Catalytic features and crystal structure of a tau class glutathione transferase from *Glycine max* specifically upregulated in response to soybean mosaic virus infections

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**A B S T R A C T**

The plant tau class glutathione transferases (GSTs) play important roles in biotic and abiotic stress tolerance in crops and weeds. In this study, we systematically examined the catalytic and structural features of a GST isoenzyme from *Glycine max* (GmGSTU10-10). GmGSTU10-10 is a unique isoenzyme in soybean that is specifically expressed in response to biotic stress caused by soybean mosaic virus (SMV) infections. GmGSTU10-10 was cloned, expressed in *Escherichia coli*, purified and characterized. The results showed that GmGSTU10-10 catalyzes several different reactions and exhibits wide substrate specificity. Of particular importance is the finding that the enzyme shows high antioxidant catalytic function and acts as hydroperoxidase. In addition, its $K_m$ for GSH is significantly lower, compared to other plant GSTs, suggesting that GmGSTU10-10 is able to perform efficient catalysis under conditions where the concentration of reduced glutathione is low (e.g. oxidative stress). The crystal structure of GmGSTU10-10 was solved by molecular replacement at 1.6 Å resolution in complex with glutathione sulfenic acid (GSOH). Structural analysis showed that GmGSTU10-10 shares the same overall fold and domain organization as other plant cytosolic GSTs; however, major variations were identified in helix H9 and the upper part of helix H4 that affect the size of the active site pockets, substrate recognition and the catalytic mechanism. The results of the present study provide new information into GST diversity and give further insights into the complex regulation and enzymatic functions of this plant gene superfamily.

**1. Introduction**

Plant glutathione S-transferases (GSTs; EC 2.5.1.18) are enzymes that catalyze the conjugation of reduced glutathione (GSH; γ-Glu-Cys–Gly) to electrophilic centers of a wide variety of, mainly hydrophobic, compounds, both endogenous and xenobiotic [1–4]. GSTs are implicated in pesticide detoxification [5–7], in responses to biotic and abiotic stress (infection, heavy metals, UV radiation, etc) [8–11], as well as in hormonal regulation and developmental change [12–16]. GSTs can be found in plants from early embryogenesis to senescence [17].

GSTs comprise a large, complex gene family in plants. Based on a variety of criteria (e.g. sequence relatedness, immunological, kinetic and structural properties), plant soluble GSTs can be subdivided to distinct classes: phi (F), tau (U), zeta (Z), theta (T), lambda (Λ), dehydroascorbate reductase (DHAR), and tetrachlorohydroquinone dehalogenase (TCHQD) [1,2,12,13]. The tau class, in particular, is the most abundant of all GST classes and its members play important roles in stress tolerance and secondary metabolism as well as catalyzing the detoxification of herbicides in crops and weeds [9,13–15, 17–19].

According to numerous crystallographic studies, GSTs display significant structural conservation [8,15,16]. The soluble plant GSTs are homo- and hetero-dimeric enzymes with 23–30 kDa subunits and average length of 200–250 amino acids [1,8,12,13,16,20]. Each subunit consists of two domains, the N-terminal domain with $\alpha$/$\beta$ topology and the C-terminal domain with $\alpha$-helical structure. Each subunit has a relatively independent active site, composed of the G-site, which is primarily responsible for binding GSH or other closely related peptides (e.g. homoglutathione), and the H-site, which is the site where hydrophobic electrophile substrates bind [8,16]. The catalytic residue of GSTs from theta, zeta, phi and tau classes is the amino acid serine [1,12,13,16]. The G- and H-sites are typically formed...
from residues of N- and C-terminal domains, respectively. Residues of the H-site are not conserved compared to those of the G-site across cytosolic GSTs [1,10–12,16,21]. GSTs, therefore, display wide substrate specificity toward electrophile molecules including organic halides, organic hydroperoxides, epoxides, arene oxides, α- and β-unsaturated carboxyls, organic nitrate esters, and organic thio-ycanates [1,10,11,22,23]. Selected GSTs from tau, theta and phi classes exhibit peroxidase activity [12–16] by reducing lipid hydroperoxides directly and by removing lipid peroxidation end products like alkenals, 4-hydroxynonenal, and other α,β-unsaturated aldehydes [3,9–11]. They also display thioltransferase and dehydroascorbate reductase activity [1,10,12]. Moreover, they are also involved in GSH-dependent isomerization reactions, the synthesis of sulfur-containing secondary metabolites, and the conjugation, transport and storage of reactive oxylipins, phenolics and flavonoids [2].

