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Antioxidation and ATPase activity in the gill of mud crab *Scylla serrata* under cold stress*

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Abstract Mud crab (*Scylla serrata*) is an important commercial crustacean in China. An experiment was designed to study the effect of cold stress on *S. serrata*. After a one-week adaptation at 28ºC, the temperature is suddenly reduced to 4ºC. The crabs were sampled every 2 h for 10 h and dissected immediately to measure the enzyme activity. The crabs at room temperature (28ºC) were used as the control group. The activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), the content of malondialdehyde (MDA) and the activity of 4 ATPases (Na^+, K^+-ATPase; Mg^{2+}-ATPase; Ca^{2+}-ATPase; Ca^{2+}, Mg^{2+}-ATPase) were measured biochemically. In contrast to the control group, the SOD activity increased significantly from 2 to 6 h after the cold stress, and then decreased. The CAT and GPX activities increased in 2 h, and then decreased gradually. The content of MDA increased gradually in 4 h. The activity of Na^+, K^+-ATPase decreased in 2 h, increased up to the top value at Hour 6, then decreased again. The activities of Mg^{2+}-ATPase, Ca^{2+}-ATPase and Ca^{2+}, Mg^{2+}-ATPase increased significantly in 6 h, insignificantly in any other hours. Under cold stress, the activity of antioxidative enzymes in *S. serrata* was reduced at first then stabilized, ROS-scavenging weakened, and MDA accumulated gradually in the gill after 6 h. The activity of the 4 ATPases in the crab decreased after 6 h, suggesting that the ability to regulate ion concentration has been paralyzed. Therefore, the maximum period to sustain healthy meat in the crab under cold stress is 6 hours.

Key words: antioxidation; ATPase; gill; cold stress; *Scylla serrata*

1 INTRODUCTION

When temperature changes suddenly, animals living in water will take a corresponding reaction to maintain homeostasis for physiological metabolism. The antioxidative enzymes for removing reactive oxygen species (ROS), being made up of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6) and glutathione peroxidase (GPX, EC 1.11.1.9), play an important role in maintaining physiological functions during temperature changes. In general, producing and scavenging of ROS are balanced dynamically (Fang and Zheng, 2002). If excess ROS is produced in metabolism and cannot be scavenged by antioxidant enzymes, it would result in oxygen stress and damage the macromolecules, such as proteins, nucleic acids and lipids. Recently, a number of studies on antioxidation in Crustaceans have already been reported, such as in crab (Gamble et al., 1995; Brouwer and Brouwer, 1998; Orbea et al., 2002; Kong et al., 2005; 2006) and shrimp (Dandapat et al., 2000). These results indicate that antioxidant effects in the organisms are closely related to environmental changes.

Adenosine triphosphatase (ATPase, EC, 3, 6, 1, 3) plays an important role in supplying energy and maintaining ion concentration. Na^+, K^+-ATPase in the gill is crucial in regulating the Na^+ and K^+ level in the body (Ahearn et al., 1999; Furriel et al., 2000; Lopez mananes et al., 2002). Mg^{2+}-ATPase, Ca^{2+}-ATPase and $Ca^{2+}Mg^{2+}$-ATPase are key enzymes to

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Abbreviation: ROS (Reactive oxygen species), SOD (superoxide dismutase), CAT (catalase), GPX (glutathione peroxidase), MDA (malondialdehyde), ATPase (Adenosine triphosphatase)

regulate Ca^{2+} and Mg^{2+} level. Once the level go beyond the homeostasis of Ca^{2+} and Mg^{2+}, it would result in malfunction and diseases.

Mud crab (*Scylla serrata*) is one of the commercial species living in estuaries, which is distributed along the coast of southeastern China. A study on changes in enzyme activity in the crab can help in understanding the physiological processes under cold stress. In this experiment, the antioxidation and ATPase activity were measured in Hours 0, 2, 4, 6, 8 and 10 under cold stress. The physiological changes under the stress were studied to give some hints for aquiculture.

2 MATERIALS AND METHODS

2.1 The animals and treatments

Healthy intermoult male mud crabs (*S. serrata*) were sampled for the experiment in subtropical coastal waters along Xiamen Island (24°26′46″ N; 118°04′04″E) south of China. The average carapace width, length and wet weight of the crabs were 8.29 ±0.34 cm, 5.85±0.26 cm and 145±20 g respectively.

