Activities of antioxidant enzymes (SOD and CAT) in the coral *Galaxea fascicularis* against increased hydrogen peroxide concentrations in seawater

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Abstract. The activities of two antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), were studied in a colony of *Galaxea fascicularis* corals with increased concentrations of hydrogen peroxide (H₂O₂) in seawater using an incubation chamber. Changes in enzyme activity were compared to those induced by increased seawater temperature. Three H₂O₂ concentrations (0, 0.3, and 3.0 µM) were tested at 27°C, and an elevated seawater temperature of 31°C was tested without added H₂O₂. CAT activities in both coral tissue and zooxanthellae increased with increased H₂O₂, but SOD activities remained relatively unchanged, suggesting that increased H₂O₂ in seawater affected coral cytosol but did not induce superoxide formation. In contrast, the elevated seawater temperature caused both SOD and CAT activities in coral tissue and zooxanthellae to increase. Coral bleaching was not observed at the levels of H₂O₂ tested during the 5-day exposure period. Although the long-term effects of H₂O₂ remain unknown, these results suggest that coral bleaching would likely not occur from short-term (5 days) exposure to increased H₂O₂ concentrations in seawater.

Key words: hydrogen peroxide, antioxidant enzyme, catalase, coral bleaching, *Galaxea fascicularis*

Introduction

In recent years, coral reefs have experienced extensive degradation throughout the world (Stone et al. 1999). This is the result of coral bleaching, whereby corals lose their symbiotic algal zooxanthellae or the photosynthetic pigments of the symbiotes are degraded. The causes of coral bleaching are thought to be abnormally high seawater temperatures, high levels of irradiance, strong ultraviolet (UV) radiation, changes in salinity, or a combination of these factors (Brown 1997; Fitt et al. 2001).

Environmental stress can induce increased production of reactive oxygen species (ROS), leading to significant oxidative damage to the coral-algae symbiotic system (Lesser et al. 1990; Dykens et al. 1992; Downs et al. 2002). The cellular response to the formation of oxygen radicals includes many defense mechanisms (Shick et al. 1995). For example, enzymes such as superoxide dismutase (SOD) and catalase (CAT) act in concert to inactivate superoxide radicals (•O₂⁻) and hydrogen peroxide (H₂O₂). This prevents the formation of the most reactive form of ROS, the hydroxyl radical (•OH), and subsequent cellular damage (Fridovich 1986). SOD catalyzes the dismutation of superoxide into oxygen and H₂O₂, and CAT is responsible for inactivating H₂O₂ into water and oxygen. These enzymes are responsible for detoxifying ROS, and their elevated activities indirectly indicate increased production of ROS in corals as a result of environmental stresses such as temperature, irradiance, and UV radiation (Lesser et al. 1990).

Smith et al. (2005) hypothesized that H₂O₂ may be the most important ROS associated with coral bleaching because it can act as an important signaling molecule between *Symbiodinium*, i.e., zooxanthellae, and their symbiotic host. In addition, Suggett et al. (2008) reported that the thermal tolerance of *Symbiodinium* is related to adaptive constraints associated with photosynthesis and that sensitive phylotypes are more vulnerable to H₂O₂. Furthermore, Mydlarz and Jacobs (2004) indicated that H₂O₂ production occurred as an oxidative burst in a physically injured *Symbiodinium* sp. that was isolated from *Pseudopterogorgia elisabethae*. Finally, Ross et al. (2006) reported that micromolar concentrations of H₂O₂ (>10 µM) induced cell death in the cyanobacterium *Microcystis aeruginosa* as measured by a caspase protease assay. The production of H₂O₂ was involved in the inhibition of the chlorophyll *a*-chlorophyll *c₂*-peridinin-protein complexes (acpPC) mRNA translation in *Symbiodinium* (Takahashi et al. 2008). Although many studies have reported the effects of in-cell H₂O₂ on zooxanthellae, few studies have examined the effects of increased H₂O₂ in the seawater surrounding a coral colony.
Here we examined the changes in activities of antioxidant enzymes (SOD and CAT) with varied concentrations of H\textsubscript{2}O\textsubscript{2} in seawater and with different seawater temperatures by using a well-controlled incubation chamber. We also compared the magnitudes of changes in enzyme levels induced by H\textsubscript{2}O\textsubscript{2} to those induced by high seawater temperature.

