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Glutathione *S*-transferase from the spruce budworm, *Choristoneura fumiferana*: identification, characterization, localization, cDNA cloning, and expression

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Abstract

A 23-kDa protein that was present at higher levels in diapausing 2nd instar larvae than in feeding 2nd instar larvae of *Choristoneura fumiferana* was purified, and polyclonal antibodies were raised against this protein. The antibodies were subsequently used to screen a cDNA library that was constructed using RNA from 2nd instar larvae. Eight identical cDNA clones were isolated. The cDNA clone had a 665-bp insert and the longest open reading frame coded for a 203-amino acid protein with a predicted molecular mass of 23.37 kDa. The deduced amino acid sequence showed high similarity to glutathione *S*-transferases and therefore, the cDNA clone was named *C. fumiferana* glutathione *S*-transferase (*Cf*GST). Identity of *Cf*GST was confirmed by using affinity-purification as well as enzyme activity assay. *Cf*GST was closer in similarity to insect GST2 members than GST1 members. The apparent V_{max} of the purified *Cf*GST towards the substrates glutathione and 1-chloro-2,4-dinitrobenezene (CDNB) were similar. However, the enzyme had a three-fold higher affinity towards CDNB than glutathione. Analyses using Northern blot, immunoblot and immunocytochemistry demonstrated that the fat body was the major tissue where the enzyme was synthesized and stored. Higher levels of *Cf*GST protein were present in diapausing 2nd instar larvae compared to feeding 2nd and 6th instar larvae, suggesting that besides detoxification *Cf*GST may have other roles during insect development that are not readily apparent at present. The *Cf*GST cDNA was expressed in a recombinant baculovirus expression system and an active enzyme was produced.

Keywords: Diapause; Insecticide; Detoxification; Resistance; Baculovirus

1. Introduction

The spruce budworm, *Choristoneura fumiferana*, is by far one of the most widely distributed destructive forest insect pests in North America. This insect overwinters as a diapausing 2nd instar larva lasting for approximately 6 months in nature and 14–30 weeks under laboratory conditions at 2°C (Grisdale, 1970). During diapause, the larvae do not feed and remain quiescent inside their hibernacula. In our work to study the biochemical and molecular processes during this obligatory diapause, we examined the proteins that are associated with the diapause phenomenon and found that several proteins accumulated in large quantities during this phase of development. Two of these diapause-associated proteins, *Cf*DAP1 and *Cf*DAP2, were recently isolated and their cDNA were cloned (Palli et al., 1998). In the present work, we have isolated another diapause-associated protein, which was identified as *Choristoneura fumiferana* glutathione *S*-transferase (*Cf*GST).

Glutathione *S*-transferases **(**GSTs) constitute a family of proteins that have long been demonstrated to be involved in: 1) detoxification of xenobiotics, 2) protection from oxidative damage, and 3) intracellular transport of hormones, endogenous metabolites and exogenous chemicals. GSTs have the capacity to conjugate reduced glutathione on the thiol of cysteine to various

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electrophiles and to bind with high affinity to a variety of hydrophobic compounds (Listowsky et al., 1988; Clark, 1989; Pickett and Lu, 1989; Armstrong, 1991; Rushmore and Pickett, 1993). Functional GSTs have been shown to be either homodimers or heterodimers. The genes encoding the subunits of mammalian GSTs have been classified into six groups, five of which code for the cytosolic subunits, alpha, mu, pi, sigma, and theta, while the sixth group codes for a microsomal subunit (Mannervik et al., 1992; Daniel, 1993; Meyer and Thomas, 1995; Jakobsson et al., 1997). Within the same gene class, sequence identities typically range from 60 to 80%, while inter-gene class identities are between 25 and 35%. Insect GSTs have been divided into two major groups, GST1 and GST2 (Grant and Matsumura, 1989; Fournier et al., 1992; Snyder et al., 1995; Franciosa and Berge, 1995) and GST1 is serologically distinct from GST2. Within each group, amino acid sequence identity can be as high as 97%, whereas amino acid sequence identities between groups range from 10 to 24%. The members of GST1 have higher identities with the theta and alpha classes of mammalian GSTs, whereas the GST2 members are closer to the mu, pi and sigma classes. The activity of insect GSTs has been found to be present in the midgut (Tate et al., 1982; Snyder et al., 1995), fat body (Chien and Dauterman, 1991), hemolymph cells and other tissues (Franciosa and Berge, 1995), but more precise sites of GSTs need to be determined.

Since GSTs play an important role in detoxification of xenobiotics, the emphasis of research on insect GSTs has historically been focused on their role in insecticide resistance. Most of these studies have examined the induction of GSTs by plant chemicals, insecticides and other xenobiotics (Motoyama and Dauterman, 1980; Yu 1982, 1989; Riskallah et al., 1986; Lee, 1991; Snyder et al., 1995) or a comparison of GST activity between insecticide resistant and susceptible strains of insects (Yu, 1984; Balabaskaran et al., 1989; Clark, 1989; Fournier et al., 1992). Earlier studies were mainly on measuring the GST activity and purification of the enzyme. More recently, molecular cloning of GSTs from insects belonging to the orders Blattodea, Diptera and Lepidoptera has been reported (Toung et al., 1990; Toung and Tu, 1992; Wang et al., 1991; Beall et al., 1992; Fournier et al., 1992; Snyder et al., 1995; Arruda et al., 1997; Prapanthadara et al., 1998; Huang et al., 1998). Such molecular information increases our understanding of the structural characteristics and functional mechanisms of insect GSTs in the detoxification process, as well as evolutionary relationships among the members of this protein family.

In this paper, we describe the identification of a *C. fumiferana* GST, which is accumulated in large quantities during diapause. We cloned the cDNA encoding this enzyme and compared the deduced amino acid sequence with other insect GSTs. Such a comparison provides useful information on the evolutionary relationships and functional mechanisms of insect GSTs at the molecular level. Also, we purified the enzyme using affinity chromatography and studied its kinetic properties. The localization of *Cf*GST within the insect larvae was studied by analyzing the protein and mRNA. The results indicate that the fat body is the major tissue where *Cf*GST is synthesized and stored. Diapausing 2nd instar larvae contained higher levels of *Cf*GST than the feeding larvae, a fact that raises questions about the function of GST in insects and implies that GSTs may have other roles besides detoxification. Finally, we expressed the cDNA in a baculovirus expression system and obtained an active enzyme, proving the identity of the *Cf*GST cDNA.

2. Materials and methods

2.1. Experimental insects

Spruce budworm (*Choristoneura fumiferana* Clem., Lepidoptera: Tortricidae) eggs were maintained at 22°C and 70% relative humidity (RH) and allowed to hatch into 1st instar larvae. The 1st instar larvae molted into 2nd instar in 7 days. The 2nd instar larvae were maintained at 16°C for 1 week, and then the diapausing 2nd instar larvae were stored at 2°C for 27 weeks to satisfy the obligatory requirement for cold treatment (Grisdale, 1970). At the end of this period, the larvae were moved from 2°C to 16°C for 1 week and then placed on an artifical diet (McMorran, 1965) at 22°C, 70% RH and a photoperiod of 12 h light and 12 h darkness and reared until they reached the pupal stage. Either entire larvae of different ages or tissues, such as the epidermis, fat body and midgut from 6th instar larvae were used for extracting proteins and RNA.

2.2. Protein isolation and purification

Either whole larvae or larval tissues of *C. fumiferana* were homogenized in a homogenization buffer (5 ml/g tissue; 50 mM Tris, 10 mM EDTA, 15% glycerol, 0.005% phenylthiourea, pH 7.8) using a motor-driven Teflon pestle in a 1.5 ml polypropylene microcentrifuge tube. The homogenate was centrifuged at 10 000***g*** for 5 min and the supernatant was re-centrifuged under the same conditions. The final supernatant was used for protein analysis, enzyme assay or further purification.

