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Studies on levels of glutathione S-transferase, its isolation and purification from *Helicoverpa armigera*

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***Helicoverpa armigera* is a polyphagus pest of agricultural importance all over the world. In insects, glutathione S-transferase (GST) provides an important defence mechanism against plant allelochemicals as well as insecticides. The present work has been initiated in lepidoptern pest, *H. armigera* related to GST and its purification, characterization and endosulphan resistance. Spectrophotometrically, GST activity was measured and endosulphan bioassay was done with field-collected *H. armigera* at Akola Central India during August 1998 to March 2000. GST was detected in the eggs; it increased throughout the larval stages and was the highest in two-day-old fifth instar larva. On purification, maximum GST was found in the 70% ammonium sulphate fraction, while by affinity chromatography maximum activity was found in the bound fraction. Electrophoresis resolved only one isozyme having a molecular weight of 30 kDa. It was concluded that GST is responsible for endosulphan resistance, as GST levels and endosulphan resistance pattern were the same.**

GLUTATHIONE S-transferase (GST EC 2.5.1.18) is a family of multifunctional isozymes found in all eukaryotes. One of the main functions of GST is to catalyse xenobiotics, including pesticides in the mercapturic acid pathway leading to the elimination of toxic compounds[1]. In insects, this family of enzymes has been implicated as one of the major mechanisms for neutralizing the toxic effects of insecticides[2–8]. In recent years, the management of *Helicoverpa armigera*, the American bollworm, has become increasingly difficult due to development of resistance to various groups of insecticides, particularly pyrethroids and cyclodyienes[9]. *H. armigera* is an important polyphagous pest of cotton and many other crops of agricultural importance all over the world. In insects, GST provides an important defence mechanism against plant allelochemicals[10] as well as insecticides[11]. Therefore, GST plays an important role in insects. In India, there is little information on insecticide detoxifying enzymes in *H. armigera*. In the present investigation purification, characterization and correlation of GST with cyclodiene and pyrethroids has been studied.

Larvae of *H. armigera* were collected weekly from a range of crops in the farmers'[1] fields within 40 km radius of Akola, Maharashtra during July 1998 to March 2000. Larvae were fed on chickpea-based semisynthetic diet[9]. All rearing procedures were carried out at 27 ± 2°C, relative humidity 78 ± 2% and photoperiod of approximately 13 : 11 light : dark.

The technical grade insecticides, viz. Fenvalerate (976 g kg^{-1}; Sumitomo, Japan), Cypermethrin (900 g kg^{-1} Zeneca Agrochemicals, UK), Endosulphan (Dhanuka Pesticides, Japan) were used for bioassay. 1-Chloro-2,4 dinitrobenzene (CDNB), reduced glutathione (GSH), phenylthiourea (PTU), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulphonyl fluoride (PMSF), Sepharose 4B and all other chemicals were of high purity, and obtained either from Sigma Chemicals, USA; Loba Chemicals, India or Himedia Chemicals, India.

Ten larvae each of first to fifth instar of *H. armigera* were dissected out separately and their midguts were removed. Dissections were carried out with the help of a sterilized dissecting needle in ice-cold sodium phosphate buffer (0.1 M, pH 7.0) containing potassium chloride (11.5 g/l). Fat bodies and food particles were removed from the midguts, which were then homogenized individually in fresh sodium phosphate buffer containing

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1 mM each of EDTA, PTU, PMSF and glycerol (200 g/l). The homogenate was centrifuged at 10,000 *g* for 15 min at 0°C and the resultant post-mitochondrial supernatant was used as the enzyme source[12]. Protein was estimated according to the procedure of Lowry and coworkers using BSA (Type V) as standard.

The assay mixture consists of 1 ml of 1 mM GSH, 1 ml of 1 mM CDNB and 1 ml of distilled water. To this reaction mixture, 20 µl of enzyme sample was added and change in optical density was recorded at 30 s intervals up to 120 s. Controls without the enzyme always accompanied each assay. Activity was calculated with an extinction coefficient of 9.6 mM/cm for CDNB. Enzyme activity was expressed as µM/min at 25°C, and the specific activity as µM/min/mg protein[13].

