Effects of chitosan on growth of an aquatic plant (*Hydrilla verticillata*) in polluted waters with different chemical oxygen demands

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Received 6 February 2006; revised 4 July 2006; accepted 3 October 2006

Abstract

Effects of chitosan on a submersed plant, *Hydrilla verticillata*, were investigated. Results indicated that *H. verticillata* could prevent ultrastructure phytotoxicities and oxidative reaction from polluted water with high chemical oxygen demand (COD). Superoxide dismutase (SOD) activity and malondialdehyde (MDA) contents in *H. verticillata* treated with 0.1% chitosan in wastewater increased with high COD (980 mg/L) and decreased with low COD (63 mg/L), respectively. Ultrastructural analysis showed that the stroma and grana of chloroplast basically remained normal. However, plant cells from the control experiment (untreated with chitosan) were vacuolated and the cell interval increased. The relict of protoplast moved to the center, with cells tending to disjoint. Our findings indicate that wastewater with high COD concentration can cause a substantial damage to submersed plant, nevertheless, chitosan probably could alleviate the membrane lipid peroxidization and ultrastructure phytotoxicities, and protect plant cells from stress of high COD concentration polluted water.

Key words: antioxidant enzymes; chitosan; *Hydrilla verticillata*; malondialdehyde; superoxide dismutase; ultrastructure

Introduction

Increased production of toxic oxygen derivatives is provoked by both normal and stress habitats. These highly cytotoxic species of oxygen can seriously disrupt normal metabolism through oxidative damage to cellular components (Halliwell, 1982). One of the most damaging effects from these molecular species or their products in cells is the peroxidation of membrane lipids (Weckx and Clijsters, 1996). Chemical oxygen demand (COD) is an index for organic pollutants which can induce lipid peroxidation and toxicity to submersed plants (Wang *et al*., 2002). Antioxidants can delay or prevent the oxidation of cellular oxidizable substrates, through scavenging active oxygen species (AOS), activating a battery of detoxifying proteins, or preventing the generation of AOS (Halliwell *et al*., 1991).

Chitosan, a natural polysaccharide, is an essential chemical component in the development of microparticulate systems owing to its excellent behavior in biodegradability, biocompatibility and non-toxicity (Felt *et al*., 1998; Shu and Zhu, 2000). Although chitosan has been recognized as a material increasing interest in medical sciences, since it can protect the human body from free radicals and retard the progress of many chronic diseases (Kinsella *et al*., 1993), the antioxidant activity of chitosan has recently attracted more attention in environmental sciences (Xue *et al*., 1998; Chiang *et al*., 2000). To control environmental pollution, for example, chitosan was widely used as a coagulating agent for removing suspended solids from various processing streams such as cheese whey and dairy wash water (Bough and Landes, 1976; Savant and Torres, 2000; Selmer-Oslen *et al*., 1996; Dyerset and *et al*., 1998), tofu manufacturing (Jun *et al*., 1994) and in the processing of poultry (Bough, 1975), seafood (Savant, 2001; Savant and Torres, 2003; No and Meyers, 1989; Shahidi *et al*., 1999; Shahidi and Synowiecki, 1991; Guerrero *et al*., 1998), wine (Lalov *et al*., 2000) and vegetables (Bough, 1975; Moore *et al*., 1987). In agriculture, particularly, chitosan is an elicitor of the lignification response in wounded (Pearce and Ride, 1982) and intact wheat leaves (Moerschbacher *et al*., 1986) as well as in suspension-cultured wheat cells (Gotthardt and Grambow, 1992).

Nevertheless, phytoremediation effects related to chitosan’s antioxidant activity on COD-induced pollutant stress of macrophytes had not previously been demonstrated. This study describes the effects of chitosan on pollution resistance of a submersed plant, *Hydrilla verticillata*. The objective of this work was to investigate the impact of chitosan on the scavenging system against active oxygen
and ultrastructure variation in *H. verticillata* growing in polluted water with high COD concentration and offer suggestions for lake restoration using aquatic macrophytes.

