Bull. Environ. Contam. Toxicol. (2002) 68:561–568

DOI: 10.1007/s00128-001-0291-8

Bulletin of Environmental Contamination and Toxicology

Induction of Phytochelatins in *Lemna aequinoctialis* in Response to Cadmium Exposure

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Received: 8 July 2001/Accepted: 10 December 2001

Plant cells when exposed to heavy metals rapidly accumulate heavy metal-binding peptides termed phytochelatins (PCs)(Grill et al. 1987; 1989). These peptides seem to be involved in the detoxification and homeostasis of excess heavy metals in plants and thus serve functions analogous to metallothioneins in animals (Grill et al. 1987; Zenk 1996). Phytochelatin-deficient *Schizosaccharomyces pombe* and *Arabidopsis thaliana* mutants are hypersensitive to cadmium (Cd) (Mutoh and Hayashi 1988; Howden et al. 1995), suggesting an important role of phytochelatins in heavy metal tolerance of higher plants and fungi. Although in an aquatic plant, *hydrilla verticillata*, the PCs under cadmium stress had been studied (Tripathi et al. 1996), the role of PCs in aquatic higher plants remains to be further demonstrated.

The aim of this study is to investigate changes in levels of PCs, glutathione, and related metabolites in *Lemna aequinoctialis,* an aquatic monocot plant, in response to Cd. Our results using a PC inhibitor support the model that PCs play a critical role in metal detoxification in plants. Consistent with this model, we also demonstrated that inhibition of PC synthesis leads to Cd hypersensitive. This provides further evidence for a general role of phytochelatins in heavy metal tolerance of higher plants.

MATERIALS AND METHODS

Lemna aequinoctialis, collected from a highly polluted water body, was sterilized with mercury chloride and 50% ethanol. A surviving single frond was brought into axenic culture to establish a clone, and maintain axenically under constant temperature and light as previously described (Yin 1994). For sample preparation, plants were

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harvested, washed three times with distilled water, frozen in liquid N_2 and stored at –80°C.

All reagents were of analytical grade. DL-Buthionine-[S.R]-sulfoximine (BSO) and Glutathione (GSH) were from Sigma; All solutions were prepared with deionized water obtained by a Milli-Q purification system (Millipore). The concentrations of cadmium used in this study were 5, 20, 30, 40, 50, and 200 μM of $CdCl_2$ (Sigma).

For acid-soluble thiols (AS-SH), 500 mg of fresh weight (FW) of frozen materials was homogenized in 6.67% 5-sulfosalicylic acid and set on ice for 15 min. After centrifugation (13,000×g, 15 min), the supernatant was diluted 10-fold with 120 mM sodium phosphate buffer (pH7.5, 5 mM EDTA, 0.5 mM 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). The absorbance was measured at 412 nm (Ellman, 1959).

For total glutathione determination, frozen materials were homogenized in sodium phosphate buffer (pH7.5, 5 mM EDTA) and 25% metaphosphoric acid (HPO_3). After centrifugation (18,000×g, 30 min) to remove all protein precipitant, the total glutathione was determined by a fluorometric method (Hissin and Hilf 1976).

Phytochelatins were assayed essentially as described (Grill et al. 1991). Briefly, *L. aequinoctialis* colonies were grown in Hutner's medium (Hutner 1953) with or without cadmium chloride added. In each day after Cd addition, plants were harvested, washed once with distilled water, and frozen in liquid N_2. The frozen materials were homogenized with 1N NaOH containing 1 mg/ml $NaBH_4$ (freshly prepared solution). After centrifugation at 13,000×g at 4°C for 15 min, the supernatant was acidified by adding HCl to a final concentration of 600 mM and left on ice for 30 min. Precipitated protein was removed by centrifugation (13,000×g, 20 min). Supernatant samples (20μl) were separated by HPLC (Waters 510) on a C18 column (3 μm, 250 mm) using an acetonitrile gradient (0-20%) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.5 ml/min. For the identification of phytochelatins, standard PC samples were run under same conditions. PC concentrations were expressed as μ mol/gFW.

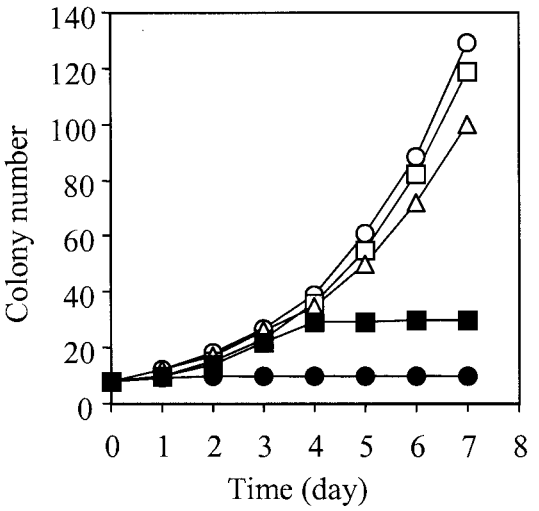

Figure 1. The growth of *Lemna aequinoctialis* under cadmium stress. The plant materials were grown for 7 days in the medium containing 0 μM (○), 20 μM (□), 30 μM (△), 50 μM (■) and 200 μM (●) of Cd, respectively. Each point represents the mean of three independent experiments.

