Expression and Characterization of a Sigma-Class Glutathione S-Transferase of the Fall Webworm, *Hyphantria cunea*

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A cDNA encoding glutathione S-transferase (GST) of the fall webworm, *Hyphantria cunea*, was cloned by reverse transcriptase-polymerase chain reaction. The resulting clone (hcGST) was sequenced and deduced for amino acid sequence, which revealed 87, 59, and 42% identities to Sigma-class GSTs from *Bombyx mori*, *Manduca sexta*, and *Blattella germanica* respectively. A recombinant hcGST protein (rhcGST) was functionally overexpressed in *Escherichia coli* cells in a soluble form and purified to homogeneity. rhcGST retained more than 75% of its original GST activity after incubation at pHs 6 to 11. Incubation for 30 min at temperatures above 75°C scarcely affected the activity. rhcGST was able to catalyze the reaction of glutathione with 1-chloro-2,4-dinitrobenzene, a universal substrate for GST, as well as with 4-hydroxynonenal, a product of lipid peroxidation. We also found that as compared to *B. mori* Sigma-class GST, rhcGST had a higher affinity for fenitrothion, an organophosphorus insecticide.

Key words: *Hyphantria cunea*; lepidoptera; glutathione S-transferase; glutathione; lipid peroxidation

Glutathione S-transferases [EC 2.5.1.18] are cytosolic enzymes widely distributed in both prokaryotic and eukaryotic cells, comprising a major family of detoxification enzymes by catalyzing the conjugation of reduced glutathione (GSH) with xenobiotics.1,2) Mammalian GSTs have been grouped into seven classes named Alpha, Mu, Pi, Sigma, Theta, Zeta, and Omega.3) In insects, GSTs are of interest for their ability to metabolize various insecticides and pesticides, and have been identified from various species. On the basis of the classification criteria for GSTs of dipteran insects, six different GST classes, designated Delta, Sigma, Epsilon, Theta, Omega, and Zeta, have been recognized.4,5) Insect Delta-class GSTs have been characterized in *Musca domestica*, *Drosophila melanogaster*, *Anopheles gambiae*, *Anopheles dirus*, and *Lucilia cuprina*.6–9) Insect Sigma-class GSTs have been found in *Manduca sexta*, *Bemisia tabaci*, *Solenopsis invicta*, *Blattella germanica*, and *D. melanogaster*.7,9–12) In addition, an insect Epsilon-class GST has been established in *D. melanogaster*.13) GSTs in lepidopteran remain poorly documented in comparison with those in other insects, particularly in dipteran species. Only recently has a major effort been made to characterize the Delta- and Sigma-class GSTs of *B. mori* in our laboratories.14,15) The present paper deals with a GST of another lepidopteran species, the fall webworm, *Hyphantria cunea*, one of the most serious insect pests of broad-leaved trees. *H. cunea* often causes damage to mulberry fields cultivated for *B. mori* rearing. We cloned and sequenced a cDNA encoding a Sigma-class GST of *H. cunea* (hcGST), and overexpressed it as a recombinant protein (rhcGST) in *Escherichia coli* cells to compare its properties with those of the *B. mori* Sigma-class homolog, GSTS1.15)

Materials and Methods

Insects. Fourth-instar larvae of the fall webworm *H. cunea* were collected in a mulberry field of the Institute of Genetic Resources, Kyushu University Graduate School, Fukuoka, Japan. They were dissected on ice, and whole bodies except for the intestines and their contents were rapidly extracted for total RNA with Sepasol-RNA 1 (Nacalai Tesque, Kyoto, Japan) according to the manufacturer’s instructions.

Cloning and sequencing of the cDNA encoding hcGST. Total RNA isolated from *H. cunea* larvae was...
subjected to the reverse transcriptase-polymerase chain reaction (RT-PCR). First-strand cDNA was produced using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and an oligo-dT primer. The resulting cDNA was used as a template to amplify a DNA fragment by PCR with the following two oligonucleotide primers: 5'-GCTACTGAACATTTTCATATTG-3' (sense) and 5'-CTTTTGTTGACAAGGAACCAATCTA-3' (antisense). These were designed based on the partial sequence of the Sigma-class GST cDNA of the silkworm (GenBank, accession no. AB206971) and the results of a search of the SilkBase EST database.\textsuperscript{16}

PCR was conducted for one cycle at 94°C for 2 min, then 35 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min, followed by one cycle at 72°C for 10 min. The resulting hcGST cDNA (hcgst) was ligated into pGEM-T Easy vector (Promega, Madison, WI) for DNA sequencing, which was carried out with a Thermo Sequenase Cycle Sequencing kit (USB, Cleveland, OH) using a DNA sequencer (LIC-4200, ALOKA, Tokyo, Japan). DNAsis software (ver. 3.4) was used for sequence analysis. Homology alignment was performed by CLUSTALW (ver. 1.83), with 10 and 0.2 to the values of the gap creation penalty and the gap extension respectively. Preparation of the phylogenetic tree was done by Neighbor-joining plot software (\url{http://www-igbmc.u-strasbg.fr/Bioinfo/ClastulX/Top.html}).

