STUDIES OF THE GLUTATHIONE S-TRANSFERASES ACTIVITIES AND THEIR LOCALISATION IN *ADALIA BIPUNCTATA* L. (COLEOPTERA: COCCINELLIDAE)

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ABSTRACT

While there is considerable evidence for inductive interaction of plant allelochemicals and xenobiotic metabolizing enzymes (XME), little is known of allelochemical-mediated induction of XME in other insect orders than Lepidoptera (Yu, 1984). The glutathione S-transferases (GSTs) catalyze the conjugation of reduced glutathione (GSH) with a wide range of electrophilic compounds and lead to the formation of products of increased water solubility and minimal toxicity (Clark, 1990). This group of enzymes plays an important role in xenobiotic detoxication.
Temperature associated to elevated concentrations of substrates or GST can influence the clear discrimination of low or high levels of enzymatic activity (Brogdon and Barber, 1989). In this study, we report the results of the investigation of a spectrophotometric method to measure GST activities in *Adalia bipunctata* L. A concentrations range of benzene substrates and GSH were tested. 2,4-Dinitro-1-iodobenzene (DNIB) and 1-chloro-2,4-dinitrobenzene (CDNB) were used to monitor the enzyme activity using each developmental stage of the beetle. The optimal concentrations of GSH and substrates have been determined for each case. Moreover, the localisation of the GST were determined in adults of ladybirds. Enzymatic activities in abdomen were more important than in the head or thorax. Significant amount of enzyme were also observed in the elytra.

INTRODUCTION

The main ways of enzymatic detoxification in animals and plants are the hydrolysis, the oxidation and the conjugation leading to hydrolysed compounds with higher hydrophility and excretability. Glutathione S-transferases (GST) catalyze the conjugation of reduced glutathione (GSH) with a broad range of electrophilic substrates which are often toxic compounds (Brogdon and Barber, 1989). The reaction is the first step in the formation of mercapturic acids, a pathway to wich lipophilic xenobiotics are metabolized and eliminated from the animal body. This group of enzymes confers resistances to numerous toxics when their activities are increased: resistances to anticancer drugs, alkylating agents, herbicides and insecticides (Clark, 1990).
In insects, GST were found in many insects as mechanism of insecticides resistance but only a reduced number of them were caracterised. Their activities increase induces resistance to organophosphates and DDT. Yu (1989) observed several isozymes in polyphagous lepidopterian species but only one form in a more specialist butterfly species. The GST are studied as induction model. Indeed, phytophagous pests, mainly lepidopterian species, display high GST acivities which were correlated to the presence of natural secondary substances (isothiocyanates, xanthotoxins) from their host plants.

Phytophagous insects have important protections against allelochemicals (displaying potential toxicity) by developping enzymatic complex. These systems can be induced at low concentrations of ingested compouds. GST induction corresponding to changes of host plant seem to be a physiological adaptation which is not heritable (Egaas *et al*, 1990). The adaptation to these compounds called allelochemicals is one aspect of the chemical ecology of pests behaviour and infestation (Pickett *et al*, 1992).
Insects with higher amount of enzymes are often more tolerant to insecticides. These inductions due to natural plant substances will be followed by differential susceptibly to insecticides selection pressure (Yu and Hsu, 1993). Nevertheless, adaptation to allelochemicals from plants could involve the selection of particular isoenzymes which have no particular specificity to insecticides.
These group of enzymes were also found in several beetles species as *Ceutorrynchus assimilis* or *Tenebrio molitor* (Kostaropoulos and Papadopoulos, 1998). Is there identical influence of the chemical environment on both species? Whether volatile compounds as isothiocyanates induced GST from *Myzus persicae* (Egaas *et al*, 1991), what is their efficacy on the natural aphids predators ? In this study, we investigated the GST activities measurements with several concentrations of benzene substrates and reduced glutathione solutions. Each developmental stages of the ladybirds were tested. Localisation of enzymatic activities was also undertaken in adults.

