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Original Article

A novel function of housefly glutathione *S*-transferase 6B—Its effect on the retention and increase of insecticidal activity of the insecticide prothiofos

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Housefly glutathione *S*-transferases 1, 3, 4, 6A and 6B were obtained from organophosphorus (OP)-resistant Yachiyo and susceptible Takatsuki strains, respectively. Over 90% homology was found between isozymes 6A and 6B but their functions differed in desethylation metabolism. Yachiyo-6A produced more desethyl product of diazinon oxon than Takatsuki-6A, thus suggesting that it plays a central role in the development of *O*-alkyl phosphate-type OP resistance. On the other hand, unlike Takatsuki-6B, Yachiyo-6B barely achieved the desethylation of prothiofos oxon and the resultant effect was more in the direction of resistance suppression. However, the chief role played by 6B with regard to prothiofos was the exclusive desethylation of *S*-oxide, which is the oxidative product of prothiofos oxon, thus giving desethyl *S*-oxide with insecticidal activity and bringing forth a novel active intermediate along with *S*-oxide.

Keywords: housefly glutathione *S*-transferase, GST6A and 6B, resistance suppression, oxidative glutathione conjugation of prothiofos oxon, desethyl *S*-oxide of prothiofos oxon.

Introduction

Glutathione *S*-transferase (GST) conjugates many chemicals to glutathione (GSH) and is generally an enzyme in detoxification metabolism. In a living organism, increase of GST and its activity is, in many cases, one of the causes of the development of resistance to chemicals. In the case of organophosphorus (OP) insecticide, most of them are exposed to desalkylation and desarylation by GST possessed by OP-resistant insects, and their insecticidal activities are lost. However, prothiofos-like *S*-propyl phosphorothiolate-type OP insecticides maintain high insecticidal activity against resistant insects. This paper is concerned with the elucidation of this mechanism. In general, thiono-type OP insecticides are oxidatively converted into their oxon to inhibit acetylcholinesterase (AChE), thus showing insecticidal activity, but certain oxons, *i.e.*, phosphorothiolates such as metamidofos, profenofos, and pyraclofos and prothiofos oxon, are weak AChE inhibitors and their *S*-oxide is an activated product which gives insecticidal activity. We[1] indicated that the active intermediate prothiofos oxon *S*-oxide (Fig. 1) not only inhibits AChE but is also further activated by GST, showing high insecticidal activity to highly resistant insects; however, we could not identify the type of reaction and the activated product. In the previous study,[2] to elucidate this question, two new activated products were predicted by computational chemistry which differ from the known activated *S*-oxide, and of the two, desethyl *S*-oxide was shown to possess insecticidal activity and AChE inhibitory activity. By further detecting its hydrolysate 2,4-dichlorophenyl phosphate in an *in vitro* approach, it became clear that desethyl *S*-oxide was produced from prothiofos oxon by partially purified housefly GST under oxidation (oxidative GSH conjugation), thus indicating the involvement of desethyl *S*-oxide along with *S*-oxide in the insecticidal activity of prothiofos. In this study, we cloned housefly GST isozymes, both from susceptible Takatsuki and resistant Yachiyo strains, ascertained which isozymes were involved in the desethylation and oxidative conjugation, and aimed to explain the activation mechanism of prothiofos oxon (LD_{50} by injection: 55 ng/fly for Takatsuki, 110 ng/fly for Yachiyo) from a GST standpoint.

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Materials and Methods

1. Chemicals

Figure 1 shows the chemical structures and abbreviations of prothiofos oxon, diazinon oxon and their metabolites used in this study, and their proposed pathways of metabolism by oxidation and/or GSH conjugation.

