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# Modulation of Expression of Oxidative Stress Genes of the Intertidal Copepod *Tigriopus japonicus* after Exposure to Environmental Chemicals

Jae-Seong LEE and Sheikh RAISUDDIN

*Department of Chemistry, College of Natural Sciences, Hanyang University, Seoul 133-791, South Korea*

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**Abstract**—The intertidal copepod *Tigriopus japonicus* has been recognized as a potential model species for toxicity testing of marine pollutants. In last five years or so sequence information and expression pattern of several genes critical in detoxification, stress, growth and reproduction have been described from *T. japonicus*. A number of genes, especially those representing antioxidant and detoxification pathways, have potential application as biomarkers in biomonitoring and risk assessment. Many of the environmental chemicals such as trace metals induce oxidative stress. Therefore, biomarkers of oxidative stress have been used in risk assessment and biomonitoring. The expression of over a dozen of genes encoding for antioxidant enzymes have been studied in *T. japonicus* exposed to prooxidant such hydrogen peroxide, redox cycling-inducing trace metals and endocrine disrupting chemicals (EDCs). Finally, we identified GST-sigma (*GSTS*) as a potential biomarker gene of oxidative stress. Most of the metals caused upregulation of *GSTS*. Therefore, *GSTS* appears to be a potential biomarker of trace metal exposure in *T. japonicus*. Currently, we are standardizing a 12k oligochip for *T. japonicus* for study of gene expression profile after exposure to trace metals and EDCs. *T. japonicus* has already been shown to be a good model for acute toxicity and two generation toxicity testing. Using this microchip we intend to test it for trace metals and endocrine disrupting chemicals (EDCs) and finally use it in the field applications. Additionally, our recent research is also focused on study of mechanism of action of toxic chemicals at molecular level. We have successfully demonstrated utility of *T. japonicus* in study of chemically-induced cytotoxicity and apoptosis. This brief review highlights the significance of *T. japonicus* in marine pollution monitoring and risk assessment.

Keywords: *Tigriopus japonicus*, trace metals, endocrine disrupting chemicals, oxidative stress, antioxidant enzymes, biomonitoring, risk assessment, oligochip

## INTRODUCTION

Use of invertebrates, especially those belonging to molluska and crustaceans is well established in aquatic pollution biomonitoring (Vindimian, 2001; Hutchinson, 2002; Porte *et al.*, 2006; Viarengo *et al.*, 2007; Zhou *et al.*, 2008). In recent years, emphasis has been given to develop and standardize newer invertebrate species, which could meet the regional regulatory requirement of environmental toxicology. In Europe some concerted efforts have been made under REACH (Registration, Evaluation, Authorisation and Restriction of Chemical substances) program. REACH deals with the regulation of chemical substances in the EU. The new law entered into force on 1 June 2007 (Angerer *et al.*, 2008; Fjodorova *et al.*, 2008). The aim of REACH is to improve the protection of human health and the environment through the better and earlier identification of the intrinsic properties of chemical substances. At the same time, innovative capability and competitiveness of the EU chemical industry has been enhanced. Therefore, it is expected that benefits of the REACH system will come gradually, as more and more substances are phased into REACH. Thus, Europe has a system in place as far as regulatory ecotoxicology is concerned. The REACH Regulation gives greater responsibility to industry to manage the risks from chemicals they produce and to provide safety information on the substances to public and policy makers. Manufacturers and importers will be required to gather information on the properties of their chemical substances, which will allow their safe handling, and to register the information in a central database run by the European Chemicals Agency (ECHA) in Helsinki. The REACH program has thus enabled a partnership between research community and industry. Unfortunately, there is lack of such system in Asia. Such an approach may be rewarding, especially to the countries who are basically heavily industrialized and there economy as well as ecology are chemical oriented, *viz.*, China, Hong Kong, Korea, Japan, Singapore and Taiwan. In this direction development of standard invertebrate test species prevalent in this region may play a pivotal role in pollution monitoring and risk assessment.