Overexpression and purification of recombinant protein. The hcgst cDNA was cloned into pGEM-T Easy vector, as described above. Using two oligonucleotide primers, 5'-TAAACCCGCATAGCCGAACGTAAAGTTCTA-3' (sense) and 5'-ATGGATCTTTAAAGTTCTGGTTCCTGGTGC-3' (antisense), PCR was carried out under the conditions described above; the underlined and double-underlined restriction sites are \textit{NdeI} and \textit{BamHI} restriction enzyme sites respectively, incorporated for the purpose of subcloning the PCR product into an expression plasmid vector. After digestion of the PCR product with \textit{NdeI} and \textit{BamHI}, the resulting fragment was subcloned into the \textit{NdeI-BamHI} site of the expression vector pET-11b (Novagen). This prepared expression plasmid harboring hcgst was transformed into competent \textit{E. coli} BL21-CodonPlus (DE3)-RIL cells (Stratagene, La Jolla, CA), which were grown at 37°C on Luria-Bertani (LB) media containing 100\,\mu g/mL ampicillin. After the cell density reached 0.7 OD\textsubscript{600}, isopropyl 1-thio-\beta-D-galactoside (IPTG) was added to a final concentration of 1\,\text{mM} to induce the production of recombinant protein. After further incubation for 3 h, cells were harvested by centrifugation, homogenized in 20\,\text{mM} Tris–HCl buffer, pH 8.0, containing 0.5 \text{mM} NaCl, 4\,\text{mg/mL} of lysozyme and 1\,\text{mM} phenylmethanesulfonyl fluoride (PMSF), and disrupted by sonication. Unless otherwise noted, all operations described below were conducted at 4°C. The supernatant was clarified by centrifugation at 10,000 \times g for 15 min and applied to ammonium sulfate fractionation between 30% and 70% saturation. The pellet was suspended in 10\,\text{mM} sodium phosphate buffer, pH 6.5, containing 0.1 \text{mM} PMSF and 1\,\text{mM} ammonium sulfate. After centrifugation, the supernatant was loaded onto a Butyl Toyopearl 650M (Tosoh, Tokyo, Japan) column equilibrated with the same buffer. After it was washed with the same buffer, the column was eluted with linear gradient of ammonium sulfate from 1 to 0\,\text{mM}. Fractions containing the enzyme were pooled, dialyzed against 10\,\text{mM} sodium phosphate buffer, pH 6.5, and subjected to anion-exchange chromatography on a CM-Sepharose (GE Healthcare, Tokyo, Japan) column, which was eluted with a linear gradient of NaCl from 0 to 0.3\,\text{M}. For estimation of the purity and molecular size of the enzyme, SDS polyacrylamide gel electrophoresis (SDS–PAGE) was done according to the method of Laemmli (1970).\textsuperscript{17} Protein bands were visualized by staining with Coomassie Brilliant Blue R250 (CBB).

**Measurements of enzyme activity and protein amount.**

GST activity was spectrophotometrically determined as described previously,\textsuperscript{18} with slight modifications. The standard assay mixture (100\,\mu l) contained 1.0 \text{mM} 1-chloro-2,4-dinitrobenzene (CDNB), 5 \text{mM} GSH, and enzyme, made up in 50\,\text{mM} Tris–HCl buffer, pH 8.0. This was incubated at 30°C and measured for the rate of change in OD\textsubscript{340} per min. A non-enzymatic reaction as a control was done using the reaction mixture in the absence of enzyme solution. The specific activity of GST was expressed as mol CDNB conjugated with GSH per min per mg protein using \(k_{340} = 9,600 \text{m}^{-1}\text{cm}^{-1}\), the molar extinction coefficient of the resulting 2,4-dinitrophenyl-glutathione.\textsuperscript{18} When 4-hydroxy-2-nonenal (4-HNE) was used in place of CDNB, the absorbance change per min was converted into mol substrate conjugated per min using a molar extinction coefficient of \(k_{224} = 13,750 \text{m}^{-1}\text{cm}^{-1}\).\textsuperscript{18–20} When ethacrynic acid (ECA) and 4-nitrophenyl acetate (4-NPA) were exploited instead of CDNB, the assay was performed by the method described in previous reports.\textsuperscript{18–20} Competitive assays were carried out with the standard assay mixture containing CDNB and GSH in the presence of various concentrations of inhibitors. Protein was determined using a Protein Assay kit (Bio-Rad, Hercules, CA) with bovine serum albumin as a standard protein.

