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Effect of Cd on GSH and GSH-related enzymes of *Chlamydomonas* sp. ICE-L existing in Antarctic ice

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Abstract: Glutathione(GSH) and GSH-related enzymes play a great role in protecting organisms from oxidative damage. The GSH level and GSH-related enzymes activities were investigated as well as the growth yield and malonyldialdehyde(MDA) content in the Antarctic ice microalga *Chlamydomonas* sp. ICE-L exposure to the different cadmium concentration in this paper. The results showed that the higher concentration Cd inhibited the growth of ICE-L significantly and Cd would induce formation of MDA. At the same time, it is clear that GSH level, glutathione peroxidases(GPx) activity and glutathione S-transferases(GST), activity were higher in ICE-L exposed to Cd than the control. But GR activity dropped notably when ICE-L were cultured in the medium containing Cd. Increase of GSH level, GPx and GST activities acclimate to oxidative stress induced by Cd and protect Antarctic ice microalga *Chlamydomonas* sp. ICE-L from toxicity caused by Cd exposure. These parameters may be used to assess the biological impact of Cd in the Antarctic pole region environment.

Keywords: antarctic ice microalgae; *Chlamydomonas* sp. ICE-L; glutathione; glutathione peroxidase; glutathione reductase; glutathione S-transferase; cadmium

Introduction

Cadmium(Cd) is one of the most toxic environmental or industrial pollutants, can interfere with respiratory, disturb metabolism and inhibit photosynthesis (Prasad, 1999). As being a secondary effect, Cd exposure stimulates formation of reactive oxygen species (ROS) and lipid peroxidation and produce oxidative stresss(Chien, 2001).

The glutathione system, including glutathione (GSH), glutathione peroxidases (GPx), glutathione S-transferases (GST), and glutathione reductase(GR), plays a major role in scavenging ROS. GSH is an essential component of the Asc-glutathione cycle, and its role in the protection of tissues from toxicants has been extensively studied(Foyer, 1991). The interaction of metals with GSH is an integral part of the toxic response(Sheweita, 1998). GPx catalyses the reduction of H_2O_2, organic hydroperoxides, and lipid hydroperoxides using GSH as a reducing agent and thus help to protect the cells against oxidative damage(Flohe, 1984). GST catalyses the conjugation of glutathione to a variety of electrophilic compounds and plays an important role in the inactivation of xenobiotics and their metabolites and the other chemicals (Batist, 1986; Kramer, 1988). GR, the key enzyme for maintaining the GSH pool in a reduced state(Rennenberg, 1982), is a disulfide oxido-reductase flavoprotein, dependent on NADPH as electron donor. It is observed that Cd can inhibit the activity of GR *in vitro*(Acan,1995). The level of GSH and the activities of antioxidant enzymes are generally increased in plants in response to abiotic stresses, and this may lead to enhanced stress tolerance(Foyer, 1997).

Antarctic ice microalgae with special characters is one of main producer of primary production and plays an important role in marine ecology system of Antarctic pole region. These microalgae living in extreme environment, which characterized by low temperature, high dissolved oxygen, presence of ice and strong seasonal changes in light intensity, are investigated more and more in recent years. They should have a more effective mechanism to remove ROS (Regoli, 1997). With the development of the human activities, it is possible that Cd could be found in Antarctic pole region. So the present study was carried out to examine the influence of heavy metal Cd on GSH level and the activities of GSH-metabolizing enzymes of the Antarctic ice microalga *Chlamydomonas* sp. ICE-L, which may become a species for assessing the biological impact of Cd in marine Antarctic environment.

1 Materials and methods

1.1 Algal culture

A unialgal strain of Antarctic ice alga *Chlamydomonas* sp. ICE-L was obtained from the key lab of marine bioactive substance of State Oceanic Administration of China and cultured in the f/2 medium of Guillard and Ryther(Guillard, 1962). Triangle flasks containing 1200 ml medium were inoculated with 300 ml of a mother culture. The alga was grown at 6—8℃ in the refrigeratory under a 12:12 light-dark cycle of 1300—1900 lux. Every flask was shaked 5 times a day.

