PARTIAL CHARACTERIZATION OF GLUTATHIONE *S*-TRANSFERASE IN TWO POPULATIONS OF THE SUNN PEST, *EURYGASTER INTEGRICEPS* PUTON (HETEROPTERA: SCUTELLARIDAE)

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[Zibaee, I., Bandani A. R., Haghani, S. & Zibaee, A. 2009. Partial characterization of glutathione *s*-transferase in two populations of the sunn pest, *Eurygaster integriceps* Puton (Heteroptera: Scutellaridae). Munis Entomology & Zoology, 4 (2): 564-571**]**

ABSTRACT: Glutathione S-transferases (GSTs) from two populations (Tehran and Shiraz) of the sunn pest *Eurygaster integriceps* Puton (Heteroptera: Scutellaridae) were characterized through in vitro colorimetric assays. GSTs showed higher activity peaks at pH 5-6. The Km-values for GSTs were different between populations in which Shiraz population had higher *Vmax* and lower *Km* values when using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate (and a fixed concentration of reduced glutathione—GSH). In addition, Native-PAGE electrophoresis showed a higher isoenzymatic patterns for Shiraz population compared with Tehran one. These results provide evidence of the involvement of enhanced GST activity as an additional organophosphorus-resistant mechanism in at least some sunn pest populations from Iran.

KEYWORDS: Metabolic detoxification, Enhanced glutathione conjugation, fenitrothione resistance

The sunn pest (*Eurygaster integriceps* Puton) (Heteroptera: Scutelleridae), is one of the most serious insect pests of wheat and barley in the wide area of the Near and Middle East, West Asia, and many of the new independent states of central Asia. It also is found in Eastern and South Europe and North Africa (Kazzazi et al., 2005). This insect has a monovoltine life cycle (one generation per year) with two different phases. The first phase (growth and development) occurs in the wheat field during the spring, whereas the second phase (diapause as an adult) usually occurs in the mountain area during the summer and winter. This pest feeds on various structures of the host plants e.g. leaves, stems and grains and as a consequence the nature of the injury that they cause is also variable. During feeding they enter their stylets into the host plant, inject their watery saliva which containing digestive enzymes, and sucking up the liquefied cells' contents (Boyd et al., 2002). Feeding punctures appeared as minute darkish spots on the seeds. Sunn pest feeding on different stages of developing seeds causes quantitative and qualitative damage because they inject enzymes into the grain that degrade gluten protein and cause rapid relaxation of dough which results in the production of bread with poor volume and texture. Most of economic loss attributed to this species is caused by nymphal and adult injury to the wheat kernels so that yield loss because of sunn pest outbreaks in some area is 100%. In Iran alone more than one million hectare of wheat field is sprayed with fenitrothione against this pest.

Since 1970's fenitrothion and sometime Cpermethrin, extensively used in order to control this insect in many countries. As a consequence of the intensive use of these pesticides, there are some complaints about the insecticide

performance, especially in areas where spraying was used consistently for some years. Insects develop resistance to insecticides primarily though three mechanisms: decreased penetration, reduced target site sensitivity and enhanced metabolism (Plapp, 1976; Oppenoorth, 1984). Enhanced metabolism of insecticides decreases the attainment of the effective amount of insecticides that can kill insects. Thus, metabolic resistance may significantly decrease the susceptibility of insects to insecticides. Three major detoxifying enzymes are associated with insecticide resistance: cytochrome P450 monooxygenases, glutathione *S*-transferases and esterases (Bull, 1981; Oppenoorth, 1984).

Glutathione *S*-transferases (GSTs) are the multigenic family of multifunctional proteins that catalyze conjugation reactions of molecules having an electrophilic site, with reduced glutathione (GSH). The effect of GSTs is generally to convert a reactive lipophilic molecule into a water-soluble, non-reactive conjugate which may easily be excreted (Habig et al., 1974). In insects, increased levels of GSTs are associated with organochlorine and organophosphorus insecticide resistance (Motoyama and Dauterman 1975; Clark et al. 1984; Hemingway et al. 1985; Grant and Matsumura 1989; Fournier et al. 1992; Lagadic et al. 1993; Zibaee et al., 2008).

Recent efforts using canonical correlation analysis to establish preliminary relationships between insecticide resistance in two populations of *E.integriceps* and their activity levels of detoxification enzymes indicated the potential effect of GSTs as a organophosphorus-resistance mechanism (Alizadeh, 2006). In previous study, it was shown that resistant ratio was 2.37 *µg/adult* and esterases had a significant role in detoxifying of fenitrothione, an organophosphorus insecticide, in wheat fields of Iran. Based on these findings and the currently unclear involvement of GSTs in fenitrothione resistance, the present study was carried out to initially characterize the activity of this enzyme in two populations of the sunn pest.