## 1 Materials and methods

### 1.1 Plant growth conditions and experimental design

*H. verticillata* was collected from an unpolluted water body in Beijing and grown in laboratory for 15 d before experiment. Four water habitats containing different pollutants or agents, e.g., (1) high COD (980 mg/L); (2) low COD (63 mg/L); (3) high COD (980 mg/L), 0.1% chitosan; (4) low COD (63 mg/L), 0.1% chitosan, were developed in the glass aquaria, with polluted water collecting from eatery sewage. A number of studies (Kamil et al., 2002; Shahidi et al., 2001) suggest that lipid oxidation inhibition by chitosan depends on its concentration (50, 100 and 200 mg/L). A 0.1% chitosan solution was used in this experiment according to a previous study (Xu et al., 2004). The starting length and wet biomass of *H. verticillata* were approximately 15 cm and 0.78 g, respectively. High COD water was taken from eatery sewage. The experiment lasted for 15 d at a 26°C environment with a photoperiod of 12 h per day with a light density of 40 μmol/(m²·s). Three replicates per treatment were used during the experiment.

### 1.2 Plant ultrastructure

Plant leaves were fixed by a 2.5% glutaraldehyde solution, washed thrice with phosphate buffer, fixed again with 2% osmium acid, dehydrated serially with acetone and embedded in EPON 812 and then cut into 20–50 nm slices with a microtome (Leica-ULTRACUT R). Specimens were examined with a transmission electron microscope (JEM2010, Japan Electronic Optics Ltd.) after staining with uranium dioxide/lead citrate.

### 1.3 Protein and lipid analysis

Soluble protein concentration was quantified according to Bradford (1976) using BSA standard. The level of lipid peroxidation, expressed in malondialdehyde (MDA) content, was determined as 2-thiobarbituric (TBA) reactive metabolites. Lipid peroxidation, expressed in malondialdehyde content, was determined as 2-thiobarbituric (TBA) reactive metabolites. The level of lipid peroxidation was expressed as nmol/mg fw.

### 1.4 Superoxide dismutase activity

Frozen leaves (0.2 g) were crushed into fine powder with a mortar and pestle which was then buffered with 3 ml of 50 mmol/L potassium phosphate (pH = 7.8, 2 mmol/L ethylene diamine tetra-acetic acid, 1 mmol/L phenylmethylsulfonyl fluoride and 1% (w/v) insoluble polyvinylpyrrolidone). Insoluble material was removed by centrifugation at 15000 r/min for 15 min at 4°C. SOD was assayed according to ISOC (1999). Reaction mixture (3 ml) composed of 60 mmol/L riboflavin, 14.5 mmol/L methionine, 2.25 mmol/L nitrotetrazolium blue chloride, and 50 mmol/L potassium phosphate buffer (pH 7.8) was obtained. The unit (min⁻¹ g⁻¹) was fresh weight.

## 1.5 Statistical analysis

Mean and standard error values of all the parameters studied were determined for each group. Student’s t-test was conducted to assess if there is significant difference between 0.1% chitosan-treated and control group. A p<0.05 was considered statistically significant.

## 2 Results and discussion

### 2.1 Ultrastructural changes

Normal chloroplast was ellipse in shape of clear structure with two layer membranes and regular array of stroma and grana. In high COD (980 mg/L) group, all of the plant cells were vacuolated, with intercellular space either increasing or falling into disintegration (Fig.1a1) or protoplasts being congealed (Fig.1a2). However, plants treated with chitosan possessed almost normal chloroplasts (Fig.1c). In the group of low COD (63 mg/L), chloroplasts had less grana and stroma (Fig.2b). If treated with chitosan, chloroplasts had a large number of both grana and stroma layers (Fig.2d1), with plenty of starch granules (Fig.2d2). A previous study indicated that cell membrane stability was affected by lipid peroxidation caused by active oxygen species under stress conditions (Sudhakar et al., 2001). Chitosan, fully or partially de-N-acetylated derivative of chitin composed of (1/4)-linked GlcNAc and 2-amino-2-deoxy-b-D-glucopyranose (GlcN) residues, is known as one of the important natural antioxidants (Kinsella et al., 1993). Results in this study also showed that chitosan can protect or alleviate the COD stress on the *H. verticillata*.

