Glutathione-Binding Site of a *Bombyx mori* Theta-Class Glutathione Transferase

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**Abstract**

The glutathione transferase (GST) superfamily plays key roles in the detoxification of various xenobiotics. Here, we report the isolation and characterization of a silkworm protein belonging to a previously reported theta-class GST family. The enzyme (bmGSTT) catalyzes the reaction of glutathione with 1-chloro-2,4-dinitrobenzene, 1,2-epoxy-3-(4-nitrophenoxyl)-propane, and 4-nitrophenethyl bromide. Mutagenesis of highly conserved residues in the catalytic site revealed that Glu66 and Ser67 are important for enzymatic function. These results provide insights into the catalysis of glutathione conjugation in silkworm by bmGSTT and into the metabolism of exogenous chemical agents.

**Introduction**

Glutathione (GSH) conjugation is essential for the detoxification of xenobiotics [1,2]. Several studies have also implicated conjugation reactions with endogenous compounds, such as α,β-unsaturated aldehydes and prostaglandin [2–4], resulting in the excretion of at least one water-soluble compound. GST transferases (GSTs, EC 2.5.1.18) are responsible for catalysis of this conjugation and are distributed ubiquitously among aerobic organisms [5]. GSTs are cytosolic enzymes, widely distributed across both prokaryotic and eukaryotic kingdoms [6]. In mammals, there are seven GST classes (α, μ, π, Ω, σ, θ, and ζ) that can be distinguished based on their primary amino acid sequence; identity is approximately 50% within a class and less than 30% between different classes [7,8]. Six GST classes (δ, ε, Ω, σ, θ, and ζ) have been identified in dipteran insects, such as *Anopheles gambiae* [9] and *Drosophila melanogaster* [10,11]. Insect GSTs can determine sensitivity to insecticides [9,12], and since the Lepidoptera are the principal insect pests in agriculture, knowledge of lepidopteran GSTs is of great importance. We have previously characterized several GSTs in the silkworm, *Bombyx mori*, a lepidopteran model insect [13–19], and a sigma-class GST in the fall webworm, *Hyphantria cunea*, one of the most serious lepidopteran pests of broad-leaved trees [16]. However, there have been no reports to date on the characterization of theta-class GSTs from silkworms.

Here, we report the identification and classification of a theta-class GST isolated from *B. mori*, which we named bmGSTT. While bmGSTT shares some common substrates with human theta-class GSTs (hGSTT), it has a distinct substrate profile when compared to other *B. mori* GSTs studied to date. Furthermore, bmGSTT does not participate in the response to agents that generate oxidative stress, in contrast to previously identified *B. mori* GSTs. The activity profile of bmGSTT sheds further light on the way in which insects deal with xenobiotic agents and contributes to a more detailed understanding of the GST system in general.

**Materials and Methods**

**Insects and tissue dissection**

Larvae of the silkworm, *B. mori*, were reared on mulberry leaves in the Institute of Genetic Resources, Kyushu University Graduate School (Fukuoka, Japan). At day -1 fifth instar larvae, fat bodies were dissected from the larvae on ice and stored at −80°C until use. Total RNA was extracted rapidly from the dissected fat bodies with the RNeasy Plus Mini Kit (Qiagen Inc., Valencia, CA), in accordance with the manufacturer’s instructions, and the resultant RNAs were subjected to RT-PCR.

**Cloning and sequencing of cDNA encoding bmGSTT**

Total RNA was processed using RT-PCR. First-strand cDNA was produced using SuperScript II Reverse Transcriptase (Life Technologies, Carlsbad, CA) and an oligo-dT primer. The resulting cDNA was used as a PCR template with the oligonucleotide primers 5′-TATACACAGTTTAAAAACTATATTTAGAT-3′ (sense) and 5′-CCGGATCTTAAAGGTTAGATA-TGCGGA-3′ (antisense), based on a sequence obtained from the SilkBase EST database [20]. Underlined and double-underlined regions in the primer sequences represent *Nol* and *BamHI* restriction enzyme sites, respectively, which were used to insert the PCR product into an expression plasmid. PCR was performed with 1 cycle at 94°C for 2 min; then 35 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min; followed by 1 cycle at 72°C for 10 min. The resulting bmGSTT cDNA (bmgstt) was ligated into the pGEM-T Easy Vector (Promega, Madison, WI), which was then used to transform *E. coli* DH5α cells. Genetyx software (ver. 14.0.12, Genetyx Corp., Tokyo, Japan) was used to
obtain the complete sequence of *bmgst* and to deduce its corresponding amino acid sequence. Homology alignment (Fig. 1) was performed using ClustalW (ver. 1.83), with 10 and 0.2 as the gap creation penalty and gap extension, respectively. A phylogenetic tree was generated using neighbor-joining plot software (http://www-igbmc.u-strasbg.fr/Bioinfo/ClustulX/Top.html).

