Available online at www.ijpab.com

DOI: http://dx.doi.org/10.18782/2320-7051.2252

ISSN: 2320 – 7051

Int. J. Pure App. Biosci. **4 (2):** 296-303 (2016)

International Journal of Pure & Applied Bioscience

Research Article

OPEN ACCESS

Humoral Immunity studies of Fresh Mud Crab Scylla Serrata Forsskal, 1775

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Received: 27.03.2016 | Revised: 12.04.2016 | Accepted: 14.04.2016

ABSTRACT

Phenoloxidase is a key enzyme in the innate immunity of the crab. It protects the animal from injury, infection, hardening of exoskeleton and melanisation. Whereas Lysozyme is a bacteriolytic enzyme secreted by host during phagocytosis. It is one of the important enzymes in the innate immunity of the crab against gram positive and gram negative bacteria. In this study we have studied that Phenoloxidase activity increased from 2hrs to 48 hrs after challenge with gram positive and gram negative bacteria in case of male crabs. In case of female crabs challenged with bacteria, phenoloxidase activity gradually increased from 2hrs to 48 hrs of post bacterial inoculation. The results of the present study revealed that the enzyme activity in hemolymph of bacterial challenged crabs was highest in all time points than control and control injured crabs. The zone of inhibition increase gradually from 2hrs to 12 hrs in Klebsiella challenged male crab and female crab and also in male and female crabs challenged with Bacillus. The protein band observed at 16 kDa was identified as Lysozyme and 67kDa as Prophenoloxidase.

***Key words**: Prophenoloxidase, lysozyme, antimicrobial studies, Scylla Serrata*

INTRODUCTION

Mud crabs in particular act as burrowers; as a result they help in aeration, mixing and nutrient flow in the soil. Being burrower mud crabs enhance aeration and nutrient flow in the soil[1,2]. Consequently, soil fertility is augmented that helps in facilitating the survival of other plants and soil dwelling animals. In addition, bioturbation structures created by crabs trap sediments and mangrove seeds[3-5] and significantly contribute to the conservation of mangrove plants. One of the important roles of crabs particularly in mangrove environment is the production of millions of meroplanktonic larvae, which serve as potential food source for a large number of planktophagus organisms including rich number of edible fishes. Crabs stabilize the complex food web in the mangrove ecosystem. Despite its high commercial value, information on its biochemistry and physiology especially in relation to free radical mediated metabolism is scanty and needs attention for its use in aquaculture. Finally, it can be concluded that crustaceans like crabs may become susceptible to various stresses when they cross over the threshold value of several environmental factors[6,7].

Cite this article: Subhashini, S. and Nagarajarao, P., Humoral Immunity studies of Fresh Mud Crab Scylla Serrata Forsskal, 1775, *Int. J. Pure App. Biosci.* **4(2):** 296-303 (2016). doi: http://dx.doi.org/10.18782/2320-7051.2252

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Therefore, *S. serrata* are rightly known for its wide range of environment stress tolerance and attenuation of physiological homoeostasis particularly with respect to changing salinity[8-11] and temperature[12]. Different mechanisms on how these marine invertebrates adapt either at physical or physiological level to avoid the environmental stress are being explored by various workers[13,14]. As evidenced from the above, studies made in past on mud crabs were basically addressed to their characterization, distribution and taxonomy. However, not much work has been done on the physiology particularly on biotic stress related changes in protein, lipids of mud crabs. This metabolism is an important aspect of aerobes. A fluctuation in metabolism due to changes in biochemical parameters may have adverse effect on the metabolism of aquatic organisms by pushing them to stress condition known as "Biotic stress". Although there are some information available on Biotic stress and antioxidant defence system in invertebrates particularly in molluscs and insects, not much information is available on crabs.