Different amounts of solid ammonium sulphate were added stepwise at 4°C to obtain 55 to 90% saturation. The precipitate obtained was centrifuged at 12,000 *g* for 15 min in cold. The precipitate was then dissolved in 0.05 M sodium phosphate buffer. The enzyme solution was dialysed against the same buffer, changing the buffer twice. Enzyme activity was determined using CDNB as a substrate.

A 1.5 cm × 10 cm column of sepharose 4B was equilibrated with sodium phosphate buffer (100 mM, pH 6.5). Then 20–50 mg enzyme protein was applied to the column and eluted with sodium phosphate buffer till no further protein was detected (absorbance was monitored at 280 nm). Next the column was eluted with phosphate buffer (50 mM, pH 9.6) containing 5 mM GSH. Three millilitre fractions were collected and tested for enzyme activity.

The enzyme which bound to the affinity column and subsequently eluted was run on SDS–PAGE containing 10% polyacrylamide. The current was maintained at 25 V on a mini-gel electrophoretic model for 2 h. Then the gel was stained with 0.25% coomassie blue R-250. The number of GST isozymes was counted and molecular weight was determined by plotting log molecular weight vs mobility (*Rf*).

When the larvae attained third instar (30–40 mg body wt), they were individually applied with a topical dose of cypermethrin 0.1 µg/larva, fenvalerate 0.2 µg/larva or endosulphan 10 µg (LD_{99} value for pyrethroid-susceptible *H. armigera*)[14]. Each week, nearly 100 larvae were treated topically on dorsal thorax with insecticides and control treated with 0.1 µl acetone. Larval mortality was assayed for 6 days after treatment, as described by Armes *et al.*[9].

The GSH S-transferase activity was determined in the eggs and various larval stages using CDNB as substrate (Table 1). CDNB activity was found in all the larval stages and the egg stage. GSH S-transferase activity was detected in the eggs two days before hatching and in various larval stages on the second day.

CDNB-conjugation activity was compared in various tissues with the fat bodies and haemolymph (Table 2). CDNB-conjugation activity per milligram of protein was 279 in the fat bodies 51.10 in decapitate fifth instar larva, 15 in the cuticle, 15.81 in the haemlymph and 86 in the midgut.

For the partial purification of GSH S-transferase, ammonium sulphate fractionation and GSH–agarose affinity chromatography were used. Different ammonium sulphate fractions contained partially purified GSH S-transferase (Table 3). The highest specific activity was found in 70% ammonium sulphate precipitate. GSH–agarose affinity chromatography showed two peaks (Table 4).

SDS–PAGE of fifth instar larva of *H. armigera* showed a single band of GST enzyme after coomassie-blue staining and had apparent molecular weight of 30 kDa in *H. armigera*, indicating a single isozyme (Figure 1). However, the purification of GST from other developmental stages and various tissues was not done.

As shown in Table 5, cypermethrin and fenvalerate resistance frequencies were high throughout the two cropping seasons; resistance frequencies to cypermethrin 0.1 µg ranged from 27.11 to 96.33% during 1998–99 and

Table 1. Levels of GST from instars

GST source	Total protein (mg/ml)	GST enzyme activity (µM/min/mg)
Eggs	0.1	0.26
Instar I	0.083	53
Instar II	0.07	76
Instar III	0.053	77
Instar IV	0.043	79
Instar V	0.052	86
Pupa	1.71	2.1
Adult	0.8	5.3

Table 2. Levels of GST from different tissues

Enzyme localization	Total protein (mg/ml)	GST enzyme activity (µM/min/mg)
Whole body	0.106	51.10
Midgut	0.052	86
Haemolymph	2.7	15.81
Fat bodies	0.025	279
Cuticle	0.73	15

Table 3. Purification by ammonium sulphate precipitation

Percentage saturation	GST activity (µM/min/mg)
55	6
60	12
65	10
70	30
75	23
80	18
85	17
90	10

