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MT-like proteins: Potential bio-indicators of *Chlorella vulgaris* for zinc contamination in water environment

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ABSTRACT

Chlorella vulgaris has been usually used to monitor the toxicity of Zn in water environment, but the biochemical responses of the algae to Zn^{2+} at different levels remain unknown to date. In the present study, the growth inhibition, the antioxidant enzymes, the subcellular Zn and the induced Zn-metallothionein-like (Zn-MT-like) proteins in *C. vulgaris* exposed to 0, 5, 10, 20, 50 and 100 μmol l^{-1} of Zn^{2+} were investigated. Results showed that the growth of *C. vulgaris* was significantly inhibited ($p < 0.05$) at Zn^{2+} level above 5 μmol l^{-1}. Compared with the control group, the activities of peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase mostly increased with the rises of Zn^{2+} concentrations ranging from 5 to 100 μmol l^{-1}, except for GSH-Px with a lower value in the 100 μmol l^{-1} group compared with that of the 50 μmol l^{-1} group, and SOD with no significant difference at the 5, 10 and 100 μmol l^{-1} groups compared with that of the control group. Contrarily, the activities of guaiacol peroxidase significantly decreased ($p < 0.05$) at Zn^{2+} concentrations from 5 to 100 μmol l^{-1} compared with that of the control group. After 72 h exposure, Zn in the algal cells was observed to mainly distribute in the form of heat stable fractions, in which Zn-binding metallothionein-like (MT-like) proteins were largely biosynthesized. In addition, the amounts of Zn-MT-like proteins induced in the algae significantly increased ($p < 0.05$) with the rises of Zn^{2+} concentrations at the assay levels. The results indicated that the activities of antioxidant enzymes and the induction of Zn-MT-like proteins in *C. vulgaris* may play important roles in dealing with the toxicity of Zn^{2+} to the algal cells, and the induction of Zn-MT-like proteins in *C. vulgaris* can be used as a potential bio-indicator to monitor the contamination of Zn^{2+} in water environment.

1. Introduction

Chlorella vulgaris (*C. vulgaris*), displaying high growth rates under autotrophic and heterotrophic conditions, is a genus of unicellular green algae, and has been widely used to monitor the contamination of heavy metals in water environment because of the algal strong bio-sorption abilities (Kajan et al., 1992). However, some biochemical responses of *C. vulgaris* to heavy metals remain unknown, in which some heavy metals pose favorable effects on the algal growth at certain concentrations, while many of them present adverse effects even at very low concentrations (Bajguz, 2011; Huang et al., 2009; Muyssen and Janssen, 2001; Wang and Wang, 2011; Wang et al., 2011). Zinc (Zn) is one of the most important metals participating in various biological functions in microalgae, but the metal may also cause severe intracellular damage by inhibiting algal metabolic processes at high concentrations (Price and Morel, 1994). For example, the median lethal concentrations (LC_{50}) of Zn^{2+} to *C. vulgaris* ranged from 2.4 mg l^{-1} to 100 mg l^{-1} (Maeda et al., 1990; Rachlin and Farran, 1974). But the toxicity of Zn^{2+} to *C. vulgaris* may also depend on the metal species, the exposure duration and the testing medium characteristics (Muyssen and Janssen, 2001; Yeh and Chang, 2012). Hence, it is important to investigate the biochemical responses of *C. vulgaris* to Zn^{2+} in water environment.

Many researches have demonstrated that heavy metals may induce the production of reactive oxygen species (ROS) in autotrophic *C. vulgaris* (Tripathi and Gaur, 2004). To prevent the ROS damages, various anti-oxidative enzymes may be induced and activated in the algal cells, among which superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and glutathione peroxidase (GSH-Px) are usually involved (Valavanidis et al., 2006). For example, the activity of SOD in *C. vulgaris* exposed to 100 μmol l^{-1} Cd^{2+} obviously increased, while the activity of CAT decreased in the

