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Effects of cadmium on glutathione synthesis in hepatopancreas of freshwater crab, *Sinopotamon yangtsekiense*

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ABSTRACT

Cadmium (Cd) is one of the most deleterious heavy metals in aquatic systems that could promote oxidative damage. To explore the effects of Cd exposure of a freshwater crab (*Sinopotamon yangtsekiense*) on hepatopancreatic glutathione (GSH) synthesis, crabs were exposed to the reagent with a dose range of 7.25–116.00 mg L^{-1} for 48 h. The concentrations of GSH, oxidized glutathione (GSSG), NADPH and $NADP^+$, as well as the activities of enzymes involved in GSH synthesis, i.e. glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PD), γ-glutamylcysteine synthetase (γ-GCS) were determined. Progressive depletion of cellular GSH content was observed with the increasing of Cd concentrations, while the level of GSSG remained constant. In response to Cd exposure, crabs showed significant induction of G6PD and NADPH, however, only up to moderate exposures. GR activity remained at a steady level at all exposure concentrations. The activity of γ-GCS was significantly positively correlated with the Cd concentration. These results suggested that GSH synthesis could be activated against reactive oxygen species induced by lower Cd exposure; under the higher Cd exposure conditions, an inhibition of NADPH-dependant redox cycling and *de novo* GSH synthesis led to significant decrease in GSH content.

1. Introduction

Cadmium (Cd), a widely used heavy metal in modern industry, is one of the most abundant, ubiquitously distributed toxic elements in aquatic systems (Novelli et al., 2000). It has been implicated in oxidative injury involving the initial formation of reactive oxygen species (ROS). ROS (such as singlet oxygen, hydrogen peroxide, and hydroxyl radicals) have the potential to generate oxidative stress within cells by reacting with macromolecules and causing damage such as mutations in DNA, destruction of protein function and structure, and peroxidation of lipids (Valko et al., 2006). Cadmium promotes oxidative damage by increasing the cellular concentration of ROS and by reducing the cellular antioxidant capacity (Corticeiro et al., 2006). As a predominant non-protein thiol compound, glutathione (GSH, *c*-glutamyl-cysteinyl-glycine) is a major intracellular antioxidant in living organisms and the first line of defense against oxidative stress. GSH is a central component in the multifaceted cellular detoxification system that constitutes an important mechanism for cellular protection against agents, such as Cd, which produce ROS. There are two mechanisms for combating metal stress that involve GSH: (i) by relieving the oxidative stress effects caused by heavy metals through the formation of oxidized glutathione (GSSG); and (ii) by forming metal complexes (Noctor and Foyer, 1998). Fluctuation of GSH levels in response to stimuli which produce oxidative stress was observed in various organisms (Świergosz-Kowalewska et al., 2006). Cellular GSH contents reflect a steady-state balance between consumption and synthesis. Glutathione synthesis inside a cell is regulated by two mechanisms, NADPH-dependant redox cycling and *de novo* synthesis of glutathione. The reduction of GSSG to GSH is catalyzed by glutathione reductase (GR), which uses NADPH as reducing potential (Romero and Canada, 1991). NADPH is the principal intracellular reductant in all cell types. An important source of NADPH is glucose-6-phosphate dehydrogenase (G6PD), the first and rate-limiting enzyme of the pentose phosphate pathway (PPP) (Beutler et al., 1996). There is abundant evidence that G6PD plays a pivotal role in NADPH and GSH production for defense against oxidative stress (Winzer et al., 2001). γ-glutamylcysteine synthetase (γ-GCS) is the rate-limiting enzyme in *de novo* synthesis of GSH and the overexpression of γ-GCS would result in enhanced cellular GSH synthesis (Noctor et al., 1996).

