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Glutathione S-Transferase Isoenzymes in the Two-Spot Ladybird, *Adalia bipunctata* (Coleoptera: Coccinellidae)

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Isoenzymes of glutathione S-transferase (GST) in adult *Adalia bipunctata*, an aphidophagous predator, were studied. Cytosolic GST activity was studied in each beetle developmental stage. The highest activities towards both 1-chloro-2,4-dinitrobenzene (CDNB) and 2,4-dinitro-1-iodobenzene (DNIB) occurred in adults. The enzyme distribution was investigated in adults. While most of the enzymatic activity was found in the abdomen (40–50 and 34–63% respectively) using several concentrations of both CDNB and DNIB, significant differences were observed for the head and the thorax depending on the substrate. Activities were more abundant in the thorax with DNIB (37–47%) compared to the 13–19% obtained with CDNB. Some GST activity was also detected in the elytra. GSTs were purified by epoxy-activated Sepharose 6B affinity chromatography and applied to an HPLC column to determine the native molecular weight (69 kDa). Three isoenzymes were separated by chromatofocusing at pH ranges 7–4. Three bands with molecular mass from 23 to 26 kDa were visualised on SDS-PAGE. Their isoelectric points were 6.66, 6.36, and 6.21. The substrate specificities and the kinetic parameters (Vm and Km) of the isoenzymes showed large differences depending on the isoenzyme. Arch. Insect Biochem. Physiol. 49:158–166, 2002.

Keywords: affinity chromatography; chromatofocusing; glutathione S-transferases; ladybird; isoenzymes; *Adalia bipunctata*

INTRODUCTION

Insects developed adaptations to protect themselves against potentially toxic compounds such as pesticides. Several defensive or xenobiotic metabolizing enzyme systems can be induced to overcome the toxicity of these chemicals. The main pathways of enzymatic detoxification in animals are the so-called phase I and phase II reactions leading to compounds with higher hydrophilicity. Glutathione S-transferases (GST; EC 2.5.1.18) are phase II enzymes that play an important role in xenobiotic detoxification. They often catalyze the conjugation of electrophilic molecules with reduced glutathione (GSH) (Boyland and Chasseaud, 1969). This family of enzymes can be induced by the administration of various xenobiotics to the animal (Pickett and Lu, 1989) and may confer resistances to numerous toxicants when their activities are increased, including alkylating agents, herbicides, and insecticides (Clark, 1990).

Glutathione S-transferases are involved in the metabolism of toxic allelochemicals from plants in phytophagous insects (Yu, 1989; Bogaards et al., 1990; Egaas et al., 1991). Feeding behavior, in terms of host plant specificity, was related to GST diversity and quantity (Yu, 1989). GST induction by allelochemicals results in altered expression patterns of isoenzyme(s), leading to the selective induction of one or some isozyme(s) in insects (Yu, 1999). The activity ratio toward different substrates (such as 1-chloro-2,4-dinitrobenzene, CDNB, and 1,2-dichloro-4-nitrobenzene, DCNB) was used in some Lepidoptera insects to suggest that GSTs differed between species (Yu, 1987). Most of the works on insect GST was performed on moth, mos-

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quito, and fly species (Yu and Hsu, 1993; Hemingway et al., 1985; Fournier et al., 1992). Only a few GSTs from Coleoptera, such as those from *Tenebrio molitor*, have been studied (Kostaropoulos et al., 1996; Kostaropoulos and Papadopoulos, 1998).

In the present work, we report on the developmental expression and the distribution of the total GST activity in *Adalia bipunctata*, a common aphidophagous ladybird. We studied the specific activity of the enzyme against three benzene substrates. Affinity chromatography followed by HPLC analysis was performed to purify and determine the molecular mass of the ladybird GST. Chromatofocusing and electrophoresis were used for further purification and characterization of GST isoenzymes. Kinetic parameters were measured with a broad range of benzene substrates and GSH concentrations to characterize each of the separated GST isoenzymes. The GST enzyme composition of the two-spot ladybird is discussed as a potential biochemical adaptation of insect to host plant allelochemicals in a tritrophic interaction way.

