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Glutathione S-transferase and metallothionein levels in the freshwater prawn *Macrobrachium malcolmsonii* exposed to mercury

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Abstract

Healthy juveniles of *M. malcolmsonii* were exposed to 24.1 µg l^{-1} of mercury (96 hr LC_{50}: 145 µg l^{-1} Hg) for a period of 21 days. The hepatopancreas and gills of the prawns were sampled on 8th, 15th and 22nd day of exposure. Accumulation and elimination of Hg, activity of glutathione S-transferase (GST), content of glutathione (GSH) and metallothionein (MT) level were studied. Mercury accumulation was found to be higher in the hepatopancreas (88.60 µg g^{-1}) and lower in the gills (67.8 µg g^{-1}). However, Hg elimination was found to be faster in the gills (62%) and slower in the hepatopancreas (58%). Therefore, the rate of Hg elimination did not match the rate of its uptake. The activity of GST was found to be higher in tissues of test prawns (5.94-9.13 nmol mg^{-1} protein min^{-1}) on all sampling days when compared with controls (3.45-4.23 nmol mg^{-1} protein min^{-1}). Similarly, the content of GSH was found to be higher in tissues of test prawns (0.80-1.43 µmol g^{-1} protein) on all sampling days when compared with controls (0.55-1.00 µmol g^{-1} protein). These results indicate the formation of glutathione conjugate in test prawns to eliminate Hg. The induction of MT level was also found to be higher in tissues of test prawns (57.50-75.76 nmol g^{-1} protein) on all sampling days when compared with control (20.24-45.22 nmol g^{-1} protein). This indicates the fact that sequestration of Hg has occurred for its easy elimination. Thus, induction of GST-GSH and MT ensured protection and adaptation of test prawns to thrive in Hg contaminated environment.

Key words

Prawn, Mercury, Glutathione S-transferase, Reduced glutathione, Metallothionein

Introduction

Mercury (Hg) is potentially toxic to aquatic animals. A recent report indicates that even safe concentration of Hg is also deleterious to fish (Masud *et al*., 2009). This non-essential metal has very wider industrial applications causing severe environmental pollution. For instance, Hg pollution in Ennore estuary, Chennai, India was due to the discharge of industrial effluents (Rajathy, 1997). The distribution of Hg in seawater along the west coast of India was reported up to 0.116 µg l^{-1} (Kaladharan *et al*., 1999). Mercury accumulation at the rate of 0.50 µg g^{-1} (wet wt.) in tissue of oysters sampled from a contaminated stream at Karwar, Karnataka coast, India has been reported (Krishnakumar *et al*., 1998). Similarly in a recent report, the distribution pattern of Hg in Ganga river at Varanasi, India was 0.0019 mg l^{-1} in water and 91.679 mg kg^{-1} in fish (Sinha *et al*., 2007). Bhattacharyya *et al*. (2008) mentioned the accumulation pattern of Hg in organisms inhabiting in different coastal regions of India. Hg accumulation in species of marketed fishes in Shillong, India has also been reported (Chakraborty *et al*., 2003).

Toxic elements are more easily eliminated from the body if their water solubility is relatively high. Thus, the body metabolic action on these substances is primarily directed towards increasing the water solubility of lipophilic material that would otherwise tend to accumulate in the body. Within cells, Hg may bind to a variety of enzyme systems, including those of microsomes and mitochondria, producing non-specific cell injury or cell death. It has particularly an affinity for ligands containing sulfydryl groups, which is extremely insoluble in water and become non-toxic (Lash *et al*., 1998). To cope with the potentially hazardous elements, organisms possess various finely-tuned mechanisms to control their concentration and availability in the body. A few enzymes and other proteins are reported to serve the detoxification function since they facilitate for the elimination of electrophiles (Burton *et al*., 1995; Elia *et al*., 2003; Chen *et al*., 2006).

