Bull Environ Contam Toxicol (2008) 81:242–244
DOI 10.1007/s00128-008-9487-5

Activity of Glutathione *S*-Transferase in the Hepatopancreas is not Influenced by the Molting Cycle in the Fiddler Crab, *Uca pugilator*

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Received: 27 November 2007 / Accepted: 4 June 2008 / Published online: 28 June 2008

Abstract Glutathione *S*-transferase (GST) in the hepatopancreas of crustaceans has been suggested as a biomarker for organic pollution. However, much of crustacean physiology is known to exhibit a cyclic characteristic because of the periodic shedding of the confining exoskeleton. The goal of this study was to determine whether hepatopancreatic GST activity varies during the molting cycle using the fiddler crab, *Uca pugilator*, as the model. Neither the molting cycle nor 20-hydroxyecdysone injection had a significant effect on hepatopancreatic GST activity, suggesting GST activity is not under control of the molting hormone in *Uca pugilator*.

Keywords Glutathione *S*-transferase · Molting cycle · Crustacean · Molting hormone

Glutathione *S*-transferase (GST) is a metabolically important phase II enzyme known for its role in the removal of xenobiotics. It conjugates potentially harmful electrophilic substances with endogenous reduced glutathione to protect other nucleophilic molecules, such as proteins and nucleic acids. Hydrophobic pollutants, such as organochlorine compounds and polycyclic aromatic hydrocarbons, can readily accumulate in the tissues of crustaceans (Ishizuka et al. 1998; Menone et al. 2000; Eickhoff et al. 2003). GST activity in the hepatopancreas of crustaceans has been found to be correlated with pollutant accumulation and inducible by organic pollutants. Ishizuka et al. (1998) reported the correlation between GST activity and the accumulation of organochlorines in the hepatopancreas of the freshwater crab, *Eriocheir japonicus*. Exposure to cypermethrin (Gowland et al. 2002), oil effluent (Arun et al. 2006), lindane and permethrin (McLoughlin et al. 2000) has been found to significantly induce GST activity in crustaceans. The correlation between enzymatic activity and contaminant accumulation and the induction of GST activity by organic contaminants have led to the suggestion of GST activity in the hepatopancreas of crustaceans as a biomarker for organic pollution (Ishizuka et al. 1998; Gowland et al. 2002; Arun et al. 2006).

Crustaceans are a group of animals with a rigid, confining exoskeleton. In order to grow, these animals must periodically shed their exoskeletons, a process known as ecdysis or molting. Because of periodic molting, much of crustacean physiology takes on a cyclic characteristic. For instance, Zou and Fingerman (1999a) found that chitobiase activity in the hepatopancreas of *Uca pugilator* varies during the molting cycle, with the peak enzymatic activity occurring in Premolt Stage D_{3-4}. The activities of digestive enzymes in the hepatopancreas of *Callinectes arcuatus* (Vega-Villasante et al. 1999) and *Artemesia longinaris* (Fernandez Gimenez et al. 2002) have been found to be influenced by the molting cycle. It has been reported that ATPase activity in the hepatopancreas of *Marsupenaeus japonicus* modulates during the different stages of the molting cycle (Zilli et al. 2003). In view of the cyclic nature of much of crustacean physiology, a question must be raised: does GST activity in crustacean hepatopancreas change with the molt stages? A result in which GST activity is found to fluctuate during the molting cycle owing to the influence of the molting hormone would call into question the use of crustacean GST activity as a biomarker endpoint if molt stages are not discriminated. In order to validate the use of GST activity in the hepatopancreas of crustaceans as a biomarker

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for organic pollution, this study was undertaken to address the above question, using the fiddler crab, *Uca pugilator*, as the model. We placed our emphasis on two lines of evidence, the variation of hepatopancreatic GST activity during the molting cycle and the induction of enzymatic activity by the molting hormone 20-hydroxyecdysone.