2. Cloning of housefly GST cDNA

The cDNAs that encode housefly GSTs were obtained by 3′-RACE. Total RNA was isolated using an RNeasy Plant mini kit (QIAGEN) from 100 mg of the abdomen of 3-day old houseflies after hatching. First strand cDNA was synthesized from 1 μg of the total RNA by Superscript II (Invitrogen) using a oligo-(dT) primer flanked with an adapter sequence at its 5′ end (5′-GGCCACGCGTCGACTAGTAC$(dT)_{17}$-3′) (Invitrogen). The cDNA encoding each housefly GST was then amplified by PCR using *Taq* polymerase with an adapter primer and a gene-specific primer designed from the 5′-UTR of the housefly GST gene, which was deposited in GenBank. The sequences of the gene-specific primers were as follows: 5′-AAGAATTCCAACCAAGCATTGCAAAGCC-3′ for GST1, 5′-CATCTTCCAGAACGGACAA-3′ for GST3, 5′-AGCAGACCGGCATATTTCCC-3′ for GST4, 5′-CAGAACTGCTGCAGCTAATT-3′ for GST6A and 5′-GCGTCGTGCAAAATTAATTCA-3′ for GST 6B. The amplified DNA fragment was introduced into the *Eco*RV recognition site of p-Bluescript SK(−) using a Blunting Kination Ligation kit (TAKARA Bio), and was transformed into DH5α-competent cells. The sequences of the DNA fragments inserted into the plasmid were analyzed more than two times to eliminate the possibility of mis-imcorporation during PCR.

3. Expression and purification of housefly GST isozymes

The DNA fragment with *Nde*I and *Sal*I recognition sites at the 5′ and 3′ ends, respectively, was prepared by PCR using each housefly GST cDNA described above as a template. After digestion by *Nde*I and *Sal*I, the DNA fragment was introduced into pET21a-competent cells (Novagen). The resulting plasmid was amplified in DH5α, followed by sequence analysis of the inserted DNA. The purified plasmid was then transformed into BL21(DE3)pLys*S*-competent cells (Novagen). LB broth (100 ml) containing 50 μg/ml of ampicillin was inoculated with a single colony of *E. coli* harboring the housefly GST gene and was incubated at 37°C and 150 rpm until the OD_{600} of the medium reached about 0.5. Protein expression was induced by adding isopropyl β-thiogalactopyranoside at a final concentration of 1 mM, and then further incubated at 37°C and 150 rpm for 3 hr. The cells were recovered by centrifugation and resuspended in 5 ml of 10 mM Tris–HCl (pH 7.4). The cells were disrupted by sonication on ice (20 sec, 100 W×5), and the resulting cell lysate was centrifuged at 15,000×*g* for 15 min to obtain the supernatant, *i.e.*, *E. coli* lysate. This was subjected to affinity chromatography on GSTrap FF column (5 ml, Amersham Biosciences) equilibrated with 50 mM Tris–HCl (pH 7.4). The GST was eluted by the same buffer containing 10 mM of GSH. The protein concentration was measured according to the method of Bradford[3] using bovine serum IgG as a standard.

4. Conjugation activity of housefly GST isozymes to CDNB and DCNB

In this trial, 0.8 ml of 100 mM phosphate buffer (pH 6.5) containing 1.25 mM GSH and 0.1 ml of purified isozyme solution diluted to an appropriate concentration with 10 mM phosphate buffer (pH 6.5) were poured into a square cell (1 cm×1 cm). After preincubating for 1 min at 25°C in a U-3200 HITACHI UV spectrophotometer with a rate assay system, 0.1 ml of 60% aqueous ethanol solution of 10 mM CDNB (1-chloro-2,4-dinitrobenzene) was added and stirred for 10 sec. At a wavelength of 340 nm, the rate of GSH conjugation of CDNB was measured for 3 min at intervals of 0.3 min from the changing absorbance, and the specific activity of the isozyme was determined. For the non-enzymatic reaction, 100 mM phosphate buffer was added instead of enzyme solution. The specific activity of DCNB (3,4-dichloronitrobenzene) was determined likewise except for the following conditions: GSH, 6.25 mM; DCNB, 50 mM; pH, 7.5; measured wavelength, 345 nm.

5. Kinetic parameters of GSH conjugation of CDNB and DCNB by housefly GST 6A and 6B

Following the rate assay system described above, CDNB (for 6A, 10, 15, 25, 39, 60, 100 μM; for 6B, 50, 75, 125, 195, 300, 500 μM) or DCNB (for both 6A and 6B, 1, 1.5, 2.5, 3.9, 6, 10 μM) was added to phosphate buffer solution of 5-fold diluted purified isozymes 6A and 6B containing GSH to measure the rate of GSH conjugation of CDNB and DCNB. Specific activity was plotted against the substrate concentration by Sigma Prot (Ver.9) and from the recurrent curve were determined the K_m and V_{max} for CDNB and DCNB of isozymes 6A and 6B.