*Cf*GST was purified using a GST-glutathione affinity chromatography system from Amersham Pharmacia Biotech (Piscataway, NJ, USA) following the manufacturer's protocol. To each milliliter of the protein extracts, 100 μl of a 50% slurry of glutathione-sepharose 4B was added. The mixture was incubated with gentle agitation

for 30 min at room temperature. The suspension was then centrifuged at 500***g*** for 5 min. The agarose matrix precipitate was collected and washed three times with 10 bed volumes of phosphate buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.5) each time. After the final wash, the matrix was suspended in 1 ml of glutathione elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0) for each 1 ml bed volume of the sedimented matrix. The suspension was incubated for 20 min at room temperature and then centrifuged at 500***g*** for 5 min. The supernatant containing the GST was collected and used for analysis by SDS-PAGE and for studies on enzyme kinetics.

2.3. SDS-PAGE

SDS-PAGE was performed according to Laemmli (1970). Protein extracts were denatured at 100°C for 5 min in an equal volume of 2× protein loading buffer (0.1 M Tris buffer, pH 6.8, 4% SDS, 0.2% β-mercaptoethanol, 40% glycerol, and 0.002% bromphenol blue) and subjected to SDS-PAGE using 15% acrylamide gels in Tris-glycine-SDS buffer (10 mM Tris, 50 mM glycine, 0.1% SDS, pH 8.0) at 100 volts for 2 h in a mini-vertical electrophoresis system (BIO-RAD Laboratories, Hercules, CA., USA). The gels were then stained with Coomassie Blue R-250.

2.4. Antibody production

Polyclonal antibodies were produced as described by Pang (1993). Proteins from 2nd instar diapausing larvae were resolved in an SDS-PAGE gel. After electrophoresis, a 23-kDa protein band was excised and the protein was eluted by electrophoresis inside a dialysis tube. The protein was mixed with Freund's adjuvant and then injected into a New Zealand white rabbit. Antiserum was collected after administering two-booster immunizations. Serum from the same rabbit collected prior to immunization was used as the control.

2.5. Immunoblot

Immunoblotting was conducted as described by Sambrook et al. (1989). After electrophoresis proteins were transferred from SDS-PAGE gels to Hybond C nylon membranes (Amersham Life Science, Oakville, Ontario, Canada). The anti-23-kDa protein antibodies were used as primary antibodies at 1:2000 dilution. The sheep anti-rabbit IgG alkaline phosphatase conjugate (Sigma Chemical Company, St. Louis, MO, USA.) was used as the secondary antibody at 1:2000 dilution. Color development of alkaline phosphatase reaction was performed using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates.

2.6. Immunocytochemistry

Preparation of tissue sections and immunodetection were conducted as described in Palli et al. (1998). The primary antibody was the same as the one used for immunoblot, but the dilution was 1:200. The secondary antibody was fluorescein-labeled sheep anti-rabbit IgG F(ab)2 fragment (Boehringer Mannheim, Montreal, Canada) at a dilution of 1:500. The sections were counter-stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) for 30 min and examined under a fluorescence microscope. The photographs were taken as double exposures using fluorescein and DAPI filters.

2.7. Northern blot

Ten micrograms of total RNA from the epidermis, fat body and midgut of 6th instar larvae as well as from the SF-21 cells infected with the recombinant baculovirus was separated on a formaldehyde-agarose (1%) gel according to Lehrach et al. (1977). The RNA was visualized by staining with ethidium bromide and photographed under UV light. The RNA was then transferred to a Hybond N nylon membrane (Amersham Life Science). The blot was hybridized with a *Cf*GST cDNA probe labeled with α-[^{32}P]dATP. Hybridization and washes were conducted as described by Palli et al. (1998).

2.8. Activity assay for CfGST

Measurement of *Cf*GST activity was conducted as described by Grant et al. (1989) using homogenized extracts of larvae or tissues of insects. Three micrograms of insect protein was used in a total volume of 300 μl of a reaction mixture in a 96-well microtiter plate. The two substrates for GST, 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione, were added to the wells. The change in absorbance of CDNB conjugate for the first minute was measured at 340 nm using an Automated Microplate Reader (BIO-TEK Instruments, Inc., Winooski, VT, USA). Protein concentrations were measured using the Bio-Rad protein reagent and bovine serum albumin as the standard. Enzyme activity is reported as μmol of CDNB conjugated min^{-1} mg^{-1} protein. The apparent K_m and V_{max} were determined using the double reciprocal plot analysis. Each data point represents the average of 12 measurements.

2.9. cDNA library construction and screening

A cDNA library was constructed in the Uni-ZAP XR vector (Stratagene, La Jolla, CA, USA) using mRNA from the diapausing 2nd instar larvae of *C. fumiferana*. Screening of the cDNA library was conducted using the *Cf*GST-specific rabbit antiserum (1:2000) and the *pico*

Blue™ Immunoscreening Kit (Stratagene). The secondary antibody was the sheep anti-rabbit IgG alkaline phosphatase conjugate (1:2000). After three rounds of purification of positive plaques, the pBluescript® SK(−) phagemid that contained inserts was excised *in vivo* following the ZAP-cDNA Gigapack Cloning Kit protocol (Stratagene).

2.10. Sequencing and analysis of sequence

Sequencing was performed using ALFexpress™ AutoRead™ Sequencing Kit and ALFexpress™ DNA Sequencer (Amersham Pharmacia Biotech) and sequences were analyzed using the MacVector DNA Analysis Program (International Biotechnologies Inc., New Haven, CT, USA). Sequences were compared to those in the GenBank database at the National Center for Biotechnology Information using the BLAST network services (Altschul et al., 1990). Alignment of amino acid sequences was performed using the Clustal Alignment Program (Higgins and Sharp, 1988).

2.11. Construction of recombinant baculovirus

The recombinant baculovirus expressing the *Cf*GST was constructed by using the BAC-TO-BAC™ Baculovirus Expression System from GIBCO BRL Life Technologies (Gaithersburg, MD, USA), following the manufacturer's instructions. The *Cf*GST cDNA was first cloned into the mini-Tn7 element of a pFASTBAC donor plasmid. The recombinant plasmid was then transformed into DH10BAC cells containing helper plasmid and *Autographa californica* multicapsid nucleopolyhedrovirus (*Ac*MNPV) bacmid. The mini-Tn7 element carrying the *Cf*GST cDNA was transposed into the *Ac*MNPV bacmid with the helper plasmid. The recombinant baculovirus with the inserted *Cf*GST cDNA, *Ac*MNPV-*Cf*GST, was selected by the disruption of the *lacZ* gene and confirmed by PCR followed by Southern hybridization.

SF-21 cells (the *Spodoptera frugiperda* ovarian cell line, IPLB-SF-21, Vaughn et al., 1977) were cultured in 2 ml of Grace's medium (Grace, 1962) supplemented with 0.25% tryptose broth and 10% fetal bovine serum in a six-well plate at a concentration of 5×10^5 cells/ml. The cells were incubated with a transfection mixture containing 300 ng of *Ac*MNPV-*Cf*GST bacmid DNA and 10 μl of CELLFECTIN™ reagent (GIBCO BRL Life Technologies) for 5 h at 28°C. After incubation, the transfection mixture was removed and the cells continued to be cultured in Grace's medium at 28°C for 4 days. Then, *Ac*MNPV-*Cf*GST was harvested and the titer of the virus was determined. The virus at 0.04 pfu was used to further infect SF-21 cells cultured in 15-ml flasks for expression of mRNA and protein. The infected cells were harvested at 0, 6, 12, 24, 48, 72, and 96 hrs post inoculation (h.p.i.) and used for analysis of mRNA, protein and enzyme activity. A recombinant baculovirus expressing a juvenile hormone esterase cDNA (*Ac*MNPV-*Cf*JHE) was constructed in the same way as *Ac*MNPV-*Cf*GST (Feng et al., unpublished) and was used as a control. Northern blot, immunoblot and enzyme activity assays were performed as described earlier.