Glutathione S-transferase (GST) is a family of intracellular multifunctional dimeric protein, plays a major role in the intracellular transport of endogenous compounds, metabolizes various electrophilic xenobiotics, ligand transport and thus protects cells

against toxic effects (George, 1994; Yu, 1996). In aquatic organisms, it is an important component of the detoxification system. GST activity has been detected in gills and hepatopancreas of *M. malcolmsonii* exposed to pesticides (Bhavan and Geraldine, 2001, 2002). It has wide substrate specificities. Glutathione (GSH, L-γ-glutamyl-cysteinyl-glycine) is a substrate in the GSH S-transferase system and the availability of GSH can be a major factor in the metabolism of xenobiotics by this enzymic system. It is capable of chelating and detoxifying metals as soon as they enter the cell (Brambila *et al*., 2002). It also forms a substrate for GSH peroxidase, an enzyme capable of both removing hydrogen peroxide (H_2O_2) from the cells and repairing peroxidatively damaged membranes (Elia *et al*., 2003; Chen *et al*., 2006).

Metallothioneins (MTs) are low molecular mass metal-binding polypeptides with high cysteine content. The sulfur atoms of MT bind strongly with thiolate bonds of metals. They scavenge hydroxyl radical (OH^-), H_2O_2 and apoptotic cells (Dalton *et al*., 1994; Amaral *et al*., 2007) and are considered valid biomarkers in medicine and environmental studies (Sheehan *et al*., 1995; Blackmore and Wang, 2004; Carpene *et al*., 2007). Activation of metallothionein has been reported in fish, prawn and marine copepod under toxic condition (Kabila *et al*., 2000; Van Cleef-Toedt *et al*., 2001; Barka, 2007).

Heavy metals induced biochemical and physiological changes in tissues of *M. malcolmsonii* have also been reported (Kabila *et al*., 2000; Yamuna *et al*., 2002; Bhavan *et al*., 2008; Yamuna *et al*., 2009). However, literature with respect to operation of detoxification cum elimination mechanisms against heavy metals and pesticides is scanty in freshwater prawns except a very few (Kabila *et al*., 2000; Bhavan and Geraldine, 2001; Yamuna *et al*., 2009). Keeping in view the importance of the GSH-GST system in the enzymatic detoxification of metals, the activity pattern of GST and the content of GSH were assessed in the hepatopancreas and gills of *M. malcolmsonii* following exposure to Hg. Since the metal binding protein, metallothionein (MT) sequestering toxic metals and facilitate protection from eliciting severe toxicological response its concentrations in tissues was also determined in the present study in order to understand whether the test prawns resorts to the synthesis of MT to protect itself from Hg toxicity. While studying the operation of detoxification cum elimination mechanism against any toxin, it is highly necessary to assess its accumulation and recovery patterns. Therefore, concentrations of Hg accumulation and elimination by *M. malcolmsonii* have also been assessed.

Materials and Methods

Acclimatization: Healthy juveniles of *M. malcolmsonii* were collected from the lower anicut of the Cauvery river, Tamilnadu (South India) and acclimatized to laboratory conditions for two weeks in cement tank (6x3x3 feet) with ground water. During acclimation the prawns were fed *ad libitum* with standard pelletized feed. The unfed feed, feces, exuviae and dead prawns if any were removed daily. Three fourth of water medium was renewed daily by siphoning method causing minimum disturbance to the prawns. No sign of distress was seen in prawns during acclimation.

Bioaccumulation study: The prawns used in bioaccumulation study were divided in to two groups. Each group comprised of 50 intermoult juveniles of *M. malcolmsonii* (length: 4.0-5.0 cm; body mass: 0.8-1.0 g) and housed in 5 aquaria of 15 l capacity, each with 10 prawns. One group served as control and the other group was exposed to the sub lethal concentration (24.1 µg l^{-1}) of mercuric chloride ($HgCl_2$) (analar grade, E Merck, India), (96 hr LC_{50}: 145 µg l^{-1}; Hg solution was prepared in de-ionized water) for a period of 21 days. The Hg water medium was gently siphoned out daily with minimal disturbance to the prawns, and replaced by freshly prepared Hg water medium. During the course of the experiment, the medium was not aerated. The hepatopancreas and gills of test prawns were sampled on 8th, 15th and 22nd day of exposure (exactly a week interval between sampling). On each sampling day 15 prawns from each group were sacrificed. Tissues from three animals were pooled to constitute a single observation and five such pooled observations were made. Thus, totally 45 animals were sacrificed in each group. The remaining 5 prawns were discarded. Tissues were wet-digested with a perchloric nitric acid mixture (1:3 v/v) as per AOAC (1984) guideline. Potassium permanganate($KMnO_4$ 5%) and acidified stannous chloride (10% stannous chloride in 10% sulphuric acid) were added to the digest and Hg content accumulated was analyzed using mercury analyzer (5800 D EC, India). The sensitivity of the instrument was 0.0017 µg ml^{-1}.

