An evaluation of glutathione transferase associated with Dichlorvos degradation in African palm weevil (Rynchophorus phoenicis) larva

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An evaluation of glutathione transferase associated with Dichlorvos degradation in African palm weevil (Rynchophorus phoenicis) larva
Olufemi Samuel Bamidele1*, Joshua Oluwafemi Ajele1 and Folasade Mayowa Olajuyigbe1

Abstract: This study was conducted to investigate the metabolic defensive mechanism in the larvae of African palm weevil (Rynchophorus phoenicis) administered with dichlorvos (2,2-dichlorovinyl dimethylphosphate) solution. Bioassay experiment with dichlorvos was conducted on the larva and glutathione-utilizing enzyme activities were determined in the major organs: fat body, gut, and head of R. phoenicis larva 48 h after treatment with 0–0.060 μg g−1 body weight dichlorvos solution. Glutathione transferase was purified from the gut of larvae by ion-exchange chromatography on diethylaminoethyl-Sephadex A50 and affinity chromatography on glutathione-Sepharose 4B columns. The purified enzyme was homogenous as revealed by sodium dodecylsulfate polyacrylamide gel electrophoresis. Initial velocity studies were carried out on the purified enzyme using standard procedures. Bioassay experiment indicated alterations of glutathione peroxidase, glutathione reductase, and glutathione transferase activities in the major organs of larva caused by dichlorvos. Glutathione transferase activity in the gut of larva was three times higher than that of glutathione peroxidase and glutathione reductase activities, an indication of possible detoxification role of glutathione transferase.

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ABOUT THE AUTHORS
The Enzymology Research Group, under the supervision of Professor Joshua O. Ajele, focuses on the understanding of the metabolic functions of various enzymes and functional proteins that drive life processes. This broad research area involves the isolation, purification, and characterization of these biomolecules from plants, micro-organisms, insects, and higher animals including humans. The group is currently focusing on the identification and heterologous expression of enzymes implicated in the antioxidant defense system of insect pest towards the development of effective and safe insecticides in the face of increasing resistance to insect pest control and other environmental health challenges.

PUBLIC INTEREST STATEMENT
Insect pest constitutes a large group of plant enemies. Palm (Elaeis guineensis), an economically important perennial crop is a host of African palm weevil (Rynchophorus phoenicis). The larva of this pest cause more harm to palm than the adult. Palm weevil is controlled by insecticides. Insecticide sometimes failed due to the defense system inherent in the insect. In order to gain insight into the biochemistry of GST in the larva, palm weevil larva was treated with a known insecticide—dichlorvos and an assessment of some important enzymes that are linked to molecular defense in the larva were carried out. Glutathione-transferase, glutathione reductase, and glutathione peroxidase were altered by dichlorvos in the larva. Glutathione transferase associated with the breakdown of dichlorvos in the gut of larva was isolated and purified. The purified enzyme from the gut of larva showed a mechanism in which two substrates interact with the enzyme to form the products in any order. This study confirmed the presence of glutathione transferase in the larva and the enzyme may enhance removal of insect poison from the larva.
transferase in the organ. A 49.7 kDa homodimeric glutathione transferase was identified from the gut of larva and was tagged rplGSTc. Mechanism of action of rplGSTc with 1-chloro-2,4-dinitrobenzene, and glutathione as substrates conformed to the random sequential mechanism. These results confirmed the presence of GST associated with the degradation of dichlorvos in the gut of R. phoenicis larva.

**Subjects: Biochemistry; Enzymology; Entomology**

**Keywords: glutathione peroxidase (GPX); glutathione reductase (GR); glutathione transferase (GST); insecticide; purification; R. phoenicis larva**