1.2 Effect of Cd on growth yield

The effects of cadmium on growth were investigated in f/2 medium under a 12:12 light-dark cycle of 1300—1900 lux. For this, all groups were cultured for 13 d in the similar medium, but with various concentrations of $CdCl_2$ (0, 40, 80, 120, 160 μmol/L). Growth was measured as cell density.

1.3 Effect of Cd on GSH level and GSH-related enzyme activities

As the above, the day 10 old Antarctic ice alga *Chlamydomonas* sp. ICE-L were further cultured for 2 d with various concentrations of $CdCl_2$ (0, 20, 40, 60, 80 μmol/L). The samples were taken per 6 h from the beginning of the test for the following assays.

1.4 Determination of malonyldialdehyde content

Lipid peroxidation level was determined in terms of 2-thiobarbituric acid (TBA) reactive metabolite, chiefly malonyldialdehyde (MDA) (Heath, 1968). Alga were extracted with 50 mmol/L PBS(pH 7.0). After centrifugation at 2000 g, 0.3 ml supernatant was added to 3 ml 20% TCA

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(including 1% TBA). The solution was quickly cooled after water-heating at 90℃. Following centrifugation at 10000 g for 10 min, the absorbance of the supernatant was measured at 532 nm. The level of lipid peroxidation is expressed as μmol of MDA formed using an extinction coefficient of 155 mmol/cm.

1.5 Preparation of enzyme extracts

Algal material treated with Cd and control for different time were powdered in liquid nitrogen. These powdered materials were further homogenized in 5—10 times volume 50 mmol/L Tris-HCl buffer (pH 7.0, including 20% (v/v) glycol, 1 mmol/L ascorbate, 1 mmol/L DTT, 1 mmol/L EDTA, 5 mmol/L $MgCl_2$ and 1% PVP) using quartz sands. The extract was centrifuged at 7000 g for 20 min and the supernatants were used for analysis of GSH, GSH-related enzymes and protein.

1.5.1 Quantitation of glutathione

Glutathione was measured on samples treated by 5% sulphosalicilic acid, centrifuged at 10000 g for 15 min. The resulting supernatants were assayed by the method of using DTNB (5, 5'-dithio-bis(2-nitrobenzoic acid)). The GSH level was calculated by the absorbance at 412 nm according to the standard curve(Ellman, 1959).

1.5.2 Assays of GSH-related enzyme activities and protein

GPx was estimated as the decrease in absorbance at 412 nm according to the change of GSH to GSSG when H_2O_2 was inverted to H_2O(Flohe, 1984). One unit of enzyme activity represents 1 μmol/L of GSH decreased min^{-1} mg^{-1} protein at 25℃. GST was assayed as the decrease in absorbance at 412 nm due to conjugation of GSH to CDNB (1-chloro-2, 4-dinitrobenzene)(Warholm, 1985). One unit of GST activity represents 1 μmol/L of GSH decreased min^{-1} mg^{-1} protein under the assay conditions of 25℃. GR activity was determined by measuring the reduction of oxidized glutathione at 340 nm. The reduction of oxidized glutathione was measured by NADPH oxidation(Guo, 2002). The reaction mixture contained 50 mmol/L Tris-HCl (pH 7.5), 0.1 mmol/L NADPH, 5 mmol/L $MgCl_2$, 0.5 mmol/L GSSG and 100 μl above extract in a final volume of 2.5 ml. One unit of enzyme activity represents 1 μmol/L of NADPH oxidized min^{-1} mg^{-1} protein at 25℃. Protein concentrations were measured by the method of Bradford(Bradford, 1976) using bovine serum albumin as a standard. All determinations are expressed as the mean ± *SD* of three separate experiments. *SD* value was calculated by Microsoft Excel 2000, and the significance test was valued by SPSS11.5 statistics software.