Material and Methods
Coral specimens
Colonies of \textit{Galaxea fascicularis} were collected from a coastal region of Okinawa Island, Japan, with permission from the Okinawa prefectural government (No. 18-11). The coral colonies were immediately transferred to an outdoor tank at the University of the Ryukyus Sesoko Station of the Tropical Biosphere Research Center (TBRC). Coral branch tips (ca. 4 cm long) from three large coral colonies were cut and attached to a polycarbonate resin bolt (ca. 1 cm in diameter and 2 cm long) using glue intended for underwater use. The branch tips were kept in the outdoor tank for 3 months, after which they were moved to an indoor tank at the University of the Ryukyus Nishihara campus (ca. 60 km from TBRC) for the enzyme experiments.

Experimental design
We studied changes in coral enzyme activities using a continuous-flow complete-mixing (CFCM) experimental system. The CFCM system consists of a water tank incubation system and a flow-through system, which can continuously supply seawater while maintaining the volume of the seawater in the incubation tank during the course of the experiments. A detailed description of the CFCM is provided by Fujimura et al. (2008).

We investigated the effects of H\textsubscript{2}O\textsubscript{2} on the activities of SOD and CAT in coral by controlling H\textsubscript{2}O\textsubscript{2} concentrations in the supplied seawater. Various concentrations of H\textsubscript{2}O\textsubscript{2} were introduced into the coral incubation tank. The H\textsubscript{2}O\textsubscript{2} stock solution was prepared with 0.7 M NaCl + 2 mM NaHCO\textsubscript{3} aqueous solution to minimize decomposition of H\textsubscript{2}O\textsubscript{2} and changes in salinity and carbonate concentration of the supplied seawater during the experiments. Concentration of H\textsubscript{2}O\textsubscript{2} in the stock solution was determined by measuring UV absorbance at 240 nm \((\varepsilon_{240} = 38.1 \text{ M}^{-1} \text{ cm}^{-1}; \text{Miller and Kester 1988})\). Immediately before the experiments, we prepared 6 and 60 \textmu M H\textsubscript{2}O\textsubscript{2} solutions that were diluted 20-fold in the incubation tank to 0.3 and 3.0 \textmu M, respectively. It should be noted that dilution of the filtered seawater was always kept at 20-fold throughout the experiments using the 0.7 M NaCl + 2 mM NaHCO\textsubscript{3} aqueous solution even when no H\textsubscript{2}O\textsubscript{2} was added.

Seawater temperature was held constant at 27°C, and three H\textsubscript{2}O\textsubscript{2} concentrations (0, 0.3, 3.0 \textmu M) were used for 1, 3, and 5-day periods, respectively. In an additional treatment, temperature was maintained at 31°C, and no H\textsubscript{2}O\textsubscript{2} (0 \textmu M) was added. Six coral branch tips were used in each treatment. Light was provided by a metal halide lamp, and photon flux density (200 or 0 \textmu mol m\textsuperscript{-2} s\textsuperscript{-1} during a 12:12 h light/dark cycle) was maintained throughout the experiments.

Isolation of zooxanthellae and analyses of antioxidant enzyme activities
Enzyme assays were conducted according to Levy et al. (2006) and Yakovleva et al. (2004). Tissue homogenates were prepared using the air-pick method, in which an air jet connected to a scuba regulator is used to strip the tissue from the coral skeleton into approximately 10 ml of 100 mmol l\textsuperscript{-1} phosphate buffer (pH=7). The volume of the homogenate was measured, and 1 ml of each subsample was used for determination of zooxanthellae density using direct counts on a Neubauer hemocytometer. A 2-ml aliquot of each homogenate sample was filtered using a GF/A filter (Whatman) and used for chlorophyll \textit{a} analysis. Chlorophyll \textit{a} was extracted from the filter overnight with acetone in the dark at 4°C. The absorbance was measured at 630 and 663 nm. We used the Jeffrey and Humphrey (1975) equation to calculate the amount of Chl. \textit{a} extracted from the filter.