6. Quantitative PCR

Each GST mRNA expressed in the housefly abdomen was quantified by two-step real-time PCR. A 1 μg of total RNA underwent reverse transcription by Superscript II with 1 μl of random primer (100 ng/μl, Invitrogen) in a final reaction volume of 20 μl. The following PCR was performed on LightCycler (Roche) using a LightCycler FastStart DNA Master SYBR Green kit (Roche) according to the manufacturer's instructions. The annealing temperature of each reaction was 64°C, and the concentration of $MgCl_2$ was adjusted to 1.6 mM. The sequences of the primers were as follows: GST1: 5′-ACCTTGGTCCCCCCCCCGATGGCGATTTCGCCT-3′, 5′-ACTGGCCAACAAAGCCAAATCGGCAAC-3′, GST3: 5′-CTACTCTGGTTTTTTGGACAATGGTTTCGCCA-3′, 5′-CAAGATGGCCAAATCGGCCAAGGTCAT-3′,

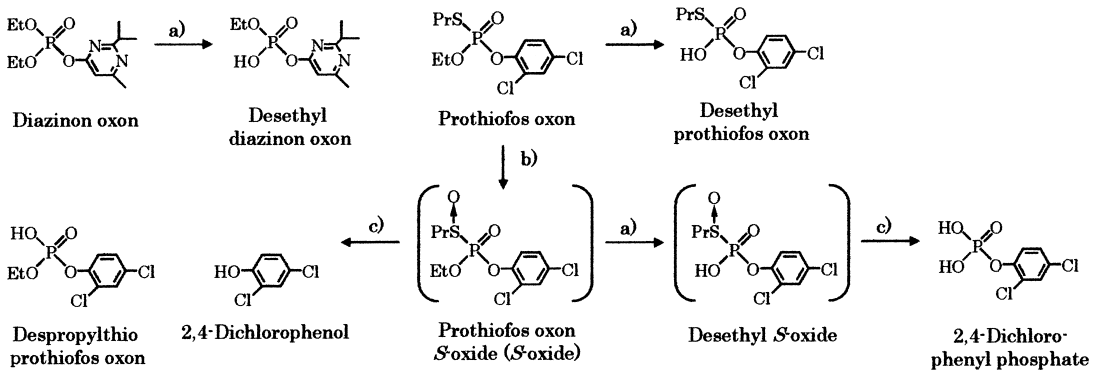

Fig. 1. Chemical structures and abbreviations of prothiofos oxon, diazinon oxon and their metabolites used in this study, and their proposed metabolism pathways by oxidation and/or GSH conjugation. a) Desethylation by GST-GSH, b) oxidation by MFO, c) hydrolysis.

GST4: 5′-ACTCTGGTGGACAATGGCTTCGCCC-3′, 5′-CTCAAAGGTACTAACCGAGGCCAAGAG-3′, GST6A: 5′-CAGTACCCACACTGGAAGATGATGGA-3′,5′-CCACCA-ATGAGGTTACCGTTGTTACG-3′, GST6B: 5′-ACATAC-AGTGCCCACACTCGAGGAG-3′, 5′-TCTTCAGCCATGC-ACTCAGCTTGGGA-3′. Each sample was quantified with respect to standard DNA (10^2–10^6 copies/reaction tube) quantified under the same conditions. The purity of the amplified DNA in every reaction was analyzed by agarose gel electrophoresis.