2 Results

There was a significant inhibition of growth yield of microalga *Chlamydomonas* sp. ICE-L when Cd concentration was more than 80 μmol/L($P < 0.05$), but the growth yield did not change significantly with 40 μmol/L and 80 μmol/L (Fig.1). The reduction compared to the control was about 25% with 120 μmol/L and 160 μmol/L at 13 d. It was clear that there was no a notable growth yield difference between 40 and 80 μmol/L or between 120 and 160 μmol/L ($P > 0.05$).

The increased accumulation of lipid peroxides is indicative of enhanced production of toxic oxygen spicies. MDA, which is one of the major TBA reactive metabolites and indicates lipid peroxidation level, was measured under various Cd concentrations. The level of MDA elevated in Cd-treated groups is shown in Fig. 2, the increase in MDA content was Cd-concentration dependent except 20 μmol/L Cd. About 42.4% and 133.3% respectively significant increase in MDA content occurred with 20 μmol/L Cd and 80 μmol/L Cd($P < 0.01$).

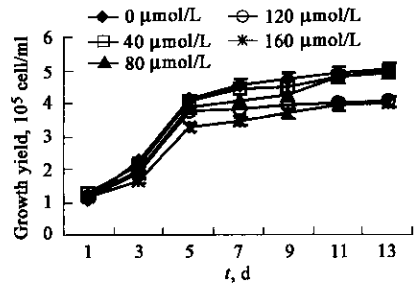

Fig. 1 Effect of Cd on the growth yield of Antarctic ice microalga *Chlamydomonas* sp.ICE-L

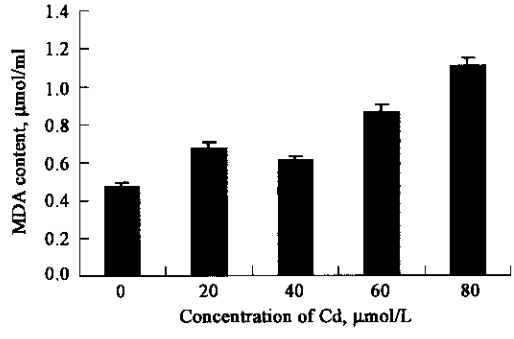

Fig. 2 Effect of Cd on MDA content of Antarctic ice microalga *Chlamydomonas* sp.ICE-L

GSH maintains the cellular redox status, showed a concentration and time-dependent change in its level in Cd-exposed algae. GSH content of control group kept between 0.50×10^{-3}—0.57×10^{-3} μg/μg protein steadily (Fig.3). But GSH of all groups treated by Cd changed significantly($P < 0.01$), which can be seen decrease at first and then increase after 18 h or 24 h treatment. GSH content of the experiment groups except 20 μmol/L was higher clearly than the control and reached 0.761×10^{-3} μg/μg protein, 0.655×10^{-3} μg/μg protein and 0.910×10^{-3} μg/μg protein respectively at 40, 60 and 80 μmol/L at 30 h. GSH content of algae treated by 20 μmol/L Cd was only 0.492×10^{-3} μg/μg protein after 30 h treatment.

GPx activity of the control did not changed notably during 30 h. On the other hand, the GPx activity of Cd-treated ICE-L increased significantly ($P < 0.01$) but 60 μmol/L Cd-treated algae at 24 h and 30 h(Fig.4). When exposure to Cd, GPx of ICE-L increased quickly and reached the maximum(909 U at 20 μmol/L, 523 U at 40 μmol/L, 155 U at 60 μmol/L, 593 U at 80 μmol/L), then began to drop, and still higher than the control but 60 μmol/L exposure. The most increase of GPx occurred from 60 U to 909 U at 20 μmol/L over a period of 18 h.