MATERIAL AND METHODS

Insects

The insects were collected from Karadj and Shiraz wheat farms in Tehran and Fars provinces, respectively. The both populations were maintained and reared on wheat plants and wheat kernels in the laboratory at 25 ±2 °C under a 14 h light: 10 h dark (LD 14:10) photoperiod.

Chemicals

All chemicals including Tris-base, HCl, reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB) and 1,2-dichloro-4-nitro-benzene (DCNB) were procured from Sigma-aldricht and Merc Companies, respectively.

Sample preparation

Bodies from 5 adults (0.490-0.589 gr) were homogenized and diluted with universal buffer containing succinate, glycine and 2-morpholinoethanesulfonic acid (pH=7.2) (Hosseinkhani and Nemat-Gorgani 2003) in weight to volume proportion and centrifuged for 10 min in 10000 *r/min*. The supernatant was transferred to new tubes and preserved at -20 ºC until the onset of the experiments. Three replicates were provided for each biochemical analysis and activity of all enzymes were measured by kinetic analysis (Qu et al., 2003).

Assay of glutathione S-transferase

For glutathione *S*-transferase activity the method reported by Oppenorth (1985) was adopted. Twenty microliters CDNB (20 mM) or DCNB (40 mM) were pipetted into the microplate wells, and then 50 µL of enzyme solution was added. The OD value at 340 nm was recorded with an interval of 9 s in 5 min.

Determination of Vmax and Km values

To determine the kinetic parameters of GST, different concentrations of CDNB and DCNB (5-20 mM) were prepared and 50 µl of each substrate were mixed with 50 µl phosphate buffer (pH 7) and the reaction was initiated and monitored at 340 at 60 s intervals.

Effect of pH on GST activity

Optimal pH for their activities was determined using universal buffer with pH set at 3, 4, 5, 6, 7 and 8 in which 50 µl of each substrate added to different tubes containing 50 µl of enzyme and read at 340 nm.

Polyacrylamide Gel Electrophoresis (PAGE)

In order to determine the comparison of GST isienzymes in two population of *E.integriceps* native polyacrylamide disc-gel electrophoresis was carried out using the method of Parish and Marchalonis (1970) using 2.7% and 7.7% polyacrylamide for the stacking and resolving gels, respectively. The gel was stained with 1.5 % (w/v) Coomassie Brilliant Blue G-250 and distained in glacial acetic acidmethanol-water (7.5: 5.0: 87.5).

Protein determination

Protein concentrations were measured according to the method of Bradford (1976), using bovine serum albumin (Bio-Rad, München, Germany) as a standard.

Statistical analysis

For determination of mortality and lethal concentration, POLO-PC software (Leora, 1987) were used. All data were compared by one-way analysis of variance (ANOVA) followed by Tukey's studentisized test when significant differences were found at P=0.05 (SAS 1997). Differences between samplings were considered statistically significant at a probability more than 5% (p<0.05). Probability levels are specified in the text.

RESULTS

Glutathione S-transferase activity

Glutathione *S*-transferase activity showed a significant difference between two populations of *E. integriceps*. When CDNB was used as substrate, activity level in Tehran and Shiraz populations was evaluated 1.49 and 2.59 *µmol/min/mg protein*, respectively and by using DCNB as the substrate, it was measured 1.03 and 0.97 *µmol/min/mg protein*, respectively (Table 1). Glutathione *S*-transferase activities in two populations were significantly different when using CDNB and DCNB as the substrate (Table 1). So that by using CDNB, GST activity level in Shiraz population was higher than Tehran population and by using DCNB as substrate it was *Vise versa*.

Determination of V_{max} and K_m values

The Michaelis–Menten equation model was derived to account for the kinetic properties of enzymes. The Michaelis constant (K_m) and the maximal reaction velocity (V_{max}) are the kinetic constants of interest. The kinetic parameters K_m and V_{max} were therefore determined using the Lineweaver–Burk (double reciprocal) transformation and are presented in table 1 and figure 1. The GST K_m-value for Tehran population using CDNB and DCNB as substrates had a significant difference to the estimated K_m-value of Shiraz population and was higher than that (Table 1). The V_{max} for Shiraz population was higher than that of Tehran and showed a significant difference (Table 1).