There are 25 GST isoenzymes in soybean (Glycine max) for which their substrate specificity has been studied towards different herbicides (diphenyl ether, chloroacetanilide, sulfonylurea) and xenobiotic substrates [16,17]. However, the results of those studies pose the question of why there are so many different GSTs in soybean with high homologous primary and secondary structures and overlapping substrate specificities. Here we report the characterization and analysis of the structural and functional features of a tau class glutathione transferase (GmGSTU10–10) from G. max. GmGSTU10–10 is differentially over-expressed in response to soybean mosaic virus (SMV) infections. In particular, among the 25 different GST isoenzymes in soybean, GmGSTU10–10 is the sole GST transcript that is substantially upregulated in SMV-infected soybean leaf tissues, suggesting that GmGSTU10–10 represents a defense-specific GST enzyme [24]. SMV is the most prevalent viral pathogen of soybean in the world. Infection by SMV usually causes yield losses of between 35 and 50% under natural field conditions and up to 50–100% in severe outbreaks [25].

Investigating plant host response at the molecular level is certainly important for control of SMV infections. GmGSTU10–10 displays an interesting expression pattern [24]. In particular, GmGSTU10–10 transcripts were significantly over-represented at 21 days post infection (p.i.), (by >2.20-fold), but not at 7 and 14 days p.i. This supports the idea that there is a delayed host defense response and at late infection stages, the soybean plant responds to SMV infection by expressing GmGSTU10–10. The same phenomenon has also been observed in other defense-related transcripts in the SMV-infected leaf [24,26]. For example, of the 24 upregulated defense-related transcripts, a subset of 17 defense-related transcripts that are involved in disease signaling, plant defense and stress responses (such as chitinase, GmGSTU10–10, heat shock protein, superoxide dismutase, peroxidase), were either downregulated or slightly affected at 7 days p.i., but substantially upregulated at 14 or 21 days p.i. [24].

The actual biological role of GmGSTU10–10 is unclear; however, additional pieces of evidence suggest that the enzyme is related to defense towards biotic stress. The biological role of GmGSTU10–10 in biotic stress is also supported by the recent finding that this enzyme is differentially expressed in soybean in response to Phytophthora pachyrhizi infections [27]. On the other hand, the enzyme is down regulated by abiotic stress (salt stress, NaCl) as demonstrated using proteomic analysis [28]. It is noteworthy that GmGSTU10–10 gene was found to be constitutively expressed in soybean, suggesting that the enzyme has housekeeping roles and presumably involved in endogenous developmental processes of soybean [29].

2. Experimental procedures

2.1. Materials

Poly(A)-mRNA purification kit, total RNA isolation kit, first-strand cDNA synthesis kits, dNTPs and restriction enzymes were obtained from Invitrogen (USA). Reduced glutathione, 1-chloro-2,4-dinitrobenzene (CDNB), and all other enzyme substrates were obtained from Sigma-Aldrich (USA). The T7 expression vector pEXP5-CT/TOPO® was obtained from Invitrogen (USA).

2.2. Methods

2.2.1. Molecular cloning

Soybean seedlings were grown in perlite in a growth chamber under controlled conditions (25 °C, 16 h light/8 h dark cycle). Poly(A)-mRNA was purified from plant seedlings using poly(A) RNA isolation kit. Purified RNA was qualitatively and quantitatively assessed by spectrophotometry and agarose gel electrophoresis. First-strand cDNA synthesis was done using oligo-p(dT)15 primer and AMV reverse transcriptase. In order to eliminate contamination of genomic DNA, RNA samples were treated with DNase I at 37 °C for 45 min. The PCR-primers 5′-ATGACA GTGAGGTGTCTTT-3′ (forward primer) and 5′-CTACTCGACTA ACTTTTCTTAT-3′ (reverse primer) were designed according to the GmGSTU10–10 gene sequence [17]. The PCR reaction was carried out in a total volume of 50 μL containing: 10 pmole of each primer, 50 ng template cDNA, 0.2 mM dNTPs, 5 μL 10 × Pfu buffer and 1 unit of Pfu extended DNA polymerase. The PCR procedure comprised 30 cycles of 1.5 min at 95 °C, 1 min at 55 °C and 2 min at 72 °C. A final extension time at 72 °C for 10 min was performed after the 30th cycle. The resulting PCR amplicons were TOPO ligated into a T7 expression vector (pEXP5-CT/TOPO®). The resulting expression constructs were sequenced along both strands and were used to transform competent Escherichia coli BL21(DE3) cells.