Most of the invertebrate ecotoxicological tests have focused on freshwater and in this regard *Daphnia* spp. has played dominant role (Iguchi *et al.*, 2006, 2007; Tatarazako and Oda, 2007). Therefore, it appears that there is general biased for freshwater moHutchinsonHutchinsondel. Many of these models can not be employed in marine ecotoxicological assessment. Marine ecosystems (estuarine, open water and sediments) are at the receiving end of chemical loading and newer chemical entities such as pharmaceuticals and nanoparticles are finding their way into the marine ecosystems (Choong *et al.*, 2006; Moore, 2006; Kim *et al.*, 2007; Cooper *et al.*, 2008; Handy *et al.*, 2008). These chemicals are likely to impact the biota of the ecosystems.

Then the question arises that which species should be ideal for ecotoxicity testing of marine pollutants. Recently, many reviews highlighted importance of copepods in ecotoxicity testing and risk assessment (Kusk and Wollenberger, 2007; Raisuddin *et al.*, 2007). The Organization for Economic Co-operation and Development (OECD) has taken up initiative in this direction. OECD highlighted the following copepod species for further development and standardization: *Acartia tonsa*, *Amphiascus tenuiremis*, *Eurytemora affinis*, *Nitocra spinipes*, *Tisbe battagliai* and *Tigriopus japonicus* (OECD, 2006). *A. tenuiremis* is also a favorite species in the USA. A standardized microplate-based full life-cycle test for *A. tenuiremis* (ASTM E-2317-04) has also been developed.

In invertebrate ecotoxicology focus is on the measurement of endpoints (mostly lethality). This generally suffices the regulatory requirements. However, some molecular events of toxicity have been studied in *Daphnia* spp. (De Coen and Janssen, 2003; Barata *et al.*, 2005; Eads *et al.*, 2007, 2008; Poynton *et al.*, 2007; Shaw *et al.*, 2007; Connon *et al.*, 2008). Microarray for *Daphnia* has also been developed recently and tested against some environmental chemical exposures. Again use of *Daphnia* is restricted to freshwater pollutants. Therefore, a need has been felt to develop and standardize organism(s) for marine ecotoxicity testing and risk assessment. There is also now a general consensus on developing test organisms for ecotoxicity testing pertaining to specific region such as mentioned above, for example, Western Pacific rim. In this regard a species that is widely distributed in a geographical region of interest may be highly useful.

*Tigriopus japonicus*, a harpacticoid intertidal copepod has been recognized as a potential model species for invertebrate marine ecotoxicity in Western Pacific (Raisuddin *et al.*, 2007). Besides *T. japonicus*, one more species of genus *Tigriopus*, *T. brevicornis* has been used in toxicities studies in Europe (Forget *et al.*, 1998, 2003; Barka *et al.*, 2001; Barka, 2007). On the other hand, *T. californicus* has mainly been studied for population genomics in the USA (Edmonds, 2001, 2008; Burton *et al.*, 2005, 2006; Rawson and Burton 2006; Ellison and Burton, 2008). Although most of the studies are confined to laboratory, *T. japonicus* has a good volume of data on acute toxicity ranges, transgenerational toxicity, gene sequences and expression of genes in exposed individuals (Lee, K.-W. *et al.*, 2007, 2008a; Raisuddin *et al.*, 2007). Laboratories in Korea and Hong Kong have shown its particular sensitivity to toxic trace metals such as arsenic, cadmium, copper, mercury, silver and zinc (Kwok and Leung, 2005; Lee, K.-W. *et al.*, 2007, 2008a; Kwok *et al.*, 2008). Many of these trace metals and endocrine-disrupting chemicals (EDCs) induce oxidative stress. Therefore, we focused our research on cloning and sequence analysis of genes involved in oxidative stress and antioxidant defense in order to study the mechanism of action of marine toxicants and also to assess the biomarker potential of those identified genes. In this paper we will mainly focus on the molecular biology of genes involved in oxidative stress and antioxidant defense and their expression in response to exposure to toxic chemicals.