Effect of pH on GST activity

The optimum pH for GST activity in Shiraz and Tehran populations were significantly different by using different substrates and universal buffer. In Shiraz population, the optimal pH value was 5.5 and 6 for CDNB and DCNB,

respectively. There were different results for Tehran population when different substrate were used i.e. the optimal pH was 6 and 5 for CDNB and DCNB, respectively. The same results achieved with Shiraz population i.e. different pH obtained when different substrate used. The activity level of GST in different pH for Shiraz population by using both CDNB and DCNB was higher than that of Tehran population (Figure 2).

Polyacrylamide Gel Electrophoresis (PAGE)

GST electrophoresis profiles are shown in Fiure 3. GST isoenzyme patterns of Shiraz population showed darker bands than Tehran populations which could be due to higher activity of GST in Shiraz population and showed a significant difference.

DISCUSSION

The sunn pest, *Eurygaster integriceps* resistance to fenitrothione was correlated with enhanced detoxifying enzymes in a previous study (Alizadeh, 2006), and here further exploration was made to provide preliminary characterization of GST activity in two geographical populations of Sunn pest. GST activity levels towards the substrate CDNB were higher in the Shiraz population compared with the Tehran one. Zibaee et al. (2008) showed that the activity level of GST in four populations of rice striped stem borer (Chilo suppressalis) was different when CDNB used as substrate. Similar results were obtained by Qu et al. (2003).

The Km, is the substrate concentration that results in the filling of one-half of the enzyme's active sites (leading to an initial velocity of $Vmax/2$) and in the simplest case, K_m is equal to "the dissociation constant" of the enzyme substrate complex, Ks. Ks = [E][S]/ [ES] Therefore, lower Km value shows higher affinity of enzyme-substrate complex. The second kinetic constant, $Vmax$, is attained when all the enzyme's active sites are filled with substrate molecules and its importance lies in allowing the estimation of the number of substrate molecules converted into product by an enzyme in a unit of time, when the enzyme is fully saturated with substrate. By taking the reciprocal of both sides of the Michaelis–Menten equation, the disadvantages of nonlinear kinetic analysis are avoided converting it into the Lineweaver–Burk relationship, which is linear. Analysis of Lineweaver-Burk plots (Tables 1 and Figure 1) provide information regarding the mode of action of GST in *E. integriceps*. In the majority of enzymes, in the resistance population the value of V_{max} increased and K_m value decreased. Since the K_m has an inverse relationship with the substrate concentration required to saturate the active sites of the enzyme, this indicates increasing of enzyme affinity for substrate (Wilson, 1986). In other words, K_m is the measurement of the stability of the enzyme-substrate complex and a low K_m would indicate strong binding and a high K_m low binding (Stryer, 1995). Resistance also increased the V_{max} value which further indicates that they interfere with the rate of breakdown of the enzyme-substrate complex (Morris, 1978). In this study, CDNB appeared to be more favorable than DCNB for glutathione *S*-transferase of *E.integriceps*, having the lower Km and higher V_{max} values in Shiraz populations.

The optimum pH value observed for GST activity in the present study was lower than the pH values more frequently used for GST characterization in insects, which are around 7.0 (Grant and Matsumura, 1989; Reidy et al., 1990; Legacid et al., 1993; Yu, 1996), but it is still within the range reported for this group of enzymes (Commandeur et al., 1995; Yu, 2002). This could be due to the hemolymph pH of *E.integriceps* which is about 6.5 and showed a type of adaptation to higher activity.

The higher catalytic activity of GSTs, particularly from the Shiraz population provides support for the hypothesis of their involvement in the resistance to fenitrothione. GSTs may act as binding proteins increasing the activity of other organophosphorus detoxification enzymes such as esterases (Grant and Matsumura, 1989; Kostaropoulos et al., 2001; Alizadeh, 2006). An alternative explanation for the GST role as a binding protein is that the higher GST activity levels in Shiraz populations of *E.integriceps*, as reported here, may be favoring their direct catalytic activity over organophosphorus as earlier recognized (Zibaee et al., 2008), or their activity as antioxidant agents decreasing the oxidative stress initiated by organophosphorus as more recently suggested (References). Either way, there seems to be an involvement of enhanced GST activity in Shiraz population of sunn pest, but this resistance mechanism is apparently secondary in importance to the altered target site (AChE).

The presented data suggest that the greater sensitivity of Tehran population to fenitrothione than Shiraz population implies higher metabolic activities which either detoxify or limit the intoxicating ability of fenitrothion. So, the present study has provided some basic information on the GST activity of these two populations that will be useful to understand the mechanisms of insecticide resistance in the *Eurygaster integriceps*.

