INTERNATIONAL JOURNAL OF PLANT, ANIMAL AND ENVIRONMENTAL SCIENCES
IJPAES
Volume-6, Issue-1, Jan-Mar-2016 Coden: IJPAJX-CAS-USA, Copyrights@2016 ISSN-2231-4490
Received: 4th Jan-2016 *Revised: 18th Jan 2016* *Accepted: 20th Jan 2016*
Research article

STUDIES ON HUMORAL IMMUNITY IN FRESH WATER CRAB *BARYTELPHUSA GUERINI*

Ch.Tulasi Mastanamma[1] and P.Nagaraja Rao[1]*

[1]Dept. of Zoology, OU College of Science, Osmania University, Hyderabad – 500 020, TS

ABSTRACT: Crustaceans contain two types of enzyme catalyzing phenol oxidation; one being hemocyanin and the other being expressed from hemocytes as its precursor prophenoloxidase. In the present work prophenoloxidase and lysozyme activity was studied in *Barytelphusa guerini*. Prophenoloxidase gradually increased from 2hrs to 24 hrs and then started decreasing from 24hrs to 48hrs after challenged 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 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 12 hrs and the decreased from 12hrs to 48hrs in case of challenged male crabs and female challenged crabs when challenged with *E.coli* and *S.aureus*. Highest enzymatic activity was observed at 12 hrs of post bacterial challenge. The highest zone of inhibition observed at 12 hrs in case of *E.coli* challenged crabs in male crab and female crabs. The zone of inhibition increase gradually from 2hrs to 12 hrs in *E.coli* challenged male crab and female crab and also in male and female crabs challenged with S.*aureus*. The protein band observed at 15 kDa was identified as Lysozyme and 66kDa as Prophenoloxidase.
Key words: Prophenoloxidase, Lysozyme, Antimicrobial studies

*Corresponding author: P.Nagaraja Rao, 1Dept. of Zoology, OU College of Science, Osmania University, Hyderabad – 500 020, TS Email: nagarajaraop@yahoo.com

INTRODUCTION

Prophenoloxidase, a Copper containing enzyme, initiates the biosynthesis of melanin and widely distributed in animals, plants fungi [1]. In crustaceans the Prophenoloxidase activation system is an important part of the host defense and functions to detect and kill invading pathogens and to synthesise melanin for wound healing and encapsulation of pathogens [2,3]. Prophenoloxidase (proPO) is complement response found in some invertebrates, including insects, crabs and worms [4].

In crustaceans, immune recognition is mediated by the prophenoloxidase cascade present in the hemocytes by the presence of non-self molecules inducing melanization reactions [2, 3, 5]. The phenoloxidase system is an efficient defense mechanism against the non-self and it can be activated by a minimum presence of microbes. This system is stored and produced by semi-granular and granular hemocytes. Melanin, a dark-brown pigment responsible for inactivating foreign particles, and preventing their spread throughout the host body, as well as for healing cuticle damages is produced on activation of the prophenoloxidase system [6].

Melanization of pathogens and damaged tissues is a major innate defense system in invertebrates controlled by the enzyme phenoloxidase (PO) [3,5].

Prophenoloxidase (proPO) from hemocytes is stimulated into phenoloxidase through a specific serine type protease, which is a latent enzyme, and is controlled by a cascade. Minute amount of cell wall components of microbes can induce this chain reaction, and invaders are destroyed by highly reactive quinines formed by activated phenoloxidase [7].

METHODOLOGY

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. The optical density values of both control and experimental heamolymph was measured at 460 nm and the absorbance was continuously monitored. Experiments were conducted at different time intervals. One unit of enzyme activity was defined as an increase of 0.001in 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 [8]. Hen egg white lysozyme (HEWL) was used as an external standard. The reduction in O.D. at 450 nm was determined over a 10 min period. The standard curve was constructed by using HEWL. The activity of lysozyme 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 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 [9].