## USEFUL ATTRIBUTES OF *T. JAPONICUS*

*T. japonicus* fulfils most of the requirements of a model test species. It is a small organism (~1 mm) with sexually dimorphic, high fecundity, short reproduction time (10–14 days) and quite hardy (can survive broad ranges of temperature, salinity, pH) characteristics (Raisuddin *et al.*, 2007) (Fig. 1). In addition, its sampling from rocky pool is easy as no specific sampling device is needed. Laboratory observations are simple as it has a distinctly pigmented body. Our experience in using this species in acute toxicity, transgenerational toxicity and gene expression demonstrated a consistent response.

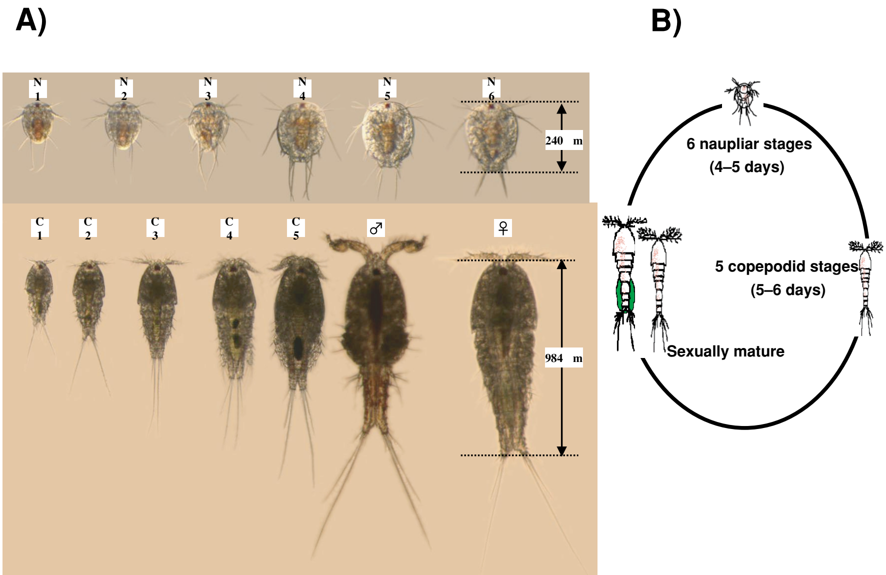


Fig. 1. A) Different nauplii, copepodid and adult stages of *Tigriopus japonicus*. Note the dimorphic characteristics of adult copepods (from Seo *et al*., 2006), B) Life cycle of *T. japonicus*.

## OXIDATIVE STRESS AND ANTIOXIDANT GENES FROM *T. JAPONICUS*

Oxidative stress and antioxidant defense biomarkers have been considered as surrogate indicators of pollution status of habitats (Pandey *et al*., 2003; Valavanidis *et al*., 2006; Vlahogianni *et al*., 2007). In this regard pioneering work in invertebrates was done on mollusks (Viarengo *et al*., 2007). Recently, these biomarkers have been studied in several other organisms and even they are also one of major target biomarkers in microarray-based studies (Shaw *et al*., 2007). In this regard, glutathione-cycle enzymes such as glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione *S*-transferase (GST) genes have been the main focus of attention. A large number of genes having important roles in oxidative stress and antioxidant defense have been identified from *T. japonicus* (Lee, Y.-M. *et al*., 2007; Raisuddin *et al*., 2007). In certain cases the recombinant protein has been made and biochemical characterization achieved. In few cases antioxidant activity of recombinant protein have been tested using transformed *Escherichia coli*. Seo *et al*. (2006) characterized cDNA sequence of GR from *T. japonicus*. Recombinant GR was purified and characterized and finally gene expression was studied in response to hydrogen peroxide and trace heavy metals (Cu and Mn). Both the metals showed upregulation of GR gene up to 48 h. However, at 96 h downregulation was observed. Besides, GR expression was significantly increased with moderately high salinity stress (24 and 40 ppt). In the case of low salt stress (0 and 12 ppt) the expression was found to be down-