There have been many reports on glutathione-mediated alleviation of metal stress in animals but studies on glutathione synthesis and its role in metal stress in Crustacea are limited. Freshwater crab (*Sinopotamon yangtsekiense*) is one of the important representatives of decapod crustacean and a species commonly found in freshwater in China. Freshwater crab has the capability of accumulating heavy metals (Reinecke et al., 2003) and is thus a suitable bioindicator for environmental contamination with these agents

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(Schuwerack et al., 2001). Hepatopancreas, the key site of Cd accumulation in Crustacea (Wang et al., 2001), is one of the most important organs that play important roles in metal detoxification (Gibson and Barker, 1979). Therefore, it is of great interest to investigate the toxicity of Cd on hepatopancreas in freshwater crabs. Previous studies in our laboratory have shown that, in metal toxic injury, hepatopancreas of *S. yangtsekiense* is an early and prominent site (Wang et al., 2003), and antioxidant enzymes activities in this tissue are impaired (Yan et al., 2007). Therefore, we hypothesized that the rate of GSH synthesis in hepatopancreas would be affected by Cd exposure. The specific aim of the present study was to assess changes in GSH levels and several other biochemical parameters in hepatopancreas that might have an effect on synthesis of GSH following exposure to different doses of Cd for 48 h. This report also investigated whether the rate of GSH synthesis was affected by Cd toxicity. Data reported in this study support the conclusions from previous studies and provide mechanistic insight.

2. Material and methods

2.1. Chemicals

All the chemicals used were analytical grade, obtained from Sigma Co. (St. Louis, MO). Assay kits for GSH and GSSG were from Beyotime Institute of Biotechnology.

2.2. Animals and treatments

Freshwater crabs, *S. yangtsekiense* were caught from Anhui province in China. Only non-damaged adult crabs with a homogeneous weight (20.0 ± 0.5 g) were collected. Prior to experiments, crabs were acclimated for 4 weeks in glass aquaria filled with dechlorinated, carbon-filtered city tap water (pH 7.5, dissolved oxygen 8.0–8.3 mg L^{-1}). A regime of 12 h light/12 h dark was applied and the temperature was kept at 20 ± 2 °C. Aquaria were shielded by a black plastic to reduce disturbance. Crabs were fed three times a week with commercial feed.

After acclimatization, crabs were randomly divided into six experimental groups of five specimens each and allocated to control and five sublethal concentrations of Cd (1/32, 1/16, 1/8, 1/4 and 1/2 of the 96 h LC_{50}): 7.25 mg L^{-1}, 14.50 mg L^{-1}, 29.00 mg L^{-1}, 58.00 mg L^{-1} and 116.00 mg L^{-1} for 48 h in glass aquaria, based on the 96 h LC_{50} of Cd for *S. yangtsekiense* (232.00 mg L^{-1}) (Yan et al., 2007). Crabs were not fed during the experimental period. All other conditions were kept the same as those used for acclimation.

2.3. Sample preparation

After the 48 h exposure period, crabs were cryoanesthesized by putting them on ice for about 15 min. Opening the cephalothorax, the tissue samples of hepatopancreas were immediately excised, washed with ice-cold saline, weighted, and homogenized (10% w/v) in 0.1 M phosphate buffered saline, pH 7.5, with a Potter-Elvehjem type motordriven homogenizer at 4 °C. The homogenates were centrifuged at 15,000*g* for 15 min at 4 °C, and the supernatants were collected and stored at −20 °C in polypropylene tubes until assay.

2.4. Measurement of GSH and GSSG

GSH and GSSG contents were measured by a commercially available kit (Beyotime Institute of Biotechnology) following the method of Anderson (1985). Briefly, total glutathione (GSH plus GSSG) was determined in the homogenates spectrophotometrically at 412 nm, after precipitation with 0.1 M HCl, using GR, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) and NADPH. GSSG was determined by the same method in the presence of 2-vinylpyridine and GSH content was calculated from the difference between total glutathione and GSSG. The levels of GSH and GSSG were expressed as mg g^{-1} protein. The ratio of GSH over GSSG was used to indicate redox status that inferences the detoxification capacity.

2.5. Measurement of NADPH and $NADP^+$

NADPH and $NADP^+$ were measured using spectrophotometric enzymatic cycling assays, by slight modifications of the method of Nisselbaum and Green (1969). To destroy $NADP^+$, a portion of the homogenates were incubated at 60 °C for 30 min and promptly chilled to 0 °C. Both the heat-treated extracts containing NADPH and the untreated preparations containing the oxidized and reduced forms of the nucleotides were immediately analyzed. The final cuvette concentrations of the reagents used in the assay were 100 mM Tris–HCl (pH 8.0), 5 mM Na_2EDTA, 2 mM phenazineethosulfate, 0.5 mM 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide, 1.3 U G6PD, 1 mM glucose 6-phosphate. After 5 min incubation at 37 °C, the reaction was started by addition of treated or untreated homogenates. The reactions were allowed to run for 5 min and the absorbance determined at A_{570}. The amounts of NADPH and $NADP^+$ in the samples were determined from standard curves.