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same testing medium (Niczyporuk et al., 2012). Cu^{2+} at the concentration of 0.5 μg ml^{-1} was also able to decrease the CAT activity in *C. vulgaris* (Mallick, 2004). However, Pb^{2+} was able to increase the activities of SOD, CAT and GSH-Px in *C. vulgaris* (Bajguz, 2010). Therefore, the activities of anti-oxidative enzymes in *C. vulgaris* may vary with different kinds of stress metals and different metal concentrations. Similarly, Zn^{2+} is also able to cause an oxidative damage to *C. vulgaris* through increasing the levels of ROS species such as the superoxide radical ($^{\bullet}O_2^-$), hydroxyl radical ($^{\bullet}OH$), and the hydrogen peroxide (H_2O_2) (Andrade et al., 2006; Mittler, 2002; White and Jahnke, 2002). However, there is insufficient scientific data to interpret the biochemical responses of the algae to the oxidative damage of Zn^{2+}.

Phytochelatins (PCs), with a general structure of (γ-Glu-Cys)*n*-Gly (*n* = 2–11), are well-known polypeptides induced in *C. vulgaris* to sequester the influx heavy metals in cells. They play important roles on the detoxification of heavy metals to the algae (Bajguz, 2002; Gekeler et al., 1988). In addition, metallothionein-like (MT-like) proteins have also been found in *C. vulgaris* capable of detoxifying the toxicities of heavy metals such as Cd^{2+} and Zn^{2+} (Huang et al., 2009; Yoshida et al., 2006). Therefore, the induction of PCs and MT-like proteins in micro-algae may depend on the algal species and the kinds of metals.

C. vulgaris has been widely used to monitor the toxicity of Zn in water environment (Kajan et al., 1992; Muyssen and Janssen, 2001). However, the study on the biochemical responses of *C. vulgaris* exposed to Zn^{2+} is rarely conducted. In the present study, some biochemical parameters, including the growth rates, the activities of anti-oxidative enzymes, the distributions of subcellular Zn, and the induction of Zn-binding MT-like proteins, in *C. vulgaris* exposed to Zn^{2+} at different concentrations are to be measured. The biochemical responses of the algae in dealing with the toxicity of Zn^{2+} are to be investigated.

2. Materials and methods

2.1. Cultivation and biomass test

C. vulgaris was donated from Fisheries College, Jimei University, China. The algae were cultured and amplified in Erlenmeyer flasks with equal volumes (v/v) of the inoculums and the culture medium consisting of 1.0 g l^{-1} $NaHCO_3$, 0.5 g l^{-1} KNO_3, 0.5 g l^{-1} $MgSO_4{\cdot}7H_2O$, 0.02 g l^{-1} K_2HPO_4, 2 μg l^{-1} vitaminB_{12} and 0.6 mg l^{-1} vitamin B_1 in sterilized seawater based on a f/2 nutritional composition (Guillard and Ryther, 1962). The cultivation temperature maintained at 26 ± 1 °C with white fluorescent illumination by a cycle of 12 h light (300 μE m^{-2} s^{-1}) per 24 h in a PGX-280B illumination cultivation cabinet (Ningbo, China). Filtered air was bubbled into the algal suspension. For the convenience, the relationship between the spectrophotometrical absorbance at 686 nm (A_{686}) (measured with LabTech-2100 spectrophotometer, Beijing, China) and the cell densities (counted by B2221P microscope, Shanghai, China) in the algal suspension were established. The initial cell densities were about 8×10^6 cells ml^{-1}. During the exponential growth phase, the regression equation between the cell densities (y) and A_{686} (x) was established as follows:

$$y = 2 \times 10^7 x + 889331, \quad R^2 = 0.997$$

2.2. Growth inhibition test

Based on the Zn criterion (15.4 μmol l^{-1}) of seawater quality standard (Level II) set in China (GB 3097-1997) and the contamination of Zn in seawater reported recently (Pan and Wang, 2012), the growth inhibition of *C. vulgaris* were tested at the concentrations of Zn^{2+} ranging from 0 to 100 μmol l^{-1}. Each of 200 ml of algal suspension, at mid-exponential growth phase, was added with $ZnCl_2$ stock solution at the concentrations of 0, 5, 10, 20, 50 and 100 μmol l^{-1} Zn^{2+}. The algal suspensions with the addition of Zn^{2+} were labeled as Zn groups accordingly and the algal suspension without adding Zn^{2+} was set as control group. Three replicates for each group were carried out. Because the concentration of Zn^{2+} in the control groups mainly from seawater was only 0.48 μmol l^{-1} based on our measurement, Zn^{2+} levels in each of Zn groups were tagged based on the concentrations of Zn^{2+} added in the corresponding algal suspensions. All the assay groups were incubated at the same cultivation conditions as described above, except for shaking five times per day by hand instead of automatically bubbling in filtered air. Cell densities (cells ml^{-1}) for each algal suspension were calculated by measuring the A_{686} values at the time intervals of 0, 12, 24, 48, 72, 96 and 120 h. After 120 h exposure, the growth rates based on the initial and final cell densities were calculated according to the method described in our previous report (Huang et al., 2009).

2.3. Measurement of antioxidant enzymes

2.3.1. Activities of superoxide dismutase (SOD)

After exposure for 72 h, each of 40 ml algal suspension was centrifuged at 4000 × *g* for 10 min. The pellets were respectively washed with 10 mmol ml^{-1} EDTA and ultrapure-water to remove Zn^{2+} absorbed on the surfaces of algal cells. The cells re-suspended with ice-chilled 10 mmol l^{-1} Tris–HCl (pH 8.0) containing 2% (w/v) of polyvinyl polypyrrolidone (PVP) were homogenized with ultrasonic disintegrator (Scientz-IID, Ningbo, China); and then, the homogenates were centrifuged at 10,000 × *g* for 20 min (4 °C). By means of pyrogallol auto-oxidation, the activities of SOD in the supernatants (defined as enzyme extracts) were measured according to the method of Marklund and Marklund (1974). Briefly, the inhibition rates of SOD in the enzyme extracts were estimated by detecting the spectrophotometrical absorbance at 325 nm in the assay mixture containing 2.8 ml of 50 mmol l^{-1} Tris–HCl (pH 8.0), 0.1 ml of 5 mmol l^{-1} pyrogallol and 0.1 ml of the enzyme extract. The enzyme causing 50% inhibition of the pyrogallol auto-oxidation rate was defined as one unit of SOD activity, expressed as the units per 10^8 cells of *C. vulgaris*. The procedural blanks, by replacing the enzyme extracts as the homogenized medium, i.e., 10 mmol l^{-1} Tris–HCl (pH 8.0) containing 2% (w/v) of PVP, were run for background subtraction.

2.3.2. Activities of glutathione peroxidase (GSH-Px)

The activities of GSH-Px were estimated based on a modified method of Rotruck et al. (1973). Briefly, 0.5 ml of enzyme extract, as described above except for the use of 10 mmol l^{-1} phosphate buffer (pH 6.2) in algal cell homogenization, was mixed with 0.35 ml of reaction solution consisting of 1 mmol l^{-1} GSH, 0.34 mmol l^{-1} EDTA, 25 mmol l^{-1} sodium azide and 0.5 mol l^{-1} phosphate buffer. After incubating at 37 °C for 5 min, 0.175 ml of 1.25 mmol l^{-1} H_2O_2 was added, and the mixture was incubated again at 30 °C for 5 min. The reaction was stopped by adding 3.25 ml of 10% (w/v) trichloroacetic acid, and then the mixture was centrifuged at 4000 × *g* for 10 min. 2 ml of the supernatant was respectively mixed with 2 ml of 0.5 mol l^{-1} disodium hydrogen phosphate, 0.15 ml of 6 mol l^{-1} NaOH and 0.3 ml of 1 mmol l^{-1} 5,5′-dithio-bis(2-nitrobenzoic acid) (in 1% sodium citrate). After reacting for 3 min, the mixture was measured at 412 nm. The GSH-Px activity was expressed as the micromole of oxidized glutathione per 10^{10} cells and per minute. The procedural blanks were run for background subtraction.