ACKNOWLEDGMENTS

This study was supported by a University of Tehran grant. We greatly appreciate M. Allahyari for his assistance.

LITERATURE CITED

Alizadeh, M. 2006. Evaluation of insecticide resistance and biochemical mechanism in two populations of Eurygaster integriceps Puton (Heteroptera: Scutelleridae). Master of science thesis. University of Tehran, 125 pp.

Boyd, D. W., Cohen, A. C. & Alverson, D. R. 2002. Digestive enzymes and stylet morphology of Deraeocoris nebulosus (Hemiptera: Miridae), a predacious plant bug. Annals of Entomological Society of America, 95: 395–401.

Bull, D. L. 1981. Factors that influence tobacco budworm, Heliothis virescens, resistance to organophosphorus insecticides. Bulletin of Entomological Society of America, 27: 193-197.

Clark, A. G., Shamaan, N. A., Dauterman, W. C. & Hayaoka, T. 1984. Characterization of multiple glutathione transferases from the housefly, Musca domestica. Pesticide Biochemistry and Physiology, 22: 51-59.

Commandeur, J. N. M., Stijntjes, G. J. & Vermeulen, N. P. E. 1995. Enzymes and transport systems involved in the formation and disposition of glutathione S-conjugates. Pharmacology Review, 47: 271–330.

Fournier, D., Bride, J. M., Poire, M., Berge, J. B. & Plapp, F. W. 1992. Insect glutathione S-transferases: Biochemical characteristics of the major forms from houseflies susceptible and resistant to insecticides. Jornal of Biological Chemistry, 267: 1840-1845.

Grant, D. F. & Matsumura, F. 1989. Glutathione S-transferase 1 and 2 in susceptible and insecticide resistant Aedes aegypti. Pesticide Biochemistry and Physiology, 33: 132-140.

Habig, W. H., Pabst, M. J. & Jakoby, W. B. 1974. Glutathione S-transferases. Jornal of Biological Chemistry, 249: 7130-7141.

Hemingway, J., Malcolm, C. A., Kissoon, K. E., Boddington, R. G., Curtis, C. F. & Hill, N. 1985. The biochemistry of insecticide resistance in Anopheles sacharovi: Comparative studies with a range of insecticide susceptible and resistance Anopheles and Culex species. Pesticide Biochemistry and Physiology, 24: 68-71.

Hosseinkhani, S. & Nemat-Gorgani, M. 2003. Partial unfolding of carbonic anhydrase provides a method for its immobilization on hydrophobic adsorbents and protects it against irreversible thermoinactivation. Enzyme Microbiology and Techology, 33: 179–184.

Kazzazi, M., Bandani, A. R. & Hosseinkhani, S. 2005. Biochemical characterization of α-amylase of the Sunn pest, Eurygaster integriceps. Entomological Sciennce, 8: 371-377.

Legacid, L., Cuany, A., Berge, J. B. & Echaubard, M. 1993. Purification and partial characterization of glutathione S-transferases from insecticideresistant and lindane-induced susceptible Spodoptera littoralis (Boisd.) larvae. Insect Biochemistry and Molecular Biology, 23: 467–474.

LeOra software, Polo-Pc. 1987. A user guide to probit or logit analysis. LeOra software, Berkeley, California.

Morris, J. G. 1978. A biologist's physical chemistry (2nd edition). Edward Arnold publishing (Limited).

Motoyama, N. & Dauterman, W. C. 1975. Inter-strain comparison of glutathione dependent reactions in susceptible and resistant houseflies. 5: 489-493.

Nouri, H. 2002. Study of the economic injury level of the Sunn pest in Qazvin province of Iran. Ph.D. thesis of Plant Protection Department, Faculty of Agriculture, University of Tehran, Karadj, Iran.

Parish, S. H. & Marchalonis, J. J. 1970. Polyacrylamide disc gel electrophoresis. Analytical Biochemistry, 34: 436-443.

Plapp, F. W. 1976. Biochemical genetics of insecticide resistance. Annual Review of Entomology, 21: 179-197.

Qu, M., Zhaojun, H., Xinjun, X. & Lina, Y. 2003. Triazophos resistance mechanisms in rice stem borer (Chilo suppressalis Walker). Pesticide Biochemistry and Physiology, 77: 99-105.

Reidy, G. F., Rose, H. A., Visetson, S. & Murray, M. 1990. Increased glutathione S-transferase activity and glutathione content in an insecticide-resistant strain of Tribolium castaneum (Herbst.). Pesticide Biochemistry and Physiology, 36: 269–276.

