Functional Analysis of an Epsilon-Class Glutathione S-Transferase From *Nilaparvata lugens* (Hemiptera: Delphacidae)

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Abstract

Glutathione conjugation is a crucial step in xenobiotic detoxification. In the current study, we have functionally characterized an epsilon-class glutathione S-transferase (GST) from a brown planthopper *Nilaparvata lugens* (nlGSTE). The amino acid sequence of nlGSTE revealed approximately 36–44% identity with epsilon-class GSTs of other species. The recombinant nlGSTE was prepared in soluble form by bacterial expression and was purified to homogeneity. Mutation experiments revealed that the putative substrate-binding sites, including Phe107, Arg112, Phe118, and Phe119, were important for glutathione transferase activity. Furthermore, inhibition study displayed that nlGSTE activity was affected by insecticides, proposing that, in brown plant hopper, nlGSTE could recognize insecticides as substrates.

Key words: *Nilaparvata lugens*, glutathione, glutathione transferase, insecticide, site-directed mutagenesis

Glutathione S-transferases (GSTs, EC 2.5.1.18) are widely present in both prokaryotic and eukaryotic cells. They are involved in glutathione (GSH) conjugation, which contributes in xenobiotic(s) detoxification and regulates endogenous compounds (Listowsky et al. 1988; Armstrong 1997). Previously, two GSTs (delta and sigma classes of GST) from *Nilaparvata lugens* have been identified (Yamamoto et al. 2015, 2017). Dipteran insects such as *Anopheles gambiae* (Diptera: Culicidae) (Ranson and Hemingway 2005) and *Drosophila melanogaster* (Diptera: Drosophilidae) (Sawicki et al. 2003; Tu and Akgul 2005) have been reported to possess six GST classes (delta, epsilon, omega, sigma, theta, and zeta). In silkworm (*Bombyx mori* [Lepidoptera: Bombycidae]), we have characterized delta, epsilon, omega, sigma, theta, zeta, and an unclassified GST isoform (Yamamoto et al. 2005, 2006, 2009a,b, 2011, 2013). Apart from this, we have also identified a sigma-class GST from the fall webworm *Hyphantria cunea* (Lepidoptera: Erebidae); a serious lepidopteran pest of the broad-leaved trees (Yamamoto et al. 2007).

Among the GST classes, delta and epsilon are insect specific. The involvement of delta-class GSTs of *A. gambiae* and *D. melanogaster* has been observed in dichlorodiphenyltrichloroethane (DDT) detoxification (Ranson et al. 1997a, b; Low et al., 2010). Expression of delta-class GST in *Liposcelis entomophila* (Psocoptera: Liposcelididae), *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae), and *Pieris rapae* was noted as upregulated by insecticide application (Han et al. 2016; Jing et al. 2017; Liu et al. 2017), whereas the activity of epsilon-class GST of *Spodoptera exigua* (Lepidoptera: Noctuidae) was inhibited by insecticides (Wan et al. 2016). In *Muscida domestica* (Diptera: Muscidae), the epsilon-class GST exhibited activity toward insecticide (Wei et al. 2001) and, in *A. gambiae*, it displayed DDT detoxification (Wang et al. 2008). So far, we have identified and characterized unclassified GST2 of *B. mori* (bmGSTu2) that catalyzes GSH conjugation to organophosphorus insecticide and is closely related to epsilon-class GST (Yamamoto and Yamada 2016). *N. lugens* is a major agricultural pest of the rice crop and, in this study, we focus on the uncharacterized epsilon-class GST (nlGSTE) of the brown planthopper (*N. lugens*). An understanding of this pest’s detoxification capacity, particularly with respect to GSTs function, could provide leads for pest control. In this study, we have identified and characterized nlGSTE complementary DNA (cDNA), which was overexpressed in *Escherichia coli* (Enterobacteriales: Enterobacteriaceae) cells and the properties of the synthesized recombinant proteins were investigated.

Materials and Methods

Insects

Adult brown planthoppers *N. lugens* (Izumo) obtained from the National Agriculture and Food Research Organization, Tsukuba, Japan, were used for total RNA isolation with RNeasy Plus Mini Kit (Qiagen, Hilden, Germany).

