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In-silico analysis and mRNA modulation of detoxification enzymes GST delta and kappa against various biotic and abiotic oxidative stressors

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ABSTRACT

This study reports the comprehensive comparative information of two different detoxification enzymes such as glutathione S-transferases (GSTs) delta and kappa from freshwater giant prawn *Macrobrachium rosenbergii* (designated as *Mr*GSTD and *Mr*GSTK) by investigating their *in-silico* characters and mRNA modulation against various biotic and abiotic oxidative stressors. The physico-chemical properties of these cDNA and their polypeptide structure were analyzed using various bioinformatics program. The analysis indicated the variation in size of the polypeptides, presence or absence of domains and motifs and structure. Homology and phylogenetic analysis revealed that *Mr*GSTD shared maximum identity (83%) with crustaceans GST delta, whereas *Mr*GSTK fell in arthropods GST kappa. It is interesting to note that *Mr*GSTD and *Mr*GSTK shared only 21% identity; it indicated their structural difference. Structural analysis indicated that *Mr*GSTD to be canonical dimer like shape and *Mr*GSTK appeared to be butterfly dimer like shape, in spite of four β-sheets being conserved in both GSTs. Tissue specific gene expression analysis showed that both *Mr*GSTD and *Mr*GSTK are highly expressed in immune organs such as haemocyte and hepatopancreas, respectively. To understand the role of mRNA modulation of *Mr*GSTD and *Mr*GSTK, the prawns were inducted with oxidative stressors such as bacteria (*Vibrio harveyi*), virus [white spot syndrome virus (WSSV)] and heavy metal, cadmium (Cd). The analysis revealed an interesting fact that both *Mr*GSTD and *Mr*GSTK showed higher ($P < 0.05$) up-regulation at 48 h post-challenge, except *Mr*GSTD stressed with bacteria, where it showed up-regulation at 24 h post-challenge. Overall, the results suggested that GSTs are diverse in their structure and possibly conferring their potential involvement in immune protection in crustaceans. However, further study is necessary to focus their functional differences at proteomic level.

1. Introduction

Glutathione S-transferases (GSTs) are a multifunctional large family of phase II detoxification/antioxidant enzymes [1]. They are involved in cellular detoxification thereby protecting the organisms from various endogenous and exogenous molecules which include therapeutic drugs, chemical carcinogens, environmental pollutant and product of oxidative stress by catalyzing the conjugation of glutathione (GSH) [2,3]. These enzymes effectively reduced many organic compounds including reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and hydroxyl radical (OH^-) by nucleophilic addition of their thiol group (GSH) to a large variety of electrophilic alkylating compounds such as hydroxyalkenals [2,4]. Such catalytic reactions eventually lead to making the end products more water soluble, less toxic and rapidly

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excretable from cell when compared to non-GSH conjugating substrates, thus protecting the cells from their potential toxic effects [5]. However, GST participates in intracellular transport and is involved in a variety of reactions which are crucial in cellular defense mechanisms against oxidative stresses caused by free radicals [6–8]. Recently, it has been reported as a potential candidate for vaccine development, due to its immune modulatory efficiency against various pathogenic infections [9].

GSTs are ubiquitously found in almost all prokaryotic and eukaryotic organisms including bacteria, animals and plants [10,11]. Based on their amino acid sequence, structure, immunological cross reactivity, evolutionary relationship, catalytic and substrate specificity, 15 different classes of GSTs (*alpha*, *beta*, *delta*, *epsilon*, *kappa*, *lambda*, *mu*, *omega*, *phi*, *pi*, *sigma*, *tau*, *theta*, *zeta* and *rho*) have been identified from diverse group of organisms till date. According to their cellular location, most GSTs are primarily found in three intracellular organelles i.e., cytosolic (soluble), mitochondrial and microsomal or MAPEG (membrane-associated protein in eicosanoid and glutathione metabolism) [12–14]. The GSTs are found to be approximately 23–28 kDa in size and exist as dimers, either homodimers or heterodimers [15]. Both structurally and functionally, most GST enzyme contain two binding sites such as G-site and H-site. The G-site binds to glutathione, found at N-terminal and is highly conserved, whereas H-site (or called substrate binding site) recognizes the hydrophobic substrate found in C-terminal. The H-site showed the highest variability among all the GST classes [12].

Among different classes of GSTs, delta and kappa are considered to be more ancient group. It has been recognized that some GSTs are structurally and functionally distinct from other mammalian GSTs and are designated separately as GST delta [16,17]. GST delta is a prominent class of GST family and it is insect specific. It exists among invertebrates and till now there is no published information on the existence in vertebrates [11,18]. In insects, GST delta shared 55% identity with their homologues in protein structure; and plays important role in detoxification of foreign compounds, insecticide resistance and cellular damage caused by oxidative stress [19,20]. Other GST classes including alpha, mu, pi, theta, zeta, omega and kappa have been widely studied in mammals [16,21]. However, among the cytosolic GSTs, kappa is the least studied. Harris et al. [22] reported the first GST kappa from rat liver mitochondrial matrix. Pemble and Taylor [21] reported that the kappa polypeptide have not shown any sequence similarities with other GSTs. GST kappa possessed a thioredoxin fold like domain dsbA and a protein disulfide isomerase from *Escherichia coli*, thus it is considered as orthologous to bacteria [23].

