Fish Aqua Sci 13(1), 71-78, 2010

KOSFAS FISHERIES AND AQUATIC SCIENCES
The Korean Society of Fisheries and Aquatic Science

Changes in Antioxidant Enzyme Activities in the Gill And Digestive Glands of the Manila Clam *Ruditapes philippinarum* exposed to Cu

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We investigated the effect of Cu exposure on the activities of protective antioxidant enzymes in the gills and digestive glands of the manila clam *Ruditapes phillippinarum* exposed to sub-chronic concentrations (0, 20, 40, and 80 μg L^{-1}) of waterborne Cu. No mortality occurred during the experimental period, and no significant condition index differences were observed in any exposure group compared with the control. No significant differences were observed in the digestive glands and gills of the clams observed during 15 days of exposure, but after 30 days, the SOD activity in the gill showed a significant difference between the 80 μg L^{-1} Cu-exposed group and the control. GPx activities in the digestive glands and gills were significantly lower after 30 days of Cu exposure. Gill GR activity in the high-exposure group (80 μg L^{-1}) was significantly elevated compared with that in the control group. GST activities in the digestive glands of all groups did not change over 30 days. However, GST activity in the gill at 80 μg L^{-1} Cu was significantly higher after 15 and 30 days of exposure. GSH activities in the gill showed patterns similar to those of GST activities during exposure periods. In the digestive glands, GSH activity was higher only at 80 μg L^{-1} after 30 days exposure. In digestive glands and gills, the MDA levels of clams exposed to 80 μg L^{-1} Cu were significantly higher after 30 days of exposure.

Key words: Copper, Antioxidant enzymes, Clam, *Ruditapes philippinarum*

Introduction

Aquatic systems are contaminated by various pollutants, including metals, as a result of human activities. Metal contamination in coastal waters has received increasing attention due to the potential for bioaccumulation in and toxicity to many aquatic organisms. Although Cu is an essential metal, its industrial and agricultural uses lead to an increase in environmental Cu concentrations that have become a threat to marine organisms.

Bivalve are suspension filter-feeding sedentary species that are known to accumulate metals, especially Cu (Romeo and Gnassia-Barelli, 1997). Consequently, they may be exposed to large amounts of chemical pollutants, even if these compounds are present at fairly dilute concentrations. Bivalve are known to accumulate high concentrations of heavy metals in their tissue and are used widely as bio-indicators for pollution in marine environments (Regoli and Orlando, 1994; Geret et al., 2003). They are also capable of bioconcentrating xenobiotics by many thousand-fold, which can facilitate chemical analysis (Sheehan and Power, 1996). The manila clam *Ruditapes phillippinarum* has been proposed as suitable for the biomonitoring of metal contamination because of its wide distribution throughout Korean coastal regions.

Antioxidant systems have been studied for some years in fish and bivalves exposed experimentally to chemicals or collected from polluted areas (Di Giulio et al., 1989; Winston and Di Giulio, 1991; Stegeman et al., 1992). The usefulness of bioindicators is enhanced greatly when chemical analyses are integrated with data on the biological effects of pollutants (Bayne et al., 1988). In this respect, oxidative stress is a common pathway of toxicity induced by several classes of pollutants (Winston and Di Giulio, 1991) that enhance the production of reactive oxygen species. Protection against the

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toxicity of oxyradicals toward cellular targets is afforded by a complex defense system consisting of both low-molecular-weight scavengers and anti-oxidant enzymes. Variations of antioxidant defenses, such as the glutathione content (one of the most important antioxidant agents) and the activity of glutathione-dependent and antioxidant enzymes have often been proposed as biomarkers of contaminant-mediated oxidative stress in several marine organisms (Di Giulio et al., 1989; Livingstone 1993; Winston and Di Giulio, 1991).

Gills and digestive glands are the main target organs for several pollutants, and these tissues have been chosen to compare the effects of various metal concentrations. The same organs have also been used for investigating the principal antioxidant defenses of bivalves, including the total glutathione concentration and the activities of several glutathione-dependent and antioxidant enzymes.

