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ESTIMATION OF PROTEIN, CARBOHYDRATE AND LIPIDS IN FEMALE MUD CRAB *SCYLLA SERRATA*: CYTOCHEMICAL STUDIES AFTER INOCULATED WITH *KLEBSIELLA PNEUMONIA AND BACILLUS CEREUS*

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ABSTRACT

Scylla serrata is having always a stress and threat from microbial organism that tries to cause infection. In the present study it was observed that the protein content increase was moderate in the hemolymph of challenged crabs during 2hrs and 4hrs but reached maximum during 12hrs and started decreasing from 12hrs to 24 hrs gradually in case of female crabs challenged with Gram positive and Gram Negative bacteria. The quantitative estimation of total carbohydrates was made in the crab, *Scylla serrata* at 2hrs, 4hrs, 8hrs, 12hrs, 16hrs, 20hrs and 24hrs in control, control injured and female crabs challenged with *Klebsiella pneumonia* and *Bacillus cereus*. Basing on the results it was observed that the carbohydrate content decreased gradually from 2 hrs to 24hrs and the percentage of decrease is highest 21.6% in female crab challenged with *Bacillus cereus.* The lipid content increased gradually from 2 hrs to 12 hrs and decrease from 12 hrs to 24hrs in female crabs challenged with *Klebsiella pneumonia* and *Bacillus cereus.* The percent of increase from 2hrs to 24 hrs is 19% in female crab challenged with *Klebsiella pneumonia* and 10% in female crab challenged with *Bacillus cereus.*

KEYWORDS: Cytochemical studies, *Scylla serrata, Klebsiella pneumonia and Bacillus cereus.*

INTRODUCTION

Scylla serrata is widely distributed in tropical and subtropical continents. A seasonal variation in fat and protein content of abdominal muscle of the species were attributed to the

changing salinity of the water.[1] It is also reported that larval survival, growth and development of *Scylla serrata* are considerably influenced by salinity.[2,3] There are reports those describe about the manifestation of physiological and biochemical variations especially that of O2 consumption of *euryhaline* crabs at altered salinity conditions.[4,6] It is reported that the excretion in *Scylla serrata* shifts from ammonotelism to ureotelism with increased salinity.[7,8] Ruscoe et al., (2004) proposed that limiting the rotifer supply as staple food to *Scylla zoea* larvae at salinity 10 to 35 ppt and temperature 30°C enhances a maximal survibility and its production. It has been demonstrated that *Scylla serrata* experiences oxidative stress in response to cold stress.[9] Also it was noticed that mitochondrial count in its different tissues considerably change by cold stress.[10] Similarly, healthy adult crabs without any hormonal or pharmacological disturbances can withstand thermal shock up to 40°C.[11] Effect of seasons Although several studies have clearly demonstrated strong correlation between the antioxidant defence system and seasons in relation to changing environmental conditions in molluscs[12,17], cephalopods[18] and other euryhaline crabs[19] not much information is available on arthropods in general and mud crabs in particular. However, Kong et al., (2008) reported that seasonal factors like high temperature with a prevalent low salinitiy during summer in tropical climate can enhance both protein and lipid oxidation in tissues of mud crabs. Effect of pollutants Pollutant like naphthalene, a fumigant, is shown to induce oxidative stress in *S. tranquebarica* in nature.[20] It also causes reproductive dysfunction by altering vitellogenesis in *Scylla serrate.*[21] It has been reported that the retention of the pharmacological compounds like enrofloxacin and ciprofloxacin are high in hepatopancreas of *S. serrata* but their rate of elimination is slow.[22] *S. serrata* has been shown to protect itself from cadmium toxicity by enhancing the level of stress resistance metal protein. Considering the crab *Scylla serrata,* Oosterom et al., (2010) opined that GST enzyme can be taken as an important biomarker for pollution stress in the aquatic environment.

METHODOLOGY

Cytochemical Studies

For the present work, female healthy adult animals (6±1 cm carapace width) were used. Healthy adult crabs (5.5 cm mean carapace width, 105 gms wt. males and 6 cm mean carapace width 120 gms wt.females) were purchased from regular animal supplier kept in the laboratory in disinfected plastic tubs, the water in the tubs was changed every day and fed with minced chicken. The crabs were acclimatized in the laboratory for 7 days.

