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Antioxidant enzymes in sea cucumber *Apostichopus japonicus* (Selenka) during aestivation

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To evaluate the effect of antioxidant defence in coelomic fluid of sea cucumber, Apostichopus japonicus *in aestivation was studied in the field from July to November 2006 in Qingdao. During the sampling period, activities of superoxide dismutase and catalase increased significantly in August and November. Activities of glutathione reductase and glutathione decreased significantly in August and increased significantly in November and activities of Se-glutathione peroxidase increased significantly in August. There were no significant differences in total glutathione peroxidase. In relation to the water temperature in the field, it is known that the oxygen consumption rate dropped and antioxidant defence was enhanced in August. The structure and function of respiratory trees of* A. japonicus *were completely vivified as normal in November, and it is suggested that antioxidant defence was enhanced because of the sharp change of oxygen consumption. Data indicate that both enzymatic and metabolite antioxidant defences in sea cucumber are adaptable systems that are modulated during pre-aestivating stage and arousing stage.*

Keywords: antioxidant defence, aestivation, *Apostichopus japonicus*

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INTRODUCTION

Sea cucumber *Apostichopus japonicus* (Selenka), a member of Echinodermata, Holothuroidea (Liao, 1980), is an epibenthile species inhabiting the temperate zone along Asian coasts. It has long been exploited as an important economic resource in China, Russia, Japan and Korea (Sloan, 1984).

Aestivation is a torpid state that is probably best defined as a survival strategy against adverse conditions including aridity, heat, or food scarcity (Pinder *et al.*, 1992; Abe, 1995; Land & Bernier, 1995; Storey, 2001). Frequently-studied vertebrates are lungfish (Sturla *et al.*, 2001), frog (Grundy & Storey, 1994; Fuery *et al.*, 1998; Hudson & Franklin, 2002; Lampert & Linsenmair, 2002), turtle (Storey, 1975; Lutz *et al.*, 1980), and toad (Stewart *et al.*, 2000; Bicego-Nahas *et al.*, 2001). Studied invertebrates are land snail (Barnhart & McMahon, 1987; Hermes-Lima & Storey, 1995; Hermes-Lima *et al.*, 1998) and sea cucumber (Choe, 1963; Li *et al.*, 1996; Liu *et al.*, 1996; Yang, 2005, 2006).

Aestivation of *A. japonicus* lasts 100 days of a year in some regions of China (Li *et al.*, 1996). It would become inactive at over 18°C and aestivate at 20–24.5°C in water temperature (Liu *et al.*, 1996), so aestivation greatly encumbers the culture of *A. japonicus*. Morphology and physiology of *A. japonicus* during the aestivation were studied (Li *et al.*, 1996; Liu *et al.*, 1996) as following. The water temperature for aestivation varied with the ages of the sea cucumber and the threshold values for the 1-year-old, 2-year-old, 2- to 3-year-old and more than 3-year-old individuals were 24°C, 22.9°C, 21.8°C and 21.8°C, respectively. There was no obvious degeneration in most individuals (77.8%) whose ages were less than 1-year-old, they were able to feed and did not aestivate. No matter what the age, the degeneration of digesting tract became a stringy gland whose length was almost the same as the body length and its diameter was less than 1 mm. During aestivation, the activity of small sea cucumbers is more frequent than large ones. The body weight decreased obviously during aestivation and gonad degenerated so quickly that it was difficult to distinguish the sex by naked eyes. The respiratory tree of sea cucumbers degenerated to half the normal and its oxygen metabolism declined (Choe & Ohshima, 1961; Choe, 1963; Sloan, 1984; Liu *et al.*, 1996). One month after aestivation, pH in coelomic fluid increased from 7.04 to 7.21, so did Po_2 from 102.94 to 135.00 mmHg, but Pco_2 decreased from 9.28 to 7.75 mmHg (K. Xing, unpublished).