MATERIALS AND METHODS

Chemicals

GSH was obtained from Janssen Chimica (Beerse, Belgium). Epoxy-activated Sepharose 6B, Polybuffer 74, and PD10 column containing 10 ml Sephadex G-25 medium came from Pharmacia (Uppsala, Sweden). Other chemicals including CDNB, DNIB, and DCNB were purchased commercially from Fluka or Merck.

Plant and Insect Rearing

Broad beans (*Vicia faba* L.) were grown in plastic pots, containing a 1:1 mixture of perlite/vermiculite, in a controlled environment room at 20 ± 2°C temperature with a 16-h photoperiod. Two-spot ladybirds, *Adalia bipunctata* L., and the aphid prey, *Acyrthosiphon pisum* Harris, were collected in Gembloux, and reared in the laboratory for several years. Aphids were mass reared on beans before being fed to the two-spot ladybirds.

Purification of Enzyme

Whole ladybirds (150 individuals) were homogenized in a blender in 3 times their volume of 22 mM sodium phosphate buffer, pH 7.0. The homogenate was ultracentrifuged (1 h at 100,000*g*) and the fat-free supernatant was applied to a PD10 column (Pharmacia) before affinity chromatography. The affinity column was packed with epoxy-activated Sepharose 6B that had been reacted with GSH as described by Simons and Vander jagt (1977). The column was eluted with 22 mM sodium phosphate, pH 7.0, until no further protein was detected by monitoring the absorbance at 280 nm. GSTs were then eluted with 5 mM GSH in 50 mM Tris-HCl buffer, pH 9.6. The fractions with GST activity were pooled, concentrated to 2.5 ml by ultrafiltration on an YM-10 membrane, equilibrated with 25 mM imidazole-HCl buffer, pH 7.4, on a PD10 column and applied to a Polybuffer exchanger 94 column (1.0 × 35 cm), equilibrated with 25 mM imidazole-HCl buffer, pH 7.4. Elution was carried out with Polybuffer 74, diluted 1/16, and adjusted to pH 4.0 with HCl. Fractions of 3 ml were collected and the pH was followed with a Pharmacia monitor.

HPLC (Hewlett Packard, 1050 model) was used to determine the native molecular weight of the GSTs purified by affinity chromatography. The sample was applied on two assembled Macrosphere (GPC 300A, 7 μ) columns, calibrated with a molecular mass standard (Biorad).

Enzyme Assays and Protein Determination

Glutathione S-transferase activity was determined according to Habig et al. (1974) in a 100 mM Sorensen phosphate buffer, pH 6.5 containing 0.25% ethanol. Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. During the purification steps, GST activity was measured with CDNB as the second substrate. A Shimadzu UV-160A spectrophotometer was used for protein and enzymatic measurements.

Enzyme Kinetics

Enzyme kinetics of purified GSTs from *A. bipunctata* were determined by recording the activity toward several concentrations (ranging from 0.5 to 0.05 mM of DNIB, CDNB, or DCNB and 1.0–0.1 mM concentrations of GSH. Maximal velocity (V_m) and Michaelis constant (K_m) values for each substrate were calculated from Lineweaver-Burk plots.

Denaturing Polyacrylamide Gel Electrophoresis (SDS/PAGE)

For analytical SDS/PAGE, samples were diluted 1:4 with a solubilizer (1% SDS; 0.02% bromophenol; 1% β-mercaptoethanol in running buffer) and boiled for 3 min before electrophoresis. Separation gels were 10% acrylamide/0.01% SDS in 0.5M Tris-HCl pH 8.8. Stacking gels were 3.5% of acrylamide in 1.5M Tris-HCl pH 6.8. The Laemmli (1970) discontinuous buffer system was used; the 10× running buffer contained 2M-glycine and 0.1% SDS in 0.4M Tris, pH 8.3. Electrophoresis was carried out overnight at 45 V and 20 mA in a Hoeffer vertical electrophoresis apparatus. Gels were stained with Coomassie Brilliant Blue.