2.2.2. Expression and purification of recombinant GmGSTU10–10

E. coli cells harboring recombinant plasmid were grown at 37 °C in 1 L LB medium containing ampicillin (100 μg/mL). The synthesis of GST was induced by the addition of 1 mM isopropyl 1-thio-β-galactopyranoside when the absorbance at 600 nm reached 0.6. Four hours after induction, cells were harvested by centrifugation at 8000 rpm for 15 min, resuspended in potassium phosphate buffer (0.1 M, pH 6.5), sonicated, and centrifuged at 13,000 rpm for 5 min. The supernatant was loaded to GSH-Sepharose column (1.4-butandiol diglycidyl ether-GSH-Sepharose-CL6B, 1 mL), which was previously equilibrated with potassium phosphate buffer (20 mM, pH 7). Non-adsorbed protein was washed off with 10 mL equilibration buffer. Bound GST was eluted with equilibration buffer containing 10 mM GSH. Protein purity was judged by SDS-PAGE.

2.2.3. Assay of enzyme activity and protein concentration

Enzyme assays for the CDNB and fluorodifen conjugation reactions were performed according to published methods [16,30,31]. Observed reaction velocities were corrected for spontaneous reaction rates when necessary. Turnover numbers were calculated on the basis of one active site per subunit. Glutathione peroxidase activity was determined according to Wilson’s method [32]. The reactions were carried out in 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 1 mM GSH, 1.5 mM cumene hydroperoxide (CuOOH) or tert-butyl peroxide (t-BuOOH), 0.2 mM NADPH, 1 unit glutathione reductase, and enzyme. Protein concentration was determined by the Bradford assay using bovine serum albumin (fraction V) as standard.

2.2.4. Viscosity dependence of kinetic parameters

The effect of viscosity on kinetic parameters was assayed based on references [30,33–35]. According to Kramer’s theory [33], enzymes that undergo conformation changes during catalysis should be inhibited by viscosity [34]. In a diffusion dependent, enzyme-catalyzed reaction, where the substrate binds to the enzyme to yield the product, the kcat value is negatively influenced by the friction of the solvent with the
### A

<table>
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<td>2. GmGSTU6-2</td>
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<td>3. GmGSTU6-3</td>
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<td>4. GmGSTU6-4</td>
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<td>20. GmGSTU16-20</td>
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</tbody>
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### B

[Diagram of GSTU1-1 to GSTU20-20 relationships]
enzyme. This is because friction increases the activation energy needed to reach the transition state [35]. In turn, friction is a function of viscosity $\eta$. Thus, the reaction rate constant depends linearly on viscosity $\eta$ (Eq. (1)) as described by Jacob and Schmid [34]:

$$k = \eta^{-1} e^{-\frac{\Delta U}{RT}}$$

(1)

where $k$ is the rate constant for the reaction ($k_{cat}$ or $V_{max}$ for enzyme catalyzed reactions), $\eta$ is the macroscopic viscosity of the solvent, $R$ is the gas constant, $T$ is the absolute temperature and $\Delta U$ is the free energy barrier imposed by solvent friction.

2.25. pH and temperature dependence of $V_{max}$

Analysis of the pH dependence of $V_{max}$ was carried out in 0.1 M potassium phosphate buffer (pH 6.0–8.5). The pH-dependent ionizations were determined by a fit of steady-state parameters to Eq. (2).

$$u = \frac{C}{1 + [H^+] K_1 + K_2 / [H^+]}$$

(2)

where $u$ is the observed rate of the reaction, $C$ is the pH-independent rate, $[H^+]$ is the concentration of hydrogen ions, and $K_1$ and $K_2$ are the ionization constants of the acid and base species, respectively [36]. $pK$ values were calculated by the program GraFit (Erithacus Software Ltd.).