As is known, the effect of reactive oxygen species (ROS) can result in irreversible chemical modification in cellular macromolecules with reduced functions (Beckman & Ames, 1997; Stadtman & Levine, 2000; Hermes-Lima & Zenteno-Savin, 2002). ROS includes superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$), singlet oxygen, ozone and lipid peroxides. As oxygen radicals can be continuously generated in various cellular processes, all organisms have developed and are equipped with antioxidant systems of enzymatic or non-enzymatic components. Key

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materials in the system against ROS include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione (GSH) and glutathione reductase (GR)(reaction (1–4)).

$$2O_2^- + 2H^+ \xrightarrow{(SOD)} H_2O_2 + O_2 \quad (1)$$

$$2H_2O_2 \xrightarrow{(CAT)} 2H_2O + O_2 \quad (2)$$

$$H_2O_2 + 2GSH \xrightarrow{(GPX)} 2H_2O + GSSG \quad (3)$$

$$GSSG + NADPH + H^+ \xrightarrow{(GR)} 2GSH + NADP^+ \quad (4)$$

Generally, enzymes (such as SOD, CAT and GPx) can scavenge excess ROS for physiological balance in the organism. Excessive ROS can offset the antioxidant capacity and build up an oxidative stress. As a defensive measure, the stressed organisms would be able to tolerate high ROS exposure by increasing the expression of antioxidant enzymes (AOEs) to suppress oxidative damage (Demple, 1999).

The role of enzymes of antioxidant defence on aestivation has been analysed in toads and snails (Hermes-Lima & Storey, 1995; Grundy & Storey, 1998; Hermes-Lima *et al.*, 1998, 2001). These researches show that these anoxia tolerant species show typically two strategies against oxidative stress during natural transitions from low to high oxygen availability: high constitutive antioxidant defences with AOEs and metabolites, and anoxia induced increases in activities of AOEs (Hermes-Lima *et al.*, 2001; Hermes-Lima & Zenteno-Savin, 2002; Storey, 2002, 2006). During the changes of oxidative metabolism, ROS formation would increase and damage the body (Storey, 2002, 2006).

When the water temperature dropped from 30°C to 20°C, the oxygen consumption rate of *A. japonicus* decreased from 400–650 $\mu g\, g^{-1}\, h^{-1}$ to 300–425 $\mu g\, g^{-1}\, h^{-1}$(Yang, 2006). Once the period of aestivation was over, oxygen uptake and consumption rebounded rapidly, so did the rate of ROS generation. In this paper, we analysed enzymatic antioxidant defence of *A. japonicus* to determine how these respond to the naturally wide variations in oxygen levels that accompany aestivation and arousal.

MATERIALS AND METHODS

Animal and sample collection

Apostichopus japonicus was collected twice per month from July to November 2006 (once in September) from aquaculture ponds (35°44′N 120°01′E) located off the Yellow Sea in Jiaonan, Shandong Province, China. At flood tide, seawater flows into the pond and at ebb tide, and the depth of water in the pond maintains at 100–120 cm. Juvenile sea cucumbers (30 g wet weight) stocked in the pond at the end of April 2005 were the source of animals for this study. Naturally-occurring detritus is the only food source for the sea cucumbers in the pond. Generally, ten sea cucumbers (94.50 ± 44.75 g, 2-year-old) were arbitrarily collected throughout ponds by a diver. To test enzymes variability among individuals, 15 samples were collected at the first sampling.

Coelomic fluid of *A. japonicus* (1.5–2.0 ml ind^{-1}) was withdrawn immediately with a syringe, frozen in sterile test tubes in liquid nitrogen, and stored at −80°C for less than one month until further analyses.

Preparation for enzyme assay

Samples of frozen coelomic fluid were thawed at 4°C in a refrigerator and then cell contents were released with ultrasonic cell disruptor. Activities of enzymes in whole coelomic fluid sample were assayed. Samples were centrifuged at 376 × *g* for 10 minutes at 4°C in a centrifuge (Sigma, German). Supernatants were collected and stored at 4°C in a refrigerator less than 5 hours before enzyme assays.

Measurement of protein concentration

Protein concentration of coelomic fluid was measured by the Coomassie blue method (Spector, 1978), using bovine serum albumin as a standard. Enzyme activities were standardized as unit per milligram protein.

Assays of antioxidant enzymes

Total superoxide dismutase (T-SOD) activity (including Mn-SOD and CuZn-SOD) was determined according to Ji (1991) with the assay kit of Nanjing Jiancheng, China. An SOD unit is defined as the amount of enzyme that inhibits the superoxide-induced oxidation in detection system (monitored at 550 nm) by 50%.