A similar trend with GPx activity also occurred in GST

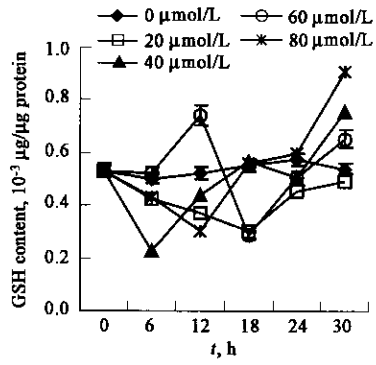

Fig. 3 Effect of Cd on the GSH level of Antarctic ice microalga *Chlamydomonas* sp. ICE-L

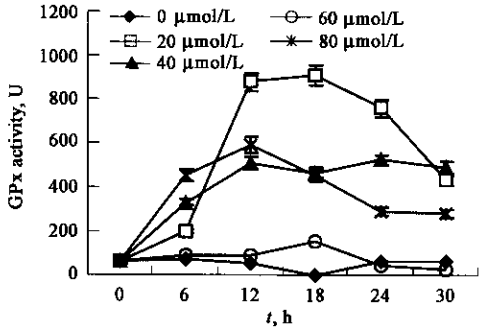

Fig. 4 Effect of Cd on the GPx activity of Antarctic ice microalga *Chlamydomonas* sp. ICE-L

activity (Fig. 5), which increased markedly and reached the maximum at 12 h or 18 h. GST activity (but 6 h at 40 μmol/L and 30 h at 20 μmol/L) was higher than the control, which was in a steady level during 30 h. The maximum of GST were 98.8 U, 88.0 U, 107.8 U and 222.6 U respectively with 20 μmol/L, 40 μmol/L, 60 μmol/L, 80 μmol/L Cd treatment. Of all, increase of GST was the most at 80 μmol/L. The changes of GST depended on the concentration of Cd more or less.

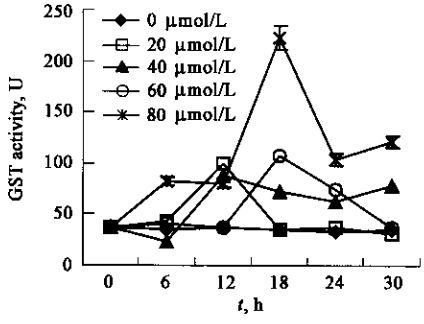

Fig. 5 Effect of Cd on the GST activity of Antarctic ice microalgae *Chlamydomonas* sp. ICE-L

GR, that catalyses the NADPH-dependent reduction of GSSG, showed a significant decrease in its activity in ICE-L but 20 μmol/L and 40 μmol/L Cd exposure at 6 h. GR activity of ICE-L treated with 60 μmol/L and 80 μmol/L Cd began to drop at 6 h and continued to the minimum 0.078 U and 0.066 U respectively (Fig. 6). However, a increase in GR activity of ICE-L exposure to 20 μmol/L and 40 μmol/L Cd was observed at 6 h. Thereafter GR activity dropped significantly on subsequent hours to a level that was lower than that of the control. The extent of GR decrease with reference to the control was higher in microalgae exposed to 60 μmol/L and 80 μmol/L Cd than in microalgae exposed to 20 μmol/L and 40 μmol/L Cd, especially exposure for 30 h.

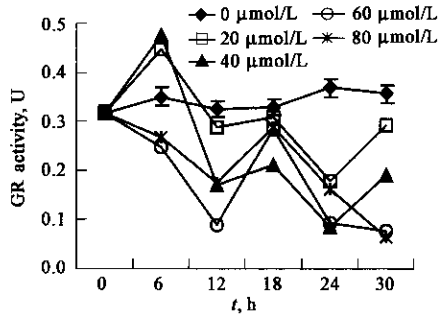

Fig. 6 Effect of Cd on the GR activity of Antarctic ice microalga *Chlamydomonas* sp. ICE-L

Compared to the control, protein concentration of *Chlamydomonas* sp. ICE-L was higher but 20 μmol/L exposure (data not shown). The change of protein level was more notable in algae treated with 40 μmol/L and 60 μmol/L Cd than that with 20 μmol/L and 80 μmol/L Cd during 30 h. As a whole, the increase or decrease of protein did not depend on the time of Cd exposure.