**Results**

**Cloning and sequencing of cDNA encoding hcGST**

The cDNA encoding hcGST was obtained by RT-PCR using total RNA from \textit{H. cunea} larvae with primers designed from the \textit{B. mori} Sigma-type GST sequences, and determined for nucleotide sequence (data were deposited in GenBank under accession no. AB223045). This contained an open reading frame of 612\,bp, encoding 203 amino acid residues (Fig. 1), whose theoretical molecular mass and pI were evaluated to be 23,204 and 8.75 respectively. The deduced amino
acid sequence of this putative GST showed 87, 59, and 42% identities to Sigma-class GSTs from *B. mori*, *M. sexta*, and *B. germanica* respectively (Fig. 2; the *B. mori* enzyme was named GSTS1 in our previous report, Yamamoto et al., 2006).\(^{15}\) We concluded that the clone hcgst represents a Sigma-class enzyme (hcGST).

Highly conserved in the protein was a tyrosine residue at position 8 of the N-terminal domain (solid triangle), which is known to be crucial for the catalytic mechanism of GST. The sites involved in the GSH-binding (G-sites) were also highly conserved in the sequence of hcGST (boxed letters).\(^{21}\) Five electrophilic binding sites were found in hcGST, whereas three such residues were seen in *B. mori* GSTS1 (bold letters).\(^{21}\)

Based on the phylogenetic tree generated from the aligned amino acid sequences of GSTs, the present hcGST sequence was closest to that of *B. mori* GSTS1 (Fig. 3), although the homology to GSTS1 was relatively low in the N-terminal region (see Fig. 2).\(^{15}\)

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**Fig. 1.** Nucleotide and Deduced Amino Acid Sequences of Sigma-Class GST of the Silkworm.

Residues of the nucleotide and the deduced amino acid sequences are numbered at the right and left sides. The asterisk indicates the termination codon. The nucleotide sequence has been registered at GeneBank under accession no. AB223045.

**Fig. 2.** Alignment of Amino Acid Sequences of Insect Sigma-Class GSTs.

Sequences of GSTs from different organisms were from Swiss-Prot databases: Hcun, *H. cunea* (determined in the present study); Bmol, *B. mori* (no. Q5CCJ4, representing GSTS1; Yamamoto et al., 2006); Msex, *M. sexta* (no. P46429); Bger, *B. germanica* (no. O18598). An asterisk represents identical amino acids and a dash denotes a deletion. Conserved G-site residues are boxed. The closed triangle indicates conserved tyrosine residue at position 8, and the bold letter shows the electrophilic binding site.
Overexpression and purification of rhcGST

hcgST was overexpressed as a recombinant protein (rhcGST) using the E. coli expression vector. SDS–PAGE analysis of E. coli cell lysate revealed that rhcGST was in a soluble form (Fig. 4A). The specific activity of GST (towards CDNB) of the lysate containing rhcGST was about nine-fold higher than that from E. coli cells without hcgst. rhcGST was purified to homogeneity by ammonium sulfate fractionation, hydrophobic-interaction chromatography, and cation-exchange chromatography. The purified protein migrated with an apparent molecular weight of 24,000 (Fig. 4B); this value was consistent with that calculated from the deduced amino acid sequence. The results of the purification procedures are summarized in Table 1. Finally we obtained 5.8 mg of highly purified rhcGST from 250 ml of LB medium. The specific activity of the final preparation toward CDNB was 4.4 \( \mu \text{mol/min/mg}. \)

Characterization of rhcGST

The enzymatic properties of hcgST were determined using the purified rhcGST preparation with CDNB and GSH as substrates, and compared with those of silk-worm GSTS1, examined in our previous study.15) The pH optimum of rhcGST was found to be about 8.0, similar to that of GSTS1 (Fig. 5A). With respect to thermostability, rhcGST differed from GSTS1; rhcGST was stable at temperatures below 50°C, whereas GSTS1 lost half of its original activity at 50°C, and was stable at temperatures below 40°C (Fig. 5B). Analysis of pH stability (Fig. 5C) indicated that rhcGST and GSTS1 retained more than 75% of their original activities at pHs 6 to 11 and 4 to 11 respectively, and that rhcGST was less stable than GSTS1 at pHs lower than 4.