Catalase (CAT) activity was measured according to Góth (1991) with the assay kit of Nanjing Jiancheng, China. One CAT unit is defined as 1 μmol of H_2O_2 consumed per minute.

Glutathione reductase (GR) activity was assayed by the method of Di Ilio *et al.* (1983). A unit of GR is defined as the amount of enzyme that can oxidize 1 nmol NADPH per minute.

The content of GSH was measured by the method of Griffith (1980). A unit of GSH is defined as the change of absorbance at 412 nm per minute according to Griffith (1980).

Selenium-dependent GPx activity was determined using the procedure of Ahmad & Pardini (1988) with a microplate reader (Tecan, Switzerland) at 340 nm. A unit of GPx is defined as the amount of enzyme causing the oxidation of 1 nmol of NADPH per minute.

Measurement of water temperature and salinity

Water temperature (°C) and salinity on the surface and at the bottom of the pond were recorded four times at ten o'clock on each sampling date throughout the study period.

Statistical analysis

Statistics were performed using statistic software SPSS 11.0 for Windows. Differences between treatments were tested using one-way analysis of variance (one-way ANOVA, Tukey) and were considered significant among treatment groups at a probability level of $P < 0.05$.

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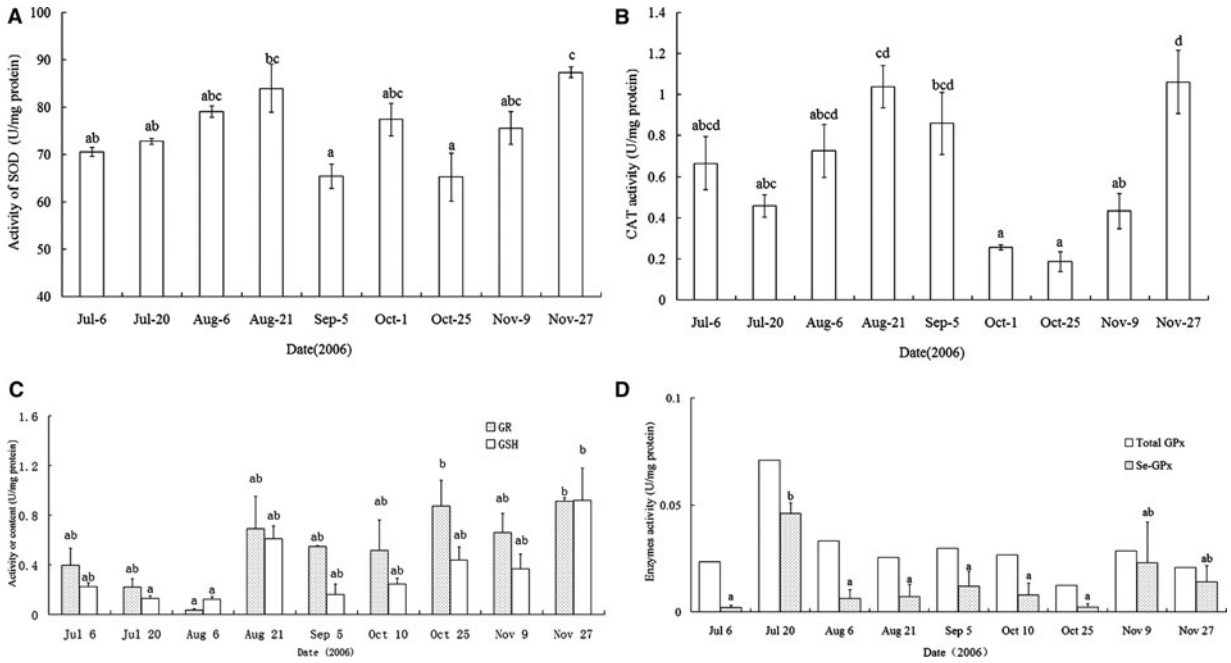

Fig. 1. Activities or contents of superoxide dismutase (SOD) (A), catalase (CAT) (B), glutathione (GSH) and glutathione reductase (GR) (C), total glutathione peroxidase (total GPx) and selenium-dependent glutathione peroxidase (Se-GPx) (D) in coelomic fluid of *Apostichopus japonicus*. Bars represent means ±SE. Data designated with different letters are significantly different from each other ($P < 0.05$) as determined by a Tukey's HSD multiple comparison test.

