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Full Length Research Paper

Purification and biochemical characterization of a novel glutathione *S*-transferase of the silkworm, *Bombyx mori*

Cheng Xiang Hou[1,2], Zhi Qiang Fu[3], Byung Rae Jin[4] and Zhong Zheng Gui[1,2]*

[1]Jiangsu University of Science and Technology, Zhenjiang 212018, China.
[2]Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, China.
[3]Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai 200232, China.
[4]College of Natural Resources and Life Science, Dong-A University, Pusan 604714, Korea.

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A novel glutathione S-transferase has been purified from *Bombyx mori* larvae using affinity chromatography on a glutathione agarose column. The purified enzyme appeared as a single band on SDS-PAGE and had a M_r of 28 kDa. Steady state kinetic assays of the enzyme were conducted with 1-chloro-2,4-dinitrobenzene as a substrate. The K_m, V_{max}, K_{cat} and K_{cat}/K_m for the purified *Bm*GST were 0.494 mM, 72.07 µmol/min/mg, 65.43 s^{-1} and 132.45 $mM^{-1}·s^{-1}$, respectively. The enzyme had a maximum activity at approximately pH 7.1 and 25ºC. *Bm*GST indicated lower inhibitory rate by some inhibitors (albendazol, praziquantel, bile acid and NaCl), suggesting that this novel *Bm*GST could differ structurally or functionally from other animal GSTs.

Key words: Purification, characterization, glutathione S-transferase, *Bombyx mori.*

INTRODUCTION

Glutathione S-transferases (GSTs, EC 2.5.1.18) are a family of multi-functional proteins that play an important role in the detoxification and biotransformation of a large array of noxious lipophilic and electrophilic compounds (Jakoby and Habig, 1980). These enzymes catalyze the conjugation of electrophilic molecules with reduced glutathione (GSH), generally making the resulting products more water soluble and excretable (Boyland and Chasseaud, 1969). The GSTs have long been demonstrated to be involved in intracellular transport of hormones, endogenous metabolites and exogenous chemicals and in the protection from oxidative damage and oxidative stress (Pickett and Lu, 1989; Enayati et al*.,* 2005).

Insects developed adaptations to protect themselves against potentially toxic compounds, such as insecticides. GSTs in insects, together with esterases and cytochrome P450 dependent monooxygenases, are focused on the metabolic detoxification of insecticides, which results in insecticide resistance. Elevated activity of these enzymes, commonly measured photometrically using artificial substrates, such as 1,2- dichloro-4-nitrobenzene (DCNB) and 1-chloro-2, 4-dinitrobenzene (CDNB), has been associated with resistance to all of the major classes of insecticides (Prapanthadara et al*.,* 1993; Huang et al*.,* 1998; Ranson et al*.,* 2001; Vontas et al., 2001; Ortelli et al*.,* 2003; Lumjuan et al*.,* 2005). GSTs can be induced by the administration of various xenobiotics to the animal (Pickett and Lu, 1989) and may confer resistance to numerous toxins when their activities are increased, including alkylating agents, herbicides and insecticides (Clark, 1990).

Insect GSTs have been divided into two major groups, GST1 and GST2 (Grant and Matsumura, 1989; Fournier et al., 1992; Snyder et al., 1995; Franciosa and Berge, 1995), and GST1 is serologically distinct from GST2 (Feng et al., 1999). The monomer size of GSTs is in general 23 - 28 kDa. The activity of insect GSTs has been determined to be present in the midgut (Tate et al., 1982; Snyder et al., 1995), fat body (Chien and Dauterman, 1991), hemolymph and other tissues (Franciosa and Berge, 1995).

The silkworm, *Bombyx mori*, is an economically important animal that has traditionally been used for silk production and also as a model animal for genetic studies. They have also been used for production of re-

*Corresponding author. E-mail: srizzgui@hotmail.com. Tel: 86-511-8561-6716.

combinant proteins through the baculovirus expression system (Maeda 1989; Lee et al., 2006). Since 70% of agricultural insect pests are lepidopteran insects, the silkworm as a model for lepidopteran insects is also useful to obtain data on its detoxification capacity for application with other pests. In spite of the particular interest in the economic importance of the silkworm, there has been poor documentation of the characterization, functional role and gene structure of *B. mori* GST (*Bm*GST). Recently, previous studies have shown the cloning of a cDNA for GST from the fat body (Yamamoto et al., 2005, 2006) and midgut (Gui et al., 2008) of the silkworm. In the present study, our initial objective is to further understand this characterization and identify the functional role of GST in *B. mori*. We purified a novel *Bm*GST enzyme from the silkworm larvae using affinity chromatography and characterized its kinetic properties. We also explored the activity of some GST inhibitors on *Bm*GST.

