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Purification, biochemical characterization, and cDNA cloning of a glutathione *S*-transferase from the red imported fire ant, *Solenopsis invicta*

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Abstract

A glutathione *S*-transferase (GST) was purified 266-fold from adult workers of the red imported fire ant, *Solenopsis invicta* (Hymenoptera: Formicidae) by affinity chromatography and preparative isoelectric focusing. The purified enzyme appeared as a single band on SDS-PAGE and had a M_r of 25.5 kDa. Steady state kinetics assays of the enzyme with 1-chloro-2,4-dinitrobenzene as substrate were conducted. The V_{max}, $K_{m\ CDNB}$, $K_{m\ GSH}$, k_{cat}, $k_{cat}/K_{m\ CDNB}$, and $k_{cat}/K_{m\ GSH}$ for the purified fire ant GST were 87.4 μmol/min/mg, 0.13 mM, 0.84 mM, 74.5 s^{-1}, 573.1 mM^{-1} s^{-1}, and 88.7 mM^{-1} s^{-1}, respectively. An internal fragment of the enzyme, released by endoproteinase Lys-C digestion, was sequenced and used to design a degenerate oligonucleotide primer. Purified cDNA from a λ-phage expression library produced from worker fire ants was used as template to amplify a fragment of the GST transcript which was subsequently cloned and sequenced. 5′ and 3′ rapid amplification of cDNA ends were subsequently conducted after cDNA production by RT-PCR of mRNA from adult worker fire ants. An open reading frame comprising 202 amino acids with a calculated molecular weight of 23,433 and a theoretical p*I* of 7.84 was located within the transcript beginning at nucleotide 85 and terminating in a stop codon at nucleotide 691. The transcript contained 5′ and 3′ untranslated regions of 84 and 296 nucleotides, respectively. The sequences of the internal fragments from the purified fire ant GST were identical to corresponding translated regions of the transcript (nucleotides 214–258, and 457–477). Comparison of the deduced amino acid sequence with GSTs from other species revealed that the enzyme was most closely related to sigma class GSTs.
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Keywords: *Solenopsis invicta*; Glutathione *S*-transferase; RT-PCR; RACE; cDNA cloning

1. Introduction

Glutathione *S*-transferase (GST) is an enzyme superfamily exhibiting a range of catalytic functions, including cellular protection from reactive oxygen species, reductive maintenance of thiolated proteins, prostaglandin synthesis, and glutathione conjugation of endogenous and exogenous ligands (Kanaoka et al., 1997; Sheehan et al., 2001). Glutathione conjugation catalyzed by GSTs is the major phase II detoxification reaction in many organisms (Yu, 1996) and has been found to be responsible for resistance of cancer cells to chemotherapeutic agents (Hayes and Pulford, 1995), antibiotic resistance in bacteria (Arca et al., 1997), herbicide resistance in plants (Hatton et al., 1999), and insecticide resistance in insects (Ranson et al., 2001). GSTs are primarily cytosolic enzymes, but microsomal forms have also been identified (Shimoji et al., 1996). They invariably comprise two subunits, either identical (homodimeric) or nonidentical (heterodimeric). Conjugation of activated hydrophobic electrophilic xenobiotics with reduced glutathione by GSTs renders the molecule more water soluble which facilitates excretion (deBethizy and Hayes, 1989).

The red imported fire ant, *Solenopsis invicta* Buren, introduced into the United States from South America in the early 1900s, has spread to more than 128 million hectares primarily in the southeastern US (Williams et al., 2001). *S. invicta* is an aggressive, territorial ant that

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adversely impacts arthropod biodiversity in infested areas (Porter and Savignano, 1990; Porter et al., 1991). Moreover, *S. invicta* poses significant risks to human health and agriculture (Williams et al., 2001). Anaphylaxis induced by red imported fire ant stings occurs in up to 6% of individuals and has resulted in 80 human deaths (deShazo et al., 1999; Williams et al., 2001). *S. invicta* causes economic losses in the cattle industry (Barr et al., 1994), damages agricultural commodities (Lofgren, 1986), and hampers interstate commerce of nursery stock imposed by quarantine regulations (Code of Federal Regulations, 2000). Although exact economic losses are difficult to determine, estimates of $0.5–1 billion annually have been proposed (Thompson et al., 1995).