Correlation coefficients of enzyme activities were tested using the Pearson correlation test (2-tailed).

RESULTS

During the sampling period, activities of SOD and CAT increased significantly in August and November. The SOD activity ranged from 65.25 U mg protein^{-1} in October 25 to 87.35 U mg protein^{-1} at the end of November (Figure 1A). The CAT activity ranged from 0.19 U mg protein^{-1} in October 25 to 1.06 U mg protein^{-1} at the end of November (Figure 1B).

During the sampling period, activities of GR and GSH decreased significantly in August and increased significantly in November. The GR activity ranged from 0.0355 U mg protein^{-1} (6 August) to 0.9130 U mg protein^{-1} (27 November) during aestivation. The content of GSH changed from 0.1222 U mg protein^{-1} (6 August) to 0.9201 U mg protein^{-1} (27 November) (Figure 1C).

Activities of Se-GPx increased significantly in August 2006. No significant difference in total GPx was shown ($P > 0.05$) (Figure 1D). The activities of total GPx ranged from 0.0124 U mg protein^{-1} (25 October) to 0.0708 U mg protein^{-1} (20 July), and those of Se-GPx were from 0.00232 U mg protein^{-1} (25 October) to 0.0459 U mg protein^{-1} (20 July) during the sampling period in 2006.

During the study period, the maximum temperature was recorded in August (29.6°C), and the minimum temperature was recorded on 27 November (11.8°C). The salinity ranged from 27.53 (at the end of July) to 33.03 (at the end of November) (Figure 2). Water temperature and salinity in the sampling pond were significantly correlated with some of the enzyme activities (Table 1). Temperature was significantly and negatively correlated with GR, and salinity was significantly and positively correlated with GSH.

DISCUSSION

Various physiological or environmental stresses can cause oxidative damage. Antioxidant defences are important safeguards in aerobic organisms, with which macromolecules are protected from damage by ROS (Winston, 1991; Grundy & Storey, 1998). Unfortunately, until recently there has been only little information about whether and how antioxidant defences operate to help marine organisms against adverse natural situations where oxygen varies widely. The organisms have different sensitivity to oxygen and its metabolites. Many species of insects, molluscs, fish, amphibians and reptiles are able to survive periods ranging from hours to months without O_2 (Pinder *et al.*, 1992; Lutz & Nilsson, 1997; Jackson, 2000; Hochachka & Lutz, 2001; Storey, 2001).

During aestivation, oxygen used for metabolism in animals decreases, resulting in reduced activity of AOEs with little ROS, as is the case in toads and snails (Hermes-Lima & Storey, 1995; Grundy & Storey, 1998; Hermes-Lima *et al.*, 2001). In aestivating toads, the activities of AOEs were often lower significantly in the first 2 months than those aroused for 10 days (Grundy & Storey, 1998). An exception was

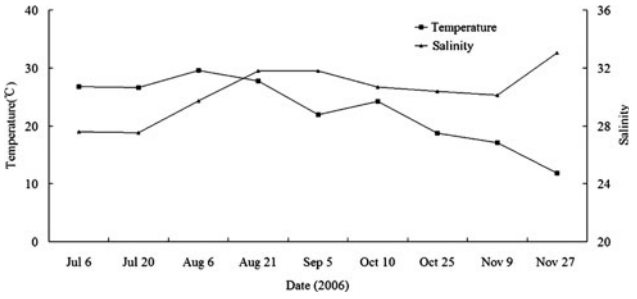

Fig. 2. Temperature and salinity conditions in the aquaculture pond where *Apostichopus japonicus* was collected.

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Table 1. Correlation coefficients of enzyme activities in the coelomic fluid of the sea cucumber, *Apostichopus japonicus*, with temperature (*T*) and salinity (*S*) from July to Novermber 2006 in aquaculture pond (35°44′N 120°01′E) located in Jiaonan, Shandong Province, China.

Pearson correlation coefficient (2-tailed)	SOD	CAT	GR	GSH	T-GPx	Se-GPx
T	−0.069	−0.098	−0.788*	−0.666	0.398	−0.037
S	0.269	0.496	0.658	0.689*	−0.543	0.340

*, correlation is significant at the 0.05 level (2-tailed).