RESULTS AND DISCUSSION

In order to understand the induction of phytochelatins in *Lemna* under the conditions of Cd stress, we first investigated the sensitivity of *Lemna aequinoctialis* to various concentrations of Cd using a colony formation assay. The growth of *Lemna* was maintained for at least 7 days at concentrations of 200 μM of Cd or less. Plant colony numbers was decreased by 10.5 percent and 22.5 percent at 20 μM and 30 μM of Cd after 7 days of incubation, respectively, while the colony multiplication ceased at 50 μM of Cd after 3 days. The growth of *Lemna* was almost completely inhibited from the first day of exposure to 200 μM Cd (Fig. 1).

To investigate the contribution of the PC synthesis to Cd induced growth inhibition of *Lemna*, we utilized an inhibitor of phytochelatin synthesis, BSO (Scheller et al. 1987), in our colony formation assay. When Cd and BSO were applied simultaneously, the inhibition of *Lemna* colony formation was much more dramatic than that obtained with Cd alone (Fig. 2). 5 μM BSO showed no inhibition to the growth of *Lemna* at all, and 20 μM Cd treatment resulted in a 10.5 percent

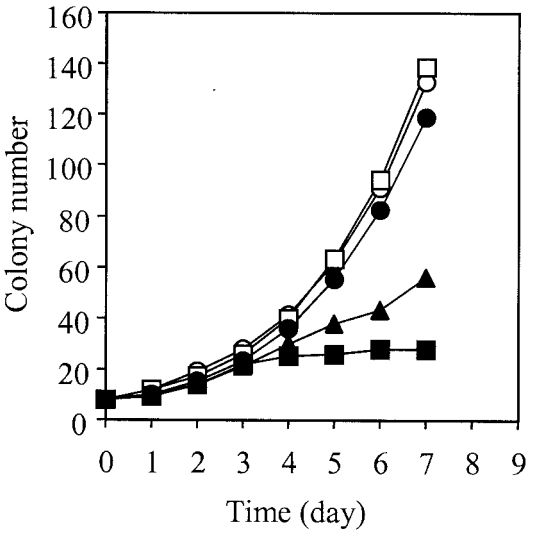

Figure 2. Effect of BSO on toxicity of Cd in *Lemna aequinoctialis*. The plants were grown for 7 days in media containing 0 μM of Cd, (○), 20 μM of Cd (●), 5 μM of BSO(□), 20 μM Cd + 5 μM BSO(■), and 20 μM Cd + 5 μM BSO + 150 μM GSH (▲) respectively. Each point represents the mean of three independent experiments.

inhibition. When 5 μM BSO and 20 μM Cd was used together, a remarkable growth inhibition on colony multiplication of *Lemna* was observed. The colony number of the sample treated with 5 μM BSO plus 20 μM Cd was only 23.5 percent or 20.5 percent of the samples treated with 5 μM BSO or 20 μM Cd alone, respectively. These data showed that BSO addition sensitized *Lemna* to Cd induced growth inhibition, implying a role of the PC synthesis in Cd tolerance of *Lemna*.

To further address the importance of the PC synthetic pathway in *Lemna* resistance to Cd, we tested the effect of GSH on *Lemna* growth inhibition by Cd. When 150 μM GSH was added to the medium, the growth inhibition of *Lemna* by 5 μM of BSO and 20 μM of Cd was partially released (Fig. 2). The enhancement effect of BSO on Cd toxicity to *Lemna* may be due to the inhibition of BSO on PC synthesis. BSO is an inhibitor of the GSH biosynthesis and GSH is the precursor of PCs (Earnshaw and Johnson 1985; Scheller et al. 1987). The partial recovery of BSO-inhibited growth by GSH supports an important role of PCs in tolerance of *Lemna* to Cd. Our data with