RESULTS

The GST activities with the three substrates CDNB, DCNB, and DNIB were determined at each developmental stage. Very low activities were observed with DCNB, commonly used for insect GST measurements but higher levels of GST were found with DNIB and CDNB. The two latter substrates were used for further measurements. The profiles of GST activity following the developmental stages were similar for the two substrates. Activity remained quite low during the larval stages, increased at the pupal stage, and reached a maximum in adults (Fig. 1). With CDNB, twofold higher GST activities were found in larvae but much lower differences were observed with pupae and adults. The adult stage was, therefore, chosen to study the localization of GSTs within the insect body.

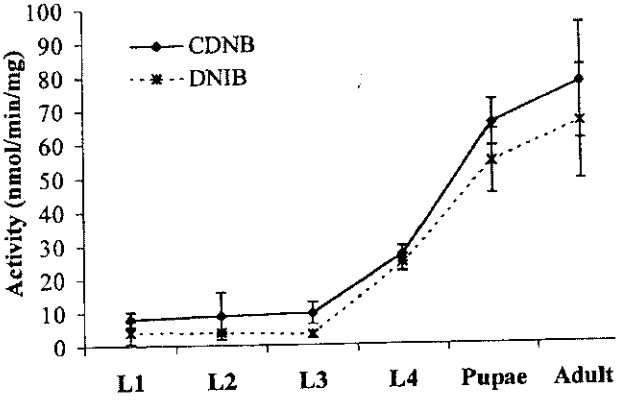

Fig. 1. Specific activity of cytosolic glutathione S-transferases from *Adalia bipunctata* toward CDNB and DNIB. Whole body homogenates were used for each developmental stage. Error bars represent the standard deviation of the mean (n = 10).

At the adult stage, individuals were cut into three parts corresponding to the head, thorax, and abdomen. The elytra were removed before dissection and were used for separate enzymatic activity measurements. Several concentrations of both substrates and GSH were utilised for each insect part (Table 1). Most of the GST activity was located in the abdomen with several concentrations of both CDNB and DNIB substrates (40–50 and 34–63% respectively). Actual values corresponding to 1% of GST activity in Table 1 were from 0.83 to 1.37 and 0.45 to 0.06 nmol/min.mg for the 0.2–0.6 mM of CDNB and DNIB concentra-

TABLE 1. Localisation of Glutathione S-Transferase Activity (in %) in *Adalia bipunctata**

Substrate concentrations (mM)	Body parts			
	Head	Thorax	Abdomen	Elytra
CDNB				
0.2	28 ± 6^{a}	19 ± 3^{c}	$50 \pm 8^{f,g}$	3 ± 0^{b}
0.4	$23 \pm 5^{a,c}$	$16 \pm 4^{a,c}$	$40 \pm 10^{d,e,g}$	$21 \pm 5^{a,c}$
0.6	27 ± 8^{a}	13 ± 3^{a}	$43 \pm 7^{d,e,g}$	17 ± 3^{a}
DNIB				
0.2	$23 \pm 7^{a,c}$	21 ± 4^{c}	34 ± 6^{d}	22 ± 2^{c}
0.4	0 ± 0^{b}	$37 \pm 9^{d,e}$	63 ± 13^{f}	0 ± 0^{b}
0.6	0 ± 0^{b}	$47 \pm 11^{d,e,g}$	$53 \pm 9^{f,g}$	0 ± 0^{b}

*Enzymatic measurements were performed with a 1 mM GSH constant concentration. Significant differences marked with different letters. Percentages were analyzed by equality tests of proportions using the angular transformation method at $\alpha = 0.05$.

tions, respectively. Significant levels of GST activity were observed in the head and the thorax depending on the benzene substrate. Higher activities were found in the thorax with DNIB (37–47%) compared to 13–19% obtained with CDNB. Around 20% of total GST activity was detected in the elytra with 0.4 and 0.6 mM CDNB and with 0.2 mM DNIB. Increasing the latter substrate concentration inhibited GST activity.