MATERIAL AND METHODS

Estimation of Prophenol Oxidase Activity

ProPhenoloxidase activity of the haemolymph in control and challenged crabs at different time intervals was assayed following the procedure of Asokan *et al* 1997. 100 µl of heamolymph was pre incubated with the same volume of L-dihydroxyphenyl-alanine (L-DOPA) or with TBS for 20 min at 22°C. All incubation experiments were performed in the dark. The O.D. of both control and experimental was measured at 460 nm and increase in the absorbance was continuously monitered. Experiments were conducted at different time intervals. One unit of enzyme activity was defined as an increase of 0.001 in absorbance/min/mg/protein.

Estimation of Lysozyme Activity

Lysozyme Activity of the haemolymph in control and challenged crabs at different time intervals was assayed by using modified Turbidometric assay. Briefly, 0.3 mg mL^{-1} suspension of freeze-dried Micrococcus lysodeikticus was prepared in 0.05 M Na_2HPO_4 buffer immediately before use and the pH adjusted to 6.0 using 1.0 M NaOH. Ten microlitres of serum were added to 250 µL of the bacterial suspension and allowed to equilibrate at 28°C. Hen egg white lysozyme (HEWL), with a specified activity of 46 200 Units mg^{-1} was used as an external standard. The reduction in O.D. at 450 nm was determined over a 10 min period at 28°C in a microplate reader. The standard curve was constructed by using HEWL. The amount of lysozyme present in the serum was calculated from the standard.

Antibacterial Activity of Haemolymph

Antibacterial activity of the haemolymph was investigated by measurement of growth inhibition by radial diffusion method. Sterile Petridishes were prepared with LB Nutrient Agar medium (Peptone -10 g, Yeast - 5 g, Sodium Chloride - 10 g and Agar 15g for 1000mL) inside laminar airflow and the bacterial cultures was spread on the petridishes and 6mm wells were prepared. The heamolymph at different time intervals was added to the discs and the plates were allowed to incubate for 24 to 48 hours with lids down at 37°c and the zone of inhibition is measured by measuring scale.

Electrophoresis

Qualitative analysis of Proteins by SDS-PAGE:

The qualitative analysis of total soluble protein was done in haemolymph by using the Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) using a 5% (w/v) acrylamide stacking gel and a 12% (w/v) acrylamide separating gel.

A 5X stock solution of electrophoresis running buffer was prepared by dissolving 15.1gm of Tris base and 94 gm of glycine in 900ml of distilled water. To this, a 50 ml of 10% SDS solution was added and volume was adjusted to 1000ml with distilled water.

A staining solution was prepared was dissolving Silver Nitrate 0.25gm of Coomassie Brilliant Blue R-250 in 100ml of methanol: acetic acid solution. The solution was filtered through Whatman No.1 filter paper to remove the particulate matter, if any.

A vertical gel apparatus from Bangalore Genie was used. The glass plates, spacers and the apparatus was cleaned and dried properly. Glass plates and spacers were assembled together using bulldog clips. Then, 2% agar was applied around the edges of the spacers to hold them in proper place and to seal the chamber between

the glass plates. 40ml of separating gel mixture was prepared. After adding ammonium per sulphate and TEMED, the solution was mixed gently and carefully. The solution was then poured in the chamber between the glass plates. The gel was allowed to polymerize for 30-45minutes. 5% stacking gel was prepared as per the above contents, and poured above the separating gel. The comb was placed in the stacking gel and the gel was allowed to polymerize for 30-45 minutes. The comb was then removed without distorting the shape of the wells. The gel was installed carefully, after removing the clips and agar, in the gel electrophoresis apparatus. The upper and lower chambers of the apparatus were filled with 1X electrophoresis running buffer.

The haemolymph from crab was dissolved in sample loading buffer and heated for 30 min at 60ºc. This ensured the complete interaction between proteins and SDS. The sample solutions were cooled and a uniform quantity of (10µl) haemolymph was loaded to slots of the gel. The gel was calibrated with broad range molecular markers. A mixture of broad range Molecular weight marker was loaded in one slot to compare the molecular weight of the proteins separated from that of the samples. Then 10-15mA current was applied for initial 15-20 minutes i.e. until the samples travelled through the stacking gel. The current was the raised at 30mA till the Bromophenol blue reached the bottom of the gel (about 3hours).