MATERIALS AND METHODS

Experimental insects

Larvae of the silkworm, *B. mori*, used in this study were F_1 hybrid Suzhen × Chunguang supplied by the Sericultural Research Institute, Chinese Academy of Agricultural Science, China. Silkworms were reared on fresh mulberry leaves.

Chemicals

Reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), bovine serum albumin (BSA) and GSH-agarose were purchased from Sigma Chemical Co. Reduced glutathione (GSH) was used as a distilled water solution. 1-chloro-2, 4-dinitrobenzene (CDNB) solution was prepared in ethanol. Other chemicals were of the highest purity commercially available.

Preparation of crude extract

Whole larvae were homogenized at 4ºC in a ratio of 1:4 (w/v) with 100 mM sodium phosphate buffer, pH 7.6. The homogenate was centrifuged at 10,000 g for 10 min and the pellet was discarded. The supernatant was filtered through glass wool and then recentrifuged at 100,000 g for 60 min. Again, the pellet was discarded, the supernatant was filtered through glass wool and the cytosolic glutathione S-transferase was obtained. The enzyme solution was stored in the cold at -20°C until needed.

Purification of GST by glutathione agarose affinity chromatography

The crude extract proteins were precipitated overnight at 4ºC with buffer A, containing 50 mM NaCl, 1 mM EDTA, 1 mM PMSF and 5 mM DTT, pH 7.0. The homogenate was centrifuged for 30 min at 9,000 *g*. Then the supernatant was filtered through two layers of cheesecloth and centrifuged for 1 h at 105,000 *g* (max). The fat-free supernatant was applied to a PD10 column (Pharmacia) before affinity chromatography. The affinity column was packed with epoxy-activated GSH-agarose. The column was eluted with buffer B (containing 100 mM H_3BO_3, pH 8.7, and 100 mM NaAc, pH 4.0), then equilibrated with buffer C (PBS, pH 7.0) and finally washed with buffer D (PBST, containing 1% Triton X-100, pH 7.0). GST was then eluted with 7 mM GSH in 50 mM Tris-HCl buffer, pH 9.6. The fractions with GST activity were pooled and subsequently used for kinetic and electrophoretic studies.

Enzyme assays and protein determination

The GST activity was determined spectrophotometrically with CDNB as a substrate by monitoring the change in absorbance at 340 nm due to thioether formation at 25ºC as described by Habig et al. (1974). Protein concentrations were determined according to the method of Bradford (1976), with Coomassie Brilliant Blue G-250 (CBB G-250) and bovine serum albumin (BSA) as a standard. One unit of transferase activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of thioether per min and the specific activity is expressed as μmol/min/mg protein.

SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970) using a Bio-Rad Mini Protean II cell. Samples were diluted with an equal volume of 100 mM Tris–HCl containing 4% SDS, 0.002% bromophenol blue, 0.2% 2-mercaptoethanol and 40% glycerol. The diluted samples were heated to 100°C for 5 min before electrophoresis. Separation gels were 12% acrylamide in 0.5 M Tris-HCl, pH 8.8. Stacking gels were 3% acrylamide in 1.5 M Tris-HCl, pH 6.8. Electrophoresis was carried out at 70 V and 20 mA for 30 min and then resolved at 110 V for 2 h in running buffer contained 2 M glycine and 0.1% SDS in 0.4 M Tris-HCl, pH 8.3. The gels were stained with Coomassie Brilliant Blue. These data were analyzed by linear regression to produce an equation that was subsequently used to estimate the molecular mass of the purified GST by plotting the migration distance of molecular weight standards (14 – 116 kDa).

Kinetics studies

The kinetic parameters K_m and V_{max} were determined for purified *Bm*GST using reduced glutathione and CDNB as substrates. When CDNB constants were measured, the GSH concentration was held at 5 mM while the CDNB concentration was varied from 0.1 to 1.6 mM. When GSH constants were measured, the CDNB concentration was held at 1 mM while the GSH concentration was varied from 0.2 to 2.4 mM. Maximal velocity (V_{max}) and Michaelis constant (K_m) values for each substrate were calculated from Lineweaver-Burk double reciprocal plots.

Effect of pH on stability

The pH optimum was determined for CDNB conjugation activity. Purified *Bm*GST was incubated at 25°C for 1 min in sodium phosphate buffer at pH 5.4, 6, 6.5, 7.1 and 7.6 and Tris–HCl buffer at pH 8.5 and 9 (all buffers were 50 mM). Conjugation activity was determined as described above. Three replicates were conducted.