SOD which showed a half activity lower in aroused toads. Results of other organs were not consistent with those of liver, but in general, for the AOEs quantified in five other tissues (heart, kidney, muscle, lung and gut), 12 instances showed higher activities in aroused toads than in aestivated ones, and only 4 were not the case (no change in 14 cases) (Grundy & Storey, 1998). Hence, the above case showed that the reduction in organ oxygen consumption during aestivation resulted in lower activities of AOEs in organs of aestivating toads. Antioxidant defences are clearly enzyme systems and would be adaptable to the changes of ROS load on them.

By contrast, a different situation occurred in aestivating *O. acteal*. Activities of CAT, SOD, glutathione peroxidase, and glutathione-S-transferase were generally higher in the hepatopancreas and foot muscle of aestivating snails than in active ones (Hermes-Lima & Storey, 1995). We know that these adjustments to antioxidant defences would minimize damage due to a burst in generation of reactive oxygen because of a rapid increase in oxygen consumption in the early minutes of arousal (Hermes-Lima *et al.*, 1998, 2001). A high oxygen uptake event for up to 5-fold boost also occurred intermittently during aestivation for snails, during which the animal hyperventilated and CO_2 was released (Barnhart & McMahon, 1987). Differently, in the case of active snails, the oxygen uptake could cause short bursts in ROS production. Good antioxidant defences are critical to preventing or minimizing the buildup of ROS damage during a long aestivation (Storey, 2006).

Li & Wang (2007) studied the physiological characteristics of the sea cucumber, *A. japonicus*, during aestivation in a laboratory. The whole process of aestivation was divided into 3 stages as early stage, middle stage and later stage based on the characteristics of shapes and the histological changes. In the first stage, the sea cucumber still fed, but had a gradually thin alimentary tract, and its structure was in degeneration. In the middle stage, the feeding activity of the sea cucumber became weak, and the alimentary tract was atrophied. More significant degeneration was observed in the structure of the alimentary tract. In the later stage, stopping feeding occurred in the sea cucumber whose alimentary tract was thread-like in shape and the tissue degenerated significantly. It is notable also, that other authors (Fankboner & Cameron, 1985; Byrne, 1986) described irregularity of a sea cucumber entering into aestivation during the summer season and attributed peak of atrophy of the visceral organs to the autumnal period. Alimentary tract and respiratory trees of *A. japonicus* reverted quickly to normal shapes in October and were completely vivified as normal in November.

According to the physiological characters (Li & Wang, 2007) and activities of AOEs, the aestivation could be divided into three stages as pre-aestivation stage (July), aestivation stage (August to September) and arousing stage (in October). For mature *A. japonicus*, oxygen consumption rate peaked at 20°C and then dropped significantly at aestivation temperatures (Yang, 2006). In relation to the water temperature in the field, we knew that the oxygen consumption rate dropped and antioxidant defence was enhanced in August. Because the structure and function of respiratory trees of *A. japonicus* were completely vivified as normal in November, it is suggested that antioxidant defence was enhanced because of the sharp change of oxygen consumption as our data showed. Our data indicate that both enzymatic and antioxidant defences in *A. japonicus* are adaptable systems during aestivation.

The level of antioxygenic property antioxidants can be modulated by a number of environmental variables (Martinez-Alvarez *et al.*, 2002; Wilhelm *et al.*, 2002). Amongst these, temperature is an important variable that has a significant influence on aquatic ectotherms (Kaur *et al.*, 2005). Prakash *et al.* (1998) have also reported ROS production in the gills of *H. fossilis* in response to temperature stress of 12°C. Heat stress induced production of ROS has also been reported in the Antartic bivalve *Laternula elliptica* King& Broderip (Heise *et al.*, 2003), and this amounted to increased oxygen demand and exaggerated oxidative stress in bivalves. In August, *A. japonicus* endured the highest water temperature of a year (Figure 2). It is presumed that ambient temperature is one reason for enhanced activities of AOEs (SOD and CAT) in August during sampling.

We propose that the activation of antioxidant defences in coelomic fluid of *A. japonicus* during aestivation is a defensive mechanism against ROS invasion once the animal arouses. It is an ideal survival strategy with self-modulation against extreme environment (high temperature in summer). Further work is needed to determine the effects of environmental effect, such as temperature grades in the laboratory, on antioxidant defences.

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