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Purification and Biochemical Characterization of Glutathione S-Transferases in Bactrocera minax (Diptera: Tephritidae)

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PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF GLUTATHIONE S-TRANSFERASES IN *BACTROCERA MINAX* (DIPTERA: TEPHRITIDAE)

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Abstract

Glutathione S-transferases (GSTs) were purified from 3 developmental stages of *Bactrocera minax* through glutathione-agarose affinity chromatography, and characterized subsequently using the Michaelis-Menten kinetics toward the artificial substrates 1-chloro-2, 4-dinitrobenzene (CDNB) and reduced glutathione (GSH), respectively. Compared to the counterparts of third instar larva and adult, the highest specific activity of the purified GSTs towards CDNB was observed in the pupae. Although the specific activities of purified enzymes varied among 3 developmental stages, the purification yields were similar. SDS-PAGE revealed only one band at 23 kDa for all 3 stages. GSTs of the adults exhibited the highest K_m value towards CDNB, while for GSH the pupae possessed the highest K_m. The optimum temperature and pH for CDNB conjugation of the 3 stages were 37 °C and 7.5, respectively. Inhibition kinetics showed that ethacrynic acid, bromosulfalein, diethyl maleate, tetraethylthiuram disulfide and curcumin possessed excellent inhibitory effects on purified GSTs in *B. minax*. Moreover, the pupa showed the highest catalytic capability based on V_{max} values for both the CDNB and GSH, which may suggest a potentially higher GSTs detoxification ability in the pupal stage than the other 2 developmental stages.

Keywords: *Bactrocera minax*, glutathione S-transferases, purification, inhibition, developmental stages

Resumen

Se purificaron las transferasas-S de glutatión (GST) de 3 estadios de desarrollo de *Bactrocera minax* por medio de la afinidad de cromatografía de glutation-agarosa, y caracterizada luego usando la kinética de Michaelis-Menten hacia el sustrato artificial 1-cloro-2, 4 dinitrobenceno-(CDNB) y glutation reducida (GSH), respectivamente. En comparación con los homólogos del tercer estadio larval y del adulto, la mayor actividad específica de las GST purificadas hacia CDNB se observó en la pupas. Aunque las actividades específicas de las enzimas purificadas varían entre los 3 estadios de desarrollo, el rendimiento de purificación fueron similares. SDS-PAGE reveló una sola banda a los 23 kDa para los 3 estadios. El GST del adulto exhibió el mayor valor de K_m hacia el CDNB, mientras que la GSH de la pupa posee la más alta K_m. La temperatura y el pH óptimo para la conjugación de CDNB de las 3 estadios fueron 37 °C y 7.5, respectivamente. La inhibición kinética mostró que el ácido etacrínico, bromosulfalein, maleato de dietilo, disulfuro de tetraetiltiuram y la cúrcuma posee excelentes efectos inhibidores sobre la GST purificada en *B. minax*. Además, la pupa presentó la mayor capacidad catalítica basado en los valores de V_{max} para el CDNB y GSH, lo que puede indicar una capacidad potencialmente mayor de desintoxicación de GSTs en el estadio de la pupa que las otros 2 estadios de desarrollo.

Fruit flies (Diptera: Tephritidae) are among the most important insect pests worldwide with the larval stages being frugivorous on a wide range of fruits and vegetables (Clarke et al. 2005). *Bactrocera* Macquart is a large genus of tephritid fruit flies, with > 500 currently described species widely distributed in the Asia-Pacific region and includes many economically important species that cause great losses in fruit and vegetable crops (Muraji and Nakahara 2002). Among these, the Chinese citrus fruit fly, *Bactrocera minax* (Enderlein), is an economically important and quarantine fruit fly pest. The host range of *B. minax* is largely restricted to cultivated and wild species of *Citrus* (Allwood et al. 1999). The female oviposits inside the fruit, where larvae feed until pupation. This often causes fruit damage and fruit drop (Fletcher et al. 1987), as well as making the fruit unmarketable. This species has been found in southern China, India (West Bengal and

Sikkim), and Bhutan (Wang & Lu 1995), *B. minax* can cause serious damage to the citrus and has been recognized as a major pest of citrus fruit in the region from Nepal to southwestern China (Drew et al. 2006), especially in China, for more than half a century (Wang & Lu 1995). For example, the eastern Himalayan kingdom of Bhutan, local land-race varieties of mandarins (*Citrus reticulata* Blanco) are one of the primary cash crops. Unfortunately, crop losses between 35% and 75% to *B. minax* infestation are common in mid and high altitude orchards (>1,100 m) and the fly is considered as one of the major limiters of production (Dorji et al. 2006). However, due to its many special features of biological characteristics, the application of effective chemical control is very difficult.

