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BIOCHEMICAL STUDIES OF FRESH WATER MALE CRAB BARYTELPHUSA GUERINI INOCULATED WITH E.COLI AND STAPHYLOCOCCUS AUREUS

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ABSTRACT

Carbohydrates, lipids and proteins are found in different forms in various cells as as degenerative material in many crustaceans. In this study total free sugar content, total protein and lipid content of *Barytelphusa guerini* were estimated using standard protocols. The protein content increase was moderate in the hemolymph of challenged crabs during 2hrs and 6hrs but reached maximum during 12hrs and started decreasing from 12hrs to 24 hrs and 48 hrs gradually in case of male crabs challenged with Gram positive and Gram Negative bacteria. Based on the results it was observed that the carbohydrate content decreased gradually from 2 hrs to 48 hrs and the percentage of decrease is highest, 26% in male crab challenged with *E.Coli. T*he lipid content increased gradually from 2 hrs to 24 hrs and decrease from 24 hrs to 48 hrs in male crabs challenged with *E.coli* and *S.aureus.*

KEYWORDS: Barytelphusa guerini, Biochemical studies, E.coli, Staphylococcus aureus.

INTRODUCTION

Proteins are large, complex molecules essential for the sustenance of life in all animals. They are required for the structure, function and regulation of the body's tissues and organs (Okada et al 1983, Chou et al 2009). Proteins are made up of hundreds or thousands of smaller units called amino acids, which are attached to one another in long chains (Ramesh et al 2009). There are 20 different types of amino acids that can be combined to make a protein. Hala Ali et al 2014 worked on protein content of Males and females marine mantis shrimp E.massavensis : male and female shrimps P. semisulcatus and both sexes of crabs P. pelagicus, both sexes of Arabian Gulf strain crustaceans, M. monoceros (shrimps) and P. pelagicus (crabs). Carbohydrates are the important components of storage and structural materials. They exist as free sugars and polysaccharides. The basic units of carbohydrates are the monosaccharides which cannot be split by hydrolysis into simpler sugars. Carbohydrates in haemocytes are present in many fonns. They may be present as mucopolysaccharides or linked to proteins fonning glycoproteins. They may also be present as energy reserves, in the fonn of glycogen (Johnson et al 1976). Glycogen in the cytoplasm of haemocytes is reported in Artemia sauna, Pachygrapsus marmoratus and Orconectes virilis (Willi et al, 2009). Cytochemical analysis of ridge back prawn, revealed that the abundant cytoplasmic deposits of agranular cells and some of the small granule haemocytes are composed of glycoproteins. Such cells are also positive for the presence of lipids. Lipids are present in all cell types as energy reserve. They are usually seen as deposits in the cytoplasm. Otherwise they accumulate in the granules of lipoprotein cells (Hose et al 1987).

MATERIALS AND METHODS

Experimental animals

The experimental animal selected for the present study was Fresh water crab, *Barytelphusa guerini*. This is a fresh water crab lives among the crevices of bunds of water bodies/Agricultural land rocks. These test organisms, the Crabs are covered with a thick exoskeleton, armed with a single pair of chelae (claws). Crabs found in all of the world's oceans, while many live in fresh water and on land.

Crabs show marked sexual dimorphism. Males often have larger claws, another conspicuous difference is the form of the abdomen, narrow and triangular in males, broader and rounded abdomen in females. When development is complete, the female releases the newly hatched free-swimming zoea larvae. They have a spine, which probably reduces the rate of predation by larger animals. These larvae increase in size through a process called moulting and develop into magalopa larva. It has little nippers and 4 jointed legs on either side. Its tail is still visible and is used for swimming. This settles at the bottom and metamorphose into a juvenile crab. The juvenile looks like adult and moults repetitively until it reaches the adult stage. *Barytelphusa guerini* is a fresh water crab lives among the crevices of rocks.