Table 4. Purification by affinity chromatography

Fraction	GST activity (μM/min/mg)
1	0
2	0
3	10.16
4	11
5	0
6	43.1
7	58.9
8	67.0
9	30.11
10	13.68

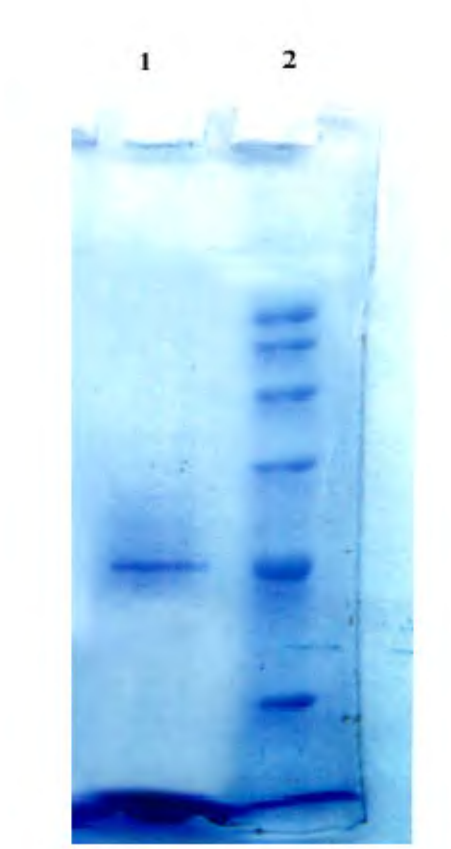

Figure 1. SDS–PAGE (discontinuous) of GST. Lane 1, with 20 μl enzyme, and Lane 2, with 10 μl standard molecular weight markers.

29.21 to 64.19% during 1999–2000. In 1998–2000, a decrease in resistance frequencies was observed between August and November, but frequencies then steadily increased over the remainder of the season. The fenvalerate 0.2 μg resistance profiles were similar to those of cypermethrin 0.1 μg during the two seasons – from 40.51 to 82.31% in 1998–99 and 50.10 to 79.11% in 1999–2000.

During 1998–99, cyclodiene resistance frequency using endosulphan was low in September (27.08%). However, it increased to 34.37% in October. The resistance frequencies declined gradually from November to March (31.73, 30.46, 26.25, 11.47, 12.15%.) On the contrary during 1999–2000, endosulphan frequencies were low all through the season (1.04 to 9.78%), except the high levels observed in October and January (18.58 and 14.34%, respectively; Table 5).

The seasonal changes in the GST titre were studied during the seasons 1998–99 and 1999–2000 (Table 6). There was a sharp increase in GST activity during 1998–1999 in the first half of the season from August (77.77) to October (161.32), while in later half, GST activity remained constant from November till March. A similar trend of enzyme pattern was evident during the 1999–2000, with increase in GST activity in first half of the season from August (76.47) to October (279.16), while in the later half, GST activity remained constant from November to March. The enzyme activity pattern was the same during 1998–99 and 1999–2000, GST titres were low over most of the season, with the exception of the September–October period (peak titre 279 μM mg^{-1} min^{-1}).

GST activity was found in various tissues and different developmental stages of *H. armigera*. From these results, it can be seen that *H. armigera* fat bodies have the highest amount of GST activity. A common feature is that the fat bodies are the major sites of metabolism of organophosphate insecticides, but the gut, cuticle and haemolymph also play an important role in the metabolism of xenobiotics. Low enzyme activity in whole body extract is probably due to the effect of endogenous inhibitors such as polyphenols and quinones released during homogenization[15]. The lowest GST activity was found in the eggs

Table 5. Levels of per cent insecticide resistance in *H. armigera* at Akola, 1998–2000