The aim of the present study was to investigate the effect of Cu exposure on the activities of protective antioxidant enzymes in the gills and digestive glands of the manila clam *R. phillippinarum* in order to explain the cytotoxic and tissue damaging effects of Cu exposure.

Materials and Methods

Exposure and depuration experiment

Manila clams *R. philippinarum* were collected in July 2005 from a clam farm in Go-heung County, Chon-nam, Korea. The clams were acclimatized for 5 days in a semi-static system. After acclimatization, clams (shell length: 35.81±2.51 mm, body weight: 10.40±2.16 g) were selected for the experiments. Fifty clams were separated into control and test tanks (50 L). The animals in the test tank were exposed to different sub-lethal concentrations (0, 20, 40, and 80 μg L^{-1}) of copper (II) nitrate hydrate (Cu, Sigma Chemical, St. Louis, MO, USA) for 30 days. Seawater was changed every 48 h (Table 1). Animals were maintained under a 12:12 h light/dark cycle. Twelve clams were sampled from each group every 15 days for 30 days, and the chemistries were determined. The condition index (CI) , derived from Duquesne et al. (2004), was calculated as CI = fresh flesh weight (g)×100/shell weight (g).

Enzymatic activities

Eight clams were removed from each tank on days 15 and 30 of the experiment. Weight and total length were recorded for each individual. Four clams were dissected to obtain gills and digestive glands and then weighed. Tissues were homogenized in 0.5 M sucrose and 0.15 M NaCl in 0.02 M Tris-HCl (pH 7.6). The homogenates were centrifuged at 500×*g* for 15 min at 4°C, and the resultant supernatant was centrifuged at 12,000×*g* for 30 min at 4°C. Antioxidant enzyme activities in the tissues were measured with a temperature-controlled spectrophotometer (DR/4000U; HACH, Germany). The assays were performed in duplicate or triplicate.

Table 1. The chemical components of seawater and experimental condition used in the experiments

Item	Value
Temperature(°C)	22.0 ± 1.0
pH	8.1 ± 0.7
Salinity(‰)	33.5 ± 0.5
Dissolved oxygen (mg L^{-1})	7.2 ± 0.2
COD (μg L^{-1})	1.1 ± 0.1
Ammonia (μg L^{-1})	12.5 ± 0.8
Nitrite (μg L^{-1})	1.3 ± 0.2
Nitrate (μg L^{-1})	11.6 ± 1.1
Hardness (mg L^{-1})	4.2 ± 0.5
Copper (μg L^{-1})	3.1 ± 0.4

Polyunsaturated fatty acid peroxides generating malondialdehyde (MDA) activities were measured in pre-centrifuged homogenate tissues according to the method of Jone and Steven (1978). The post-centrifuged supernatant was analyzed for superoxide dismutase (SOD) activity using the xanthine oxidase-cytochrome *c* method (Flohe and Otting, 1984), glutathione reductase (GR; Carlberg and Mannervik, 1985), glutathione-S-transferase (GST; Habig and Jacoby, 1981a, b), and glutathione peroxidase (GPx; Paglia and Valentine, 1967) activities.

Reduced glutathione (GSH) activity was measured in the other four clams. Tissues were dissected, homogenized in 10% $HClO_4$, and centrifuged at 5,000× *g* for 15 min. The resultant supernatants were used for GSH determination (Richardson and Murphy, 1975). The reaction mixture contained 0.01 M 5,5'-dithiobis, 2-nitrobenzoix acid (DTNB), and 0.1 M PBS (pH 8.0). The GSH standard curve was generated up to 10 nM using a reduced glutathione standard solution. The linear increase in absorbance was recorded at 412 nm. Total protein concentrations (Bradford, 1976) were determined using bovine serum albumin (BSA) as a standard.

Statistical analysis

Data are expressed as the mean ± standard deviation (S.D.). Prior to analysis, all data were tested for homogeneity of variances among groups using the Bartlett test. Statistical analysis was performed using the SPSS/PC$^+$ statistical package. Comparisons of normalized data between control and treatment

groups were made by one-way analysis of variance (ANOVA) followed by Duncan's multiple comparisons test of mean values if significant differences ($P<0.05$) were found.