GSTs from *A. bipunctata* adults were purified by affinity chromatography on a Sepharose column. The elution profile of the enzyme is shown in Figure 2 and the purification protocol is presented in Table 2. More than 77% of the total GST activity toward CDNB was bound to the column. The native molecular mass of *A. bipunctata* GSTs was estimated by HPLC to be 69 kDa (Fig. 3). Fractions with purified GSTs were pooled and chromatofocused at pH range 7–4. Three isoenzymes were recovered and called C_1, C_2, and C_3 in function of their decreasing pI (Fig. 4). These results show the existence of multiple GST isoenzymes in the cytosolic extract from the two-spot ladybird. An additional small peak of GST activity was not bound to the column. No further GST activity was eluted when rinsing the column with 1M NaCl after chromatofocusing. The activity of the isoenzymes isolated from the chromatofocusing were with CDNB and DNIB. Kinetic parameters were determined for each isoenzyme towards a broad range (0.05 to 0.5 mM) of benzene substrate (CDNB or DNIB) and GSH concentrations (Table 3). Specific activity of C_1, C_2, and C_3 using 1 mM of both GSH and CDNB was 1.78, 1.65, and 1.83 µmol/min.mg, respectively. SDS-PAGE using the affinity bound GSTs revealed the presence of three bands of 23–26 kDa molecular mass, respectively (see Fig. 5).

DISCUSSION

Total GST activity towards two substrates was studied at each developmental stage. The highest amounts of GST activity were found in pupae and adults of *A. bipunctata*. Some insect species, such

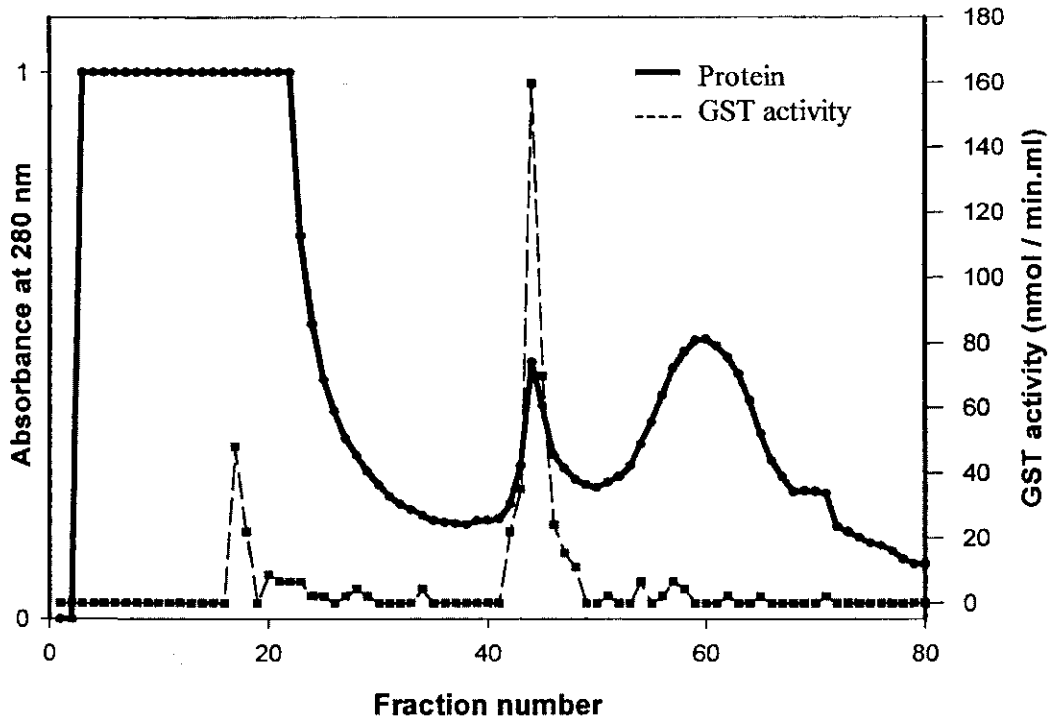

Fig. 2. Elution profile of *Adalia bipunctata* glutathione S-transferases from an epoxy-activated Sepharose 6B affinity column.

