Microcystin-RR-induced accumulation of reactive oxygen species and alteration of antioxidant systems in tobacco BY-2 cells

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Abstract

Microcystins are cyclic heptapeptide hepatotoxins produced by cyanobacteria. It has been shown that microcystins have adverse effects on animals and on plants as well. Previous researches also indicated that microcystins were capable of inducing oxidative damage in animals both in vivo and in vitro. In this study, tobacco BY-2 suspension cell line was applied to examine the effects of microcystin-RR on plant cells. Cell viability and five biochemical parameters including reactive oxygen species (ROS), superoxide dismutase (SOD), catalase (CAT), glutathione peroxide (GPX) and peroxide dismutase (POD) were investigated when cells were exposed to 50 mg/L microcystin-RR. Results showed that microcystin-RR evoked decline of the cell viability to approximately 80\% after treating for 144 h. ROS levels, POD and GPX activities of the treated cells were gradually increased with a time dependent manner. Changes of SOD and CAT activities were also detected in BY-2 cells. After 168 h recovery, ROS contents, POD, GPX and CAT activities returned to normal levels. These results suggest that the microcystin-RR can cause the increase of ROS contents in plant cells and these changes led to oxidant stress, at the same time, the plant cells would improve their antioxidant abilities to combat microcystin-RR induced oxidative injury.

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1. Introduction

The occurrences of Microcystis blooms have been frequently reported in many freshwater bodies all over the world (Ueno et al., 1996; Codd et al., 1999). The blooms not only decrease water quality, but also increase the risk to animals, humans and plants because many species of Microcystis (mainly Microcystis aeruginosa) can produce cyclic peptide hepatotoxins called microcystins (Codd et al., 1989; Carmichael and Falconer, 1993).

The toxic effects and mechanism of microcystins on animals have been well studied (Gehringer, 2004). Microcystins can potently inhibit protein phosphatase type-1 and 2A after the toxin was transported to cytoplasm by the bile acid transporter in the cell membrane of hepatocytes (MacKintosh et al., 1990; Yoshizawa et al., 1990; Eriksson et al., 1990; Runnegar et al., 1993, 1995). Microcystins can also induce the oxidative stress in in vitro cultured rat and fish cells (Li et al., 2003; Ding et al., 1998, 2001; Codd et al., 1999).
Recently, some attentions also have been paid to the toxic effects of microcystins on plants. The growth inhibitory phenomenon of microcystins on plant was firstly observed in Sinapis alba (Kos et al., 1995). Thereafter, many reports demonstrated that the growth, development and physiological pathway of plants could be affected by microcystins (Abe et al., 1996; Kurki-Helasmo and Meriluoto, 1998; McElhiney et al., 2001; Yin et al., 2005). The commonly accepted toxic mechanism of microcystins on plants is that microcystins inhibit protein phosphatase type-1 and 2A. At present, there has some evidence suggesting that oxidative stress might be involved in the toxicity of microcystins on plants. It was reported that the activity of peroxidase (POD) and superoxide dismutase (SOD), two of antioxidant enzymes, were changed in rape (SOD), two of antioxidant enzymes, were changed in rape activity of peroxidase (POD) and superoxide dismutase (SOD), two of antioxidant enzymes, were changed in rape toxicity of microcystins on plants. It was reported that the activity of peroxidase (POD) and superoxide dismutase (SOD), two of antioxidant enzymes, were changed in rape (Brassica napus L.) and rice (Oryza sativa L.) seedlings when exposed to microcystins (Chen et al., 2004). The oxidative stress induced by microcystins in the aquatic macrophyte Ceratophyllum demersum also was reported (Pflugmacher, 2004). However, the information about microcystins induced oxidative stress of plants is still very limited.

In our experiment, tobacco suspension cells were used for their small volumes, which is useful because of the limited availability of microcystins. More importantly, the effects of microcystins on the in vitro cultured cells makes easy both (a) the investigation of their primary interaction with plant cells and (b) the study of the mechanisms which plants use to eliminate/decrease microcystins induced toxicity.

The present work focused on the microcystin-RR stimulated reactive oxygen species (ROS) accumulation in tobacco suspension cells, the antioxidant systems to eliminate the ROS factors as well as the changes of cell viability.

2. Materials and methods

2.1. Toxin and reagents

M. aeruginosa bloom material was collected from Lake Dianchi, Kunming in southwestern China, in August 2001. Microcystin-RR was extracted and purified with the improved HPLC method (Harada et al., 1988; Yin et al., 2005). All reagents, obtained from various commercial sources, were analytical or higher grades.