Despite reliance on insecticides for control of fire ants, detoxification in these species is poorly understood. Thus, we report for the first time, the purification, characterization, and molecular cloning of a GST from the red imported fire ant.

2. Materials and methods

2.1. Ants

Queenright polygynous *S. invicta* colonies were excavated from areas in Gainesville, FL. Ants were separated from the soil and transferred to rearing trays using the floating technique described previously (Jouvenaz et al., 1977).

2.2. GST purification

GST purification was followed by the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) to glutathione (GSH) (Habig et al., 1974). Soluble fraction was prepared by homogenizing 25 g of *S. invicta* workers in 200 ml of phosphate-buffered saline (PBS, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 150 mM NaCl), pH 7.3, with a Teflon pestle and glass mortar. The homogenate was filtered through two layers of cheesecloth and centrifuged for 1 h at 105,000 *g* (max). The supernatant was clarified by filtering it through a 0.45 μm filter. This entire procedure was repeated five times and the clarified soluble fractions were combined. The clarified solution was diluted 1:1 with PBS + 1% Triton X-100 and applied in 100 ml volumes to an equilibrated glutathione sepharose 4B affinity column (Amersham Pharmacia, Uppsala, Sweden). Following the manufacturer's directions, GSTs were eluted from the column with 5 mM glutathione in 50 mM Tris–HCl, pH 8. After regeneration and equilibration, the remaining clarified soluble fraction was likewise applied to the column. The most active fractions from all affinity purifications were pooled and mixed with 0.3 ml of ampholytes in the 3–10 pH range (BioRad, Hercules, CA). This solution was placed into the mini sample cell of a preparative isoelectric focusing rotofor (BioRad, Hercules, CA) using 0.1 M NaOH and 0.1 M H_3PO_4 in the cathode and anode electrode chambers, respectively. The solution was focused at 12 W constant power for 2.5 h. Twenty 0.75 ml fractions were harvested from the chamber by vacuum, and subsequently tested for pH and CDNB conjugating activity.

2.3. SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli (1970) using a BioRad Mini Protean II cell with 15% resolving and 5% stacking gel (0.75 mm thick). Samples were diluted with an equal volume of 60 mM Tris–HCl containing 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, and 0.1% bromophenol blue. The diluted samples were heated to 100 °C for 3 min, applied to the SDS polyacrylamide gel, stacked at 50 V for 15 min, then resolved at 200 V for 40 min. After electrophoresis, gels were washed in deionized water and subsequently silver stained with GelCode SilverSNAP protein staining reagent (Pierce, Rockford, IL) according to the manufacturer's instructions. SDS-PAGE was used to estimate molecular mass by plotting the migration distance of molecular weight standards (16–98 kDa) vs. log molecular mass. These data were analyzed by linear regression to produce an equation which was subsequently used to estimate the molecular mass of the purified GST.

2.4. Enzyme analysis, kinetics, and pH optimum

GST activity was determined with CDNB as substrate. The 3 ml reaction mixture contained 1 ml of 15 mM glutathione and an enzyme source in 2 ml of homogenization buffer. The mixture was incubated at 25 °C for 3 min, then 20 μl of 150 mM CDNB in ethylene glycol monomethyl ether was added and mixed. The change in absorbance at 340 nm was measured on a Varian Model 3 Bio uv/vis spectrophotometer against a reference containing CDNB and glutathione. Enzyme activity was expressed as μmol CDNB conjugated per min per mg protein using the molar extinction coefficient of 9.6 mM^{-1} cm^{-1} for the product, *S*-(2,4-dinitrophenyl)glutathione.

The kinetic parameters K_m and V_{max} were determined for purified fire ant 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 0.047 to 1.49 mM. When GSH constants were measured, the CDNB concentration was held at 1 mM while the GSH concentration was varied from 0.25 to 8 mM. K_m and V_{max} were determined by linear regression of a double reciprocal plot of velocity (μmol *S*-(2,4-dinitrophenyl)glutathione/min/mg protein) and substrate concentration (mM).