B. guerini attains sexual maturity on reaching a carapace width (CW) of about 45 mm in both the sexes. Studies

on growth rate revealed that the animals attain a CW of 35 to 40 mm in the first year, 50-55 mm in the second year and up to 65-70 mm in the third year. The intermediate sized animals with a CW of 45-60 mm are reproductively more active and the reproductive activity declines in older animals with a CW of 60 mm and above (Reddy et al 1982).

Adult specimens of fresh water male crabs *Barytelphusa gureini* were purchased from regular animal supplier and brought to department of zoology, Osmania university. They were acclimatized in the laboratory for seven days before they were used for experimentation. Only healthy crabs weighing between 30-40 grams and almost equal in size were selected for experimentation. The animals were fed with small pieces of goat flesh and uncooked oats.

Bacterial Preparations

The Bacterial strains were obtained from MTCC (Microbial Type Culture Collection), Chandigarh, India. *Escherichia coli (Gram -ve) and Staphyloccoccus aureus (Gram +ve)* ***t***wo different strains of bacteria, were used for inoculation during the study.

Escherichia coli is a Gram-negative, facultatively anaerobic, rod-shaped bacterium of the genus *Escherichia* that is commonly found in intestine.

Staphylococcus aureus is a gram-positive coccal bacterium that is a member of the Firmicutes and is frequently found in the nose, respiratory tract, and on the skin. It is often positive for catalase and nitrate reduction.

A known amount of bacterial culture, Gram negative (*Escherichia Coli*, MTCC-1687) and Gram Positive (*Staphyloccocus aureaus*, MTCC-3160) were chosen based on their clinical and pharmacological importance. The bacterial strains obtained from Department of Microbiology, Osmania University, were used for evaluating antimicrobial activity. The bacterial stock cultures were incubated for 24 hours at 37°C on nutrient agar medium following refrigeration storage at 4°C. The bacteria were grown on Mueller-Hinton agar plates at 37°C. The stock cultures were maintained at 4°C.

Preparation of LB Media

2.5gms Peptone, 1.25gms Yeast and 2.5gms Nacl were make up to 250 ml Milli Q Water and autoclave at 15 Psi for 25 minutes and cool it to room temperature.

Preparation of Nutrient Agar Media (NAM)

2.5gms Peptone, 1.25gms Yeast, 2.5gms Nacl and 3.75gms Agar were make up to 250 ml of distilled water and autoclave at 15 Psi for 25 minutes and cool it to room temperature.

Experimental Groups

The Test animals were grouped into following classes

1. Group I: Control crabs without any bacterial inoculation
2. Group II: Control injured crabs, these crabs were injected with saline/ 0.9% Nacl
3. Group III: Male Crabs challenged with *Staphylococcus aureaus,* Gram +ve bacteria
4. Group V: Male Crabs challenged with *E.coli,* Gram -ve bacteria

Haemolymph extraction

Haemolymph samples can be extracted using a 1 ml syringe from the ventral sinus at the basis of either fifth pereopods (Shields et al., 2003; Uhlmann et al., 2009), or from under the abdomen (Stewart et al., 2004) at different time intervals 2h, 6h, 12h, 24h and 48 hrs. Samples for haemocyte counts are withdrawn into a chilled syringe (on ice) pre-filled with a suitable anti-coagulant (e.g. 1% formalin, at 9 parts formalin to 1 part haemolymph) (Malev et al., 2010). Before storage, the actual volume of haemolymph extracted must be noted, for subsequent volume adjustment (to account for dilution).

Anticoagulant Preparation

For 100 ml of Anticoagulant 9.8 ml of 1M NaoH, 18.6 ml of 1MNacl, 17 ml of 0.01 M EDTA and 41 ml of 0.01 M Citric acid was taken mixed together and pH was adjusted to 4.5 then final volume was made up to 100 ml and autoclaved at 15 PSI (Pounds per square Inch) for 20 min.

Cytochemical Studies

Cytochemical analysis were conducted in the haemocytes of *Barytelphusa gureini* cytochemical studies Cytochemical staining methods were employed to demonstrate indicus. Carbohydrates, lipids and the protein in haemolymph.