After one week of adaptation at room temperature (28ºC), the crabs were directly transferred to a container with a temperature of 4ºC. The sudden drop from 28ºC to 4ºC created cold stress on the crabs. We randomly took 6 crabs every 2 h and dissected them immediately for enzyme activity analysis, naming the crabs at room temperature as the Hour 0 (the control), the others as Hours 2, 4, 6, 8, and 10, respectively.

2.2 Sample preparation

The bi-hourly sampled crabs were dissected, having the gills removed and rinsed in 0.9% sodium chloride and cleaned carefully, then stored at –80ºC for analysis.

The 0.2 g frozen gill was rinsed in 1.8 ml ice cold 0.9% sodium chloride solution and homogenized by a hand-driven glass homogenizer. The homogenates were centrifuged at 3 824 g (RCF) at 4ºC for 15 min. The supernatant was stored in Eppendorf tubes at –20ºC for chemical analyses on antioxidative enzymes, MDA and ATPase.

2.3 Enzyme and MDA analyses

2.3.1 Protein, enzyme and MDA

The protein concentration of the prepared supernatant was determined according to Bradford (1976). The bovine serum albumin (BSA) (AMRESCO) was used as the standard.

The CAT activity was determined by using the molybdate colorimetric method according to Goth (1991) and Cheng and Meng (1994), which was based on yellow complex resulting from surplus H_2O_2 and molybdate reaction, with the largest absorbance at 405 nm.

The activity of SOD, GPX and ATPase and the content of MDA were measured spectrometrically with the test kits supplied by Jian-Cheng Bio-engineering Institute of China. The test kits were made of reagents from Sigma-Aldrich Co. All the assays were preformed in triplicate following the supplier's instructions.

2.3.2 Definition of enzyme activity

A unit of SOD activity (U/mg) is defined as a nitrite unit of enzyme activity, under which 50% of the SOD activity can be inhibited in 1 mg protein in a 1 ml reaction solution. A unit of GPX activity (U/mg) is defined as 1 μmol/L of GSH (supposing zero enzyme action), under which 1 mg protein in reaction solution can decrease in 1 min. A unit of CAT activity (U/mg) is defined as a unit that can decompose 1 μmol H_2O_2 in 1 mg protein in reaction solution per minute. A unit of ATPase activity (μmol/h·mg) is defined as a unit in which 1 μmol Pi can be produced during ATP decomposition by ATPase in 1 mg protein per hour.

2.4 Statistical analysis

Statistical analysis was performed using one-way ANOVA and Student's *T*-test at p=0.05 or 0.01 significance.

3 RESULTS

3.1 Antioxidation in different hours of cold stress

In the cold condition, enzyme activity against oxidation changed in different ways in the gill of *S. serrata* (Fig.1). The SOD activity fluctuated in different periods of cold stress, which increased significantly in Hours 2 to 4 (p<0.05), maximum at