Materials and Methods

Female fiddler crabs, *Uca pugilator*, were purchased from the Gulf Specimen Marine Laboratories (Panacea, FL). Upon arrival crabs were distributed into tanks containing artificial seawater made with Instant Ocean synthetic sea salt (Aquarium Systems, Mentor, OH). The animals were fed uncooked oatmeal once a week and maintained under the natural light regime of approximately 14 h light/10 h dark at a temperature of 19–21°C. The animals were allowed to acclimate to laboratory conditions at least 5 days before use in an experiment. Molt staging was determined according to the extent of epidermal retraction in pleopods as well as morphological characteristics of the pleopod setae using the system of Drach (1939) as adapted to *Uca pugilator* by Vigh and Fingerman (1985). This setogenic staging technique can distinguish female *Uca pugilator* in Postmolt Stage A–B, Intermolt Stage C, and Premolt Stages D_0, D_1 and $D_{3–4}$. Because of cannibalism in crab tanks, it was very difficult to obtain the soft-shelled, postmolt crabs. Only crabs representing Intermolt Stage C and Premolt Stages D_0, D_1 and $D_{3–4}$ were used in the experiment for alterations in GST activity during the molting cycle. Hepatopancreatic tissue of each crab was dissected out, snap-frozen with liquid nitrogen and stored at −80°C until enzymatic analysis.

Hepatopancreatic tissue was homogenized on ice in a buffer containing 10 mM pH 7.4 PBS, 1 mM EDTA, 0.15 mg/mL DTT, 1 mg/mL trypsin inhibitor, and 0.1 mM phenanthroline. After centrifugation at 10,000*g* for 20 min at 4°C, the supernatant was collected and kept on ice. Protein concentration in the supernatant was determined according to the method of Bradford (1976). GST activity assay essentially followed the protocols provided by the kit supplier (Sigma, St. Louis, MO) with slight modifications. The reaction mixture was prepared by mixing 980 μL of 10 mM pH 7.4 PBS, 10 μL of 200 mM reduced glutathione, 10 μL of 100 mM 1-chloro-2,4-dinitrobenzene and 40 μg of protein. The absorbance was measured at 340 nm and at a temperature of 21°C. The increase in absorbance was directly proportional to the GST activity, and the change in absorbance per min was calculated for the blank and the sample. Enzymatic activity was reported as nmol/min/μg protein.

To study the effect of injection of the exogenous molting hormone on hepatopancreatic GST activity, a total of 80 female intermolt crabs were used. These crabs were divided into two groups, control and hormone-treatment groups. The molting hormone 20-hydroxyecdsyone was first dissolved in ethanol at a concentration of 10 mg/mL. One aliquot of this stock solution was mixed with nine aliquots of Pantin's crustacean saline to produce a final injection solution of 1 mg/mL 20-hydroxyecdysone and 10% v/v ethanol. One group of 48 crabs received an injection of 20-hydroxyecdsyone at a dose of 25 μg/g live mass through the arthrodial membrane at the base of the walking leg on day 0. Thirty-two control crabs were injected with an appropriate amount of 10% v/v ethanol in Pantin's crustacean saline on day 0. On day 2 all survivors were sacrificed. Hepatopancreatic tissue of each crab was removed, snap-frozen with liquid nitrogen and stored at −80°C until analysis of GST activity. Enzymatic assay was carried out as described above.

One-way analysis of variance (ANOVA) (SAS 9.1) was used to determine whether GST activity in the hepatopancreas varies significantly with different molt stages, while Student's *t*-test was employed to determine whether treatment with the exogenous molting hormone induced hepatopancreatic GST activity. A probability value of less than 0.05 was considered significant.