Biochemical Parameters

Estimation of Total Protein

Total protein was determined following the method of Lowly et al. (1951). The carbamyl groups of protein molecules react with copper and sodium reagent to form a biurette complex. This complex reacts with tyrosine and phenolic compounds present in the protein and reduces the phosphomolybdate of the folin phenol reagent to a blue color. Eighteen crabs were exposed to the three sub lethal concentrations of mercury for three time periods of 1, 7 and 15 days. Six crabs served as the controls. At the end of each time period, six crabs from the experimentals were sacrificed and tissue samples of abdominal muscle, chelate muscle, hepatopancreas, and gill were removed and processed for protein, carbohydrate and lipid analysis. The controls were also sacrificed on the 15"‘ day and tissue samples processed for biochemical analysis as done in the case of experimentals. The samples were oven-dried at 110°C for 24h. A sample of l0 mg dry weight of each tissue was transferred into a test tube, added 5 ml of lN NaOH, and warmed slightly for 10 minutes, but not overheating.

After warming, the tubes were cooled and their volumes adjusted to 10 ml with distilled water, and then centrifuged for 5 minutes at 3500 rpm. From this 1 ml of the supernatant was taken, then 5 ml of alkaline copper reagent added and mixed well, incubated for 10 minutes, 0 .5 ml of Folin's phenol reagent was added, and mixed well. The optical density of the sample was determined spectrophotometrically at 500 nm, after 30 minutes. The concentration of total protein is expressed as mg/100gm dry weight of the tissues.

Estimation of Total Carbohydrates

The total free sugar content was estimated by anthrone method (Roe, 1955). Sulphuric acid hydrolyses disaccharides and oligosaccharides into monosaccharides and converts monosaccharides into furfuryl derivatives, which react with anthrone and produce the colored product. A 10mg dry weight sample of each tissue was weighed out into a. test tube, and hydrolyzed by keeping it in a boiling water bath for 3 h with 2.5 N Hcl, and cooled to room temperature, neutralized with solid Na_2Co_3 until the effervescence ceased. The volume was then made up to 10 ml and centrifuged at 3500 rpm for 5 minutes, the supernatant was pipetted out, and l ml of the aliquot was taken for analysis. To that added 4 ml of anthrone reagent, and boiled for 8 min in a boiling water bath. The optical density of the sample was determined spectrophotometrically at 620 nm. The concentration of total free sugar content is expressed as mg total sugar/100 g dry weight of the tissue.

Estimation of Lipid (Barnes and Blackstock, 1973) (Sulphophospho vanillin method)

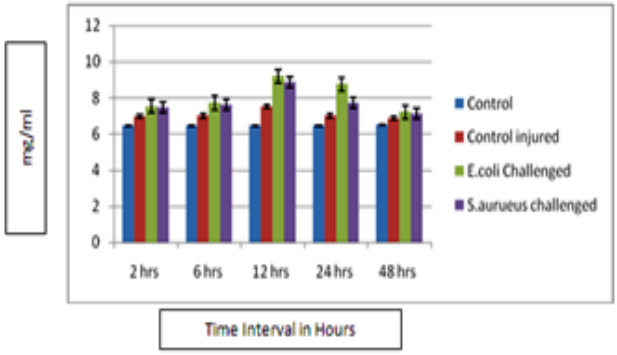
The quantitative estimation of lipid by sulphophospho vanillin method depends on the reaction of lipids extracted from the sample using chloroform--methanol with sulphuric acid, phosphoric acid and vanillin to give a red complex. To a 10 mg dry weight sample of each tissue were added 10 ml of chloroform-methanol (2:l v/v), and mixed well. Filtered the homogenate through a Whatman No.1 filter paper, added 2ml of sodium chloride solution (0.9% Nacl), shaken well, transferred the mixture to a small separating funnel, and allowed to stand overnight at 0°C, to obtain a clear biphasic layer. The lower phase contains lipids. Removed the lower phase and adjusted the volume to 10 ml by addition of chloroform. A 0.5 ml sample of this extract was taken into a clean dry test tube, dried in a vacuum desiccator over silica gel, then dissolved in 0.5 ml of concentrated sulphuric acid, mixed well, and the test tubes were plugged with non absorbent cotton wool. Placed in a boiling water bath for 10 minutes and then cooled the tubes to room temperature. To 0.2 ml extract of this acid digest, pipetted 5 ml of vanillin reagent, mixed well and allowed to stand for half an hour, and measured the developed color at 520 nm in a spectrophotometer.