After electrophoresis, the gel was removed from between the glass plates and fixed in 12% trichloroacetic acid for 30 minutes, and then in 10% acetic acid for another 30 minutes. The gel was stained in 0.25% Coomassie brilliant blue R-250, overnight, with uniform shaking. Proteins absorbed the Coomassie brilliant blue dye. After appropriate staining, it was destained in a mixture of distilled water, methanol and acetic acid (9:6:1 v/v). The dye that was not bound to proteins was thus removed. The destainer was changed frequently (30 minutes) particularly during initial periods, until the background of the gel was colorless and bands could be distinctly seen. The gel was then photographed.

Statistical Analysis

The SPSS software version 11.0 for Windows was used for the statistical analysis. Results are reported as mean ± S.D. of three individuals per group per time point. The data were processed by two-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison post hoc test to identify statistical differences. Pearson correlation coefficient was used in the correlation matrix.

RESULTS AND DISCUSSION

Estimation of Phenoloxidase Activity in the heamolymph of crab

Phenoloxidase activity was evaluated in control, control injured and male and female crabs challenged with Gram negative (*Klebsiella pneumonia)* and Gram positive (*Bacillus cereus*) bacteria at 2hrs, 6hrs, 12hrs, 24hrs and 48 hrs. The phenoloxidase enzyme activity levels were observed to be higher in challenged crabs than control and control injured crabs at all time intervals. Phenoloxidase activity gradually increased from 2hrs to 48 hrs in case of challenged male crabs and but in case of female challenged crabs the phenoloxidase activity constantly increased from 2hrs to 48 hrs of post bacterial inoculation (Fig 1, 2).