	1998–99			1999–2000		
Month	Cypermethrin (0.1 μg)	Fenvalerate (0.2 μg)	Endosulphan (10 μg)	Cypermethrin (0.1 μg)	Fenvalerate (0.2 μg)	Endosulphan (10 μg)
August	27.11	40.51	0	29.21	50.10	1.04
September	60.13	49.99	27.08	64.19	51.11	7
October	64.21	68.11	34.37	50.26	71.22	18.58
November	40.50	74.12	31.37	37.12	67.22	9.78
December	44.63	70.19	30.46	55.29	68.27	5.2
January	95.21	82.31	26.25	56.39	73.33	14.34
February	96.33	80.21	11.47	63.15	75.19	3.12
March	82.16	81.20	12.15	50.26	79.11	5.2

Table 6. Levels of detoxification enzymes in *H. armigera* at Akola, 1999–2000

Month	1998–99 GST ($\mu M\ min^{-1}\ mg^{-1}$)	1999–2000 GST ($\mu M\ min^{-1}\ mg^{-1}$)
August	77.77	76.47
September	79.60	156.96
October	161.32	279.16
November	67.91	71.32
December	70.13	78.21
January	65.26	78.89
February	77.19	65.23
March	64.66	68.63

of *H. armigera*. These differences in the specific activity might be due to different isozymes present in the various tissues and different developmental stages of *H. armigera*, and their preference for binding to CDNB or any inhibitor. The reported specific activity for *H. armigera* larvae is similar to that of *H. zea*[16]. *Drosophila* eggs exhibit the highest specific activity of the enzyme, while the adults show the lowest specific activity. Few studies reported a much higher specific activity for the pupae of yellow-fever mosquito[17].

From day-wise data, it was found that maximum activity was observed in two-day-old first to fifth instar larvae. Maximum GST activity was found in two-day-old fifth instar larvae. In *H. zea*, GST activity was detected in the eggs; it increased throughout the larval stages and was the highest in two-day-old fifth instar larvae[16]. This indicates that only the quantity of enzyme increased as the larvae grew older.

In our studies, a relatively pure enzyme from the midgut of *H. armigera* was obtained by glutathione sepharose 4B affinity chromatography showing two peaks (Table 4). The enzyme responsible for CDNB conjugation in *H. zea* was purified 48-fold by GSH–agarose affinity column[16]. Similarly, a relatively pure enzyme was obtained from various developmental stages of *Drosophila* using a GSH–agarose affinity column[18]. After ammonium sulphate precipitation, S-hexyl glutathione affinity chromatography was done in *Tribolium castaneum* (Herbst). The GST activity was approximately four to six-fold higher[19]. Using a glutathione sepharose 4B affinity column, GST was purified in *B. microplus*. It was found that purified GST was more active in catalysing conjugation of reduced glutathione to CDNB than to DCNB[20].

Purification by ammonium sulphate precipitation shows maximum GST activity in 70% ammonium sulphate fraction in *H. armigera*, while partial purification of GST in an insecticide-resistant strain of *T. castaneum* (Herbst) was done by ammonium sulphate precipitation, 660 g/l (ref. 19). In *H. zea* the highest specific activity for CDNB-conjugation was found in the 65–70% ammonium sulphate precipitate[16].

The soluble fraction of five phytophagous lepidoptera was first fractionated with ammonium sulphate to obtain a protein fraction which corresponded to 45–70% saturation. It yielded 54–80% of the initial enzyme activity towards CDNB and purification of 1.5–3.0 fold[21].

In nonreducing condition, SDS–PAGE showed a single band of GST enzyme after coomassie-blue staining and had apparent molecular weight of 30 kDa in *H. armigera*, while in the case of *B. microplus*, a single band of GST enzymes showed a molecular weight of 25 kDa[20]. In *Drosophila melanogaster*, GST enzyme shows one protein band in 12% SDS gel electrophoresis of purified enzyme from larvae, pupae and adults. The molecular weight of this band is 23.5 kDa. Another protein band with molecular weight of 35 kDa was also observed in the purified adult enzyme. It is obvious from these and previous results that larval enzymes are homodimeric proteins, while the adult enzyme may be heterodimeric[18]. Similar results were obtained from the third instar larvae of *Costelytra zealandic*[22].