Glutathione S-transferases (GSTs; E.C. 2.5.1.18) are a large family of multifunctional enzymes found ubiquitously in aerobic organisms (Ranson et al. 2001). They play important roles in phase II detoxification of several chemical insecticide classes, i.e., pyrethroids (Lumjuan et al. 2011), organophosphates (Melo-Santos et al. 2010), and chlorinated hydrocarbons such as DDT (Low et al. 2010). In insects, high levels of GSTs activity are associated with the expression of metabolic resistance to insecticides (Clark 1990; Fukami & Shishido 1996). Efforts to control *B. minax* often result in development of insecticide resistance. However, very little is known about the characteristics of glutathione S-transferases in *B. minax*, despite their significance in the detoxification xenobiotics and insecticide resistance. Age-dependent alteration of GSTs activities has been demonstrated in both invertebrates (Kostaropoulos et al. 1996; Papadopoulos et al. 2004) and vertebrates (Gregus et al. 1985). Recently, the GSTs from 4 field populations of the oriental fruit fly, *Bactrocera dorsalis* (Hendel) have been successfully purified and investigated (Hu et al. 2011). As part of our ongoing research on resistance mechanisms and management of tephritid fruit flies to different insecticides, we initiated a project to investigate the biochemical and toxicological characteristics of GSTs purified from 3 developmental stages of *B. minax*. The results may contribute to an advanced understanding of the sensitivity of the various developmental stages of *B. minax* to xenobiotics and provide some basic information about the potential GSTs detoxification system in each developmental stage of this pest.

MATERIALS AND METHODS

Insects

Larvae of *B. minax* were collected from a citrus orchard in Jiangyou, Sichuan province, China in 2010. After collection, insects were reared on fruit in plastic basins with sand until pupation.

All instars or life stages were kept in a temperature controlled room at 27 ± 1 °C, 70 ± 5% RH and 14:10 h L:D throughout experiment. Larvae, pupae and adults were sampled and stored at -80 °C for further analysis.

Chemicals and Reagents

Reduced glutathione (GSH, Sigma, St. Louis, Missouri, USA), 1-chloro-2, 4-dinitrobenzene (CDNB, Shanghai Chem. Ltd., China), Bovine serum albumin (BSA, Shanghai Bio Life Science & Technology Co. Ltd., China), and other biochemical reagents were of analytical grade. The xenobiotic compounds used for the inhibition bioassays were: bromosulfalein (Dow AgroSciences LLC, Indiana, USA), ethacrynic acid, 97% diethyl maleate, 97% tetraethylthiuram disulfide, and 94% curcumin (all from Sigma, St. Louis, Missouri, USA).

Enzyme Preparation and Protein Assays

For GSTs, 120 mg (3-4 individuals) of *B. minax* from each developmental stage were homogenized manually on ice in 4 mL sodium phosphate buffer (20 mM, pH 7.3). The homogenates were centrifuged for 5 min at 5,000 × g at 4 °C. The pellets were discarded and the supernatants were again centrifuged at 17,500 × g and 4°C for 15 min. Finally, the supernatants were used as the enzyme source for assays of GSTs activity. Protein content of the homogenate was determined according to the method of Bradford (1976) with bovine serum albumin as a standard. The color of the reaction was measured spectrophotometrically at 595 nm wavelength at 25 °C.

Purification of Enzymes

The GSTs from 3 developmental stages of *B. minax* were purified using Prepacked Glutathione SepharoseTM 4B (GE Healthcare Biosciences, Uppsala, Sweden) according to the protocol supplied by Amersham Biosciences and Wu et al. (2009). The column was pre-washed with 10-20 mL of sodium phosphate buffer (20 mM, pH 7.3). Then, the gel bed was equilibrated with 6 mL PBS + 1% Triton X-100. The homogenates from larva, pupa, and adult of *B. minax* were each prepared as described above. The supernatant was filtered through a 0.45 µm Millex-HV (Millipore, Massachusetts, USA) and loaded on the GSH-agarose affinity gel column with a bed volume of 4 mL. Then, the column was washed with 2 × 10 mL PBS. Finally, the bound GSTs enzyme was eluted with 10 mL of elution buffer (5 mM Glutathione in 50 mM Tris-HCl, pH 8.0) and 1-2 mL fractions were collected. Those fractions containing GST activity were pooled for further analysis.