7. Desethylation and oxidative conjugation of the oxon form by housefly GST isozymes

In the trial, 0.5 ml of 20% rat liver microsome and 0.5 ml of *E. coli* lysate having each housefly GST isozyme (to make 1 μmol/min/ml of CDNB conjugate, dilution 6-fold for GST1, 2-fold for GST3, 1.5-fold for GST4 of both strains, 5-fold for GST6A and 6B of both strains) were added to 2.0 ml of 10 mM phosphate buffer (pH 7.4) in ice water. For the GSH-added group, 0.5 ml of 10 mM GSH aqueous solution was added. For the NADPH-added group, 0.5 ml of 25 mM $NADP^+$ in the same buffer and 0.5 ml of 50 mM glucose-6-phosphate, 5 units/ml glucose-6-phosphate dehydrogenase and 60 mM $MgCl_2$ in the same buffer were added as the NADPH-generating system. For the NADPH+GSH-added group, both the NADPH-generating system and GSH were added. Each reaction solution including a co-enzyme-free group was prepared to 4.5 ml with the same buffer, and lastly, 0.5 ml of each 30% aqueous acetone solution of 7.5×10^{-4} M of diazinon oxon or 7.5×10^{-4} M of prothiofos oxon was added to all these solutions, which were then incubated at 37°C for 1 hr (diazinon oxon was added only to the GSH-added and co-enzyme-free groups). To stop the enzyme reaction, 0.5 ml of 6 M hydrochloric acid was added to the reactant, and the denatured protein was precipitated by centrifugation at 10,000 rpm for 10 min. The supernatant was filtered using a SamplePrep-LC13R (MILLIPORE) filter (0.22 μml), extracted with ethyl acetate (5 ml×3), dried and concentrated to 1 ml, and diazinon oxon, prothiofos oxon, their desethyl forms, despropylthio prothiofos oxon, 2,4-dichlorophenol and 2,4-dichlorophenyl phosphate were determined using a Shimadzu LC10A high performance liquid chromatograph. Operating conditions: wavelength 270 nm; immobile phase, C8 column, 4.6×250 mm; mobile phase, mixture of 0.1% trifluoracetic acid (TFA) aqueous solution and methanol. The ratio of TFA/methanol in case of desethyl diazinon oxon (t_R, 15.0 min), desethyl prothiophos oxon (t_R, 20.0 min), despropylthio prothiophos oxon (t_R, 12.5 min) and 2,4-dichlorophenyl phosphate (t_R, 7.2 min) was 58 : 42. The ratio in case of 2,4-dichlorophenol (t_R, 7.0 min), non-reacted diazinon oxon and prothiophos oxon (t_Rs, 14.0 min and 21.0 min) was 37 : 64.

Results and Discussion

1. Preparation of housefly GST isozymes

For cDNA encoding housefly GST isozymes, the cloning of MdGST 1, 2, 3C, 4, 6A and 6B has been reported so far. These were isolated from the Cornell-HR strain housefly which has shown high resistance to insecticides through repeated selection caused by 2-chloro-1-(2,4,5-trichlorophenyl)vinyl dimethyl phosphate.[4–6] In this study, mRNA of the housefly of susceptible Takatsuki and resistant Yachiyo strains was prepared, and cDNA of GST1, 3, 4, 6A and 6B was obtained by RT-PCR. Table 1 shows the difference between the reduced amino acid sequences of our GSTs and Cornell-HR MdGSTs deposited in a database. Our GST1 differed from GST1 of Cornell-HR in a residue in comparison to the sequences for both strains, but no difference between Takatsuki and Yachiyo strains was observed. With GST3, three kinds of cDNA were obtained for Takatsuki and Yachiyo strains, respectively. All three sequences were confirmed in both strains and no specific sequence was found. Still, these amino acid sequences differed from Cornell-HR MdGST3C at two or three locations. With GST4, the amino acid sequence differed from Takatsuki and Yachiyo strains, *i.e.*, there was amino acid mutation at three locations. GST5 is reported

Table 1. The difference between amino acid sequences of GST isozymes from houseflies of susceptible Takatsuki and resistant Yachiyo strains, and Cornell-HR MdGSTs deposited in a database

	Cornell-HR	Takatsuki	Yachiyo
MdGST1	F173	I173	I173
MdGST3(C)	F128, Q151, A195, F208	L128, E151, A195, F208	L128, E151, A195, F208
MdGST4	K32, G37, D151, I172	N32, G37, D151, T172	N32, E37, G151, I172
MdGST6A	A204	T204	A204
MdGST6B	P70, F121, S144, N148, P196	A70, L121, A144, D148, A196	A70, F121, S144, N148, P196

GenBank accession numbers are: MdGST-1, X61302; MdGST-3C, X73575; MdGST-4, X73576; MdGST-6A, AF147205; and MdGST-6B, AF147206.

only from screening of the genome library and is not isolated as cDNA. This study was unable to obtain a GST5 clone by RT-PCR using GST5-specific primers. With GST6A and GST6B, there was a difference in the primary structure between the strains. There was amino acid mutation at one location with GST6A and four locations with GST6B. Of special interest are isozymes 6A and 6B showing over 90% homology apart from other isozymes. With GST isozymes, differences in the primary structure observed in Takatsuki and Yachiyo strains were found in GST4, 6A and 6B. In either case, such mutation was concentrated at the C-end of the GST sequence. This result coincides with the general theory concerning GST that diversification is less likely at the N-end of the GST due to functional restriction compared to the C-end.