**Overexpression and purification of recombinant protein**

The *bmgst* clone was digested with NcoI and BamHI and subcloned into the expression vector pET-11b, which was then used to transform competent *E. coli* Rosetta (DE3) pLysS cells (Novagen, EMD Biosciences, Inc., Darmstadt, Germany). Cells were then cultured at 37°C in Luria-Bertani media containing 100 µg/mL ampicillin. After cell density reached an OD600 of 0.7, isopropyl-1-thio-D-galactoside was added at a final concentration of 1 mM to induce recombinant protein production. The culture was further incubated for 3 h, and cells were harvested by centrifugation. Bacteria were resuspended in 20 mM Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl, 4 mg/mL lysozyme, and 1 mM phenylmethanesulfonyl fluoride, and cells were subsequently disrupted by sonication. Unless otherwise stated, all operations for purification described below were conducted at 4°C.
supernatant containing the recombinant protein was clarified by centrifugation at 10,000 x g for 15 min and subjected to ammonium sulfate fractionation. The pellet obtained by ammonium sulfate fractionation was resuspended in 20 mM Tris-HCl buffer, pH 8.5. After dialysis against the same buffer, samples were subjected to anion-exchange chromatography on a DEAE-Sepharose column (GE Healthcare Bio-Sciences, Uppsala, Sweden) and eluted with a linear gradient of 0–0.3 M NaCl. The enzyme-containing fractions, assayed as described below, were pooled, concentrated using a centrifugal filter (Millipore Corp., Billerica, MA), and applied to a Superdex 200 column (GE Healthcare Bio-Sciences, Buckinghamshire, UK) equilibrated with the same buffer, but with the addition of 0.2 M NaCl. The purity of the pooled material was analyzed by SDS-PAGE using a 15% polyacrylamide slab gel containing 0.1% SDS, according to the method of Laemmli [21]. Protein bands were visualized by Coomassie Brilliant Blue R250 staining, and protein concentrations were measured using a Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA), with bovine serum albumin as a standard.

Molecular modeling

A structural model of bmGSTT was constructed by SWISS-MODEL [22] using the amino acid sequence. The model showed a GMQE (Global Model Quality Estimation) score of 0.69 [23]. The construction of the bmGSTT model was based on the structure of hGSTT1-1 (PDB code: 2C3T). The secondary structure assignments were produced with DSSP [24]. The Superpose program [25] revealed structural homology between bmGSTT and hGSTT1-1 with a root-mean-square deviation of 2.412 Å/214 residues for all atoms. Figures were prepared using Coot [26] and PyMOL (http://pymol.sourceforge.net).

Site-directed mutagenesis

Amino acid-substituted mutants of bmGSTT were constructed using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene Corp., La Jolla, CA), according to the manufacturer’s recommendations. An expression plasmid containing bmgstt was used as a template, and full-length mutated cDNAs were verified by DNA sequencing.

Measurements of enzyme activity

GST activity was spectrophotometrically measured using 1-chloro-2,4-dinitrobenzene (CDNB) and 5 mM GSH as standard substrates [27]. Enzymatic activity was expressed as mol CDNB conjugated with GSH per min per mg of protein. Alternatively, other substrates listed in Table 1 were used instead of CDNB [28,29]. Kinetic parameters (k_m and k_a) were assessed with a nonlinear least-squares data fit under assay conditions with different substrate concentrations in the presence of 5 mM GSH. The kinetic parameters toward GSH were measured in the presence of 1 mM CDNB. Thermostability of bmGSTT was determined by pre-incubation of enzyme solutions at various temperatures for 30 min before a residual activity assay. The pH stability of bmGSTT was assessed by pre-incubation of enzyme solutions at various pH values at 4°C for 24 h before a residual activity assay. Optimal pH for bmGSTT activity was determined using citrate-phosphate-borate buffer at various pH values with a fixed ionic strength of 0.25.

