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ROS RESPONSE OF THE TOXIC DINOFLAGELLATE ALEXANDRIUM MONILATUM ON THREE ECOLOGICALLY IMPORTANT SHELLFISH SPECIES

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ABSTRACT

Harmful Algal Blooms (HABs) are becoming an increasing problem to human health and environment (including effects on natural and cultured resources, tourism and ecosystems) all over the world. There are about 70 species of planktonic and non-planktonic microalgae considered harmful in world wide coasts. The most important toxin-producing species are the dinoflagellates Alaxandrium monilatum, Gymnodinium catenatum and Pyrodinium bahamense var. compressum, in the world, and consequently the poisonings documented in india are Paralytic Shellfish Poisoning (PSP) and Neurotoxic Shellfish Poisoning (NSP). Toxic A. monilatum produces endotoxins with hemolytic and neurotoxic properties, and has been linked to major fish and invertebrate kills. The responses of three ecologically important shellfish species to A. monilatum (toxic strain AMO3) were experimentally assessed. In the first set of experiments, grazing studies were conducted with adult and juvenile eastern oysters (Crassostrea virginica Gmelin), northern quahogs (Mercenaria mercenaria Linnaeus), and green mussels (Perna viridis Linnaeus), which inhabit areas where A. monilatum blooms occur. The type of phytoplankton and epiphytic microalgae, their toxins and harmful effects as well as current methodology used to study these phenomena are presented in this paper. As an experienced group of workers, we include descriptions of monitoring and mitigation programs, our proposals for collaborative projects and perspectives on future research.

KEY WORDS : Algal Blooms, planktonic, Paralytic Shellfish Poisoning (PSP), Neurotoxic Shellfish Poisoning (NSP)

INTRODUCTION

Generally, Free-radicals are highly reactive chemicals that attack molecules by capturing electrons and thus modifying the chemical structures and also make it unstable. Leibfritz, *et al*., (2007) stated under normal physiological condition, animals maintained a balance between generation and neutralization of reactive oxygen species (ROS). However when organisms are subjected to xenobiotic compounds, rate of production of ROS in cells get increased along with hydrogen peroxide (H2O2), hypochlorous acid (HClO), and free radicals including hydroxyl radical (OH) and superoxide anion (O2 .–). They are normally neutralized in the body employing the following enzymes.

Catalases are enzymes that catalyse the conversion of hydrogen peroxide to water and oxygen, using either an iron or manganese cofactor (Zamocky and Koller, 2004; Chelikani, 1999).

Superoxide dismutase (SOD) are a class of closely related enzymes that catalyse the breakdown of the superoxide anion into oxygen and hydrogen peroxide (Johnson and Giulivi, 2005; Grayck, *et al*., 2005). SOD enzymes are present in almost all aerobic cells and in extra cellular fluids.

Glutathione reductase is an enzyme which reduces glutathione disulphide (GSSG) to the sulfhydryl form GSH, which is an important cellular antioxidant (Meister, 1988 and Mannervi, 1987) For every mole of GSSG one mole of NADPH is required for reduction reaction. Where as in the cells exposed to high levels of oxidative stress (eg., red blood cells) need up to 10% of the available glucose for the production of the NADPH, in this reaction (Mannervi, 1987).

MECHANISM: All organisms respire to survive, thus have their own cellular antioxidative defense system, involving both enzymatic as well as non-enzymatic components. Enzymatic pathway consists of SOD, CAT, GSH, GPX, etc. SOD dismutate the O2 ● − in to H2O2 which is reduced to water and molecular oxygen by CAT or is neutralized by GPX, that catalyzes the reduction of H2O2 to water and organic peroxide to alcohols using GSH as a source of reducing equivalent. Glutathione reductase (GSH) regenerated from oxidized glutathione (GSSG), which is a scavenger for ROS as well as a substrate of the other enzymes. GST conjugates xenobiotics with GSH for its excretion (Livingstone *et.al.,* 1992; Arun and Subramanian, 1998; Halliwell, 1999).

CHEMICALS

Thiobarbituric acid (TBA), bovine serum albumin (BSA), cumene-hydroperoxide, 5,5`- dithiobis (2-nitrobenzoic acid) (DTNB), nicotinamide adenine dinucleotide reduced (NADH), nicotinamide adenine dinucleotide phosphate reduced (NADPH), GSSG, GSH, 1-chloro-2,4-dinitrobenzene (CDNB), dithiothreitol (DTT), butlylated hydroxytoluene (BHT) and phenyl methyl sulphonyl fluoride (PMSF). All other chemicals were analar grade.

