Comparative Biochemistry and Physiology, Part A 164 (2013) 605–611

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Daily variations of the antioxidant defense system of the lithodid crab *Lithodes santolla*

Natasha Schvezov *, Gustavo A. Lovrich, Federico Tapella, M. Carolina Romero

Consejo Nacional de Investigaciones Científicas y Técnicas, CONICET. Centro Austral de Investigaciones Científicas, CADIC, Houssay 200, V9410CAB Ushuaia, Tierra del Fuego, Argentina

ARTICLE INFO

Article history:
Received 12 October 2012
Received in revised form 5 January 2013
Accepted 21 January 2013
Available online 30 January 2013

Keywords:
Beagle channel
Sub-Antarctic crustaceans
Reactive oxygen species
Enzyme activities
Oxyradicals
Daily variations

ABSTRACT

Several physiological processes can induce daily variations in aerobic metabolism. *Lithodes santolla* is a decapod crustacean of special concern since it is a sub-Antarctic species of commercial interest. The aim of this work was to study in *L. santolla* the daily variations in levels of enzymatic and non-enzymatic antioxidants, lipid peroxidation and protein oxidation, and haemolymphatic pH. Males of *L. santolla* of commercial size were randomly dissected every 4 h during a period of 24 h. Enzymatic activities of superoxide dismutase (SOD), catalase (CAT), glutathione-*S*-transferase and glutathione peroxidase were determined in samples of gills, muscle, hepatopancreas and haemolymph. Ascorbic acid, total glutathione, lipid peroxidation and protein oxidation were also determined in all tissues. Gills showed the highest enzymatic activity and hepatopancreas the highest concentration of non-enzymatic antioxidants. Maximum antioxidant activity was during the dark phase in gills and during the photophase in the haemolymph. Muscle showed significant daily variations, with peaks during the photophase and scotophase. Overall, an antioxidant protective mechanism is present in all tissues, where SOD and CAT represent the first line of defense. The defense mechanism in *L. santolla* seems to be more active during the dark phase, with slight differences among the analyzed tissues, indicating a higher metabolic rate.

1. Introduction

There are several physiological processes that induce daily variations in aerobic metabolism. Physiological parameters like oxygen consumption and locomotor activity vary rhythmically along the day/night cycle, and imply alterations in aerobic metabolism (Maciel et al., 2010). Of special importance in crustacean species are photoperiodically controlled phenomena in locomotor activity, moulting, and reproduction including the release of eggs or dispersal of larvae, not at last interesting for fisheries in terms of predictions of catches (Strauss and Dircksen, 2010). In the crab *Neohelice granulata* oxygen consumption was augmented at night in laboratory conditions, which coincided with the circadian rhythm of the locomotor activity reported for this species (Pereyra et al., 1996; Maciel et al., 2004).

Alteration in aerobic metabolism produces changes in radical oxygen species (ROS) production (Maciel et al., 2004) which affects macromolecules such as proteins, carbohydrates, nucleic acids and lipids (Kong et al., 2008). Organisms have developed mechanisms to defend against these ROS constituted by enzymatic and non-enzymatic molecules. Among the antioxidant enzymes are superoxide dismutase (SOD) that converts the superoxide anion (O^{2-}) to hydrogen peroxide (H_2O_2), catalase (CAT) that converts H_2O_2 to water, glutathione peroxidase (GPx) that reduces lipid hydroperoxides to their corresponding alcohols and free H_2O_2 to water, and glutathione-S-transferase (GST) that is involved in the biotransformation of numerous xenobiotic compounds. Furthermore, low molecular weight antioxidants such as reduced glutathione and vitamins C and E, act together with these enzymatic defenses (Halliwell and Gutteridge, 1999; Hermes-Lima, 2004).

Studies on daily changes in the antioxidant system have been done in vertebrate and invertebrate species. CAT activity in mouse has been studied, and showed the existence of time-related changes along a 24-h period (Sani et al., 2006). In rats daily variations in lipid peroxidation levels were correlated with daily variations in aerobic metabolism (Baydas et al., 2002). In contrast, Fanjul-Moles et al. (2003, 2009) who studied the glutathione system of the crayfish *Procambarus clarki*, demonstrated daily variations in the oxidative status of the animal. The involvement of rhythmicity in free radical formation, detoxification and, perhaps, also radical generation avoidance, in fact, suggests a particular adaptive value for programmed, anticipatory temporal organization (Hardeland et al., 2003).