Electrophoresis:

Qualitative analysis of Proteins by SDS-PAGE:

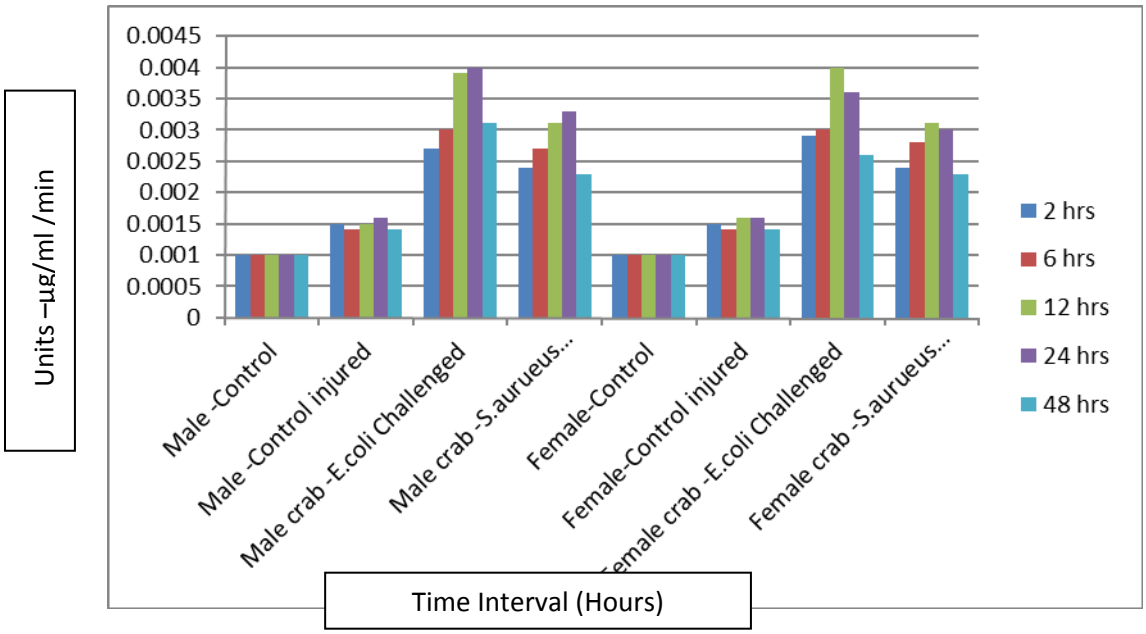
The qualitative analysis of total soluble protein was done in haemolymph by using the Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) as described by Laemmii Nursel G.L, Cevat Ayvali. The staining solution was prepared was dissolving Silver Nitrate 0.25gm of Coomassie Brilliant Blue R-250 in 100ml of methanol: acetic acid solution.

Proteins absorbed the Coomassie brilliant blue dye. After appropriate staining, it was de-stained and the gel was then photographed.

RESULTS AND DISCUSSION

Estimation of Phenoloxidase Activity in the heamolymph of crab:

Phenoloxidase is a key enzyme in the innate immunity of the crab. It protects the animal from injury, infection, hardening of exoskeleton and melanisation. Phenoloxidase activity was evaluated in control, control injured and male and female crabs challenged with Gram negative (*E.coli)* and Gram positive (*S.aureus*) 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 24 hrs and the decreased from 24hrs to 48hrs in case of challenged male crabs and but in case of female challenged crabs the phenoloxidase activity constantly increased from 2hrs to 12 hrs and highest at 12hrs interval and started decreasing from 12hrs to 48 hrs of post bacterial inoculation.