SAS institute. 1997. SAS/STAT User's Guide for Personal Computers. SAS Institute, Cary, Nc.

Stryer, L. 1995. Biochemistry. W. H. Freeman and Company, New York.

Wilson, K. 1986. Principle and techniques of practical biochemistry, (Eds K. Wilson and Goulding, K. H.). Third edition. Edward Arnold.

Yu, S. J. 1996. Insect glutathione S-transferases. Zoological Studies. 35: 9–19.

Yu, S. J. 2002. Substrate specificity of glutathione S-transferases from the fall armyworm. Pesticide Biochemistry and Physiology. 74: 41–51.

Zibaee, A., Jalali Sendi, J., Alinia, F. & Ghadamyari, M. 2008. Diazinon resistance in different selected strains of Chilo suppressalis Walker (Lepidoptera: Pyralidae), rice striped stem borer, in the north of Iran. Journal of Economic Entomology. (In press).

Table 1. Activity ($\mu mol/min/mg\ protein$) and Kinetic parameters (V_{max} and K_m) of glutathione S-transferases in two populations of *E. integriceps* adults on two substrates (CDNB, DCNB) combined with the conjugating tripeptide glutathione (GSH).

Populations	CDNB ($\mu mol/min/mg\ protein$)	DCNB ($\mu mol/min/mg\ protein$)	V_{max} ($\mu mol/min/mg protein$)		K_m (mM)	
			CDNB	DCNB	CDNB	DCNB
Shiraz	2.49±0.17a	0.97±0.017b	1.44±0.23b	7.19±1.48a	33.72±10.25b	41.42±5.17b
Tehran	1.59±0.026b	1.03±0.013a	1.78±0.19a	1.49±0.23b	68.71±23.36a	70.77±32.47a

*. Results are reported as means±standard error. Different letters indicate that the activity and kinetic parameter of the populations are significantly different from each other by Tukey's test (p<0:05).

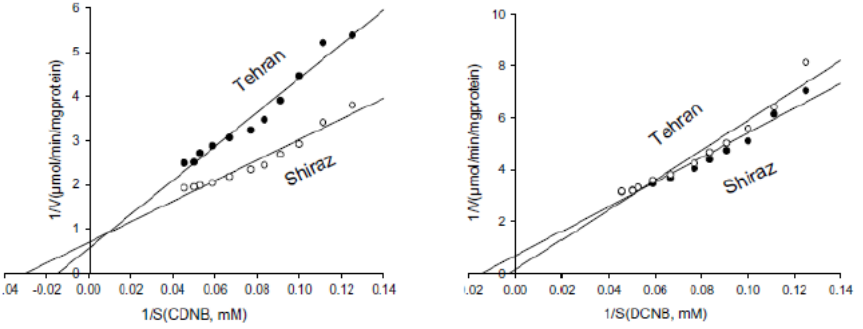

Figure 1. Lineweaver_Burk plots of CDNB and DCNB in two populations (Tehran and Shiraz) of *E. integriceps* adults. For measurements related to GSH and benzene substrates, CDNB or DCNB, varying concentrations of benzene substrates 5-20 mM. were used.

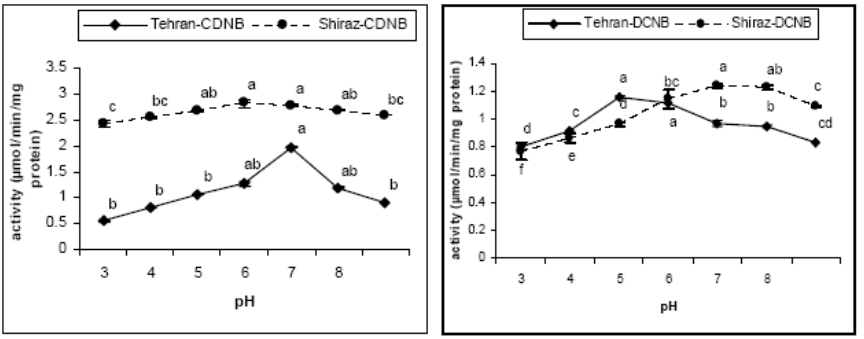

Figure 2. Effect of pH on activity of glutathione *S*-transferase extracted from *E. integriceps* adults.

Figure 3. Native-PAGE of whole insect homogenates from two populations (Tehran and Shiraz) of *E. integriceps* adults on 8% polyacrylamide gels.