Recovery study: Prawns were exposed to sub lethal concentration of Hg for 21 days in a similar experimental set up described above and consequently allowed for elimination in Hg free test water for 21 days. Sampling was performed essentially similar to that explained previously. The content of Hg present in tissues of test prawns during (on day 8th and 15th) and immediately after recovery period (on day 22nd) was estimated essentially similar to that described previously and percentage of eliminated Hg was calculated.

Estimation of GST, GSH and metallothionein: The intermoult juvenile prawns used for estimation of activity of GST, contents of GSH and metallothionein were also divided into two groups (control + experiment), each comprised of 150 prawns and housed appropriately for a period of 21 days in a similar experimental conditions described previously. Sampling was done on the hepatopancreas and gills of test prawns on 8th, 15th and 22nd day of exposure. On each sampling day, 45 prawns from each group were sacrificed for estimation of GST activity, contents of GSH and MT. For each parameter, tissues from three prawns were pooled to constitute a single observation and five such pooled observations were made (3x5=15x3=45). Thus, totally 135 prawns (45x3) out of 150 were sacrificed in each group. Tissues were dissected out and immediately subjected to analytical procedure. However, the tissues were stored at -20°C during the latent period taken to follow the analytical procedure. Chemicals, reagents, substrates, standards, enzymes and co-enzymes used were all of AR grade. The data were analyzed statistically by adopting 'student t-test' (Zar, 1984) to determine the significance between control and experiment using Microsoft Excel.

GST (1-chloro-2,4,-dinitrobenzene (CDNB) and reduced glutathione (GSH): The activity of GST was estimated as described by Habig *et al*. (1974). GST activity was assayed by using 1-chloro-2,4,-dinitrobenzene (CDNB) and reduced glutathione (GSH). Calculations were performed taking the mM extinction coefficient value to be 9.6. The values were expressed as nmol mg^{-1} protein min^{-1}. The concentration of GSH content was estimated by the method of Anderson (1985) and the values were expressed in µmol g^{-1} protein.

Metallothionein (^{203}Hg radio assay): The content of metallothionein (MT) was estimated by the ^{203}Hg radio assay, as modified by Kotsonis and Klaassen (1977). Tissue homogenates (6%) of the hepatopancreas and gills were separately prepared with ice-cold 1.15% KCl solution. To 1 ml of homogenate, increasing concentration of ^{203}Hg were added (0.166-12.266 µg of 203 Hg g^{-1}) and allowed to react for 10 min at room temperature. Following this, 1 ml of 10% TCA was added to each of the concentrations and allowed to stand for 10 min at room temperature to ensure complete precipitation of high molecular weight proteins (Lobel and Payne, 1987). The mixture was then centrifuged carefully and radioactivity was measured in the gamma counter (LKB Wallac 1270 Rack gamma II). A graph was plotted with the amount of ^{203}Hg in the TCA supernatant (nmol of MT) noted on the Y axis. The concentration of ^{203}Hg that produced a plateau region in the curve was taken as the specific concentration permitting estimation of MT content in tissue homogenate.