### 1. Introduction

Palm (*Elaeis guineensis*) is an economically important plant cultivated in Nigeria. The primary products of palm are palm oil, palm kernel oil, and palm kernel cake (Soyebo, Farinde, & Dionco-Adetayo, 2005). The full yield potentials of palm products are affected by insect pests. African palm weevil, *Rynchophorus phoenicis* (Coleoptera: Curculionidae) is identified as one of the major insect pests of palm (Al-Ayied, Alswailem, Shair, & Al Jabr, 2006). Similar hosts of this insect are date palm (*Phoenix dactylifera* L.), raffia palm (*Raphia* spp.), and coconut palm (*Cocos nucifera* L.) (Bong, Er, Yiu, & Rajan, 2008; Gries et al., 1994). The life cycle of *R. phoenicis* is similar to other *Rynchophorus* species (Giblin-Davis et al., 1996). Damage to the host is caused by the grubs (larvae). These larvae make tunnels in the trunk and feed on the tissues of the palm. Decaying of the tissues results in the production of a foul smell and if unchecked, leads to death of palm (Mariau, Chenon, Julia, & Philippe, 1981). Huge loss of palm products and revenue may be caused by infestation of palm by *R. phoenicis* specie (Faleiro, 2010). However, insecticides are employed for the control of *R. pheonicis* and other related insect pests of palm (Barranco, de la Peña, Martín, & Cabello, 1998; Cabello, de la Pena, Barranco, Belda, & de la Pena, 1997).

Generally, insects can metabolize and thereby degrade toxic or otherwise detrimental chemicals for surviving in a chemically unfriendly environment. While all insects probably possess capacity to detoxify toxic chemicals, the amount can be expected to vary among species, with developmental stage, and with the nature of insect’s recent environment (Sívori, Casabé, Zerba, & Wood, 1997). Versatility in the adaptation of insects to environment is provided by the phenomenon of induction of detoxification systems in insects (Liu, Zhu, Xu, Pridgeon, & Gao, 2006; Sívori et al., 1997). It is obvious from reports on resistance to insecticides in various species of insects that the most important factor in the defensive system of insects is an increased capacity to detoxify insecticides, most likely as a result of the production of additional enzymes of detoxification (Syvanen, Zhou, Wharton, Goldsburry, & Clark, 1996).

Glutathione (GSH)-related enzymes play pivotal role in the protection of biological cell against damage by toxic compound. The main protective roles of glutathione against oxidative stress consist of glutathione acting as a cofactor of several detoxifying enzymes such as glutathione peroxidase (GPX) and glutathione transferase (GST), participation in amino acid transport through the plasma membrane; scavenging hydroxyl radical and singlet oxygen directly, detoxifying hydrogen peroxide, and lipid peroxides by the catalytic action of glutathione peroxidase; regeneration of the most important antioxidants, Vitamins C and E, back to their active forms and reducing the tocopherol radical of Vitamin E directly, or indirectly, via reduction of semidehydroascorbate to ascorbate (Masella, Di Benedetto, Vari, Filesi, & Giovannini, 2005). The capacity of glutathione to regenerate the most important antioxidants is linked with the redox state of the glutathione disulfide-glutathione couple (GSSG/2GSH) (Pastore, Federici, Bertini, & Plemonte, 2003).

GSTs (EC 2.5.1.18) catalyze the conjugation of glutathione to electrophilic centers of non-polar compounds, making them more water soluble and eliminated from the cells (Hayes, Flanagan, & Jowsey, 2005; Salinas & Wong, 1999). They are involved in the detoxification of various endogenous and xenobiotic compounds, such as drugs, insecticides, organic pollutants, secondary metabolites, and other toxins (Blanchette, Feng, & Singh, 2007; Hayes et al., 2005). They are also involved in the
biosynthesis and intracellular transport of hormones and protection against oxidative stress (Cnubben, Rietjens, Wortelboer, van Zanden, & van Bladeren, 2001; Hayes et al., 2005). GSTs are known to exist in dimers (homo- or heterodimers) (Vanhaelen, Francis, & Haubruege, 2004). Each GST subunit consists of two different domains, i.e. one-third of the N-terminal protein which provides the binding site for GSH (domain I), and two-third of the C-terminus which determines the substrate specificity (Armstrong, 1997). To date, at least nine classes of GSTs have been identified in mammals, eight cytosolic, and one microsomal class. In insects, first two distinct classes were described, class I and class II GSTs with 40–90% similarity between members of the same class (Chelvanayagam, Parker, & Board, 2001; Hemingway, 2000). Class I insect GSTs has also been referred to as the Delta class and class II as Sigma (Chelvanayagam et al., 2001). Another insect GST class was established (class III), which is also named Epsilon class GST (Sawicki, Singh, Mondal, Beneš, & Zimniak, 2003). GSTs have been described to play a major role as a detoxification mechanism for insecticides, thus contributing to insecticide resistance in economically important pest species in diverse agronomic cropping systems (Huang et al., 1998; Vontas, Small, & Hemingway, 2001; Vontas, Small, Nikou, Ranson, & Hemingway, 2002; Yu, 2002). GSTs have been shown to be involved in the detoxification of several classes of insecticides, i.e. organophosphates, pyrethroids, carbamates, and chlorinated hydrocarbons such as DDT (Ranson, Prapanthadara, & Hemingway, 1997). GST-based resistance to insecticides was described to be facilitated by the increase in the level of expression of one or more GSTs (Hemingway, 2000).