Enzyme Activity and Kinetics

The GSTs activity was measured using CDNB and reduced GSH as substrates, respectively, according to Habig et al. (1974) with slight modification. The total reaction volume per well of a 96-well microplate was 300 µL, consisting of 100 µL supernatant, CDNB [containing 2% (v/v) ethanol] and GSH in 0.05 M Tris-HCl, pH 7.5, giving a final concentration of 0.6 and 6 mM of CDNB and GSH, respectively. The non-enzymatic reaction of CDNB with GSH measured without supernatant served as the control. The changes in absorbance were measured continuously at 30 s intervals for 5 min at 340 nm and 37 °C in a microplate spectrophotometer (XMarkTM, BIO-RAD). Changes in absorbance per min were converted into nmol CDNB conjugated min^{-1} mg^{-1} protein using the extinction coefficient of the resulting 2, 4-dinitrophenyl-glutathione: $\varepsilon_{340nm} = 9.6$ $mM^{-1}cm^{-1}$ (Habig et al. 1974).

The Michaelis constant (K_m) and maximum velocity (V_{max}) of the purified GSTs from the three developmental stages were determined for GSH or CDNB by recording the activity towards a range of GSH (2 to 24 mM) or CDNB (0.2 to 2.4 mM), as substrates, respectively. The K_m and V_{max} values were calculated by the Michaelis-Menten equation in SPSS13.0 (SPSS, Inc., Chicago, USA).

Temperature and pH Optimum

Temperature dependence was assayed at reaction temperatures of 25, 28, 31, 34, 37, and 41 °C, respectively. The pH optimum was determined for CDNB conjugation activity. Purified GSTs were incubated at 37 °C for 5 min in 0.05 M sodium phosphate buffer (pH 6.0, 6.5, and 7.0), and Tris-HCl buffer (pH 7.5, 8.0, and 8.5), respectively. Conjugation activity was determined as described above.

Electrophoresis

Electrophoresis was performed according to the method of Laemmli (1970) using a Bio-Rad Mini Protean 3 cell and a Bio-Rad PowerPacTM HV (Bio-Rad Laboratories, Hercules, California, USA). The enzyme samples were diluted 1:4 with a solubilizer (5% SDS; 0.006% bromophenol blue; 1.25% β-mercaptoethanol in running buffer) and heated to 100 °C before electrophoresis. Separation gels were 12.5% acrylamide in 0.375 M Tris-HCl (pH 8.9). Stacking gels contained 4% acrylamide in 0.125 M Tris-HCl (pH 6.8). The samples were then run into the stacking gel at 15 mA for 30 min, and into the resolving gel at 30 mA for 90 min. The gels were stained with silver according to the manufacturer's instruction (Bio-Rad). The molecular weight of GSTs was calculated by the Gel Doc™ XR System and Quantity One 1-D

Analysis Software (Bio-Rad Laboratories, Hercules, California, USA) after scanning the stained the stained gel.

In vitro Inhibition Assay

The *in vitro* inhibition assay was performed using the GSTs assay conditions in the absence or presence of various concentrations of inhibitors. Stock solutions of the inhibitors, including ethacrynic acid, bromodulfalein, diethyl maleate, tetraethylthiuram disulfide, and curcumin were prepared in ethanol and diluted with Tris-HCl (50 mM, pH 7.5). The highest final concentration was 1% in the test solutions. Twenty five µL of enzyme source and 25 µL inhibitor solutions with appropriate concentrations were incubated for 10 min at 37 °C and then added to CDNB/GSH substrate mixture as described above. Reactions without the inhibitor were included as controls. Acetone with an appropriate concentration (up to 1%) was also added in control reactions to exclude the potential inhibition of GSTs by this solvent. The median inhibition concentration (I_{50}) for each inhibitor was determined based on the log-concentration vs. probit (% inhibition) regression analysis.

Statistical Analysis

All the data concerning the 3 developmental stages were analyzed using analysis of variance (ANOVA), and the means were separated by Duncan's Multiple Range Test or LSD Test for significance ($P = 0.05$). SPSS 13.0 for Windows. Regression analyses were also performed to calculate the GSTs kinetic parameters, purification profiles, and I_{50} values of various inhibitors.

RESULTS

Assay of Crude GSTs Activity

The specific activities of crude GSTs from larvae and adults were similar, while that of the larvae was significantly lower (Table 1). The statistical analysis showed that crude GSTs from pupa and adult of *B. minax* possessed a similar affinity to CDNB, for the V_{max} value, GSTs from adult was the lowest. For GSH, K_m values of GSTs from the 3 developmental stages varied significantly. Larva possessed the highest K_m value while pupa possessed lowest. There was no significant difference of V_{max} values among various developmental stages.