Results and Discussion

Accumulation and elimination of Hg: In the present study, Hg accumulation in the hepatopancreas and gills of test prawns was detected when compared with control prawn (Table 1). Uptake of Hg by test prawns increased from 8th to 22nd day. Therefore, this was gradual and continuous uptake. This indicates the fact that Hg uptake was directly proportional to duration of exposure. The gills are the principal route of entry of toxicants. However, Hg accumulation was found to be higher in the hepatopancreas and less in the gills of test prawns. This is because since the crustacean hepatopancreas is the metabolic centre of xenobiotic substances. During recovery phase, Hg level in test prawn was found to decrease on all sampling days when compared to that of the concentration recorded on 22nd day of accumulation phase (Table 1). Therefore, there was gradual and continuous elimination. Total quantum of Hg elimination was found to be higher in the gills (62%) followed by the hepatopancreas (58%). This indicates the fact that inter-organ transport of Hg has occurred to the hepatopnacreas for elimination. In the present study, the data indicates the fact that both the process of Hg uptake and elimination were gradual and continuous. However, the rate/ quantum of Hg elimination did not match with its uptake. Therefore, no complete elimination was recorded. This indicates the fact that the test prawn were able to regulate only a part of accumulated Hg. Similar pattern of accumulation and recovery has previously been reported in *M. malcolmsonii* exposed to pesticides and heavy metal (Bhavan *et al.,* 1997; Kabila *et al.,* 1999).

Glutathione and glutathione S-transferase: This system represents detoxification and elimination of metals. GST catalyze the nucleophilic addition of the thiol group of glutathione to toxic compounds to form a glutathione-conjugate. This conjugate are then transformed to more water soluble for easy elimination/ excretion through urine and feces (Al-Ghais and Ali, 1995; Gadagbui and James, 2000; Huang *et al*., 2008). GST is found to be present in tissues which interact with the external environment and are highly metabolic in nature (Al-Ghais and Ali, 1995; Mohamed *et al*., 2008). In the present study, the activity of GST was found to significantly higher ($p<0.1$) in both the gills (which directly interact with toxic medium) and the hepatopancreas (which is the metabolic centre of xenobiotics) of test prawns exposed to Hg on all sampling days when compared with control. However, maximum activity was seen on 22nd day of exposure (Table 1). An increase in GST activity has also been reported in mussel, *Mytilus edulis* exposed to sediments contaminated with pollutants (Sheehan *et al*., 1995), in *M. malcolmsonii* exposed to endosulfan and carbaryl (Bhavan and Geraldine, 2001, 2002), in the toad *Chaunus schneideri* inhabiting in agro ecosystem (Attademo *et al*., 2007) and in fish exposed to cadmium (Mohamed *et al*., 2008).

GSH forms the substrate in the GSH S-transferase system, therefore its availability is the major factor in detoxification. In this study, the content of GSH was found to significantly higher ($p<0.1$) in tissues of test prawns exposed to Hg on all sampling days when compared with control (Table 1). This elevation was higher on 8th day (43% in the hepatopancreas and 64% in the gills) followed by 15th and 22nd days of exposures. This indicates an induction of GSH synthesis against Hg was operational in test prawns as a toxicological response. Therefore, the availability of GSH for activation of GST was ample in test prawns. An elevation in GSH level has also been reported in fish exposed to cadmium (Mohamed *et al*., 2008). GSH level and GST activity is directly proportional to each other. The increased GST activity indicates utilization of GSH for formation of glutathione conjugate. Therefore, the elevation of GSH was recorded in decreasing trend from 8th day to 22nd day of exposures, while, the elevation of GST activity was recorded in increasing trend (Table 1). Thus, the present study confirms operation of GST-GSH system in tissues of *M. malcolmsonii* for elimination of Hg.

Metallothionein: MT is important in the regulation of intracellular availability of essential metals (Brouwer and Brouwer, 1992) since they prevent inappropriate and potentially deleterious intracellular interactions. In the case of non-essential metal it serves protective (detoxification) function by sequestering the metal which might otherwise bind to sensitive cellular sites and exerts toxic effects (Roesijadi, 1992). The enhanced metal tolerance is associated with the induction/ over expression of MT and the amplification of MT gene (Liu *et al*., 1995; Amaral *et al*., 2007). In this study, the content of MT was found to be significantly higher ($p<0.1$) in tissues of test prawns exposed to Hg on all sampling days when compared with control (Table 1). The elevation was maximum on 22nd day of exposure. In control prawns, the hepatopancreas showed higher