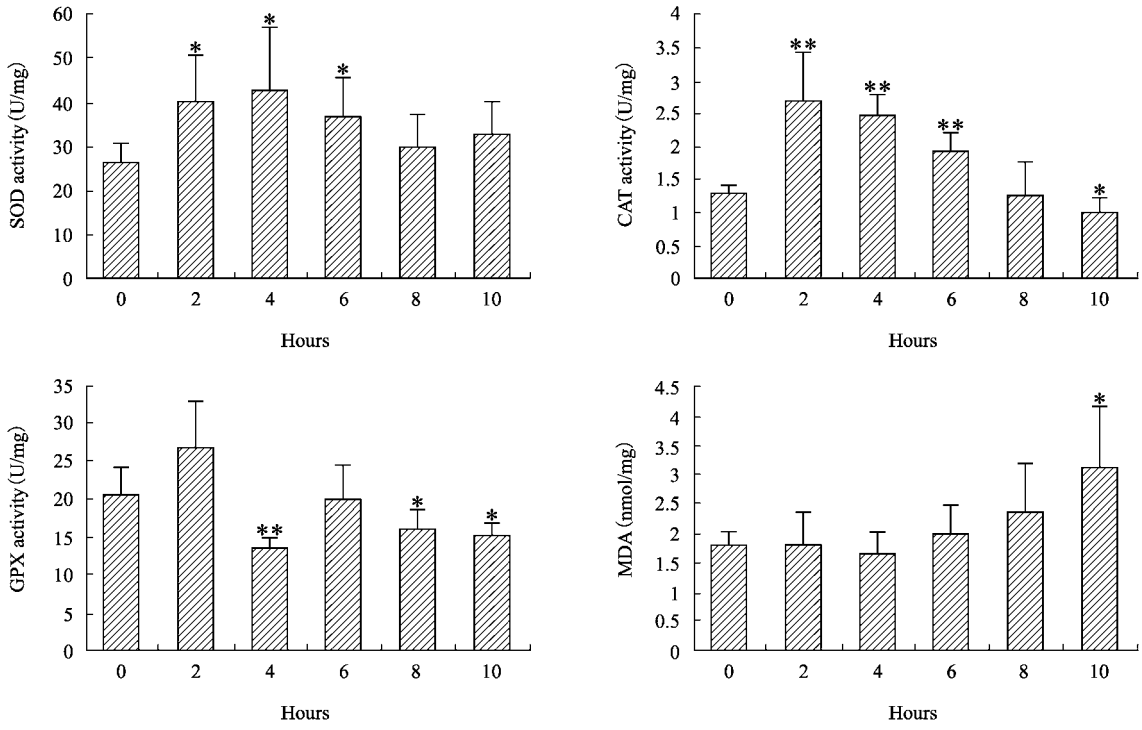

Fig.1 The activity of SOD, CAT and GPX and the content of MDA in the gill of *S. serrata* in different hours of cold stress

All values are in M±SD. *: significant difference ($p<0.05$) compared to the control; **: very significant difference ($p<0.01$)

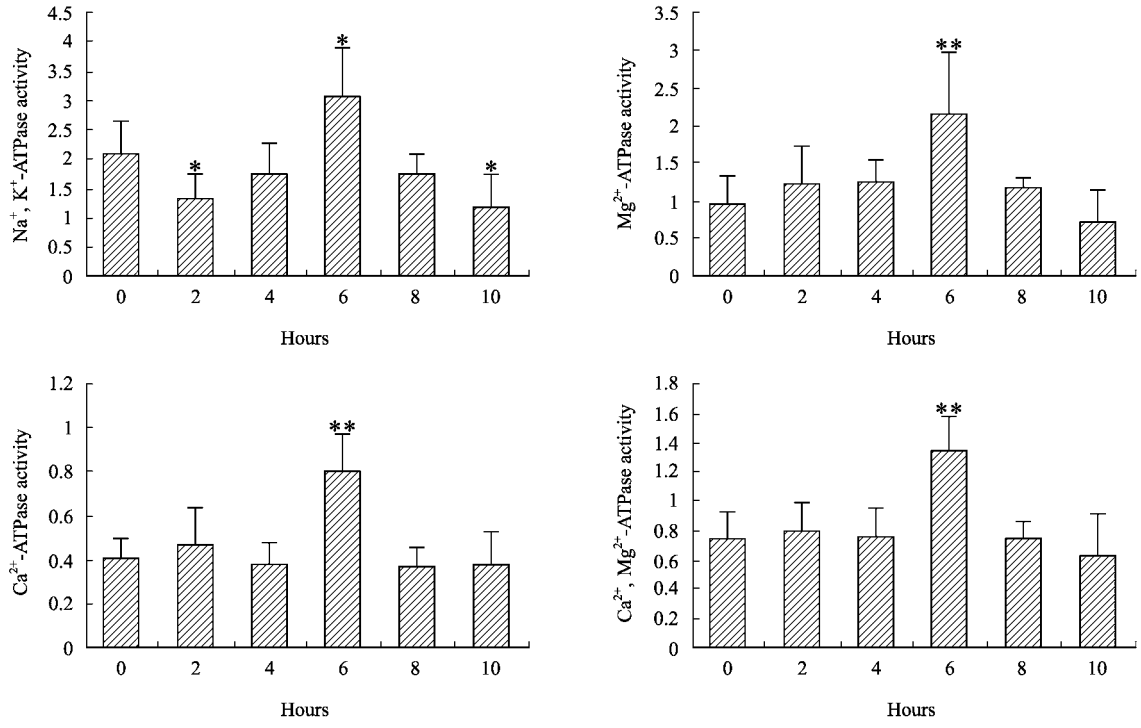

Fig.2 The activity of the 4 ATPases in the gill of *S.serrata* in different hours of cold stress

All values are in M±SD; ATPase activity unit is μmol/h·mg; *: significant difference ($p<0.05$) compared to the control; **: very significant difference ($p<0.01$)

42.83±14.26 U/mg at Hour 4. After that, SOD activity slowed down until Hour 6 but still above that of the control group, and went down further to the minimum of 29.88±7.50 U/mg at Hour 8, close to that of the control group.