The pH optimum was determined for CDNB conjugation activity. Purified fire ant GST (0.5–2 μg) was incubated at 25 °C for 3 min in citric acid–sodium phosphate buffer, pH 5 and 5.5; sodium phosphate buffer, pH 6, 6.5, 7, 7.5, and 8; or Tris–HCl buffer, pH 8.5 and 9 (all buffers were 50 mM). Conjugation activity was determined as described above. Three replications were conducted.

2.5. Glycosylation, phosphorylation assessment

Glycosylation of fire ant GST was assessed by affinity to Concanavalin A (ConA, Amersham Pharmacia, Pascataway, NJ). Fire ant GST was diluted 50-fold (1.5 μg protein) in binding buffer (20 mM Tris–HCl containing 0.5 M NaCl and 1 mM $MnCl_2$) and applied to a ConA column equilibrated with 10 ml of binding buffer at a flow rate of 0.3 ml/min. Fractions (1 ml) were collected for 15 min. Bound enzyme was then eluted from the column with 0.5 M methyl-α-D-glucopyranoside, 20 mM Tris–HCl, 0.5 M NaCl, pH 7.4. Each fraction was analyzed for CDNB conjugating activity as described above.

The purified fire ant GST was evaluated to determine if the protein was phosphorylated. Fire ant GST (2 μg) was subjected to SDS-PAGE on a 15% separating gel, 5% stacking gel as described above. The gel was stained using the GelCode phosphoprotein staining kit following the manufacturer's instructions (Pierce, Rockford, IL).

2.6. N-terminal sequencing

The purified fire ant GST (50 pmol) was electroblotted onto a polyvinylidene fluoride membrane (Immobilon-P^{SQ}; Millipore, Bedford, MA) and submitted to the Interdisciplinary Center for Biotechnology Research (ICBR, University of Florida, Gainesville, FL) for N-terminal sequence analysis.

2.7. Expression library construction, PCR, cloning, 5′ and 3′ RACE, sequence analysis

A complementary DNA expression library was constructed from worker ants of polygynous *S. invicta* using the HybriZAP-2.1 XR Library Construction Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Briefly, first strand cDNA was synthesized from polyadenylated mRNA purified from worker ants with 5-methyl dCTP and an oligonucleotide primer with an 18-base poly(dT) region and an *Xho* I restriction site (RTXho 1, Table 1). Second strand cDNA synthesis was conducted with DNA polymerase I and unmodified dNTPs. An *Eco*RI adaptor was ligated onto the hemimethylated double-stranded cDNA which was subsequently digested with *Xho* I producing unidirectional cDNA comprising an *EcoR* I restriction site at the 5′ terminus and an *Xho* I restriction site at the 3′ terminus. cDNA was cloned into the HybriZAP-2.1 cloning vector, packaged using GigaPack III-Gold packaging extracts, and titered using the XL1-Blue MRF' *E. coli* strain. The initial library titer was 1.4×10^6 pfu/ml. The primary library was amplified once to yield a titer of 13×10^9 pfu/ml.

A degenerate oligonucleotide primer (3SiGSTSDg, Table 1) was designed using a sequence of an internal fire ant GST peptide fragment released by endoproteinase Lys-C digestion (Table 1). An antisense oligonucleotide primer (4SiGSTASXhoI, Table 1) was designed toward the *Xho* I restriction site of the vector (common to all constructs) of the *S. invicta* cDNA library. Polymerase chain reaction (PCR) was conducted (one cycle at 94 °C for 2 min, 35 cycles at 94 °C for 15 s, 54 °C for 15 s, and 68 °C for 30 s) with this primer pair (3SiGSTSDg, 4SiGSTASXhoI) using cDNA purified from the *S. invicta* expression library as template. An 800 bp amplicon was produced which was subsequently ligated into pGEM-T Easy, transformed into JM109 competent cells (Promega, Madison, WI), and sequenced by the Interdisciplinary Center for Biotechnology Research (University of Florida). This sequence was used to generate oligonucleotide primers (17SiGST5RaceAS, 21SiGST3RaceS, Table 1) to conduct 5′ and 3′ rapid amplification of cDNA ends (RACE) of the fire ant GST transcript with the GeneRacer kit according to the manufacturer's directions (Invitrogen, Carlsbad, CA). Total RNA was isolated from 0.1 g of polygyne worker ants with TRIZOL Reagent (Invitrogen, Carlsbad, CA). The total RNA was decapped, and ligated to the GeneRacer RNA 5′ oligonucleotide provided with the kit and reverse transcribed to produce cDNA. PCR was conducted on the cDNA with the GeneRacer 5′ oligonucleotide primer and the gene-specific primer, 17SiGST5RaceAS (Table 1). The amplicon (~500 bp) was ligated into pGEM-T easy, transformed into JM109 competent cells, and sequenced.