Insecticide metabolism assay

The ability of bmGSTT to metabolize each insecticide was determined by high performance liquid chromatography (HPLC) [14]. Reaction mixtures (500 μL) contained 120 μM PM, bmGSTT (12 μg), and 5 mM GSH in 50 mM Tris-HCl buffer at pH 8.0. Dehydrochlorinase activity for 1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane (DDT) was assayed by incubating the purified bmGSTT with 0.1 mM DDT and 5 mM GSH in 20 mM Tris buffer (pH 8.0) at 30°C for 2 h. DDT and its metabolites were analyzed by HPLC, as described below, according to a previous report [14,30].

Reaction mixtures were extracted with three 500 μL portions of ethyl acetate for analysis by HPLC. After removing ethyl acetate, the amounts of each insecticide were determined by HPLC. An HPLC instrument (Prominence, Shimadzu Corp., Kyoto, Japan) was fitted with a 250×4.6 mm Mightysil RP-18 column (Kanto Chemical Co., Inc., Tokyo, Japan) with a flow rate of 1.0 mL/min at 40°C. The mobile phases employed were methanol (MeOH)/acetonitrile/H2O (70/15/15, v/v/v), MeOH/0.1% acetic acid (70/30, v/v), and MeOH/0.1% acetic acid (85/15, v/v) for detection of DDT, chlorfenapyr (CP), and permethrin (PM), respectively. The concentrations of each insecticide were determined from the corresponding peak areas identified by its authentic sample.

Results

Sequence of cDNA encoding bmGSTT

We isolated a cDNA, bmgstt, from silkworm fat bodies, the nucleotide sequence of which is deposited in GenBank under Accession No. AB848737. A BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the Swiss-Prot database (http://expasy.org/sprot/) showed that the sequence corresponds to theta-class GSTs. The sequence contains an open reading frame of 690 base pairs, encoding 229 amino acid residues (Fig. 1). The deduced amino acid sequence shows 34% and 31% identities to hGSTT1-1 and hGSTT2-2, respectively. In A. gambiae, there are two isoforms of the theta class (GenBank under accession numbers: AF515525 and AF515526), whereas, in D. melanogaster, 4 isoforms of the theta class are known (flybase (http://flybase.org) under accession numbers: CG1681, CG1702, CG30000, and CG30005). The amino acid sequence of bmgstt reveals identities of 39%, 45%, 30%, 47%, 35%, and 35% to AF515525, AF515526, CG1681, CG1702, CG30000, and CG30005, respectively (Fig. 1). Secondary structural elements of bmGSTT were predicted using SWISS-MODEL (http://swissmodel.expasy.org/workspace/). Additionally, the DSSP program [24] revealed that the bmGSTT monomer includes 9 α-helices and 4 β-strands (Fig. 1). In hGSTTs, there are additional α-helices, in comparison to bmGSTT, which would be present as α2α, α4c, and α9. The locations for the other α-helices and β-strands are conserved among the three GSTs (Fig. 1). Construction of a phylogenetic tree clustered bmGSTT in the same clade as all current theta members (Fig. 2). The theoretical molecular mass and isoelectric point (26,915 Da and 8.91, respectively) of bmGSTT are similar to those of zeta- and delta-class GSTs from B. mori (Table 2).

Putative GSH-binding site (G-site)

The G-site identified in hGSTT1-1 include His40, Val54, Lys53, Glu66, Ser67, and Thr104; whereas in hGSTT2-2, they are Lys41, Leu54, Glu66, Ser67, Asp104, and Arg107 [31]. Superimposition of modeled bmGSTT on hGSTT1-1 indicates that equivalent B. mori residues include His40, Arg53, Val54, Glu55, Ser67, and Ile104 (Fig. 3A). In this model, the distance is
large between the oxygen atom in the cysteinyl region of θ- hexylglutathione (GTX), an inhibitor of GST, and the side-chain of Arg33 of bmGSTT (3.54 Å), and there is a large distance between the amino group in the θ-glutamyl region of GTX and the side-chain of Ile104 (8.39 Å between the amino group in the θ-glutamyl region of GTX and the side-chain of Ile104) in bmGSTT. In Fig. 3B, GSH is far away from Tyr41 (3.41 Å between oxygen atom in the glycine portion of GSH and the hydroxy-group of Tyr41) and Ile104 (8.39 Å between the amino group in the θ-glutamyl region of GSH and the side-chain of Ile104) in bmGSTT. Taken together, the structural data indicate that five residues (His40, Val54, Glu66, Ser67, and Arg107) in bmGSTT participate in the interaction with GSH.