In CAT activity, the highest value of 2.70±0.75 U/mg occurred at Hour 2, then gradually decreased to the bottom at 1.01±0.23 U/mg at Hour 10. Statistics indicate that the CAT activity is boosted more significantly ($p<0.01$) in Hours 2, 4 and 6 than that in the control, but decreased significantly at Hour 10 ($p<0.05$).

The GPX activity increased to its highest value at 26.79±6.28 U/mg at Hour 2, at a level close to that of the control group. Then, it decreased to a level below that of the control, and further slid significantly ($p<0.01$ or $p<0.05$) at Hours 4, 8 and 10.

However, no clear change was found in MDA in Hours 2, 4 and 6. After Hour 6, MDA increased gradually to 3.13±1.01 μmol/mg at Hour 10, which was much higher than that in the control.

3.2 Changes in ATPase activity in different hours of stress

The changes in the activity of 4 ATPases in the gill varied in different hours of cold stress (Fig.2). Na^+, K^+-ATPase activity decreased at Hour 2, and peaked at 3.09±0.82 μmol/h·mg at Hour 6, and then, slowly approached the bottom at Hour 10. Na^+, K^+-ATPase activity was significantly higher ($p<0.01$) at Hour 6, while much lower ($p<0.01$) in Hours 2 and 10 than that of the control. The Mg^{2+}- ATPase activity was enhanced gradually before Hour 6, reaching a maximum of 2.17±0.80 μmol/h·mg, then went down. Statistics indicate that Mg^{2+}-ATPase activity at Hour 6 was much higher than that of the control group ($p<0.01$). Ca^{2+}-ATPase activity however fluctuated during the course, maximized at Hour 6, which was much higher than that of the control group ($p<0.01$). Then obviously fell. So did the Ca^{2+}, Mg^{2+}-ATPase similarly.

4 DISCUSSION

4.1 Antioxidation in gill under cold stress

In aquiculture, the physiological function of *S. serrata* is often affected by abrupt temperature change, often resulting in serious diseases. However, the problem has not been addressed by scientific communities. The study on the relationship between oxygen stress and environmental changes was rare until recent years (Orbea et al., 2002; Brouwer, 1998). However, most of them focused on antioxidation on environmental pollution.

As mentioned above, the SOD activity in the gill increased significantly at Hours 2, 4 and 6 of low temperature stress. In general, enzyme activity should increase at low temperatures to compensate the need from physiological metabolism. Meanwhile, during the process of metabolism, ROS would be built up to induce SOD activity that is then temperature and ROS dependent. The quick rise at the beginning and fall in the CAT activity showed that it had the ability to remove ROS and improve the cold-stressed situation. The GPX activity variation was similar to that of SOD, increased as temperature dropped, resulting in ROS accumulation. Therefore, the enzymes can regulate the metabolism under cold stress by reducing their own activities, especially after Hour 6.

Oxidation can damage the organism, which would be reflected by MDA produced by oxidation stress on membrane lipid and fatty acid. The accumulation of MDA (Li and Yang, 1990; Wang et al., 1998) or ROS (Lin and Wang, 2001; Yang and Gao, 2002) would damage cells and trigger apoptosis. As shown in this study, the MDA in the gill increased gradually as cold stress lasted. Under the stress, the ability of removing excess would be weakened, resulting in the accumulation of MDA, and sometimes the death of the experimental animals (in this experiment, a few crabs died at Hour 10).

The above results showed that changes in antioxidative enzymes were related to temperature and ROS. At low temperatures, the metabolism rate of the animal would be reduced. As a counter-measure, enzyme activity would increase for weak metabolism. Meanwhile, ROS could be produced during the metabolism to stabilize antioxidative enzymes. MDA was built up during stress, indicating that ROS could not be scavenged in time. Antioxidative enzyme activity increased before Hour 6, but decreased afterwards, showing on the other hand that the inability to scavenge ROS may build up MDA, which is harmful to the animal cells.