Till date, many GSTs have been reported from non-mammalian organisms [14], but the reports on GSTs from aquatic invertebrates are limited. Freshwater giant prawn, *Macrobrachium rosenbergii* is farmed on large scale worldwide, especially in Southeast Asian countries including China, Malaysia, India, etc. It has become a potential candidate for aquaculture industry due to its great commercial importance [24]. In recent years, the intensive culture of this shrimp species has been extremely affected by various bacterial and viral infectious diseases such as vibriosis, tail rot disease, white tail disease (WTD), white spot syndrome disease (WSSD) and so on [25], resulting in huge amount of economic loss every year [26]. Like most arthropods, shrimp relies on a series of innate immune reaction to protect themselves and combat the threat against infectious pathogens [27]. Many immune genes have been identified and characterized from *M. rosenbergii* [28], however, sparse information are available about prawn GST [29]. Few isoforms of GSTs have been identified and characterized from aquatic invertebrates which include marine shrimp *Litopenaeus vannamei* [30], crayfish *Macrobrachium vollenhovenii* [31], blue mussels *Mytilus edulis* [32], bivalve mollusks *Atactodea striata* [33] and gastropods *Bulinus truncates* [34]. Considering the importance of GST in the immune system of prawn, it is necessary to study the GST mediated prawn defense mechanism which would help to develop a disease control strategy at molecular level.

Therefore, the objective of this study was designed to study the molecular characterization of two different GSTs namely delta and kappa from *M. rosenbergii* (designated as *Mr*GSTD and *Mr*GSTK) to understand its immune involvement in a comprehensive and comparative approach. Also, we have reported a detailed account on relative *Mr*GSTD and *Mr*GSTK mRNA regulation upon bacterial, viral and heavy metal challenges.

2. Materials and methods

2.1. Identification and sequencing of MrGSTD and MrGSTK cDNAs

A normalized cDNA library of *M. rosenbergii* was developed from the tissue pool of muscle, gill, haemocyte, hepatopancreas and brain [35] and sequenced using Genome Sequencer FLX. The library was annotated on Blast2GO program and two partial sequences of *Mr*GSTD and *Mr*GSTK were identified. Then, applying internal sequencing technique using ABI Prism-Bigdye Terminator Cycle Sequencing Ready Reaction kit, *Mr*GSTD and *Mr*GSTK sequences were received from the library. For internal sequencing of *Mr*GSTD sense (F1) CGC TAA AGC TGT TGG GTT AGA and antisense (R2) TTG GCC TTC CTT ATG GTG ATG and for *Mr*GSTK, sense (F3) GAG GTT CTG ATG CGG TAC AA and antisense (R4) CTA CAA GAT GGA CGA CCC AAA were used. The full length sequences *Mr*GSTD and *Mr*GSTK received from the assay were deposited under EMBL gene bank database.

2.2. In-silico characterization of MrGSTD and MrGSTK

MrGSTD and *MrGSTK* cDNA and polypeptide sequences were characterized using various *in-silico* tools as reported in our earlier findings [36–38]. DNAssist program was used to analyze the physico-chemical properties of cDNA and polypeptide [39]. Also, we considered the following analysis or program for *in-silico* characterization of *MrGSTD* and *MrGSTK*: transmembrane [40], signal peptide [41], domains or motifs [42], multiple sequence alignments [43], phylogenetic tree [44], secondary structure (http://polyview.cchmc.org), 3D structure [45] and Ramachandran plot (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php).

2.3. Oxidative stress and collection of tissues

M. rosenbergii (21.5 ± 3.5 g) were collected from local commercial prawn farm and transported to the laboratory and maintained in the aquaria. A week after acclimatization, the prawns were induced with various oxidative stresses such as *Vibrio harveyi* or white spot syndrome virus (WSSV); and exposure to heavy metal, cadmium (Cd) as demonstrated in our earlier study [29]. The oxidative stress challenge study was performed in three duplications. For WSSV injection, PCR confirmed viral infected *M. rosenbergii* tail tissue homogenized in 2% NaCl was injected (200 μl per 20 g animal). For control, tissue homogenate from healthy prawn tail muscle was injected. Similarly, *V. harveyi* (5×10^6 CFU/ml) suspended in PBS was injected (75 μl per 20 g prawn) intraperitoneally to the prawn. Same quantity of PBS was injected to the control groups. For cadmium challenge, the individuals were allowed in the aquaria dissolved with cadmium at the concentration of 50 μg/L. For control, UV treated freshwater was considered.

Tissue samples were collected from the control and experimental individuals at 0, 3, 6, 12, 24, 48 and 72 h and stored