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Identification of a developmentally and hormonally regulated Delta-Class glutathione *S*-transferase in rice moth *Corcyra cephalonica*☆

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ABSTRACT

Glutathione *S*-transferases (GSTs) are a large family of multifunctional enzymes, known for their role in cellular detoxification. Here we report a cytosolic GST with optimal activity at alkaline pH (8.3) from the visceral fat body of late-last instar (LLI) larvae of a lepidopteran insect rice moth *Corcyra cephalonica*. All previously known GSTs are active between pH 6.0 to 6.5. Purification and characterization revealed the *Corcyra cephalonica* GST (CcGST) as a 23-kDa protein. HPLC and 2D analysis showed a single isoform of the protein in the LLI visceral fat body. Degenerate primer based method identified a 701-nucleotide cDNA and the longest open reading frame contained 216 amino acids. Multiple sequence and structural alignment showed close similarity with delta-class GSTs. CcGST is present mainly in the fat body with highest activity at the late-last instar larval stage. Juvenile hormone (JH) negatively inhibits the CcGST activity both *ex vivo* and *in vivo*. We speculate that high expression and activity of CcGST in the fat body of the late-last instar larvae, when endogenous JH titer is low may have role in the insect post-embryonic development unrelated to their previously known function.

1. Introduction

GSTs (EC 2.5.1.18) in insects are a ubiquitously present superfamily of enzymes that play key roles in detoxification of xenobiotics (including insecticides) and protection of cells against damage due to oxidative stress (Enayati et al., 2005; Giovanini et al., 2006; Li et al., 2008; Salinas and Wong, 1999; Udomsinprasert et al., 2005). They catalyze the conjugation of electrophilic compounds with thiol groups making the xenobiotics more water-soluble (Habig et al., 1974). GSTs also catalyze reactions in metabolic pathways not associated with detoxification (Sheehan et al., 2001). Most GSTs are cytosolic enzymes, present in both homo and heterodimeric forms with subunit masses of 23- to 28-kDa. Each subunit contains a specific glutathione (GSH)-binding site (G-site) in the highly conserved N-terminal region next to a non-specific electrophilic ligand-binding site (H-site). Phylogenetic analysis revealed the presence of at least six distinct classes of cytosolic GSTs in insects along with several unclassified genes (Enayati et al., 2005). GSTs are classified into two major subfamilies characterized by Ser (delta and theta classes) or Tyr (alpha, mu, pi and sigma classes), which plays an important role in lowering the p*Ka* of the thiol group of bound GSH from 9.0, to approximately 6.0 to enhance the rate of nucleophilic attack of GSH towards electrophilic substrates (Caccuri et al., 1999; Wongsantichon and Ketterman, 2006). The majority of insect GSTs belongs to the delta and epsilon classes and lack alpha, mu and pi classes. In spite of low sequence homology among GST classes they have fairly similar tertiary structures, topography of active site and G-sites, and are induced after insecticide application for detoxification (Dirr et al., 1994; Enayati et al., 2005). Recent elucidation of the structure of an epsilon class GST from *Anopheles gambaie* provided critical explanation to high DDT-detoxification activity (Wang et al., 2008).

The fat body of insects is the main metabolic centre, and performs a large number of complex cellular functions (Keeley, 1985). It is involved in detoxification/degradation of xenobiotics and protection from oxidative stress (Motoyama and Dauterman, 1980; Clark et al., 1984; Sawicki et al., 2003). Due to the role of GSTs in insecticide detoxification and other detoxifications such as *O*-dealkylation or *O*-dearylation of organophosphorous insecticides (Hayes and Pulford, 1995; Hayes et al., 1998), there is a significant increase in the number of well characterized insect GSTs. However, little is known about the role of GSTs of stored-grain pests, which are exposed to a variety of chemical formulations. As these pests live successfully under high stress conditions, characterization of these enzymes is critical to ascertain the basis for stress management. Studies on these groups of GSTs will yield important

Abbreviations: GST, glutathione *S*-transferase; VFB, visceral fat body; HGLFB, hind-gut associated lobular fat body.

