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Physiological responses of swimming crab *Portunus trituberculatus* under cold acclimation: Antioxidant defense and heat shock proteins

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ABSTRACT

Cold temperature can lead to large-scale mortality of overwinter cultured swimming crab *Portunus trituberculatus*; however, little is currently known about the physiology of this species under low temperature. The present study was carried out to investigate the effects of cold exposure on physiology of *P. trituberculatus*. Activities of antioxidant enzymes (SOD and CAT), oxidative damage to lipid and protein (indexed by contents of malondialdehyde (MDA) and protein carbonyl (PC), respectively), and expression of heat shock protein genes (*hsp70*, *hsp90*-1 and *hsp90-2*) and a marker of apoptosis, *caspase-3*, in muscle and hepatopancreas tissues of the crabs acclimated at the temperatures of 3, 6, 9, 12, 15 and 22 °C (control) for four weeks were measured. SOD and CAT activities in the crabs increased significantly at 9, 12 and 15 °C, but decreased markedly at 3 and 6 °C, compared with those in control crabs. Accumulation of MDA and PC in both muscle and hepatopancreas tissues was observed when the crabs were acclimated at 3 and 6 °C. Abundance of *hsp70*, *hsp90-1* and *hsp90-2* mRNA increased with decreasing temperature, reached the maximum levels at 6 °C and then decreased sharply at 3 °C. *Caspase-3* expression, in coincidence with MDA and PC contents, increased significantly at 3 and 6 °C. These results suggested that at low temperatures antioxidant defense system and heat shock proteins in swimming crabs are enhanced to prevent cellular damage and can effectively protect cells against apoptosis; however, cold stress beyond a critical level results in depression of the protective mechanisms, which in turn leads to cellular components damage, and elevated levels of apoptotic cell death. Based on the results of this study, overwintering *P. trituberculatus* should be cultured at temperatures above 6 °C.

1. Introduction

The swimming crab *Portunus trituberculatus* (Crustacea: Decapoda: Brachyura) is widely distributed in the estuary and coastal areas of Korea, Japan, China, and Southeast Asia (Dai et al., 1986). This species is dominant in portunid crabs fisheries around the world and supports a large aquaculture industry in China. In 2010, *P. trituberculatus* production in China reached up to 91,050 tons and valued more than AUS$2.5 billion (China Fishery Statistical Yearbook, 2011). Because wild-caught broodstock vary in both quality and quantity and are a common source of pathogen introduction into farming system (Coman et al., 2006; Meng et al., 2009a), many swimming crab farms in China utilize domesticated stocks for hatchery production. The farms select parent crabs at harvest in late autumn and maintain them over winter for seed production in the next year. Cold temperatures in winter often lead to large-scale mortality of the overwintering broodstock. Thus, it is very important to investigate the effects of cold temperature on physiology of *P. trituberculatus*.

Exposure of poikilothermic organisms to thermal stress can result in increases in endogenous reactive oxygen species (ROS), such as superoxide, peroxyl radical and hydroxyl radicals, which leads to imbalance between the production and elimination of ROS and consequently to oxidative stress (An and Choi, 2010; Kong et al., 2007, 2012; Qiu et al., 2011; Vinagre et al., 2012). Oxidative stress can damage critical biological molecules, such as lipid, protein and DNA, and initiate a cascade of events, bringing about impaired cellular function or apoptosis (Green and Reed, 1998; Halliwell, 1999; Stadtman and Levine, 2003). To prevent oxidative stress and keep cellular redox state in balance, aerobic organisms have evolved efficient antioxidant defense system (Yu, 1995), which consists of both nonenzymatic small antioxidant molecules (such as reduced glutathione (GSH), ascorbic acid (AA), carotenoids, etc.) and a cascade of enzymes, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Halliwell, 1999). Recently, the roles of enzymatic antioxidant system in thermal adaptation have received much attention in marine poikilothermic organism, such as molluscs (An and Choi, 2010; Park et al., 2009), crustaceans (Kong et al., 2012; Zhou et al., 2010), echinoderms (Dong et al., 2008; Wang et al., 2013), and fishes (Cui et al., 2013; Vinagre et al., 2012).

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Thermal stress can cause enhanced synthesis of heat shock proteins (Lindquist, 1986; Parsell and Lindquist, 1993). Acting as molecular chaperones, Hsps protect proteins from denaturation, and assist in refolding misfolded proteins and removing irreversibly damaged proteins (Feige et al., 1996; Gething, 1996; Hartl, 1996; Morimoto and Santoro, 1998). Among the different families of Hsps, Hsp70 and Hsp90 play critical roles in protection of cells from stresses and maintenance of cellular homeostasis (Gething and Sambrook, 1992; Morimoto and Santoro, 1998; Parsell and Lindquist, 1993). Hsp70 and Hsp90 are found to be up-regulated under cold stress in previous studies on poikilothermic animals (Ali et al., 2003; Deane and Woo, 2005; Kelley et al., 2013; Urian et al., 2011) and are considered to be critically important for cold survival (Koštál and Tollarová-Borovanská, 2009; Rinehart et al., 2007).