3. Results

3.1. Isolation of a diapause-associated protein

In an attempt to identify diapause-associated proteins, we compared the protein profiles of 2nd instar larvae that were in pre-diapause (1 week at 16°C, PrDW1), diapause (1 and 15 weeks at 4°C, DW1 and DW15), post-diapause (4 days at 16°C, PtDD4), and those that were feeding and growing (3 days after moving them to 22°C PtDD10) using SDS-PAGE (Fig. 1A). We observed that several proteins were present in large quantities in pre-diapausing, diapausing and post-diapausing larvae, and their abundance decreased at the termination of diapause and low levels were detected in the 2nd instar larvae that were actively feeding. One of these is a 23-kDa protein present at high levels in the pre-diapause, diapause and post-diapause larvae, but at low levels in the 2nd instar larvae that were actively feeding (Fig. 1A).

We excised the 23-kDa protein band from a SDS-PAGE gel and produced an antiserum against this protein in order to clone the corresponding cDNA. Prior to using the antibody to screen a cDNA library, we examined the specificity of the antiserum for the 23-kDa protein by using immunoblots (Fig. 1B). The results of the immunoblots showed that the antiserum specifically bound to the 23-kDa protein. Immunoblots also confirmed the SDS-PAGE results indicating that much higher levels of the 23-kDa protein were present in the diapausing larvae than in the feeding 2nd instar larvae. The antibody also bound weakly to a high molecular weight protein.

3.2. Cloning and sequence analysis of CfGST cDNA

To clone the cDNA encoding the 23-kDa protein, we used the antiserum produced against this protein to screen a cDNA library made from mRNA isolated from diapausing 2nd instar larvae. Eight positive clones were obtained by screening 2×10^4 recombinant Uni-ZAP λ phage plaques. Restriction enzyme analysis and partial sequencing showed that all these clones were identical and contained a 665-bp insert. One of these 8 clones was then selected for complete sequencing on both strands.

Sequencing results revealed that this cDNA clone was

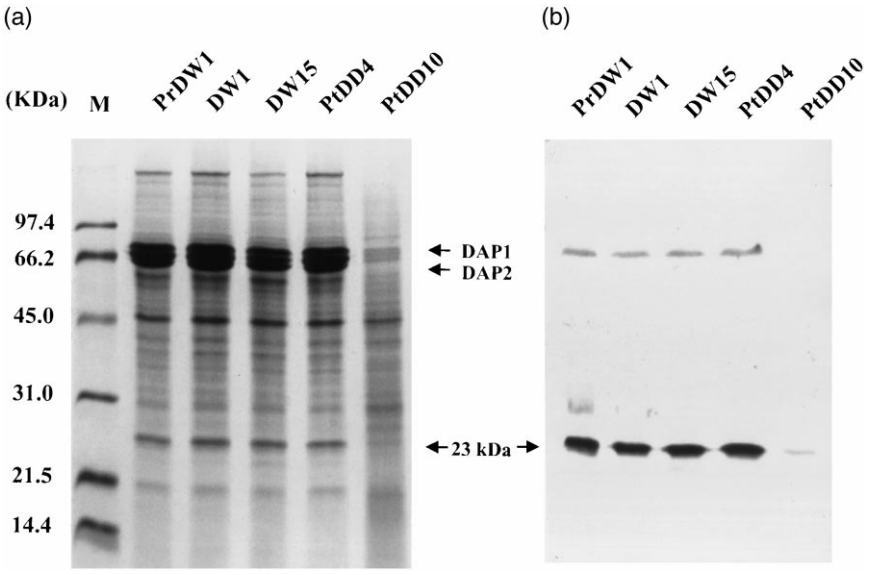

Fig. 1. Analysis of *C. fumiferana* 2nd instar larval proteins using SDS-PAGE (a) and immunoblot (b). Five micrograms of protein was loaded into each lane. PrDW1, pre-diapause 1 week at 16°C; DW1 and DW15, diapause 1 and 15 weeks at 4°C, respectively; PtDD4, post-diapause 4 days at 16°C; PtDD10, 2nd instar feeding larvae day 3 after diapause at 22°C. DAP1 and DAP2, diapause-associated protein 1 and 2; M, molecular weight marker.

665 nucleotides long and the longest open reading frame (ORF) encoded a protein of 203 amino acid residues (Fig. 2). The predicted molecular weight of the translated protein was 23 371, which agrees well with the protein detected on the SDS-PAGE (Fig. 1). The predicted isoelectric point (pI) was 8.91. A putative polyadenylation signal, AATAAA, was located at the 626 nucleotide, followed by a poly $(A)_{18}$ tract. This polyadenylation signal overlapped the translation stop codon, TAA, by one base. Comparison of the deduced amino acid sequence of this cDNA with the protein sequences in GenBank indicated that the deduced amino acid sequence matched well with the glutathione *S*-transferases from other organisms and hence the 23-kDa protein was referred to as *C. fumiferana* glutathione *S*-transferase (*Cf*GST).

The deduced amino acid sequence of *Cf*GST did not contain any hydrophobic sequence that could act as a signal peptide (Fig. 2). There were two putative *N*-glycosylation sites, Asn_{63}-Gln_{64}-Ser_{65} and Asn_{124}-Glu_{125}-Ser_{126}. Nine (Tyr_7, Gly_{12}, Trp_{38}, Lys_{44}, Gln_{51}, Pro_{53}, Gln_{64}, Ser_{65} and Asp_{98}) of the 11 amino acid residues that made up the glutathione binding site and were