MATERIALS AND METHODS

CULTURING OF DINOFLAGELLATE STRAINS

Dinoflagellates were cultured for 28 days in 150 ml of f/2-enriched seawater (22) without copper and with added selenium (Na2SeO3) at a final concentration of 1028 M. Cultures were exposed to a 12-h light-dark cycle at 158C (ca. 125 mmol m22 s21 [irradiance]; cool white fluorescent tubes). Cells obtained by centrifugation (2,000 3 *g* for 20 min) of a 50-ml culture of *Alexandrium* sp. were sonicated with 1 ml of 0.05 M acetic acid followed by centrifugation (10,000 3 *g* for 10 min) to remove cell debris. The resultant supernatant was stored frozen at 220°C until used.

ISOLATION AND GROWTH OF BACTERIA FROM DINOFLAGELLATE CULTURES.

Tenfold dilutions of each *Alexandrium* culture were prepared in marine broth, and 0.1ml of each was spread onto marine agar plates. These plates were incubated at 20°C for 7 days, and the total viable count was calculated. Bacteria from the plates containing approximately 50 colonies were isolated and replated individually onto marine agar plates, followed by incubation at 208C for 48 h. The resultant pure colonies were subsequently inoculated into 30 ml of marine broth. After incubation at 20°C for 24 h on a rotary incubator (120 oscillations per min), the supernatants were collected by centrifugation (10,000 3 *g* for 20 min) and stored at 220°C until required. These samples were used for ROS activity checking.

EXPERIMENTAL ANIMALS

Green-lipped mussels (*Perna viridis*), length 8-10 cm were brought in March from an unpolluted area of beach. This place is relatively far less polluted because of the absence of any industrial or major fishing activity around it. Mussels were transported to the laboratory in the polythene bags filled with natural habitat seawater. The ambient temperature at which mussels were collected was 26 ± 1oC. Fouling organisms were removed from the external part of the shells. Mussels were acclimatized in aerated seawater for 2-3 week. To avoid faecal

contamination, the water was drained at every 24 hours the water was drained and filled with fresh sand filtered seawater. Animals were fed daily with culture algae (*A.monilatum*).

SAMPLE PREPARATIONS:-

The digestive gland was carefully excised, surface dried with filter paper, thoroughly washed with 50 mM phosphate buffer pH 7.4 and homogenized with 50 mM phosphate buffer pH 7.4 containing, 1 mM EDTA, 1 mM DTT, 0.15 M KCl, 0.01 % PMSF. Homogenization was carried out at 4°C using 12-15 strokes of a motor driven Teflon Potter homogenizer and centrifuge at 10,000 x g for 20 min at 4oC. Supernatant was used for biochemical studies.

BIOCHEMICAL BIOMARKERS:-

The activity of total SOD determined by the method of Paoletti et al. The EDTA / MnCl2, *β*-mercaptoethanol and oxygen were used to generate O2 ●−. This anion produced the oxidation of NADH. The SOD of the sample removed the O2 ●− and brought an inhibition of NADH oxidation. One unit of SOD activity was defined as 50 % inhibition of oxidation process. The absorbance was recorded at 340 nm and expressed as U / mg of protein. CAT activity was determined according to Aebi by monitoring decrease in absorbance of H2O2 at 240 nm and enzyme activity was expressed as nkat / mg protein. The activity of GPX was determined by the method of Pagilia and Valentine. GPX catalyses the oxidation of GSH to GSSG in the presence of H2O2. This reaction was coupled to NADPH oxidation in presence of exogenous GR to maintain GSH concentration. Total GPX activity was measured taking cumene-hydroperoxide as the substrate. The absorbance was recorded at 340 nm. Enzyme activity was expressed as nmol NADPH oxidized / min/ mg proteins using a molar extinction coefficient of 6.22 mM-1 cm-1. The activity of GR was assayed following the rate of NADPH oxidation at 340 in the presence of GSSG. Enzyme activity was expressed as nmol NADPH oxidized / min/ mg proteins using a molar extinction coefficient of 6.22 mM-1 cm-1. GST activity was measured according to Habig et al., using CDNB as a substrate. The change in absorbance was recorded at 340 nm and enzyme activity was expressed as nmol CDNB conjugate formed / min / mg protein using a molar extinction coefficient of 9.6 mM-1 cm-1. GSH was measured according to Ellman, using DTNB and the formation of thiol anion was measured at 412 nm.

Protein content was estimated by Folin-Phenol method of Lowry et al., using Bovine serum albumin as a standard.

RESULTS

CATALASE ACTIVITY:

During the study period (2005-2007) the liver, foot and gill of *all shellfish species* from the study stations were analyzed. Among the analysis of liver S9 fraction catalase activity was higher in all the *p.virdis* species (114 ± 15.25 µmol / mg-1 protein/min-1, followed by 2nd species that is C*rassostrea virginica* Gmelin , 53.66±8.26 µmol / mg-1 protein/min-1 and 3rd species *Mercenaria mercenaria* Linnaeus, 23±2.58 µmol / mg-1 protein/min-1 respectively).

SOD ACTIVITY:

The SOD activity showed a peak for *p.virdis*. The observed higher activity was (86.82±11.36Unit /mg-1 protein/min-1) followed by C*rassostrea virginica* Gmelin(36.43±5.25 Unit /mg-1 protein min-1) and then *Mercenaria mercenaria* Linnaeus(13.21±2.24 Unit /mg-1 protein/min-1).

