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Combined Effect of Copper and Cadmium on Heavy Metal Ion Bioaccumulation and Antioxidant Enzymes Induction in *Chlorella vulgaris*

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Abstract The relationships between metal uptake and antioxidant enzyme activities or a response to membrane lipid peroxidation (i.e., malondialdehyde production) in *Chlorella vulgaris* exposed to Cu and Cd compounds singly and in combination were investigated. The results showed that bioaccumulation of a single metal was influenced by the presence of the other metal. The activities of superoxide dismutase and peroxidase increased to more than fivefold of the control after exposure to Cu(1.5 μM) alone or to Cu(1.5 μM) with Cd mixtures. Malondialdehyde levels in *C. vulgaris* also increased to approximately twofold of the control after exposure to high concentration of Cu(1.5 μM) alone or to Cu and Cd mixtures. However, Cd alone did not significantly increase the levels of antioxidant enzymes or malondialdehyde.

Keywords Antioxidant enzyme · Bioaccumulation · Cadmium · Copper

Copper (Cu) is a micronutrients essential at low concentrations for normal plant growth. It is associated with a large number of enzymes, which catalyze oxidative reactions in a variety of metabolic pathways (Marschner 1995). However, high concentrations of Cu induce strong phytotoxic responses that affect a wide range of biochemical and physiological processes, including photosynthesis, pigment synthesis, nitrogen and protein metabolism, and mineral uptake through cell membranes (Shen et al. 1998; Nielsen et al. 2003; Demirevska-Kepova et al. 2004). Excessive Cu may be toxic not only to plants but also to human beings via the food chain and may thus pose a potential threat to human health. Unlike Cu, Cadmium (Cd) is a non-essential element and extremely toxic to humans, animals and plants. Studies on different plants revealed that Cd can interfere with antioxidant system or nutritional status and a number of metabolic processes such as photosynthesis, respiration (Sandalio et al. 2001; Romero-Puertas et al. 2007; Qian et al. 2009).

Traditionally, information on the toxicity of heavy metal pollutants to the aquatic environment has been gained from tests involving single pollutants. However, in metal-polluted sites, multiple metals commonly occur together. Since natural pools of water are normally polluted by several metal compounds, they may exert toxicity simultaneously (Shuhaimi-Othman and Pascoe 2007). A few studies have been conducted to investigate the combined effect of metal compounds on plant species, and synergistic or antagonistic effects on cell division rate, cell membrane lysis, photosynthesis and chlorophyll synthesis was observed in different species (Franklin et al. 2002; Sacan et al. 2007). Metal compounds are known to be absorbed by plants and to affect their normal growth. It has also been demonstrated that metal compounds are able to induce the formation of reactive oxygen species (ROS) (Schützendübel and Polle 2002; Qian et al. 2009). The balance between ROS production and its scavengers, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) and glutathione-S-transferase (GST) may be upset following exposure to metal compounds. If these ROS scavengers are not produced in sufficient quantities to reduce oxidative stress, MDA levels may increase within the plant cells. MDA is the major aldehyde that is generated from lipid peroxidation, and is considered to be a biomarker for

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oxidative stress. The level of MDA in the cell is indicative of the degree of balance between ROS and antioxidative substances.

In the present study, *Chlorella vulgaris* was chosen as a representative green microalga to evaluate the effects of Cu and Cd singly and in combination on the bioaccumulation of these metals, the induction of antioxidant enzymes, and the production of MDA.

Materials and Methods

Chlorella vulgaris was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences. The alga was cultured in 250 mL flasks containing 70 mL of sterilized shuisheng-4 medium (Zhou and Zhang 1989) at 25 ± 0.5°C on a 14:10-h light: dark cycle and a light intensity of approximately 46 μmol m^{-2} s^{-1}. Cells in the exponential growth phase were used for all experiments, and the initial cell density for each experiment was about 3.5×10^5 cells mL^{-1}.

Cadmium chloride ($CdCl_2$, reagent grade, 99.9% purity) and copper sulfate ($CuSO_4$, reagent grade, 99.0% purity) were used. Metals were added as aqueous solutions. Based on our previous report that the 48-h EC_{50} values of $CuSO_4$ and $CdCl_2$ were 2.63 and 4.68 μM, respectively (Qian et al., 2009), two concentrations of $CuSO_4$ (0.5 and 1.5 μM) and $CdCl_2$ (1.0 and 2.0 μM) were adopted, thus four single treatments (Cu0.5, Cu1.5, Cd1.0 and Cd2.0) and four combined treatments [Cu(0.5) + Cd(1.0), Cu(0.5) + Cd(2.0), Cu(1.5) + Cd(1.0), and Cu(1.5) + Cd(2.0)] were used to research metal compound bioaccumulation, antioxidant enzyme induction and MDA production, after 48 h of exposure. Three replicates were made of each bioassay.