The kinetic parameters of rhcGST and GSTS1 were evaluated using CDNB and 4-HNE as GSH-conjugating substrates. As summarized in Table 2, for each substrate, the \( K_m \) value of rhcGST was almost one-quarter of that of GSTS1. When CDNB was used as a substrate, there was only a small difference in \( V_{\text{max}} \) values between rhcGST and GSTS1. When 4-HNE was used as a substrate, however, the \( V_{\text{max}} \) value of rhcGST was one-sixth of that of GSTS1. Based on a comparison of \( V_{\text{max}}/K_m \) values, the catalytic efficiency of rhcGST for

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**Table 1. Summary of Purification of rhcGST from E. coli Cells**

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Total protein (mg)</th>
<th>Total activity (( \mu \text{mol/min} ))</th>
<th>Specific activity (( \mu \text{mol/min/mg} ))</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude lysate</td>
<td>104</td>
<td>83.2</td>
<td>0.8</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>48</td>
<td>52.8</td>
<td>1.1</td>
<td>63.5</td>
</tr>
<tr>
<td>Butyl Toyopearl</td>
<td>20.3</td>
<td>36.6</td>
<td>1.8</td>
<td>44.0</td>
</tr>
<tr>
<td>CM-Sepharose</td>
<td>5.8</td>
<td>25.4</td>
<td>4.4</td>
<td>30.5</td>
</tr>
</tbody>
</table>

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**Fig. 3. Phylogenetic Analysis of GST Amino Acid Sequences.**

The phylogenetic tree was made with neighbour-joining plot software using GST sequences cited from Swiss-Prot: these include delta-class GSTs from B. mori (no. Q60GK5), A. gambiae (no. Q93113), D. melanogaster (no. P20432), and M. domestica (no. P28338), and sigma-class GSTs from A. gambiae (no. P46428) and the other organisms cited in the legend to Fig. 2. Numbers attached indicates branch length.
CDNB was found to be notably high. Both ECA and 4-NPA were unfavorable substrates of rhcGST and GSTS1 (data not shown).

Since CDNB was a favorable substrate for both rhcGST and GSTS1 (Table 2), using this compound as a substrate, the inhibitory effects of model insecticides on the enzymes were examined. As shown in Fig. 6, the effects of fenitrothion on the activity of rhcGST was remarkably different from those of the activity of GSTS1. An increase in the amounts of fenitrothion from 10 nM to 10 μM halved the activity of rhcGST, but was mostly ineffective against the activity of GSTS1.

![Fig. 4. Electropherograms of rhcGST Overexpressed in E. coli Cells and after Purification.](image1)

![Fig. 5. Basic Enzymatic Properties of rhcGST Assayed with CDNB and GSH as Substrates.](image2)

**Table 2.** Comparison of Kinetic Parameters between rhcGST and GSTS1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>CDNB</th>
<th>4-HNE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$</td>
<td>$V_{max}$</td>
</tr>
<tr>
<td>rhcGST</td>
<td>0.41 ± 0.07</td>
<td>6.99 ± 0.78</td>
</tr>
<tr>
<td>GSTS1</td>
<td>1.57 ± 0.12</td>
<td>4.76 ± 0.36</td>
</tr>
</tbody>
</table>

*a* All values except those of $V_{max}/K_m$ are means of three independent experiments.

*b* Expressed in units of μM.

*c* Expressed in units of mmol/mg/min.

*d* Calculated from the corresponding values of $K_m$ and $V_{max}$. 

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On the other hand, the effects of permethrin and deltamethrin on the activities of rhcGST and GSTS1 were similar. In the presence of 1 mM permethrin, both of the enzymes showed 25% of their original activities. In the presence of 1 mM deltamethrin, the activities of both the enzymes were halved.

**Discussion**

To our knowledge, the present study is the first to report GST from *H. cunea*. Molecular cloning of the cDNA encoding hcGST was done. The deduced amino acid sequence from the cDNA clone was found to be related to those of Sigma-class insect GSTs, as seen in the phylogenetic tree: 87, 59, and 42% identities to the enzymes from *B. mori*, *M. sexta*, and *B. germanica* respectively. In contrast, the sequence of hcGST showed lower homology to Delta-class GST: 29, 30, and 32% homologies to the enzymes from *B. mori*, *D. melanogaster*, and *M. domestica* respectively. It has been reported that the tyrosine residue in the N-terminal region is catalytically essential and that it is well conserved in Sigma-class GST and mammalian Alpha, Mu, and Pi-class GSTs, whereas serine is substituted for tyrosine in Theta-class GST. 22–24) It was confirmed that the N-terminal tyrosine residue is conserved in hcGST.