In order to amplify the 3′ end of the GST cDNA, PCR was conducted with the GeneRacer 3′ primer and gene-specific primer, 21SiGST3RaceS (Table 1). The amplicon (~400 bp) was ligated into pGEM-T easy, transformed into JM109 competent cells, and sequenced.

Lastly, the entire transcript was amplified with oligonucleotide primers (27SiGSTCDS5S and 28SiGSTCDS3AS, Table 1) designed toward the 5′ and 3′ termini. PCR was conducted with cDNA synthesized for RACE under the following conditions: one cycle at 94 °C for 2 min, 35 cycles at 94 °C for 15 s, 49 °C for 15 s, and 68 °C for 75 s, a final elongation step of 68 °C for 5 min. Purified PCR product was ligated into pGEM-T easy, transformed into JM109 competent cells, and sequenced. Three randomly selected clones were sequenced entirely.

Table 1
List of oligonucleotide primers used in this study

Primer name	Sequence
RTXhoI	5′GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTTTTTTTTTTTTTTT
3SiGSTSDg	5′ATGCCNATGGGNCARATGC
4SiGSTASXhoI	5′GCAGTAATACGACTCACTATAGGGC
17SiGST5RaceAS	5′CATCAATCTCCATGGCCTCAAGTTCGTCGG
21SiGST3RaceS	5′GCTACGGGAGAAAGTTCGCGCTTTGCCC
27SiGSTCDS5S	5′AGTCQTTCACGATTCACGAAC
28SiGSTCDS3AS	5′ATTGAATGAAAAAGATTTTATTTCC

Table 2
Purification table for glutathione *S*-transferase from *Solenopsis invicta*[a]

Step	Specific activity (μmol/min/mg)	Total protein (mg)	Total activity (μmol/min)	Purification (-fold)	Yield (%)
Crude homogenate	0.32	14550	4656	1	100
Soluble fraction	0.63	6340	3994	2	86
GSH affinity purified	21.40	7.2	154	67	3.3
Rotofor purified	85.10	0.14	12	266	0.3

[a] Based on the conjugation of CDNB and GSH.

3. Results

3.1. GST purification

GST specific activity (CDNB conjugation) increased twofold after separation of the soluble fraction by subcellular fractionation from the crude homogenate (Table 2). GSH affinity chromatography of the soluble fraction increased GST specific activity 67-fold. A large portion of material with CDNB conjugating activity passed through the GSH affinity column. Nearly 83% of the total CDNB activity passed through the column during the binding step (Table 2). Further purification was achieved by preparative isoelectric focusing of the GSH affinity purified fraction (Fig. 1). The most active fractions (open circles in Fig. 1), corresponding to pH values 7.8–9, were pooled and found to be 266-fold more active than the crude homogenate. SDS-PAGE of the pooled fractions produced a single band with a molecular retention at 25,500 Da (Fig. 2).

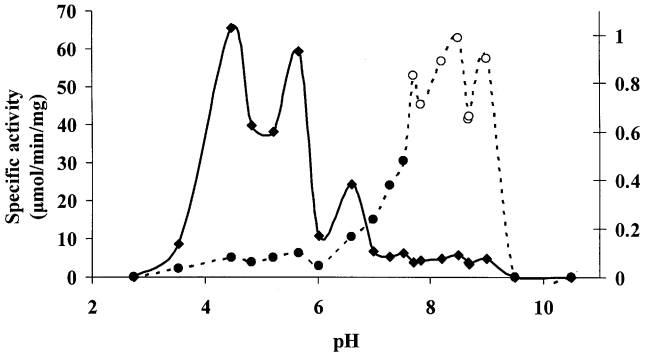

Fig. 1. Fraction profiles for protein (◆) and CDNB activity (●) after separation of affinity purified GSTs by preparative isoelectric focusing. Open circles represent the fractions that were pooled.

