Effects of low temperature acclimation on antioxidant defenses and ATPase activities in the muscle of mud crab (*Scylla paramamosain*)

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remove ROS is weakened, excess ROS initiates lipid peroxidation and generates malondialdehyde (MDA) as a final product, which has been used as a biomarker for oxidative stress (Doyotte et al., 1997; Viarengo et al., 1990, 1991a). Numerous studies on antioxidant defense systems have been carried out in aquatic animals, such as fish (e.g., Ronisz et al., 1999), crustaceans (e.g., Dandapat et al., 2000; Kong et al., 2004a, 2005, 2007a,b; Niyogi et al., 2001), cephalopods (e.g., Zielinski and Portner, 2000), and bivalve mollusks (e.g., Sheehan and Power, 1999). The effects of antioxidant defenses are closely correlated with temperature variations (Power and Sheehan, 1996; Ronisz et al., 1999; Viarengo et al., 1991a; Wilhelm et al., 2001), reproduction and food availability (Cancio et al., 1999), ontogenetic development (Livingstone et al., 1992; Rudneva, 1999; Viarengo et al., 1989, 1991b;), and xenobiotics (Di Guilio et al., 1989; Livingstone et al., 1989, 1992; Sheehan and Power, 1999; Viarengo et al., 1990; Winston, 1991). However, a majority of these studies have focused on bivalve mollusks, particularly in mussels *Mytilus edulis* and *Mytilus galloprovincialis* (e.g., Di Guilio et al., 1989; Livingstone et al., 1989, 1992; Sheehan and Power, 1999; Viarengo et al., 1990; Winston, 1991). Some studies on antioxidant defenses have been reported in crabs (e.g., Gamble et al., 1995; Kong et al., 2004a, 2005, 2007a,b; Orbea et al., 2002). However, few studies have analyzed the responses of antioxidant defenses to low temperature acclimation in the muscle of crabs.

Another set of enzymes important for aquatic animals in managing temperature change is adenosine triphosphatase (ATPase, EC 3.6.1.3). Transmembrane ATPase maintains ion equilibrium across cell membranes, and the disruption of this equilibrium often results in physiological dysfunction (Kong et al., 2007b). Na^+/K^+

Ca^{2+}. Ca^{2+} was omitted from the standard mixture to measure Mg^{2+}-ATPase activity. Mg^{2+} and EGTA were omitted to determine Ca^{2+}-ATPase activity. EGTA was omitted to measure Ca^{2+}/Mg^{2+}-ATPase activity. The activities of SOD and GPX were measured according to the methods reported by Orbea et al. (2002). SOD activity was determined by measuring the degree of inhibition of the reduction of cytochrome *c* by superoxide anion radicals generated by the xanthine:xanthine oxidase system. The rate of reduction was obtained spectrophotometrically at 550 nm (Spectrophotometer 752N, Shanghai Cany Precision instrument Co., Ltd, China). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 50 μM hypoxanthine, 1.87 mU/mL xanthine oxidase, and 10 μM cytochrome *c*. Using a spectrophotometer at 340 nm, GPX activity was measured by determining the decrease in NADPH during the formation of reduced glutathione via glutathione reductase. In this procedure, 0.2 mM H_2O_2 in 100 mM potassium phosphate buffer (2 mM glutathione, 0.5 mM sodium azide, 2 U/mL glutathione reductase, and NADPH 120 μM, pH 7.0) was used as the substrate. CAT activity was measured using the molybdate colorimetric method described by Goth (1991). All enzyme assays were performed at 37 °C in triplicates. MDA content was determined via the protocol provided by Draper and Hadley (1990). One unit of ATPase activity was defined as micromoles of inorganic phosphate (Pi) produced by ATP decomposition per milligram protein per hour. One unit of SOD activity is defined as the inhibition of 50% SOD activity in 1 mg protein. One unit of GPX activity is defined as the decrease of 1 μmol/L GSH (deducted non-enzyme action) in 1 mg protein per minute. One unit of CAT activity is defined as the decomposition of 1 μmol H_2O_2 in 1 mg protein in 1 min.

Protein concentration was determined according to the method described by Bradford (1976). Bovine serum albumin (AMRESCO Inc., USA) was used as the standard protein for the standard curve of protein concentration. The samples were diluted so that OD values are less than 0.5.

2.5. Statistical analysis

Statistical analysis was performed using the statistical analysis tools of EXCEL 2007. Homogeneity of variance was measured using one-way ANOVA. Significant differences between the acclimated group and the control were determined using Student's *t*-test. The significance levels were assigned at $P = 0.01$ and 0.05.

3. Results

3.1. Antioxidant defense in the muscle of S. paramamosain under low temperature acclimation

In the muscle of *S. paramamosain*, the changes in the activities of antioxidant enzymes SOD, CAT, and GPX at low temperatures are similar. In general, when measured at 37 °C, enzymatic activities in crab muscles collected at lower temperatures are lower than those in tissues collected at higher temperatures. The activity of SOD was 62.8 U/mg at 27 °C, a temperature similar to the native habitat of *S. paramamosain*. At 15 °C, SOD activity was 56.5 U/mg, slightly lower but not statistically significant. The SOD activities measured at 10 and 5 °C were 50.6 and 45.9 U/mg, respectively. Both values were significantly lower than those obtained at 27 °C ($P<0.05$ and $P<0.01$, respectively). The patterns of CAT and GPX activities are similar (Fig. 1). This result is consistent with what was previously observed in the gills (Kong et al., 2007a). The measurement of MDA content confirmed the build-up of oxidative stress. At 27 °C, approximately 0.8 nmol/mg of MDA was present in the muscle homogenate of *S. paramamosain*. A slightly higher MDA concentration (i.e., above 1 nmol/mg) was detected in the muscle tissue of *S. paramamosain* acclimated at 15 °C. The contents of MDA in the muscle of the 5 and 10 °C groups were 1.6 and 2.6 nmol/mg, respectively. Both values are significantly higher than that of the 27 °C group ($P<0.01$).