2.2. Plant material and toxin treatment

The tobacco BY-2 cell line (Nicotiana tabacum L. cv. Bright Yellow 2) was kindly provided by Dr Chen, Huazhong Agricultural University, China. The cells were cultured at KCMS liquid medium: MS (Murashige and Skoog, 1962) salts, 0.5 mg/L myo-inositol, 1.3 mg/L thiamine HCl, 200 mg/L KH₂PO₄ and 3% (W/V) sucrose supplemented with 0.2 mg/L 2, 4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg/L Kinetin (KT). The media were adjusted to pH 5.6 with a KOH solution prior to autoclaving. The cells were subcultured once a week and maintained at 25 °C in darkness on an orbital shaker at 130 rpm. For microcystin-RR toxicity studies, the cells in the exponential phase of growth (3 days old cell culture) were used. The media contained 0 (control) and 50 mg/L microcystin-RR (1.113359 mg/mL stock solution of microcystin-RR in milli-Q water was filter sterilized, then added to the medium to yield a final concentration of 50 mg/L). Control and toxin-treated cells were harvested for biochemical measurements and cell viability assessments after culturing for 12, 24, 48, 72, 96, 120 and 144 h. For recovery experiments, both the control and toxin-treated cells were washed three times with sterilized KCMS liquid medium, and then suspended at a density of 2.0 × 10⁵ cells/mL in fresh KCMS liquid medium for 168 h.

All the experiments were repeated four times.

2.3. Cell viability assessment

0.025% w/v Evan’s blue was used to determine cell viability. The viability percentage was calculated 5 min later based on Evan’s blue exclusion.

2.4. In vivo detection of ROS

ROS production was detected by 2′,7′-dichlorofluorescein diacetate (DCFH-DA) according to He and Häder (2002) with slight modifications. DCFH-DA (final concentration 5 µM from stock solution of 2 mM) was immediately added to the cells and incubated on a shaker at 25 °C in the dark for 1 h. Then the cells were immediately washed three times with 0.1 M PBS (pH = 7.8). The fluorescence of the samples was measured with a spectrofluorometer (SFM 25, Kontron, Switzerland) at room temperature, with an excitation wavelength of 485 nm and an emission band of 520 nm. The fluorescence intensity at 520 nm normalized to the wavelength of 485 nm and an emission band of 520 nm. The fluorescence intensity at 520 nm normalized to the protein content was used to determine the relative ROS production.

Soluble protein was assayed according to Bradford (1976), using bovine serum albumin as standard.

2.5. Antioxidant enzyme activities assays

About 0.5 g of cells was ground into slurry with a mortar and pestle with 2 ml of phosphate buffer (pH 7.8) in an ice bath. The homogenates were centrifuged at 12,000 × g at 4 °C for 10 min, and the supernatants were kept at 4 °C prior to use.

POD activity was determined by measuring the rate of increase in absorbance at 470 nm of a mixture containing 1 ml of 50 mM sodium phosphate buffer (pH 7.0), 0.95 ml of 0.2% 2-methoxyphenol, 1 ml of 0.2% hydrogen peroxide and 0.05 ml of enzyme extract.
Glutathione peroxide (GPX) activity was measured according to Drotar et al. (1985) using glutathione as substrate.

Catalase (CAT) activity was detected according to Cakmak and Marschner (1992) with slight modifications. The reaction mixture in a total volume of 3 mL containing 50 mM sodium phosphate buffer (pH 7.0) 1.9 mL and 0.2% H$_2$O$_2$ 1 mL. The reaction was initiated by the addition of 0.1 mL of enzyme extract and activity was determined by measuring the initial rate of disappearance of H$_2$O$_2$ at 240 nm.

SOD activity assay was based on the method described by Giannopotitis and Ries (1977). One unit of the enzyme activity was defined as the amount of enzyme required to result in a 50% inhibition of the rate of nitro blue tetrazolium reduction measured at 560 nm.

2.6. Statistics

All data were evaluated by one-way ANOVA (Spss 6.0.1 for windows, tests: least significant difference, Tukey’s honestly significant difference).

3. Results

3.1. Cell viability

The percentage of viable cells cultured in the control medium was sustainingly high over the whole culture period (about 95%), while that of toxin-treated cells significantly decreased after 72 h exposure (Fig. 1). After 168 h recovery treatment, the viability percentage of toxin-treated cells increased from 80 to 89%.

3.2. ROS accumulation induced by microcystin-RR

Significant induction of intracellular ROS formation was found in cells after 48 h exposure to 50 mg/L microcystin-RR ($p<0.01$). With the increment of exposure time, ROS content of treated cells increased gradually while that of the control constantly remained at low level. ROS level of the toxin-treated cells was almost twenty-times higher than the control after 144 h exposure (Fig. 2). While after 168 h recovery treatment, toxin-treated cells had low levels of ROS, which was not significantly different from the control.

3.3. Effects of microcystin-RR on antioxidant enzymes

A time-dependent increase in the activity of POD was observed in tobacco BY-2 cells when treated with 50 mg/L microcystin-RR (Fig. 3). Similar to that of ROS, POD activity in treated cells was significantly higher than that of the control after 48 h treatment, and it was almost ten-times higher after 144 h exposure. In addition, after 168 h recovery treatment, POD activity of toxin-treated cells decreased dramatically and had no significant difference compared with the control.

Significant increase of GPX activity was found in toxin-treated cells after 12 h of exposure to 50 mg/L microcystin-RR (Fig. 4). The increase of the GPX activity of the treated cells was almost two-times higher than that of the control within 12 h treatment and thereafter. GPX activity in toxin-treated cells decreased after 168 h recovery treatment and was not significant different from the control.