3 Discussion

In the present study we can observe clearly that 80 μmol/L and 160 μmol/L Cd inhibit the growth yield of Antarctic ice alga *Chlamydomonas* sp. ICE-L notably ($P < 0.05$, Fig. 1). Cd can bind to proteins and nucleic acids and thus is highly toxic to the most organisms (Prasad, 1999). So it will destroy photosynthesis and respiratory functions. The adverse effect of Cd on growth was examined in many other Cd-treated plants. 5 μmol/L Cd could decrease biomass of the marine algae *N. oculata* by 10% during 4 d (Lee, 2003). 5 μmol/L Cd would induce the growth of *Populus canescens*, but 50 μmol/L Cd inhibited it by 50% (Schützendübel, 2002). *Hordeum vulgare*, bean and pea treated with 0—40 μmol/L Cd grew slower than the control (Finkemeier, 2003; Dixit, 2001; Sandalio, 2001). It is apparent that resistance ability of *Chlamydomonas* sp. ICE-L to Cd is stronger than that of other organisms. The growth yield of ICE-L treated with 40 μmol/L Cd did not change pronouncedly, however, growth of other organisms exposed to less than 5 μmol/L Cd decreased greatly.

Many studies have been focused on the enhancement of plant tolerance to oxidative stress by modifying the plant antioxidant defense system (Anderson, 1995). The glutathione metabolic pathways contribute to the protection against peroxides in a complex manner. GSH and GSH-related enzymes have been demonstrated to be involved in the climination of peroxides, in the protection against toxic agents released during lipid peroxidation and in the restoration of GSH (Bielawske, 1986). Detailed information concerning the function of GSH and GSH-related enzymes on the metal Cd in Antarctic ice microalga has not been found.

An elevated level of MDA in ICE-L exposed to Cd in this study (Fig. 2) indicates that the metal caused oxidative

damage possibly by generating ROS, which is evident from the increased GPx activity in Cd treated ICE-L(Fig.4). All ROS are extremely reactive and rapidly disrupt normal cell metabolism if they have not been cleaned off in time(Dawes, 2000). Like the present study, Cd was reported to increase lipid peroxidation via ROS generation in many plants. The MDA concentration of rice leaves enhanced when rice exposed to 5 μmol/L Cd (Chien, 2001). Pea treated with 0—50 μmol/L Cd would lead to elevation of lipid peroxidation (Dixit, 2001), and increasing lipid peroxidation occurred when bean exposed to only 5 μmol/L Cd(Chaoui, 1997). In sunflower leaves exposed to Cd, it was shown that Cd-induced oxidative stress is mediated by ROS (Gallego, 1996). Cd can also induce enhancement of lipid peroxidation in mouse, which depended on time(Karmakar, 1998).

GSH has been suggested to act in scavenging of H_2O_2 and in the maintenance of the redox in the cells (Foyer, 1991). Various levels of Cd-induced depletion of GSH have been reported in different plant species, such as *Hordeum vulgare*, pea, rice, sunflower, and *Arabidopsis* (Finkemeier, 2003; Xiang, 1998; Dixit, 2001; Gallego, 1996). Leaves and roots of 40 μmol/L Cd-treated pea plants showed a maximum decrease of 29% and 34%, respectively, in GSH content (Dixit, 2001). De Vos *et al*. (De Vos, 1992) reported more than a 50% decrease in GSH level in roots of Cd-treated *Silene cucubalis*, while Gallego *et al*. (Gallego, 1996) found only a 20% reduction in leaves of Cd-exposed sunflower. In general, the glutathione pool recovered after prolonged Cd-exposure frenquently to levels near or above those of controls(Arisi, 2000). Decreased level of GSH in rice treated with 10 μmol/L Cd began to enhance after 8 d, and GSH content dropped at first, then restored to the level of control in *Arabidopsis* exposed to 100—400 μmol/L Cd (Xiang, 1998). In the present study, the change of Antarctic ice microalga ICE-L in GSH level is similar to the above theory, which is verified by our results (Fig. 3). According to the results, the changes in GSH level are dependent on species of organisms used in the experiment. We can conclude that ICE-L is more resistible to Cd than other plants from the above data because decreased GSH recovered quickly and decreased the deleterious effects of Cd. By contrast, the level of GSH in the roots of phragmites exposure to 50 μmol/L Cd elevated and was higher than the control(Iannelli, 2002). Decrease of GSH in both ICE-L and other organisms was found at first, but the following increase of GSH is probably correlated with GSH-related enzymes which could restore GSH level, so the changes of these enzymes activities were determinated in the following part of this paper.