☆ GenBank submission. The CcGST nucleotide sequences discussed in the paper have been submitted to the GenBank with accession number DQ234273.

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information about insecticide resistance. Our endeavor to understand the role of GSTs in stored-grain pest resulted in the identification and characterization of a single isoform from the visceral fat body of LLI larvae of the rice moth, *Corcyra cephalonica* (termed CcGST). The 23-kDa CcGST has unusual optimal activity at alkaline pH 8.3, whereas most other previously reported GSTs have optimal activity between pH 6.0–6.5 (Clark et al., 1989).

2. Materials and methods

2.1. Experimental insect

The larval forms of rice moth, *Corcyra cephalonica* (Stainton), a serious stored-grain lepidopteran pest were reared on crushed sorghum seeds and maintained at 26 ± 1 °C, 65 ± 5% relative humidity and 14:10 light-dark period. In the present study the last (=Vth) instar larvae, which were further classified into early-last instar (ELI), mid-last instar (MLI) late-last instar (LLI) larvae and prepupae (PP) (Lakshmi and Dutta-Gupta, 1990) were used.

2.2. Sample preparation and GST activity assay

The larval stage of interest was narcotized on ice. The prolegs were cut with a fine sharp scissors and the oozing hemolymph was removed using an absorbent filter paper. The hemolymph-free larvae were washed several times in cold insect Ringer's (130 mM NaCl, 5 mM KCl, 0.1 mM $CaCl_2$ and 1 mM phenymethylsulfonyl fluoride), crushed in liquid N_2 and suspended in homogenization buffer (20% w/v) containing 50 mM Tris–HCl (pH-7.8), 10 mM EDTA, 15% glycerol and 0.005% phenylthiourea. The homogenate was centrifuged at 13,000 ×*g* for 5 min at 4 °C and the supernatant was used as whole body extract. Specific tissue homogenates were also prepared in a similar manner. Protein estimation was done according to Bradford's micro protein assay method (Bradford, 1976) using bovine serum albumin (fraction V) as standard. GST enzyme activity was assayed as described previously (Habig et al., 1974; Arif et al., 2004). The reaction mixture contained 0.1 mM 1-chloro-2,4-dinitrobenzene (CDNB), 0.1 mM GSH in 100 mM potassium phosphate buffer (for pH range, 5–7) or 100 mM Tris–HCl buffer (for pH range, 7–9). The reaction was initiated by the addition of 10 μg of homogenate protein and the formation of thio-ether conjugate was measured at 340 nm on a time scan of 0–60 s. V_{max} and K_m of purified CcGST were determined by fitting the activity data into a Michaelis–Menten equation and non-linear regression analysis using GraphPad Prism (GraphPad software, San Diego, CA, USA).

2.3. GST purification

Visceral fat bodies (VFB) were isolated from the LLI larvae of *Corcyra cephalonica* and the extract was prepared as described above. The VFB extract was centrifuged at 13,000 ×*g* for 45 min at 4 °C. The resulting supernatant was centrifuged at 100,000 ×*g* for 1 h to isolate the cytosolic (supernatant) and microsomal fractions (pellet). Cytosolic fraction was passed through a Sephadex G-25 gel filtration matrix and the active fractions were pooled for purification on a DEAE cellulose column pre-equilibrated with 50 mM Tris–HCl (pH 7.8). The flow through fractions that contained majority of GST activity were pooled and dialyzed. Samples were then applied to a GSH-cross-linked with epoxy-activated Sepharose 6B affinity column, pre-equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 2 mM DTT. CcGST-bound-affinity resin was eluted with 50 mM Tris–HCl (pH 8.0) containing 1 mM EDTA, 10 mM GSH and 2 mM DTT. The purity of CcGST was determined both by activity and SDS-PAGE (12% polyacrylamide) assays. The CcGST purity was also determined by reversed-phase HPLC on a Waters-1525 system (USA) using BioSuite C18 column. Mobile phases were mixtures of 25% (v/v) acetonitrile and 0.1% TFA. Elution was done using a linear increase of acetonitrile. 2D analysis of the affinity pure VFB-CcGST was performed according to O'Farrell (1975) with slight modifications.