For measuring intracellular metal concentrations, collected algal bodies were thoroughly washed with cultured medium (without heavy metal ion) and digested with HNO_3:$HClO_4$ at 160°C. Digested material was diluted with de-ionized water, and Cu and Cd concentrations were determined using an Inductively Coupled Plasma Mass Spectrometer (ICP-MS, Agilent 7500a, USA). Analyses of selected enzyme activity for SOD, POD and CAT, and MDA content were carried out according to our previous report (Qian et al. 2009).

Experimental data were checked for normality and homogeneity of variance using the Kolmogorov–Smirnov one-sample test and Levene's test, respectively. When necessary, data were transformed for normalization and to reduce heterogeneity of variance. Intergroup differences were assessed by one-way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test. In order to assess the individual effects of Cu and Cd, as well as their interaction, on accumulation, antioxidant enzymes induction, and MDA production, a factorial design experiment consisting of two concentrations of Cu and Cd, was performed, and followed by two-way ANOVA. All statistical analyses were carried out using SPSS 13.0 (SPSS, Chicago, IL, USA). The critical value for statistical significance was $p < 0.05$.

Results and Discussion

Copper ion concentrations in the algals cell increased with exposure concentration in treatments with Cu alone (Table 1). At the treatment of Cu(0.5) and Cu(1.5), Cu ion in algal cell increased to 2.7- and 14-fold of the control, respectively. Bioaccumulation of Cu ion in the treatment of Cu(0.5) and Cu(1.5) significantly increased compared with the control (Tables 1 and 2). The effect of bioaccumulation of Cd ion in *C. vulgaris* was dosage-dependent, which was similar to Cu ion bioaccumulation. In the treatment of Cd(1.0) and Cd(2.0), Cd ion bioaccumulation increased to 65.7- and 115.8-fold of the control. Bioaccumulation of Cd ion significantly increased compared with the control (Table 2). These results are consistent with the report of An et al. (2004) showing that bioaccumulation of Cu, Cd, and Pb was concentration-dependent in *Cucumis sativus*, and the report of Benimeli et al. (2010) showing that bioaccumulations of Cu by *Z. mays* increased concomitantly to Cu concentration. This phenomenon also agrees well with our previous report that increased inhibition of both algal growth and chlorophyll content occurred with increasing concentrations of Cu and Cd (Qian et al. 2009).

The co-existence of Cd and Cu ion in the culture medium affected the bioaccumulation of each other in *C. vulgaris*. A 14-fold level of bioaccumulation in the Cu(1.5)-treatment group was greatly reduced to 5.3- and 7.2-fold levels in the combined treatment groups of Cu(1.5) + Cd(1.0) and Cu(1.5) + Cd(2.0), respectively. Cd ion significantly decreased Cu ion bioaccumulation (Table 2). In addition, the existence of Cu ion in cultured medium also affected Cd ion bioaccumulation. In the existence of low concentration of Cu ion (0.5 μM), Cd ion bioaccumulation in the treatments of Cu(0.5) + Cd(1.0) and Cu(0.5) + Cd(2.0) were increased to 90.9 and 124.5-fold of the control, respectively. However, Cd ion bioaccumulation was greatly reduced by the presence of Cu ion at the concentration of 1.5 μM.

The phenomenon of bioaccumulation of a single metal being influenced by the presence of other metals has been reported by others. An et al. (2004) found that accumulation of Cu in shoots of *C. sativus* was negatively influenced by the presence of Cd in soil, and Cd uptake to shoots decreased by Cu addition, similar to our current results. Similarly, other researchers reported that the existence of Cr(VI) was observed to reduce the bioaccumulation of Mn,

Table 1 Bioaccumulated copper and cadmium content in *Chlorella vulgaris* after 48 h of exposure to Cu, Cd or Cu + Cd

Metal ion content (10^{-16} g cell^{-1})	Treated groups								
	Cont.	Cu(0.5)	Cu(1.5)	Cd(1.0)	Cd(2.0)	Cu(0.5) + Cd(1.0)	Cu(0.5) + Cd(2.0)	Cu(1.5) + Cd(1.0)	Cu(1.5) + Cd(2.0)
Cu	5.8 ± 0.1	15.5 ± 0.4	81.3 ± 9.6	3.2 ± 0.3	4.7 ± 0.4	12.8 ± 1.1	14.0 ± 1.4	30. 6 ± 4.2	41.5 ± 3.7
Cd	1.0 ± 0.2	2.0 ± 0.5	10.3 ± 2.7	68.7 ± 3.9	121.0 ± 13.6	95.0 ± 4.2	130.1 ± 9.8	67.5 ± 11.2	66.4 ± 1.4

Data are presented as mean ± standard error of the mean (SEM) of 3 individuals

Cu, Zn or Fe (Liu et al. 2008; Doncheva et al. 2009; Mallick et al. 2010). However, the bioaccumulation pattern in mixtures is not consistent with algal growth responses, given our previous report showing that the combination of Cu and Cd exhibited a strong synergistic interaction on algal growth inhibition. This phenomenon was also reported by An et al. (2004), who observed that Pb reduced the accumulation of Cu in plant shoots, but had no direct relationship with the growth data.