Bacterial strains and culture

Two different strains of bacteria, *Klebsiella pneumonia (Gram -ve), Bacillus cereus (Gram +ve)* were used for inoculation during the study. *Klebsiella pneumonia,* Gram Negative bacteria do not retain crystal violet dye. The cells are typically rod shaped, facultative anaerobic bacteria can live on wide variety of substrates. The optimal growth is at 37°C. *Bacillus cereus,* Gram Positive coccal bacterium retains crystal violet dye during Gram staining. The bacterium frequently found in human respiratory tract and on the skin and causes common skin and respiratory diseases.

The Bacterial strains were obtained from MTCC (Microbial Type Culture Collection), Chandigarh, India. Small amount of bacterial culture, Gram negative (Klebsiella pneumonia MTCC-4030) and Bacillus cereus (MTCC 430) were taken from the Glycerol stock and spread on to the Luria- Bertani agar Plates. The Agar plates were incubated for 24 hours at 37^0C. Pure colonies were picked from the Overnight culture and inoculated into an autoclaved broth for 12 hours and incubated at 37^0 C on a rotor shaker at 150 rpm. 1 ml of this culture was introduced for 3 hrs culture and incubate for 3 hrs at 37º C in shaker and 1 ml of 3 hrs culture was taken, centrifuged at 2000rpm for 10 min at 4º C and the pellet was taken. 100 ml Milli Q water was added to the pellet and subjected to serial dilutions (9 times) and 1 ml of 10^3 cultures was used for inoculation.

LB Media Composition and Preparation

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

Nutrient Agar Media (NAM) Preparation

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

Experimental Groups

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: Female Crabs challenged with *Bacillus cereus,* Gram +ve bacteria
4. Group IV: Female Crabs challenged with *Klebsiella pneumonia,* Gram -ve bacteria

Collection of Hemolymph

Hemolymph was collected from unsclerotized membrane from the ventral side with Insulin syringe and each crab was subjected to a single bleed amounting to 1–2 ml of Hemolymph at different time intervals 2h, 4h, 8h, 12h, 16h, 20h and 24 hrs. The collected hemolymph was immediately diluted with 1:1 ice cold anticoagulant solution for further biochemical studies.

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 + 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.

The hemolymph was collected and diluted with anticoagulant and other chemicals (1ml Hemolymph + 1ml Anticoagulant + 10 µl Phenylthiourea + 10 µl of Aprotin) and centrifuged at 2000 rpm for 15 min at 4ºC and the supernatant was used for assessment of biochemical parameters (G. Rameshkumar et al., 2009). In the citrate–EDTA buffer used, citric acid serves to delay cell break down while EDTA inhibits prophenoloxidase (proPO) activation and prevents the clotting reaction, (Hall et al., 1999), and this buffer at low pH, in combination with citrate, glucose and NaCl, provides a medium optimal for maintenance of cell integrity without significant loss of cell viability.

Biochemical Parameters

The hemolymph was collected and diluted with anticoagulant and other chemicals (1ml Hemolymph + 1ml Anticoagulant + 10 µl Phenylthiourea + 10 µl of Aprotin) and centrifuged at 2000 rpm for 15 min at 4ºC and the supernatant was used for assessment of biochemical parameters.

Estimation of Total proteins (Bradford method)

The total protein was estimated by the metthod described by Dr. Marion Bradford in 1976 (Bradford, M. (1976). Proteins bind to coomassie brilliant blue G-250 to form a blue coloured complex having greater extinction coefficient than the free dye. In the acidic environment of the reagent, protein binds to the coomassie dye. This results in a spectral shift from the reddish/brown form of the dye (absorbance maximum at 465nm) to the blue form of the dye (absorbance maximum at 610nm). The difference between the two forms of the dye is greatest at 595nm (Bradford, M. (1976). Acidic coomassie-dye reagent changes color from

brown to blue in proportion the amount of protein present in the sample. Protein determinations are made by comparison to the color response of protein assay standards, usually prepared as a series of known dilutions of Bovine Serum Albumin (BSA). The total protein was estimated in control and challenged crabs as per the protocol given in Bradford method, using Bovine Serum Albumin (BSA) as standard. 250 µl of Bradford reagent was added to 5µl of hemolymph and incubated for 10 minutes at room temperature and absorbance was measured at 595 nm.