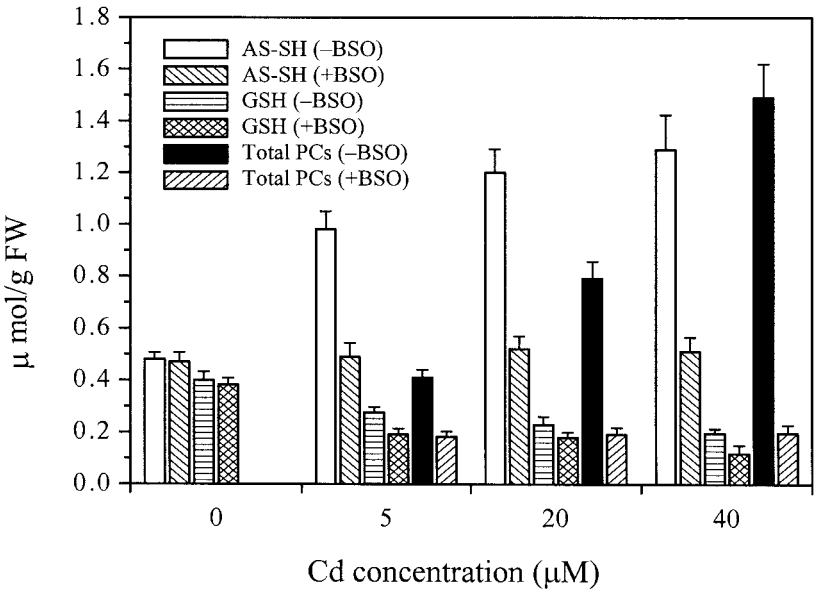

Figure 3. Effect of BSO on depletion of GSH and total PCs and accumulation of AS-SH under three days Cd treatment in *Lemna aequinoctialis*. Each column represents the mean of three independent experiments. Bars indicate ±S.E.

Lemna are consistent with reports that BSO prevented plants from metal-induced adaptive responses (Panda et al. 1994; Tripathi et al. 1996).

To directly evaluate the effect of BSO on levels of AS-SH, GSH and total PC in response to Cd, we determined these metabolites of *Lemna* under different treatment conditions (Fig. 3). *Lemna* samples were grown in medium containing various concentration of Cd for 3 days and harvested for AS-SH, GSH and total PC determination. The endogenous AS-SH and total PC levels increased as the exogenous Cd concentration increased, while the GSH content declined. When 5 μM BSO was present in the culture, all AS-SH, GSH and Total PC levels were declined in comparison to that in controls without BSO. It is interesting to note that with 5 μM BSO, the levels of AS-SH, GSH and total PC were almost stable in selected concentrations of Cd. Without Cd, 5 μM BSO showed no effects on the titers of AS-SH and GSH. The AS-SH contents with 5 μM BSO at four concentrations of Cd (0, 5, 20 and 40 μM Cd) remained the same as that in control

(-BSO, -Cd). Depletion of GSH with BSO (Fig. 3) was correlated with increased sensitivity of *Lemna* to Cd (Fig. 2), and exogenous GSH can partially release the growth inhibition induced by Cd (Fig. 2). Taken together, our findings suggest that GSH seems to play a role in the defense of *Lemna* against Cd. This notion is further supported by our evidence that BSO also inhibited the rise of the AS-SH level in *Lemna* treated with Cd (Fig. 3). Cd at comparable doses was known to induce rapid accumulation of phytochelatins in plant cells *in vivo* and in *vitro* (Steffens, 1990). To check whether this is true in *Lemna*, we determined the PC levels as a function of Cd and glutathione concentrations (Table 1). The PC_2 and PC_3 contents increased with Cd concentrations from 5 μM to 40 μM, while both PC_2 and PC_3 was undetectable in the control sample without Cd. Furthermore, the maximum increase for PC_2 was noticed at the 40 μM Cd together with 10 μM GSH. Another point noteworthy is that GSH did not increase the PC_3 level at 40 μM Cd, and on the contrary, PC_3 levels decreased with GSH at 40 μM Cd. One possible explanation for this is that the PC_3 was used to synthesize PC_4 or longer chain of PCs, and the PC assay method we used in this experiment was not sensitive enough to detect PCs longer than PC_4. GSH stimulates the accumulation of PCs in *Lemna* exposed to Cd, indicating that availability of endogenous GSH can limit synthesis of phytochelatins. However, it should be pointed out that cadmium exposures employed in our laboratory study is likely maximize any cadmium effects on PCs, because our

Table 1. Phytochelatin levels in *Lemna aequinoctialis* as a function of glutathione and Cd concentration.

Treatment	PC_2 (n mol/g FW)	PC_3 (n mol/g FW)
Control (-Cd)	0	0
Cd 5 μM	374 ± 23	66 ± 5.33
Cd 20 μM	658 ± 56	118 ± 7.32
Cd 40 μM	867 ± 39	158 ± 10.67
Cd 40 μM + GSH 10 μM	1770 ± 53	102 ± 9.43
Cd 40 μM + GSH 100 μM	1320 ± 68	84 ± 9.13

For experimental procedures see Materials and Methods and references therein. Values represent mean value ± standard error (n=3); $p<0.05$ (t-test).

laboratory solution is devoid of any complications as found in natural waters where other natural organic matters and inorganic particulates may bind metals, thus decreasing the effective concentration of cadmium.

Acknowledgments. We thank Dr Haian Fu in Emory University, Atlanta, USA, for the kind gift of BSO and GSH used in these studies.

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