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  1  GGCACGAGGCAACAAAATGGCCAAGAAACTACATTATTTCCATTTGAACGGGCTTGCTGAG
                     M  A  K  K  L  H  Y  F  H  L  N  G  L  A  E      15
 62  TCCATCAGGTACATCCTGCACTACGGCGGACAAAAGTTCGAGGATGTCAGATACGATCTG
      S  I  R  Y  I  L  H  Y  G  G  Q  K  F  E  D  V  R  Y  D  L      35
122  AAAAGCTGGCCCATCAAGAGTGTGAAAGACACTCTCCCATACGGCCAGCTGCCACTCTAC
      K  S  W  P  I  K  S  V  K  D  T  L  P  Y  G  Q  L  P  L  Y      55
182  GAGGAGGGAAATAAGACCCTAAACCAGTCACTGGCCATCGCGCGCTACGTAGCTGCCCAG
      E  E  G  N  K  T  L  N  Q  S  L  A  I  A  R  Y  V  A  A  Q      75
242  GTCCACCTCCTGCCCACCGATCCCTGGGAGCAGGCCGTCCTGGATGCCATCGTCTTCAAC
      V  H  L  L  P  T  D  P  W  E  Q  A  V  L  D  A  I  V  F  N      95
302  ATCTATGACTTCTGGGGAAAGATTCTGGTCTTCATCAAGGAGAATGATGCTGCTAAGAAG
      I  Y  D  F  W  G  K  I  L  V  F  I  K  E  N  D  A  A  K  K     115
362  GAGGTAATCAAGAAGGAGATCATAAACGAATCCGTTGACTTCTTCTTCTCCCGATTTGAG
      E  V  I  K  K  E  I  I  N  E  S  V  D  F  F  F  S  R  F  E     135
422  AAGGAACTTAAGGCCAACAAGGGATTCTTCAACGGAAAGCTGAGCTGGGCTGACTTCGTC
      K  E  L  K  A  N  K  G  F  F  N  G  K  L  S  W  A  D  F  V     155
482  CTTGTGGGCATCGTCGAGTCTGCCAACCTGTTCCTTGGCACCGAGATTGAGAAGAAATAC
      L  V  G  I  V  E  S  A  N  L  F  L  G  T  E  I  E  K  K  Y     175
542  CCCACCGTGCTCGTGCTCGTCCAGAAAATCCGCACCCTCCCTGGAGTGAAGGAATACATC
      P  T  V  L  V  L  V  Q  K  I  R  T  L  P  G  V  K  E  Y  I     195
602  GCGACTAGGAAACCATATGCTCTATAAATAAAGTACACGTTACTGTAAAAAAAAAAAAAA
      A  T  R  K  P  Y  A  L  ***                                     203
662  AAAA
```

Fig. 2. Nucleotide and deduced amino acid sequences of the *Cf*GST cDNA. The stop codon, TAA, is marked with asterisks. The putative polyadenylation signal, AATAAA, is double-underlined. Two putative N-glycosylation sites are underlined. The nine amino acid residues that are known to be involved in glutathione binding or enzyme activity are shaded. The GenBank accession number for the sequence is AF128867.

involved in specific activity of mammalian mu, pi and sigma GSTs (Reinemer et al., 1991; Ji et al. 1992, 1995) were found to be present in the deduced amino acid sequence of *Cf*GST. Of these residues, Gln_{64} and Ser_{65} overlapped with one of the putative *N*-glycosylation sites. There was no cysteine present in the sequence of *Cf*GST.

3.3. Purification of CfGST protein

We used GST-glutathione affinity chromatography to purify the *Cf*GST protein and to confirm the identity of *Cf*GST. The affinity-purified *Cf*GST protein from 2nd instar larvae in diapause for 20 weeks appeared as a single band in SDS-PAGE gels (Fig. 3A) that was immunologically recognized by the antibody produced against the 23-kDa protein (Fig. 3B). GST activity assays indicated that the affinity-purified *Cf*GST protein from 2nd instar diapausing larvae exhibited high GST enzyme activity (65 μmol CDNB conjugated min^{-1} mg^{-1} protein), which was 130 fold higher than that (0.5 μmol CDNB conjugated min^{-1} mg^{-1} protein) in crude extracts of diapausing 2nd instar larvae. These results confirmed that the 23-kDa protein, that we had isolated, was indeed *Cf*GST.

As shown in Fig. 1, the levels of *Cf*GST were much higher in the diapausing than in the feeding 2nd instar larvae. We also examined the levels of *Cf*GST in 6th instar larvae because some diapause-associated proteins such as *Cf*DAP1 and *Cf*DAP2 have been shown to be present at high levels at this stage. The results indicated that 6th instar larvae contained lower levels of *Cf*GST than diapausing 2nd instar larvae (Figs. 3A and B). For example, 6th instar larvae contained 18 μg of affinity-purifed *Cf*GST per gram of tissues (or 0.04% of total protein), whereas diapausing 2nd instar larvae contained 485 μg of *Cf*GST per gram of tissues (or 0.5% of total protein).

3.4. Kinetic properties of CfGST

The steady state kinetic properties of the affinity-purified *Cf*GST from diapausing 2nd instar larvae were determined. Double reciprocal plots of enzyme-catalyzed reactions with varying concentrations of glutathione (0.01–3 mM) or CDNB (0.01–2 mM) at a fixed concentration of CDNB (3 mM) or glutathione (5 mM), respectively, are shown in Fig. 4. The apparent V_{max} values for glutathione and CDNB appeared to be similar, while the apparent K_m value for glutathione was three times higher than that for CDNB. This suggests that *Cf*GST had a higher affinity for CDNB than for glutathione. This result was consistent with observations on other insect GSTs (Clark, 1989). Given a molecular mass of 23.37-kDa for *Cf*GST, the catalytic constants, K_{cat}, were 1.6×10^3 min^{-1} and 1.7×10^3 min^{-1} for glutathione and CDNB, respectively. Thus, the enzyme's catalytic efficiencies (K_{cat}/K_m) were 5.9×10^3 min^{-1} mM^{-1} and 2.1×10^4 min^{-1} mM^{-1} for glutathione and CDNB, respectively.

3.5. Localization of CfGST within the larvae