Characterization of bmGSTT

Bacterially produced bmGSTT was purified to homogeneity, yielding a single band in SDS-PAGE with a molecular size of approximately 26,000 Da (Fig. 4). This size is close to the estimated size based on the deduced amino acid sequence.

The enzymatic properties of purified bmGSTT were studied using CDNB and GSH as substrates. The enzyme was stable at temperatures <50°C and retained >75% of its original activity over a pH range of 5–11, similar to other B. mori GSTs. The pH optimum of bmGSTT was 8.0, which is identical to the optima of epsilon-, delta-, and sigma-class B. mori GSTs.

Various substrates were then used to profile the activity of bmGSTT. Table 1 shows that bmGSTT was active toward CDNB, 1,2-epoxy-3-(4-nitrophenoxy)-propane (EPNP), and 4-nitrophenethyl bromide (4NPB), but it was not active toward 4-nitrobenzyl chloride (4NBC), 4-hydroxynonenal (4HNE), ethacrynic acid (ECA), or 4-nitrophenyl acetate (4NPA). The activity of bmGSTT toward CDNB is higher than that of GSTT-1 from rats or 5 mM GSH. Data are expressed as means of three independent experiments. NA represents no activity. Wavelength and Δε represent maximum wavelength of the absorption and molecular coefficient, respectively. —: not applicable.

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Table 1. Substrate specificity of bmGSTT.

| Substrate | Concentration (mM) | Activity (μmol/min/mg) | Wavelength (nm) | Δε (mM⁻¹ cm⁻¹) |
|-----------|-------------------|------------------------|----------------|----------------|
| CDNB      | 1.0               | 0.03                   | 340            | 9.6            |
| EPNP      | 1.0               | 2.57                   | 260            | 0.5            |
| 4NBC      | 1.0               | NA                     | 310            | 1.9            |
| 4NPB      | 1.0               | 3.56                   | 310            | 1.2            |
| 4HNE      | 0.1               | NA                     | 224            | 13.8           |
| ECA       | 1.0               | NA                     | 270            | 5.0            |
| 4NPA      | 1.0               | NA                     | 400            | 8.3            |
| H2O2      | 0.2               | NA                     | 340            | −6.2           |
| PM        | 0.25              | NA                     | ---            | ---            |
| DDT       | 0.1               | NA                     | ---            | ---            |
| CP        | 0.25              | NA                     | ---            | ---            |

Activity was measured at pH 8 in the presence of 5 mM GSH. Data are expressed as means of three independent experiments. NA represents no activity. Wavelength and Δε represent maximum wavelength of the absorption and molecular coefficient, respectively. —: not applicable.

Amino acid residues involved in catalytic function

Based on the G-site of hGSTT1-1 and hGSTT2-2, we identified His40, Val54, Glu66, Ser67, Asp104, and Arg107 (Fig. 3B), which are superimposed upon Tyr41, Val54, Glu66, Ser67, Ile104, and Arg107 in bmGSTT. In Fig. 3B, GSH is far away from Tyr41 (3.41 Å between oxygen atom in the glycine portion of GSH and the hydroxy-group of Tyr41) and Ile104 (8.39 Å between the amino group in the θ-glutamyl region of GSH and the side-chain of Ile104) in bmGSTT. Taken together, the structural data indicate that five residues (His40, Val54, Glu66, Ser67, and Arg107) in bmGSTT participate in the interaction with GSH.
Discussion

Although many GSTs have been identified in *B. mori*, the theta class remains poorly understood. This is a critical gap in our knowledge, because understanding the metabolic profile of theta-class GSTs may provide novel insecticide-targeting strategies. According to the silkworm genome sequence, there could be 23 homologs of GSTs: delta-class (4 isoforms), epsilon-class (8 isoforms), omega-class (4 isoforms), sigma-class (2 isoforms), theta-class (1 isoform), zeta-class (2 isoforms), and unclassified (2 isoforms).

