



Salinity-induced oxidative stress and regulation of antioxidant defense system in the marine macroalga *Ulva prolifera*

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ABSTRACT

The physiological and biochemical responses to salinity stresses in *Ulva prolifera* were investigated, including growth rate, level of oxidative stress and regulation of antioxidant defense system. A six-day exposure to hyposaline (10‰) and hypersaline (60‰) conditions resulted in a significant decrease in growth rate and maximum photosynthetic quantum efficiency (Fv/Fm) compared with the control (30‰). Increases in H₂O₂ contents correlated to the level of lipid peroxidation, which suggested that oxidative damage occurred in salinity stress and was more severe at 60‰ than at 10‰. The amount of total soluble protein (TSP) significantly increased in a hypersaline condition. The fluctuations of four antioxidant substrates and four antioxidant enzymes were determined after the long-term salinity stress. Compared to growth at 30‰, low salinities led to a major increase in activities of catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR), coupled with an increase in contents of ascorbate, glutathione and β-carotenoid. Thalli exposed to hypersaline conditions rapidly accumulated glutathione and did not affect the content of ascorbate, α-tocopherol and β-carotenoid. The activities of CAT, SOD, ascorbate peroxidase (APX) and GR increased in hypersaline conditions, suggesting that reactive oxygen scavenging enzymes played an important role in *U. prolifera* for adapting to the hypersaline condition. The alterations in antioxidant enzymes and substrates are not consistent between hyposaline and hypersaline stresses in *U. prolifera*, but the regulation of antioxidant defense system was a vital tolerance mechanism involved in the oxidative stress.

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1. Introduction

Salinity is one of the primary factors restricting growth and development of macroalgae distributed in the intertidal zones and estuaries (Jahnke and White, 2003; Parida and Das, 2005). Biochemical and physiological response mechanisms are extensively investigated to understand how *Ulva* algal species respond and adapt to hyposaline and hypersaline conditions (Table 1). Some reports have indicated that when macroalgae are subjected to salinity stress, reactive oxygen species (ROS) accumulate rapidly and cause oxidative stress (Collén and Davison, 2001; Dring, 2006; Liu and Pang, 2010). These free radicals such as hydrogen peroxide (H₂O₂), superoxide radical (O₂^{•−}), singlet oxygen (¹O₂) and hydroxyl radical (OH•), could disrupt normal metabolism through peroxidizing membrane lipids and denaturing proteins and nucleic acids (Apel and Hirt, 2004).

However, macroalgae have evolved antioxidant defense mechanisms to cope with the potential damage of ROS (Lesser, 2006). It has been reported that higher antioxidant contents and antioxidant enzyme

activities are associated with higher stress tolerance in various algae (Choo et al., 2004; Lu et al., 2006). Antioxidant contents and enzymatic activity reflecting the status of an organelle's oxidative machinery may be taken as an indicator of cell acclimation and tolerance (Miller-Morey and Van Dolah, 2004; Ross and Van Alstyne, 2007).

Ulva prolifera (syn. *Enteromorpha prolifera*, Hayden et al., 2003) is one of the most common bloom-forming macroalgae. From 2007 to 2011, green tides formed by *U. prolifera* have broken out for 5 years in Yellow Sea of China (Leliaert et al., 2009; Liu et al., 2010; Wang et al., 2010). In the Jiangsu coast of China, *U. prolifera* is distributed broadly in the nearshore ponds, estuaries and the intertidal niches (Ding and Luan, 2009; Liu et al., 2009), where salinities were significantly different because of freshwater inputs from rivers, rainfall and tidal period. However, it is unknown how *U. prolifera* responses physiologically to the salinity stress.

In order to improve the understanding about acclimation of this opportunistic alga to hyposaline and hypersaline conditions, the study was designed to investigate the strategies to cope with salinity stress in this alga. Three questions would be answered in this paper:

- (1) to what extent do hyposaline and hypersaline stresses cause the decline in growth and photosystem II (PSII) activity in *U. prolifera*;

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Table 1
Recent studies on responses of different *Ulva* species to hyposaline and hypersaline stresses.

Algae species	Salinity	Treatment time	Main results	Literature
<i>Ulva fasciata</i>	55 psu	12 h	The induction of free proline accumulation by hypersalinity was observed only with a change in the concentration of NaCl in the medium.	Liu et al. (2000)
<i>Ulva fenestrata</i>	5–95 psu	24 h	<i>U. fenestrata</i> can tolerate salinity changes, but uses metabolites other than DMSP for osmotic acclimation.	Van Alstyne et al. (2003)
<i>Ulva lactuca</i>	48–128‰	12 h	The main targets in PSII affected by elevated salinity were inactivation of reaction centers and inhibition of the electron transport at the acceptor side.	Xia et al. (2004)
<i>Ulva fasciata</i>	5–150‰	4 days	The availability of antioxidants and the activities of antioxidant enzymes are increased to cope with the oxidative stress occurring in hyposaline and hypersaline conditions.	Lu et al. (2006)
<i>Ulva pertusa</i>	6–60‰	24 h	Total pigment contents increase in response to low salinity. The total carbon content significantly increased in response to high-salinity treatment. <i>U. pertusa</i> exposed to hypersaline conditions rapidly accumulated proline.	Kakinuma et al. (2006)
<i>Ulva fasciata</i>	90‰	12 h	Oxidative damage did not occur in 90‰ condition. Enzyme activity is transcriptionally regulated by 90‰ except a fast increase in FeSOD, APX, and GR activities during 1 h. APX is responsible for early H ₂ O ₂ decomposition while CAT scavenges H ₂ O ₂ in the later period.	Sung et al. (2009)

- (2) whether oxidative stress occurs in this alga, exposed to hyposaline and hypersaline stresses for a long term (6-day);
- (3) how *U. prolifera* regulates antioxidant defense system (antioxidants and antioxidative enzymes) to cope with hyposaline and hypersaline stresses.