Results

No mortality occurred during the experimental periods. No significant differences in CI values were observed between any exposure group and the controls (Fig. 1).

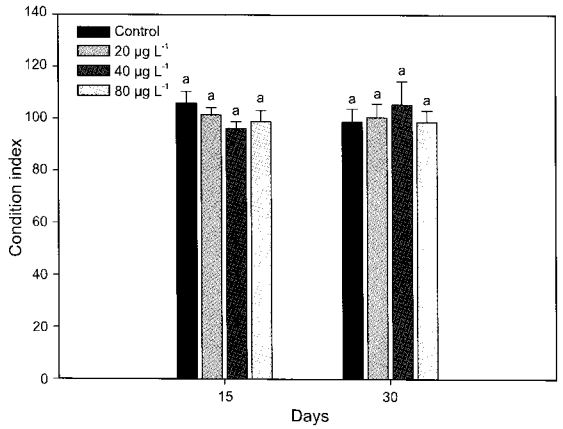

Fig. 1. Changes of condition index in the *Ruditapes philippinarum* exposed to Cd for 2 weeks and followed by a depuration periods of 1 week. Vertical bar denotes one standard deviation of the mean.

SOD activities in the digestive glands and gill of *R. phillippinarum* exposed to Cu (control, 20, 40 and 80 μg L^{-1}) are presented in Fig. 2 as a function of exposure time and exposure concentrations. After 30 days of exposure, no significant differences in SOD activity in the digestive glands of the clams were found; however, the SOD activity in the gill was significantly higher in the 80 μg L^{-1} Cu-exposed group compared with the controls.

Changes in GPx activity in the digestive gland did not vary significantly after 15 days of Cu exposure compared with the control group, but it was significantly less in the 40 and 80 μg L^{-1} exposed groups after 30 days. GPx activity in the gill was significantly less in the 80 μg L^{-1} Cu-exposed group after 30 days (Fig. 3).

GR activity levels in the clam tissues are presented in Fig. 4. Although the GR activity in the digestive glands showed a slight increase with Cu concentration compared with the controls, the difference was not significant. GR activity in the gill with high exposure (80 μg L^{-1}) was significantly higher than that of the control group.

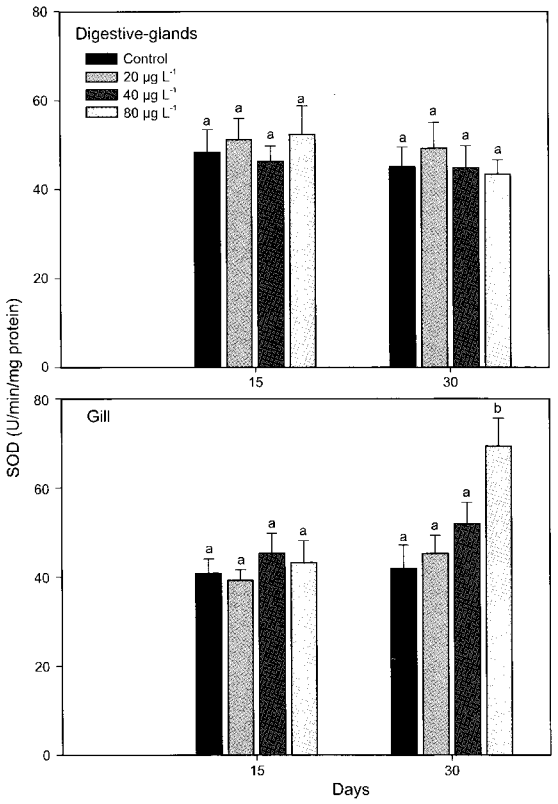

Fig. 2. Variation of SOD activities in the digestive glands and gill of clam, *Ruditapes philippinarum* exposed to Cd for 2 weeks and followed by a depuration periods of 1 week. Vertical bar denotes one standard deviation of the mean. Different superscripts on the bar are significantly different ($P<0.05$).