Sequencing of nlGSTE cDNA

First-strand cDNA was synthesized from the total RNA, derived by reverse transcription-polymerase chain reaction (RT-PCR)
using SuperScript II reverse transcriptase (Thermo Fisher Scientific, Carlsbad, CA) and oligo-dT primers (Thermo Fisher Scientific, Carlsbad, CA). The resulting cDNA was used as a PCR template along with the oligonucleotide primers: 5'-GCA GAG CAT ATG ACA ATC GAC TTT TAT TAC-3' (forward) and 5'-AAG GAT CCT TAT TCA GAC TGT AGA ATG TTC-3' (reverse). The primers were synthesized based on the partial sequence registered in the genomic database for brown planthopper (http://150.26.71.109/bph/Nlugens). The underlined and double-underlined parts of the primers correspond to the restriction sites of NdeI and BamHI, respectively. These would aid PCR product’s insertion into the expression vector (plasmid). PCR was performed, starting with 1 cycle at 94°C (2 min), followed by 35 cycles at 94°C (1 min), 57°C (1 min), and 72°C (2 min), and 1 cycle at 72°C (10 min). The niGSTE cDNA (nlgste) was ligated into the pGEM-T Easy Vector (Promega, Wisconsin), which was transformed into E. coli DH5α cells. GENETYX-MAC software (ver. 14.0.12, GENETYX Corporation, Tokyo, Japan) was used for deducing the amino acid sequence. Further, sequence alignment was carried out using ClustalW (ver. 1.83; https://www.genome.jp/tools-bin/clustalw) and a phylogenetic tree was constructed by the neighbor-joining plot method (Saitou and Nei 1987).

Recombinant Protein Preparation

The nlgste clone was cleaved with the restriction endonucleases NdeI and BamHI, and its insertion into the expression vector pET-15b (Novagen, EMD Biosciences, Inc.) was carried out. This was followed by transformation into the competent E. coli Rosetta (DE3) pLyS5 cells (Novagen; EMD Millipore, Germany). The E. coli cells were incubated at 37°C in Luria–Bertani (LB) media, including ampicillin (100 mg/ml). When the optical density of the former at 600 nm corresponded to 0.5 (cell density), the cells were stored on ice for 30 min before adding isopropyl-1-thio-β-D-galactoside to a final concentration of 1 mM. Following this, the cultures were incubated overnight at 30°C, and the cells (transformed with nlgste) were later harvested, homogenized with 20-mM Tris–HCl buffer (pH 8.0) containing NaCl (0.5 M) and lysozyme (4 mg/ml), and then disrupted by sonication. Following centrifugation at 10,000 × g (15 min), the obtained supernatant was applied to a resin equilibrated with 20-mM Tris–HCl buffer (pH 8.0) containing 0.2 M NaCl for Ni²⁺-affinity chromatography. This was followed by washings with the same buffer and bound protein(s) elution with 0.5-M imidazole. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on a 15% polyacrylamide slab gel containing 0.1% SDS (Laemmli 1970) to confirm the purity of the eluted protein. Protein bands were imaged after Coomassie brilliant blue R250 (Sigma–Aldrich, St. Louis, MO) staining.

Assay of niGSTE

niGSTE activity was measured spectrophotometrically at 340 nm with 1-chloro-2,4-dinitrobenzene (CDNB) and GSH as standard substrates. (Habig et al. 1974) The assay mixture (50 µl) comprised of 20 mM Tris–HCl (pH 8.0), 5 mM GSH, and several CDNB concentrations. niGSTE activity is stated as mole CDNB conjugated with GSH per minute per milligram of protein. Alternatively, 4-hydroxyxenonanal (4-HNE), ethacrynic acid (ECA), 4-nitrobenzyl chloride (4-NBC), 4-nitrophenyl acetate (4-NPA), 4-nitrophenethyl bromide (4-NPB), and 1,2-epoxy-3-(4-nitrophenoxyl)-propane (EPNP) were used instead of CDNB. The influence of different inhibitors on GST activity was examined by adding their various amounts in the presence of CDNB and GSH. Kinetic parameters (Km and kcat) were calculated by double-reciprocal plot with the data obtained under the assay conditions with different concentrations of CDNB or other compounds in the presence of 5 mM GSH.