RESULTS

Quantitative Estimation of Total Protein

At different time intervals of 2hrs, 6hrs, 12hrs, 24hrs and 48 hrs, estimation of Total Protein were measured in control, control injured and Gram positive and Gram Negative bacteria challenged crabs. The total protein content was observed to be slightly more at all time intervals in Bacterial challenged crabs than control and control injured crabs.

During 2hrs and 6hrs it was observed that the protein content increase was moderate in the hemolymph of challenged crabs, but reached maximum during 12hrs and started decreasing from 12hrs to 24 hrs and 48 hrs gradually in case of male crabs challenged with Gram positive and Gram Negative bacteria (Fig 1).

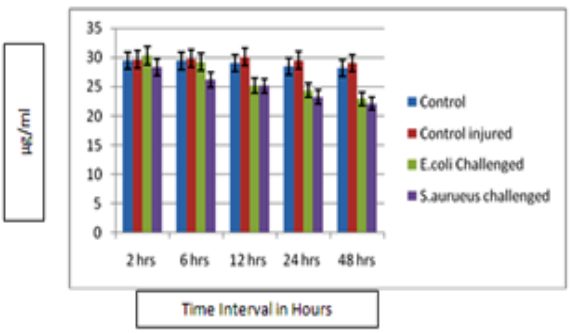


Values are mean ±S.D. Values that do not share a common superscript (a, b and c) differ significantly from each other ($p<0.05$, Duncan's multiple range test).

Figure 1: Total protein content in the haemolymph of control and challenged male crabs.

Estimation of Total Carbohydrates

At 2hrs, 6hrs, 12hrs, 24hrs and 48 hrs, the estimation of total carbohydrates was made in control, control injured and male crabs challenged with Gram negative (*E.coli)* and Gram positive (*S.aureus*) bacteria. Basing on the results from 2 hrs to 48 hrs it was observed that the carbohydrate content decreased gradually and the percentage of decrease is highest, i.e 26% in male crab challenged with *E.Coli* (Fig 2).



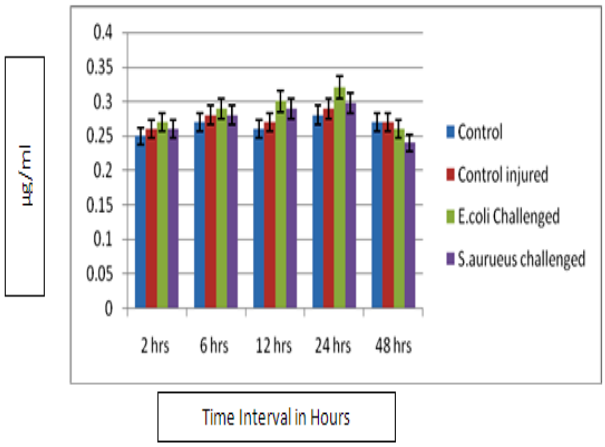
Values are mean ±S.D. Values that do not share a common superscript (a, b and c) differ significantly from each other ($p<0.05$, Duncan's multiple range test).

Figure 2: Total carbohydrate in the haemolymph of control and challenged male crabs.

Estimation of Total Lipids

At 2hrs, 6hrs, 12hrs, 24hrs and 48 hrs the total lipid content was estimated in control, control injured and male crabs challenged with Gram negative (*E.coli)* and Gram positive (*S.aureus*) bacteria.