### 2.2 Effects on plant growth

Wastewater is normally considered as a habitat with low or no dissolved oxygen which makes the aquatic plants difficult to grow and develop. Anoxia-induced changes in plants are therefore characterized by a combined effect of both stresses-anoxic stress itself and oxidative stress, which occur during polluted water influx. Effects of COD on growth, expressed as average increases in weight, length and soluble protein of plant, are shown in Table 1. Chitosan

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Soluble protein (mg/g fw)</th>
<th>Increased weight (mg)</th>
<th>Increased length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>15.2 ± 1.4</td>
<td>-0.171</td>
<td>-0.019</td>
</tr>
<tr>
<td>B</td>
<td>24.9 ± 3.2</td>
<td>-0.105</td>
<td>-0.012</td>
</tr>
<tr>
<td>C</td>
<td>18.8 ± 1.1</td>
<td>0.216</td>
<td>0.024</td>
</tr>
<tr>
<td>D</td>
<td>37.6 ± 9.8*</td>
<td>0.395*</td>
<td>0.044*</td>
</tr>
</tbody>
</table>

*Significance (P<0.05): A: COD 980 mg/L; B: COD 980 mg/L and 0.1% chitosan; C: COD 63 mg/L; D: COD 63 mg/L and 0.1% chitosan.
can stimulate plant growth, and show significant in low COD treatment \( (P<0.05) \).

2.3 Responses of antioxidant enzyme activity

A major cause for the oxidative stress might be the disability of the scavenging system to metabolize the toxic active oxygen owing to either increased reactive oxygen species (ROS) formation or decreased activity of the scavenging enzymes (Foyer et al., 1994). Scavenging system itself comprises several enzymes often with synergetic action. SOD, the first major enzyme, can catalyze the disproportion of O\(_2\) to H\(_2\)O\(_2\), and is an important protective enzyme against active oxygen. It plays a significant role in eliminating free radical, alleviating lipid peroxidation and membrane breakage (Asada and Takahashi, 1987). Effect of chitosan on SOD activity participating in the scavenging of oxidative stress is shown in Fig.3. We noted that SOD activity was largely elevated in plants treated with chitosan compared with the control. Oxygen free radical (O\(_2^-\) or \(\cdot\)OH) is produced by chloroplast and mitochondria during photosynthesis and respiration (Elstner, 1982), while SOD can remove anion free radical of superoxide and produce H\(_2\)O\(_2\). Therefore, SOD controls the density of O\(_2^-\) and H\(_2\)O\(_2\) in plants to a certain extent. As an induced enzyme, SOD can be induced to a certain degree by O\(_2^-\) in the plant under stress thus helping the plant maintain its function to remove the free radical. Therefore the improvement of SOD activation equals to emergent to detoxication by increasing the content of O\(_2^-\). It is an accommodative
reaction of a plant to protect itself from being poisoned (Luo et al., 1987). In this study we found that polluted water containing either high or low COD was resulted in a discernible change in SOD activity. In the high COD group, for example, the SOD activity in *H. verticillata* pretreated with chitosan was 1748.20 U/g fw, whereas the control was 1147.50 U/g fw (t=6.11, p<0.01, Fig.3). In the low COD group, the SOD activity in the plants pretreated with chitosan was 1793.98 U/g fw, while the control was 1454.58 U/g fw (t=10.57, p<0.01). Results showed that chitosan could promote SOD activity. Similar studies reported that chitosan with low molecular weight could scavenge superoxide radical with the scavenging activity of 80.3% at 0.5 g/L (Yin et al., 1998); Esumi et al. (2003) also noted that the gold-chitosan nanocomposites had an ability to depress the activity of hydroxyl radicals.

### 2.4 Responses of MDA content

Lipid peroxidation can be evaluated by the determination of malondialdehyde (MDA) concentration in leaf tissues which can damage the structure of plant cells (Huang and Luo, 1997). MDA content was lower in plants treated with chitosan than that of the control (Fig.4). Sewage with both high and low COD led to a discernible change in MDA contents. If the aquatic plants were treated with chitosan than that of the control (Fig.4). The results from the present study indicated that feasible chitosan may have profound application in lake management using aquatic macrophytes as a restoration tool. Chitosan not only purify the water as coagulating agent also promote the growth of macrophytes as antioxidant in polluted water.

#### Acknowledgements:
The authors are indebted to Professor Zheng-wen Liu for his suggestions and comments.

#### References


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