TABLE 2. Purification of Glutathione S-Transferases From *Adalia bipunctata*

	Protein (mg/ml)	Activity (µmol/min/ml)	Specific activity (µmol/min/mg)	% Recovery	Fold purification
Cytosolic fraction	3.94	0.368	0.093	100	1
After PD10	2.59	0.679	0.261	98.5	2.79
Affinity chrom. bound fraction	0.91	0.960	1.053	77.3	11.25

as *Musca domestica*, did not show increases of enzyme activity from larval to pupal stage (Saleh et al., 1978) but other studies on Coleoptera were in accordance with the results of this work. *Tenebrio molitor* had higher GST activities in adult than pupae, while larvae only showed low GST activities (Kostaropoulos et al., 1995). Gillott (1980) suggested that the increasing amounts of GST activity

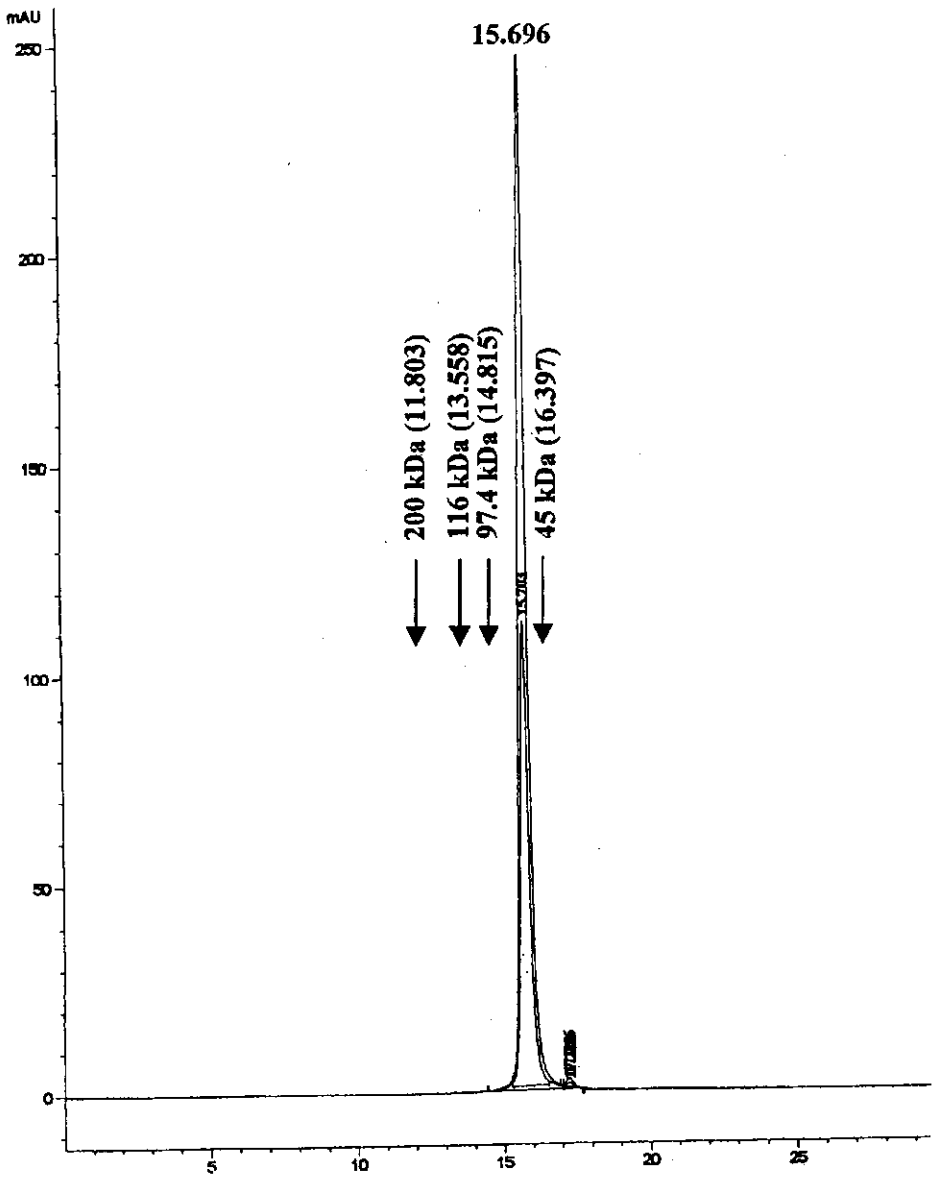

Fig. 3. HPLC chromatogram of affinity purified glutathione S-transferases from *Adalia bipunctata*. The elution of the standard proteins is given by arrows followed by their molecular weights and retention times (in brackets), respectively.