2. Materials and methods

2.1. Algal material and treatment

Algal materials of *U. prolifera*, which were visually healthy, were collected in July 2009 from Rushan Beach (36°48'39.75"N; 121°38'10.88"E), Shandong Province, China. According to satellite data, the algae were transferred by force of wind and tide from the open sea of Jiangsu Province to the north of Yellow Sea (Liu et al., 2009), and eventually accumulated in Rushan nearshore of Shandong Province. The drifting algae collected were washed with natural seawater to remove sands and epiphytes. Then thalli were transported to the lab in a cool box (4–8 °C) within 48 h and cultured at 16 °C in SPX-GB-250 intelligent illumination incubators (Botai, Shanghai, China) for 4 days in f/2 culture medium prepared by adding nutrients to 0.51 M NaCl solution (30 ± 1‰, pH = 8.0). The NaCl solution was prepared by adding NaCl to sterile distilled water. Salinity was determined by salinometer (Dongke, China) and pH was adjusted by acidimeter (Leici, Shanghai, China). Light was provided by a halogen lamp at PAR of 100 μmol m⁻² s⁻¹ and light time was 12 h. The nutrient-enriched medium was renewed every 2 days and salinity stress experiments were performed after pretreatment.

2.2. Determination of growth rates and PS II activity

Salinity effect on growth rates of *U. prolifera* was tested over a 6-day period. A standard algal weight of 0.1 g (fresh weight, about 5–6 thallus fragments) was cultured in the different saline mediums (10, 30 and 60‰) in 1-L glass flask in the incubators. Nutrients were added to reach to the level of f/2 medium. The temperature was 16 °C, the irradiance was 100 μmol photon m⁻² s⁻¹ and the photoperiod was 12 h light:12 h dark. Each treatment was started in five flasks. After 6 days, algal material was reweighed and relative growth rates (RGR) were calculated: $RGR = (\ln w_2 - \ln w_1) / \Delta t$, where w_1 is the initial fresh weight and w_2 the fresh weight after Δt days. Algal thalli were dried for 72 h at 60 °C to evaluate the dry weight in loft drier (Yiheng, Shanghai, China) and then water content was determined.

Resistance against hyposaline and hypersaline stresses was measured by evaluating changes in maximum photosynthetic quantum efficiency (Fv/Fm) after exposure to different salinities for 6 days. For each salinity treatment, algal thallus fragments in every one of five

flasks were randomly selected for measurement. Fragments were transferred to a Petri dish (9 cm diameter) with 30 mL f/2 media in the same salinity and incubated in darkness for 15 min (Liu and Pang, 2010). The Fv/Fm ratio of each thallus fragments was determined with the pulse amplitude modulated fluorometer (DIVING-PAM, Walz, Germany).

2.3. Determination of H₂O₂ content and lipid peroxidation

Algal thalli samples (0.1 fresh weight) were ground to powder in liquid nitrogen and 1 mL of 5% (w/v) trichloroacetic acid (TCA) was added. The mixture was centrifuged at 12,000 g for 10 min at 4 °C, and the supernatant was collected for determination of H₂O₂ and lipid peroxidation contents.

Algal H₂O₂ contents were determined based on the decomposition of H₂O₂ by peroxidase as described by Okuda et al. (1991). The reaction mixture contained 0.2 mL of supernatant and 11.5 μL of 4 M KOH (pH = 7.5). After having been centrifuged at 12,000 g for 1 min at 4 °C, the supernatant was transferred to a 1-mL column of Amberlite IRA-410, and residual H₂O₂ was washed out by 0.8 mL of distilled water. For the determination of H₂O₂, 0.4 mL of 12.5 mM 3-dimethylamino-benzoic acid, 0.4 mL of 10 mM 3-methyl-2-benzothiazoline hydrazone, and 20 μL of 0.25 U mL⁻¹ horseradish peroxidase (Sigma, USA) were added to the eluate. Then the mixture was detected by evaluating the absorbance at 590 nm. A standard curve was prepared based on a series of solutions with different concentrations of H₂O₂. The concentrations of thallus H₂O₂ were estimated from the H₂O₂ standard curve.

The level of lipid peroxidation was determined by measuring the content of malondialdehyde (MDA) using thiobarbituric acid (TBA) reacting substance contents as determined according to Buege and Aust (1978). To each 0.2 mL aliquot of the supernatant from each treatment, 0.2 mL of 0.6% (v/v) TBA was added. The mixtures were heated at 95 °C for 40 min and then quickly cooled in the ice bath. After centrifugation at 4000 g for 10 min, the absorbance of the supernatant was recorded at 532 nm and 450 nm.

2.4. Analysis of antioxidant contents

Ascorbate content was measured spectrophotometrically after addition of ascorbate oxidase according to Foyer et al. (1983). Algal samples (0.1 g fresh weight) were ground in liquid nitrogen and mixed with 1 mL of 2.5 M HClO₄. After incubation on ice for 30 min, the extracts were neutralized with 1.25 M K₂CO₃ and centrifuged at 5000 g for 10 min. Reaction mixture contained 50 μL sample, 5 U mL⁻¹ ascorbate oxidase and 700 μL 0.1 M sodium phosphate buffer (pH = 5.6) with 1% (w/v) polyvinylpyrrolidone-40 (PVP-40). The ascorbate content was determined by recording the decrease in absorbance at 265 nm.

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