth inhibition against *E. coli*.

Well diffusion assay: Tested against multiple bacterial strains (*E. coli*, *K. pneumoniae*, *S. aureus*, *B. subtilis*). Zones of inhibition measured.

Antifungal activity: Assessed against yeast strains (*C. albicans*, *C. tropicalis*, *C. glabrata*) by monitoring optical density after incubation with peptides.

Haemolytic assay

Tested haemolytic activity of antimicrobial concentrate on human erythrocytes. Calculated percentage hemolysis using spectrophotometric absorbance at 450 nm.

Haemagglutination activity

Evaluated red blood cell agglutination in response to antimicrobial concentrate.

Antimicrobial peptide identification: Tricine SDS-PAGE: Used silver nitrate staining to visualize protein bands.

Zymogram analysis: Conducted electrophoresis followed by incubation with bacterial strains (*E. coli*, *B. subtilis*) to detect antimicrobial zones.

This comprehensive study involved isolating haemolymph, preparing it for testing, and evaluating its antimicrobial and cytotoxic properties

Results and discussion

Sample collection and rearing

Eupterotemollifera larvae were collected from Moringa fields in Tiruchirappalli, Tamil Nadu, and reared on Moringa leaves in plastic containers under natural conditions.

Immune challenge

The larvae were injected with *E. coli* D31 to induce antimicrobial activity. After 48 hours, hemolymph was collected and separated into two components: retentate (>3 kDa) and filtrate (<3 kDa).

Protein estimation (Bradford method)

Protein concentrations in the retentate and filtrate were determined to be 33.12 mg/ml and 23.34 mg/ml, respectively.

Colony counting assay

The bacterial colony count of *E. coli* cultures showed higher colony formation in the filtrate (<3 kDa) compared to the retentate (>3 kDa), with the control group exhibiting the highest CFU.

Antibacterial activity (Well diffusion assay)

The retentate demonstrated antibacterial activity against *E. coli* and *B. subtilis*, with inhibition zones observed at various concentrations. The filtrate showed activity against *B. subtilis*.

Antifungal activity assay

The peptides showed varying antifungal activity: 3 kDa inhibited *C. albicans* growth by 34%. <3 kDa showed minimal activity.

Hemolytic assay

The protein/peptide exhibited low cytotoxicity, with only marginal hemolysis (1.9%) at concentrations up to 3.3 mg/ml, indicating its safety for use.

Hemagglutination assay

The protein at 3.3 mg/ml showed hemagglutination

activity, while lower concentrations did not.

SDS-PAGE analysis

Tricine SDS-PAGE revealed the antimicrobial peptides to have molecular weights between 20-23 kDa.

Conclusions

The present investigation was carried out *Eupterote mollifera* moth larvae. They were collected from the *Morniga oleifera* fields. The rearing was carried out in the natural environment. The larvae were immune-challenged by an injection of live *Escherichia Coli* D31. After the treatment, hemolymph was collected after 48 hours in sterile condition. Hemocyte free hemolymph was prepared, and the sample was concentrated using ultra centrifugation process. The peptide concentration determined by Bradford method. The concentration of the peptide is 33.12mg/ml. The cell count was carried out by using 24hrs of *E.coli* culture. The final average CFU values of two different samples (>3kDa and <3kDa) were calculated by two different dilution ratio (10^{-5} and 10^3). For >3kDa, no of colonies were found in 10^{-5} ratio is 204 and 10^3 is 256. For <3kDa, no of colonies were found in 10^{-5} ratio is 304 and 10^3 is 392.

The hemolymph of *E.mollifera* moth antibacterial activities were tested by Gram Positive and Gram Negative Bacteria. The selective bacteria's are *B.subtilis*, *E-coli*, *S. aureus*, *K. pnemoniae*. After incubation, the inhibition diameters were observed in *E-coli* and *B. subtilis*. The hemolymph of *E.mollifera* moth antifungal activities were tested by using the following fungi, *C. albicans*, *C.tropicals* and *C.olahrata*. After 24hr incubation the fungal activity was determined by measuring the optical density (OD) values at 450nm. The *E. mollifera* peptides were also effective in inhibition of filamentous fungi growth. In >3kDa protein/peptide sample, there is an inhibition growth in *C.albicans*- 34%, *C.tropicalis*-6.46% and *C.olahrata*-11.65%. The cytotoxic effect of the protein/peptide was tested at different concentrations.

The results of toxicity experiments revealed that the concentrate protein/ peptide of *E.mollifera* investigated in the present study exerted in significant. toxicity and only marginal haemolysis (1.9%) was detected at the concentrations up to 3.3 mg/ ml. The protein of concentration 3.3mg/ml was found to be haemagglutinating the human RBCs. The

concentrations from 1.6mg/ml-0.002µg/ml were not found to be haemagglutinating. SDS-PAGE was performed by the method of (Laemmli, 1970). The molecular weight of the peptide was found to be 20-23kDa by Tricine SDS-PAGE of Silver Nitrate staining.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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