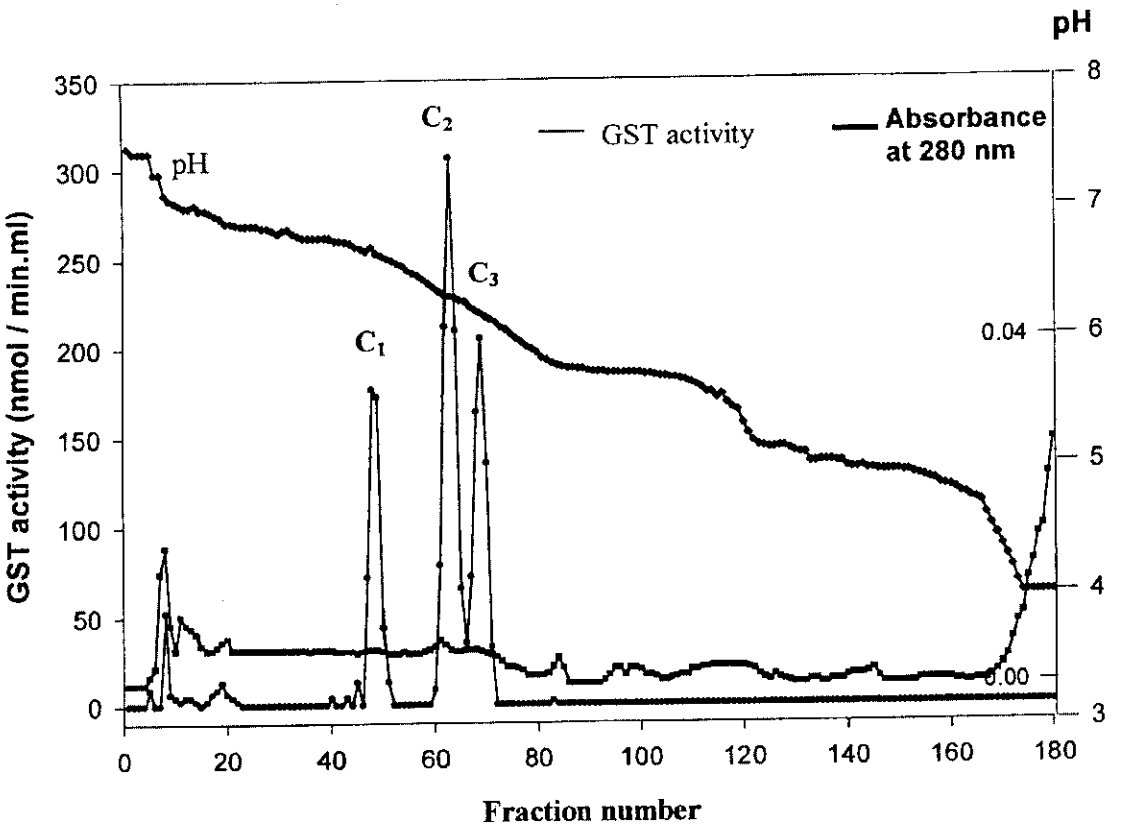

Fig. 4. Elution profile of glutathione S-transferase isoenzymes from *Adalia bipunctata* by chromatofocusing at a pH range 7–4 using the affinity bound fraction.

at the pupal stage afforded protection against potential environmental xenobiotics related to the vulnerable immobility of this stage. Indeed, pupae are not able to move and avoid toxicants such as pesticide application. Moreover, the construction of adult tissues and the elevated biosynthesis in the pupae are involved in the higher ability of the insect to detoxify potential toxic compounds (Doctor and Fristrom, 1985).