Localization of *Cf*GST within the larvae was determined using three methods: SDS-PAGE followed by immunoblot (Fig. 5A), immunocytochemistry (Fig. 5B) and Northern blot (Fig. 5C). *Cf*GST protein was detected in the fat body and epidermis, but not in the midgut of

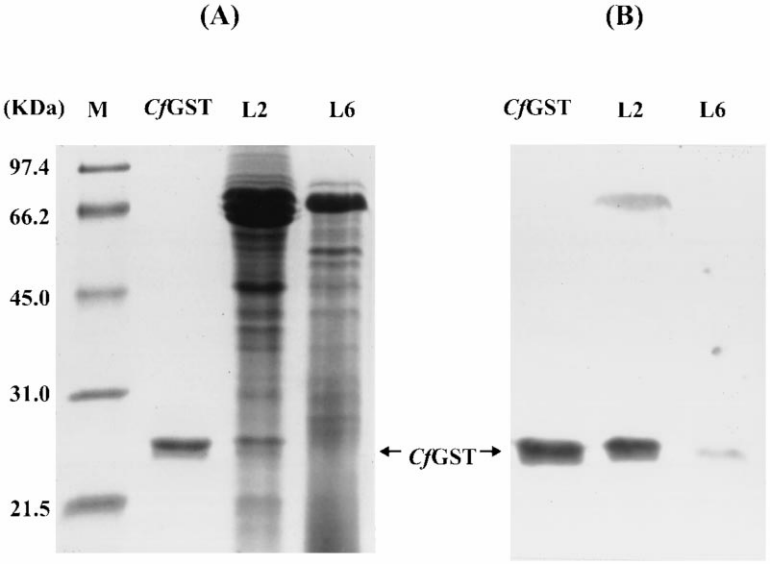

Fig. 3. Analysis of *Cf*GST in 2nd instar larvae in diapause for 20 weeks and 4-day old 6th instar larvae using SDS-PAGE (A) and immunoblot (B). The purified *Cf*GST was from 2nd instar larvae in diapause for 20 weeks. Five micrograms of protein was loaded into each lane. L2, 2nd instar larvae in diapause for 20 weeks; L6, 4-day old 6th instar larvae; M, molecular weight marker.

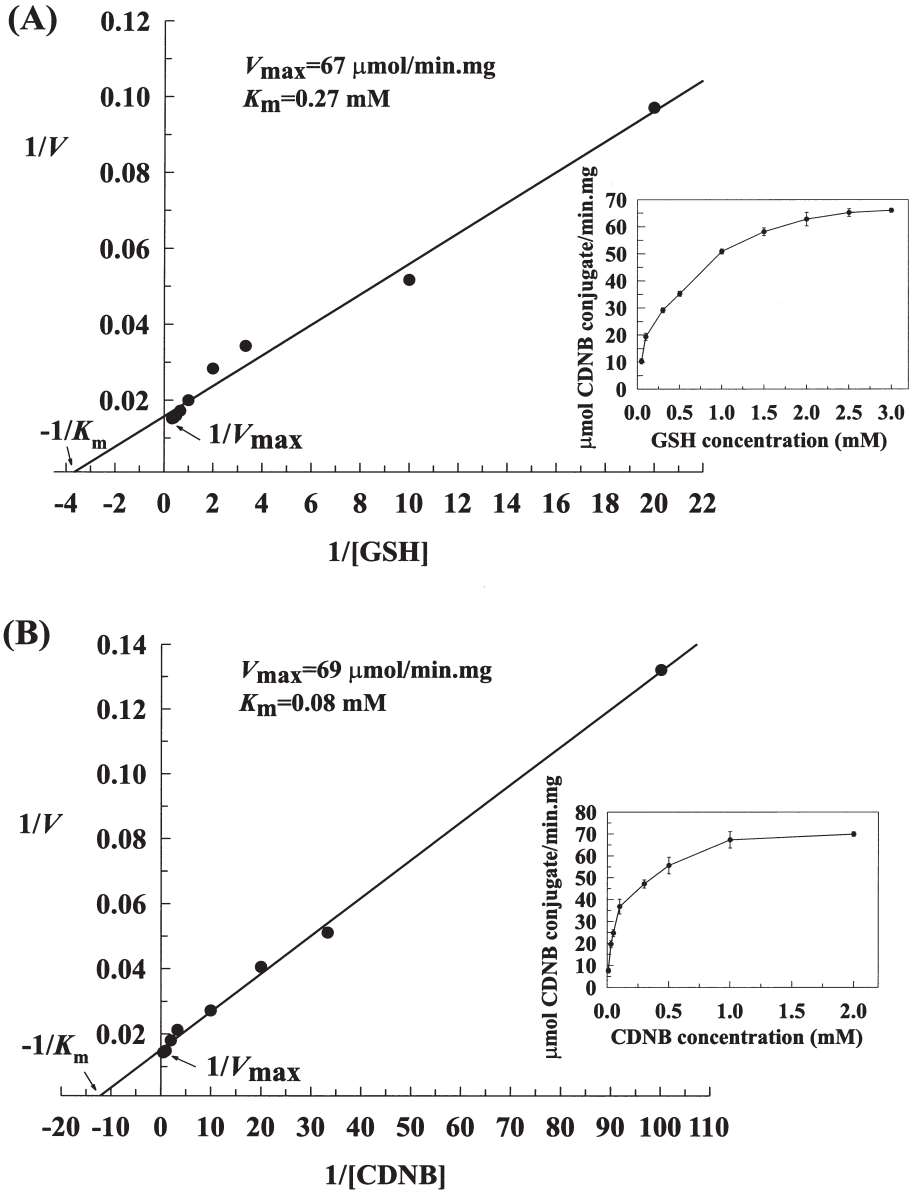

Fig. 4. Double reciprocal plots of enzyme activity with varying concentrations of (A) glutathione (0.01–3 mM) or (B) CDNB (0.01–2 mM) at a fixed concentration of CDNB (3 mM) or glutathione (5 mM), respectively.

6th instar larvae using SDS-PAGE gels and immunoblots (Fig. 5A). Immunocytochemistry on sections of 2nd and 6th instar larvae revealed the abundant presence of *Cf*GST protein in the fat body [Fig. 5B(a and c)]. Immunostaining with non-immune serum as a control did not show any positive stain in the fat body [Fig. 5B(b and d)]. No *Cf*GST protein was detected in the epidermis [Fig. 5B(a and c)]. Sections of the 6th instar larval midgut stained with anti-*Cf*GST antibodies showed a weak positive signal in the epithelial cells of the midgut [Fig. 5B(e)]. This positive reaction was not detected in the control where non-immune serum was used [Fig. 5B(f)].

Northern blot analysis for spatial expression of *Cf*GST mRNA in the epidermis, fat body and midgut of 4-day-old 6th instar larvae showed that a 0.7 kb *Cf*GST mRNA was abundant in the fat body and present in a small amount in the epidermis, and absent in the midgut (Fig. 5C).

3.6. Expression of CfGST in a recombinant baculovirus expression system

To verify that the *Cf*GST cDNA encodes an active enzyme, we inserted the cDNA into a polyhedrin minus *Ac*MNPV genome and obtained the recombinant baculovirus, *Ac*MNPV-*Cf*GST which was then used to infect SF-21 cells. Northern blot hybridized with a *Cf*GST cDNA probe showed that a 0.7 kb *Cf*GST mRNA started to appear at 24 h.p.i. in the cells infected with *Ac*MNPV-

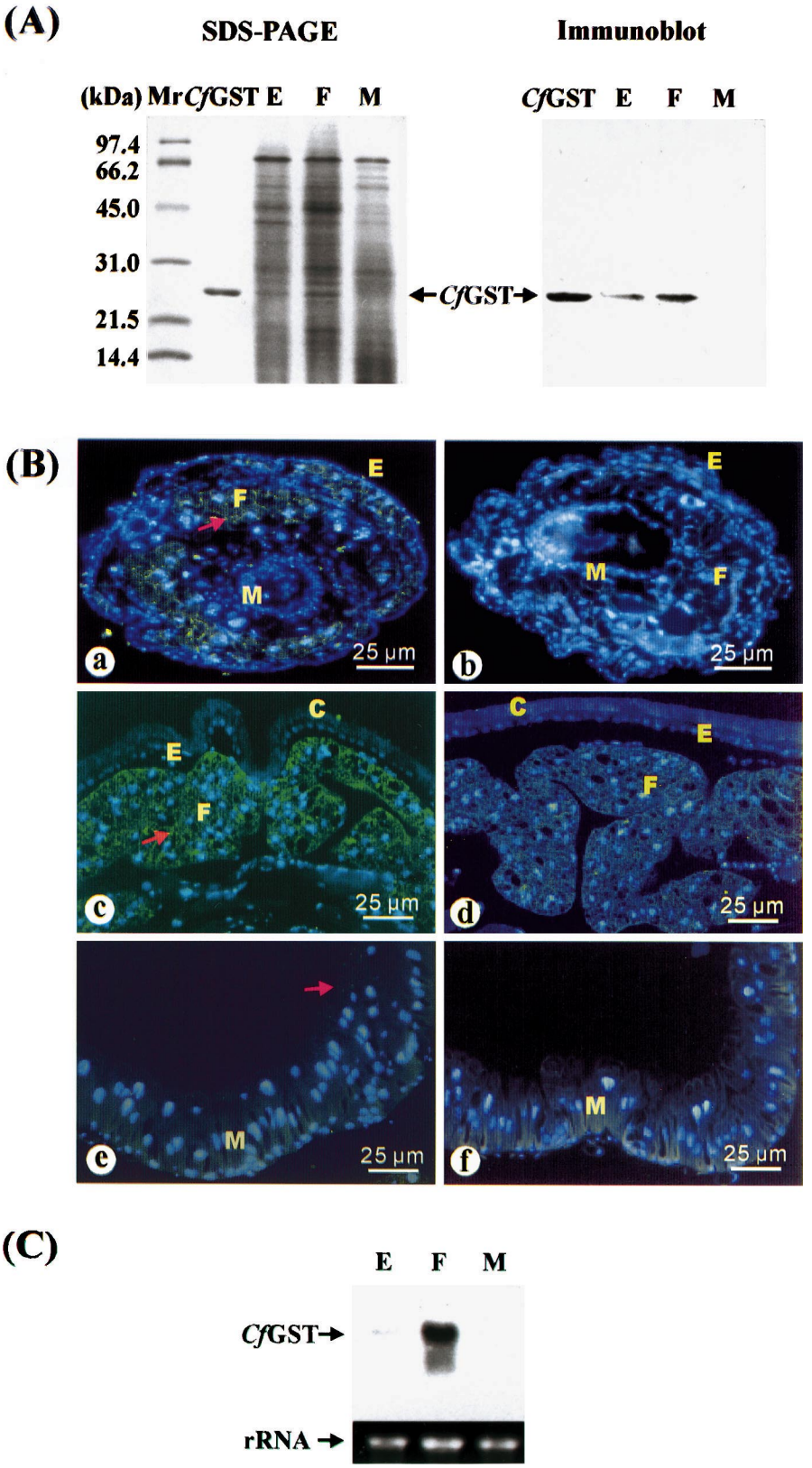

Fig. 5. Localization of *Cf*GST in the spruce budworm. (A) Detection of *Cf*GST in 6th instar larvae using SDS-PAGE and immunoblot. Five micrograms of protein was loaded into each lane. The purified *Cf*GST was from 2nd instar larvae that were in diapause for 20 weeks. (B) Immunocytochemistry of *Cf*GST in 2nd (a–b) and 6th (c–f) instar larvae. Five micron sections were immunostained with anti-*Cf*GST antibodies (a, c and e), or non-immune serum from the same rabbit prior to immunization (b, d and f), followed by fluorescein-labeled sheep anti-rabbit IgG Fluorescein F(ab)2 fragment and counter-stained with DAPI. The sections were observed under a fluorescence microscope and the photographs were taken as double exposures under fluorescein and DAPI filters. Arrows show *Cf*GST protein stained with fluorescein. (C) Expression of *Cf*GST mRNA in the epidermis, fat body and midgut of 3-day-old 6th instar larvae. The top panel shows the Northern blot containing 10 μg of total RNA per lane hybridized with a *Cf*GST cDNA probe. The bottom panel shows rRNA stained with ethidium bromide, indicating equal loading of total RNA. C, cuticle; E, epidermis; F, fat body; M, midgut. Mr, molecular weight marker;

*Cf*GST (Fig. 6A, left). The mRNA level greatly increased by 48 h.p.i. This mRNA was not present in the control samples, where the cells were infected with the recombinant baculovirus containing a cDNA encoding juvenile hormone esterase (*Ac*MNPV-*Cf*JHE, Fig. 6A, right).

Protein analysis using SDS-PAGE revealed that *Cf*GST protein started to appear at 48 h.p.i. and reached maximum levels by 72 h.p.i. (Fig. 6B, left). The molecular mass of the expressed protein was exactly the same as that of *Cf*GST protein purified from 2nd instar larvae. Immunoblots showed that the expressed protein was recognized by the anti-*Cf*GST antibodies (Fig. 6B, right). The expressed protein was examined for GST activity using glutathione and CDNB as substrates (Fig. 6C). The appearance of GST activity correlated well with the increase in concentration of the expressed *Cf*GST protein. GST activity was first detected at 48 h.p.i. and it reached maximum levels by 72 h.p.i. The enzyme activity of the crude extract of *Cf*GST expressed in the infected cells was as high as one-fourth the equivalent of the affinity-purified enzyme from the diapausing 2nd instar larvae.

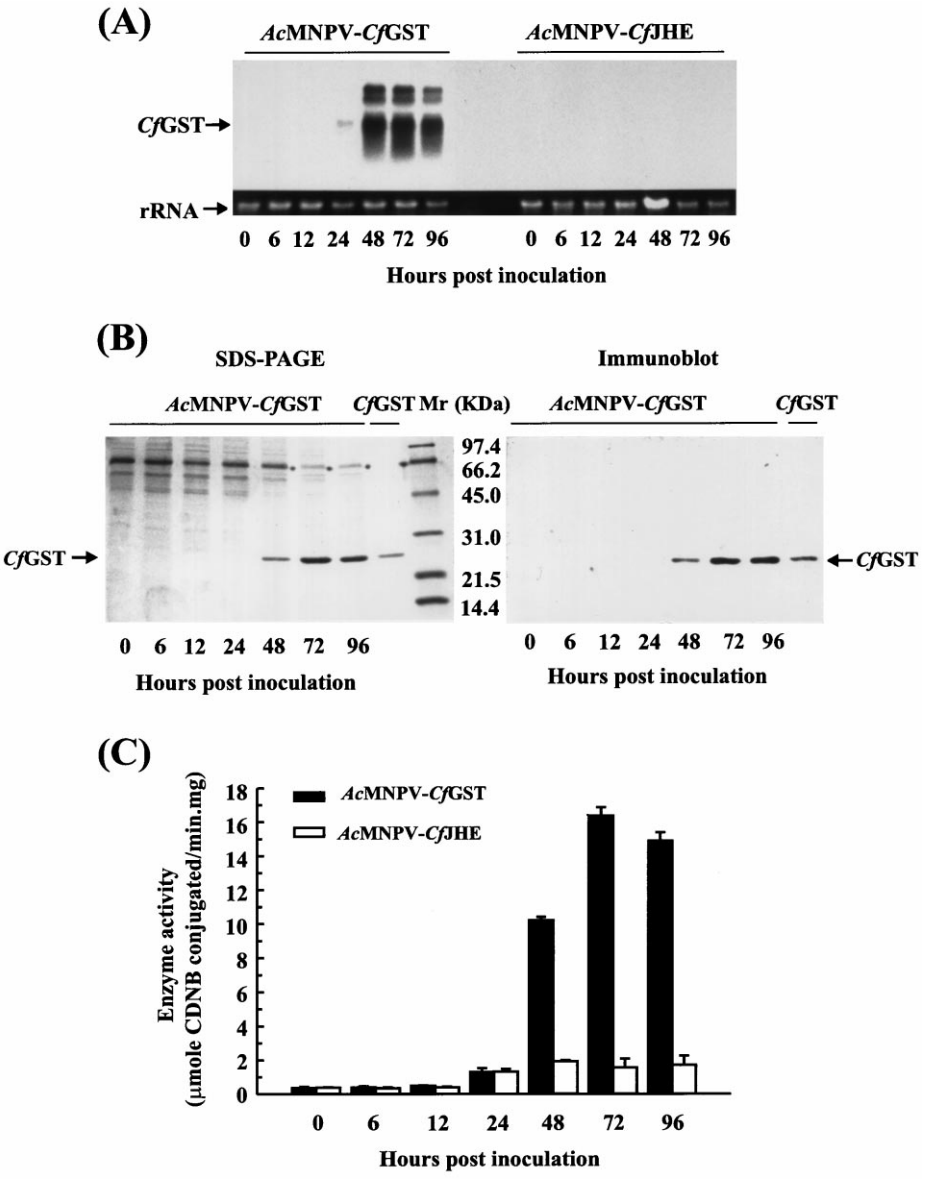