GST activity in clam tissues is presented in Fig. 5. GST activity in both the gill and digestive glands was higher after 30 days with 80 μg L^{-1} Cu ($P>0.05$). At other Cu concentrations, the GST activity did not change significantly compared with the control group.

In the digestive gland, GSH activity showed a pattern similar to that of the GST activity during the exposure period, and only the 80 μg L^{-1} Cu-exposed group showed a difference when compared with the control group (Fig. 6). In the gill, GSH activity was significantly higher in the 80 μg L^{-1} exposed group during the experiment. In the digestive gland and gill, the MDA level of clams exposed to Cu was significantly higher with 80 μg L^{-1} Cu exposure after 30 days, although no differences were observed with the other Cu concentrations after 15 and 30 days (Fig. 7).

Discussion

Laboratory studies are a useful tool to evaluate the

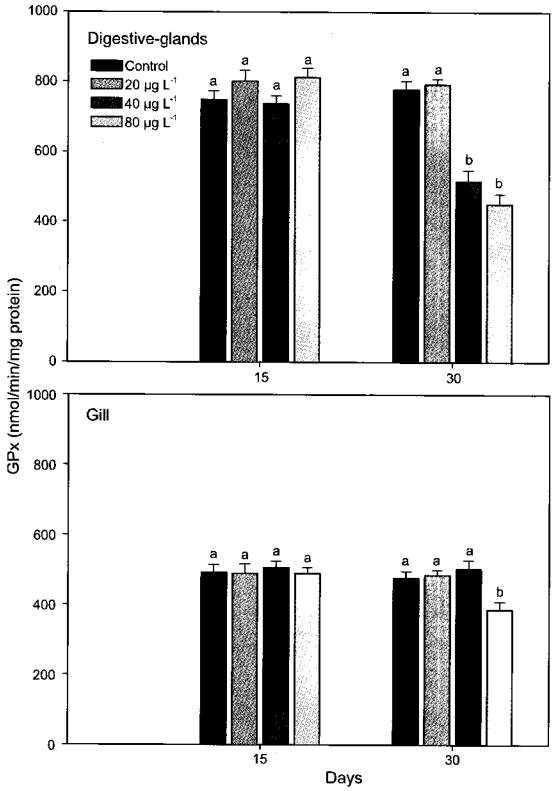

Fig. 3. Variation of GPx activities in the digestive glands and gill of clam, *Ruditapes philippinarum* exposed to Cd for 2 weeks and followed by a depuration periods of 1 week. Vertical bar denotes one standard deviation of the mean. Different superscripts on the bar are significantly different ($P<0.05$).

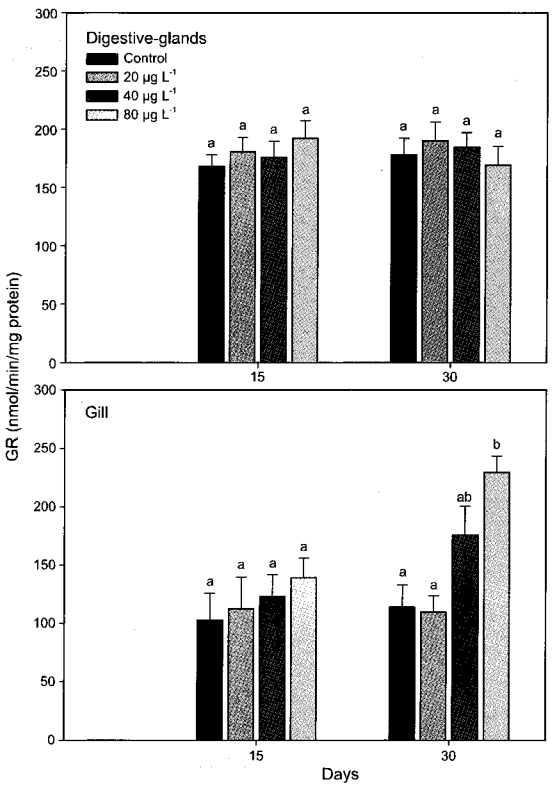

Fig. 4. Variation of GR activities in the digestive glands and gill of clam, *Ruditapes philippinarum* exposed to Cd for 2 weeks and followed by a depuration periods of 1 week. Vertical bar denotes one standard deviation of the mean. Different superscripts on the bar are significantly different ($P<0.05$).