3.2. Biochemical enzyme analysis, kinetics, pH optimum, and N-terminal sequencing

The purified fire ant GST was kinetically examined with CDNB and GSH as substrates. Enzyme kinetic con-

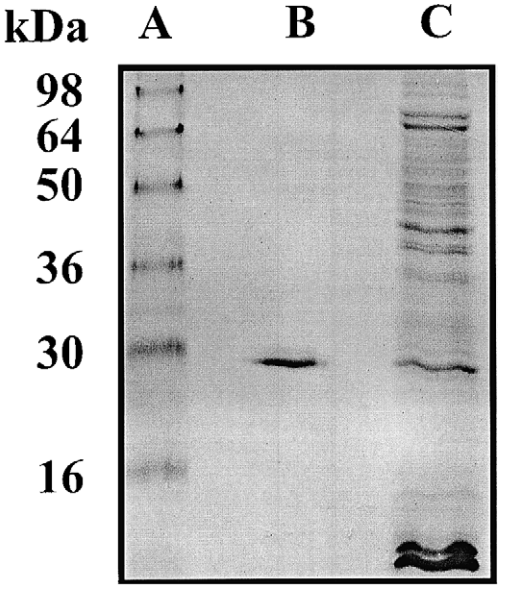

Fig. 2. SDS-PAGE of purified fire ant GST. Lane A, molecular weight markers; lane B, purified fire ant GST (2 μg); lane C, soluble fraction.

Table 3
Biochemical and kinetic constants for *S. invicta* GST with GSH and CDNB substrates

Constant	Fire ant GST
V_{max} (μmol/min/mg)	87.4 ± 5.1
$K_{m\ GSH}$ (mM)	0.84 ± 0.06
$K_{m\ CDNB}$ (mM)	0.13 ± 0.01
k_{cat} (s^{-1})[a]	74.5
$k_{cat}/K_{m\ GSH}$ ($mM^{-1}\ s^{-1}$)	88.7
$k_{cat}/K_{m\ CDNB}$ ($mM^{-1}\ s^{-1}$)	573.1
p*I* (PIEF)[b]	8.50
p*I* (predicted)	7.84
M_r (by SDS-PAGE)	25500 ± 900
Molecular weight (predicted)[c]	23,433

[a] k_{cat} was calculated using the empirically derived homodimer molecular weight (i.e. 51,000).
[b] Corresponds to the preparative isoelectric focusing (PIEF) fraction containing the highest CDNB activity.
[c] Predicted molecular weight derived from the translated open reading frame.

stants are summarized in Table 3. The pH optimum for the fire ant GST with CDNB as substrate was found to be 7 (Fig. 3).

The fire ant GST was N-terminally blocked. Therefore, the enzyme was digested with endoproteinase Lys-C, an endopeptidase that cleaves on the carboxyl side of lysine residues. Two fragments were produced providing unambiguous sequences, PNMPMGQMPILEIDG, and LPFYLD. The larger fragment was used to design a forward degenerate oligonucleotide primer (3SiGSTSDg, Table 1).