Some previous reports have shown bio-pesticide as an alternative to synthetic insecticide for the control of similar insect pest (Abuhussein, 2008; Faleiro, 2006; Ghoneim, Beam, Tanani, & Nassar, 2001; Nassar & Abdllahi, 2001). Although success had been recorded with the adult, but the R. phoenicis larvae inhabiting the core of the host calls for concern. The metabolic adaptive feature of this larva to its host may be connected to the activity of detoxifying enzyme present in the larva. Evolvement of adult R. phoenicis from its larva having resistant trait may be a reason why an alternative control method is sought. Bamidele, Ajele, Kolawole, and Akinkuolere (2013) previously reported the alteration of endogenous antioxidant enzymes and non-antioxidant enzymes activities in the major organs of R. phoenicis larvae caused by dichlorvos (DDVP). A further step is to gain deeper insight into the biochemistry of GST from R. phoenicis larva by elucidating the mechanism of defense against insecticide inherent in the larva. This study is designed to investigate organ distribution of some GSH-utilizing enzymes as well as to establish the involvement of glutathione transferase in the degradation of dichlorvos in the gut of larvae.

2. Materials and methods

2.1. Materials

Reduced glutathione (GSH), oxidized glutathione (GSSG), reduced nicotinamide adenine dinucleotide phosphate (NADPH), 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), bovine serum albumin (BSA), GSTrap 4B (1 × 1 cm) column, and molecular weight marker were purchased from Sigma Chemicals Company, St. Louis, USA. Diethylaminoethyl (DEAE)-Sephadex A50 was from Pharmacia Fine Chemicals, Uppsala, Sweden. Insecticide: “Sniper 1000 EC” containing 2,2-dichlorovinyl dimethylphosphate (DDVP, 1,000 g L⁻¹) was manufactured by Hubei Sanonda Co. Ltd, Shashi Hubei, China. Other chemicals were of the highest purity commercially available.

2.2. Collection of insect larvae

African palm weevil (R. phoenicis) larvae (of 10.08 g mean body weight) were collected from Igbokoda palm plantation, Ondo State (7.2°N, 5.1°E) in June, 2014. Larvae were transported to the Enzymology Research Laboratory, The Federal University of Technology, Akure (FUTA) in an insect box constructed with iron-wire mesh (25 × 40 × 45 cm) and acclimatized to an air-conditioned laboratory room at 25°C, relative humidity (75%), and exposed to 12 h L: D cycle for one week prior to the experiment. The larvae were reared on degraded palm fiber collected from infested palm tree. Identification of larva was carried out in the Entomology Research Laboratory, Department of Biology, FUTA.
2.3. Larvae grouping

Individual weight of larva was taken and recorded prior to the experiment. Random selection method was used (based on recorded weights) to place active and stress-free larvae in groups. The larvae were divided into six groups consisting of 25 larvae per group. Group one was used as the control. The larvae were of an average weight of 10.08 g and the body length range of 7.0–8.0 mm in each group.