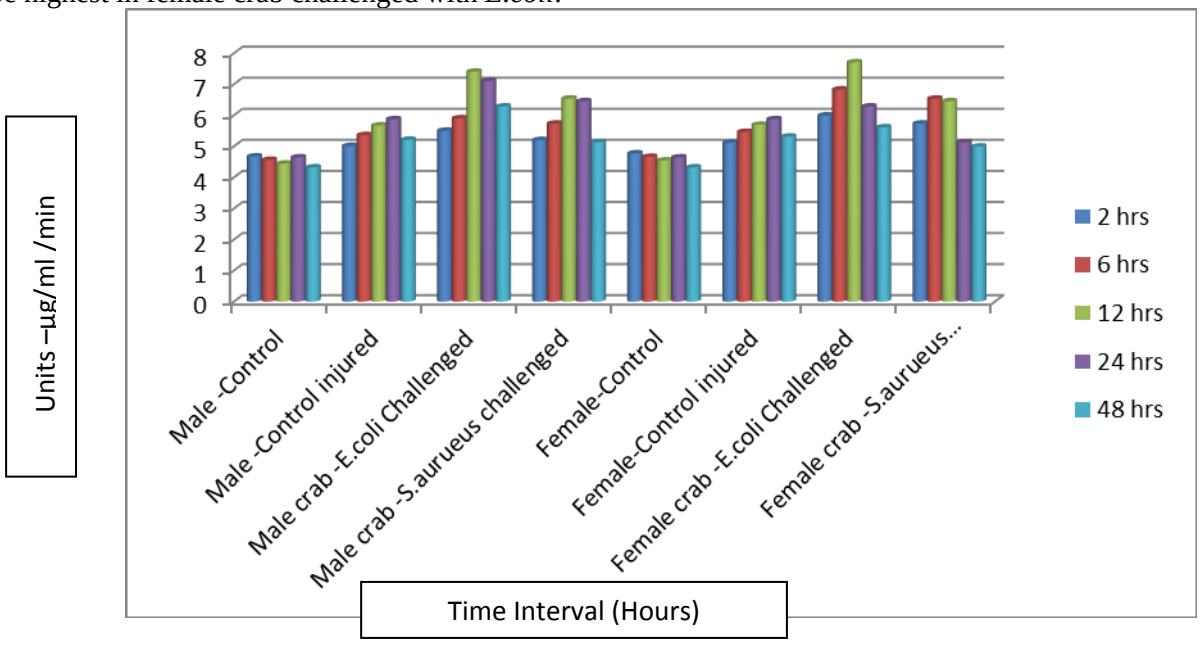


Graph – 1 : Prophenoloxidase at different time intervals in the haemolymph of challenged crabs

Estimation of Lysozyme Activity in the heamolymph of crab:

Lysozyme is a bacteriolytic enzyme secreted by host during pahgocytosis. It is one of the important enzymes in the innate immunity of the crab against gram positive and gram negative bacteria. 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 (*E.coli)* and Gram positive (*S.aureus*) 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 12 hrs and the decreased from 12hrs to 48hrs in case of challenged male crabs and female challenged crabs of post bacterial inoculation. Highest enzymatic activity was observed at 12 hrs of post bacterial challenge. The enzyme activity levels were observed to be highest in female crab challenged with *E.coli*.

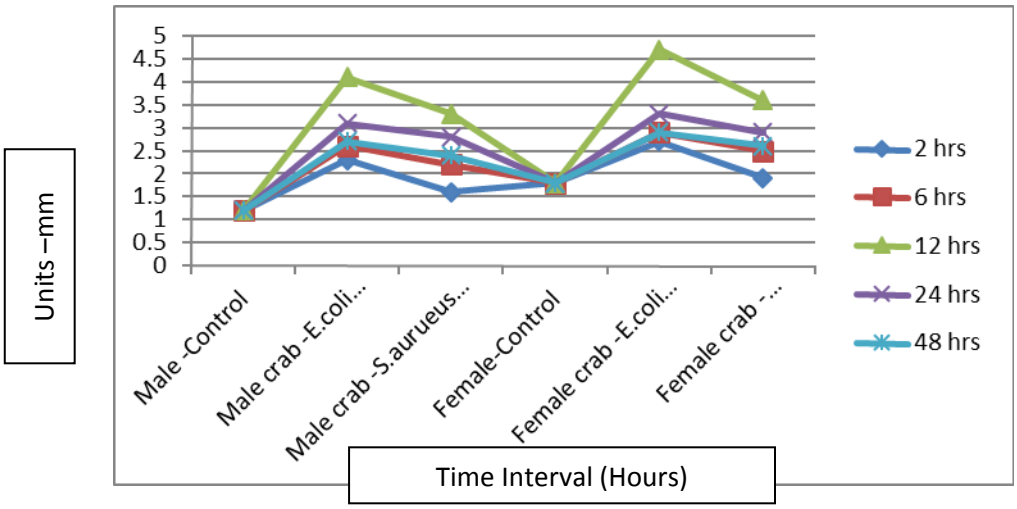


Graph – 2 : Lysozyme activity at different time intervals in the haemolymph of challenged crabs