**Figure 2. Phylogenetic analysis of amino acid sequences of GSTs.** The phylogenetic tree was produced by neighbor-joining plot software using GST sequences obtained from NCBI (http://www.ncbi.nlm.nih.gov/), SwissProt (http://expasy.org/sprot/), and flybase (http://flybase.org/); each entry contains the species name, GST class, and accession number. Numbers represent branch length; Ag, *A. gambiae*; Md, *Musca domestica*; Dm, *D. melanogaster*; Ms, *Manduca sexta*; Hc, *Hyphantria cunea*; Hs, *Homo sapiens*; bm, *B. mori*; Ae, *Aedes aegypti*; Sl, *Spodoptera litura*; Px, *Papilio xuthus*; and numbers attached to nodes indicate bootstrap values. The unclassified GST group does not include GST class.

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Table 2. Properties of GSTs as determined in the present and previous studies.

| Class | Calculated Molecular Weight (Da) | Stable pH Range | Stable Temperature Range |
|-------|----------------------------------|-----------------|-------------------------|
| theta | 26,913                           | 5–11            | <50°C                   |
| epsilon | 25,296                         | 5–10            | <50°C                   |
| unclassified | 24,457                      | 5–9             | <50°C                   |
| delta | 24,225                           | 3–9             | <50°C                   |
| omega | 29,806                           | 2–12            | 4–10                    |
| sigma | 23,338                           | 4–11            | 4–10                    |
| zeta | 24,727                           | <50°C           | CDNB                    |

Substrate Specificity: CDNB, EPNP, 4NPB, CDNB, ECA, GPx, CDNB, CDNB, 4HNE, 4NPA, CDNB, 4HNE, GPx, CDNB, DCA.

B. mori delta-class GST; epsilon, B. mori epsilon-class GST; omega, B. mori omega-class GST; sigma, B. mori sigma-class GST; unclassified, B. mori unclassified GST; zeta, B. mori zeta-class GST. Data were obtained from previous reports ([13] for delta; [14] for epsilon; [15] for omega; [16] for sigma; [18] for unclassified; [17] for zeta). DCA, dichloroacetic acid.

GSTs catalyze a broad range of reactions, and each family member has its own discrete substrate specificity. This characteristic is also true for B. mori GSTs (Table 2). bmGSTT possesses GSH-conjugation activities toward EPNP and 4NPB, a property shared with mammalian theta-class GSTs. In contrast to hGSTT1-1, bmGSTT was not reactive with 4NBC and H$_2$O$_2$, suggesting that the catalytic properties of the bmGSTT enzyme are unique. bmGSTT did not recognize 4HNE, a cytosolic product of lipid peroxidation [33], or H$_2$O$_2$ as substrates, indicating that the enzyme is unlikely to participate in the response to oxidative stress. Intriguingly, although bmGSTT shares some substrate preferences with mammalian GSTT1s, it appears to have very different substrate specificity compared to other B. mori GSTs. Epsilon-class GSTs of mosquito could be involved in resistance to DDT and pyrethroid insecticides [34,35]. This resistance is particularly relevant given that HPLC analyses revealed that bmGSTT was unable to degrade the insecticides tested, in contrast to the results with other B. mori GSTs.

The GST amino acid sequence is divided into two regions, the N- and C-terminal domains [5]. The N-terminal domain includes the G-site, and the C-terminal domain has a hydrophobic substrate-binding site (H-site). The sequence diversity of the H-site dictates substrate selectivity [5]; moreover, this diversity likely explains the varied substrate specificity of B. mori GSTs, because there is considerable divergence between their C-terminal regions (alignments not shown). Our mutagenesis results suggest that residues Glu66 and Ser67 in bmGSTT play important roles in its catalytic functions. Notably, while mutation of His40 in bmGSTT did not alter the kinetics of catalysis, the equivalent residue in hGSTT1-1, bmGSTT was not reactive with 4NBC and H$_2$O$_2$, indicating that the enzyme is unlikely to participate in the response to oxidative stress. Intriguingly, although bmGSTT shares some substrate preferences with mammalian GSTT1s, it appears to have very different substrate specificity compared to other B. mori GSTs. Epsilon-class GSTs of mosquito could be involved in resistance to DDT and pyrethroid insecticides [34,35].