2.4. Administration of larvae with DDVP solution

Administration of larvae with DDVP solution was carried out according to Kostaropoulos, Papadopoulos, Metaxakis, Boukouvala, and Papadopoulou-Mourkidou (2001). Stock DDVP solution was prepared by dissolving “Sniper” solution in acetone and was diluted with normal saline (0.9% NaCl) to the desired concentrations of DDVP before administration. *R. phoenicis* larvae were administered 2 μL with different concentrations of DDVP (0, 0.20, 0.30, 0.40, 0.50, and 0.60 μg g⁻¹ body weight) at two to three abdominal segments using a microsyringe. Care was taken to avoid puncturing the alimentary tract. Control larvae received 2 μL of normal saline containing 40% acetone (saline/acetone). Each DDVP concentration was administered into 25 individuals. Knocking down effect was recorded 48 h after treatment. The remaining live larvae were used for the analysis of GPX, GR, and GST. After the insects had been separated, they were immediately stored at −4°C. All tests were conducted at room temperature.

2.5. Dissection and preparation of larvae cytosolic fraction

Surviving larvae (*n* = 5) 48 h after exposure were demobilized by freezing, quickly dissected, and separated into three fractions; fat body (FB), gut (GT), and head (H) using dissecting kit. Fractions were stored below −20°C until use. The tissues were thereafter homogenized 1:3 (w/v) in ice-cold buffer: (25 mM potassium phosphate buffer, pH 7.2 containing 1 mM EDTA, and 1 mM 2-mercaptoethanol). Crude cytosolic enzyme was prepared subsequently by differential centrifugation. Homogenate was centrifuged at 10,000 g for 30 min at 4°C using Eppendorf Table Top Centrifuge model 5418R (Germany); floating lipid was carefully removed from supernatant through a funnel plugged with glass wool. The supernatant obtained after filtration was stored in aliquots at below −4°C and subsequently used as crude enzyme. Protein concentration was determined by the method of Bradford, (1976) using bovine serum albumin (BSA) as the standard.

2.6. Glutathione peroxidase (GPX) activity assay

The GPX (EC 1.11.1.9) activity was measured with H₂O₂ as substrate according to Paglia and Valentine (1987). This reaction was monitored indirectly as the oxidation rate of NADPH at 340 nm for 3 min using Jenway 6280 UV/Visible spectrophotometer (USA). Enzyme activity was expressed as micromoles of NADPH consumed per minute per milligram of protein, using an extinction coefficient of 6.220 M⁻¹ cm⁻¹. A blank without homogenate was used as a control for the non-enzymatic oxidation of NADPH upon addition of hydrogen peroxide in 100 mM Tris–HCl buffer, pH 8.0.

2.7. Glutathione reductase (GR) activity assay

Glutathione reductase (GR) was assayed according to the method described by Saydam, Kirb, and Demir (1997). The assay mixture (3.0 mL) consisted in final concentration of 100 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 1 mM GSSG, 0.16 mM NADPH, and 30 μL of the crude enzyme. NADPH oxidation was monitored at 340 nm for 3 min at 25°C using Jenway 6280 UV/Visible spectrophotometer (USA) and the enzyme activity was expressed as μmol min⁻¹ mg⁻¹ protein.

2.8. Glutathione transferase (GST) activity assay

Measurement of GST activity was conducted according to the procedure described by Habig, Pabst, and Jakoby (1974). The GSH conjugation reaction was initiated by the addition of enzyme solution to a reaction medium consisting of CDNB (1 mM) and GSH (1 mM) as substrates in 100 mM phosphate buffer pH 6.5. The change in absorbance of product for 3 min was measured at 340 nm and 25°C using Jenway 6280 UV/Visible spectrophotometer (USA). The amount of conjugated product formed
was calculated using the extinction coefficient 9.6 mM\(^{-1}\) cm\(^{-1}\). Enzyme activity was defined as \(\mu\)mol of product formed per min per mg protein.