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 *E.coli* and *S.aurueus*. 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 *E.coli* challenged crabs in male (4.1 mm) and female crabs(4.7 mm). The increase of zone from 2hrs to 12 hrs is by 1.8 mm in *E.coli* challenged male crab and 2.0 mm in female crab where as the increase of zone from 2hrs to 12 hrs is by 1.8 mm in S.*aureus* challenged male crab and 1.6 mm in female crab.



Graph – 3: Anti bacterial activity at different time intervals in the haemolymph of challenged male crabs

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

Electrophoresis is a technique for separating proteins in a mixture under the influence of an applied electric field. SDS PAGE is rapid and sensitive. On SDS PAGE protein bands belong to different molecular weight ranging from 10 kDa to 70 kDa were observed. In control male heamolymph sample the bands were observed at 15 kDa, 23 kDa, 45 kDa, 66kDa and 70 kDa. The bands observed in challenged male crab hemolymph are at 15 kDa, 23 kDa and at 66 kDa. In the haemolymph of female crab the protein bands were observed at 15 kDa, 23 kDa and 66 kDa. The protein band observed at 15 kDa is Lysozyme and 66kDa is phenoloxidase.

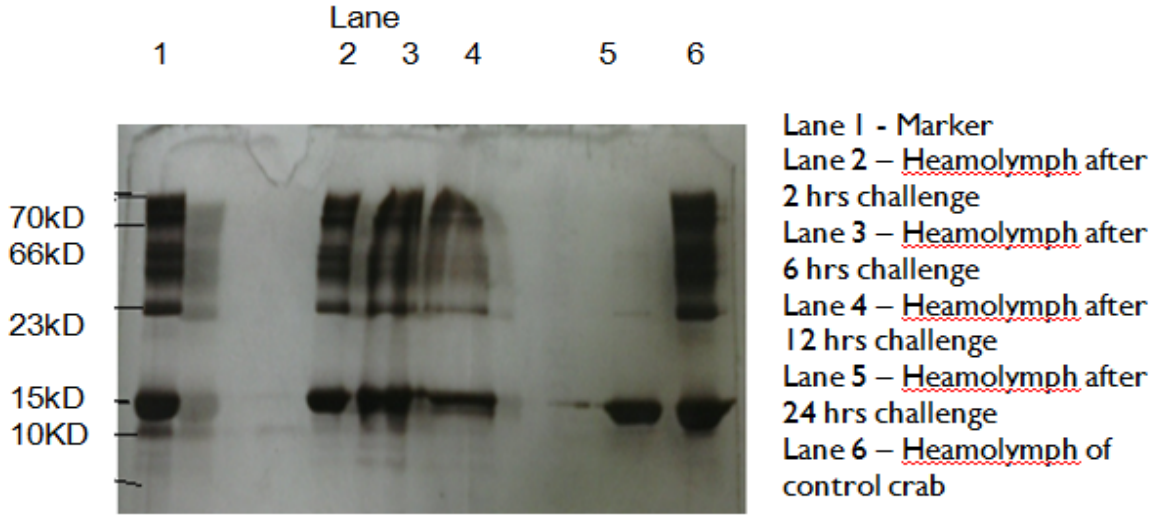


Fig 1: Heamolymph of challenged Male crab, SDS PAGE

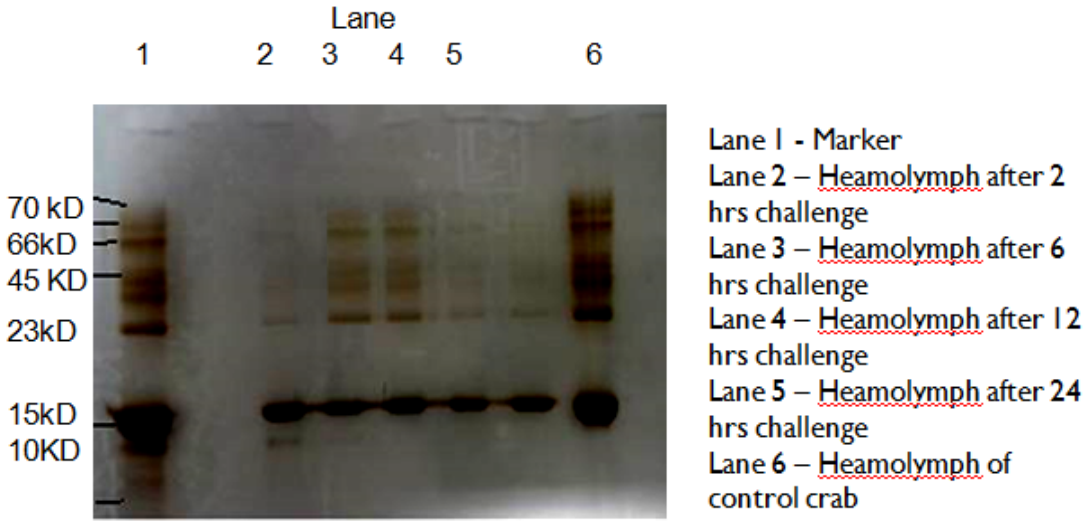


Figure 3 –Hemolymph challenged female crab, SDS PAGE

CONCLUSION

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. 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 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 *E.coli* challenged male crab and female crab and also in male and female crabs challenged with S.*aureus*. The protein band observed at 15 kDa was identified as Lysozyme and 66kDa as Prophenoloxidase.