As mentioned above, the diversity of amino acids at the N- and C-terminal binding domains of GST is associated with substrate selectivity. hGSTT1-1 contains an H-site formed by Leu7, Leu35, Ile36, His40, Leu11, Trp115, Met119, Phe123, His176, Leu231, Thr234, Val235, and Met238 [32]. We found that only 3 of these 13 residues were conserved in the H-site of bmGSTT, which may explain the difference in substrate specificity between bmGSTT and hGSTT1-1. Additionally, a C-terminal helix in theta-class GSTs and residue 234 in the amino acid sequence of hGSTT1-1 play important roles in substrate specificity and catalysis.
respectively [31,32] [38]. There is no corresponding region, including the residue at position 234, in bmGSTT (Fig. 1), which may explain why it exhibits lower activity than rat, mouse, and human theta-class GSTs [39].

Recently, the electron-sharing network that contributes to the catalytic activity of GST has been described [40,41]. Based on an amino acid residue at position 64 that is functionally conserved in the GST classes [40], this network can be divided into type I and II classes. The type I electron-sharing network is exemplified by delta-, theta-, omega-, and tau-class GSTs, which contain an acidic amino acid residue at position 64, whereas the type II network GSTs (alpha, mu, and pi classes) have a polar amino acid residue. Glu66 is conserved in the sequence of bmGSTT; thus, this enzyme resembles a member of the type I network. The electron-
sharing network in hGSTT2-2 was proposed to contain Ser67 as one of residues involved in the network [41]. The equivalent residue in bmGSTT (Ser67) is conserved (Fig. 1). Glu66 and Ser67 in bmGSTT could be part of an electron-sharing network and the G-site via direct interaction with GSH. Thus, mutation of the residues may result in a decrease in GSH-conjugation activity. Thus, mutation of the residues may result in a decrease in GSH-conjugation activity. The equivalent sharing network in hGSTT2-2 was proposed to contain Ser67 as one of residues involved in the network [41]. The equivalent residue in bmGSTT is Ser11 (Fig. 1). In other GST classes, mutagenesis of amino acid residues in electron-sharing networks results in decreased activity [40,41]. Investigation of putative catalytic residues using site-directed mutagenesis is now underway in our laboratories.

Our results suggest that bmGSTT might play a role in detoxification of xenobiotics in *B. mori*. Together with bmGSTT, the roles of other GSTs in *B. mori* should be further examined to understand the mechanisms underlying insecticide detoxification. In turn, such studies will aid the design and implementation of insecticide-resistance management strategies for agricultural pests.

**Author Contributions**

Conceived and designed the experiments: KY NY. Performed the experiments: MDTH NY KY. Analyzed the data: MDTH NY KY. Contributed reagents/materials/analysis tools: MDTH NY KY. Wrote the paper: KY.

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**Table 3.** Comparison of kinetic data from bmGSTT and mutant forms.

|          | bmGSTT mutants |          |
|----------|----------------|----------|
|          | WT            | H40A     | V54A | E66A | S67A | R107A |
| **Km**a | 1.5 (0.25)    | 0.52 (0.08) | 28 (3.6) | 12 (2.1) | 4.3 (0.72) | 0.96 (0.11) |
| **kcat**b | 0.27 (0.020) | 0.12 (0.036) | 3.9 (0.84) | 0.88 (0.85) | 0.35 (0.022) | 0.16 (0.042) |
| **kcat/Km**c | 0.18 | 0.23 | 0.22 | 0.073 | 0.051 | 0.17 |
| **GSH** |          |          |          |          |          |          |
| **Km**a | 3.6 (0.56) | 1.8 (0.34) | 11 (1.9) | 12.9 (2.2) | ND | 3.9 (0.35) |
| **kcat**b | 0.15 (0.034) | 0.065 (0.018) | 0.52 (0.082) | 0.28 (0.077) | ND | 0.13 (0.022) |
| **kcat/Km**c | 0.041 | 0.036 | 0.049 | 0.022 | ND | 0.033 |
| **4NPB** |          |          |          |          |          |          |
| **Km**a | 0.13 (0.03) | 2.3 (0.22) | 0.50 (0.083) | ND | ND | 0.52 (0.071) |
| **kcat**b | 0.78 (0.17) | 3.1 (0.56) | 3.7 (0.61) | ND | ND | 1.3 (0.16) |
| **kcat/Km**c | 6.0 | 1.3 | 7.4 | ND | ND | 2.4 |

Values, except those of **kcat/Km** are expressed as means of three independent experiments.

*Expressed in units of mM; †expressed in units of μmol/L/min; ‡expressed in units of/min/mM. ND represents ‘not detected’.

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