Fig. 6. Expression of *Cf*GST in a baculovirus expression system. (A) Expression of *Cf*GST mRNA in SF-21 cells inoculated with *Ac*MNPV-*Cf*GST (left) or *Ac*MNPV-*Cf*JHE as a control (right). The top panel shows the Northern blot containing 10 μg of total RNA per lane hybridized with a *Cf*GST cDNA probe. The bottom panel shows rRNA stained with ethidium bromide, indicating equal loading of total RNA. (B) Analysis of *Cf*GST protein expressed in SF-21 cells inoculated with *Ac*MNPV-*Cf*GST using SDS-PAGE and immunoblot. Three micrograms of protein was loaded into each lane. The purified *Cf*GST was from 2nd instar larvae in diapause for 20 weeks. (C) Enzyme activity assay of cytosolic *Cf*GST expressed in SF-21 cells inoculated with *Ac*MNPV-*Cf*GST or *Ac*MNPV-*Cf*JHE as a control. Glutathione (3 mM) and CDNB (2 mM) were used as substrates for a reaction. Bars represent mean ±SE (n=6).

4. Discussion

Among the objectives of this study is the identification of proteins that were associated with the overwintering diapause in the spruce budworm. A 23-kDa protein was found to be present at high levels in the diapausing 2nd instar larvae, but at low levels in the feeding 2nd instar larvae. By screening a cDNA library with antibodies raised against the 23-kDa protein, a cDNA clone was isolated and identified as GST based on sequence identities with other known GSTs. Its identity was confirmed by affinity purification and enzyme activity of the purified and expressed protein.

Because the antibodies were raised against the 23-kDa protein band from the SDS-PAGE gel, some confirmation is needed of their specificity for *Cf*GST. There are two lines of evidence which supports their specificity. First, all 8 clones isolated by screening cDNA library using these antibodies were identical. Second, these antibodies detected a single band on immunoblots of proteins isolated from tissue extract. Given this degree of specificity, we can use these antibodies to compare the levels of *Cf*GST during larval development.

These data indicate that the diapausing 2nd instar larvae contain a much higher level of *Cf*GST than feeding 2nd and 6th instar larvae. Diapausing larvae do not feed and therefore they would contain fewer plant chemicals and xenobiotics than the larvae that are actively feeding. Larvae that are actively feeding have the potential to ingest contaminating toxic chemicals along with the diet and therefore one would expect these insects to contain a higher level of GST. Since GST has been linked to detoxifying ingested chemicals that are toxic, it raises the question as to why the diapausing 2nd instar larvae contain a higher level of GST than the feeding 2nd and 6th instar larvae. This can be seen clearly in Fig. 1, which shows the dramatic decrease in the levels of *Cf*GST in 2nd instar larvae just 3 days after emerging from diapause. These findings suggest that, in addition to the classical detoxification function, insect GSTs must play some other important role. Perhaps accumulation of GST during diapause may be intended to deal with the post diapause prefeeding metabolism. It is possible that material accumulated during diapause in the fat body contains potentially toxic substances and these insects use GST for detoxification. In rat ovary and liver GST is involved in intracellular binding and transport of steroid and thyroid hormones (Ishigaki et al., 1989; Singh and Pandey, 1996). The role of insect GSTs in functions other than detoxification needs to be critically examined.

The three-dimensional structure of the mammalian homodimeric GSTs, pi, mu and sigma, revealed that each of the two subunits of GSTs contains two domains (Reinemer et al., 1991; Ji et al., 1992, 1995). Domain I is close to the *N*-terminal end and is the glutathione binding site (G-site) whereas domain II is at the *C*-terminal end and is the hydrophobic site (H-site) responsible for the electrophilic substrate binding. It appears from the alignment of *Cf*GST with mammalian GSTs (data not shown) that *Cf*GST is closer to pi, mu and sigma GSTs than to alpha and theta GSTs. In the G-site of the *Cf*GST, 19 out of the 29 amino acid residues conserved in pi, mu and sigma GSTs, including the 8 residues involved in glutathione binding, were identical to pi, mu and sigma GSTs. In the H-site of *Cf*GST, 10 out of the 23 conserved amino acid residues were identical to pi, mu and sigma GSTs.

We compared the deduced amino acid sequence of *Cf*GST with that of 36 insect GSTs that have been cloned and sequenced to date (Table 1). The amino acid identities between *Cf*GST and the other insect GSTs varied from 10.8 to 32.5%. It is obvious that *Cf*GST is closer to the GST2 group than the GST1 group of insect GSTs because the amino acid identities between *Cf*GST and the GST2 members are 26.1–32.5%, whereas the identities between *Cf*GST and the GST1 members are 10.8–16.7%.