CAT activity increase was observed after cells were exposed to 50 mg/L microcystin-RR for 24 and 72 h, and there was significant difference compared with the control.
After 96 h, CAT activity of the treatment and control both had a slight increase while there were no significant differences detected (Fig. 5).

At the initial time of toxin treatment, enhancement of SOD activity of the tobacco suspension cells was detected, and there were significant differences between the treatment and the control ($p<0.05$) at 24, 48 and 72 h, respectively. However, when the time of exposure prolonged, SOD activity of the treatment decreased while that of the control increased. After recovering for 168 h, SOD activity in the treatment remained low while that of the control remained high and significant difference was detected (Fig. 6).

4. Discussion

Results from the present study indicate that microcystin-RR induced oxidative stress of in vitro cultured plant cells manifested by ROS accumulation and increased activity of antioxidant enzymes.

It is known that a variety of abiotic stresses such as drought, salinity, extreme temperatures, high irradiance, UV light, nutrient deficiency, air pollutants, etc. cause oxidative damage to plants either directly or indirectly through the formation of ROS (He and Häder, 2002; Noctor and Foyer, 1998; Lin and Kao, 2000). Our present work showed that microcystin-RR resulted in significant induction of
intracellular ROS formation in plant cells after 48 h treatment with 50 mg/L microcystin-RR ($p < 0.01$) (Fig. 2). The exponential increase of ROS factors may be attributed to microcystin-RR induced toxicity as well as stress condition. The drastically increased ROS content suggests that the cells were under an oxidative stress and it may cause plant cell damage, similar to that of primary cultured rat (Ding et al., 1998, 2000) and fish (Li et al., 2003) hepatocytes damage induced by microcystin.

To scavenge ROS and to avoid oxidative damage, plants possess the antioxidative enzymes including SOD, CAT, POD and GPX. By dismutation of O$_2^-$ to O$_2$ and H$_2$O$_2$, SOD blocks O$_2^-$ driven cell damage (Giannopotitis and Ries, 1977). Subsequently, CAT and POD as well as GPX break down H$_2$O$_2$ to H$_2$O and O$_2$ (Cakmak and Marschner, 1992), avoiding the production of OH$,^+$ which is highly reactive, potentially hazard to all biological molecules without enzymatic mechanism to eliminate it (Vranova et al., 2002).

The results of this work indicate that in microcystin-RR treated cells, there was a very high activity of POD and GPX (Figs. 3 and 4), indicating that POD and GPX were initiated after microcystin-RR exposure, and these enzymes helped to scavenge the ROS. CAT activities in toxin-treated cells were significantly higher than that of the control at 48 and 72 h (Fig. 5). It indicated that CAT also took part in eliminating ROS. However, when the cells were cultured after 96 h, there were no significant differences of CAT activity between treatment and control (Fig. 5). It may suggest that POD and GPX can serve as more effective intrinsic defense tool than CAT to resist microcystin-RR induced oxidative damage in BY-2 cells. Similar patterns of POD and CAT activity have been observed in plant tissues under NaCl salinity (Mittal and Dubey, 1991) and Cadmium toxicity (Shah et al., 2001).

SOD is an essential component of plants antioxidative defense system. Increased SOD activity in transgenic plants has been shown to confer increased protection from oxidative damage (Allen et al., 1997). It was reported that lower concentrations of microcystins (0.024–0.12 mg/mL) induced a large increase in the activity of SOD in rice seedlings, while only a slight increase was observed at higher toxin concentrations (0.6–3 mg/mL), and SOD activity of rape seedlings decreased as the concentration of microcystins increased (Chen et al., 2004). Our results show SOD activity significantly increased at 24, 48 and 72 h of toxin treatment (Fig. 6). It indicated that SOD played an important role in eliminating ROS. However, when the toxin treatment prolonged, SOD activity of the treated cells decreased, and it was significantly lower than that of the control even after recovery treatment. Decline in SOD activity may be due to two factors: (1) a change in the assembly of enzyme subunits; (2) inhibition of enzyme synthesis due to the elevated H$_2$O$_2$ level, which was deemed to inhibit the SOD activity (Bowler et al., 1994).

With the prolonged toxin application, the cell viability also decreased gradually (Fig. 1). However, the surviving rate was about 80% even after 144 h, indicating that most of in vitro grown cells adapted this toxic condition. The increased activities of the antioxidative enzymes such as POD, CAT and GPX also supported this statement. Moreover, after 168 h recovery, the viability of the plant cells almost returned to 90%, similar to the control (Fig. 1). At the same time, the antioxidant enzymes declined to the normal level. This is quite different from that of in vitro cultured animal cells, in which the cell viability was almost reduced to zero after 7 d exposure of 100 μM microcystins (McDermott et al., 1998).

In summary, the adverse effect of microcystin-RR can induce ROS formation in tobacco suspension cells. On the other hand, these cells can eliminate ROS through the enhancement of antioxidative enzymes. The oxidative stress on cells mainly depends on the balance of ROS content and antioxidative system. If the former prevail over the later, the cell will be damaged and this will ultimately lead to the cell death, reflected by cell viability.

**References**