impact of heavy metals on antioxidant enzyme systems and to explain the intervention and relationship of the different antioxidant enzyme mechanisms as a function of metal exposure. Antioxidant enzymes such as glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), glutathione (GSH), lipid peroxidation (LPO), and superoxide dismutase (SOD) were monitored to evaluate the impact of exposure to waterborne Cu.

GPx catalyzes the reduction of hydrogen peroxide to water or organic peroxides to their corresponding stable alcohols by oxidizing the reduced glutathione (GSH) to its oxidized form (GSSG), whereas glutathione reductase (GR) regenerates GSH by catalyzing the reduction of GSSG into GSH. Thereby, GR is of utmost importance, as its inhibition is a factor in sensitivity to chemical stress (Regoli and Principato, 1995; Cossu et al., 1997; Doyotte et al., 1997). GST, which catalyzes a conjugation reaction with the tripeptide glutathione, is quantitatively the most important phase II enzyme (Mannervik and Danielson, 1988). These enzymes also play a role in protection against oxidative stress by catalyzing a selenium-independent glutathione peroxide activity (Prohaska, 1980). The tripeptide glutathione (GSH) is one of the most intensively studied intracellular solutes due to its critical role in cell biochemistry and physiology. GSH acts in the protection against oxidative damage, detoxification of endogenous and exogenous reactive metals and electrophiles, and storage and transport of cysteine, as well as for protein and DNA synthesis, cell cycle regulation, and cell differentiation (Meister and Anderson, 1983; Meister, 1984; Wang and Ballatori, 1998; DeLeve and Kaplowitz, 1990).

SOD is an oxido-reductase that catalyzes the dismutation of the superoxide anion into molecular oxygen and hydrogen peroxide (Fridovich, 1989). The SOD activity results in the current study showed changes in bivalves not only at high exposure (80 μg L^{-1}) to Cu, but also at lower doses below 40 μg L^{-1} Cu. These changes were more pronounced in the gills than in the digestive glands. A change in SOD activity was found in bivalves after Cu 30 days of

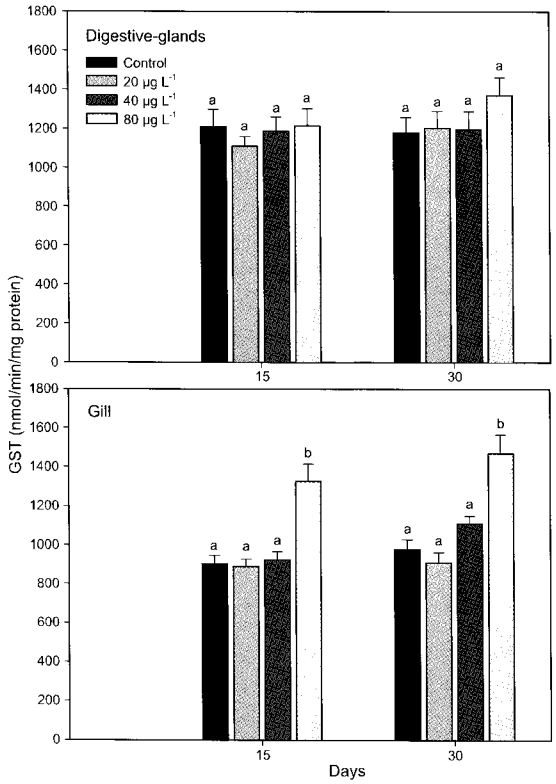

Fig. 5. Variation of GST activities in the digestive glands and gill of clam, *Ruditapes philippinarum* exposed to Cd for 2 weeks and followed by a depuration periods of 1 week. Vertical bar denotes one standard deviation of the mean. Different superscripts on the bar are significantly different ($P<0.05$).