Results and Discussion

The results of the present investigation show that the molting cycle did not have a significant effect on GST activity in the hepatopancreas of *Uca pugilator* ($p = 0.987$, Fig. 1). In crustaceans a cyclic variation in enzymatic activity during the molting cycle is usually an indication that the activity of this enzyme is under control of the molting hormone; or in other words, this enzyme is a product of the gene regulated by the molting hormone. For instance, Zou and Fingerman (1999a) found that chitobiase activity in the epidermis and hepatopancreas of *Uca pugilator* varies during the molting cycle. In a follow-up study these investigators demonstrated that injection of 20-hydroxyecdysone at 25 μg/g live mass, a dose also used in the present study, resulted in a significant increase in epidermal chitobiase

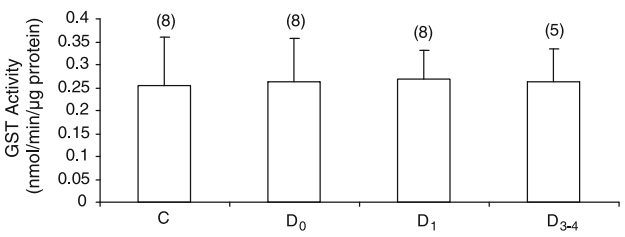

Fig. 1 Glutathione *S*-transferase (GST) activity in the hepatopancreas of *Uca pugilator* during the molting cycle. Sample size is shown in brackets. Error bars represent standard deviation

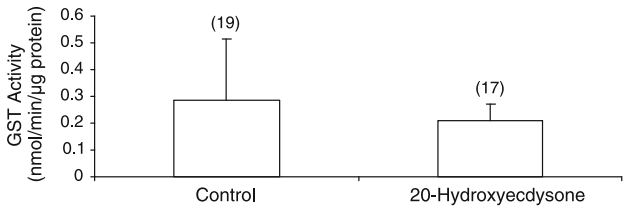

Fig. 2 Effect of 20-hydroxyecdysone injection on glutathione *S*-transferase (GST) activity in the hepatopancreas of *Uca pugilator*. Sample size is shown in brackets. Error bars represent standard deviation

activity and a trend of induction of enzymatic activity in the hepatopancreas of the fiddler crab (Zou and Fingerman 1999b). The invariant characteristic of GST activity during the molting cycle suggests that GST activity in the hepatopancreas is not regulated by the molting hormone in *Uca pugilator*. This is further corroborated by the absence of a significant change in hepatopancreatic GST activity following 20-hydroxyecdysone administration ($p = 0.162$, Fig. 2). The consistency of GST activity during the molting cycle, along with the absence of induction of GST activity by the molting hormone, unequivocally suggests that hepatopancreatic GST activity is not under control of the molting hormone in *Uca pugilator*. The implication of the findings described herein is that there is no need to distinguish different molt stages when it comes to GST activity in the hepatopancreas of crustaceans being used as a biomarker for organic pollution. Therefore, our results bring validity to the use of GST activity in the hepatopancreas of crustaceans as a biomarker for organic pollution.

Under the hypothesis that cyclic changes in GST could influence an animal's susceptibility to a toxicant with GST upregulation providing increased protection and a decrease in GST activity increasing an animal's susceptibility, there has been a concern that the sensitivity of an exposed crustacean could change during the molting cycle. Our findings that GST activity is neither a function of the molting cycle nor influenced by the molting hormone do not support such a concern.

Although the soft-shelled, postmolt crabs were not included in the present investigation, it is unlikely that GST activity of the hepatopancreas of postmolt crabs is much different from that for crabs at another molt stage considering the fact that hepatopancreatic GST activity is apparently not influenced by the molting hormone. After all, hard-shelled, intermolt and premolt crustaceans are most likely to be used in practice.

Additionally, hepatopancreatic GST activity not being inducible by the molting hormone is an indication that GST is not involved in ecdysteroid metabolism in the fiddler crab. Or in other words, glutathione conjugation is not a part of the mechanism for ecdysteroid elimination in *Uca pugilator*.

Acknowledgments Appreciation is expressed to Ms. Yanling Meng for her technical assistance. This investigation was supported by grant LEQSF (2005-08)-RD-A-26 from the Louisiana Board of Regents. We wish to thank the anonymous reviewers for suggestions that lead to the improvement of this manuscript.

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