In *H. zea*, the findings indicated the presence of one isozyme with a pI of 8.0. Based on SDS electrophoresis, the isozyme is a homodimer consisting of two equal size subunits having a molecular weight of 23,900 (ref. 16). Multiple forms of GST have been reported for other insect species such as *Musca domestica* and the moth, *Wiseana cervinate*[22].

The low cypermethrin resistance frequencies observed in the present study might be due to low insecticide selection pressure at the beginning of the season. The present results also revealed high cypermethrin resistance frequencies during September and low frequencies of cypermethrin during October–December, which corroborates with work of Sekhar *et al.*[23], who also reported low frequencies of resistance in *H. armigera* in Andhra Pradesh during October. Armes *et al.*[24] and Kranthi *et al.*[25] monitored the pyrethroid resistance in Andhra Pradesh and Central India, respectively. They also reported moderate resistance against pyrethroid during October. The probable reason for the high resistance during the later period of the cropping season might be due to excessive use of pyrethroids on pigeon pea and chickpea during the winter period for control of *H. armigera*, indicating high selection pressure. Rapid increase in resistance frequencies was observed during January–February. The seasonal fluctuations in the resistance frequencies observed during the present study were also confirmed by the findings of Pasupathy and Regupathy[26] in Tamil Nadu, where the pyrethroid resistance increased over the cropping seasons reaching peak levels during the winter period. Resistance to pyrethroids is also frequently accompanied by resistance to endosulphan, organophosphates such as quinolphos and monocrotophos, and oxime carbadamate methomyl[9,27].

Resistance to cypermethrin and fenvalerate is now widespread in *H. armigera* population in the country, even in the regions where insecticides are little used. It is

suggested that to control further spread of resistance to these molecules, implementation of strategies is essential. The high migration rate from Andhra Pradesh and genetic mixing of *H. armigera* population in the Akola region is responsible for the resistance. Even low immigration rate can facilate the rapid spread of resistance alleles among isolated populations[28].

The resistance frequencies for endosulphan (10 µg/larva) in *H. armigera* population at Akola, were in the lower range. Probably, the over-use of biologically safer insecticides such as endosulphan might have created increased selection pressure, resulting in higher resistance in successive seasons. From two-year data, the resistance frequencies for endosulphan were found at a lower level in the month of August, which increased in October and declined later. The seasonal variation in endosulphan resistance can be attributed to the general recommendation of endosulphan application during the early period of cropping season, which may create selection pressure resulting in higher resistance in October. In later periods, the incidence of other bollworms tends to be more and therefore use of endosulphan is usually replaced with application of pyrethroid and organophosphoric insecticides on cotton and pigeon pea. These result in the low selection pressure for endosulphan which correlates with moderate level of resistance, as observed during the present investigation. However, on chickpea endosulphan is again the most preferred insecticide, used largely for control of *H. armigera*, resulting in higher selection pressure and obviously increasing resistance frequencies. The above findings regarding endosulphan resistance frequencies observed at Akola could be well compared with those at other locations in India. Thus, under a country-wide monitoring programme, moderate endosulphan resistance of 20–50% was reported at different locations, including Nagpur where 50% resistance was observed towards the end of April[29]. Armes *et al.*[27] also found incipient endosulphan resistance present in *H. armigera* in India. However, high endosulphan resistance frequencies in *H. armigera* populations was found in South India[23,27,30,31]. Similarly, Kranthi *et al.*[32] also communicated high survial (up to 80%) of *H. armigera* larvae to 10 µg topically-applied, discriminating dose of endosulphan over two seasons (1993–94 and 1994–95). This indicates a serious cyclodine resistance problem in central India. However, the present findings at Akola differed from the above, and endosulphan resistance was moderate.

Low levels of pyrethroid resistance were observed at the start of the season during both the years. However, from December to March during both the years, cypermethrin and fenvalerate resistances had increased. The pattern of GST activity in general was followed in both the cropping seasons, whereas increased levels of GST at the start of the season and decreased levels during the later half were correlated with endosulphan resistance and not with pyrethroid resistance.