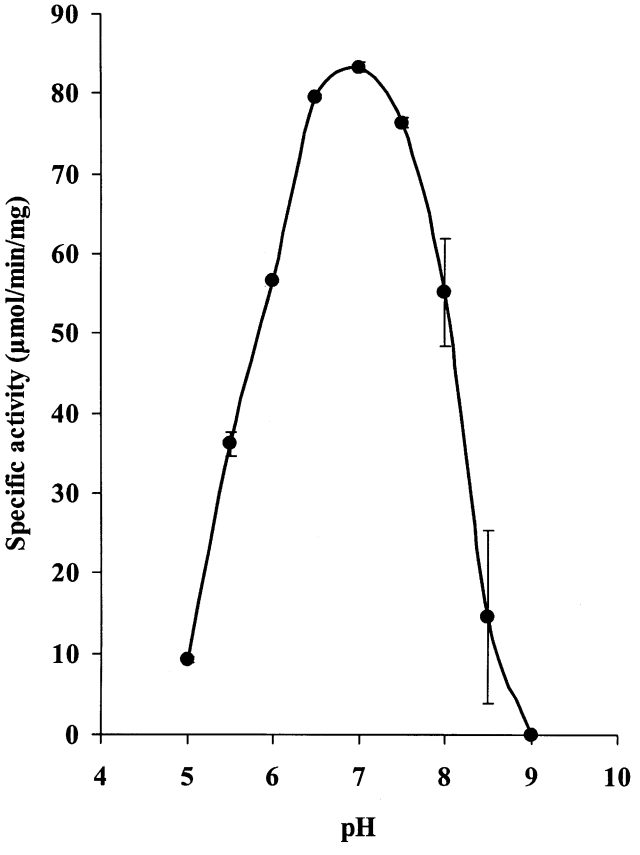

Fig. 3. Effect of pH on the CDNB conjugating activity of the purified fire ant GST. All buffers were 50 mM, citric acid–sodium phosphate buffer, pH 5 and 5.5; sodium phosphate buffer, pH 6, 6.5, 7, 7.5, and 8; or Tris–HCl buffer, pH 8.5 and 9.

Fire ant GST did not bind to ConA indicating that it was not glycosylated. Also, the enzyme was not stained by the phosphoprotein staining kit indicating that it was not phosphorylated.

3.3. Fire ant GST sequence analysis

PCR conducted with the degenerate primer, 3SiGSTSDg, and the primer specific to the cloning vector at the 3′ end of each cDNA insert in the expression library, 4SiGSTASXhoI, yielded an amplicon of approximately 800 bp which was subsequently sequenced. 5′ and 3′ RACE yielded the remaining portions of the fire ant GST transcript. The entire transcript, amplified from cDNA with primers 27SiGSTCDS5S and 28SiGSTCDS3AS, yielded a transcript 962 bp in length (excluding 3′ polyadenylation which varied in length from 5 to 42 residues; Fig. 4). A putative polyadenylation signal was located at positions 941–946 (17 bp upstream of the poly A^+ tail). An open reading frame comprising 202 codons was located within the transcript beginning at nucleotide 85 and terminating in a stop codon (TAA) at nucleotide 691. The transcript contained 5′ and 3′ untranslated regions of 84 and 296 nucleotides, respectively. The open reading frame encoded a protein composed of 202 amino acids with a calculated molecular weight of 23,433 and a theoretical p*I* of 7.84. The sequence of the internal fragments from the purified fire ant GST was identical to the corresponding translated regions of the transcript (nucleotides 214–258, and 457–477).

4. Discussion

Affinity chromatography and preparative isoelectric focusing were used to purify a GST from adult workers of *S. invicta* 266-fold. SDS-PAGE (with and without β-mercaptoethanol) of the purified preparation revealed a single band indicating that the fire ant GST was a homodimer with a M_r of 25,500. The primary structure of the fire ant GST, deduced from the cDNA sequence, contained 202 amino acids with a predicted molecular weight of 23,433. This value agrees well with the empirically determined molecular weight. Furthermore, Edman N-terminal sequencing of two fragments from the purified GST were identical to corresponding deduced amino acid sequences of the cloned transcript.