The homogenate was further centrifuged twice at 1500 \times g for 15 min to separate the supernatant and pellets. The supernatant was used to analyze protein and enzyme activities of the host coral. The pellets containing the zooxanthellae were suspended in 2 ml of 100 mmol l\textsuperscript{-1} phosphate buffer, and were then dissolved by sonication for 30 min in an ice bath. A 0.05% Triton X-100 solution was added to the sonicated suspension. After incubation for 10 min, the suspension was centrifuged at 14000 \times g for 30 min and used as the algal solution for protein and enzyme assays.

SOD activity was assayed spectrophotometrically as described by Elstner and Heupel (1976) and Oyanagui (1984). Standards for activity were prepared using bovine erythrocytic SOD (Sigma) for each set of samples. CAT activity was measured by the depletion of H\textsubscript{2}O\textsubscript{2} at 240 nm (0.052 cm\textsuperscript{-1} M\textsuperscript{-1}; Beers and Sizer 1952). All assays were conducted at 25°C, and enzyme activity was expressed as units (U) per mg protein. Protein content was determined by the Bradford assay (Bradford 1976). Dunnett’s test was used for the statistical analyses for comparing the data under various conditions against the control data (JMP 6.0.2, SAS).
Results and Discussion

Effects on SOD activity

Figure 1 shows the effects of H$_2$O$_2$ and seawater temperature on the activity of SOD in the coral tissue and in the isolated zooxanthellae. With H$_2$O$_2$ exposure, there were no significant differences in SOD activity in either coral tissue or zooxanthellae during the 5-day exposure period. Similarly, with a high seawater temperature of 31°C and without added H$_2$O$_2$, SOD activity of the host coral was relatively unchanged on day 1 and day 5, even though SOD activity of coral tissue on day 3 was significantly high. Conversely, with high seawater temperature, SOD activity in the zooxanthellae increased significantly by approximately 2-fold on day 3 and day 5 ($P<0.01$). Increases in antioxidant enzyme activities are indicative of increased concentrations of ROS (Lesser et al. 1990). Given that changes in SOD activity reflect changes in •O$_2^-$ concentrations, the results suggested that H$_2$O$_2$ exposure did not induce •O$_2^-$ formation in the coral tissue or zooxanthellae during the incubation period.

Figure 1: Effects of hydrogen peroxide and/or temperature on the activity of superoxide dismutase (SOD) in (a) coral tissue and (b) zooxanthellae. Means ± S.D. (n=6). **: $P<0.01$, *: $P<0.05$ significantly different with Dunnett’s test for comparing treatments to a control.

Yakovleva et al. (2004) showed that SOD activity of coral tissue and the zooxanthellae in *Stylophora pistillata* and *Platygrya ryukyuensis* increased under high seawater temperatures, while SOD activity of the coral tissue in *P. ryukyuensis* showed little change. Dykens et al. (1992) suggested that the SOD activity in host coral changed in response to the amount of photosynthetically generated O$_2$ by its algal symbiotes in the sea anemone *Anthopleura elegantissima*. Our results show a greater increase in the SOD activity of zooxanthellae compared to the coral tissue, which is in accordance with these studies and suggests that the zooxanthellae of the coral *Galaxea fascicularis* are more susceptible to temperature changes than the coral host is. In addition, Takahashi and Asada (1983) reported that •O$_2^-$ exhibits low permeability through the cell membrane. Therefore, it is probable that the •O$_2^-$ formed in the zooxanthellae did not penetrate into the coral tissues.

Effects on CAT activity

Figure 2 shows the effects of H$_2$O$_2$ and/or high water temperature on the activity of CAT in the coral tissue and in the isolated zooxanthellae. CAT activity was significantly increased by both H$_2$O$_2$ and high water temperature conditions. Levy et al. (2006) reported that CAT activities increased within a few minutes to several hours. Moreover, as shown in Fig. 2, CAT activities in the coral tissue increased gradually each day with either H$_2$O$_2$ exposure or high temperature conditions. It is possible that the coral tissue acclimatized day by day against increased H$_2$O$_2$ or high temperature. Yakovleva et al. (2004) also reported that the CAT activities in the coral tissue of *P. ryukyuensis* and *S. pistillata* exposed to high water temperature were higher after 12 h than after 6 h of exposure. CAT activities in zooxanthellae also increased quickly within the first day of the experiments, but CAT activities in zooxanthellae appeared to level off for the remainder of the incubation period. Yakovleva et al. (2004) also reported that CAT activities of the zooxanthellae were slightly lower after 12 h than after 6 h of exposure, which is different from the response of the host coral.