The inhibitory actions of 5 inhibitors (ethacrynic acid, bromosulfalein, diethylmaleate, disulfiram and curcumin) against the crude GSTs from 3 developmental stages of *B. minax* are presented in Table 2. The efficiencies of the tested inhibitors

TABLE 1. COMPARISON OF THE ACTIVITIES AND KINETIC PARAMETERS OF CRUDE GSTS TOWARDS CDNB FROM DIFFERENT DEVELOPMENTAL STAGES OF *BACTROCERA MINAX*.

| Stage | Specific activity (nmol·min^{-1} μg^{-1}) | CDNB | | GSH | |
		K_m (mM)	V_{max} (nmol·min^{-1})	K_m (mM)	V_{max} (nmol·min^{-1})
Larva	0.058 ± 0.002 a	0.061 ± 0.005 a	0.076 ± 0.003 b	1.450 ± 0.110 c	0.119 ± 0.002 a
Pupa	0.117 ± 0.013 b	0.152 ± 0.060 b	0.080 ± 0.002 b	0.139 ± 0.015 a	0.224 ± 0.171 a
Adult	0.043 ± 0.003 a	0.139 ± 0.015 b	0.055 ± 0.003 a	0.600 ± 0.023 b	0.061 ± 0.001 a

Each value represents the mean (M ± SE) of three replications. Means within the same column followed by different letters are significantly different ($P < 0.05$).

TABLE 2. THE *I50* INHIBITION CONSTANTS OF FIVE XENOBIOTIC COMPOUNDS ON THE CRUDE GSTS FROM DIFFERENT DEVELOPMENTAL STAGES OF *BACTROCERA MINAX*.

| Effector (*I50*) | Developmental stages | | |
	Larva	Pupa	Adult
Ethacrynic acid (μM)	33.3 ± 0.70 a	45.9 ± 0.47 a	161.7 ± 5.71 b
Bromosulfalein (μM)	121.6 ± 24.5 b	47.5 ± 9.10 a	99.3 ± 21.55 b
Diethyl maleate (μM)	241.5 ± 2.06 b	77.8 ± 1.03 a	201.3 ± 4.94 b
Disulfiram (μM)	57.74 ± 10.50 a	205.8 ± 1.52 b	182.1 ± 2.28 b

Each value represents the mean (M ± SE) of three replications. Means within the same row followed by different letters are significantly different ($P < 0.05$). The curcumin had least inhibitory effects on crude GSTs and the efficiency did not reach 50%.

were compared based on their I_{50}s (the concentration required to inhibit 50% of GSTs activity). The adult possessed the highest values toward ethacrynic acid. The *I50* value of bromosulfale and diethyl maleate against the pupa was significantly lower than the other 2 developmental stages. The larva possessed the lower values toward disulfiram than pupa and adult. The inhibition kinetics showed that curcumin exhibited poor inhibitory effects on crude GSTs from 3 developmental stages of *B. minax*, and no significant difference were observed among various concentrations.

GSTs Purification

GSTs were purified from the 3 developmental stages of *B. minax* using GSH-affinity chromatography. The chromatography elution profiles are shown in Fig. 1. In total, 9 tubes of solution eluted through the chromatography column (1 mL eluted solution per collection tube) were collected and assayed, and the 3rd tube solution from 3 developmental stages exhibited the highest specific activity of GSTs. The total activity and specific activity of GSTs purified from the pupa were much higher than the other 2 stages. Moreover, the purification fold of the pupa was significant higher than the larva. However, the yields of the purified GSTs were similar among the 3 developmental stages (Table 3).

The purity of the final enzyme preparation from 3 developmental stages of *B. minax* was examined by SDS-PAGE. The purified enzyme of larva, pupa and adult of *B. minax* all appeared as one GST band after silver staining on SDS-PAGE (Fig. 2). The molecular weight of the purified GSTs was estimated at approximately 23 kDa for each developmental stage of *B. minax*.

GSTs Characterization

The kinetic parameters of purified GSTs are presented in Table 4. For the catalytic activity of GSTs towards CDNB, the K_m values in the larva (1.10 mM) and pupa (0.89 mM) were significant higher than in the adult (0.28 mM). The V_{max} value of purified GSTs from the pupa (37.31 nmol min-1) was the highest among the 3 developmental stages. For the catalytic activity of GSTs towards GSH, the K_m (0.20 mM) value from the larva was significantly higher than those from the pupa and adult. The V_{max} (13.98 nmol min^{-1}) value from the pupa was the highest.

