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Proteomic identification, cDNA cloning and enzymatic activity of glutathione *S*-transferases from the generalist marine gastropod, *Cyphoma gibbosum* ☆

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ABSTRACT

Glutathione *S*-transferases (GST) were characterized from the digestive gland of *Cyphoma gibbosum* (Mollusca; Gastropoda), to investigate the possible role of these detoxification enzymes in conferring resistance to allelochemicals present in its gorgonian coral diet. We identified the collection of expressed cytosolic *Cyphoma* GST classes using a proteomic approach involving affinity chromatography, HPLC and nano-spray liquid chromatography–tandem mass spectrometry (LC–MS/MS). Two major GST subunits were identified as putative mu-class GSTs; while one minor GST subunit was identified as a putative theta-class GST, apparently the first theta-class GST identified from a mollusc. Two *Cyphoma* GST cDNAs (CgGSTM1 and CgGSTM2) were isolated by RT-PCR using primers derived from peptide sequences. Phylogenetic analyses established both cDNAs as mu-class GSTs and revealed a mollusc-specific subclass of the GST-mu clade. These results provide new insights into metazoan GST diversity and the biochemical mechanisms used by marine organisms to cope with their chemically defended prey.

Glutathione *S*-transferases (GST,[2] EC 2.5.1.18) form a large superfamily of multifunctional enzymes capable of conjugating a broad range of toxic electrophilic xenobiotics with glutathione [1]. GSTs function primarily as detoxification enzymes, generally rendering the resultant products more water soluble (non-reactive conjugate), thereby facilitating excretion. The catalytic versatility and diversity of GSTs can be attributed to the non-specific nature of the hydrophobic substrate binding site (H-site), and the extensive gene duplication and divergence that has occurred in this superfamily [2,3]. The soluble GSTs in metazoans are divided into eight classes (alpha, kappa, mu, pi, sigma, theta, omega, and zeta) based on sequence identity, immunological and kinetic properties [1,4,5]. While the majority of GST enzymatic characterization has focused on mammalian forms, non-vertebrate models offer an exciting opportunity to examine the evolution of GSTs and their adaptive responses to environmental chemicals, including natural products.

Biochemical adaptations involving xenobiotic response genes, such as GSTs, may help explain consumer resistance to dietary chemical threats and provide further insight into the enzymatic mechanisms underlying foraging decisions. For example, the ability of terrestrial invertebrates (insects) to tolerate naturally occurring dietary toxins (i.e., allelochemicals in their host plants) has been linked in part to high constitutive activity of their GSTs (reviewed in [6]). The induction of GSTs in response to dietary allelochemicals may serve as an additional adaptive mechanism to protect against toxicity. While the role of GSTs in plant–herbivore interactions has clearly influenced the ecology of terrestrial consumers, far less is known about the detoxification enzymes that allow marine invertebrates to exploit allelochemically defended prey.

Only a few studies have addressed the induction of GSTs upon exposure to allelochemicals in marine invertebrates [7–9]. One such study [9] examined GST activity in the digestive gland of a generalist gastropod, *Cyphoma gibbosum*, which exclusively feeds on several families of chemically defended gorgonian corals. The authors reported significantly higher GST activity in field-collected *C. gibbosum* feeding on gorgonians *Gorgonia ventalina* and *Briareum asbestinum*, suggesting that GST expression varies in response to different suites of gorgonian allelochemicals. Additionally, GST activity from *C. gibbosum* cytosolic preparations was among the

☆ The nucleotide and translated amino acid sequences for *C. gibbosum* GSTs have been deposited in GenBank with the following Accession Nos. EU008563 (CgGSTM1) and EU008562 (CgGSTM2).

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[2] *Abbreviations used:* CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione *S*-transferase; nrDB, non-redundant database; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase-polymerase chain reaction.

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highest ever reported from a molluscan digestive gland and was similar to values described from *Papilio polyxenes*, a specialist insect that feeds solely on chemically defended plants [10,11]. In a subsequent study [12], thin-layer chromatographic profiles of non-polar tissue extracts from *C. gibbosum* feeding on *G. ventalina* did not mirror those of its octocoral prey, lending further support to the idea that this gastropod predator has the capacity to biotransform dietary compounds to readily excretable metabolites. However, the GST isoforms responsible for the detoxification of gorgonian allelochemicals are not known, and in general GST diversity in molluscs, including marine gastropods, is poorly understood.

GST cDNA sequences representing alpha, mu, pi, omega, and sigma GST classes have been identified in molluscs. The majority of sequences are grouped within the pi-class, with representatives from cephalopods (squid, octopus) and seven bivalve species ([13–20]; GenBank Accession Nos. EF194203, EF520700, DQ530213, and DQ530212). The endogenous function of pi-class GSTs in molluscs has yet to be defined; however, studies in mammalian systems indicate that pi GSTs are more involved than other GST classes in the detoxification of prostaglandins and other electrophilic α,β-unsaturated carbonyl compounds [21,22]. The highest concentrations of prostaglandins in nature have been found in Caribbean gorgonians [23], where the acetoxy acids, hydroxyl methyl esters and hydroxyl acids of 15(*R*)-prostaglandin A_2 function as feeding deterrents against generalist reef fish [24,25]. Prostaglandins in the A series can significantly induce GST activity in mammalian cells [26]. We hypothesized that *C. gibbosum* GSTs may conjugate gorgonian allelochemicals, like prostaglandins, potentially alleviating their toxicity.