4.2 Variation in ATPase activity under cold stress

ATPase is a type of lipid-protein, an important component in cell membrane. ATPase can catalyze ATP to release energy, driving ion transport across membranes and maintaining a constant ion level in the animal body. Na^+, K^+-ATPase is responsible for regulating the Na^+ and K^+ level in the body. Ca^{2+} in the body plays an important role in keeping the membrane functioning normally and releasing nervous medium. Ca^{2+} concentration in cells is maintained at a low level by ejecting Ca^{2+} out of the cell or absorbing Ca^{2+} into the endoplasmic reticulum and mitochondria across the membrane via Ca^{2+}-ATPase. Mg^{2+}-ATPase is used to regulate Mg^{2+} level, which is important for the physiological activity of membrane protein (Yang and Huang, 1996). Ca^{2+}, Mg^{2+}-ATPase is Mg^{2+}-dependent Ca^{2+}-ATPase, which is also involved in the regulation of Ca^{2+} level (Zylinska and Legutko, 1998). If Ca^{2+}-ATPase and $Ca^{2+}Mg^{2+}$- ATPase activities decreased, Ca^{2+} would be accumulated in the cytoplasm, resulting in physiological malfunction as well as cell apoptosis.

In different periods of cold stress, the activity of the 4 ATPases showed similar changes. In our experiment, they decreased at Hour 2 and then increased, reaching the highest point at Hour 6, then decreasing gradually again. Finally, it reached the lowest point at Hour 10 indicating that the compensation for enzyme activity for weak metabolism under cold stress fluctuated (Hochachka and Somero, 1984). For the crabs under cold stress at Hour 2, the decrease in enzyme activity might be due to enzyme decomposition. After Hour 2, the enzyme activity increased, which might be due to compensation of the activity for low metabolism. Under the stress, the ability to compensate decreased. This fluctuation in enzyme activity demonstrated a dynamic reaction of the synthesis and decomposition of enzymes in the crab, which is a complicated process, of course. In addition to the change in enzyme activity, stress proteins (e.g., HSP70, HSP90) that were used to recover damage proteins, were probably involved during the course of stress (Oberdorster et al., 1998; Frankenberg et al., 2000). Therefore, if the condition was back to normal in time, the animal could resume its normal state using its internal physiological system and recover from any harm caused by the stress.

4.3 Coordination of various enzymes on metabolism

As the results show, antioxidative enzymes SOD activity decreased after Hour 6 under cold stress, and CAT and GPX activities decreased after Hour 2, indicating the reduction of the ability to remove ROS in the crab, which in turn caused ROS accumulation and oxidation stress within the first 6 h of cold stress. As a reaction, the MDA in *S. serrata* increased gradually after Hour 6, and the activity of the 4 ATPases started to decrease after Hour 6, showing a weakening ability of regulating ion level; so Ca^{2+} might be built up in the cell. In this case, antioxidative enzymes and ATPases coordinated well in keeping normal physiological functioning.

To sum up, *S. serrata* could recover itself from cold stress by regulating enzyme activity in certain periods. If cold stress continues, the ability to regulate metabolism would decrease. Once the stress goes beyond the animal's regulation ability, cell apoptosis could occur. As shown in this case, the ability of *S. serrata* to regulate their metabolism decreased at 4ºC after Hour 6, being at a low temperature of 4ºC for over 6 h would be harmful to the crab, which should be avoided in practice of aquaculture for the crabs.

5 CONCLUSION

1. Under the cold stress, the activity of antioxidative enzymes in *S. serrata* was reduced. As a result, accumulated ROS boosted up the enzyme activity for physiological compensation by increasing enzyme concentration.

2. With cold stress going on, the activity of antioxidative enzymes slowed down, ROS scavenging weakened, and MDA accumulated gradually in the gill of *S. serrata* after Hour 6.

3. The activity of the 4 ATPases in *S. serrata* decreased after Hour 6 under cold stress, suggesting that the ability to regulate body ion level was reduced. In order to sustain the crab, the stress must be removed after Hour 6.

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