The increased GPx activity(Fig.4) can explained well the remarkable resistance of ICE-L to the oxidative stress caused by high concentration of Cd. The study about effect of Cd on the GPx of other lives is scarce. Finkemeier *et al*. (Finkemeier, 2003) found GPx gene expression enhanced in *Hordeum vulgare* treated with Cd, whereas, Liu Xiaolin *et al*. (Liu, 2003) reported the enzyme activity decreased during 24 h, then elevated after 48 h and restored to the level of control in *Eriocheir sinenses* exposure to Cd. In the present study, it is easy to conclude that the GPx activity of ICE-L treated with Cd increased compared to the control. Although the activity droped at last, it was still higher than the control. It is clear that GPx plays a great role in protection against toxicity of Cd in Antarctic ice microalgae. These can be explained by the increase of MDA. High activity of GPx can clean lipid peroxide quickly and effectively. The increase of GPx activity may be caused by activation of existed GPx protein, or enhance of GPx gene expression, or increase of GPx protein synthesis.

In our study, GST activity like GPx increased early, and then dropped to a level that was still in a high condition (Fig. 5). It could be hypothesized that the need for detoxification of the toxic products of peroxidation processes induces GST in ICE-L. This change of GST might be an adaptive mechanism of *Chlamydomonas* sp. ICE-L. The same results were observed in phragmites or pea exposed to Cd (Iannelli, 2002; Dixit, 2001). But decrease of GST activity occurred early in mouse exposure to Cd, and began to increase after 74 h(Karmakar, 1998). It is evident that GST can also be induced by Cd in order to protect Antarctic ice microalgae from deleterious effects caused by oxidative stress from Cd. In agreement with the reported data(Boyer, 1989), GSH level does not appear to directly regulate GST activity since GST and GSH did not change synchronously.

GR is crucial for the regeneration of GSH from GSSG (Foyer, 1997). Its higher activity has been related to resistance to oxidative stress in plants. Transgenic plants overexpressing the gene for GR showed greater resistance to oxidative stress (Creissen, 1994). Increase of GR activity was reported in pea, bean, *Phragmite*, *Arabidopsis* and mouce (Iannelli, 2002; Ferreira, 2002; Dixit, 2001; Xiang, 1998; Karmakar, 1998). In this study, only elevated GR activity can be seen in the cell of *Chlamydomonas* sp. ICE-L treated with 20 μmol/L and 40 μmol/L at 6 h(Fig.6). As a whole, GR activity of ICE-L exposed to Cd decreased signicantly, which was in agreement with the data reported in rice and *Populus canescens* exposure to Cd (Schützendübel, 2002; Yu, 2000). Different stress has a distinct effect on GR. The divarication can be interpreted that a low dose Cd would induce GR, however, a higher dose Cd would inhibit GR activity. Inhibition effect of Cd on GR has been proved in sheep (Acan, 1995) and marine alga *Nannochloropsis oculata* (Lee, 2003). GR activity generally follows the pattern of GPx. In fact, it seemed that decrease of GR activity had no effect on the reduction of GSSG because the level of GSH can keep a high level in the present study. It is possible that GR activity of Antarctic ice microalgae, which is higher than that of other lives under the same condition, is enough to reduce GSSG to compensate for the depleted GSH level. At the same time, the synthesis ability of GSH in Antarctic ice microalgae may be also responsible for maintaining the GSH level.

4 Conclusions

It is concluded that changes of growth yield, MDA content, GSH level and GSH-related enzymes activities occurred in Antarctic ice alga *Chlamydomonas* sp. ICE-L treated with Cd. Increase of GSH level and GPx and GST activities acclimate to oxidative stress induced by Cd and

protect ICE-L from toxicity caused by Cd exposure. The changes of enzymes activities should be correlated to either the expression of enzymes or the activation of the existed enzymes. These parameters may be used to assess the biological impact of Cd contaminant in Antarctic pole region environment.

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