

ANTIFUNGAL AND CYTOTOXICITY EFFECT OF IMMUNE HEMOLYMPH PROTEIN FROM EUPTEROTE MOLLIFERA LARVAE

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ABSTRACT - 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. Immune challenged Hemolymph were collected from the larvae and prepared hemocyte free hemolymph by centrifugation process. The Antifungal activity and minimum inhibitory concentration (MIC) of purified hemolymph was tested against *Candida albicans*, *Candida tropicalis*, *Candida olahraga* were grown in YPD medium. The haemolytic and haemagglutination activity of the concentrate protein/peptide of the extracellular proteins obtained from Eupterote Mollifera was studied against human RBCs. 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.

Key Words: Eupterote Mollifera, Hemolymph, Antifungal, MIC, Haemolytic, Haemagglutination, Human RBCs, Tricine SDS-PAGE, Antimicrobial peptides.

INTRODUCTION

Insects are particularly resistant to microbial infections, although they do not have an acquired immune system that is capable of specifically recognizing and selectively eliminating foreign microorganisms and molecules (i.e., foreign antigens). The present investigation was taken up to study of haemolymph from Eupterote molifera moth (Eupterotidae: Lepidoptera), a common pest of *Moringa oleifera* tree. The haemolymph was chosen as a biological agent susceptible and modified by the pollutants changes in the protein content of the haemolymph may reflect specialization and adaptation in the organisms based on subtle metabolic alterations, and may possible be used as taxonomic tool (Marynard, 1960). Antimicrobial peptides are synthesized in response to microbial infection or septic body injury mainly in insect fat body (functional equivalent of mammalian liver) and in certain blood cells, and then rapidly released into hemolymph where they act synergistically against microorganisms (Hetru., et al, 1998; Tzou., et al, 2002; Irving.,et al,2004). Peptides exhibiting antimicrobial activity are mainly small (≈ 5 kDa), amphipathic and cationic molecules (Bulet et al., 1999).

Generally, AMPs are assumed in the near future as an alternative for the nowadays classical antibiotics. The advantages of AMPs are: selectivity, fast killing, broad antimicrobial spectra and no resistance development (Boman, 2003; Matsuzaki, 1999). These peptides possess broad-spectrum antifungal and minimum inhibitory concentration activity against *Candida albicans*, *Candida tropicalis*, *Candida olahraga* were grown overnight in YPD medium (Abhishek Mathur et al., 2011). Ultra-centrifuged concentrate exhibits haemagglutination activity studied against human RBCs (Liu et al., 2007). SDS-Polyacrylamide gel electrophoresis was performed on slab gel with separating and stacking gels (10 & 5 % w/v) by the method of (Laemmli, 1970). 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 insignificant toxicity and only marginal haemolysis was detected.

MATERIAL AND METHODS

HEMOLYMPH COLLECTION

Hemolymph samples were collected by puncturing larval head with sterile needle. Out-flowing hemolymph was immediately transferred into sterile and chilled Eppendorf tubes containing an equal volume of an ice cold anti-coagulant (pH 4.5).

PREPARATION OF HEMOCYTE FREE HEMOLYMPH

The hemocyte free hemolymph was obtained by centrifugation for 5 minutes to pellet hemocytes and subsequently the supernatant was spun down for 15 minutes at 4°C to pellet cell debris. Finally 5ml of hemolymph samples were collected. The obtained hemocyte free hemolymph was stored at -20°C until use.

ULTRA-CENTRIFUGATION

Ultra-centrifugation of hemolymph was done using amicon ultra-centrifugal filter (MWCO-3kDa). Sample centrifuged at 7000g for 45min at 4°C. Centrifugation resulted in (>3kDa) of retentate (<3kDa) of filtrate.

ANTI-FUNGAL ASSAY

For antifungal activity assays, yeasts (*C.albicans*, *C.tropicalis*, *C.olahrata*) were grown overnight in YPD medium (0.1% yeast extract, 0.05% peptone, 0.2% dextrose) at 30 °C. Yeast suspensions were diluted with a fresh YPD medium, grown for an additional 6 h and diluted to A600 = 0.002. Aliquots (10 ml) of the culture were incubated with purified peptides (1 ml) for 24 or 48 h at the proper temperature, diluted 10 times and their optical density was measured. (Malgorzata et al., 2007).