2.6. Enzyme activity assays

2.6.1. G6PD activity

G6PD activity was determined according to the method developed by Deutsch et al. (1983). The final cuvette concentrations of the reagents used in the assay were 0.49 M Tris–HCl buffer pH 7.5, 3.20 mM glucose 6-phosphate, 0.37 mM $NADP^+$ as cofactor and 61.70 mM $MgCl_2$. Addition of maleimide (0.50 g L^{-1} in cuvette) inhibited further oxidation of reaction products by 6-phosphogluconate dehydrogenase. The rate of increase in absorbance at 340 nm is a measure of G6PDH activity. One unit is equivalent to 1 νmol of $NADP^+$ reduced mg^{-1} protein min^{-1}.

2.6.2. GR activity

GR activity was assayed by the method developed by Goldberg and Spooner (1987). The oxidation of NADPH during the reduction of GSSG was monitored at 340 nm. The final cuvette concentrations of the reagents used in the assay were 100 mM phosphate buffer pH 7.2, 0.49 mM EDTA, 5.10 mM FAD, 2.14 mM GSSG, and 0.16 mM NADPH. The decrease in absorbance at 340 nm was recorded at 60 s intervals for 6 min. Enzyme units were expressed as nmol NADPH oxidized mg^{-1} protein min^{-1}.

2.6.3. γ-GCS activity

γ-GCS activity was measured by the method developed by Seelig and Meister (1985), which utilizes the coupled reaction of pyruvate kinase-lactate dehydrogenase (PK-LDH) in reaction mixture containing Tris–HCl buffer 100 mM (pH 8.0), 150 mM KCl, 20 mM $MgCl_2$, 5 mM ATP, 2 mM phosphoenolpyruate, 10 mM glutamate, 10 mM L-γ-aminobutyrrate, 0.2 mM NADH, 5 U PK, and 10 U LDH. Change in absorbance per minute at 340 nm was recorded at 37 °C. γ-GCS activity is expressed as nmol mg^{-1} protein min^{-1}.

2.7. Protein concentration

The protein concentration was determined using the Biuret method (Gornall et al., 1949), with bovine serum albumin as a standard.

2.8. Statistics

All data presented are the mean values of five independent sets of experiments. Each value was presented as means ± standard deviation (SD). Statistical analysis were carried out by one-way ANOVA using the Dunnet's test to evaluate whether the means were significantly different, taking $P < 0.05$ as minimal significant. Statistical computations were performed with SPSS 13.0 for Windows (SPSS Inc.).

3. Results

After in vivo exposure to different Cd concentrations for 48 h, crab hepatopancreatic GSH and GSSG contents were measured. As seen in Table 1, there were no significant changes in GSH levels up to 14.50 mg L^{-1}, but a dose-dependent decrease in GSH contents was observed in crabs exposed to higher Cd concentrations. At 116.00 mg L^{-1} Cd exposure, GSH reached the lowest level, with a concentration of 30.536 mg g^{-1} protein. This represented a 33% decrease in GSH level. However, Cd treatment did not cause significant changes in GSSG from the control group (0.198 mg g^{-1} protein). Similar to GSH, at lower Cd exposure (7.25 and 14.50 mg L^{-1}), no significant induction in the T-GSH (total GSH = GSH + 2GSSG) content was noticed, while the reduction of this index was marked at each of the dose levels beyond 29.00 mg L^{-1}. The ratio of GSH/GSSG decreased continuously and reached the lowest level of 156.207 in the group treated with the highest concentration of Cd (116.00 mg L^{-1}).

Intracellular specific activities of enzymes and content of the cofactor NADPH involved in the redox cycling of GSH were also assayed. Despite Cd stress significantly affecting GSH content, GR activity remained at a steady level (0.416 U mg^{-1} protein) (Fig. 1A). Initially up to 14.50 mg L^{-1} Cd resulted in a concentration-dependent increase in the activity of G6PD, and maximal increase of 99.2% was observed as compared to control. G6PD activity showed decrease in crabs exposed to 29.00 mg L^{-1} Cd but still above control levels, and it returned to control values after exposed to 58.00 mg L^{-1} Cd. In the 116.00 mg L^{-1} Cd treated group, a significant decline of G6PD activity as compared to control was observed (Fig. 1B). Fig. 1C and D shows the relationship between Cd concentrations and changes in NADPH and $NADP^+$, respectively. Similar to G6PD activity, the concentrations of NADPH were elevated at the lower concentrations of Cd. The NADPH level was reduced in the 116 mg L^{-1} Cd treatment group compared with that of the control group. On the contrary, $NADP^+$ levels had a clear reduction at 14.50 and 29.00 mg L^{-1} Cd concentration, however, significant elevation was noticed in the group treated with the highest concentration.