Lithodes santolla, commonly known as Southern king crab, is a decapod crustacean of special concern since it is a sub-Antarctic species with a commercial interest. The fishery of *L. santolla* has developed south to 40°S since the 1930s, especially in the Beagle Channel and Straits of Magellan. As for the last few years, total annual landings for the Argentinean and Chilean fisheries have totaled ca. 64,000 t.

Abbreviations: SOD, Superoxide dismutase; CAT, Catalase; GPx, Glutathione peroxidase; GST, Glutathione-S-transferase; AA, Ascorbic acid; TG, Total glutathione; LPO, Lipid peroxidation; PO, Protein oxidation; ROS, Reactive Oxygen Species; HL, Haemolymph; Mc, Muscle; HP, Hepatopancreas.

* Corresponding author at: Bernardo Houssay 200, V9410CAB Ushuaia – Tierra del Fuego, Argentina. Tel.: +54 2901 422310, +54 2901 422314, +54 2901 422278x130; fax: +54 2901 430644.
E-mail address: nschvezov@cadic-conicet.gob.ar (N. Schvezov).

1095-6433/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cbpa.2013.01.013

Regulations impose that only males above 110 mm of carapace length can be retained and commercialized. To our knowledge, studies on daily variations of the antioxidant defense system have been done previously in crustaceans, only in species of Cambariidae (Fanjul-Moles and Prieto-Sagredo, 2003) and in the estuarine crab *N. granulata* (Maciel et al., 2004). Even though several studies have been done in different aspects of *L. santolla* (*e.g.* Vinuesa et al., 1990; Lovrich and Vinuesa, 1999; Lovrich et al., 2003; Thatje et al., 2003; Anger et al., 2004; Calcagno et al., 2005), only a few of them were focused on its physiological aspect (Comoglio and Amin, 2008; Paschke et al., 2009). Rhythmicity as an ecophysiological adaptation involves almost every facet of the crustacean biology and comprises developmental, physiological, sensory, and behavioural aspects (Strauss and Dircksen, 2010) that could eventually affect the biology. Commercial animals suffer different physiological changes due to air exposure or handling that could change the flesh quality (c.f. Romero et al., 2011). This article is part of a borader study on dry transportation of commercial crabs.

The aim of the present work was to analyze in *L. santolla* the daily variations in *i*) activities of the enzymes: superoxide dismutase (SOD); catalase (CAT); glutathione peroxidase (GPx); and glutathione-S-transferase (GST); *ii*) concentration of non-enzymatic antioxidant molecules (ascorbic acid and glutathione); *iii*) concentration of oxidative damage parameters (lipid peroxidation and protein oxidation); and *iv*) haemolymphatic pH. Results of this study will help in understanding the physiological and biochemical basis of *L. santolla*, as well as providing additional contribution to the field of oxidative stress in decapod crustaceans.

2. Material and methods

2.1. Acclimation and assay condition

Male *L. santolla* were captured in Beagle Channel (ca. 55ºS; 68ºW) in August 2010 by baited commercial traps and transported to the laboratory for 3 weeks of acclimation. Animals were sorted into 6 groups of 8 animals each and kept in tanks. Animals were fed with fresh squid mantle and they were maintained in aquaria with filtered sea water at 6 ± 0.5 °C, pH 8 and under 12:12 h light cycles, with the onset of light at 8:00 h. Selected photoperiodic conditions resembled the natural period of L/D hours in the Beagle Channel during late August, and used as a standard throughout the literature. All crabs were in intermoult stage and of legal size (119 ± 5 mm of carapace length). One group of animals was dissected randomly every 4 h during a 24 h cycle.

Before dissection, samples of haemolymph were withdrawn from the ventral sinus via the arthrodial membrane at the base of the 3rd or 4th pair of pereiopods using 10 mL disposable plastic syringes. Two subsamples of 1 mL were transferred to pre-chilled 1.5 mL centrifuge tubes that contained 500 μL of ice cold Tris–HCl buffer (pH 6.8, 125 mm Tris, 1 mM 2-mercaptoethanol, and 0.1 mm PMSF) as was described by Vijavayel et al. (2004), to avoid haemolymph clotting. The rest of haemolymph sample was used to analyze haemolymph pH in situ (THERMO pHMETER Model ALFA5, Fields Instrumental). After haemolymph sampling, the 7th gill, hepatopancreas and the muscular mass from the 4th pair of pereiopods were dissected and frozen at −80 °C. The chocie of the 7th gill and the 4th pair of pereiopods were selected to standardize the protocol and to make results more comparable.