HAEMOLYTIC ASSAY

The haemolytic activity of the antimicrobial concentrate on human erythrocytes was determined using the method described by Yadav et al., 2005.

CALCULATION OF PERCENTAGE OF INTACT ERYTHROCYTES

$$\% \text{ of intact erythrocytes} = 1 - \left[\frac{\text{Absorbance of sample} - \text{Absorbance of PBS}}{\text{Absorbance of lysis buffer} - \text{Absorbance of PBS}} \times 100 \right]$$

Formula obtained from the study carried out by Lupetti et al., 2008

CALCULATION OF PERCENTAGE OF HEMOLYSIS

$$\% \text{ of hemolysis} = 100 - (\% \text{ of intact erythrocytes})$$

HAEMAGGULTINATION ACTIVITY ASSAY

In view of the findings the concentrate exhibits haemagglutination activity (Liu et al., 2007), a serial 2-fold dilution of a solution of the antimicrobial concentrate (3.3 to 0.025 mg ml⁻¹) was added in microtitre plates, wherein 50 µl was mixed with 50 µl of a 2.0% suspension of human red blood cells in PBS (pH 7.2) at 20°C. The results were observed after about 1 h when the blank without antimicrobial concentrate was fully sedimented to inspect whether the red blood cells had agglutinated in response to the antimicrobial peptide/protein.

IDENTIFICATION OF ANTIMICROBIAL PEPTIDE BY TRICINE SDS-PAGE WITH SILVER NITRATE STAINING

SDS-Polyacrylamide gel electrophoresis was performed on slab gel with separating and stacking gels (10 & 5 % w/v) by the method of (Laemmli, 1970).

RESULTS AND DISCUSSION

ANTIFUNGAL ACTIVITY ASSAY:

The hemolymph of *E.mollifera* moth antifungal activities were tested by using the following fungi, *C. albicans*, *C.tropicalis* and *C.olahrata*. After 24hr incubation the fungal activity was determined by measuring the optical density (OD) values at 450nm. Inhibition percentage value was calculated by the following formula,

$$\text{Inhibition (\%)} = \left[\frac{\text{control OD} - \text{Sample OD}}{\text{Control OD}} \right] * 100$$

The *E.mollifera* peptides were also effective in inhibition of filamentous fungi growth (Table 4.4). In >3kDa protein/peptide sample, there is an inhibition growth in *C.albicans*- 34%, *C.tropicalis*-6.46% and *C.olahrata*-11.65%. In <3kDa protein/peptide sample, there is an inhibition growth in *C.albicans*- 23.4%, *C.tropicalis*-0.5% and *C.olahrata*-1.07%.

Table 1: Antifungal Activity Assay-OD value

S. No	Sample	OD		
		C. albicans	C. tropicalis	C. olahrata
1	<3KDa	0.729	1.013	0.987
2	>3KDa	0.849	1.077	1.105
3	Control	1.108	1.083	1.117

Table 2: Antifungal activity Assay- Inhibition value

S. No	Sample	Inhibition %		
		C. albicans	C. tropicalis	C. olahrata
1	<3KDa	34	6.46	11.64
2	>3KDa	23.4	0.5	1.07

HAEMOLYTIC ASSAY

The cytotoxic effect of the concentrate protein/peptide of the extracellular proteins obtained from Eupterote Mollifera was studied against human RBCs. As mentioned in the table:3, the protein samples were treated with human RBCs for 1hr at 37 °C and the absorbance of the supernatant was measured at 450nm using UV spectrophotometer.

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 insignificant toxicity and only marginal haemolysis (1.9%) was detected at the concentrations up to 3.3 mg/ ml. The obtained % of hemolysis were mentioned below,