MATERIAL AND METHODS

Rearing of ladybirds

Broad beans (*Vicia fabae*) were raised in 20 x 30cm tray including a mixture of perlite /vermiculite in a controlled environment room at 20±2°C temperature and 16 hours daylight photoperiod. Plants were only watered when the substrate was dry. *Acyrthosiphon pisum* (Harris) were mass reared on bean which were inoculated when they had 2-3 true leaves.
Larvae of *Adalia bipunctata* were raised in rectangular plastic boxes at 15±1°C and 16 hours daylight photoperiod. After hatching, 15 first instar larvae were placed per box. At emergence, 25 to 30 adults of ladybirds were pooled in larger plastic boxes (3,5 litres). Every other day, the ladybirds were fed with plenty of fresh aphids. Section of a stem of broad bean was put in each rearing box to keep the aphids alive as long as possible and to provide a humidity source.
Corrugated filter papers were used in plastic boxes to increase contact areas and to receive laying of eggs once at the adult stage. Every day, filter papers were removed to collect the eggs laying. The latter were placed at 15±1°C in 5 cm Petri dish to allow hatching. Plastic boxes were changed every week.

Chemicals

1-Chloro-2,4-dichloro-4-nitrobenzene (CDNB), 2,4-dinitro-1-iodobenzene (DNIB), 4-nitrobenzoyl chloride (NBC) and 1,2-dichloro-4-nitrobenzene (DCNB) were prepared in ethanol at 200 mM solutions. Reducted Glutathione (GSH)

was used as distilled water solution at 100 mM concentration. All products were purchased commercially from Fluka Chemical or Vel company.

Determination of Glutathione S-transferases activity

Surviving individuals of *Adalia bipunctata* were crushed in 1,5 ml eppendorf tube containing 600 µl of phosphate buffer (pH= 7). Homogenates centrifugation were carried out in a Rotor Sigma 2K15 for 15 minutes at 4°C and 15000 g. Fifty microlitres of supernatant were added to 930 µl of phosphate buffer, 10 µl of GSH solution and 10 µl of benzene substrate. Control were composed of 950 µl of buffer and 50 µl of supernatant. The protein concentration of homogenates was determined by the method of Lowry. Serial dilutions of bovine albumin were used for the construction of a standard curvethat provided the extinction coefficient. A thermostated Shimadzu UV-160A spectropho-tometer was used for enzyme activity (at 340 nm) and protein concentration (at 750 nm) measurements.

RESULTS

Benzene substrates selection

Between the four benzene substrates which were tested, only two gave significant absorbances for the spectrophotometric measurements (Figure 1). The continuation of the experiment was made using only DNIB and CDNB.

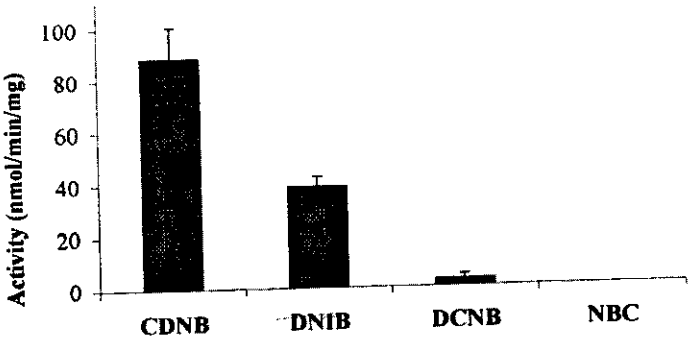

Figure 1: Substrates selection on *Adalia bipunctata* adults

Developmental expression of GST

First, each stage of the ladybirds were used to determine the evolution of the enzymatic activity. All the larval stages corresponded to quite low GST activities while adults displayed much higher values (Figure 2). This result confirmed the choice of the adult stage to study the localisation of GST in insect body.