Phylogenetic tree analysis of genetic relationships indicated that insect GSTs could be grouped into six clusters (Fig. 7A). Clusters I and IV consist of *Drosophila* GSTs. Cluster I includes the six members of a multigene family found in different species of *Drosophila* (GenBank Accession No. P30104-P30108), while Cluster IV includes the members of a D gene family in *D. melanogaster* (Toung et al., 1993). Clusters II and III include *Lucilia* and *Musca* GSTs (Board et al., 1994; Syvanen et al., 1994; Franciosa and Berge, 1995; Zhou and Syvanen, 1997). Cluster V comprises of mosquito GSTs, three of which (*Ag*GST1-1, *Ag*GST1-5 and *Ag*GST1-6) belong to a gene family of *Anopheles gambiae* (Ranson et al., 1997a,b). The most interesting cluster is Cluster VI, which consists of 9 GSTs from different insect species, four of which are from Lepidoptera, four from Diptera and the other one from Blattodea. Six members (*Cf*GST, *Ag*GST2-1, *Bg*GST, *Md*GST, *Ms*GST2, and *Dm*GST2) of this cluster belong to the GST2 group, whereas the other three members of this cluster and all the members of other clusters belong to the GST1 group.

Fig. 7B shows the alignment of amino acid sequences of insect GSTs. The sequences of GST2 members of Cluster VI, to which *Cf*GST belongs, are different from GST1 members of Clusters I-VI. While the members of Clusters I–V have amino acid identities of 30-99%, the ones from the members of Cluster VI range from 26 to 78%. But the identities between members of the two sets of clusters are less than 25%. Forty-seven percent of the amino acid residues are conserved among the GST2 sequences (Fig. 7B, blue). Twenty-five amino acids are identical in all six GST2 sequences of this cluster. Eight of the nine amino acids that have been suggested to be involved in glutathione binding or catalytic activity in

Table 1
Glutathione *S*-transferases from various insect species

Common name	Species	Sequence	Amino acid no.	Identity with *Cf*GST (%)	Insect group	Mammalian group	Reference
Lepidoptera							
Spruce budworm	*Choristoneura fumiferana*	*Cf*GST	203		2		This study
Silkworm	*Bombyx mori*	*Bm*GST	218	10.8	1		GenBank, AJ006502
Tobacco hornworm	*Manduca sexta*	*Ms*GST1	217	16.7	1	Theta	Snyder et al., 1995
Tobacco hornworm	*Manduca sexta*	*Ms*GST2	203	26.1	2	Sigma	Snyder et al., 1995
Diamondback moth	*Plutella xylostella*	*Px*GST3	216	16.7	1	Theta	Huang et al., 1998
Blattodea							
German cockroach	*Blattella germanica*	*Bg*GST	200	31.0	2		Arruda et al., 1997
Diptera							
Thai malaria mosquito	*Anopheles dirus*	*Ad*GST1-1	209	13.3	1		Prapanthadara et al., 1998
African malaria mosquito	*Anopheles gambiae*	*Ag*GST2-1	218	32.5	2	Sigma	Reiss and James, 1993
African malaria mosquito	*Anopheles gambiae*	*Ag*GST1-1	140	13.6	1		Ranson et al., 1997a
African malaria mosquito	*Anopheles gambiae*	*Ag*GST1-2	140	11.8	1		Ranson et al., 1997a
African malaria mosquito	*Anopheles gambiae*	*Ag*GST1-5	209	12.3	1	Theta	Ranson et al., 1997b
African malaria mosquito	*Anopheles gambiae*	*Ag*GST1-6	209	10.8	1	Theta	Ranson et al., 1997b
Biting midge	*Culicoides variipennis*	*Cv*GST1	219	11.3	1		GenBank, U879458
Fruit fly	*Drosophila erecta*	*De*GST1-1	200	13.0	1	Theta	GenBank, P30104
Fruit fly	*Drosophila melanogaster*	*Dm*GST1-1	209	13.8	1	Theta	Toung et al., 1990
Fruit fly	*Drosophila melanogaster*	*Dm*-GST2	247	26.1	2	Sigma	Beall et al., 1992
Fruit fly	*Drosophila melanogaster*	*Dm*GST27	212	12.3	1		Toung and Tu, 1992
Fruit fly	*Drosophila melanogaster*	*Dm*GSTD21	214	11.8	1		Toung et al., 1993
Fruit fly	*Drosophila melanogaster*	*Dm*GSTD22	199	10.6	1		Toung et al., 1993
Fruit fly	*Drosophila melanogaster*	*Dm*GSTD23	214	13.3	1		Toung et al., 1993
Fruit fly	*Drosophila melanogaster*	*Dm*GSTD24	215	12.3	1		Toung et al., 1993
Fruit fly	*Drosophila melanogaster*	*Dm*GSTD25	214	11.8	1		Toung et al., 1993
Fruit fly	*Drosophila melanogaster*	*Dm*GSTD26	170	14.1	1		Toung et al., 1993
Fruit fly	*Drosophila sechellia*	*Dse*GST1-1	200	14.0	1	Theta	GenBank, P30106
Fruit fly	*Drosophila simulans*	*Dsi*GST1-1	200	14.0	1	Theta	GenBank, P30105
Fruit fly	*Drosophila teissieri*	*Dt*GST1-1	200	13.5	1	Theta	GenBank, P30107
Fruit fly	*Drosophila yakuba*	*Dy*GST1-1	200	13.6	1	Theta	GenBank, P30108
Greenbottle fly	*Lucilia cuprina*	*Lc*GST	208	13.8	1		Board et al., 1994
Sheep blowfly	*Lucilia cuprina*	*Lc*GST1-1	208	13.8	1	Theta	Board et al., 1994
House fly	*Musca domestica*	*Md*GST	241	31.0	2	Sigma	Franciosa and Berge, 1995
House fly	*Musca domestica*	*Md*GST1	208	14.3	1	Theta	Fournier et al., 1992
House fly	*Musca domestica*	*Md*GST2	210	13.3	1	Theta	Syvanen et al., 1994
House fly	*Musca domestica*	*Md*GST3a	210	11.3	1	Theta	Syvanen et al., 1994
House fly	*Musca domestica*	*Md*GST3b	210	13.3	1		Zhou and Syvanen, 1997
House fly	*Musca domestica*	*Md*GST3-5	210	11.3	1		Zhou and Syvanen, 1997
House fly	*Musca domestica*	*Md*GST4	210	12.8	1	Theta	Syvanen et al., 1994
House fly	*Musca domestica*	*Md*GST5	215	12.3	1		Zhou and Syvanen, 1997

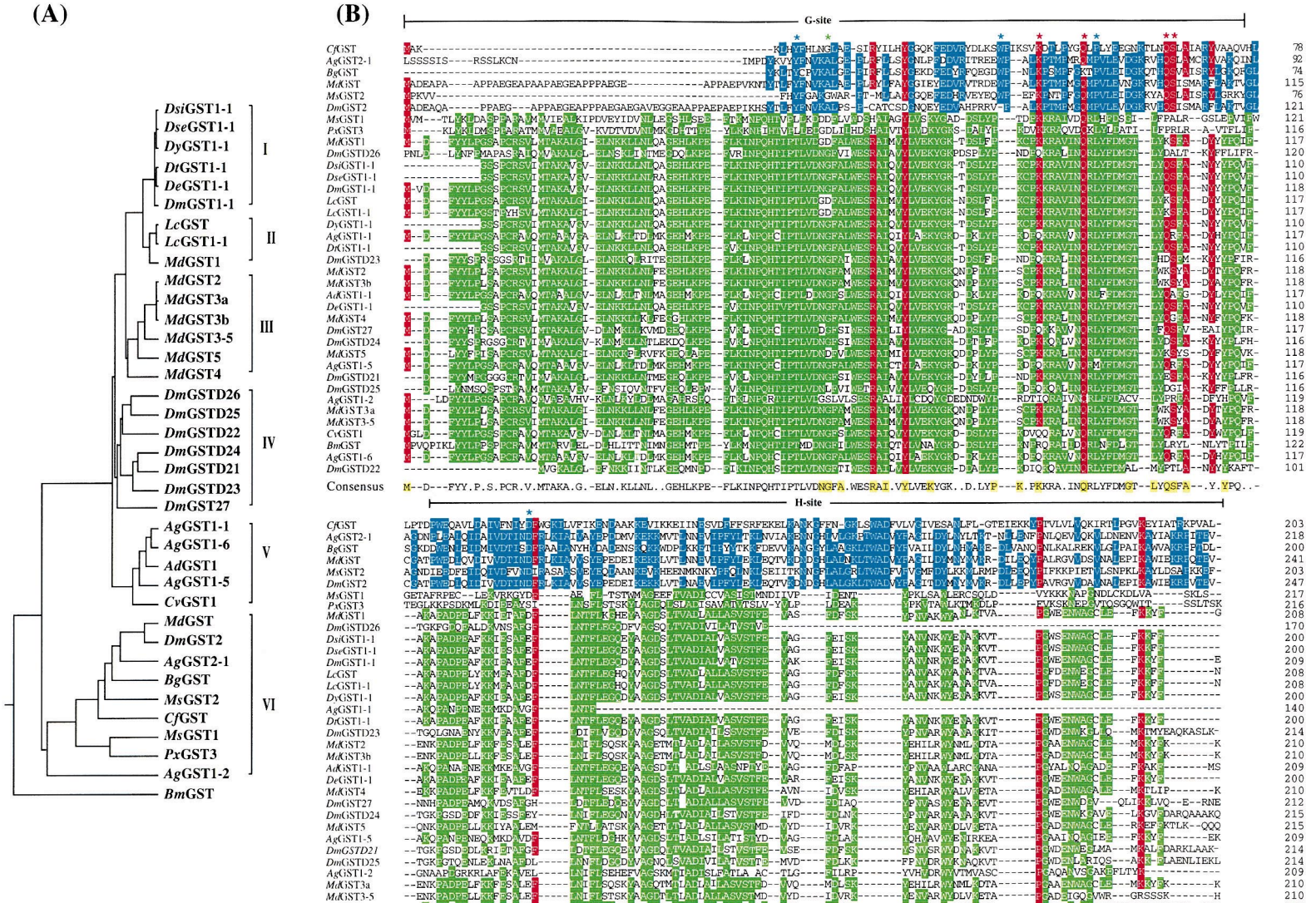