Effect of temperature

The effect of temperature on CDNB conjugation reaction catalyzed by *Bm*GST was examined under the standard assay conditions. The reaction was carried out at temperatures ranging from 15 to 70ºC and the residual activity of the enzyme was measured.

Table 1. Purification of glutathione S-transferase from *Bombyx mori*.

Step	Total protein (μg/ml)	Total activity (μmol/min/ml)	Specific activity (μmol/min/mg)	Fold purification	Recovery (%)
Crude enzyme extract	1106.380 ± 14.948	0.806 ± 0.068	0.728 ± 0.062	1	100
GSH affinity purified	56.204 ± 4.902	0.259 ± 0.012	4.610 ± 0.217	6.332	32.134

Inhibition studies

The enzyme activity of purified *Bm*GST was performed for inhibition patterns according to the methods of Ge and Singh (1996). Albendazol, Praziquantel and bile acid were dissolved in the assay buffer and NaCl was dissolved in diluted water. Experiments were repeated at least twice and each assay was run in triplicate.

RESULTS

Purification of BmGST

The results of the purification of glutathione transferase from the silkworm are summarized in Table 1. GST specific activity (CDNB conjugation) increased 6.33-fold after separation by GSH affinity chromatography from the crude homogenate. A large portion of material with CDNB conjugating activity passed through the GSH affinity column. The specific activity of the purified glutathione transferase was 4.61 μmol/min/mg. The purified enzyme was homogeneous, as determined by SDS-PAGE with silver staining (Figure 1). SDS-PAGE of the pooled fractions produced a single band with a molecular retention at 28,000 Da.

Kinetic properties of BmGST

The kinetic properties of affinity purified glutathione transferase from *B. mori* were studied. Line weaver-Burk plot of enzyme-catalyzed reactions with varying concentrations of glutathione (0.2-2.4 mM) or CDNB (0.1-1.6 mM) at a fixed concentration of CDNB (1 mM) or glutathione (5 mM), respectively, are shown in Figure 2. The K_m and V_{max} values were calculated and are represented in Table 2. Given a molecular mass of 28 kDa for *Bm*GST, the catalytic constants, K_{cat}, were 57.87s^{-1} and 65.43 s^{-1} for glutathione and CDNB, respectively.Thus, the enzyme's catalytic efficiencies (K_{cat}/K_m) were 118.10 and 132.45 for glutathione and CDNB, respectively (Table 2).

pH stability

The effect of pH on the stability of *Bm*GST from pH 5.4 to 7.6 (sodium phosphate buffer) and pH 8.5 and 9 (Tris–HCl buffer) are shown in Figure 3. The pH optimum for the *Bm*GST with CDNB as a substrate was found to be 7.1.

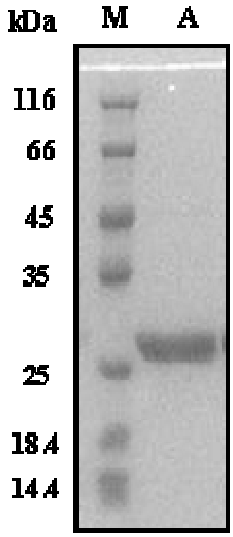

Figure 1. SDS-polyacrylamide gel electrophoresis of the purified *Bm*GST enzyme. Lane M, standard molecular weight marker; Lane A, purified *Bm*GST.

Effect of temperature

At temperatures ranging from 15 to 70ºC, the enzymatic activity of *Bm*GST increased with increasing temperature up to 25ºC, then the activity starts to decrease with increasing temperature (Figure 4).

Inhibition studies

Inhibition studies were performed with purified *Bm*GST in order to better understand the structure-function relationship with the relative inhibition of *Bm*GST. The *Bm*GST inhibition pattern is shown in Table 3. The strongest inhibition of 18% was observed with albendazol and the weakest of 42% with bile acid. *Bm*GST from different sources showed differences in inhibition.

DISCUSSION

All of the insect GST was retained on the GSH-affinity column and the reconstituted homodimers demonstrated activity towards CDNB. The results of the present study clearly demonstrate that the silkworm glutathione transferase can be purified to apparent homogeneity effec-

Table 2. Kinetic constants for *Bm*GST with GSH and CDNB substrates.

Substrate	K_m (mM)	V_{max} (μmol/min/mg)	K_{cat} / K_m ($mM^{-1}.s^{-1}$)
CDNB	0.494	72.07	132.45
GSH	0.490	64.30	118.10

* K_{cat} was calculated using the empirically derived homodimer molecular weight (i.e. 54,000).