2.4. Western analysis and production of CcGST antibody

Purified CcGST protein (200 μg) was emulsified with Freund's complete adjuvant and injected subcutaneously into two 6-month old male rabbits. Booster injections of CcGST, emulsified with Freund's incomplete adjuvant were also administered. Serum was collected after the second booster and IgG was purified using Protein-A-affinity chromatography (BioRad). SDS-PAGE and silver staining was carried following Laemmli (1970) and Blum et al. (1987). Western blot analysis was performed using anti-CcGST antibody as described (Towbin et al., 1979) and the cross-reactivity was detected using anti-rabbit IgG coupled to alkaline phosphatase followed by an NBT/BCIP color reaction.

2.5. Isolation and characterization of CcGST cDNA

Total RNA isolated from LLI VFB was used to synthesize cDNA using SuperScriptII first strand synthesis system (Invitrogen). The cDNA was used as template for specific amplification of GST from *Corcyra* using degenerate primers (5′-ATG MCC ATC GAY CTS TAC-3′ and 5′-TTR AGC ATG CTG ACR A-3′) designed on the basis of available lepidopteran GST cDNA sequences. We also used degenerate primer sets for other classes of insect GSTs, but only the primers for Delta class showed successful amplification of the fragment. PCR product was cloned in TOPO-TA vector (Invitrogen) and sequenced. CcGST cDNA was amplified using specific primers (5′-CAT ATG ACC ATT CGA TCT GTA CT-3′ and 5′-GAA TTC TTA CTT TTG AGC AAT GCT GT-3′) and cloned between Nde I and EcoR I restriction sites of pRSETa vector (Invitrogen). Expression of pRSETa-CcGST was tested by IPTG induction after transformation into *E. coli* BL21-DE3 cells.

2.6. Homology modeling of CcGST

Based on the high sequence identity with *Anopheles dirus* GST (AdGST), its crystal structure (IJLV) was selected as template to build the CcGST homology model. Using the homology module of Accelrys (San Diego, CA, USA), an initial model of CcGST (Met^1-Lys^{216}) was generated. The structurally conserved regions were determined by multiple sequence alignment based on the Needleman and Wunsch Algorithm. The loop-search algorithm was used to construct the structure of LOOPs. The initial model was improved by energy minimization. Layers of water with a thickness of 5 Å were added to the whole protein using the SOAK program of the Insight-II that allowed the hydrophilic residues to interact with water instead of interacting with each other. The coordinates were also analyzed by PROCHECK for allowed or disallowed regions in a Ramachandran plot. Docking of GSH with the GST active site was performed using the GOLD program to understand the interactions of the ligand.

2.7. Organ culture and hormonal regulation studies

Visceral fat bodies from two thorax-ligated LLI larvae (Arif et al., 2004) were dissected under sterile conditions and washed with TC-100 insect culture media (Sigma) with streptomycin sulfate (1 μg/200 μL). The tissues were transferred to fresh media and incubated in absence or presence of 70 nM of juvenile hormone I (JHI) (Hoffman-LaRoche) or 80 nM 20-hydroxyecdysone (20E) (Sigma) for 4, 8 and 12 h. The hormone stocks were prepared as described in Kramer et al., (1974) and Arif et al. (2003). Equal volume of carrier solvent (0.05% ethanol) that was used to prepare hormone stock was added to the control cultures. After incubation with hormone, tissue was washed with insect Ringer's, homogenized and used for activity and western blot assays. For *in vivo* studies, 5 μL of 250 nM JHI was topically applied, while 250 nM 20E was