Table - 1: Concentrations of mercury (Hg) accumulation, elimination during recovery, GST activity, GSH and MT content in tissues of *M. malcolmsonii* on different sampling days

Parameter	Days	Hepatoapancreas		Gills	
		Control	Exposed	Control	Exposed
Accumulation (µg g^{-1} wet tissue)	8	ND	52.93 ±5.98	ND	50.02 ± 4.10
	15	ND	56.90 ± 3.95	ND	52.20 ± 3.75
	22	ND	88.60 ± 8.53	ND	67.80 ± 5.42
Recovery (µg g^{-1} wet tissue)	8	ND	54.60 ± 3.55	ND	36.90 ± 3.10
	15	ND	50.00 ± 4.65	ND	33.42 ± 3.36
	22	ND	37.56 ± 2.33	ND	25.90 ± 2.75
Total elimination %	—	—	58 %	—	62 %
GST (nmol mg^{-1} protein min^{-1})	8	3.45 ± 0.20	5.94 ± 0.52 (72)	4.16 ± 0.40	7.43 ± 0.55 (79)
	15	3.58 ± 0.22	6.29 ± 0.50 (76)	4.14 ± 0.30	8.29 ± 0.72 (100)
	22	3.59 ± 0.20	7.45 ± 0.70 (108)	4.23 ± 0.39	9.13 ± 0.76 (116)
GSH (µmol g^{-1} protein)	8	1.00 ± 0.14	1.43 ± 0.22 (43)	0.55 ± 0.07	0.90 ± 0.05 (64)
	15	1.00 ± 0.14	1.40 ± 0.24 (40)	0.55 ± 0.05	0.83 ± 0.06 (51)
	22	1.00 ± 0.14	1.35 ± 0.17 (35)	0.56 ± 0.05	0.80 ± 0.08 (43)
MT(nmol g^{-1} protein)	8	46.12 ± 4.09	69.43 ± 5.70 (51)	20.54 ± 2.18	57.50 ± 4.50 (180)
	15	45.22 ± 4.00	71.83 ± 6.30 (59)	20.24 ± 1.93	69.61 ± 5.80 (244)
	22	46.50 ± 4.10	75.76 ± 6.50 (63)	21.10 ± 2.30	70.18 ± 5.75 (233)

Each value is the mean ± S.D. of five pooled individual observations. ND = Not detected. All other the values are significant at $p<0.1$ (10%). Values in parentheses are percent increase over control

level of MT than that of the gills. The extent to which MT is increased in a particular organ is believed to be related to the presence of the inducer (Choudhuri *et al*., 1993). The percentage induction of MT was higher in the gills (180-233%) of test prawns followed by the hepatopancreas (51-63%). Therefore, the gills represent the most efficient site of MT induction in test prawns exposed to Hg as it is directly contact to the external environment. Further, this is supported by our previous observation that metal-rich inorganic deposits or granules in the gills of *M. malcolmsonii* and such deposits are believed to represent the detoxified dumps of Hg (Yamuna *et al*., 2009). In this study, the recorded increase in MT level indicates operation of Hg elimination mechanism. Similar increase in MT level has previously been reported in *M. malcolmsonii* exposed to Ni (Kabila *et al*., 2000). Increase in MT has also been reported in freshwater bivalves exposed to Cd and Zn (Marie *et al*., 2006) and in the euryhaline crustacean, *Neomysis integer* exposed to Cd (Erk *et al*., 2008).

Metal toxicity ensues when the rate of it uptake exceeds the rate of elimination (Depledge and Rainbow, 1990; Rainbow, 2007). This statement is valid in the present study. Further, Hg elimination was confirmed through recoded elevation of GST-GSH and MT levels. It has also been reported that MT binds Hg in the cytosol with limited capacity, and the rest was redistributed to the high molecular weight protein, which is important than the storage or transformation of Hg by MT (Chen *et al*., 2006). Similar conditions prevailed in the present study. Therefore, considerable quantum of Hg was persistent in test prawns even after recovery. In this study, Hg dependent activations of GST-GSH and MT systems may represent specific adaptative responses of *M. malcolmsonii* for elimination of Hg poisoning in order to prevent tissue damage.

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