The effect of temperature on $k_{cat}$ of the CDNB conjugation reaction, the activity data were analyzed by plotting the logarithm of activity versus the reciprocal of the absolute temperature. Arrhenius equation was fitted to the experimental data (Eq. (3)) [37,38]:

$$\log k_{cat} = \log Z - \frac{E_a}{2.303RT}$$

(3)

where $E_a$ is the energy of activation, $R$ is the gas constant, and $Z$ is the preexponential factor. The Eyring equation was fitted to the activity data (Eq. (4)) [39]:

$$k_{cat} = k_0 \frac{T}{h} e^{-\frac{\Delta H^*}{RT}} = k_0 \frac{T}{h} e^{-\frac{\Delta S^*}{RT}}$$

(4)

where $k_0$ is the Boltzmann’s constant, $h$ is Planck’s constant, $R$ is the gas constant, and $\Delta G^*$, $\Delta H^*$, and $\Delta S^*$ are the free energy, enthalpy, and entropy of activation of the rate limiting step in the reaction, respectively.

The data were plotted as logarithm of $k_{cat}/T$ versus the reciprocal of the absolute temperature.

2.2.6. Thermal stability

GmGSTU10-10 was incubated at different temperatures at a protein concentration of 0.02 mg mL$^{-1}$ in 0.1 M potassium phosphate buffer pH 7. The samples were incubated at different temperatures (20 to 85 °C) for 5 min and subsequently assayed for residual activity. $T_m$ values were determined from the plot of relative inactivation (%) versus temperature (°C). The $T_m$ value is the temperature at which 50% of the initial enzyme activity is lost after heat treatment.

2.2.7. Crystallization and data collection and processing

GmGSTU10-10 was concentrated to ~10 mg/mL in buffer HEPES-NaOH pH 7.0 prior to crystallization. Initial crystallization conditions were established using the INDEX crystallization screen (Hampton Research) in the presence of 10 mM spirodilcolofen dissolved in acetone solution. Condition No. 82 (0.2 M MgCl$_2$, 0.1 M Bis-Tris, pH 5.5, PEG 3350 25% (w/v)) resulted in the appearance of small crystals. After optimization, crystals of maximum size of 0.4 mm × 0.2 mm × 0.2 mm were grown within 2 days in 0.2 M MgCl$_2$, 0.1 M Bis–Tris, pH 5.6, PEG 3350 22% (w/v) using the hanging drop vapor diffusion method. X-ray diffraction data to 1.6 Å resolution were collected from a single crystal at 100 K on station X13 (λ = 0.8123 Å) at EMBL Hamburg, c/o DESY, Germany, using a 165 mm MARCCD detector. All data were autoindexed, integrated, and scaled using the XDS software package [40].

2.2.8. Structure determination and refinement

The structure was determined by molecular replacement using PHASER [41] in the CCP4 program suite [42]. The subunit A of GST from G. max in complex with GSH (PDB ID: 3HFS, 91% sequence identity with GmGSTU10-10 [43]) was used as a search model after the waters,
side chains and the ligand were removed. The resulting top solution from PHASER showed a Z-score of 50.7. After calculation of an initial electron-density map, most of the side chains were visible in the model and manual building was carried out. The program COOT [44] was used for inspection of $2|F_o| - |F_c|$ and $|F_o| - |F_c|$ maps, and rebuilding of the structure. Refinement was carried out with PHENIX [45]. A randomly selected subset (5%) of the total number of reflections was set aside for cross-validation analysis to monitor the progress of refinement using the $R_{\text{free}}$ factor. The quality of the final structure was assessed with MOLPROBITY [46], PROCHECK [47], and validation tools in COOT.

2.2.9. Protein Data Bank accession code

The refined coordinates of GmGSTU10-10 and the structure factors have been deposited with the Protein Data Bank (PDB ID: 4CHS).

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**Fig. 3.** Kinetic analysis of GmGSTU10-10. A: The effect of viscosity on $k_{\text{cat}}$ for the CDNB-GSH reaction. Plot of the reciprocal of the relative turnover number ($k_{\text{cat}}/k_{\text{cat}}$) as a function of relative viscosity ($\eta/\eta^0$) with glycerol as cosolvent. Lines were calculated by least-squares regression analysis. B: Dependence of $V_{\text{max}}$ on pH for the CDNB-GSH reaction. Buffer used was 0.1 M potassium phosphate. C: Effects of temperature on GmGSTU10-10 catalytic activity. The Arrhenius equation was fitted to the experimental data. D: Effects of temperature on catalytic activity. The Eyring equation was fitted to the experimental data. E: Thermal inactivation curves. The residual activities were measured after heat treatment at various temperatures (°C) for 5 min.
2.2.10. Bioinformatics and structural analysis

The superimposed structures were visually inspected using COOT. Contacts were measured with the program CONTACT in CCP4 [48]. The interface analysis was done with PDBePISA server (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html) [49]. The secondary structure assignments were produced with DSSP [50]. Calculation of enzyme area and volume occupied by each active site was carried out using CASTp [51].