Table 3: Haemolytic assay

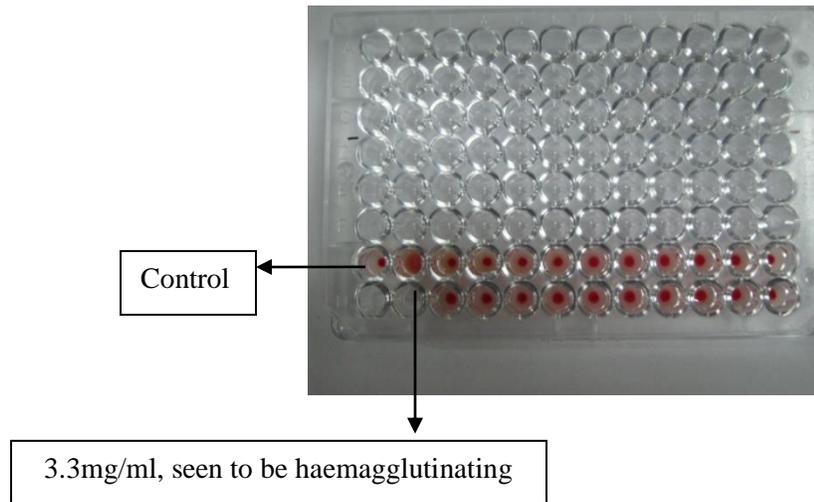
Sample	Concentration (µg/ml)	% of intact Erythrocytes	% of hemolysis
S1	0.33	98.09	1.91
S2	0.16	98.18	1.82
S3	0.082	98.19	1.81
S4	0.041	98.52	1.48
S5	0.020	98.67	1.33
S6	0.010	99.91	0.086
S7	0.0051	99.92	0.072
S8	0.0025	99.96	0.035

Since the concentrate protein/peptide of E.mollifera investigated in the present study exerted insignificant toxicity and only marginal haemolysis (1.9%) was detected at the concentrations up to 3.3 mg/ ml.

HAEMAGGULTINATION ASSAY

The concentrate protein/peptide obtained after the centrifugal filtration of the concentrate protein/peptide (concentration of the protein used 3.3mg/ml-0.002µg/ml) was subjected to haemagglutination test. PBS was used as a negative control (first well). The protein of concentration 3.3mg/ml was found to be haemagglutinating the human RBCs (RBCs have evenly coated the bottom of the well). The concentrations from 1.6mg/ml-0.002µg/ml were not found to be haemagglutinating (pellet was seen at the bottom of the well).

Figure 1: Haemagglutination assay

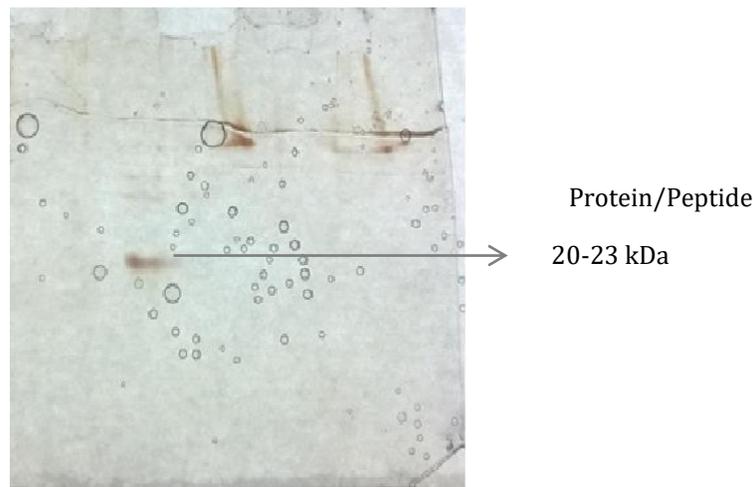


Microtiter U-plate showing the haemagglutination results (the last two rows). Here, the first well is a negative control. The second well shows the haemagglutination exhibited by 3.3mg/ml of the dialysed concentrate. Further wells of protein which were subjected to serial two fold dilution are not showing haemagglutination

IDENTIFICATION OF ANTIMICROBIAL PROTEIN/PEPTIDE BY TRICINE SDS-PAGE WITH SILVER NITRATE STAINING

The protein/peptide was successfully eluted through the gel and silver nitrate staining was done. The molecular weight of the protein/peptide was found to be 20-23kDa.

Figure 2: TRICINE SDS-PAGE with Silver nitrate staining



SUMMARY AND CONCLUSION

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 hemolymph of E.mollifera moth antifungal activities were tested by using the following fungi, C. albicans, C.tropicalis 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 insignificant 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 from the desired sample. The molecular weight of the peptide was found to be 20-23kDa by Tricine SDS-PAGE of Silver Nitrate staining.

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