Although cells have diverse protective mechanisms against stresses, an enhancement of stress beyond the ability of cells to cope with may lead to cell signaling disruption, cellular components damage, and ultimately lead to cell death (Chandra et al., 2000; Rauen et al., 1999; Ron and Walter, 2007). Cell death often occurs through an ordered pathway of self-destruction termed apoptosis, which is mediated by the sequential activation of a set of cysteine proteases (Nicholson, 1999). Caspases is a family of cysteine proteases that play essential roles at various stages of the apoptotic cascade. Caspase-3 is one of the key executioners of apoptosis, being responsible for the proteolytic cleavage of many critical cellular proteins (Elmore, 2007; Nicholson, 1999) and has been widely used as biomarker of apoptosis (Abu-Qare and Abou-Donia, 2000; Chiang et al., 2001; Davis et al., 2003). Extensive studies have been focused on apoptosis in marine invertebrates exposed to toxins (Foster et al., 2011; Leaver et al., 2010; Liu et al., 2011) and pathogens (Hughes et al., 2010; Pirarat et al., 2007; Vale et al., 2003). However, little is currently known about thermal stress-induced apoptosis in marine poikilotherms (Chang et al., 2009; Li et al., 2014).

In the present study, we aimed to elucidate the effects of cold temperature acclimation on the physiological responses of *P. trituberculatus*. Specifically, we examined (I) activity of antioxidant enzymes; (II) levels of oxidative damage; (III) expression of *heat shock proteins*; and (IV) expression of *caspase-3*. Results of this study were not only expected to help in understanding physiological basis of adaptation of *P. trituberculatus* to cold temperature but also to provide valuable information to overwintering management of this species.

2. Materials and methods

2.1. Animals and experimental treatments

Adult *P. trituberculatus* with an average weight of 213.84 ± 21.55 g (means ± S.D.) were obtained from Kaihang Farm, Rizhao, China. The water temperature is 21.6 °C when the crabs were collected. After transferred to the laboratory, seventy-two crabs without visible damage were reared at the ambient temperature of 22 °C which is among the optimum temperatures for 10 days. Then the swimming crabs were randomly allocated into seventy-two tanks (one individual per tank), and the water in the tanks were adjusted to the temperatures of 3, 6, 9, 12, 15 and 22 °C (control) (twelve tanks per temperature) gradually with the rate of 2 °C per day and maintained for a period of four weeks. The crabs at different temperatures displayed different behavior: at 3 °C, the swimming crabs entered a state of torpor and ceased feeding; at 6 and 9 °C, the crabs seldom moved, but still can feed; and at 12 and 15 °C, they are active and feed avidly. To investigate the effects of cold acclimation on the physiology of *P. trituberculatus*, after the four-week temperature acclimation, the muscles of swimming legs and hepatopancreas were dissected from the crabs, snap frozen in liquid nitrogen and stored at −80 °C for biochemical assays and gene expression analysis.

During the period of experiment, all the crabs were fed daily at 17:00 with polychaete *Perinereis aibuhitensis*, and the feces and leftover feed were removed prior to feeding. Aeration was provided continuously and the photoperiod was 12 h light:12 h dark. Seawater was filtered using a sand filter, and the salinity was 30–32 psu. One third to one half of the rearing water was exchanged using fresh equi-temperature seawater. Water pH was around 7.6 and ammonia was less than 0.22 mg L^{-1}. Water salinity, pH and ammonia were determined with salinity refractometer (AIAGO, Japan), pH meter (PH3150i, WTW, Germany), and hypobromite methods, respectively.

2.2. Enzyme assays

The muscle and hepatopancreas tissues of eight crabs from each temperature treatment were used for antioxidant enzyme assays, respectively, using commercial diagnostic reagent kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China), according to the manufacturer's instructions. Each sample was homogenized in ice-cold buffer (50 mM Tris–Cl, 1 mM EDTA, 1 mM DTT, 0.5 mM sucrose, 150 mM KCl and 1 mM PMSF, pH 7.8) in a proportion of 0.1 g of tissue to 1 ml of buffer. The homogenate was centrifuged at 12,000 *g* for 15 min at 4 °C and the supernatant was collected, divided into aliquots and stored at −20 °C for enzyme assays. All the assays were completed within one week after preparation. Protein concentration of the tissue extracts was determined using the BCA protein assay reagent (Pierce, IL, USA).

2.2.1. SOD activity

The SOD activity was assayed based on its inhibitory effect on the rate of superoxide anion generating by xanthine and xanthime oxidase reaction system (Oyanagui, 1984). In brief, the tissue extract was mixed with reaction solution containing xanthine, xanthime oxidase, hydroxylamine, anilinesulfonic acid and alpha naphthylamine. Nitrites could be produced during the reaction between superoxide anion and hydroxylamine. The mixture of nitrites, anilinesulfonic acid and alpha naphthylamine would appear purple–red color. After 20 min of incubation at 37 °C, the absorbance at 450 nm of the mixture was measured and converted into activity unit according to the manufacturer's instruction. One unit of SOD was defined as the activity amount that resulted in 50% inhibition of the production of superoxide anion in 200 μl of reaction solution. Results were expressed as specific activity in the unit of U mg^{-1} protein (U/mg prot).

2.2.2. CAT activity

CAT activity was measured according to the ammonium molybdate spectrophotometric method (Góth, 1991). The tissue extract was incubated with hydrogen peroxide for 1 min, then the reaction was terminated by adding ammonium molybdate, and the remaining hydrogen peroxide combined with ammonium molybdate to form a yellow compound. The absorbance was measured spectrophotometrically at 405 nm, where it had its maximum absorbance. One unit of CAT catalytic activity is defined as the amount of enzyme required to decompose of 1 μmol hydrogen peroxide per second and the enzyme activity was expressed as U/mg prot.

2.3. Oxidative damage parameters

Malondialdehyde (MDA), an end product derived from peroxidation of polyunsaturated fatty acid, and protein carbonyls (PC) were measured to assess oxidative damage to lipid and protein. Tissues of eight individuals from each temperature acclimation treatment were used in oxidative damage assays. Protein concentration of the samples was determined with the BCA protein assay reagent (Pierce, IL, USA).