3. Results and discussion

3.1. Cloning, expression and kinetic characterization of the recombinant GmGSTU10-10 enzyme

The coding sequence of GmGSTU10-10 with complete open reading frame was isolated using RT-PCR. GmGSTU10-10 contained an open reading frame (ORF) of 660 bp, coding for a polypeptide of 219 amino acid residues with a predicted molecular mass of 25,578.55 Da and a theoretical pl of 5.71. In silico analysis, using iPSORT, TargetP and SignalP algorithms, revealed the absence of putative N-terminal transit peptide, suggesting that it is a cytosolic enzyme. The deduced amino acid sequence shares high homology with the tau class plant GSTs. Fig. 1 describes the phylogenetic relationship of GmGSTU10-10 with other tau class GSTs from G. max. The GmGSTU10-10 is phylogenetically the closest with the isoenzymes GmGSTU2-2 and GmGSTU4-4 and shares >85% sequence identity.

The coding sequence of GmGSTU10-10 was TOPO ligated into a pT7 expression vector. The resulting expression construct was used to transform competent E. coli BL21(DE3) cells. The recombinant enzyme was purified to homogeneity by affinity chromatography on GSH- Sepharose column (Fig. 2). The substrate specificity of the purified GmGSTU10-10 was investigated using steady-state kinetic analysis. The enzyme was assayed for activities as transferase and peroxidase, using four selected substrates (CDNB, cumene hydroperoxide and tert-butyl hydroperoxide). The catalytic efficiency of GmGSTU10-10 towards organic hydroperoxides may provide a link to the in vivo functional role of the enzyme towards oxidative-stress tolerance. Reactive or activated oxygen species have been suggested to be key mediators of local and systemic resistance responses in incompatible plant–pathogen reactions and to be involved in symptom development and pathogenesis in compatible plant–virus interactions [52]. The expression of GmGSTU10-10 [24] is suppressed significantly in the SMV-infected leaf at 14 days p.i. and significantly over-represented at 21 days p.i. (by 2.20-fold). On the other hand, the enzyme peroxidase is induced significantly 14 days p.i. (by 4-fold) and downregulated (>9-fold) at 21 days p.i. This mechanism of upregulation of peroxidase transcripts and downregulation of GST transcripts may provide adjustment to the oxidative stress in the early or late infection process. Such an antioxidative metabolism imbalance may be associated with the progression of SMV infection and symptom development, as suggested for the plum pox virus–peach interaction [52].

The Km values for GSH, using different electrophilic substrates, lie between a relative narrow range (28.6–87.01 μM). The Km values for GSH are about 5–10-times lower compared to other tau class GST isoenzymes, as for example the isoenzyme GmGSTU4-4 [16] or the Phi class enzyme ZmGSTF1-1 [30,53], but is close to that observed for the biotic resistant response of GmGSTU10-10 towards organic hydroperoxides (Fig. 3B). The activity of GmGSTU10-10 shows linear dependence on the relative viscosity, the activity data kcat/Km were plotted against the solvent relative viscosity, defined as η/ηf (see Eq. (1)). In these calculations, kcat and ηf are respectively the kcat and the viscosity in the absence of glycerol and km and η are the observed values at each glycerol concentration. A plot of the inverse relative rate constant kcat/km versus the relative viscosity η/ηf should be linear, with a slope equals to unity when the product release is limited by a strict diffusional barrier or close to zero if the catalytic reaction chemistry is rate-limiting [16,53]. The inverse relative rate constant kcat/km for GmGSTU10-10 shows linear dependence on the relative viscosity with a slope 1.106 ± 0.007 (Fig. 3A). The slope is close to unity, suggesting that the product release is limited by a strict diffusional barrier. In the case of the homologue enzyme GmGSTU4-4, the dependence on the relative viscosity showed a slope 0.87 ± 0.1 suggesting that the rate-limiting step in the GmGSTU4-4 is not dependent on a diffusional barrier and other viscosity-dependent motions or conformational changes of the protein contribute to the rate-limiting step of the catalytic reaction [3].