3.2. Four ATPase activities in S. paramamosain muscle under low temperature acclimation

Changes in ATPase activity are shown in Fig. 2. The Na^+/K^+-ATPase activity of the tissue samples at 10 and 15 °C was significantly higher ($P<0.05$) than that of the control at 27 °C. The Na^+/K^+-ATPase activity of the sample at 5 °C was similar to that of the control ($P>0.05$). For

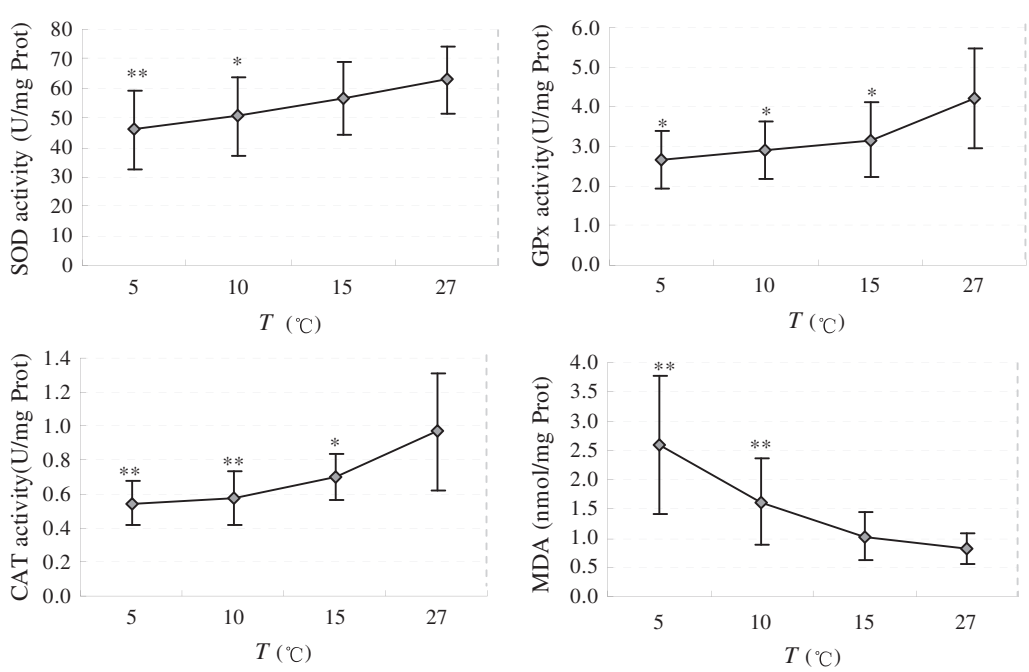

Fig. 1. Changes in SOD, CAT, and GPX activities, as well as MDA content in the muscle of *Scylla paramamosain* under low temperature acclimation. Note: All values in the figures are presented as means ± standard deviation (n = 10). '*' represents the significant difference between the acclimation and the 27 °C group ($P<0.05$); '**' represents highly significant difference ($P<0.01$).

Mg^{2+}-ATPase, significantly higher enzymatic activity was detected only from the sample taken from the crabs acclimated at 10 °C ($P<0.01$). Similarly, Ca^{2+}/Mg^{2+}-ATPase and Ca^{2+}-ATPase activities were significantly higher in the samples from the 10 °C group compared with those of the samples from the 27 °C group. However, the activities of these two enzymes in the tissue samples from crabs acclimated at 5 °C were both significantly lower compared with those in the control group ($P<0.01$).

studies suggest that Ca^{2+} accumulation in the cytoplasm disturbs Ca^{2+} ion homeostasis, subsequently causing the dysfunction of mitochondria and endoplasmic reticulum and then finally leading to the apoptosis of muscle cells (Kong, 2004). At 5 °C, muscle cells cannot synthesize more Ca^{2+}-ATPase and Ca^{2+}/Mg^{2+}-ATPase to maintain a stable physiological level of Ca^{2+}. However, in the hepatopancreas, a more essential organ for the survival of crabs, an additional mechanism may be present to regulate Ca^{2+} level and thus ensure the integrity of physiological functions.

For mud crabs, the muscle plays an important role in activities, such as preying, mating, and fighting. The movement of crabs weakens at low temperatures because of insufficient energy. In the current study, mud crabs acclimated at 15 °C could move under the disturbance. At 10 °C, they could only move slightly when touched. However, at 5 °C, they became motionless even after being touched. After acclimation, the crabs kept at 5 °C were transferred back to room temperature. The crabs still could not move, except for the slight trembling of appendages after being kept at room temperature for more than 2 h. Behavioral observation indicated that the mud crabs at 5 °C were in the state of anesthesia. Therefore, 5 °C was suggested beyond a critical limit of low temperature adaptation for mud crabs. Results from biochemistry assays also support this suggestion. At 5 °C, the declined antioxidative enzymes result in remarkable MDA accumulation. Furthermore, Ca^{2+}-ATPase and Ca^{2+}/Mg^{2+}-ATPase activities at 5 °C also significantly decreased. These results, taken together, suggest that a temperature of 5 °C is very low for mud crabs and that it is beyond the critical value of adaptive low temperatures. At this temperature, mud crabs would not survive because of physiological function disturbance (Kong, 2004). The biochemistry analysis of a set of enzymes, which play vital roles in cellular function, not

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