Point Mutation of Amino Acid Residue

Amino acid-substituted niGSTE mutants were constructed by using QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) in accordance with the manufacturer’s recommendations. Full-length mutated cDNAs were prepared using an expression plasmid pET-15b harboring nlgste cDNA and were confirmed by DNA sequencing using DNA Analyzer (Applied Biosystems, Foster City, CA).

Results

Sequencing of niGSTE cDNA

The cDNA encoding niGSTE was isolated by RT-PCR using total RNA of N. lugens. The nucleotide sequence was determined and has been deposited in DDBJ/ENA/GenBank under the accession number (LC430994). The open reading frame (ORF) of nlgste comprised of 217 amino acids (Fig. 1) with a calculated molecular weight of 24,994 and an isoelectric point of 5.22 (Table 1). The deduced amino acid sequence of niGSTE shared approximately 36–44% identity with the epsilon-class GSTs of insects (Fig. 1).

A phylogenetic tree, generated with the amino acid sequences of epsilon-class GSTs (aligned in Fig. 1) revealed the closeness between niGSTE and bmGSTE (Fig. 2).

Amino acid-substituted nlGSTE mutants were constructed by using mutagenesis kit (Agilent Technologies, Santa Clara, CA) in accordance with the manufacturer’s recommendations. Full-length mutated cDNAs were prepared using an expression plasmid pET-15b harboring nlgste cDNA and were confirmed by DNA sequencing using DNA Analyzer (Applied Biosystems, Foster City, CA).

Recombinant niGSTE Preparation

The recombinant niGSTE was overexpressed in an E. coli expression vector. The recombinant protein obtained in soluble form was purified using affinity chromatography. An insignificant difference was noted in the molecular weights predicted by the deduced amino acid sequence and by SDS–PAGE measurement (Fig. 3). The calculated molecular weight (24,994) exhibited similarity with that of the isolated insect GSTs (~23,536–25,296 [Table 1]). The specific activities of the final enzyme preparation toward CDNB were 0.28 µmol/min/mg. The purified niGSTE (6.7 mg) was obtained from 1 liter of LB culture.

Functional Characterization of niGSTE

niGSTE was functionally characterized by using CDNB and GSH as standard substrates. The pH optimum of niGSTE was found as 8 (Table 1), which corresponds to that of bmGSTE. The stability analyses (Table 1) suggested that niGSTE retained more than 75% of its original activity in the pH range 6–8 and at temperatures below 50°C. This pH range was narrower than that of bmGSTE (Table 1), which corresponds to that of bmGSTE and two epsilon-class GSTs of Spodoptera litura (sLGST7 and sLGST20). Stable temperature range of niGSTE was same as that of bmGSTE (Table 1).

The specificity of niGSTE for various substrates was investigated at pH 8.0 and 30°C and is listed in Table 2. niGSTE was observed to possess detectable activities toward CDNB (0.42 µmol/min/mg), EPNP (0.71 µmol/min/mg), 4-NBC (0.30 µmol/min/mg), and CDNB or other compounds in the presence of 5 mM GSH.
4-NPB (0.31 µmol/min/mg), 4-HNE (0.26 µmol/min/mg), ECA (1.4 µmol/min/mg), and 4-NPA (0.72 µmol/min/mg; Table 2).

The effect(s) of frequently used insecticides on nlGSTE activity were investigated with CDNB as a substrate. The inhibition assay indicated that nlGSTE activity was affected by permethrin, bendiocarb, diazinon, and chlorfenapyr as substrates (Fig. 4). The remaining activities were noted to decrease with an increase in the quantity of each insecticide. In the presence of 0.1 mM bendiocarb, nlGSTE activity decreased to 36% of its original activity, whereas, with 0.1 mM chlorfenapyr, nlGSTE activity exhibited 84% of its original activity (Fig. 4).