Active rhcGST was successfully overexpressed in a soluble form in *E. coli* cells. There were insignificant differences in the molecular sizes of rhcGST as between the value calculated from the deduced amino acid sequence and that measured by SDS-PAGE: 23,204 and 24,000 from sequence and SDS-PAGE respectively. These sizes were similar to those of GSTs isolated from other insects: *M. sexta*, *B. tabaci*, and *S. invicta*. 7,10,11) Although the pH-activity profile of rhcGST did not completely overlap that of GSTS1, there was no difference in optimum pHs. rhcGST was different from GSTS1 with respect to temperature and pH stabilities. Especially, the residual activity of rhcGST after incubation at 50°C was almost twice that of GSTS1. Since the silkworm has long been domesticated, this might reflect the difference in living environments between the two insects. In contrast, GSTS1 was more stable than rhcGST in the acidic pH region. There were differences in two N-terminal motifs between hcGST and GSTS1; these corresponded to motifs of GSTS1 from alanine-13 to asparagine-31 and from lysine-43 to glycine-49. It was inferred that the difference in stability between hcGST and GSTS1 is partly to be attributed to the difference in their N-terminal structures. rhcGST showed GSH-conjugating activity towards CDNB, and its catalytic efficiency was greater than those of other Sigma-class GSTs. 10,11,21) ECA is a substrate for Pi, Mu, and Alpha-class GSTs, but not for Sigma-class GST of *D. melanogaster*. 21) On the other hand, the Delta-class GST of the silkworm conjugated GSH to ECA. 14) ECA was an unfavorable substrate for rhcGST. These results also indicate that hcGST is to be classified in the Sigma-class GSTs. 4-HNE is a cytotoxic product of lipid peroxidation under conditions of oxidative stress, 25) but it is associated with signalling functions, cell proliferation, and apoptosis. 26,27) From the standpoint of catalytic efficiency, CDNB was a much better substrate than 4-HNE, but both were favorable substrates. It is thus possible that hcGST plays an important role related to 4-HNE metabolism in the fall webworm. Living organisms are exposed to foreign chemical substances, including herbicides, chemotherapeutic agents, and insecticides, and they require protection mechanisms from such toxic materials. It has been reported that GST is induced in physiological responses to perturbation and functions as a stress response protein, 9,26) being involved, e.g., in protection of cells from hydroperoxides generated by oxidative stress. 28) Hence it was interesting that the amounts of GST mRNA increase in the midgut of *M. sexta* fed diets containing xenobiotics. 29)
Results from inhibition experiments on rhcGST and GSTS1 with fenitrothion, permethrin, and deltamethrin indicated that all these insecticides at around 1 mM inhibit the enzymes considerably, and that residual activity decreases similarly with increasing amounts of insecticide, except for inhibition of GSTS1 by fenitrothion. Although not enough information on the inhibitory effects of these insecticides on GST is yet available, previous reports indicate that GSTs participate in resistance to various organophosphorous compounds and pyrethroids in insect species such as Haemaphysalis longicornis and Rhipicephalus appendiculatus;\(^1\)^ and that the GST activities of these species were reduced to 85% and 60% respectively by 0.1 mM fenitrothion. Although not enough information on the inhibitory effects of these insecticides on GST is yet available, previous reports indicate that GSTs participate in resistance to various organophosphorous compounds and pyrethroids in insect species such as Haemaphysalis longicornis and Rhipicephalus appendiculatus;\(^1\)^ and that the GST activities of these species were reduced to 85% and 60% respectively by 0.1 mM fenitrothion,\(^3\) H. cunea and B. mori enzymes hcGST and GSTS1 were found to exhibit about 50% and 70% maximum activity respectively under the same conditions. The gap in inhibition rate between hcGST and GSTS1 was largest at the 0.01 mM fenitrothion. This may be of relevance to the difference in numbers of electrophilic binding sites (see Fig. 2), which are responsible for the affinity of a protein to a substance. Fenitrothion is an organophosphorus insecticide popular worldwide, and this situation has promoted the occurrence of insect species that have acquired high resistance.\(^3\) In this context, the prospects for chemical control are concerned.

Plural GSTs, including the currently cloned Sigma-class enzyme, exist in H. cunea, and all of them must be examined to understand their correlation to the insecticide detoxification system in this species. It may be of importance to compare detailed properties, such as expression rates, activities, substrate specificity, and resistance spectrum, among GSTs as well as related enzymes of H. cunea and other insects, in particular B. mori. Investigation along these lines is now underway in our laboratories.

#### Acknowledgments

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#### References