2. *Enzyme activity in GSH conjugation of CDNB and DCNB by housefly GST isozymes*

Enzyme activity of the purified housefly GST isozymes derived from Takatsuki and Yachiyo strains expressed in *E. coli* was determined by means of one concentration per one substrate. The result is shown in Table 2. The specific activity of all isozymes with CDNB differed from each other at most 1.5–3 fold but, in all, remained on the same level. Toward DCNB, GST1, 3 and 4 showed no or little activity compared to CDNB activities, whereas GST6A showed the same level of activity with DCNB as with CDNB. The current data coincide with those of previous studies.[4,6,7] Moreover, GST6B, which had previously not been reported, also showed the same activity as GST6A. No difference between the strains was observed in the conjugation activity of GST4, 6A and 6B. A further study on the reactivity of GSH conjugation to CDNB and DCNB by GST6A and 6B of both strains was carried out by selecting six substrate concentrations to obtain K_m and V_{max} from the reaction curve. The result is shown in Table 3. V_{max} from the conjugation by both isozymes showed no difference between the strains but K_m gave an interesting result, *i.e.*, isozyme 6B of the Takatsuki strain had higher affinity than that of Yachiyo strain 6B; with CDNB and DCNB reactions by 6B, the difference was 2.4-fold and 1.4-fold, respectively. K_m from the reaction by isozyme 6A to both substrates in both strains showed no difference to V_{max}.

Table 2. GSH conjugation activity of CDNB, DCNB by GST isozymes from houseflies of susceptible Takatsuki and resistant Yachiyo strains

Isozyme	Activity (μmol/min/mg)	
	CDNB	DCNB
GST1	17.8	0.02
GST3AF[a)]	6.0	0.1
GST3DF[b)]	6.0	0.09
GST3DL[c)]	6.9	0.12
Takatsuki-GST4	11.0	0.8
Yachiyo-GST4	13.0	0.6
Takatsuki-GST6A	16.0	15.0
Yachiyo-GST6A	15.0	15.0
Takatsuki-GST6B	17.0	14.0
Yachiyo-GST6B	17.0	13.0

[a)] GST3 (L128, E151, A195, F208). [b)] GST3 (L128, E151, D195, F208). [c)] GST3 (F128, E151, D195, L208).

Table 3. Kinetic parameters in the GSH conjugation of CDNB, DCNB by GST 6A and 6B of susceptible Takatsuki and resistant Yachiyo strains

	K_m (μM)[a)]		V_{max} (μmol/min/mg)[a)]	
	Takatsuki	Yachiyo	Takatsuki	Yachiyo
GST6A				
CDNB	2.5±0.92	2.6±0.81	18.5±1.1	17.6±1.5
DCNB	392±25	367±29	21.7±1.9	20.9±2.1
GST6B				
CDNB	9.1±1.02[b)]	22±1.3	18.2±2.3	19.8±1.6
DCNB	230±21[b)]	330±32	23.1±1.7	23.2±2.2

[a)] Means±SD (n=3). [b)] K_m values of CDNB and DCNB are significantly different between two strains by t-test ($P<0.05$).

Table 4. Expression level of housefly GST isozyme genes from susceptible Takatsuki and resistant Yachiyo strains

Isozyme	Level of expression[a)]	
	Takatsuki	Yachiyo
GST1	100	100
GST3	0.6	1.9
GST4	12.9	6.7
GST6A	7.9	22.0
GST6B	1.7	4.7

[a)] The expression level of each isozyme is noted while setting the level of the GST1 gene from Takatsuki and Yachiyo as 100.