As the limiting enzyme in the *de novo* synthesis of GSH, the activity of γ-GCS was monitored under different treatments. The activity of γ-GCS increased gradually in dose-dependent manner and was significantly positively correlated with the Cd stress. The maximal increase in γ-GCS at 116.00 mg L^{-1} Cd treatment was 63.1% greater than the control (Fig. 2).

4. Discussion

In previous studies (Li et al., 2005; Yan et al., 2007), we have demonstrated that the administration of Cd leads to significant alterations in antioxidant status and activities of some antioxidant enzymes. The toxicity of Cd is associated with oxidative damage caused by the production of ROS. As the most abundant non-protein thiol and prominent cellular antioxidant in living organisms, GSH plays a key role in protection against oxidative stress. A decline in GSH content has been reported in different tissues of aquatic animals (Chelomin et al., 2005; Ivanina et al., 2008), which is consistent with the findings of the present study. GSH is considered a primary defense mechanism against Cd, since its cysteine thiol group rapidly reduces the metal by forming a stable GS–Cd complex. The important antioxidant function of this tripeptide is also due to its involvement in scavenging oxyradicals and participation as a substrate in detoxification reactions catalyzed by glutathione peroxidase and glutathione S-transferases (GST). Thus, the excessive consumption of GSH in metal reduction, chelation and oxidation by ROS leads to its significant depletion following exposure to high levels of Cd, compromising detoxification. As a very important indicator of redox status in cells (Rana et al., 2002), the GSH/GSSG ratio decreased with the increasing concentration of Cd in the present study, demonstrating that the exposure to Cd did induce oxidative stress. The synthesis of sufficient concentrations of GSH is of vital importance to the protection of cells from oxidative stress during Cd exposure. Glutathione homeostasis is maintained by two mechanisms, NADPH-dependant redox cycling and *de novo* glutathione synthesis. The results obtained in the present study show that the activities of enzymes involved in GSH synthesis vary considerably in response to Cd stress.

GSH is consumed in reactions that protect cells by removing deleterious compounds and hydroperoxides, with the formation of GSSG. GR is the key enzyme that plays an important role in maintaining glutathione in its reduced form by recycling GSSG to GSH at the expense of cofactor NADPH, which is provided by G6PD reaction (Filosa et al., 2003; Leopold et al., 2003). Changes in GR activity may affect the cellular antioxidant capacity and the ability to withstand oxidative stress. Although decreased activity of this enzyme has been reported under different kinds of stresses (Kim et al., 1998), the activity of GR in hepatopancreas of *S. yangtsekiense* did not change during Cd exposure in the present study. Due to the presence of thiol groups at the active site, GR is very sensitive to inhibition by heavy metals such as Cd, Zn, Cu and Fe, and by compounds that react with thiol groups (Smith et al., 1989). An increased gene expression of GR has been observed in liver during the response to conditions of oxidative stress (Diaz-Flores et al., 2006). It is possible that similarly to the above two mechanisms, GR activity reflected homeostasis between synthesis and loss in the present study. Because a steady state of GR activity was found during administration of Cd and GR is highly efficient in GSSG reduction, GR may not be the limiting factor for the regeneration of GSH.