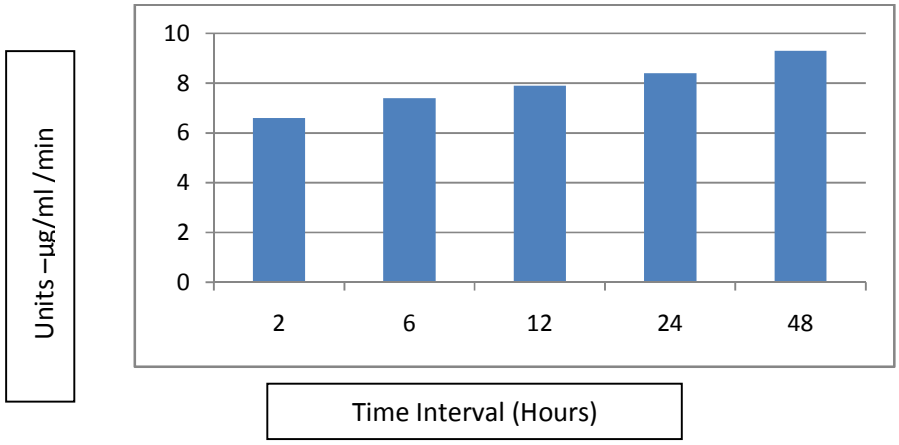

Fig 1: Amount of Prophenoloxidase (µg /protein/ml) in male crab

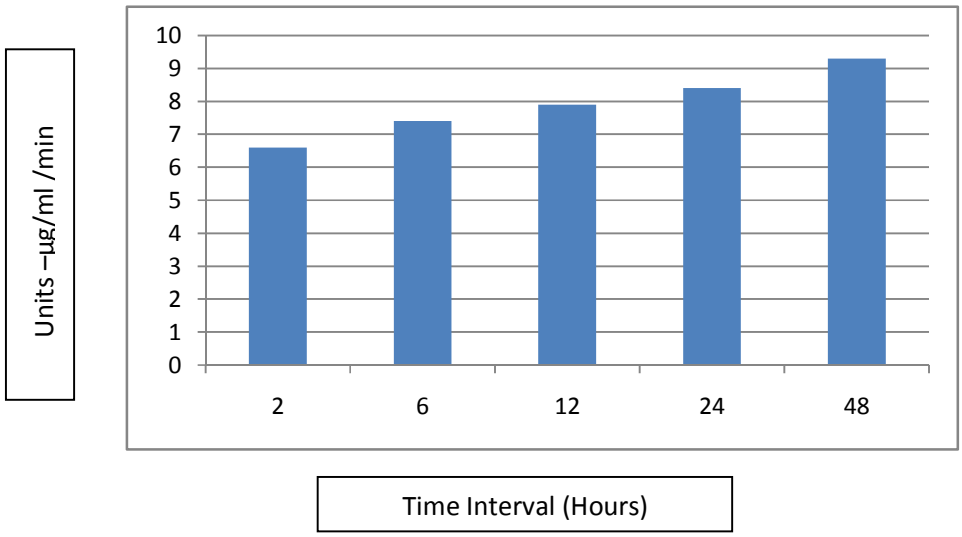

Fig 2: Amount of Prophenoloxidase (µg /protein/ml) in female crab

Estimation of Lysozyme Activity in the heamolymph of crab

Lysozyme activity was evaluated at 2hrs, 6hrs, 12hrs, 24hrs and 48 hrs in control, control injured and male and female crabs challenged with Gram negative (*Klebsiella pneumonia)* and Gram positive (*Bacillus cereus*) bacteria.

The enzyme activity in hemolymph of bacterial challenged crabs was highest in all time points than control and control injured crabs. Enzymatic activity gradually increased from 2hrs to 48 hrs in case of challenged male crabs and female challenged crabs of post bacterial inoculation. Highest enzymatic activity was observed at 48 hrs of post bacterial challenge (Fig 3, 4).