3. Expression level of housefly GST isozyme genes

Table 4 shows the level of expression of each isozyme when setting the level of the GST1 gene from Takatsuki and Yachiyo strains as 100. Comparison of the expression level of isozymes in the same strain is possible but it is not possible to compare the expression level of isozymes between the strains. The specific activity of the isozyme to CDNB was more or less the same for GST1, 6A and 6B, and was slightly smaller for GST4 but even less for GST3 (Table 2). The specific activity of the crude enzyme solution of housefly GST prepared from the abdomen of both strains was 588, 545 μmol/min/mg, showing almost the same value (no details mentioned), thus indicating that the expression level of GST1 from Takatsuki and Yachiyo strains did not differ much from the other. Therefore, using the isozyme values between the strains for comparison does not result in a huge error. MdGST1 is the foremost isozyme first isolated by screening of the cDNA expression library.[8,9)] Furthermore, when the cDNA library was screened using the MdGST1 gene as a probe, the MdGST1 clone was obtained at much higher frequency compared to MdGST3 and MdGST4.[4)] Our results coincide with this report. The expression of MdGST3 and MdGST4 is reported to be at the same level by Northern analysis,[4)] however, our study showed a higher expression of GST4 in both strains, notably in the Takatsuki strain. No explanation for this discrepancy is available at present. Because of the high degree of homology between GST3 and GST4, 85%, they may have cross-reacted even under the highly stringent conditions of hybridization by Northern analysis. The level of expression of Yachiyo strain 6A was higher than that of Takatsuki strain 6A. Of the total GST expression, Takatsuki was 6.4% and Yachiyo 16.3%. Of the gene expression of GST6B in total GST, Takatsuki was 1.4% and Yachiyo 3.5%, approximately 20% of that of 6A, respectively. The level went up with the resistant Yachiyo strain, similar to GST6A. Thus, it is highly interesting to note that GST6A and 6B, which exist at different loci from GST 1, 3 and 4, are expressed at a higher rate in the Yachiyo strain.[6)]

4. GSH conjugation of the oxon form by housefly GST isozymes

4.1. GST isozymes in desethylation

O-Alkyl phosphate-type diazinon oxon and *S*-propyl phosphothiolate-type prothiofos oxon were desethylated by housefly GST isozymes to determine the produced desethyl oxons, respectively. The result is shown in Table 5. As stated in section *3*, when the conjugation activity of CDNB by isozymes gives more or less the same value, the protein in the isozymes will be approximately the same amount. Thereby, it will be feasible to compare the reactivity of the desalkylation of an isozyme from its product between isozymes and between strains. In this study, an enzyme solution diluted for an

Table 5. *In vitro* desethylation of diazinon oxon and prothiofos oxon by the housefly GST isozymes of susceptible Takatsuki and resistant Yachiyo strains

GST isozyme	Amount of substance (%)[a)]			
	Diazinon oxon		Prothiofos oxon	
	Desethyl product	Residual SM[b)]	Desethyl product	Residual SM[b)]
GST1	3.6±1.8	76±2.3	N.D[c)]	92±3.3
GST3	4.2±1.6	70±2.5	3.7±1.5	93±3.2
Takatsuki-GST4	3.2±1.3	77±2.8	3.7±1.2	83±3.4
Yachiyo-GST4	3.8±1.3	76±3.1	3.6±1.1	84±3.1
Takatsuki-GST6A	12.8±1.5	76±2.2	7.5±1.3	84±2.9
Yachiyo-GST6A	17.3±2.1	69±3.2	8.2±1.6	83±3.6
Takatsuki-GST6B	9.8±1.7	77±3.1	10.5±1.9	84±3.1
Yachiyo-GST6B	7.5±1.4	82±2.5	5.8±1.3	87±3.5

[a)] Means±SD (n=3). [b)] SM: starting material. [c)] N.D: not determined.

isozyme to produce CDNB conjugate at a rate of 1 μmol/min/ml was used. Except for GST1, which did not carry out the desethylation of prothiofos oxon, 8 kinds of GST isozymes performed desethylation of the oxons. Still, there was a difference in the reaction, which could be divided into two groups. One group included GST1, 3 and 4. These isozymes produced only 2–3% of the desethyl product from both oxons, which is an inefficient conjugation process. In GSH-free groups, 1–2% of desethyl product was detected, but this was deemed insignificant because a small amount of GSH was already present in the *E. coli* lysate. The other group included GST6A and 6B, which, except for the desethylation of the prothiofos oxon by Yachiyo strain 6B, produced the desethyl product of the oxons in a large amount. With 6A, the resistant-strain isozyme tended to produce more desethyl forms whereas with 6B, the susceptible-strain isozyme produced more. Significant differences between strains were seen in the diazinon oxon reaction by 6A and the prothiofos oxon reaction by 6B. In the former reaction, isozymes in both strains gave the most desethyl form in this desethylation study (13–17%) and the Yachiyo strain 6A produced 1.3 times more than Takatsuki strain 6A. As stated in Table 3, V_{max} and K_m in the GSH conjugation of CDNB and DCNB by 6A showed no difference between the strains, indicating no significant difference between the strains with regard to the function of 6A with these substrates. Considering that expression of 6A was significantly higher for the resistant strain than for the susceptible strain (Table 4), the level of activity of isozyme 6A (desethylated by 6A) is thought to be dependant on the level of expression between the strains. In the case of *O*-alkyl phosphate-type OP insecticides, this strongly indicates that GST6A plays the central role in promoting resistance by desalkylation. In the latter reaction, in contrast to the former, the desethyl product decreased significantly by Yachiyo strain 6B. The expression level of 6B was 1/5 that of 6A in either strain (Table 4). From K_m in the GSH conjugation of CDNB and DCNB by 6B, we have stated that the affinity of these substrates to the Yachiyo strain isozyme was lower than that for the Takatsuki-strain isozyme (Table 3). Therefore, if prothiofos oxon fits into the active site of Yachiyo strain 6B with difficulty compared to these substrates, desalkylation will not proceed easily. This indicates that Yachiyo strain 6B represses the desethylation of prothiofos oxon, *i.e.*, represses the detoxification in insects.