TABLE 3. Some Properties of the GST Isoenzymes Purified From the Two Spot Ladybird, *Adalia bipunctata**

Property	Isoenzymes		
	C1	C2	C3
pI	6.66	6.36	6.21
K_m towards CDNB (mM)	0.154	2.550	1.556
V_{max} towards CDNB (µmol/min/mg protein)	0.285	0.841	0.249
K_m towards DNIB (mM)	—	—	0.682
V_{max} towards DNIB (µmol/min/mg protein)	—	—	0.026
K_m towards GSH (mM)	0.269	0.338	0.471
V_{max} towards GSH (µmol/min/mg protein)	0.413	0.051	0.005

*V_{max} and K_m were measured at a fixed concentration of 1 mM of GSH or benzene substrate.

Large differences were observed in the distribution of GST activity in the various body segments of the ladybird. Most of the GST activity occurred in the abdomen. In *M. domestica*, more than 60% of total GST activity was detected in the abdomen. High levels of GST were found in fat body and guts in various insect species including house fly (Konno and Shishido, 1992). The head of the two-spot ladybird also showed high GST activity toward CDNB while the elytra also had GST activity. More than a protection against physical stresses, this body part can also constitute an efficient barrier of chemical compound detoxication. The thorax

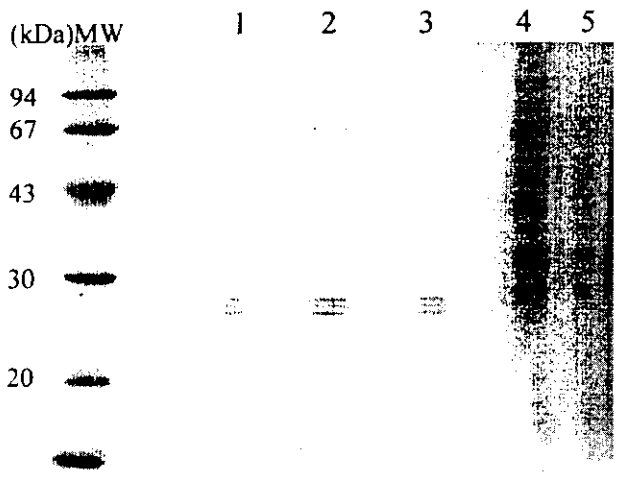

Fig. 5. SDS-PAGE of the bound glutathione S-transferase fraction from adults of *Adalia bipunctata*, obtained after affinity chromatography: Molecular weight markers (MW), three replicates of GST samples (**lanes 1–3**), cytosolic fraction after ultracentrifugation (**lane 4**), and after elution on a PD10 column (**lane 5**).

also contained GST enzymes but with lower activities than in the head when CDNB was used as the substrate. Changes of GST distribution were observed when the latter was replaced by DNIB. Indeed, higher activities were found in the thorax while no activity was observed in the head. The differences in substrate specificity suggest that at least two GST isoenzymes are present in *A. bipunctata*. Moreover, changes of GST response in relation to the benzene substrate depending on the body part also suggested that the isoenzymes are differently distributed in the beetle body. The heterogeneity of substrate specificity between GST isoforms has significant physiologic and pathophysiologic importance in the detoxication of endogenous and exogenous compounds (Beckett and Hayes, 1993).