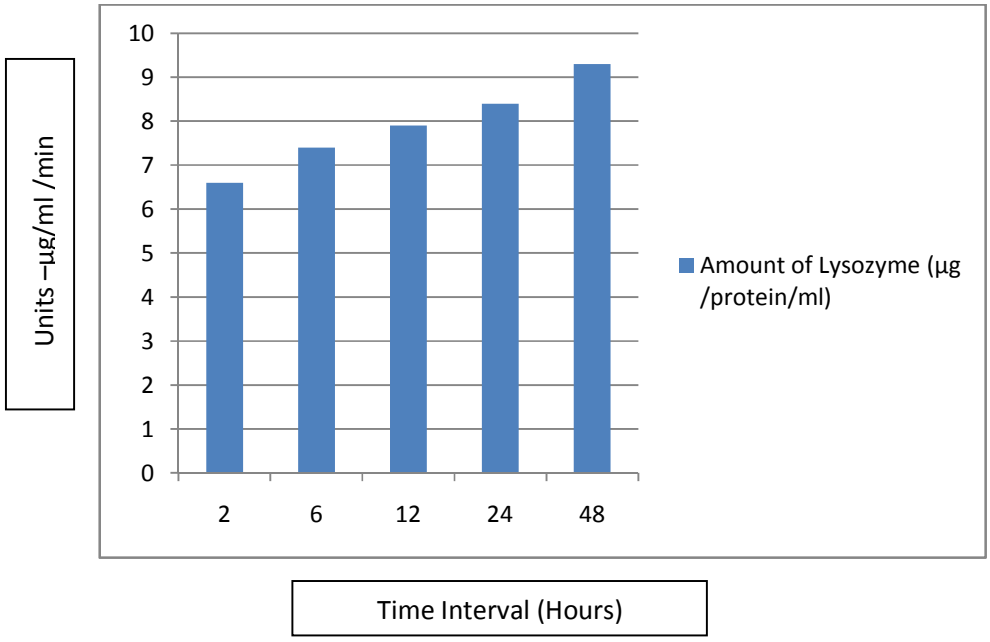

Fig 3: Amount of Lysozyme (µg /protein/ml) in challenged male

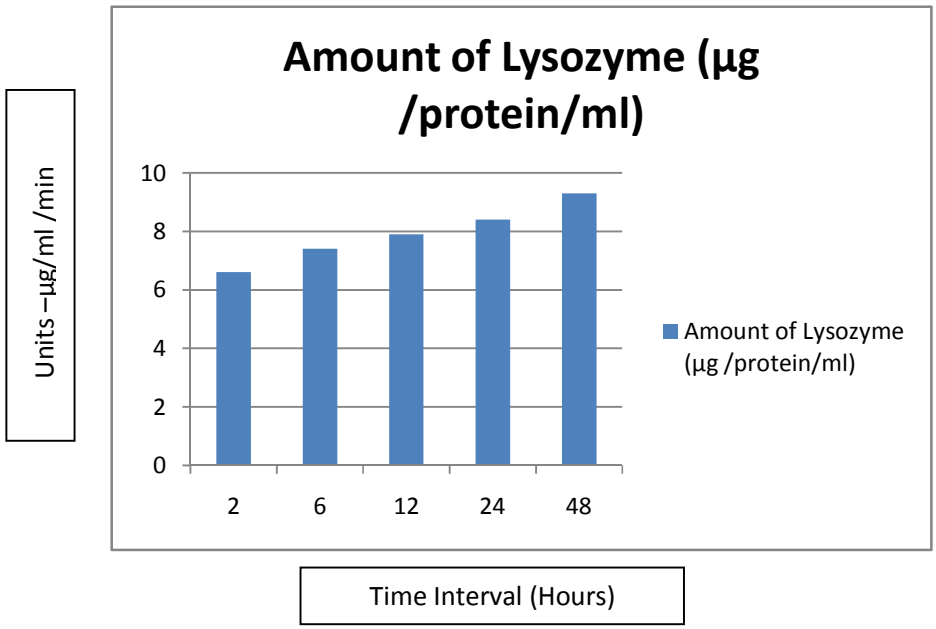

Fig 4: Estimation of Lysozyme in challenged Female crab

Estimation of Antibacterial Activity in the heamolymph of crab

Antibacterial activity was estimated by radial diffusion method in control, control injured and male and female cabs challenged with *Klebsiella pneumonia* and *Bacillus cereus.* The heamolymph of challenged crabs showed higher antibacterial activity when compared with control and control injured crabs at all time points from 2hrs to 48 hrs of post bacterial challenge.

The Result indicated that highest zone of inhibition observed at 12 hrs in case of *Klebsiella pneumonia* challenged crabs in male (3.6mm) and female crabs (4.1mm) (Fig 5, 6).

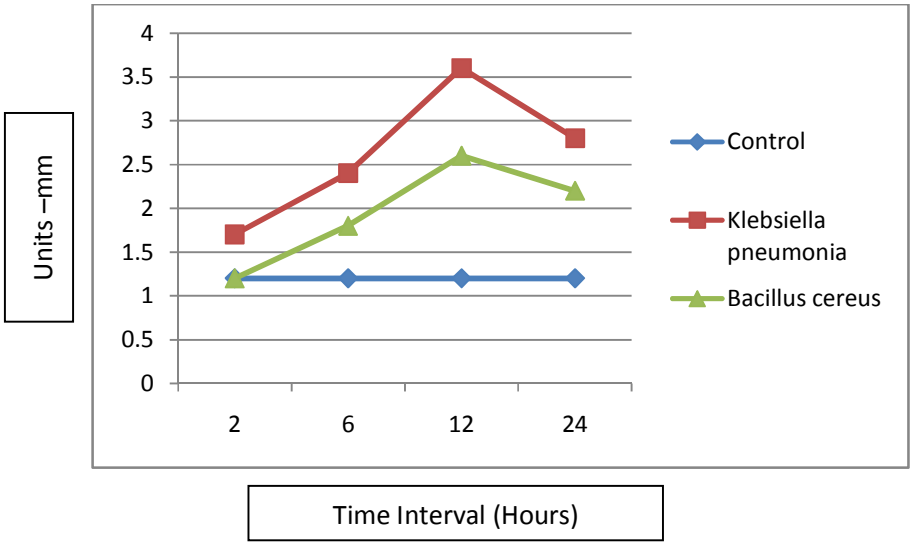

Fig. 5: Anti bacterial activity at different time intervals in the haemolymph of challenged male crabs