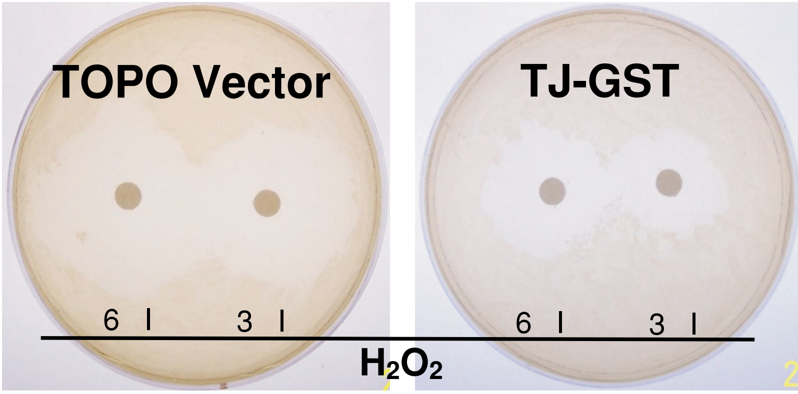


Fig. 2. Demonstration of in vitro antioxidant activity of GST-Sigma (*GSTS*) of *T. japonicus* in transformed *E. coli* (from Lee, Y.-M. *et al*., 2007).

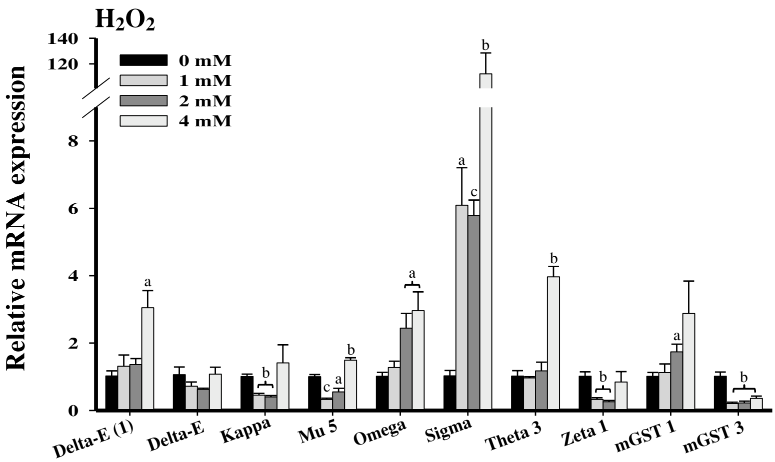


Fig. 3. Expression of profile of mRNA of various GST isoforms of *T. japonicus* exposed to hydrogen peroxide (from Lee *et al*., 2008b).

regulated.

Similarly, Lee *et al*. (2006) reported on a cDNA sequence of GST gene and studied its expression in EDC-exposed *T. japonicus*. The two EDCs tested were 4,4′-octylphenol (OP) and polychlorinated biphenyl (PCB). Both the compounds showed a differential response; while OP caused upregulation of *GST* gene PCB caused downregulation. This may be due to the fact that both compounds belong to different class. Recently, Lee, Y.-M. *et al*. (2007) characterized a Sigma class GST (*GSTS*) gene from *T. japonicus* and studied its expression in response to