Estimation of Total Carbohydrates (Anthrone method)

The total carbohydrates were estimated by the metthod described by Anthrone. Carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxymethyl furfural. This compound forms with anthrone a green coloured product with an absorption maximum at 630 nm. The total carbohydrate was estimated in control and challenged crabs as per the protocol given in Anthrone method by using standard Glucose as standard.

Total Lipids (Zaks method)

The total carbohydrates were estimated by the metthod described by Zaks. The lipids present in the sample are first precipitated by adding FeCl3-CH_3COOH reagent. The lipid free filtrate is treated with conc. H_2SO_4. In the presence of conc H_2SO_4, cholesterol present in the sample gets dehydrated to form cholesterol 3, 5 diene in presence of excess and by the H_2SO_4. catalytic action of Fe3+ ions a red coloured complex is formed. The intensity of red colour is measured at 560 nm.

The total lipid was estimated in control and challenged crabs as per the protocol given in Zaks method. Pipette out 1-5 ml of standard solution in a series of test tubes. The volume in each test tube is made upto 5ml $FeCl_3$-CH_3COOH with reagent. 3ml of conc. H_2SO_4 is added to all the test tubes and mix well. Standards are incubated for about 20-30 minutes at room temperature. The intensity of standards is measured at 560 nm against blank.

5 ml of reagent, $FeCl_3$-CH_3COOH 3ml H_2SO_4 of are taken in a test tube, mixed well and used as a blank. In the centrifuged tube 0.1ml of serum and 10ml of Ferric chloride-acetic acid reagent reagents are taken, mixed well for 5 minutes and then centrifuged. 5 ml of supernatant is collected and added with 3ml of H_2SO_4. Test is incubated at room temperature to 20-30 Intensity is measured at 560nm against blank.

RESULTS AND DISCUSSION

Quantitative Estimation of Total Protein

Quantitative Estimation of Total Protein in control, control injured and Gram positive and Gram Negative bacteria challenged crabs were made at different time intervals of 2hrs, 4hrs, 8hrs, 12hrs, 16hrs, 20hrs and 24hrs. The results indicated that the total protein content was observed to be slightly more at all time intervals in Bacterial challenged crabs than control and control injured crabs (Figure 1). It was observed that the protein content increase was moderate in the hemolymph of challenged crabs during 2hrs and 4hrs but reached maximum during 12hrs and started decreasing from 12hrs to 24 hrs gradually in case of female crabs challenged with Gram positive and Gram Negative bacteria (Table 1).

Table 1: Total protein in the haemolymph of control and bacterial challenged female crabs.

	2hours (µg/ml)	4hours (µg/ml)	8hours (µg/ml)	12hours (µg/ml)	16hours (µg/ml)	20hours (µg/ml)	24hours (µg/ml)
control	8.45 ± 0.658	8.45 ± 0.658	8.45 ± 0.658	8.45 ± 0.658	8.45 ± 0.658	8.45 ± 0.658	8.45 ± 0.658
Control Injured	8.85± 0.583	9.15± 0.263	9.45± 0.259	9.87± 0.583	8.92± 0.637	8.75± 0.253	8.15± 0.347
Klebsiella pneumonia	8.58 ± 0.539	9.23± 0.157	9.58 ± 0.637	9.97± 0.583	8.84 ± 0.389	8.46± 0.583	8.32 ± 0.457
Bacillus cereus	8.71±0.257	9.18± 0.246	9.38 ± 0.368	9.79± 0.427	8.92 ± 0.424	8.85± 0.433	8.54± 0.352