Since recycling of GSSG consumes NADPH, the cellular levels of NADPH and its synthesis may represent the rate-limiting factor for

Table 1
Effect of different Cd concentrations on glutathione redox state in hepatopancreas of *S. yangtsekiense*

Parameters	Control	7.25 mg L^{-1}	14.50 mg L^{-1}	29.00 mg L^{-1}	58.00 mg L^{-1}	116.00 mg L^{-1}
GSH	45.672 ± 0.917	44.896 ± 1.222 (−1.7)	43.228 ± 1.520 (−5.4)	40.874 ± 1.075** (−10.5)	36.120 ± 1.028** (−20.9)	30.536 ± 0.760** (−33.1)
GSSG	0.198 ± 0.016	0.195 ± 0.011 (−1.5)	0.188 ± 0.027 (−5.1)	0.184 ± 0.031 (−7.1)	0.203 ± 0.010 (+2.5)	0.197 ± 0.022 (−0.5)
T-GSH	46.068 ± 0.949	45.286 ± 1.244 (−1.7)	43.604 ± 1.574 (−5.3)	41.242 ± 1.137** (−10.5)	36.526 ± 1.048** (−20.7)	30.930 ± 0.804** (−32.9)
GSH/GSSG	231.385 ± 21.195	230.500 ± 18.203 (−0.4)	232.457 ± 27.178 (+0.5)	226.085 ± 40.093 (−2.3)	178.620 ± 12.924 (−22.8)	156.207 ± 20.124* (−32.5)

The contents of GSH, GSSG and T-GSH (GSH + 2GSSG) are expressed as mg g^{-1} protein; data represent means ± SD of five animals; values in parenthesis indicate percent change from control; statistical significance was denoted by $^*P < 0.05$, $^{**}P < 0.001$ versus the respective control crabs.

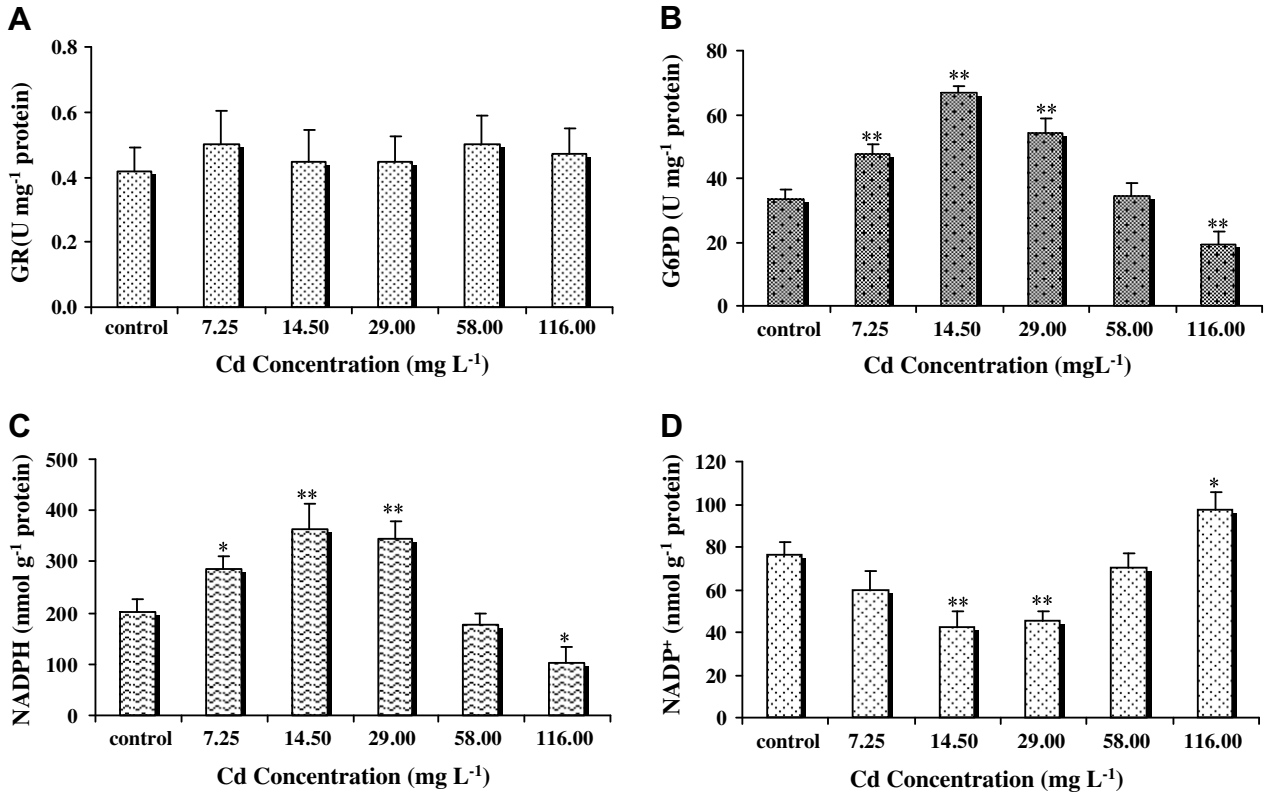

Fig. 1. Effect of different Cd concentrations on activities of GR (A) and G6PD (B), as well as contents of NADPH (C) and $NADP^+$ (D) in hepatopancreas of *S. yangtsekiense*. Data represent means ± SD of five animals. Statistical significance was denoted by $^{*}P < 0.05$, $^{**}P < 0.001$ versus the respective control crabs.