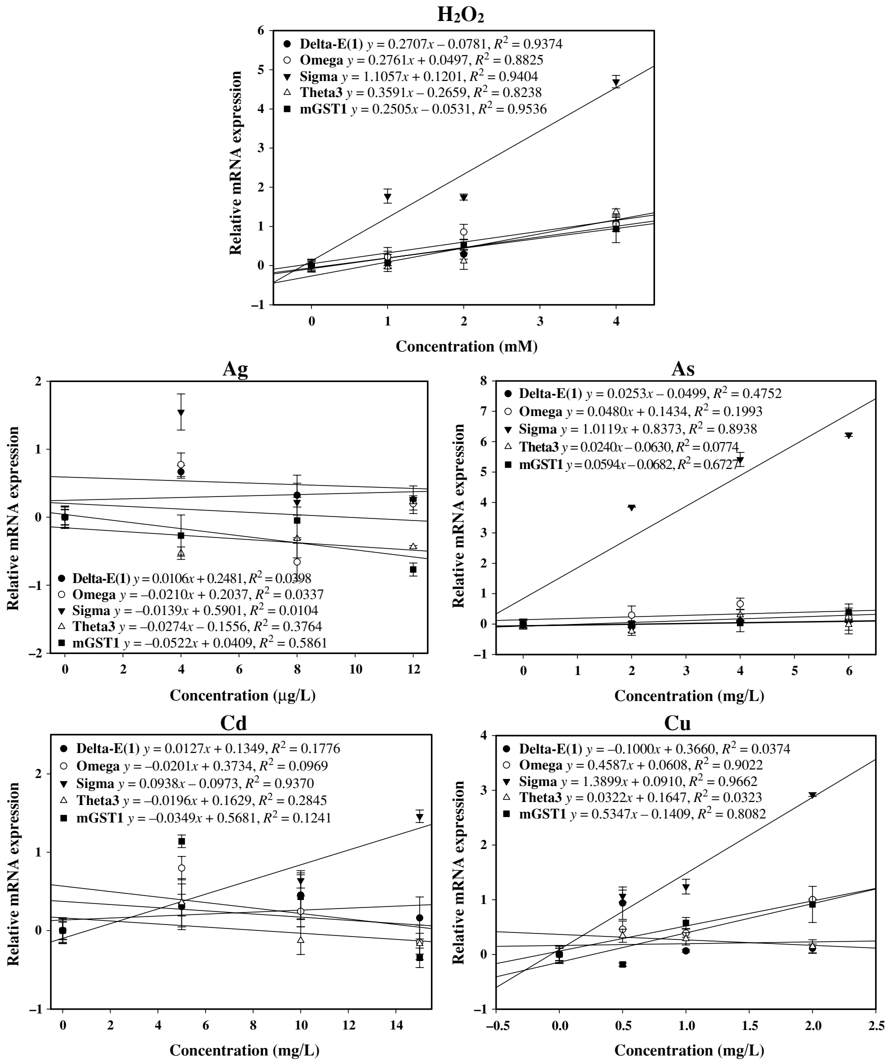


Fig. 4. Relative expression of selected *GSTS* in response to exposure to hydrogen peroxide and trace metals. Vertical lines indicate ±standard error of the mean. Note high correlation for *GSTS* (from Lee *et al.*, 2008b).

exposure to two oxidative stresses-inducing agents, *viz.*, hydrogen peroxide and heavy metals (Cu and Mn). $H_2O_2$ down-regulated *GSTS* expression at the initial stage. However, there was recovery and up-regulation shortly afterwards. Trace metal exposure caused a concentration-dependent increase in *GSTS* expression up to 24 h. Using plate assay test, transformed *E. coli* with GST showed higher