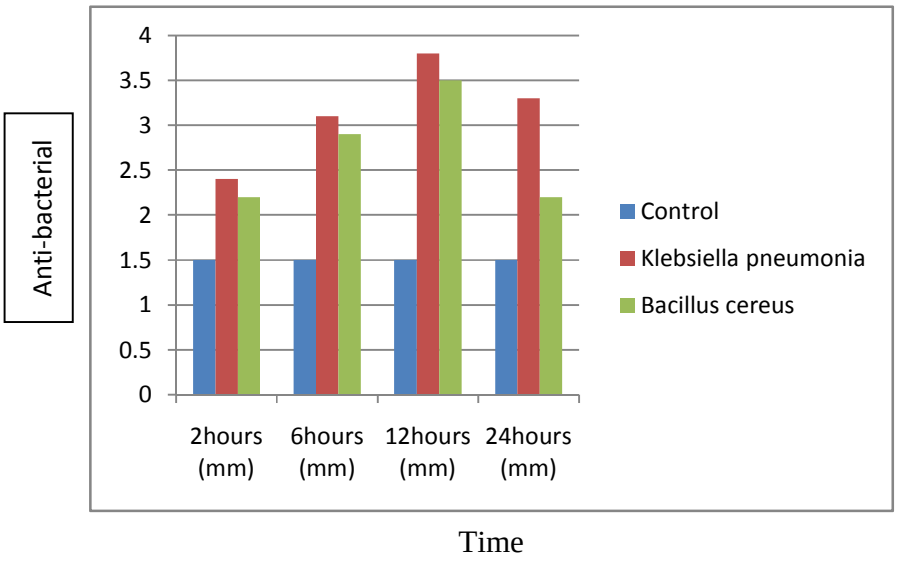

Fig. 6: Anti bacterial activity at different time intervals in the haemolymph of challenged female crabs

Identification of Antimicrobial Peptides by SDS PAGE (Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis)

In SDS PAGE it was observed that protein bands belong to different molecular weight ranging from 16 kDa to 205 kDa were obtained. In control male heamolymph sample the bands were observed at 16 kDa, 67kDa 120kDa to 205kDa. The bands observed in challenged male crab hemolymph are at 16kDa, 67kDa 120kDa to 205kDa. In the haemolymph of male crab the protein bands were observed at 16kDa, 67kDa 120kDa to 205kDa. The protein band observed at 16 kDa is Lysozyme and 67kDa is phenoloxidase (Fig 7).

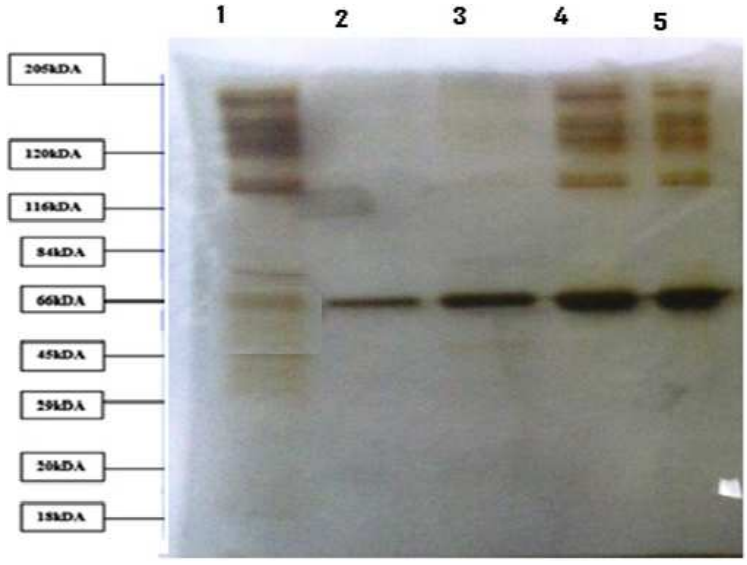

Fig. 7: Heamolymph of challenged Male crab, SDS PAGE

Lane 6 - Marker
Lane 5 – Heamolymph after 2 hrs challenge
Lane 4 – Heamolymph after 6 hrs challenge
Lane 3 – Heamolymph after 12 hrs challenge
Lane 2 – Heamolymph after 24 hrs challenge
Lane 1 – Heamolymph of control crab