Site-Directed Mutagenesis

Amino acid residues Phe107, Arg112, Phe118, and Phe119 were identified as important for nlGSTE activity. GSTE protein sequence alignment displayed the substrate-binding site (including these amino acid residues) to be conserved within epsilon-class GSTs. To confirm whether these residues were crucial for nlGSTE activity, we converted Phe107, Arg112, and Phe119 to Ala and Phe118 to Lue (Fig. 1).

The resulting nlGSTE mutants were named as F107A, R112A, F118L, and F119A and were purified using the same method as for the wild-type (WT) enzyme. A single band was observed for each purified mutant enzyme in SDS–PAGE. Under optimum condition(s) for nlGSTE activity, the mutant enzymes were assayed and their kinetic values were compared with that of WT enzyme (Table 3). The catalytic efficiency ($k_{cat}/K_m$) for CDNB with WT nlGSTE was noted as 18 mM/s. This value was 2.3-, 2.6-, 1.9-, and 1.5-fold higher than that for F107A, R112A, F118L, and F119A mutants, respectively (Table 3).

Discussion

GSTs are involved in the detoxification reaction via GSH conjugation with exogenous compounds. We have already characterized the delta-class GST of N. lugens (one of insect-specific GSTs) and its functional characterization has provided insights into insecticide(s) detoxification. In the present study, the deduced amino acid sequence was revealed to be homologous to the epsilon-class GSTs of insects (Fig. 1). Moreover, their relatedness was also indicated by the phylogenetic tree (Fig. 2). A corresponding residue is located at the N-terminus region of the epsilon-class GSTs (Fig. 1). A corresponding residue is reported to be found in the N-terminus region of theta-class GSTs, whereas, in mammalian sigma-, alpha-, mu-, and pi-class GSTs, a

**Table 1.** Properties of glutathione S-transferases (GSTs) of as determined in the present and previous studies

| Class       | nlGSTE | bmGSTE | nlGSTD | nlGSTS |
|-------------|--------|--------|--------|--------|
| Calculated molecular weight (Da) | 24,994 | 25,296 | 23,836 | 23,536 |
| Calculated isoelectric point | 5.22 | 5.98 | 5.20 | 6.00 |
| Optimum pH | 8 | 8 | 8 | 8 |
| Stable pH range | 6–8 | 5–10 | 6–12 | 4–12 |
| Stable temperature range | <50°C | <50°C | <50°C | <40°C |

nlGSTE, Nilaparvata lugens epsilon-class GST; bmGSTE, Bombyx mori epsilon-class GST; nlGSTD, N. lugens delta-class GST; nlGSTS, N. lugens sigma-class GST.
tyrosine residue substitutes serine (Board et al. 1995; Reinemer et al. 1996; Rossjohn et al. 1998). The amino acid sequence of nlGSTE possessed a GSH-binding site (Fig. 1), which was also noted in bmGSTE, bmGSTD (delta class of bmGST), and other GSTs. The recombinant nlGSTE was successfully overproduced in a soluble state by bacterial expression and was efficiently purified to homogeneity. The calculated molecular size for nlGSTE was indistinguishable from the SDS–PAGE-determined size and was consistent with the results of GSTs isolated from B. mori and H. cunea. nlGSTE was observed to possess broad substrate specificity (Table 2). We have previously reported bmGSTE to exhibit substrate specificity toward CDNB, ECA, and hydrogen peroxide (H₂O₂) with weak activity; however, we were unable to recognize 4-HNE and 4-NPA as substrates (Yamamoto et al. 2013). CDNB serves as a general GST substrate, whereas 4-NPA is frequently used for GSTs substrate specificity. ECA on the other hand, is a substrate for pi, mu, and alpha classes of GSTs. However, bmGSTE and bmGSTD were reported to conjugate GSH to ECA (Yamamoto et al. 2005). 4-HNE is a cytotoxic product of the lipid peroxidation generated under oxidative stress conditions (Singh et al. 2001). H₂O₂ is a product of active oxygen. The results indicated that nlGSTE was not involved in metabolism of active oxygen. When the substrate-binding sites between