The pH dependence of Vmax for GmGSTU10-10 was investigated in an attempt to compare the acid/base properties of the ionizable groups upon substrate binding and catalysis. The pH dependence of Vmax was determined over the pH range of 6.0 to 8.5. The pH–Vmax profiles are shown in (Fig. 3B). The activity of GmGSTU10-10 was greatest between pH 7 and 8, indicating a broad pH optimum. Eq. (2) was fitted to the

Table 2

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<tr>
<td>Unique reflections</td>
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<td>Wilson B-factor (Å²)</td>
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</table>

Numbers in parentheses correspond to the highest resolution shell (1.7–1.6 Å).
data, giving $pK_1 = 6.5 \pm 0.2$ and $pK_2 = 8.4 \pm 0.2$. The $pK_1$ is close to the value expected for a carboxylate side chain, or for the imidazole group of a His residue [57], whereas, the $pK_2$ is close to the value expected for Lys or Cys residues [57]. The crucial property of GSTs is their ability to lower the $pK_0$ of the thiol group of the bound GSH. In the tau class GSTs this is accomplished by the active site Ser whose hydroxyl group of the side-chain forms a hydrogen with the $–SH$ group. Published work shows that the $pK_a$ of GSH in the active site ranges from 6.0 to 6.5 [30]. Presumably, the observed $pK_1$ may reflect the ionization of the thiol group of the bound GSH.

To study the effect of temperature on $k_{cat}$, the Arrhenius and Eyring equations (Fig. 3C and D) were fitted to the experimental data and typical linear relationships were obtained indicating that the same kinetic step remains rate limiting throughout the different temperatures. The activation energy, a measure of the energy barrier that the reactants must overcome before products can be formed, was estimated equals to $19.1 \pm 1.4 \text{ kJ/mol}$, close to that observed for other GSTs [58]. The enzyme exhibits large negative $\Delta S^*$ value ($-0.17 \pm 0.01 \text{ kJ/mol K}$), which is characteristic of transition states that are more ordered than the corresponding ground states with minimal disruption of the water network in the active center of the enzymes. As expected, a positive $\Delta H^*$ was observed ($12.4 \pm 1.1 \text{ kJ/mol}$) because the transition state involves the breaking of bonds. The fairly positive value of $\Delta H^*$ indicates that the transition state is highly solvated.

The thermal stability of the GmGSTU10-10 enzyme was assessed by measuring their residual activity after heat treatment for 5 min at various temperatures (20–85 °C, Fig. 3E). The $T_m$ was measured equal to 62.43 ± 0.62 °C. This $T_m$ value is similar to that determined for other tau class GST isoenzymes [3]. The absence of substantial differences in $T_m$ values suggests that the isoenzymes display similar structural stability and suggest that the potential of a GST gene to evolve new catalytic function upon evolution may depend on its ability to accept mutations without losing the stability of the protein domain that is encoded.

3.1.1. Structure determination of GmGSTU10-10 and quality of the final model

The structure of GmGSTU10-10 was determined to 1.6 Å resolution in complex with GSOH. The final statistics for data collection and structure refinement are summarized in Table 2. Although crystals were produced in the presence of spirodiclofen, no binding of spirodiclofen was found after inspection of the electron density maps. Instead, a GSH molecule was initially placed at the active site based on the electron density. At the later stages of refinement, GSH was replaced by GSOH owing to extra electron density near the sulfur atom. At the N-terminus, the first residue (Met1) from subunit A, the first three residues (Met1, Thr2 and Asp3) from subunit B, the last 3 residues at the C-terminus (Val217, Ile218 and Glu219) from subunit A and the last residue (Glu219) from subunit B could not be modeled in the structure owing to lack of sufficient density.