The temperature and the pH dependence of the purified GSTs from the 3 developmental stages of *B. minax* are presented in Fig. 3 and Fig. 4, respectively. The optimal temperature was 37 °C for the purified GSTs from 3 developmental stages. The activities varied slightly at lower temperatures in the larva and adult, but varied more in the pupa. The optimal pH for purified GSTs activity was 7.5, and the activities varied slightly in the pH range of 6.0-7.5 and decreased abruptly between pH 8.0 to 8.5 for the pupa. In contrast, the specific activity of GSTs from the other 2 developmental stages varied greatly in the pH range of 6.0-7.5.
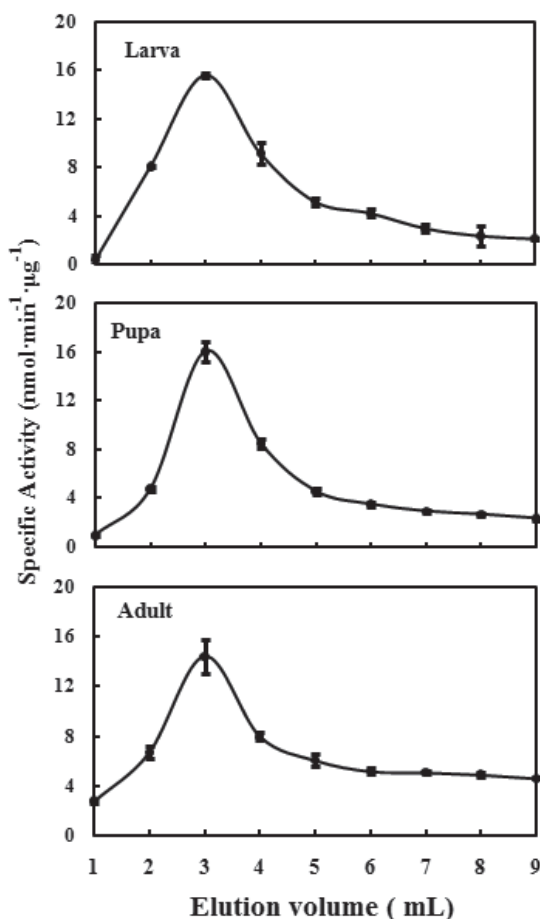
Fig. 1. Elution profiles for GST activities from 3 different developmental stages of *Bactrocera minax* for GSH-affinity chromatography.. Each value represents the mean ± SE of 3 independent experiments.

In Vitro Inhibition of GSTs

The inhibitory kinetics of 5 inhibitors against the purified GSTs from the 3 developmental stages of *B. minax* are presented in Table 5. The statistical analyses showed that the adult possessed the highest I_{50} value toward ethacrynic acid (49.54 μM). The pupa expressed the highest I_{50} value toward curcumin (88.02 μM) (Table 5).The I_{50} value of disulfiram against the larva (425.40 μM) was significantly higher than those of the larva and pupa, and the pupa possessed the lowest values (5.98 μM) toward disulfiram. The I_{50} value of diethylmaleate against the larva (42.60 μM) was significantly higher than those of the other 2 developmental stages. The I_{50} values of bromosulfalein against the 3 stages of *B. minax* were not significantly different from each other.

DISCUSSION

GSTs are present in almost all eukaryotes where they occur in multiple isoenzymic forms, constituting a significant intracellular mechanism of detoxification (Freitas et al. 2007). A total of 23, 28, and 37 GSTs genes from *Bombyx mori* (L.), *Anopheles gambiae* Giles, and *Drosophila melanogaster* Megen have been sequenced. (Enayati et al. 2005; Tu & Akgül 2005; Yu et al. 2008). The current study describes the purification and the biochemical characterization of GSTs from larvae, pupae and adults of *B. minax*. The SDS-PAGE revealed only 1 band was detected at 23 kDa in each stage of *B. minax*, which is the same as that of GSTs from *B. dorsalis* (Hu et al. 2011), and in the same range as those in the house flies, *Musca domestica* L.; Diptera: Muscidae (Fournier et al. 1992), the two-spotted ladybeetle, *Adalia bipunctata* L.; Coleoptera: Coccinellidae (Francis et al. 2002), the fall webworm, *Hyphantria cunea* Drury; Lepdioptera: Arctiidae (Yamamoto et al. 2007) and the psocid, *Liposcelis bostrychophila* Badonnel; Psocodea: Liposcelididae (Dou et al. 2009, 2010) and *Liposcelis paeta* Pearman (Wu et al. 2009). The specific activities and yields of purified GSTs from *B. minax* were much lower while the purification folds were much higher than those from *B. dorsalis*, (Hu et al. 2011).