As known, one of the biochemical changes in plants subjected to heavy metals stress is the over-production of reactive oxygen species, ROS (Qian et al. 2009; Bajguz 2010). Plants have been demonstrated to induce various antioxidant enzymes or low molecular weight antioxidants to control ROS levels (Chaoui et al. 1997; Lombardi and Sebastiani 2005; Dazy et al. 2008). The concentrations of Cu (1.5 μM) enhanced the activity of SOD to 5.2-fold of the control, while 1.0 and 2.0 μM concentrations of Cd did not induce SOD activity (Fig. 1a). The combined treatments of two metal compounds with higher dosage of Cu present stimulated SOD activity, reaching 5.4- and 9.6-fold of the control after exposure to Cu(1.5) + Cd(1.0) and Cu(1.5) + Cd(2.0), respectively. Cu alone had an inductive effect on SOD activity (Table 2), however, there was no interaction between Cu and Cd (Table 2).

The induced effects of Cu and Cd, singly and in combination, on POD activity in *C.vulgaris* cells after 48 h of exposure are shown in Fig. 1b. Cu (1.5 μM) enhanced the activity of POD to 2.1-fold of the control, while the 1.0 and 2.0 μM concentrations of Cd did not induce POD activity. The combined treatments of two metal compounds stimulated POD activity, reaching 2.5- and 3.2-fold of that of the control after exposure to Cu(1.5) + Cd(1.0) and Cu(1.5) + Cd(2.0), respectively. Cu alone induced POD activity (Table 2), and there was interaction between Cu and Cd (Table 2).

CAT activity was slightly stimulated by Cu or Cd treatments (Table 2). The combinations of Cu and Cd had no influence on CAT activity, except for Cu(1.5) + Cd(1.0) combination Both Cu and Cd alone had no significant effect on the activity of CAT (Fig. 1c). Furthermore, we also observed no interaction between Cd and Cu on CAT activity (Table 2). Our results show that both SOD and POD were sensitive to Cu, consistent with previous reports (Dazy et al. 2008). This meant that SOD and POD may be the main enzymes involved in ROS-quenching mechanisms.

MDA content was measured to evaluate lipid peroxidation. Administration of Cu (1.5 μM) stimulated MDA levels 2.0-fold greater than the control, while Cd in this study did not enhance MDA levels. The combined administration of Cu and Cd also stimulated MDA levels, and the level of stimulation were 2.0-, 1.8-, 2.9- and 3.2-fold greater than the control when after treatment with

Table 2 Results of 2 × 2 factorial analysis of variance (ANOVA) between copper and cadmium on bioaccumulated copper and cadmium content, SOD, POD, CAT activity, and MDA content in *Chlorella vulgaris* after 48 h exposure

Factors	F(*p*)					
	Cu content	Cd content	SOD activity	POD activity	CAT activity	MDA activity
Cu	110.169 (< 0.001)**	8.813 (0.003)**	19.718 (< 0.001)**	57.209 (< 0.001)**	1.345 (0.283)	30.642 (< 0.001)**
Cd	17.335 (< 0.001)**	123.852 (< 0.001)**	2.747 (0.085)	2.980 (0.074)	2.477 (0.109)	8.971 (0.002)**
Cu + Cd	13.700 (< 0.001)**	6.698 (0.003)**	1.705 (0.183)	4.427 (0.010)*	1.380 (0.276)	1.697 (0.188)

Data are presented as mean ± standard error of the mean (SEM) of 3 individuals. Statistically significant differences are shown by * $p < 0.05$ and ** $p < 0.01$