Fig. 2. Phylogenetic tree with the epsilon-class glutathione S-transferases (GST) amino acid sequences. The phylogenetic analysis was performed by a neighbor-joining method using various GST sequences from Swiss-Prot database (http://web.expasy.org/docs/swiss-prot_guideline.html). Each entry contained an accession number and species name or protein name. Anopheles cracens, Anopheles funestus, Anopheles gambiae, and Musca domestica. The number attached represents the distance and the arrow indicates epsilon-class GSTs of Nilaparvata lugens (nlGSTE).
nlGSTE and bmGSTE were compared (Fig. 1), three of the six amino acid residues were observed to be nonidentical. This difference could influence the substrate specificities between nlGSTE and bmGSTE. Using CDNB as a substrate, the $k_{cat}/K_m$ values of nlGSTE was higher than the value for nlGSTD (delta class of N. lugens; 0.19/min/mM) and bmGSTE (3.4/min/mM), respectively (Yamamoto et al. 2013). However, the value was lower than that for sigma-class GST of N. lugens (102/min/mM; Yamamoto et al. 2015). Site-directed mutagenesis aided in examining the role of Phe107, Arg112, Phe118, and Phe119. The G-site of GSTs is highly conserved, whereas its substrate-binding site is diverse with respect to the amino acid sequences. Since, a few studies on the function of substrate-binding site have been reported previously, we mutated the putative substrate-binding site (Phe107, Arg112, Phe118, and Lue119). Alanine scanning was employed to determine the contribution of Phe107, Arg112, and Phe118 in nlGSTE’s function. In epsilon-class GST of Anopheles funestus, mutation of Lue119 to Phe was noted to affect insecticide metabolization. Therefore, we changed the corresponding residue Phe118 in nlGSTE to Lue. The results displayed a decrease in the $k_{cat}/K_m$ values of all the mutants, which suggested that four residues were involved in nlGSTE catalysis. To express GST activity, other amino acid residues have been identified in amino acid sequence of GST.

GSTs play an important role in the resistance to organophosphates and pyrethroids (Hemingway 2000). A competitive assay was performed to investigate the interaction(s) between insecticides and nlGSTE. In the present study, nlGSTE activity inhibition with various insecticides was displayed and the results revealed bendiocarb as the most prominent inhibitor. Crystallization of nlGSTE complexed with bendiocarb is required to figure out the structure-function relationship to ascertain whether bendiocarb binds to nlGSTE’s substrate-binding site.

In this study, based on the presence of epsilon-class GSTs in brown planthopper N. lugens, we hypothesize that nlGSTE plays a role in detoxification of the xenobiotic-including insecticides. Examining the nlGSTE difference between a susceptible and resistant N. lugens strain would help in understanding their correlation with insecticide detoxification in the species.

### Table 2. Substrate specificity of nlGSTE

| Substrate | Concentration (mM) | Activity (µmol/min/mg) | Wavelength (nm) | Δε (per mM/cm) |
|-----------|-------------------|-----------------------|----------------|----------------|
| CDNB      | 1.0               | 0.42 ± 0.037         | 340            | 9.6            |
| EPNP      | 1.0               | 0.71 ± 0.41          | 260            | 0.5            |
| 4-NBC     | 1.0               | 0.30 ± 0.048         | 310            | 1.9            |
| 4-NPB     | 0.5               | 0.31 ± 0.029         | 310            | 1.2            |
| 4-HNE     | 0.1               | 0.26 ± 0.0042        | 224            | 13.8           |
| ECA       | 1.0               | 1.4 ± 0.12           | 270            | 5.0            |
| 4-NPA     | 0.25              | 0.72 ± 0.0069        | 400            | 8.3            |
| H₂O₂      | 0.2               | NA                   | 340            | −6.2           |

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

nlGSTE, Nilaparvata lugens epsilon-class GST; CDNB, 1-chloro-2,4-dinitrobenzene; EPNP, 1,2-epoxy-3-(4-nitrophenoxy)-propane; 4-NBC, 4-nitrobenzyl chloride; 4-NPB, 4-nitrophenethyl bromide; 4-HNE, 4-hydroxynonenal; ECA, ethacrynic acid; 4-NPA, 4-nitrophenyl acetate; H₂O₂, hydrogen peroxide.
of nLGSTE crystal structure is underway in our laboratories, which would make it possible to focus on the catalytic site.

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Declarations of interest: none

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