Several ontogenetic studies have been reported for various rodents (Gregus et al. 1985), and the toad, *Bufo bufo* (L.) (Del Boccio et al. 1987) that extended over considerable fractions of their life spans. Ontogenetic models in insects, including all 3 developmental stages, i.e., larvae, pupae and adults, have been reported for relatively few species and these include the Australia blow fly, *Lucilia cuprina* Wiedemann (Diptera: Calliphoridae), the yellow mealworm beetle, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) and the European honey bee, *Apis mellifera macedonica* Ruttner (Hymenoptera: Apidae) (Kotze & Rose 1987; Kostaropoulos & Papadopoulos 1998; Papadopoulos et al. 2004). The highest purified GSTs activity of above mentioned insects is found in the pupal stage except in *Apis mellifera macedonica* in which expressed the highest activity is found in the adult. *B. minax* exhibits an ontogenetic model similar to that of *Lucilia cuprina* and *Tenebrio molitor*. In this study, the highest purified specific activity appeared in the pupal stage, which is 2.18-fold and 2.27-fold higher than in larvae and adults, respectively. This may be explained in 2 different ways. Firstly, elevated biosynthesis and construction of adult tissues in the pupa and high specific activity could mean that a high detoxification ability is needed for the protection in adults of crucial and important biosynthetic pathways from inhibition by toxic substances (Kostaropoulos et al. 1996). Secondly, the immobility of these pupae are immobile and their elevated vulner-

TABLE 3. COMPARISON OF THE ACTIVITIES OF PURIFIED GSTS FROM DIFFERENT DEVELOPMENTAL STAGES OF *BACTROCERA MINAX*.

Stage	Protein (μg·mL^{-1})	GSTs activity		Purification (-fold)	Yield (%)
		Total activity (nmol·min^{-1})	Specific activity (nmol·min^{-1}·μg^{-1})		
Larva	7.52 ± 1.67 b	178.75 ± 17.97 b	5.64 ± 3.03 a	96.62 ± 5.34 a	17.93 ± 2.23 a
Pupa	3.62 ± 0.64 a	236.42 ± 20.62 c	12.35 ± 1.08 b	174.07 ± 10.40 b	19.63 ± 2.42 a
Adult	3.76 ± 0.83 ab	104.65 ± 4.37 a	5.45 ± 0.89 a	129.02 ± 23.30 ab	21.79 ± 1.98 a

Each value represents the mean (M ± SE) of three replications. Means within the same column followed by different letters are significantly different ($P < 0.05$).

TABLE 4. KINETIC PARAMETERS OF GSTS PURIFIED FROM DIFFERENT DEVELOPMENTAL STAGES OF *BACTROCERA MINAX*.

Stage	CDNB		GSH	
	K_m (mM)	V_{max} (nmol·min^{-1})	K_m (mM)	V_{max} (nmol·min^{-1})
Larva	1.10 ± 0.11 b	24.37 ± 0.72 b	0.20 ± 0.01 b	6.52 ± 0.28 a
Pupa	0.89 ± 0.05 b	37.31 ± 5.31 c	0.10 ± 0.01 a	13.98 ± 2.03 b
Adult	0.28 ± 0.02 a	10.62 ± 1.67 a	0.11 ± 0.01 a	2.96 ± 0.29 a

Each value represents the mean (M ± SE) of three replications. Means within the same column followed by different letters are significantly different ($P < 0.05$).

ability to unfavorable environmental conditions, including the presence of toxic substances, requires high detoxification abilities (Kostaropoulos et al. 1996).
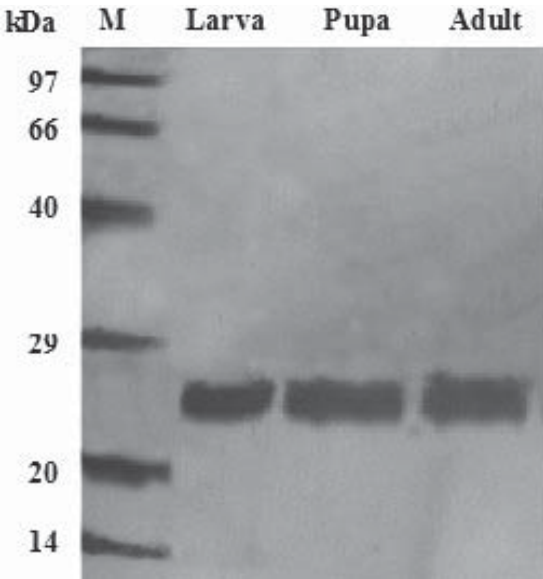

Fig. 2. SDS-PAGE of purified GSTs is from 3 different developmental stages of *Bactrocera minax*. Lane M, molecular weight markers; Lane Larva, purified GSTs from larva of *Bactrocera minax*; Lane Pupa, purified GSTs from pupa stage of *B. minax*; Lane Adult, purified GSTs from adult stage of *B. minax*.