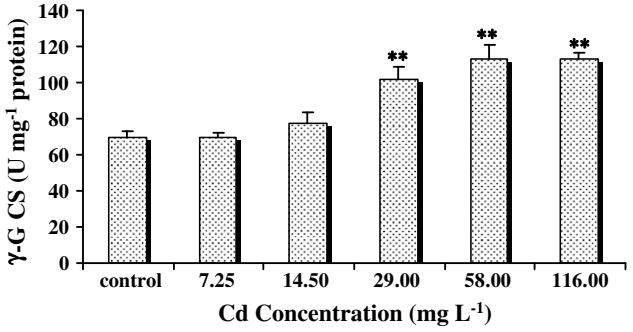

Fig. 2. Effect of different Cd concentrations on γ-GCS activity in hepatopancreas of *S. yangtsekiense*. Data represent means ± SD of five animals. Statistical significance was denoted by $^{*}P < 0.05$, $^{**}P < 0.001$ versus the respective control crabs.

the reaction and operation of the mechanisms of detoxification. Although there are other metabolic pathways that produce NADPH, studies have shown that PPP is the principal source of NADPH required to defend against oxidative stress (Tian et al., 1999). As the rate-limiting enzyme of PPP, sufficient G6PD activity is required for cells to properly defend against ROS. Increased G6PD activity prevents cell death under different conditions, particularly during oxidative stress. Organisms or cells lacking or deficient in G6PD are especially susceptible to free radical damage (Filosa et al., 2003). Our results indicated that, following the increase of concentration of Cd (from control to 14.50 mg L^{-1}), G6PD activity was upregulated, directly resulting in the enhancement of NADPH content. Agents that produce oxygen radicals are known to induce G6PD through the soxR regulon, which controls G6PD and eight other genes needed to protect cells from free radical damage (Kletzien et al., 1994). The generation of oxygen radicals after Cd administration may be responsible for the induction of hepatopancreatic G6PD. Although G6PD activity in the hepatopancreas increased significantly up to 14.50 mg L^{-1} Cd, its activity decreased at higher Cd levels (29.00, 58.00 and 116.00 mg L^{-1}). G6PD can be activated by sufficient concentrations of Mg and Ca, but Cd, at the concentration of 10 mg L^{-1}, markedly inhibited this enzyme (Sharma et al., 1998). In the present study, exposure to higher Cd concentration led to a decrease in G6PD activity. The down-regulated G6PD activity in crabs exposed to higher exposure levels could be due to either an altered enzyme gene expression or a post-translational enzyme modification (protein oxidation), or a combination of both, although the precise mechanism involved merits further investigation.

The antioxidant defense mechanisms ultimately rely on the adequate production of NADPH for reducing equivalents during oxidative stress (Martini and Ursini, 1996). Thus, cells with higher activity of G6PD induced by the mild concentration of Cd were capable of responding rapidly to the need of NADPH for maintenance of the cellular redox state, antioxidant system, and the normal level of GSH. The decreased GSH content (see Table 1) following exposure to higher doses of Cd are likely due, at least in part, to decreased G6DP activity and NADPH concentration. Lack of NADPH was due to the fact that higher concentrations of Cd may inhibit G6PD activity and thus made cells more susceptible to oxidant injury. An important revelation of the present study was the striking parallelism between the G6PD activity, NADPH concentration and GSH content in groups exposed to higher Cd concentrations. This is in agreement with Diaz-Flores' study (Diaz-Flores et al., 2006), in which a regulatory role of G6PD in maintaining NADPH and GSH levels has been shown. This indicates that NADPH-dependant redox cycling plays a crucial role in providing

GSH and, hence, maintaining the normal level of GSH and a natural redox state in cells.