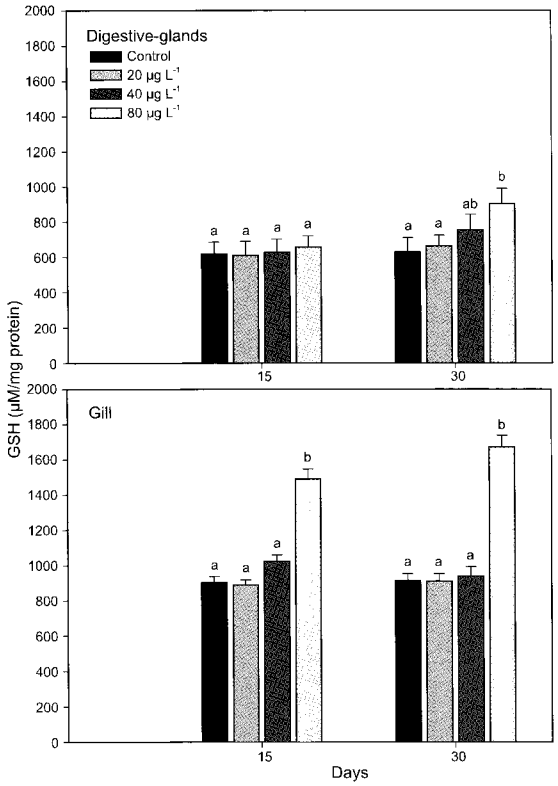

Fig. 6. Variation of GSH activities in the digestive glands and gill of clam, *Ruditapes philippinarum* exposed to Cd for 2 weeks and followed by a depuration periods of 1 week. Vertical bar denotes one standard deviation of the mean. Different superscripts on the bar are significantly different ($P<0.05$).

treatment, especially at 80 μg L^{-1} Cu. This suggested that Cu causes oxidative damage in bivalves, possibly by generating reactive oxygen stress in the body.

GPx activity may play a protective role for gill tissues against oxidative stress when the activity of antioxidant enzymes is reduced (Power and Sheehan, 1996; Sheehan and Power, 1999). In this study, GPx activity in the digestive gland and gill was significantly reduced after 30 days of exposure to 80 μg L^{-1} Cu. GPx is considered to be an efficient protective enzyme against lipid peroxidation (Winston and Di Giulio, 1991). In general, this inhibition is Cu dependent. Cu is known to generate oxyradicals. Cu^{2+} ions also react with ROOH, leading to the formation of $ROO^{\cdot}$ and Cu^{+}. Cu can react with H_2O_2, leading to the formation of $OH^{\cdot}$. H_2O_2 is the substrate for catalase and GPx. Therefore, an inhibitory effect of these enzymes may be due to Cu. A similar effect was observed for the gills of the freshwater bivalve, *Unio tumidus*, after 3 days of Cu exposure (30 μg L^{-1}) (Doyotte et al., 1997). In other studies that recorded the induction of GPx activity, this increase was not efficient enough to prevent oxidative damage (Di Giulio et al., 1993).

In the present study, the GR activity was higher in the gill than in the digestive glands. A higher sensitivity of the gills to oxidative stress was also indicated by the inhibition of antioxidant parameters, often more pronounced in this tissue than in the digestive glands. It was concluded that the gills were more sensitive than the digestive glands because lipid peroxidation was found exclusively in the gills (Cossu et al., 1997). GR activity is rarely investigated, and few researchers have studied this enzyme in field experiments on the oxidative stress of aquatic species (Hasspieler et al., 1994 a, b; Di Giulio et al., 1995; Regoli and Principato, 1995).