REFFERENCES

[3] Ashida, M., Brey, P.T, 1997. Recent advances in research on the insect prophenoloxidase cascade. In: Brey, P.T., Hultmark, D. (Eds.), Molecular Mechanisms of Immune Responses in Insects. Chapman and Hall, London, pp. 135–172.

[7] Ashida, M. and K. Soderhall, 1984. The prophenoloxidase activating system in crayfish. Comp. Biochem. Physiol, 77B-21-26.

[4] Beck, Gregory and Habicht, Gail S. 1996. Immunity and the Invertebrates Scientific American. November: 60-66.

[5] Cerenius, L, Söderhäll, K 2004. "The prophenoloxidase-activating system in invertebrates". Immunological reviews 198: 116–26.

[9] Chapelle M. Girard PA, Cousserans F. Volkoff NA, Duvie B 2009. Lysozymes and lysozymes like proteins from the fall army worm, *Spodoptera frugiperda* . Mol Immunol.47:261-9

[8] T. H. Hutchinson and M. J. Manning, 1996. "Seasonal trends in serum lysozyme activity and total protein concentration in dab (Limanda limanda L.) Sampled from Lyme Bay, U.K," Fish and Shellfish Immunology, vol. 6, no. 7, pp. 473–482.

[2] Soderhall, K., Johansson, M. W. and Smith, V. J. 1988. Internal Defense Mechanisms (In freshwater Crayfish. Biology. Management and Exploitation. Ed. By Holdrich and Lowery) London. 213-234.

[1] Solomon EI, Sundaram UM, Machonkin TE. 1996. Multicopperoxidases andoxygenases. Chem Rev: 2563-2605

[6] Sritunyalucksana, K. and Söderhäll, K. 2000. The proPo and clotting system in crustaceans. Aquaculture, 191:53-69.

INTERNATIONAL JOURNAL OF PLANT, ANIMAL AND ENVIRONMENTAL SCIENCES

ISSN 2231-4490

International Journal of Plant, Animal and Environmental Sciences