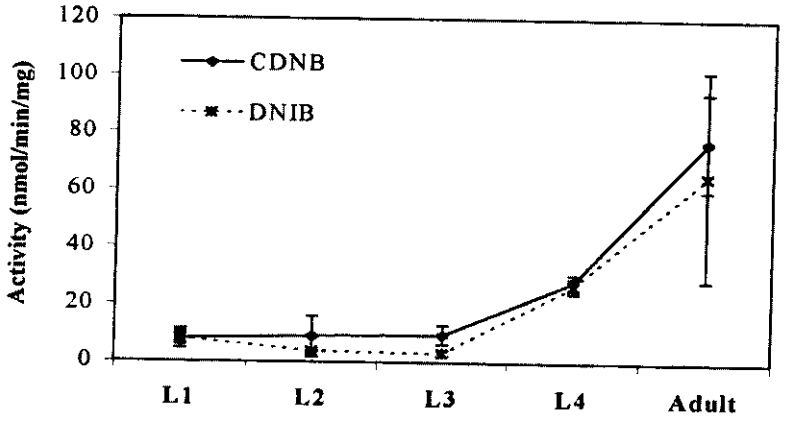

Figure 2: Evolution of Glutathione S-transferases activity following the developmental stages of *A. bipunctata*

Optimisation of substrates concentrations

After having determined the number of larvae to use for GST activity measurements at each developmental stage, different concentrations of GSH and benzene substrates were tested to lead to an optimisation of classical methods. Results are presented in Figure 3 and 4.

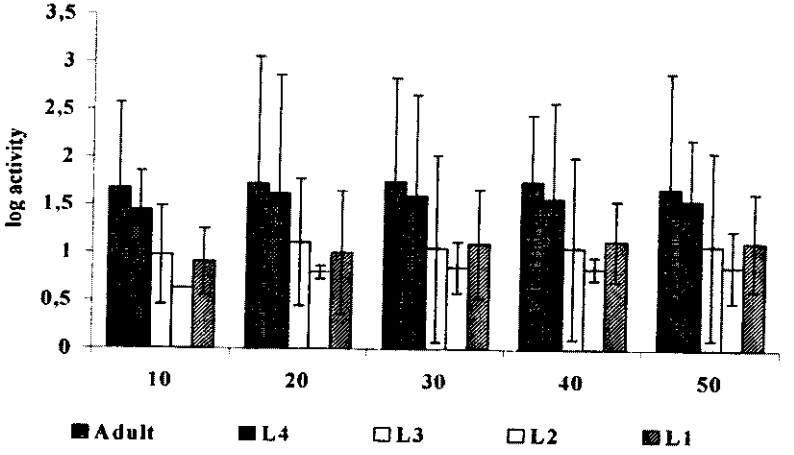

Figure 3: Glutathione S-transferases expression at each developmental stage following CDNB variations. Numbers 10 to 50 corresponded to CDNB volumes (in μl)

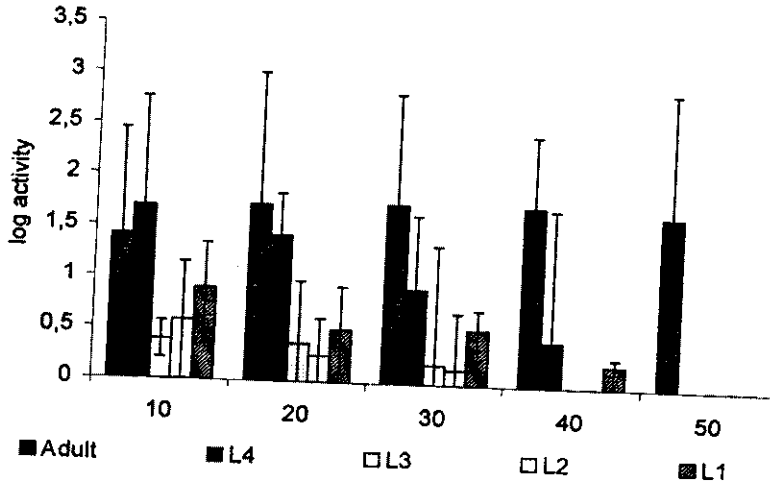

Figure 4: Glutathione S-transferases expression at each developmental stage following DNIB variations. Numbers 10 to 50 corresponded to DNIB volumes (in μl)