Fig. 7. Comparison of *Cf*GST with other insect GSTs. (A) Phylogenetic tree analysis of genetic relationships among 37 insect GSTs. (B) Alignment of the deduced amino acid sequence of *Cf*GST cDNA with other insect GSTs. The residues that are conserved (at least 4 out of 6) among members of GST2 are shaded solid blue. The residues that are conserved (at least 16 out of 31) among the members of GST1 are shaded solid green. The residues that are conserved among the members of both GST1 and GST2 are shaded solid red. The consensus residues are given in lines below the alignment panels when at least 19 out of 37 residues among all sequences match; otherwise '.' is shown. The consensus residues that are found in *Cf*GST are shaded solid yellow. The nine amino acid residues in *Cf*GST sequence that are known to be involved in glutathione binding or enzyme catalytic activity are marked with asterisks. The putative G-site and H-site regions are based on an alignment of *Cf*GST with the mammalian pi, mu, and sigma GSTs (Reinemer et al., 1991; Ji et al., 1992, 1995).

mammalian GSTs are conserved in the GST2 members of Cluster VI. Four of these 8 amino acid residues (equivalent to Tyr_7, Trp_{38}, Pro_{53} and Asp_{98} for *Cf*GST, respectively) are present only in the GST2 members of this cluster, and are absent in the GST1 members of all clusters. Seventy-two percent of amino acid residues are conserved among the GST1 members (Fig. 7B, green). In the G-site of insect GSTs, 9 amino acid residues are conserved in all the sequences, while in the H-site, 3 amino acid residues are conserved in all insect GSTs (Fig. 7B, red).

The location of GSTs within the insect body might shed some light on their physiological roles. A major function of GSTs has long been believed to be detoxification of xenobiotics. Because the larval gut is the place where insects digest food and take up chemical substances, it is reasonable to assume that the gut is the first barrier to xenobiotics. Many reports have shown GST activity in the insect midgut. For example, Tate et al. (1982) and Snyder et al. (1995) observed a higher GST activity in the midgut of the tobacco hornworm, *Manduca sexta*, than in the fat body, but the difference was minimal. Chien and Dauterman (1991) found that the fat body of *Helicoverpa zea* contained the highest GST enzyme activity, although enzyme activity was also detected in the midgut. Lee (1991) observed the same levels of GST activity in the midgut and fat body of *Papilio polyxenes*. Midgut GST cDNA have been cloned from *M. sexta* (Snyder et al., 1995) and *Plutella xylostella* (Huang et al., 1998). Most of the work on locating the site of insect GSTs was done by measuring GST catalytic activity towards the substrates glutathione and CDNB (or 1,2-dichloro 4-nitrobenzen, DCNB). However, the conjugation reaction between glutathione and CDNB can occur even in the absence of the enzyme (Abdel-Aal and Roe, 1990). Thus, a more precise method to locate GST appears to be the detection of GST protein and its mRNA. Our data from protein analysis using SDS-PAGE followed by immunoblot, immunocytochemistry and mRNA Northern blot clearly indicated that the fat body is the major source of *Cf*GST protein and mRNA. Because immunocytochemistry did not show *Cf*GST in the epidermis, *Cf*GST protein and mRNA detected in the epidermis by immunoblot and Northern blot were probably due to contamination with fat body cells that are difficult to be removed completely during dissection. In mammals, it has been found that the liver (equivalent to the fat body of insects) contains the highest amount of GSTs and is the major organ where detoxification takes place (Vos and van Bladeren, 1990). We also detected the presence of *Cf*GST protein in the epithelial cells of the midgut by immunocytochemistry, although the reaction was minimal. *Cf*GST mRNA was not detected in the midgut. It is possible that the mRNA levels in the midgut are below the threshold of our Northern blot detection. It is also possible that *Cf*GST detected in the epithelial cells of the midgut may be transported from some other tissues, such as the fat body. Further work is needed to resolve this problem.

As shown in Figs. 1 and 3, the polyclonal antibodies raised against *Cf*GST immunologically cross-reacted with one other high molecular weight protein. To eliminate the possibility that *Cf*GST and this high molecular weight protein are derived from a dimer, larval proteins were resolved in a native gel and then an immunoblot was performed. The two proteins were separated in the native gel and the antibody bound to both the native proteins (data not shown). Thus, *Cf*GST does not appear to be derived from the denaturation of a higher molecular weight protein. In addition, glutathione affinity chromatography did not purify this high molecular weight protein from the larval protein extracts. Although the observed cross-reaction between *Cf*GST and the high molecular weight protein may be simply due to some common antigenic determinant that these two proteins may share, further work is necessary to elucidate the relationship between these two proteins.

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