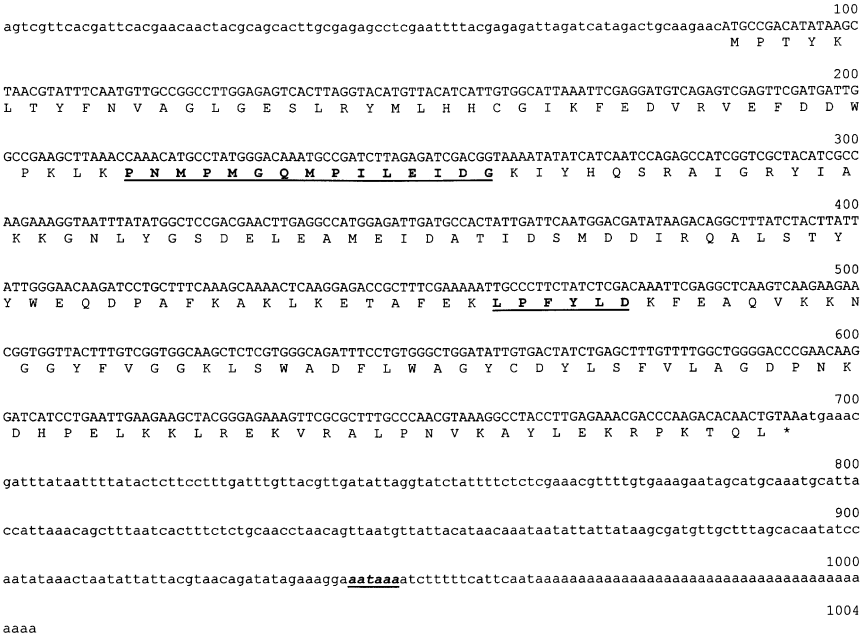

Fig. 4. cDNA sequence and deduced amino acid sequence of the fire ant GST. The bold, underlined amino acid sequences are the internal sequences determined by Edman degradation. Lower case letters indicate the untranslated regions. The putative polyadenylation signal is indicated by bold font and underlining, and the asterisk denotes the stop codon. This sequence has been submitted to GenBank and assigned the accession number AY255670.

Fractions from preparative isoelectric focusing were pooled over a range of pH values (7.8–9) suggesting that multiple charge-isoforms, perhaps as a result of post-translational modification, may have been present. The predicted p*I* of fire ant GST (7.84) was within this pH range.

As observed elsewhere (Board et al., 1994), a large portion of material with CDNB conjugating activity passed through the affinity column without binding. Additional experiments were conducted in which small quantities of soluble fraction were applied to the affinity column. In every case, the effluent contained significant CDNB conjugating activity ruling out column overloading as a possible cause of the bypass. Therefore, it is highly likely that additional GST isozymes from *S. invicta* exist.

The kinetic constants for the fire ant GST toward CDNB and GSH were comparable to values reported from mammalian (Alin et al., 1985) and other insect GSTs (Prapanthadara et al., 1996). The $K_{m\,CDNB}$ and V_{max} of the fire ant GST were most similar to corresponding constants reported in a GST purified from *Blattella germanica* (Yu and Huang, 2000). Interestingly, the amino acid sequence of a GST reported from another strain of *B. germanica* exhibited the highest identity (46%) with that of the fire ant GST (Arruda et al., 1997). Although these studies were conducted independently with different cockroach strains, the GSTs possessed identical N-terminal sequences.

GSTs are a large superfamily comprised currently of at least nine classes among mammals (Sheehan et al., 2001), three classes among insects (Chelvanayagam et al., 2001), four classes among plants (Edwards et al., 2000), and a single bacterial class (Chelvanayagam et al., 2001). Although nomenclature assignments for insect GSTs are currently in a state of flux, generally GSTs sharing greater than 40% identity are considered within the same class (Agianian et al., 2003). A BLAST (Altschul et al., 1997) search of GenBank revealed that the fire ant GST shared greatest identity with sigma class GSTs (Table 4, Fig. 5); the highest identity was found toward a sigma class GST from *B. germanica* (Arruda et al., 1997). Further evidence for the sigma class assignment is offered by presence of R99 and Y206. These residues are thought to be involved in catalysis among sigma class GSTs (Agianian et al., 2003). Using the naming convention posited by Chelvanayagam et al. (2001) for insects, and Edwards et al. (2000) for plants, the fire ant GST should be assigned the name, SiGSTS1 *(Solenopsis invicta*, glutathione *S*-transferase, sigma class, enzyme 1).