Glutathione S-transferases distribution

At the adult stage, individuals were cut in three parts corresponding to the head, thorax and abdomen. Before dissection, the elytra were removed and also used separately in enzymatic activity measurements. Several concentrations of both substrates and reduced glutathione were utilised for each insect part (see Table 1).

Table 1: Glutathione S-transferases distribution (in %) in *Adalia bipunctata*

Substrates variations (µl)	Body parts			
	Head	*Thorax*	*Abdomen*	*Elytra*
CDNB				
10	28 *a*	19 *c*	50 *feg*	3 *b*
20	23 *ac*	16 *c*	40 *deg*	21 *c*
30	27 *a*	13 *c*	43 *deg*	17 *c*
DNIB				
10	23 *ac*	21 *c*	34 *d*	22 *c*
20	0 *b*	37 *de*	63 *f*	0 *b*
30	0 *b*	47 *deg*	53 *fg*	0 *b*

Significant differences are marked with different letters. Percentages were analysed by equality tests of proportions using the angular transformation method at $\alpha = 0.05$.

DISCUSSION - CONCLUSION

Substrates selection and developmental stage

Most of the GST studies in insects used CDNB or DCNB as benzene substrates (Franciosa and Bergé, 1995). In this study, DCNB gave no significant activities but DNIB corresponded to higher activities at all ladybirds stages and was quite original when reported to classical method. Moreover, while higher concentrations of DNIB displayed inhibition effects on larval stages, elevated GST activities were constantly observed at adult stage. Dierieckx (1987) and Shiotsuki et *al* (1990) already reported GST inhibitions respectively to 2,4-dichlorophenoxyacetic acid, 1,4-benzoquinone and saligenin cyclic phosphate. The differential distribution of the GST activities when DNIB or CDNB were used could suggest that the ladybird *Adalia bipunctata* possess several GST isozymes. Their expression would also depend on the developmental age.
As in housefly (Saleh *et al*, 1978), the GST increase occured through the larval, pupal and adult stages. While the amount of GST increased slowly from a 5 day-old to a 9 day-old larvae, the enzymatic activity augmented following a two to three times factor from larvae to adult stage.

Glutathione S-transferases distribution

While some variations were underlined following the substrate nature, no relevant difference was seen in the GSH concentrations range we used ($P>0,05$). The enzymatic activity was predominantly present in the abdomen with 22 to 63 % of the total activity. This results corresponded to other GST localisation in insects. Indeed, in *Shistocerca gregaria*, most of the GST activity was found in the boby fats, the Malpighi and digestive tubes. The lower rate of enzymatic activities were detected in muscles. Malpighi tubes seem to play a role in xenobiotics metabolism in insects (Konno and Shishido, 1992).
There was a high difference in the GST distribution in the various body regions. Beside abdominal presence, GST activity was found in the head (21 to 33%) and in thorax (13 to 21 %). In housefly, approximately 60-65 % of the glutathione S-transferases were found in the abdomen. GST activity was also found in head, which contains a larger proportion of nervous tissue than does the thorax and abdomen (Saleh *et al*, 1978). In *Musca domestica*, 80-90 % of the GST activity were found in thorax, about 10 % in the head and the remaining in abdomen (Franciosa and Bergé, 1994).
It would be interesting to use the insecticides resistance model, which is due in some species to increasing amounts of GST. Both differenciation of localisation and determination of catabolic capabilities of each form would be considered regarding to the tolerated insecticides. Indeed, this could lead to the understanding of such enzymes distribution by selective strenghts in evolution.

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