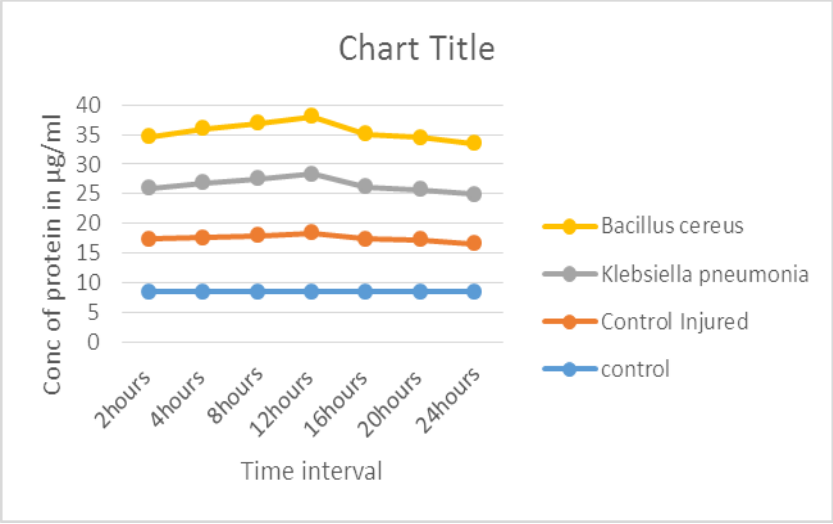

Figure 1: Total protein in the haemolymph of control and bacterial challenged female crabs.

Estimation of Total Carbohydrates

The quantitative estimation of total carbohydrates was made at 2hrs, 4hrs, 8hrs, 12hrs 16hrs, 20hrs and 24hrs in control, control injured and female crabs challenged with Gram negative (*Klebsiella pneumonia)* and Gram positive (*Bacillus cereus*) bacteria. Basing on the results it

was observed that the carbohydrate content decreased gradually from 2 hrs to 24 hrs and the percentage of decrease is highest, 24% in female crab challenged with *Bacillus cereus* (Figure 2, Table 2).

Table 2: Total carbohydrate in the haemolymph of control and bacterial challenged female crabs.

	2hours (µg/ml)	4hours (µg/ml)	8hours (µg/ml)	12hours (µg/ml)	16hours (µg/ml)	20hours (µg/ml)	24hours (µg/ml)
Control	40.64±0.184	40.64±0.184	40.64±0.184	40.64±0.184	40.64±0.184	40.64±0.184	40.64±0.184
Control injured	41.27±0.325	40.64±0.184	38.17±0.367	36.64±0.584	34.94±0.325	32.64±0.264	30.85±0.127
Klebsiella pneumonia	41.56±0.263	40.64±0.265	38.32±0.4284	36.64±0.153	34.15±0.267	32.64±0.287	30.68±0.385
Bacillus cereus	41.42±0.436	40.64±0.268	38.22±0.263	36.64±0.375	34.02±0.278	32.64±0.296	30.43±0.186

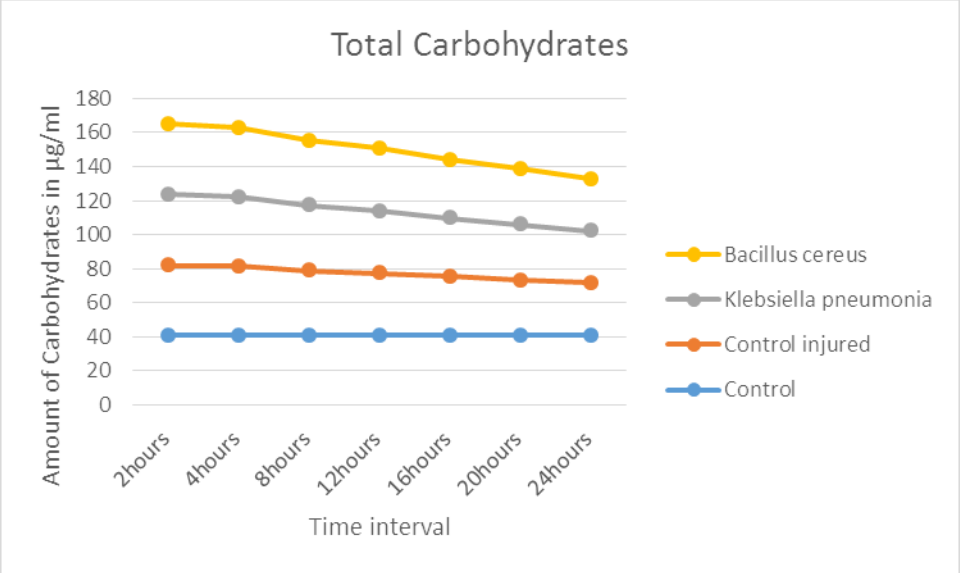

Figure 2: Total carbohydrate in the haemolymph of control and bacterial challenged female crabs.