Considering the kinetic parameters, important differences were observed according to the 2 tested substrates. The results may demonstrate differential isoenzymes expression among the 3 different developmental stages, or the expression of the same isoenzymes at a different percentage. Moreover, after purification, there is a significantly increase of V_{max} from larvae to pupae followed by a significant decrease in the adults. This pattern results in a significant increase in the total amount of GSTs, and in a higher catalytic capability in the pupal stage. In adults both the amount of enzyme and its affinity are low as indicated by the low V_{max} and high K_m values determined. This suggests that the GST detoxification system may be of potentially moderate importance in the adult stage. To illustrate the environmental adaptability of the 3 developmental stages of *B. minax*, the effects of different temperatures and pH values on specific activities of the GSTs were subsequently investigated. Each stage was characterized by a temperature optimum of 37 °C. Similar phenomena were also recorded for *B. dorsalis* (Hu et al. 2011), the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae) (Valles et al. 2003) and the rice leaf roller *Cnaphalocrocis medinalis* Guenée (Lepidoptera: Crambidae) (Yamamoto et al. 2008); and the GST activities varied slightly at lower temperatures in the larvae and adults but varied more in the pupae. This may be because fruit fly pupae live in the soil, where the environmental temperature was relatively stable, and, therefore, the pupae are more sensitive to temperature changes. Expression of GSTs isolated from each developmental stage was highest at pH 7.5, which is in agreement with the
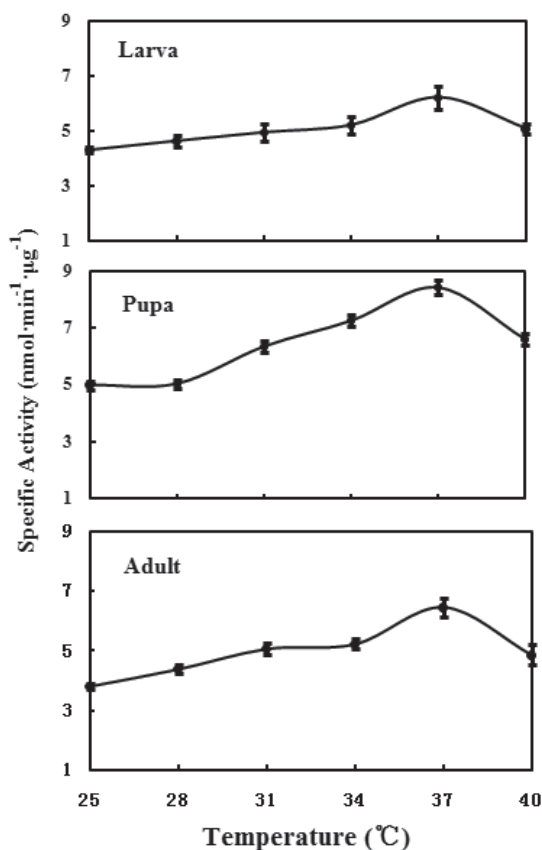
Fig. 3. Effect of temperature on specific activities of purified GSTs from 3 different developmental stages of *Bactrocera minax*. Each value represents the mean ± SE of 3 independent experiments.
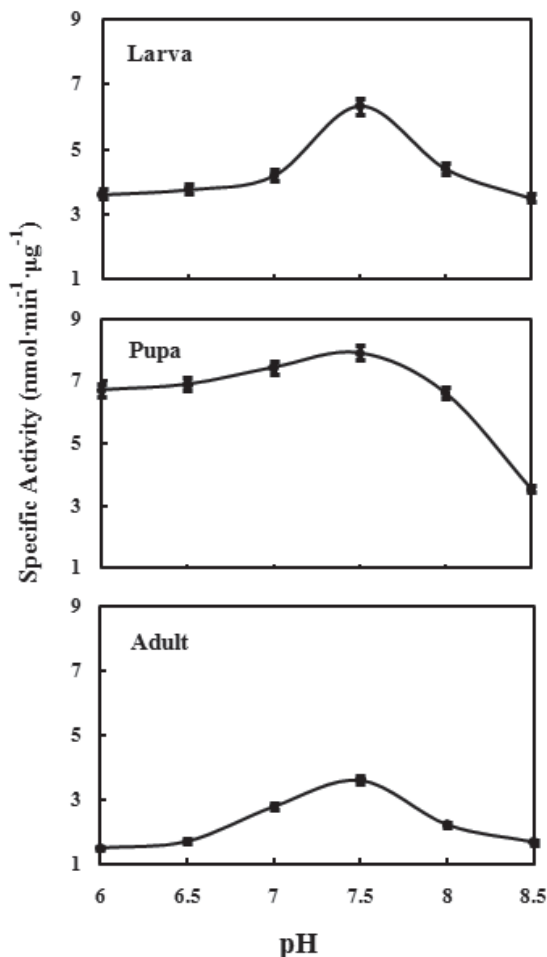