GLUTATHIONE REDUCTASE (GSH):

Gulathione reductase activity behaves differently from the other two (catalase & SOD) antioxidant enzyme. Here the minimum level of GSH activity was noted in liver s9 fraction for both C*rassostrea virginica* Gmelin and *Mercenaria mercenaria* Linnaeus. The GSH activity of shellfish species -1,2 & 3 were 68.45±10.36 µmol /mg-1 protein/ min-1, 36.78 ±6.22 µmol / mg-1 protein/min-1 and 5.67±1.21 µmol / mg-1 protein/min-1 respectively.

DISCUSSION

In the oxidative metabolism the role of antioxidants are very essential. The antioxidants are mainly divided into enzymatic and non enzymatic. Antioxidant requirements are mostly derived from the food sources. Present study gives emphasis the enzymatic antioxidants and thus evaluated the same in *shell fish species* under environmental and experimental stress conditions. Interactions between abiotic and biotic factors are more common in the aquatic environments. Among abiotic factors, saline and thermal stress leads physiological disorder in animals, which primarily affects the metabolism, resulting in the accumulation of ROS (Rajagopal, *et. al.,* 2005; Bhat and Desai, 1998). Thereby the season oriented three antioxidant enzyme activity was primarily observed in this study.

CATALASE

The present study evidenced that the catalase activity was tissues specific and environmental parameter sensitive. The maximum catalase activity was recorded in the order of digestive glands (liver), foot and gill respectively. In the case of long term exposure to toxins affect the activity of scavenging enzyme catalase. Identical trend was demonstrated in the digestive gland, foot and gill tissues of zebra mussel (Parihar, 1997; Khessiba, *et. al.,* 2005).

SUPEROXIDE DISMUTASE (SOD):

SOD was designated as an index for a range of contaminants (Cossu, et al, 2000). The SOD exerts an elevated level in liver than foot and gill. Similar finding was reported in another species of bivalves, *Scapharca inaequivalvis* and *Tapes philippinarum*, (Santovito *et.al.,* 2005; Lushchak *et.al.,* 2006; Irato *et. al.,* 2001). Further, who stated the experience of some species-specific differences that could be attributed to their different adaptation and habitat. In that the recorded high SOD activities in the gills of both the species may be related to their physiological role in respiration (Irato *et. al.,* 2001). In the present study the SOD activity increased in liver for all species. It could be attributed to the higher hydrocarbon level. The gill showed second place for the enzyme activity but foot exhibited very lower activity than other tissues. So liver is the ideal organ to evaluate the activity of SOD against the xenobiotics exposure. In the xenobiotics exposure profile SOD showed a positive correlation with catalase ($r = 0.888$, $P < 0.05$). SOD activity in the gills of *H. fossilis* on short-term temperature showed similar enhancement (Parihar, *et. al.,* 1997; Zhang*, et. al.,* 2003). The same conditions of higher activity in *Perna viridis* also noted in long term exposure in this study.

GLUTATHIONE REDUCTASE (GSH):

Among the three antioxidants studied GSH seems to be highly sensitive to environmental dynamic such as dissolved oxygen, salinity and temperatures, besides xenobiotics. The seasonal change in environmental parameters modulates the induction of antioxidant enzymes as a protective measure against stress including potential toxicity which increased the ROS formation (Santovito *et. al.,* 2005). This GSH enzyme catalyzes the reduced nicotinamide adenine dinucleotide phosphate (NADPH)–dependent reduction of the disulfide bond of oxidized glutathione. A major function of GSH is to serve as a reductant in the oxidationreduction processes; a function resulting in the formation of glutathione disulfide (GSSG). Glutathione S-transferases (GSTs) are ubiquitous multifunctional enzymes, which play a key role in cellular detoxification. The enzymes protect the cells against toxicants by conjugating them to glutathione, thereby neutralizing

their electrophilic sites, and rendering the products more water-soluble for elimination. The temperature stress activates increased level of total glutathione, initially which serves as a compensatory mechanism, to allow mussels to maintain constant GSH / GSSG ratio despite heat induced oxidative stress (Lushchak *et. al.,* 2006). In this study the toxins exert stress to the GSH activity. Similar effect in the long term exposure to toxin was recorded by Zhang *et. al., (*2003). The liver showed higher enzyme activity.

The instant depletion of GSH level would probably reveals enhanced risk of oxidative stress in digestive gland, gills and other parts of the body that induces the membrane and cellular damages. Identical higher productions were obtained in rain bow trout fish exposed Phenobarbital, p,p-di chloro-diphenyl -dichloro-ethylene (DDE), or the prototypal oxidation-reduction cycling compound 2,3-dimethoxynaphthoquinone (Petrivalsky *et. al.,* 1997).

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