Induction of GST has been noted in *Mugil* sp. (Rodriguez-Ariza *et al.*, 1993) and *M. edulis* (Suteau et al., 1988) collected from polluted coasts. The detoxification enzyme GST exhibited the maximum activity in the gill with 80 μg L^{-1} Cu exposure after 15

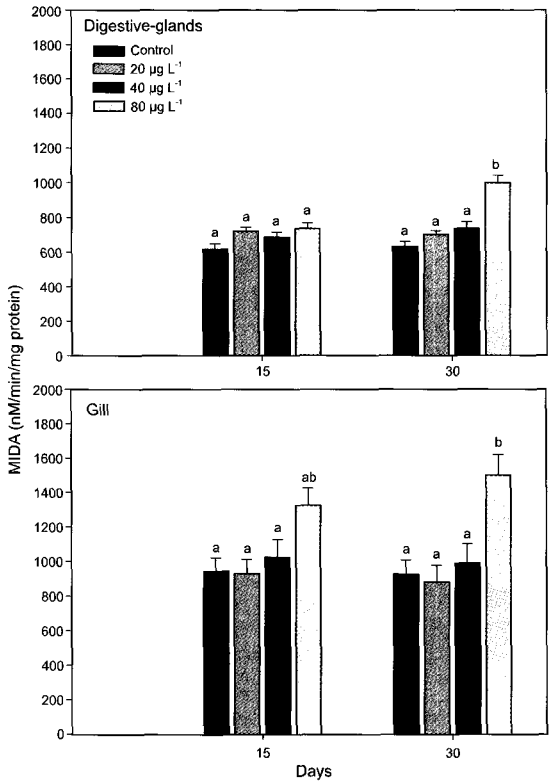

Fig. 7. Variation of MDA activities in the digestive glands and gill of clam, *Ruditapes philippinarum* exposed to Cd for 2 weeks and followed by a depuration periods of 1 week. Vertical bar denotes one standard deviation of the mean. Different superscripts on the bar are significantly different ($P<0.05$).

and 30 days. GSTs are distributed widely in nature, and their induction of expression could form the basis for the early detection of stress responses in organisms exposed to toxicants. This enzyme activity has been assessed as a potential indicator of exposure to chemical pollutants in both Cork Harbor and Venice Lagoon using the closely related species, *M. galloprovincilais* (Buetler and Eaton, 1992).

GSH is an essential non-protein antioxidant that acts either directly as a reductant or indirectly as a substrate for enzymes such as glutathione peroxidases and glutathione transferases. GSH ensures the reduction of oxidants, quenching of free radicals, neutralization of organic peroxides, and elimination of hydrocarbons by conjugation. It can also bind directly to metals. Low GSH levels made the cells more sensitive to prooxidants and were found to be associated with increased MDA levels. Much lower GSH concentrations have also been measured in *M. galloprovincialis* living in polluted water compared with those from unpolluted areas and in mussels transplanted from clean to contaminated sites (Regoli and Principato, 1995). Variation in GSH levels seemed to be a valuable indicator of exposure to Cu. The effect on toxicity will depend on the capacity of cells to maintain sufficient turnover of GSH and/or to synthesize new pools of reduced glutathione.

MDA is considered to be an important feature in cellular injury and results largely from free radical reactions in membranes, which are rich in polyunsaturated fatty acids. The MDA results in this study demonstrated that exposure to sub-lethal Cu concentrations stimulates lipid peroxidation in the tissues of bivalves after 30 days of exposure. An increase in lipid peroxidation was also observed in *in vitro* studies with the giant fresh water prawn *Macrobrachium rasenbergii* after exposure of the crude homogenate to 500 μM $CdCl_2$ for 30 min (Dandapat et al., 1999). An increase in MDA was also observed when the supernatant of gill of the clam *R. decussatus* was exposed to 25 μg L^{-1} Cu for 30 days (Geret et al., 2002). An MDA deficiency of aquatic species has been reported previously by several authors, whether the deficiency was caused by environmental contaminants (Livingstrone et al., 1993), seasonal factors (Viarengo et al., 1991a, b), or spawning (Ribera et al., 1989; Solé et al., 1995). These results suggest that the gills appear to be more susceptible to oxidative stress than the digestive glands and that a relationship may exist between the degree of deficiency of antioxidant defenses, lipid peroxidation, and toxicity in bivalves.

Acknowledgements

This research was supported by the Pukyong National University Research Fund in 2007 (PK-2007-052).

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(Received 4 December 2008; Revised 29 January 2010; Accepted 12 March 2010)