To identify the number of isoenzymes, adults of *A. bipunctata* were used to purify the GST by affinity chromatography and subsequently by chromatofocusing. Using an epoxy-activated Sepharose 6B column, more than 77% of the GST activity toward CDNB was found in the bound fraction. The purification yield was quite high compared to the purification of GST enzyme from other insect species (from 3 to 26% in several lepidopterous species (Yu, 1989); 34% in *Tenebrio molitor* L. (Kostaropoulos and Papadopoulos, 1998). An 11.25-fold-purification was obtained by affinity chromatography. Although this is low, it agrees with other purification procedures in insects. GST from *Trichoplusia ni* (Hübner), *Spodoptera frugiperda* (Smith), and *T. molitor* were purified with a 9–15-fold factor (Yu, 1989; Kostaropoulos et al., 1996). After the first step, the GST affinity bound fraction was studied by SDS-PAGE and three bands of 26–29 kDa were observed on the gel. Yu (1989) demonstrated that a two-step procedure (ammonium sulfate fractionation followed by affinity chromatography) already gave purified glutathione S-transferases with apparent homogeneity. Multiple glutathione S-transferases have been reported in cockroach (Usui et al., 1977), house flies (Clark et al., 1984; Fournier et al., 1992), and mosquito species (Prapanthadara et al., 2000). The subunit molecular weights of GST found in five phytophagous Lepidoptera species were 27.5 to 32 kDa (Yu, 1989). As in *A. bipunctata*, *Heliothis virescens* (Fabricius) was shown to possess three subunits on SDS-PAGE. Glutathione S-transferases are known to exist in dimers (homodimers and heterodimers) (Grant and Matsumura, 1988). The molecular weight of the native enzyme was estimated to be about 69 kDa by HPLC on a Macrosphere column. This result was a bit higher than in previous works that estimated the size of the insect enzyme at 50–60 kDa by gel filtration and electrophoresis (Fournier et al., 1992).

The specific activities of the chromatofocused GST isoenzymes, namely C_1 to C_3, were similar (from 1.650 to 1.835 µmol/min.mg) and corresponded to a 17.7–19.7 purification factor. The kinetic parameters were calculated for the three isolated GST isoenzymes. Very large differences of Km toward CDNB were observed depending on the isoenzyme. The Km for C_1 was lower than that for the two other forms (10 and 16 times compared to C_2 and C_3, respectively). Low variations were only found when the Km values toward GSH of each isoenzyme were compared (1.75-fold higher with C_3 compared to C_1). The variations of the cata-

lytic efficiency for glutathione conjugation with CDNB were more than 3-fold between GST isoenzymes from *Anopheles dirus* (Prapanthadara et al., 2000). With *T. molitor*, the Km toward GSH and CDNB increased with a factor of 1 to 5 depending on one of the four isolated isoenzymes (Kostaropoulos and Papadopoulos, 1998). In regard to the Vmax values of *A. bipunctata* GSTs, one form differed from the others with either CDNB or GSH as the variable substrate. While the Vmax toward CDNB for C_2 was 4-fold higher than those for of both C_1 and C_3, the Vmax toward GSH of C_1 was 8.1- and 81-fold higher than those for C_2 and C_3, respectively. CDNB conjugating activity of one of the *T. molitor* isoenzymes was 15-fold higher than some other isoenzymes (Kostaropoulos and Papadopoulos, 1998). Kinetic parameters for DNIB were only calculated for C3 as DNIB was not recognized as substrate nor inhibited the activity of C_1 and C_2 isoenzymes. The observation of this different substrate specificity must be coupled to our results on GST distribution in the ladybird body parts. Observed differences in GSTs showed that enzyme expression can be influenced by environmental factors such that qualitatively distinct forms can be selected at the genetic level and differentially expressed (Prapanthadara et al., 2000).

Several xenobiotics, such as insecticides, herbicides, or allelochemicals from different crop plants, are inducers of xenobiotic metabolizing enzymes in insects (Yu, 1982; Clark, 1989; Dauterman, 1989). The GST system is affected by secondary substances (glucosinolates and isothiocyanates) from Brassicaceae plant species (Egaas et al., 1991; Wadleigh and Yu, 1988). Francis et al. (1999) demonstrated that the total GST activity of the aphidophagous *A. bipunctata* was induced when the ladybirds were exposed to isothiocyanates. Feeding the aphid predators with prey reared on Brassica plant induced GST levels in *A. bipunctata* (Francis, 1999). Glucosinolates and isothiocyanates were found in the aphid prey when *Myzus persicae* Sultzer and *Brevicoryne brassicae* L. were analysed by HPLC and GC-MS, respectively (Francis et al., 2001). Induction of the GST system may be an adaptation of the two spot-ladybird to the presence of plant secondary substances in their prey. The responses of insect detoxication enzymes, such as the GST system, to xenobiotic compounds must be further investigated in order to understand the chemical ecology of the plant-insect interactions in such multitrophic models.

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