### 2.9. Purification of GST

Palm weevil (R. phoenicis) larvae were administered 2 \(\mu\)L sub-lethal DDVP solution (0.35 \(\mu\)g g\(^{-1}\)) at two to three abdominal segments using a microsyringe. Control larvae received 2 \(\mu\)L of normal saline containing 40% acetone (saline/acetone). Larvae were collected at 2 h intervals for a period of 12 h from the control and treated larvae. They were immediately stored at \(-4^\circ\)C and were later used for GST assay and SDS-PAGE analysis. Treatment of larvae was conducted at room temperature. Crude cytosolic enzyme was prepared subsequently by differential centrifugation as earlier described. Crude enzyme solution (6 mL; 30.45 mg mL\(^{-1}\)) was applied to an ion-exchange column of DEAE-Sephadex A50 (1.5 \(\times\) 25 cm) previously equilibrated with 20 mM phosphate buffer pH 7.0 containing 1 mM EDTA and 1 mM 2-mercaptoethanol. Flow-through fractions were collected at a flow rate of 1 mL min\(^{-1}\). After washing the column, the bound protein was eluted with a gradient of (0–1.0 M) sodium chloride in the elution buffer. Fractions with GST activity were pooled, desalted by dialysis, and concentrated by ultrafiltration on Amicon PM membrane. Concentrated protein was further purified on pre-packed GSH-Sepharose 4B affinity (1 \(\times\) 1 cm) column (GSTrap 4B) previously equilibrated with phosphate buffer saline (PBS), pH 7.4 (140 mM NaCl, 2.7 mM KCl, 10 mM Na\(_2\)HPO\(_4\), 1.8 mM KH\(_2\)PO\(_4\)) according to the manufacturer instruction. Protein was eluted with the same buffer at a flow rate of 1 mL min\(^{-1}\) until no protein was detected in the flow through-fractions. Bound protein was eluted with 10 mM GSH in 50 mM Tris–HCl buffer, pH 8.0 (Konishi et al., 2005). The fractions with GST activity were pooled and subsequently used for electrophoretic and inhibition studies. All purification steps were conducted at 4\(^{\circ}\)C.

### 2.10. Gel profile of GST

Purified GST was analyzed by SDS-PAGE using omniPAGE Vertical Electrophoresis system (England) according to the method of Laemmli (1970). Purified GST was mixed with protein sample buffer (0.0625 M Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% \(\beta\)-mercaptoethanol and 0.125% Bromophenol blue) and boiled for 2 min. A total of 21.50 \(\mu\)g of the mixture was subjected to 10% SDS-PAGE. After electrophoresis, gel was stained with 0.1% Coomassie Brilliant Blue R-250. Gels were thereafter destained in a destaining solution overnight.

### 2.11. Initial velocity of GST

Initial velocity rate measurements were performed in 100 mM potassium phosphate buffer pH 6.5 containing EDTA and 2-mercaptoethanol at 25\(^\circ\)C according to Bastien, Fournier, Baudras, and Baudras (1999). The reaction mixture (3 mL) consisted of buffer, varied concentrations of GSH (0.25–1 mM), and different concentrations of CDNB (0.25–1 mM) which were kept constant. Reaction rate measurements were also carried out with varied concentrations of CDNB (0.25–1 mM) at constant varied concentrations of GSH (0.25–1 mM) which were kept constant. Substrates were freshly prepared during the experiment and ethanol concentration was kept at 4% in the reaction medium. Enzyme solution (30 \(\mu\)L) was added last to initiate the reaction. The appearance of product was monitored at 340 nm for 3 min. Reaction mixture without enzyme was used as a reference to correct non-enzymatic reaction. Each reaction rate was measured at least three times and then averaged. Data were fitted to the rectangular hyperbola equation and analyzed using Graphpad prism 6 (Graphpad Software, San Diego, CA, USA). The kinetic constants (\(V_{max}\) and \(K_m\)) were also determined.