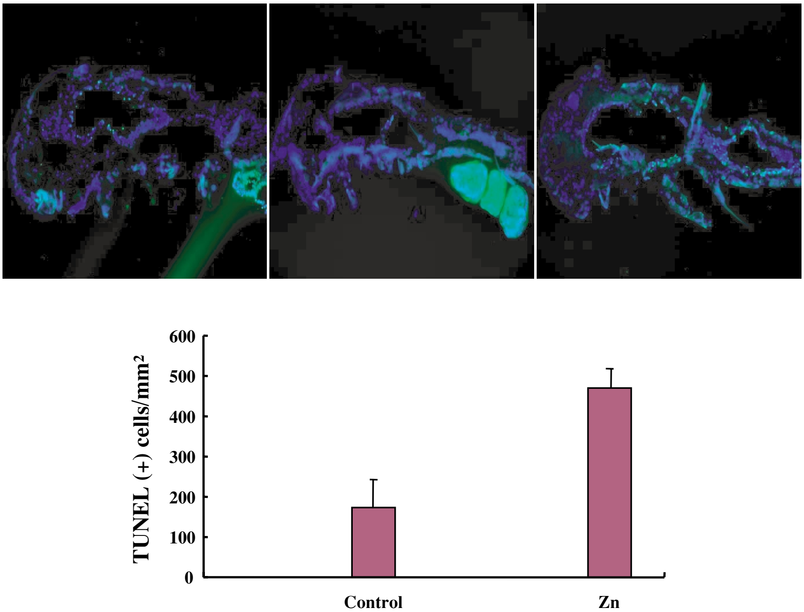


Fig. 5. Demonstration of TUNEL in zinc exposed *T. japonicus* (unpublished data).

survival under $H_2O_2$ exposure than the control bacteria (Fig. 2). This study demonstrated an antioxidant role for *GSTS* in *T. japonicus*.

## IDENTIFICATION OF POTENTIAL ANTIOXIDANT BIOMARKER GENE

With the use of newer technological development such as Gene Sequencer 20 (GS20) we identified a large of expressed sequence tags (ESTs) from *T. japonicus*. Later, we used this strategy to identify potential gene biomarker from oxidative stress and antioxidant pathway. The expression of ten glutathione *S*-transferase (GST) genes was studied in the copepods exposed to various trace metals. These genes included *GSTs* belonging to class *Delta-E(1)*, *Delta-E*, *Kappa*, *Mu5*, *Omega*, *Sigma*, *Theta3*, *Zeta1*, *mGST1*, *mGST3* (Lee *et al*., 2008b). Expression of these genes was also studied against exposure to hydrogen peroxide ($H_2O_2$). This study revealed that of all genes, expression of GST-Sigma (*GSTS*) was highly upregulated in $H_2O_2$ as well as trace metal-exposed copepods (Figs. 3 and 4). Additionally, in a time-series study, expression of *GSTS* mRNA was more consistent compared to other *GSTS*. So far *GSTS* is predominantly reported from the insects. Moreover, expression of many of the genes mentioned above was studied for the first time in copepod species. This study established an antioxidant role for *GSTS* in *T. japonicus* and highlighted its importance as a biomarker of exposure to trace metals.

## RECENT DEVELOPMENTS AND FUTURE DIRECTIONS

Our current research on *T. japonicus* is focused on three divergent aspects. Firstly, we want to standardize this species to a level so as it become a candidate species for toxicity testing in the OECD framework, if not at global level, at least in Western Pacific region. In this regard, we seek active collaboration from research groups of Japan and Hong Kong. Recently, we have established a collaborative program with researchers at City University Hong Kong and Hong Kong University. We believe that there is a strong possibility of having partnership with Global Centre of Excellence (G-COE) program of Japan with special focus on *T. japonicus*. Secondly, we have developed a 12k microchip for *T. japonicus* and currently testing is in progress for gene profiling of trace metal and EDC-exposed copepods. The final goal of this microchip development is to use it in field for pollution monitoring and risk assessment. Lastly, we are exploring possibility of using this tiny organism in study of complex biological processes. For example, we have successfully performed TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) assay in *T. japonicus*. TUNEL is the most commonly used method of apoptosis detection, which has so far generally been used in the mammalian system. Preliminary study involving use of TUNEL assay in *T. japonicus* showed that Zn induced significant increase in apoptotic cells after 96 h of exposure (Fig. 5). However, effect of Cu was not so severe. These observation open up new possibility of using *T. japonicus* in toxicity mechanisms and even in drug testing.

## CONCLUSIONS

Among copepod test species, *T. japonicus* has shown its potential in toxicity testing and molecular mechanism study of toxicity and toxicogenomics. In future its use will intensify after we publish our data on DNA chip. Furthermore, we also want to develop consortium for fullest exploration and exploitation of sequence database of this marine species. It is also proposed to develop a seed bank of this species for use by other researchers.

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J.-S. Lee (e-mail: jslee2@hanyang.ac.kr) and S. Raisuddin (e-mail: sheikhraisuddin@yahoo.com)