Crabs exposed to lower Cd (7.25 and 14.50 mg L^{-1}) did not show significant changes in GSH, GSSG and T-GSH levels in comparison with control (Table 1). This phenomenon indicated that GSH concentrations were kept at a balance between synthesis and consumption, and there was no net loss of GSH at lower Cd exposure. But, in our model, exposure to higher levels of Cd decreased the activity of G6PD and reduced the supplement of NADPH to inhibit the GSH regeneration, and we hypothesized that abundant GSSG would begin to accumulate in cells. However, in this study, the accumulation of GSSG had not been observed and its levels had no significant changes in all the five sublethal concentrations of Cd compared to control (Table 1). The reason for the phenomenon is that GSSG would be preferentially secreted from cells to decrease the level of cellular GSSG. This efflux appears to be a part of the protection of cells and tissues from oxidative stress. A potential reason for the efflux of GSSG may help maintain the half-cell reduction potential of the GSH/GSSG couple and a favorable redox environment in cells (Schafer and Buettner, 2001). But the efflux of GSSG to maintain the redox status during oxidative stress will result in loss of glutathione from cells. Since GSSG is taken up poorly if at all by cells, loss of GSSG from cells under conditions of oxidative stress induced by Cd increases the cellular requirement for *de novo* GSH synthesis.

As the rate-limiting enzyme in *de novo* synthesis of GSH, γ-GCS is a heterodimer comprised of a catalytically active heavy subunit that includes all substrate binding sites and a light subunit that modulates the affinity of the heavy subunit for substrates and inhibitors (Huang et al., 1995). Since the 5′-flanking region of the γ-GCS heavy subunit gene contains antioxidant and electrophile response elements binding site within the transcription start site (Mulcahy et al., 1997), increase in ROS would activate the gene expression of γ-GCS. Interestingly, though the activity of γ-GCS in the hepatopancreas showed a significant dose-dependent increase in the presence of high levels of Cd (Fig. 2), glutathione accumulated only to a limited degree and then significantly decreased at the higher Cd concentrations (Table 1). In contrast to other studies (Noctor et al, 1996; Zhu et al., 2006), during an increase of γ-GCS, the enhanced *de novo* synthesis of GSH was not observed in the present study. *De novo* GSH synthesis is regulated by at least three factors: (i) the level of γ-GCS present in the cell; (ii) the availability of its substrates, particularly L-cysteine; and (iii) feedback inhibition of GSH (Griffith, 1999). Since, γ-GCS activity was significantly enhanced after the exposure to Cd and *de novo* synthesis would not be feedback-inhibited by decreased GSH concentration, we hypothesized that *de novo* synthesis of GSH was limited by L-cysteine availability following the administration of higher doses ($\geqslant$29 mg L^{-1}).

In the previous study, we observed that the activity of GST was at a steady state in response to the increasing Cd concentrations (Yan et al., 2007). GST could detoxify endogenously produced electrophiles by conjugation with GSH. The initial products are chemically stable sulfides of GSH, but further metabolism removes the L-glutamate and glycine residues, forming S-substituted L-cysteines. S-substituted cysteines are metabolized to form a mercapturic acid, which is excreted in the urine. Such metabolism and the decrease in T-GSH (Table 1) result in the irreversible loss of the L-cysteine residue of GSH. Based on our present study, we suspected that the decreased GSH contents in groups treated with higher doses of Cd were partially due to the inhibition of *de novo* synthesis caused by the depletion of the limiting amino acid L-cysteine.

Considering the highly significant increase in NADPH concentration, G6PD activity as well as constant activities of GR and γ-GCS at lower exposure concentrations (7.25 and 14.50 mg L^{-1}), it could be concluded that GSH synthesis was enhanced against ROS induced by Cd. At the higher Cd concentrations, the cellular oxidative stress was likely to have been intensified. Under the higher Cd exposure conditions (29.00, 58.00 and 116.00 mg L^{-1}), more GSH was needed to eliminate hydroxyl radical, the cytotoxic Fenton reaction product, cytotoxic products formed by the reaction of nitric oxide (NO) with O_2 and superoxide, respectively. Therefore, the insufficient NADPH supply due to reduced G6PD activity, the inhibition of *de novo* synthesis caused by the depletion of the limiting amino acid L-cysteine, and the increased GSH consumption during the antioxidant defense state could explain the gradually decreased GSH content observed in hepatopancreas of *S. yangtsekiense* exposed to high levels of Cd.

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