The objective of the present study was to isolate and characterize the GSTs in *C. gibbosum* digestive gland that may protect this marine consumer from allelochemicals found in its prey. Initially, we targeted pi-class GSTs because of their role in prostaglandin metabolism. However, our attempts to obtain pi-class GSTs from *C. gibbosum* using RT-PCR with degenerate primers designed from an alignment of molluscan GST pi cDNA sequences [15] failed to yield any GST sequences. Therefore, we initiated a proteomic approach involving affinity chromatography coupled with HPLC and mass spectrometry to provide an unbiased assessment of GST protein diversity in the digestive gland of *C. gibbosum*. Here we report the identification of several mu-class GST proteins in *C. gibbosum* digestive gland, the cloning and phylogenetic characterization of two complete mu-class GST cDNAs from *C. gibbosum*, and partial peptide sequences of a theta-class GST, apparently the first member of this class identified from a mollusc. These results contribute to a better understanding of GST diversity in molluscs and of the biochemical resistance mechanisms used by marine consumers to cope with their allelochemically defended prey.

Materials and methods

Reagents

CDNB, dithiothreitol (DTT), potassium phosphate, potassium chloride, EDTA, protease inhibitor cocktail (4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, bestatin hydrochloride, E-64, leupeptin, and pepstatin A), SDS, boric acid, NaCl, sodium acetate, GSH, GSH–agarose (G4510) was purchased from Sigma (St. Louis, MO). Bradford reagents and molecular weight standards for protein gels were purchased from Bio-Rad (Hercules, CA). Novex® Tris–glycine gels and sample treatment buffer were purchased from Invitrogen (Carlsbad, CA). SilverSNAP® stain for mass spectrometry was purchased from Pierce (Rockford, IL).

Animals

A total of 42 adult *C. gibbosum* (ca. 2–3 cm length) were collected from five shallow reefs (<20 m) near the Perry Institute of Marine Science (PIMS), Lee Stocking Island, Exuma Cays, Bahamas in January 2006 and transported to wet laboratory facilities provided by PIMS. Individuals were allowed to feed on a control diet (e.g., alginic acid and freeze-dried squid paste prepared as described in [27], or one of six gorgonian diets—*Briareum asbestinum*, *Eunicea mammosa, Gorgonia ventalina, Pseudopterogorgia acerosa, Pseudopterogorgia americana, Plexaura homomalla*) for 4 days. Upon completion of the feeding assay the digestive glands were immediately dissected and either preserved in RNALater® (Ambion, Austin, TX) or frozen in liquid nitrogen and transported back to the Woods Hole Oceanographic Institution. Tissues were maintained at −80 °C until further analysis.

Enzyme purification

Individual digestive glands ($n = 42$) were homogenized 1:4 (w/v) in ice-cold homogenization buffer (0.1 M potassium phosphate, 1 mM EDTA, 1 mM DTT, 1.15% potassium chloride, protease inhibitor cocktail (1×); pH 7.5) with an IKA Ultra Turrax T8 homogenizer (Wilmington, NC) for 30 s on ice. All subsequent steps were carried out at 4 °C. Cytosol was isolated by centrifugation of the crude homogenate at 750*g* for 10 min then, without stopping, at 12,000*g* for 10 min using a Beckman J2-21 centrifuge (Fullerton, CA). The supernatant was carefully removed, avoiding both the fatty layer and the pellet and transferred to an ultracentrifuge tube and centrifuged at 100,000*g* for 70 min using a Beckman L8-60M ultracentrifuge (Fullerton, CA). An aliquot of the supernatant was taken for cytosolic protein determination using the BCA protein assay method [28] with BSA as the standard and the remaining cytosol was flash frozen in liquid nitrogen until further analysis. Samples were stored for several months in this manner suffered no apparent loss of enzymatic activity.

GSTs were purified using size-exclusion chromatography, affinity chromatography and HPLC following a modified method from [29]. Cytosolic samples from individual snail digestive glands were purified separately. Initially, a subset of cytosol samples ($n = 8$) were used to determine the optimal buffer conditions and volumes for GST purification. Eluted 1-mL fractions from both size-exclusion and affinity columns were sampled for activity towards CDNB and assayed for protein using the Bradford assay [30]. These results helped streamline the GST purification process for the remaining cytosol samples.

A PD-10 desalting column (bed volume 8.3 mL, bed height 5 cm, 5K NMWL; GE Healthcare, Piscataway, NJ) containing Sephadex G-25 matrix was equilibrated in Buffer A (50 mM Tris buffer, 1 mM EDTA, 1 mM DTT; pH 6.0) and 2.5 mL of crude cytosol was applied to the column. Cytosolic samples less than 2.5 mL were brought up to this volume with Buffer A and then applied to the column. GST proteins were eluted by gravity with Buffer A and fractions containing GST activity were combined (approx. 6 mL of elute) and then applied to a GSH–agarose affinity column (bed volume 0.5 mL, 0.8 × 4 cm i.d.) equilibrated in Buffer A. The affinity column was washed with 7 mL of Buffer B (Buffer A + 0.5 M NaCl) to rinse away non-specific proteins. Retained GSTs were then eluted with 5 mL of Buffer C (50 mM Tris–base, 1 mM EDTA, 1 mM DTT, 0.5 M NaCl, 50 mM glutathione; pH 9.5) and fractions containing GST activity were then combined, buffer exchanged to low salt concentration, and concentrated with Amicon Ultra-4 centrifugational filters (5K NMWL membrane; Millipore, Billerica, MA). Protein concentrations of Amicon concentrates were determined with the NanoOrange protein quantitation kit (Molecular Probes, Eugene, OR).