3.1.2. Overall structure

The structure of GmGSTU10-10 is a homodimer with a 2-fold symmetry between the subunits (Fig. 4). The dimer has a globular shape with a solvent-accessible deep V-shaped crevice at the inter-subunit interface. Hydrogen bonds, salt bridges and hydrophobic interactions are observed in the subunit–subunit interface. Structural comparison of the subunits showed only minor differences (RMSD = 0.63 Å) with respect to their secondary structure elements. Each subunit folds into two distinct domains, namely, the N-terminal domain [residues 1–77] and the all-helical C-terminal domain [residues 89–219]. The N-terminal domain adopts a thioredoxin-like fold (Fig. 4B) as in previously reported structures [16,43]. Helix H2 and strand J3 are connected by a loop containing a cis-Pro, which is highly conserved in all GSTs (Fig. 1A). The cis-Pro loop, although not directly involved in catalysis, is important in maintaining the protein in a catalytically competent conformation [59]. The C-terminal domain consists entirely of α-helices positioned downstream the thioredoxin structure and is connected to the N-terminal domain by a short linker sequence.

Subunit A lacks the C-terminal residues and subunit B lacks the N-terminal residues due to their flexible character that results in weak or no electron density at all. GSOH was found bound in both subunits. An acetone molecule was located on the surface of subunit B, in a pocket formed by Glu178, Lys126, and Ile129. This compound was used during co-crystalization in the presence of the pesticide spirodiclofen.
3.1.3. GSH binding site (G-site)

In each subunit, one molecule of glutathione sulfenic acid (GSOH) was placed (Fig. 5). The GSOH was presumably formed during crystallization after oxidation of the SH group of GSH. Naturally, GSOH is formed as an intermediate by the reaction of GSH with hydroperoxides [60]. In particular, the reaction of GSH with hydroperoxides is achieved in two steps. The first step involves the nucleophilic attack by GS− on hydroperoxide that results in the formation of GSOH. This sulfenic acid then reacts non-enzymically with GSH to produce glutathione disulfide (GSSG). Therefore, GSOH may be considered as a possible intermediate in the catalytic reaction with hydroperoxides.

As shown in Fig. 5, GSOH binds in the same conformation in both subunits. The glycine moiety of GSH is located in a polar region, formed by the beginning of helices H1 [Pro14–Lys26], H2 [Pro42–Met47], and H3 [Ser67–Val77] in the N-terminal domain. There is no direct interaction between the GSH portion and residues of the C-terminal domain. Its γ-Glu moiety points downwards to the internal cavity and its glycine moiety is oriented upwards and projects into the bulk solvent. The γ-Glu moiety of GSOH forms hydrogen bonds with Glu66 and the hydroxyl group of Ser67. The complete conservation of this Ser residue (Fig. 1A) is consistent with its critical role in GSH binding [58]. The cysteinyl moiety of GSOH forms a hydrogen bond with the peptide bond of Ile54. The glycyl moiety of GSOH forms a hydrogen bond with the side chain of Lys40. In addition, the hydroxyl group of Ser13 is 4.6 Å away from the sulfur group of the GSOH, indicating that Ser13 corresponds to the catalytic residue as in other tau class GSTs [3]. Ser13 is highly conserved and it is well established that it plays a crucial role in the mechanism of GSH activation as a catalytically essential residue [3, 10, 16]. In particular, the Ser hydroxyl group acts as hydrogen bond donor to the thiol group of GSH, contributing to stabilization of the reactive thiolate anion, which is the nucleophile group for the electrophilic substrate [16,43].

3.1.4. Electrophilic binding site (H-site)

The H-site of GmGSTU10-10 resides next to the G-site and is formed by residues from the C-terminal domain. In general, the H-site of GSTs displays a low degree of sequence identity. This reflects differences in substrate specificity, compared to other tau class plant enzymes [3, 10, 11,16], and suggests that the C-terminal domain of these enzymes may have evolved under differential selective pressures. The H-site of GmGSTU10-10 is typically hydrophobic and built mainly by residues from the C-terminal domain: H4 (His107, Lys111, Trp114, Thr115), H6 (Trp163), H9 (Phe208 and Leu212), and Phe10 from the N-terminal domain. All these residues are oriented towards the center of the active site. These residues are not conserved among other GSTs and may play a role in regulation of substrate recognition by influencing the
isoenzyme a non-conserved residue, replaced by either polar or non-polar residues. An interesting characteristic of this site is the position of His107, which is involved in the binding mechanisms and physiochemical properties of the H-site. The role of the enzyme is to cope with the anti-oxidative metabolism balance at late infection stages. Structural analysis showed that GmGSTU10-10 shares the same overall fold and domain organization as other tau class GSTs, with major differences in the size of the active site pockets and the structure of the H-site that lead to different kinetic properties and rate-limiting step. Our results shed light on understanding better the GST structural and catalytic diversity and highlight the pivotal role of GSTs used by plants to cope with biotic stress.