On SDS PAGE it was observed that protein bands belong to different molecular weight ranging from 16 kDa to 205 kDa. In control female heamolymph sample the bands were observed at 16 kDa, 67kDa 120kDa to 205kDa. The bands observed in challenged female crab hemolymph are at 16kDa, 67kDa 120kDa to 205kDa. In the haemolymph of female crab the protein bands were observed at 16kDa, 67kDa 120kDa to 205kDa. The protein band observed at 16 kDa is Lysozyme and 67kDa is phenoloxidase (Fig 8).

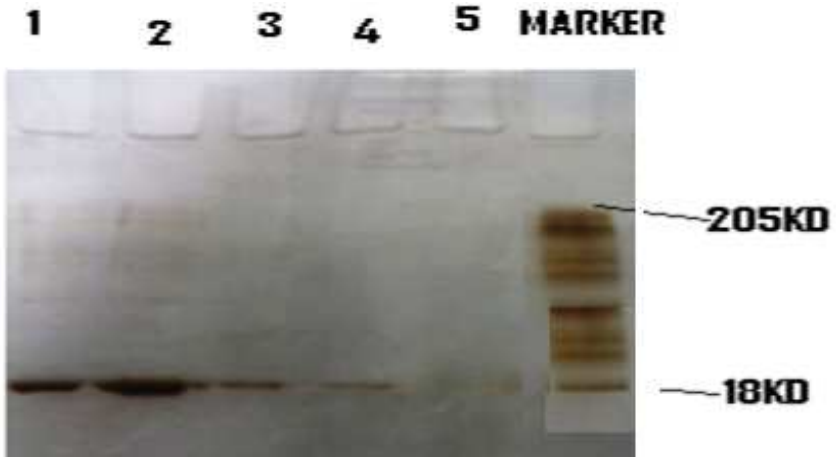

Fig.8: Hemolymph challenged female crab, SDS PAGE

Lane 6 - Marker
Lane 5 – Heamolymph after 2 hrs challenge
Lane 4 – Heamolymph after 6 hrs challenge
Lane 3 – Heamolymph after 12 hrs challenge
Lane 2 – Heamolymph after 24 hrs challenge
Lane 1 – Heamolymph of control crab

CONCLUSION

In the present study also similar results were observed, the Phenoloxidase activity gradually increased from 2hrs to 24 hrs and then started decreasing from 24hrs to 48hrs after challenge with gram positive and gram negative bacteria in case of male crabs. In case of female crabs challenged with bacteria, phenoloxidase activity gradually increased from 2hrs to 12 hrs and highest at 12hrs interval after challenge and started decreasing from 12hrs to 48 hrs of post bacterial inoculation. The results of the present study revealed that the Lysozyme activity in hemolymph of bacterial challenged crabs was highest in all time points than control and control injured crabs. Enzymatic activity gradually increased from 2hrs to 12 hrs and the decreased from 12hrs to 48hrs in case of challenged male crabs and female challenged crabs when challenged with *Klebsiella pneumonia* and *Bacillus cereus*. Highest enzymatic activity was observed at 12 hrs of post bacterial challenge. The Result in the present study indicated that highest zone of inhibition observed at 12 hrs in case of *Klebsiella pneumonia* challenged crabs in male crab and female crabs. The zone of inhibition increase gradually from 2hrs to 12 hrs in *Klebsiella pneumonia* challenged male crab and female crab and also in male and female crabs challenged with Bacillus cereus. In the present study on SDS PAGE it was observed that protein bands belong to different molecular weight ranging from 16 kDa to 205 kDa. The bands were observed at 16 kDa, 45 kDa, 67kDa 120kDa to 205kDa in male hemolymph sample. This study indicates that the haemolymph of crab would be a good source of antimicrobial agents and may be useful for provision of cost effective antibiotics. Further research on crab

immunology may lead to identification and synthesis of more useful antimicrobial peptides.

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