Fig. 4. Effect of pH on specific activities of purified GSTs from 3 different developmental stages of *Bactrocera minax*. Each value represents the mean ± SE of 3 independent experiments.

reports for *B. dorsalis* (Hu et al. 2011), and *H. cunea* (Yamamoto et al. 2007). Isoforms of GSTs may require specific conditions such as substrate concentration, temperature, and pH, for maximum activity (Yu 2002).

Both the crude and purified GSTs of *B. minax* were shown to be inhibited by various xenobiotic compounds (ethacrynic acid, bromosulfalein, diethyl maleate, disulfiram, and curcumin), and these compounds all had greater inhibitory effects on GSTs purified from larvae, pupae and adults with I_{50} at micromolar levels. The I_{50} values of these compounds—except disulfiram—against the crude GSTs from the 3 different stages were significantly higher than those of purified GSTs. These results are similar to those found in previous investigations of *B. dorsalis* (Hu et al. 2011), *Liposcelis bostrychophila* and *L. entomophila* (Dou et al. 2010). Moreover, curcumin exhibited poor inhibitory effects on crude GSTs with an efficiency below to 50%; on the other hand, the curcumin was more effective against purified GSTs. Ethacrynic acid is considered to be one of the

specific inhibitors of GSTs (Wu et al. 2009) and expressed the most excellent inhibitory effects with I_{50} values at sub-micromolar levels. The GSTs purified from *B. minax* were much more tolerant to ethacrynic acid than the GSTs from *B. dorsalis* (Hu et al. 2011). However, GSTs purified from some other insects exhibited I_{50} values for ethacrynic acid at even lower concentrations, e.g., the brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae) (40 nM), and the fall armyworm, *Spodoptera frugiperda* J. E. Smith (Lepidoptera: Noctuidae) (150 nM) (Yu & Huang 2000; Yu 2002).

In conclusion, the present study has provided some basic biochemical and identification information of GSTs from 3 different developmental stages of *B. minax*. These results indicated that GST enzymes are differentially regulated in a developmental stage-specific manner. Specifically,

TABLE 5. THE *I50* INHIBITION CONSTANTS OF FIVE XENOBIOTIC COMPOUNDS ON THE PURIFIED GSTS FROM DIFFERENT DEVELOPMENTAL STAGES OF *BACTROCERA MINAX*.

Effector (*I50*)	Developmental stages		
	Larva	Pupa	Adult
Ethacrynic acid (μM)	4.42 ± 1.20 a	3.21 ± 0.17 a	49.54 ± 1.32 b
Bromosulfalein (μM)	40.83 ± 3.79 a	20.66 ± 1.51 a	36.64 ± 1.99 a
Diethylmaleate (μM)	42.60 ± 5.99 b	7.20 ± 0.74 a	8.73 ± 0.44 a
Disulfiram (μM)	425.40 ± 14.40 c	5.98 ± 1.91 a	37.96 ± 2.33 b
Curcumin (μM)	48.00 ± 1.92 a	88.02 ± 5.38 b	39.98 ± 4.13 a

Each value represents the mean (M ± SE) of three replications. Means within the same row followed by different letters are significantly different ($P < 0.05$).

our study could serve as an initial step in the detailed elucidation of the characteristics of GSTs of *B. minax*. There is no doubt that techniques, such as genomics and proteomics, would help in the classification and identification of *B. minax* GSTs from the pest's 3 developmental stages. Further studies will be necessary to embark on subsequent explorations of GSTs regulation in response to various inducers or environmental signals, or in a tissue-manner, or even molecular genetic mechanisms responsible for those differences.

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