Fig. 7. A: Structural superposition of GmGSTU10-10 (magenta), GmGSTU4-4 (coral), and GmGSTU4-4-Nb-GSH (dark cyan). B: Close-up view of the side chains of GmGSTU10-10 (magenta), GmGSTU4-4 (coral), and GmGSTU4-4-Nb-GSH (dark cyan) at the active site. GSH, GSOH and Nb-GSH are shown as sticks and colored according to atom type. Labels for Tyr107, His107, Arg111, Lys111 and Lys215 are colored according to the respective structure.

3.2. Structure comparison of GmGSTU10-10 and GmGSTU4-4

Recently, we reported the structural and kinetic properties of the isoenzyme GmGSTU4-4 from G. max [3,16,43]. This isoenzyme together with GmGSTU10-10 forms a subgroup of highly homologues enzymes (>88% sequence identity) (Fig. 1) with distinct structural and functional features. Comparative structural analysis of GmGSTU10-10 and GmGSTU4-4 (in complex with GSH (GmGSTU4-4-GSH) [43] and S-p-nitrobenzyl-GSH (GmGSTU4-4-Nb-GSH) [16]) was used to identify key structural characteristics and key amino acid residues in the G- and H-site as well as to provide insights into the mechanism of molecular recognition (Fig. 6). Both enzymes share the same overall fold and domain organization as other plant tau class GSTs (Fig. 6). The RMSD and Q-score between GmGSTU10-10 and GmGSTU4-4-GSH were 0.52 Å and 0.92, respectively, indicating no significant differences in the structure. G-site residues are highly conserved and show no alterations in their positions.

However, significant variations that may contribute to different binding properties were identified in the H-site of the active site, more specifically in helix H9 at the C-terminal domain (Fig. 7). The C-terminal residues 208-219 at H9 of GmGSTU10-10 and GmGSTU4-4-GSH form a more open conformation as compared to GmGSTU4-4-Nb-GSH. Importantly, helix H9 (residues 202-211) of GmGSTU4-4-Nb-GSH was shown to fold back over the top of the N-terminal domain forming a lid to block the active site pocket in agreement with the induced fit mechanism [16]. A key residue Lys215 at H9, which acts as a lid over the entrance to the site in GmGSTU4-4-Nb-GSH, is observed to point away from the active site in the GmGSTU10-10 and GmGSTU4-4-GSH structures.

Calculation of the area and volume occupied by each active site using CASTp [51] shows that GmGSTU4-4-GSH and GmGSTU4-4-Nb-GSH have comparable active site pockets (453.5 Å², 698.3 Å³; 483 Å², 788.5 Å³) but significantly larger than that of GmGSTU10-10 (341.9 Å², 531.4 Å³), suggesting a difference that may affect GSH and xenobiotic recognition. The smaller size of active site in GmGSTU10-10 may reflect a more compact binding of GSH and probably explains the higher affinity of the enzyme for GSH. Additionally, it is observed that D103KKIY107 at the upper part of helix H4 (part of the H-site) in GmGSTU10-10 may restructure the structure. G-site residues are highly conserved and show no alterations in their positions.

4. Conclusions

Members of tau GSTs in G. max overlap in their functions, thus providing the plant a broad range of protection. GST function cannot infer from their high sequence similarity to other members of known function. Thus, to understand at molecular level the functional role of GSTs, each member should be characterized individually by biochemical and structural analysis. In the present work, we describe the characterization of a tau class GST isoenzyme from G. max, which is specifically induced following SMV infection. The results showed that GmGSTU10-10 exhibits high antioxidant catalytic function and significantly lower Km for GSH compared to other plant GSTs, suggesting that the biological role of the enzyme is to cope with the anti-oxidative metabolism balance at late infection stages. Structural analysis showed that GmGSTU10-10 shares the same overall fold and domain organization of other tau class plant cytosolic GSTs, with major differences in the size of the active site pockets and the structure of the H-site that lead to different kinetic properties and rate-limiting step. Our findings shed light on understanding better the GST structural and catalytic diversity.
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