Increases in CAT activities were a result of increased H$_2$O$_2$ concentrations in the host coral and zooxanthellae, which suggests that H$_2$O$_2$ in the surrounding seawater reached the cytosol of the coral. Dykens and Shick (1982) reported that enzymatic defenses in the animal host occur in proportion to the
potential for photooxidative damage in symbiotic cnidarians. Our results showed that exposing corals to ten-fold differences in H₂O₂ concentrations did not result in significant differences in CAT activities in zooxanthellae, which suggested that zooxanthellae probably had a limit in increasing CAT activities, unless penetration of H₂O₂ into the zooxanthellae was controlled by the cell membrane. Once inside the coral cytosol, H₂O₂ should be inactivated by protective enzymes such as CAT, or could be converted into OH radicals, which could damage the photosynthesis system and can eventually lead to coral bleaching (Levy et al. 2006). Higuchi et al. (2009) showed the photosynthesis rate of the coral *G. fascicularis* was reduced by the higher concentration of H₂O₂. It was suggested that higher H₂O₂ in the coral cytosol remained unless all the H₂O₂ was neutralized by the enzymes because of the limitation of increased CAT activity.

**Relationship between ROS and coral bleaching**

Figure 3 shows the changes in the density of zooxanthellae, measured as the number of cells per cm² of the surface area of the coral colonies, during the 5-day incubation period. There were no significant differences in the density of zooxanthellae with increased concentrations of H₂O₂ during the exposures, even with an addition of 3 µM H₂O₂ (Fig. 3). Thus, coral bleaching was not observed with the elevated H₂O₂ exposure within the 5-day experiments. Downs et al. (2002) suggested that bleaching is a final defense of corals against oxidative stress. As shown in Fig. 2, corals (both host coral and zooxanthellae) were under oxidative stress with increased H₂O₂, but coral was not bleached. This could be because the oxidative stress was not due to the in situ formation of H₂O₂ in the symbiotic algae, and therefore the corals did not expel zooxanthellae. Alternatively, it is possible that the oxidative stress posed by the 3 µM H₂O₂ in seawater was within the tolerance level for the tested corals.

Figure 3 also shows the effects of increased seawater temperature on the density of the zooxanthellae. When seawater temperature was elevated from 27 to 31°C, the density of zooxanthellae decreased rapidly by approximately 50% within 1 day, and leveled off for the rest of the 4-day exposure. Significant differences in zooxanthellae density were observed between the corals kept at 27°C and those kept at 31°C during the 5-day high temperature conditions (*P*<0.05). The amount of Chl. *a* per surface area was also decreased by high temperature (data not shown).

**Figure 2:** Effects of hydrogen peroxide and/or temperature on the activity of catalase (CAT) in (a) coral tissue and (b) zooxanthellae. Means ± S.D. (n=6). **: *P*<0.01, *: *P*<0.05 significantly different with Dunnett’s test for comparing treatments to a control.

**Figure 3:** Effects of hydrogen peroxide and/or temperature on the density of zooxanthellae per coral surface area. Means ± S.D. (n=6). **: *P*<0.01, *: *P*<0.05 significantly different with Dunnett’s test for comparing treatments to a control.
Summary
We studied the effects of increased H$_2$O$_2$ in the surrounding seawater and/or high seawater temperature on antioxidant enzyme activities of SOD and CAT in the coral G. fascicularis. With increased concentrations of H$_2$O$_2$, CAT activities in both coral tissue and zooxanthellae were increased, but SOD activities were relatively unaffected. In contrast, with high seawater temperature, both SOD and CAT activities in coral tissue and zooxanthellae were increased, while CAT activities in zooxanthellae were not significantly different even with a ten-fold difference in H$_2$O$_2$ concentration. It is possible that the coral tissue and zooxanthellae reached a limit of CAT activity. Coral bleaching was not observed at the levels of H$_2$O$_2$ used for our experiments during the 5-day exposure period. Although the long-term effects of H$_2$O$_2$ remain unknown, our results suggest that coral bleaching would likely not occur as a result of short-term (5 days) exposure to increased H$_2$O$_2$ in the seawater, and that high seawater temperature had a greater impact on the enzymatic activities of the coral.

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