2.2. Sample preparation

The homogenates were prepared using 0.3 g of gills or muscle or 0.15 g of hepatopancreas tissue in 1.5 mL of cold (4 °C) Tris–HCl buffer (0.125 M, pH 6.8). Samples were homogenized between 0 and 4 °C, and then they were centrifuged at 11,000 *g* at 4 °C for 15 min. The supernatants were collected and employed as antioxidant and oxidative damage source.

The enzymatic activity of superoxide dismutase (EC 1.15.1.1; SOD), catalase (EC 1.11.1.6; CAT), glutathione peroxidase (EC 1.11.1.9; GPx) and glutathione-S-transferase (EC 2.5.1.18; GST) and the antioxidants ascorbate (AA) and total glutathione (TG) were determined in each sample. Additionally, lipid peroxidation (LPO) and protein oxidation (PO) were determined as indicators of oxidative stress. All enzymatic analyses were expressed in relation to total proteins in the sample, and non-enzymatic analyses in relation to wet tissue weight or mL haemolymph.

2.3. Apparatus and reagents

All analyses were performed in a GBC Cintra 10e Spectrophotometer. Enzymes and compounds used to determine enzymatic activities were from Sigma, solvents and acids used were from Cicarelli and reagents used for buffers were from Anedra. All reagents were pro-analysis quality.

2.4. Antioxidant enzymes assays

The activity of CAT was determined according to the decrease in the concentration of hydrogen peroxide at 240 nm (Aebi, 1984). The reaction mixture contained 30 mM H_2O_2 and 50 mM phosphate buffer (pH 7.0). The activity of SOD was quantified measuring the inhibition of the reaction of the superoxide anion (O_2^-) with cytochrome c, which forms reduced cytochrome c and has a maximum of absorbance at 550 nm (McCord and Fridovich, 1969). The superoxide anion is generated by 50 μM xanthine and 6 nM xanthine oxidase, and reacts with 20 μM cytochrome c in a phosphate buffer (50 mM, pH 7.8, 0.1 mM EDTA). GST activity was measured by monitoring the rate of conjugation of glutathione (GSH) to 1-chloro-2.4-dinitrobenzene (CDNB) at 340 nm (Habig et al., 1974). The reaction mixture contained 0.1 M phosphate buffer (pH 6.5), 1 mM GSH and 1 mM CNDB. GPx activity was determined measuring the velocity by which 0.2 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) diminishes in a system containing 1 mM reduced glutathione (GSH), in the presence of glutathione reductase. The determination was done in a buffer containing 2 mM EDTA, 1 mM azide and 50 mM Tris–HCl, pH 7.6. The lectures were performed at 340 nm ($\varepsilon_{340} = 6{,}23\ mM^{-1}\ cm^{-1}$, Flohé and Gunzler, 1984). GPx activity in haemolymph and hepatopancreas could not be measured due to loss of sample. Enzymatic activities of GST, GPx and CAT were expressed as U mg protein^{-1}, where 1 U is equivalent to 1 μmol of substrate consumed per minute at 25 °C. One unit of SOD (USOD) was defined as the amount of enzyme inhibiting the oxidation of product by 50% at 30 °C.

2.5. Non-enzymatic antioxidant assays

The determination of total glutathione (TG) was based on the reaction generated by 5,5′-dithiobis-2 nitrobenzoic acid (DTNB), which resulted on oxidized glutathione (GSSG) and 2-nitrobenzoate (TNB), this last one having an absorbance at 412 nm. Previously, samples were deproteinized with perchloric acid 2 M. Results are expressed in nmol GSH · mg wet tissue^{-1} (Beutler et al., 1963). In hepatopancreas and haemolymph TG could not be analyzed because measurements were non detectable with the used technique.

Ascorbic acid (AA) was determined according to the method of Mitsui and Ohta (1961). Samples were deproteinized with TCA 5%, and mixed with sodium molybdate 0.66% p/V, sulfuric acid 25 mM and sodium phosphate 0.025 mM. This mixture was heated for 40 min at 60 °C, then centrifuged at 8000 *g* for 5 min, and measured at 660 nm. Ascorbic acid was used for the calibration curve, and data is presented as ng AA · g wet tissue^{-1}.