### 2.12. Statistical analysis

Data obtained from the experiments were presented as mean \(\pm\) SD of the results from three independent experiments. Kinetic data were analyzed using Graphpad prism 6 (Graphpad Software, San Diego, CA, USA). Data were statistically analyzed using one-way analysis (ANOVA) followed by Duncan’s New Multiple Test where appropriate.
3. Results

3.1. GPX activity
Effect of DDVP on glutathione peroxidase activities in the FB, GT, and H tissues of *R. phoenicis* larva is shown in Figure 1. In the FB of DDVP-treated *R. phoenicis* larvae, GPX activities increased significantly \((p < 0.05)\) with increased DDVP concentration when compared with the control. GPX activity of 24.94 μmol min\(^{-1}\) mg\(^{-1}\) protein in the FB of larva was produced by 0.33 μg g\(^{-1}\) DDVP. In the GT of larva, initial DDVP concentrations (0.22 and 0.33 μg g\(^{-1}\)) caused increased GPX activity while higher concentrations caused a decline in GPX activity when compared with the control. GPX activity in the H tissue of larva decreased significantly \((p < 0.05)\) with increased DDVP concentrations when compared with the control. Highest GPX activity was recorded in the GT of larva.

3.2. GR activity
Effect of DDVP on glutathione reductase activity in the FB, GT, and H of *R. phoenicis* larva is shown in Figure 2. GR activity was DDVP-dose dependent in the three organs of *R. phoenicis* larva. GR activity was significantly increased in the gut of larva but declined at DDVP concentration greater than 0.44 μg g\(^{-1}\) when compared with the control. Similar result was observed in the head of *R. phoenicis* larva.

3.3. GST activity
Figure 3 shows the effect of DDVP on glutathione transferase activity in the FB, GT, and H of *R. phoenicis* larva. GST activity in the GT and H increased significantly \((p < 0.05)\) with increased DDVP concentration when compared with the control. Due to significant increase in GST activity in the gut of larva, further investigation of the effect of sub-lethal DDVP concentration (0.35 μg g\(^{-1}\)) on GST activity in the GT for duration of eight hours was done. Bioassay results showed an increase in GST activity in the gut tissue of *R. phoenicis* larva as a function of time of DDVP treatment (Figure 4). The initial GST activity was similar for DDVP-treated and control larvae. The specific activity of crude GST from the gut of larvae treated with DDVP after 2, 4, 6, and 8 h were 20.49, 21.43, 25.34 and 22.52 μmol min\(^{-1}\) mg\(^{-1}\) protein, respectively. GST activity in the treated larva was four times higher than in the control. GST was detected by SDS-PAGE (Figure 5).
3.4. Purification of DDVP-induced GST

The elution profile of the crude enzymes from the gut of control and DDVP-treated larvae on DEAE-sephadexA50 are shown in Figure 6(A) and (B), respectively. Glutathione transferase activity was detected in the flow-through fractions as indicated by the two major activity peaks “a” and “b” in Figure 6(A). Minor GST activity peak “c” indicated bound GST eluted with a linear gradient of NaCl in buffer solution. Elution profile of GST from gut of DDVP-treated larva (Figure 6(B)) was similar to the control (Figure 6(A)) but an observable difference existed with the bound protein. After protein was eluted with a (0–1.0 M) gradient of NaCl in buffer solution, GST activity detected in the fractions obtained from bound protein was indicated as the GST activity peaks “c” and “d” in Figure 6(B). GST activity peak “c” was considered one of the GST isoenzymes associated with DDVP degradation in the gut of *R. phoenicis* larva. The specific activity of this ion-exchange purified GST was 11.65 ± 0.72 μmol min⁻¹ mg⁻¹ protein. Further purification by GSH-Sepharose 4B affinity gel chromatography revealed a major GST activity peak eluted with 10 mM GSH in buffer (Figure 7). The yield of purification of GST was 8.37 ± 1.61% of the total GST activity obtained from GSH-affinity
chromatography (Table 1). The specific activity and purification fold of purified GST were 52.16 ± 5.28 μmol min⁻¹ mg⁻¹ protein and 20.37 ± 2.43, respectively (Table 1). The affinity bound protein was tagged rplGSTc and used for the SDS-PAGE. rplGSTc showed a single band on a 10% gel and the estimated molecular weight was approximately 26.2 kDa (Figure 8(A)). Native gel electrophoresis revealed an estimated molecular weight of 49.7 kDa (Figure 8(B)).