Basing on the results it was observed that the Lipid content increased gradually from 2 hrs to 24 hrs and decrease from 24 hrs to 48 hrs in male crabs challenged with *E.coli* and *S.aureus.* From 2hrs to 24 hrs the percent of increase is is 14 % in male crab challenged with *S.aureus*, 18% in male crab challenged with *E.Coli.*



Values are mean ±S.D. Values that do not share a common superscript (a, b and c) differ significantly from each other ($p<0.05$, Duncan's multiple range test).

Figure 3: Total Lipid content in the haemolymph of control and challenged male crabs.

DISCUSSION

*Barytelphusa Guerin*i revealed the presence of carbohydrates, Lipids, in the haemolymph. Not all haemocytes were positive to the various cytochemical tests, implying that the haemocytes of *Barytelphusa Guerin*i vary in their physiological role (Chandrakala et al 2008). Carbohydrate positive granules were observed in less number of haemocytes of *Barytelphusa Guerin*i. The nature of the haemocytes positive to PAS staining and the nature of the carbohydrate could not be ascertained. Carbohydrates are found in different forms in various cells as mucopolysaccharides, gIycoproteins, and energy reserves or as degenerative material from phagocytosed microorganisms (Hose et al., 1987; 1990; 1992). Carbohydrate in the form of the energy reserve, glycogen, has been detected in many crustaceans as in A. salina, P. marmoratus and 0. virilis and is usually seen in the cytoplasm as deposits. In C. maenas, glycogen was seen as cytoplasmic deposits in hyaline cells and in between the granules of granulocytes (Johnson et al., 2000). But, William and Lutz (1975) observed glycogen containing granules and non-glycogen polysaccharide granules in C. maenas. In E. sinensis, the granules of the haemocytes contained the structural polysaccharide, chitin (Bauchau et al., 1975).

In the present study it was observed that the protein content increase was moderate in the hemolymph of challenged crabs during 2hrs and 6hrs but reached maximum during 12hrs and started decreasing from 12hrs to 24 hrs and 48 hrs gradually in case of both male crabs challenged with Gram positive and Gram Negative bacteria. This rise in the protein levels during first 12 hrs after bacterial inoculation might be associated with the release of other humoral defense factors involved in clearance of bacteria.

The quantitative estimation of total carbohydrates was made in the crab, *Barutelphusa guerini*at 2hrs, 6hrs, 12hrs, 24hrs and 48 hrs in control, control injured and male crabs challenged with Gram negative (*E.coli)* and Gram positive (*S.aureus*) bacteria. Basing on the results it was observed that the carbohydrate content decreased gradually from 2 hrs to 48 hrs and the percentage of decrease is highest, i.e 26% in male crab challenged with *E.Coli* with *S.aureus.* The depletion of total carbohydrate may be due to its rapid utilization to meet the energy demands under the impact of bacterial inoculation.

In the present study it was observed that the lipid content increased gradually from 2 hrs to 24 hrs and decrease from 24 hrs to 48 hrs in male crabs challenged with *E.coli* and *S.aureus.* The percent of increase from 2hrs to 24 hrs is is 18% in male crab challenged with *E.Coli* and 14 % in male crab challenged with *S.aureus.*

CONCLUSION

The protein content increase was moderate in the hemolymph of challenged crabs during 2hrs and 6hrs but reached maximum during 12hrs and started decreasing from 12hrs to 24 hrs and 48 hrs gradually in case of both male crabs challenged with Gram positive and Gram Negative bacteria. Based on the results it was observed that the carbohydrate content decreased gradually from 2 hrs to 48 hrs and the percentage of decrease is highest, 26% in male crab challenged with *E.Coli. T*he lipid content increased gradually from 2 hrs to 24 hrs and decrease from 24 hrs to 48 hrs in male crabs challenged with *E.coli* and *S.aureus.*

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