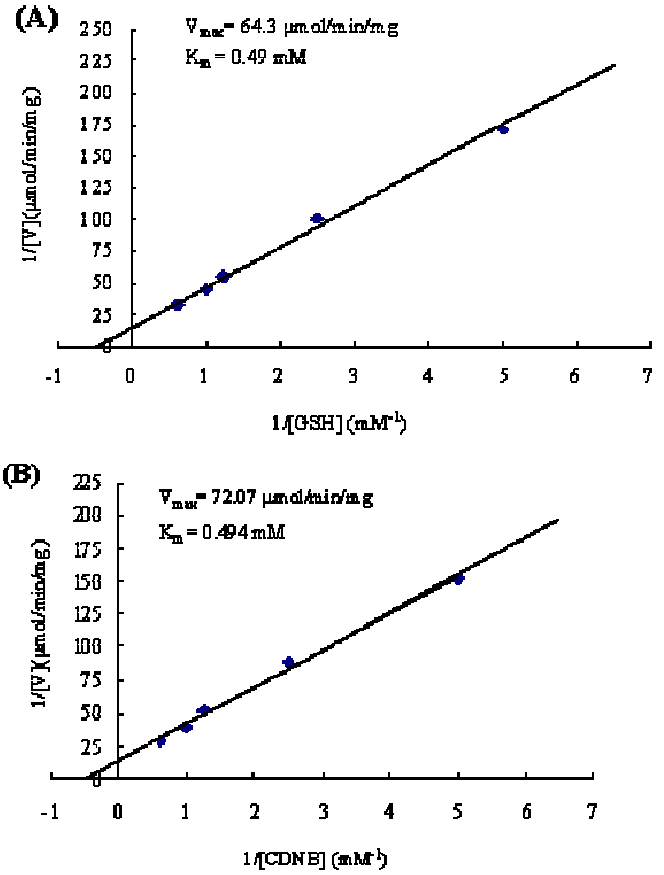

Figure 2. Lineweaver-Burk plot of enzyme activity of the purified *Bm*GST with varying concentrations of (A) glutathione (0.2-2.4 mM) or (B) CDNB (0.1-1.6 mM) at a fixed concentration of CDNB (1 mM) or glutathione (5 mM), respectively. Values are means based on at least 3 replicates.

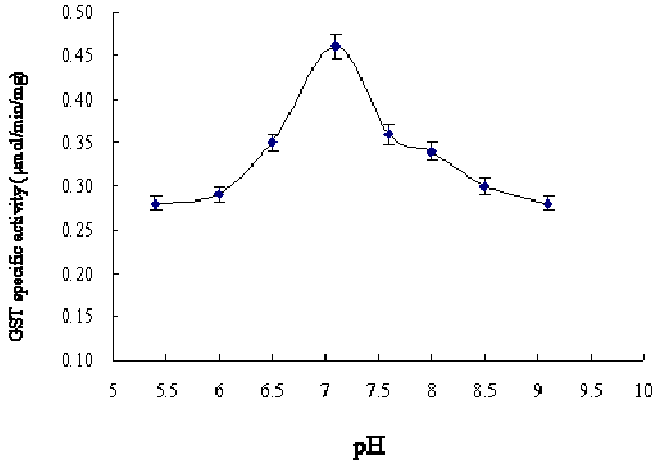

Figure 3. Effect of pH on the purified *Bm*GST activity. All buffers were 50 mM and consisted of sodium phosphate buffer for pH 5.4, 6, 6.5, 7.1 and 7.6 or Tris–HCl buffer for pH 8.5 and 9.

tively by affinity chromatography with GSH-agarose, resulting in as much as 6.33-fold purification. Although this is low, it is similar to values obtained with other purification procedures in other insects, such as *Trichoplusia ni* (Hübner), *Spodoptera frugiperda* (Smith) and *Tenebrio molitor* (Yu, 1989; Kostaropoulos et al., 1996). The yield was 32% of the cytosolic glutathione transferase activity. It was quite high compared to the purification of GST enzyme from other insect species, which were from 3 to 26% in *Hyphantria cunea* (Yamamoto, 2007) and other lepidopterous species (Yu, 1989) and similar to 34% in *T. molitor* (Kostaropoulos and Papadopoulos, 1998). It is reported that several factors have caused the low recovery rates of glutathione transferases, such as excessive amounts of the enzyme applied to each column, aging of the GSH-agarose and inactivation of the enzyme during chromatography (Yu, 1989). The specific activity of the purified glutathione transferase from the silkworm was 4.61 μmol/min/mg protein, which is higher than *Anticarsiu gemmatalis* (Yu, 1989) and two syrphid flies (Vanhaelen et al., 2004) and lower than other reported insect species from several lepidopteran insects (Yu, 1989) and *Solenopsis invicta* (Valles et al., 2003).