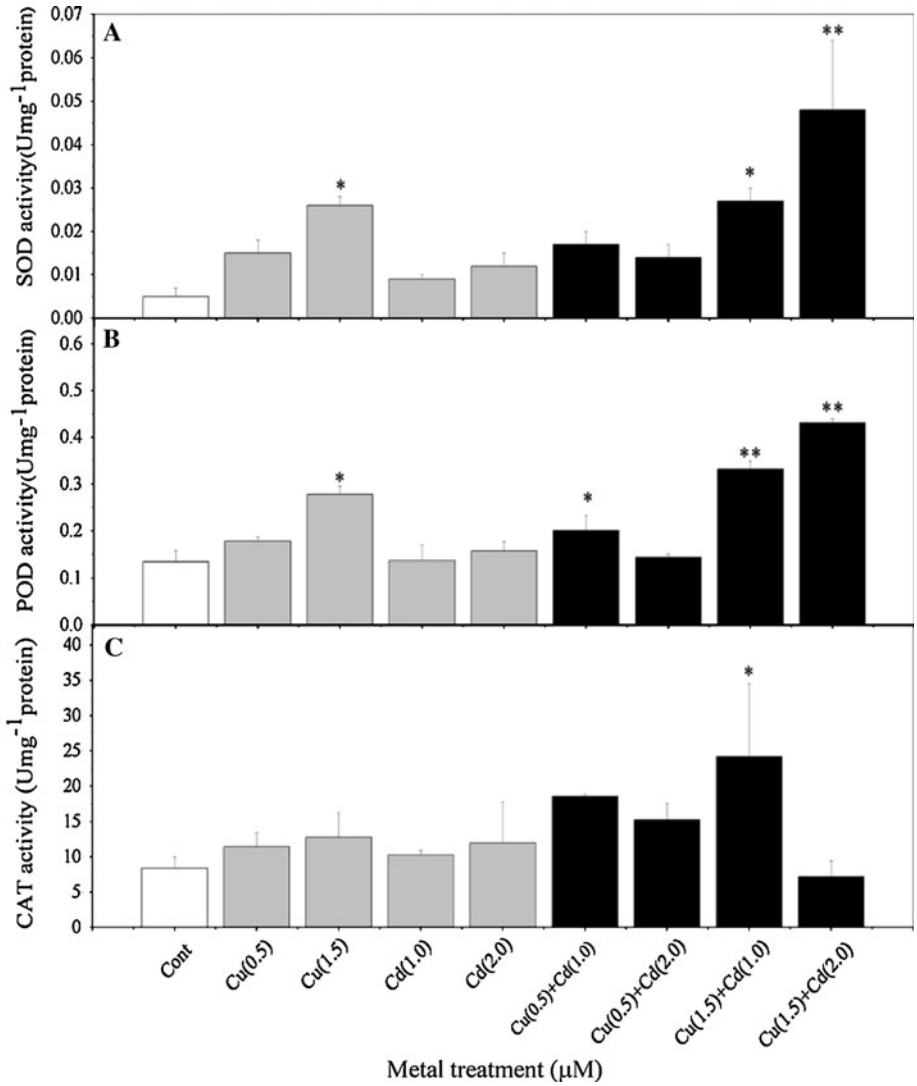

Fig. 1 Effects of copper and cadmium, singly and in combination, on the SOD **a**, POD **b** and CAT **c** activity of *Chlorella vulgaris* after 48 h of exposure. Data are presented as mean ± standard error of the mean (SEM) of 3 individuals. Statistically significant differences are shown by * $p < 0.05$ and ** $p < 0.01$ from two-way ANOVA

Cu(0.5) + Cd(1.0, 2.0), Cu (0.5) + Cd(1.0, 2.0), Cu(1.5) + Cd(1.0), and Cu(1.5) + Cd(2.0), respectively. Both Cu and Cd alone stimulated MDA formation (Table 2), although only Cu(1.5) exposure increased the level significantly (Fig. 2). No interaction between Cu and Cd was evident here (Table 2).

From these results, we speculated that Cu or Cd and their combination when reached certain concentrations had an impact on *C. vulgaris* physiology by inducing cellular damage by lipid peroxidation. This kind of metal compound effect has already been proven by other reports (Chaoui et al. 1997; Dazy et al. 2009). In the present work,

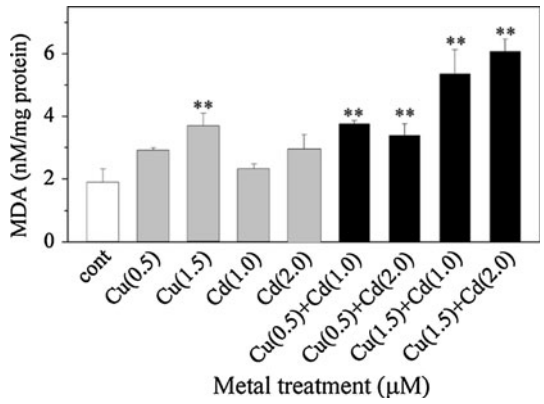

Fig. 2 Effects of copper and cadmium, singly and in combination, on MDA content of *Chlorella vulgaris* after 48 h of exposure. Data are presented as mean ± standard error of the mean (SEM) of 3 individuals. Statistically significant differences are shown by * $p < 0.05$ and ** $p < 0.01$ from two-way ANOVA

the observed relationships between antioxidant enzymes and MDA level suggested that the protective reaction to oxidative stress via antioxidant enzyme production was not sufficient for cell protection in the case of the higher treatment of Cu alone, or in the combined treatments of Cu and Cd.

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