3.5. Initial velocity of rplGSTc

Double reciprocal plot of rplGSTc-catalyzed reaction with respect to GSH is shown in Figure 9(A). The plot of 1/v vs. 1/[CDNB] at constant varied concentrations of GSH were linear and the point of intersection or convergence of lines is on the 1/[CDNB] axis. The slopes and intercepts of double reciprocal

Figure 4. Time-course glutathione transferase activity (GST) in the gut tissue of Rynchophorous phoenicis larva.

Notes: Each value is the mean of three repetitions ± SD. Experiment was conducted at room temperature.

Figure 5. Gel profile of crude extracts of gut of DDVP-treated Rynchophorous phoenicis larva.

Notes: STD: Standard molecular mass, L1: Crude extract from control larva at 0 h and crude extract from larva L2–2 h, L3–4 h, L4–6 h, L5–8 h, L6–10 h, L7–12 h after treatment with DDVP.

Figure 6. Elution profiles of glutathione transferase from the gut of (A) control R. phoenicis larva and (B) DDVP-treated R. phoenicis larva using ion exchange chromatography.

Notes: Crude enzyme was applied to a DEAE-Sephadex A50 (1.5 × 25 cm) column previously equilibrated with 25 mM potassium phosphate buffer, pH 7.2 containing 1 mM EDTA and 1 mM 2-mercaptoethanol. The protein was eluted at a flow rate of 1 mL min⁻¹ and fractions of 5 mL each were collected. Fractions pooled: 33–37 (6B).
Figure 7. Elution profile of glutathione transferase on affinity chromatography-GSTrap 4B. Ion-exchange-purified enzyme was applied to a GSH-Sepharose 4B (1 x 1 cm) column previously equilibrated with PBS, pH 7.4.

Notes: The protein was eluted at a flow rate of 1 mL min⁻¹ and fractions of 1 mL each were collected. Bound protein was eluted with 10 mM GSH in 50 mM Tris–HCl buffer, pH 8.0. Fractions pooled: 13–18.

Table 1. Summary of purification of rplGSTc

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (μmol min⁻¹)</th>
<th>Specific activity (μmol min⁻¹ mg⁻¹)</th>
<th>Yield (%)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude GST</td>
<td>3,110.01 ± 194.2</td>
<td>8,011.22 ± 367.12</td>
<td>2.57 ± 0.16</td>
<td>100.00 ± 0.00</td>
<td>1.00 ± 0.00</td>
</tr>
<tr>
<td>Ion exchange chromatography</td>
<td>185.62 ± 11.5</td>
<td>2,164.25 ± 99.17</td>
<td>11.65 ± 0.72</td>
<td>27.10 ± 1.69</td>
<td>4.54 ± 0.28</td>
</tr>
<tr>
<td>GSTrap 4B</td>
<td>12.75 ± 2.34</td>
<td>665.05 ± 18.15</td>
<td>52.16 ± 5.28</td>
<td>8.32 ± 1.61</td>
<td>20.37 ± 2.43</td>
</tr>
</tbody>
</table>

Notes: Purification was conducted at 4°C. Values are mean ± SD of three repetitions.

plot in Figure 9(A) vs. 1/[GSH] were also linear (Figure 9(a)). Similarly, the plot of 1/v₀ vs. 1/[GSH] at constant varied concentrations of CDNB is shown in Figure 9(B). Double reciprocal plot of rplGSTc-catalyzed reaction with respect to CDNB were linear and the point of convergence of lines is on the 1/[GSH] axis. Secondary replots of intercepts and slopes in Figure 9(B) vs. 1/[CDNB] were linear (Figure 9(b)). The estimated Kᵣₐ₅₄ and Kᵣₐ₁₇ from the secondary replots were 0.191 ± 0.03 and 0.136 ± 0.02 mM, respectively, while maximum velocity (V_max) was 46.21 ± 7.23 μmol min⁻¹ mL⁻¹.
4. Discussion