Since insect glutathione transferases exist as dimers (homodimers and heterodimers) (Cohen, 1987; Cochrane et al., 1987; Grant and Matsumura, 1988), the molecular weights of the native glutathione transferases would likely range from 50,000 to 60,000 based on their subunit molecular weights (Motoyama and Dauterman, 1977, 1978; Cochrane et al., 1987; Toung et al., 1990). These estimates are close to the sum of two subunits. Our previous finding has shown that two subunits, GST1 and GST2, were cloned from the silkworm midgut (Gui et al., 2008) and the molecular weight of native *Bm*GST is about 54,000. In this study, the molecular weight of glutathione transferase purified from the silkworm is 28,000 Da, which is similar to those isolated from different lepidopteran insect species (Yu, 1989). It suggests that this is possibly a novel *Bm*GST. Due to the small quantities of purified glutathione transferase abtain-

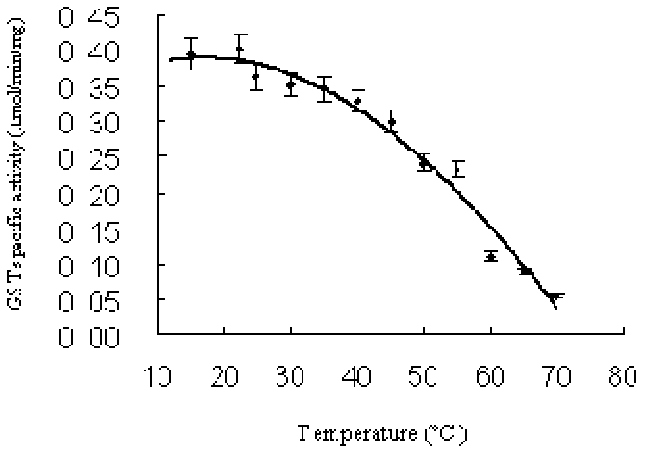

Figure 4. Effect of temperature on CDNB-GSH conjugation catalyzed by *B. mori* GST. Values are means based on at least 3 replicants.

ed from the silkworm, attempts to determine the molecular weights of their native forms using Sephadex G-100 were unsuccessful.

The purified *Bm*GST was enzymatically characterized with CDNB and GSH as substrates. The pH optimum for activity of *Bm*GST was found to be 7.1. It is slightly different from GSTS1 and GSTT1 reported by Yamamoto et al. (2006, 2007). This may partly be attributed to differences in the amino acid sequences between these GSTs. The K_m, V_{max}, K_{cat} and K_{cat}/K_m for the silkworm GST toward CDNB and GSH were comparable to values reported from other insect GSTs (Prapanthadara et al., 1996; Ma et al., 1999; Valles et al., 2003). The K_m and V_{max} of *Bm*GST were higher than other lepidopterous insects (Yu, 1989) and most similar to corresponding constants reported in GST_3 from *Bulinus truncates* (Abdalla et al., 2006). Comparing the K_{cat}/K_m values with other insect GSTs, the *Bm*GST has a lower catalytic efficiency for both GSH and CDNB. The apparent K_m values for glutathione and CDNB appeared to be similar, while the apparent V_{max} value for CDNB was higher than that for glutathione. This suggests that *Bm*GST had a higher affinity for glutathione than for CDNB.

Glutathione S-transferases play a crucial role in resistance to organophosphate (fenitrothion) and pyrethriods (deltamethrin and permethrin) (Hemingway, 2000). In addition to the interaction between inhibitors and *Bm*GST, we performed inhibition assays and found that the activity of *Bm*GST was inhibited to various extents by several inhibitors. This result is similar to those found in the Northern Quahog, *Mercinarin mercinaria* (Blanchette and Singh, 1999), and other insects (Prapanthadara et al., 1998; Da Silva Vaz et al., 2004).

In conclusion, we have purified and characterized a novel GST from silkworm larvae. Our study broadens the biochemical information on insect GSTs by demonstrating the role for *Bm*GST in defense mechanisms against some inhibitors. It could serve as a basis for future studies on GST in insects by providing the biochemical characterization of *Bm*GST.

Table 3. Comparison of inhibition patterns of purified *Bm*GST.

Condition	Inhibition (%)
Albendazol	17.87
NaCl	26.22
Praziquantel	31.31
Bile acid	41.78

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