In conclusion, we have purified, characterized, cloned and sequenced the first GST from an insect in the Hymenoptera (Formicidae). SiGSTS1 was a basic protein with a deduced molecular mass of 23,433. It also appeared to be a homodimer which readily conjugated GSH to CDNB. BLAST analysis of the translated open reading frame placed the enzyme in the sigma class with closest identity to a GST from *B. germanica* (46%). The coding sequence possessed 5′ and 3′ untranslated

Table 4
Sequence identities of other species as compared with the translated fire ant GST

GenBank designation	GST source	Accession number	GST class	Identity with fire ant GST (%)
GTS1 (Bla g5)	*Blattella germanica*	O18598	Sigma	46
GTS	*Anopheles gambiae*	P46428	Sigma	44
GTS	*Musca domestica*	P46437	Sigma	42
GTS2	*Manduca sexta*	P46429	Sigma	42
PGD2	*Gallus gallus*	O73888	Prostaglandin D synthase	42
PGD2	*Rattus norvegicus*	O35543	Prostaglandin D synthase	39
PGD2	*Homo sapiens*	O60760	Prostaglandin D synthase	38
GTA1	*Caenorhabditis elegans*	Q09596	Alpha	35

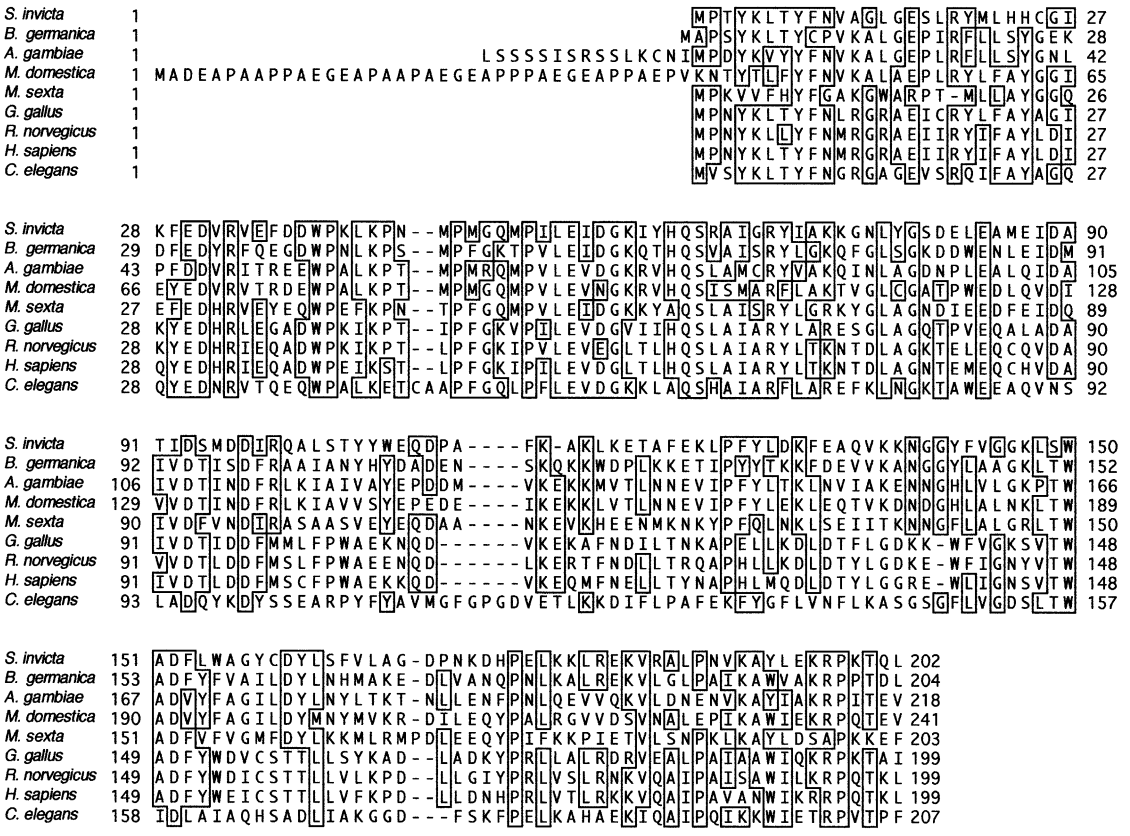

Fig. 5. Sequence alignment of fire ant GST and GST enzymes with the greatest identities retrieved from GenBank. Refer to Table 4 for corresponding accession numbers, GST classification, and identity values. Boxed sections indicate identity.

regions, a putative polyadenylation signal, and a variable length polyadenylated 3′ terminus.

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