Estimation of Total Lipids

The total lipid content was estimated at 2hrs, 4hrs, 8hrs, 12hrs, 16hrs, 20hrs and 24hrs in control, control injured and female crabs challenged with Gram negative (*Klebsiella pneumonia)* and Gram positive (*Bacillus cereus*) bacteria. Basing on the results it was observed that the Lipid content increased gradually from 2 hrs to 12 hrs and decrease from 12 hrs to 24 hrs female crabs challenged with *Klebsiella pneumonia* and *Bacillus cereus.* The percent of increase from 2hrs to 12 hrs is 17% in female crab challenged with *Klebsiella pneumonia* and 11% in female crab challenged with *Bacillus cereus* (Figure 3, Table 3).

Table 3: Estimation of Total Lipids in challenged Female crab.

	2hours (µg/ml)	4hours (µg/ml)	8hours (µg/ml)	12hours (µg/ml)	16hours (µg/ml)	20hours (µg/ml)	24hours (µg/ml)
Control	0.62±0.046	0.62±0.046	0.62±0.046	0.62±0.046	0.62±0.046	0.62±0.046	0.62±0.046
Control injured	0.65±0.014	0.68±0.026	0.72±0.043	0.75±0.021	0.77±0.025	0.70±0.036	0.64±0.042
Klebsiella pneumonia	0.67±0.022	0.69±0.034	0.74±0.025	0.77±0.022	0.79±0.036	0.74±0.026	0.66±0.034
Bacillus cereus	0.69±0.023	0.72±0.057	0.76±0.017	0.79±0.036	0.82±0.016	0.76±0.028	0.68±0.027

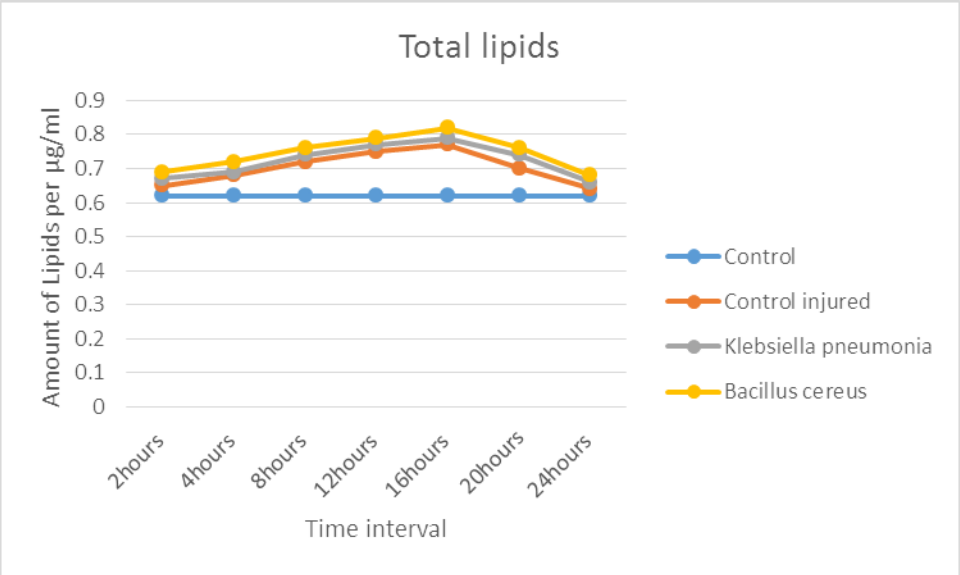

Figure 3: Total Lipids in the haemolymph of control and bacterial challenged female crabs.

CONCLUSION

In this work, protein content increase was moderate in the hemolymph of challenged *Scylla serrata* during first 12hrs and started decreasing from 12hrs to 24 hrs in case of female crabs challenged with Gram positive and Gram Negative bacteria. Basing on the results it was observed that the carbohydrate content decreased gradually from 2 hrs to 24hrs and the percentage of decrease is highest 21.6% in female crab challenged with *Bacillus cereus.* The lipid content increased gradually from 2 hrs to 12 hrs and decrease from 12 hrs to 24hrs in female crabs challenged with *Klebsiella pneumonia* and *Bacillus cereus.*

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