In this report, alteration of activities of some GSH-utilizing enzymes was observed in the fat body, gut, and head of DDVP-treated larva. This suggestively indicated the presence of GPX, GR and GST in the organs of the larva. Dose-dependent responses of GSH-utilizing enzymes against the concentration of DDVP were recorded. However, linearity was not observed with the increasing concentration of DDVP and the corresponding levels of GPX, GR, and GST activities in the organs. This observation might be on the account of sensitivity of individual larva to DDVP, genetic makeup, and different rate of elimination of the insecticide. Similar finding have been well documented by some authors (Bamidele et al., 2013; Vanhaelen et al., 2004; Zhang, Rashid, Ji, Rashid, & Wang, 2013). Due to the significantly increased activities of GPX, GR, and GST in the gut of larva, GSH-utilizing enzymes seem to play significant role in the gut of larva by probably degrading and detoxifying insecticide and its metabolites leading to removal of toxic compounds from the larval system. Also the various levels of glutathione-utilizing enzymes in the three organs showed that the individual organ handled the chemical assault differently. This is partly responsible for the degree of free radical scavenging and detoxifying activities in the larva.

Analysis of crude enzyme preparation from gut of treated larva by SDS-PAGE revealed increased intensity and broadness of GST bands at different time intervals after exposure. This observation conforms to the increased specific activity of GST measured over the period of exposure to the insecticide. This also suggested the capacity of GST in the gut of larva to degrade the chemical insecticide. Earlier reports by some authors suggested that increased level of GSTs is one of the major reasons for the development of resistance by metabolic detoxification of insecticides (Zhang et al., 2013). Development of insecticide resistance is caused by increased levels of GSTs in diamondback moth and H. armigera (Furlong & Wright, 1994; Mohan & Gujar, 2003). Enhanced activities of GSTs that confer insecticide resistance from both qualitative and quantitative alterations in gene expression are provided. Evidence for over-expression of one or more GST isoenzymes in resistant insects was observed in M. domestica (Fournier, Bride, Poire, Berge, & Plapp, 1992; Zhang et al., 2013).
Purification processes revealed the possible GST associated with DDVP degradation in the gut of treated larva. It is evident therefore that GST among other GSH-utilizing enzymes is linked with the detoxification of insecticides and removal of possible metabolites from the gut of larva. The yield of the purified rplGSTc was significantly lower than that obtained in Myalopa flariae (77%), Syrphus ribesii (65%) (Vanhaelen et al., 2004; L. paeta (4.1%) (Wu, Dou, Wu, & Wang, 2009). This might be due to loss of GST activity and unrecovered enzyme bound to resin during the purification steps. But, the yield of the purified rplGSTc was higher than S. inneta (3.3%) (Valles, Perera, & Strong, 2003). The purification step in this report were previously used by Konishi et al. (2005) and Shukor, Wajidi, Avicor, and Jaal (2014). The estimated molecular weights of rplGSTc from native gel and SDS-PAGE revealed that the soluble cytosolic GST might be homodimeric enzyme and the result is similar to the size of GST of many other insect species such as S. invicta (Valles et al., 2003); fall webworm H. cunea (Yamamoto, Mika, & Aso, 2007) and some Psocids (Dou, Wu, Hassan, & Wang, 2009; Wu et al., 2009). The mechanism of rplGSTc appears to conform to the random sequential model. This is indicated by the pattern of convergence of lines of double reciprocal plots in both cases of GSH and CDNB as variable substrates. This pattern would permit greater flexibility in the conjugation of various xenobiotics to water soluble, less toxic products (Adewale & Afolayan, 2005).

Conclusively, Dichlorvos (DDVP) caused the alteration of some glutathione-utilizing enzymes in the major organs of R. phoenicis larva. The elevated levels of these enzymes suggested possible protective handling of the toxic tissue-damaging insect poisons in the gut of the larva. A novel 49.2 kDa homodimeric GST isoenzyme (rplGSTc) associated with DDVP degradation was purified and the enzyme revealed a random sequential mechanism toward its substrates. The present results indicate that GST might be involved in the degradation of dichlorvos in the gut of R. phoenicis larvae.