In our study, GST activity did not respond to pyrethroid resistance. Similar results were obtained by McCaffery *et al.*[33], who showed that GST did not appear to be involved in pyrethroid resistance. The role of GST is well-documented in organophosphate resistance in *H. armigera*[34], but not in pyrethroid resistance. In conclusion, the results presented here have confirmed the presence of GST activity in *H. armigera* eggs, larval, pupal and adult stages. GST activity towards CDNB was detected in the eggs, it increased throughout the larval stages and was the highest in two-days-old fifth instar larvae. The enzyme responsible for CDNB-conjugation was purified by ammonium sulphate precipitation, with maximum activity in the 70% fraction. While in affinity chromatography, maximum GST activity was found in the bound fraction. Also, the study revealed that the GST enzyme has a molecular weight of 30 kDa. Our insecticide resistance results revealed that GST is responsible for endosulphan resistance. Similar results were observed by Kranthi[12].

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Indicators of gas hydrates: Role of velocity and amplitude

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Methane hydrates/gas hydrates are now viewed as a promising alternate energy source in the near future. Studies are now more focused to find the indicators of these deposits and aid in exploration purposes. Bottom simulating reflectors (BSRs) identified on the seismic reflection records are the most important indicators of gas hydrates. BSRs are recognized on the seismic records as strong negative-polarity reflectors mimicking the seafloor. Velocity and amplitude (VAMP) are essentially the pseudo-structures that are associated with the massive hydrate deposits and are also observable on the seismic records as a series of pull-ups directly above the push-downs. These features have been used to delineate free gas in the Aleutian, Bowers and Berings basin. Investigations are being carried out in Indian offshore regions to evaluate the presence of gas hydrates. Therefore studies regarding the occurrence of VAMPs gain importance. In the present communication we studied VAMP anomalies identified in the marine reflection data off Kerala–Konkan region, western India.

METHANE hydrate/gas hydrates are increasingly recognized as being a potential future energy source. Initial estimates indicated that the energy available from hydrate reserves far exceeds all conventional forms of energy. Gas hydrates, also called gas clathrates, are naturally-occurring solids comprised of water molecules forming a rigid lattice of cages, with most of the cages containing a molecule of natural gas, mainly methane[1]. These gas hydrates are formed at high pressures and low temperatures. In marine environments, these conditions are met within continental margin sediments at water depths greater than 500 m. Gas hydrates have become a major focus of international research because of the increasing demand for hydrocarbons. According to Hovland and Judd[2], the favourable areas occupy more than 90% of the ocean basins. Gas hydrates are found mostly on the continental slopes, which are characterized by thick sediments, rich in organic content. It may also be noted that gas hydrates occur near the places of discharge of deep fluids and gas-escape features such as gas seepages, gas vents, mud volcanoes, etc. The high tectonic activity in these regions also favours the release of fluids. Two models have been proposed to account for the formation of hydrate and development of bottom simulating reflector (BSR). In the first model, the BSR is caused by the hydrate overlying gas-saturated sediment, wherein the impedance contrast at the base of the hydrated zone and the top of the gas layer provides the required strong reflection. In the second model, the BSR is caused by the hydrate overlying brine-saturated sediment, and impedance contrast between the overlying sediments containing substantial amounts of hydrate and the underlying brine-saturated sediments gives the required reflection.

Seismic reflection technique is the main tool to infer natural gas hydrates within the marine sediments. The presence of gas hydrates is mainly inferred from the appearance of the BSR, a strong negative-polarity reflector mimicking the seafloor, as well as bright spot anomalies in combination with the velocity and amplitude (VAMP) features. Another special reflection seems to be acoustic blanking[2]. In addition, gas-venting features sometimes also testify the presence of gas hydrates below the seabed. Several publications of the last decade highlighted the presence and significance of these gas hydrates. However, for ready reference, some of the frequently used terms with details are presented below.

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