4.2. GST isozymes in oxidative GSH conjugation

Prothiofos oxon was reacted with NADPH or NADPH+GSH in an enzyme mixture of rat liver microsome and each housefly GST isozyme to identify the isozyme which acts as a mediator in oxidative conjugation. Due to the natural inhibitor[10] present in the housefly microsomal fraction which interferes with the oxidative reaction, rat liver microsome was used for oxidation. The result is shown in Table 6. When oxidation and

Table 6. *In vitro* oxidative GSH conjugation of prothiofos oxon by housefly GST isozymes of susceptible Takatsuki and resistant Yachiyo strains under rat liver microsome oxidation

Housefly GST isozyme	with GSH and/or NADPH	% of metabolite[a]			Residual prothiofos oxon (%)[a]
		Despropylthio prothiofos oxon	2,4-Dichlorophenol	Phenyl phosphate	
GST1	+NADPH	32±2.8	22±2.3	N.D[b]	11 ±2.8
	+NADPH+GSH	31±2.1	22±2.5	N.D	13 ±3.3
GST3	+NADPH	31±2.8	23±2.3	N.D	8.9±4.1
	+NADPH+GSH	30±2.1	21±2.5	N.D	12 ±2.4
Takatsuki-GST4	+NADPH	33±2.5	22±1.7	N.D	11 ±2.8
	+NADPH+GSH	32±2.1	21±2.3	N.D	13 ±3.3
Yachiyo-GST4	+NADPH	32±2.8	23±2.1	N.D	13 ±2.6
	+NADPH+GSH	31±1.9	21±1.9	N.D	9.8±2.9
Takatsuki-GST6A	+NADPH	32±2.1	24±2.3	N.D	10.2±3.2
	+NADPH+GSH	28±2.4	20±2.5	N.D	9.7±2.8
Yachiyo-GST6A	+NADPH	33±2.6	24±2.8	N.D	13.0±2.8
	+NADPH+GSH	30±2.2	20±2.7	N.D	11.0±3.1
Takatsuki-GST6B	+NADPH	32±3.2	24±2.7	N.D	10.2±3.2
	+NADPH+GSH	25±2.8	22±2.5	4.1±1.3	9.8±3.6
Yachiyo-GST6B	+NADPH	31±2.3	23±2.8	N.D	11.0±2.8
	+NADPH+GSH	16±2.1	20±2.3	7.8±1.7	11.0±3.1

[a] Means±SD (n=3). [b] N.D: not determined.

the known desethylation proceed independently in the NADPH+GSH-added group, the amount of *S*-oxide will decrease in proportion to the production of desethyl prothiofos oxon; thereby both produced amounts of despropylthio prothiofos oxon and 2,4-dichlorophenol will decrease although the product ratio should not differ from that of both compounds in the NADPH-added group. The GST1 reaction did not change in the produced amount of both compounds in both groups. With the reaction of GST3 and 4, some decrease was seen in the amount produced in both groups but the production ratio did not show any significant change. With the GST6A reaction, the amount in the NADPH+GSH-added group decreased compared to that in the NADPH-added group but the production ratio did not show any change, whereas with the GST6B reaction, the compounds in both groups decreased and the production ratio changed, and Yachiyo strain 6B showed more significant change compared to Takatsuki strain 6B. That is to say, reviewing all the data so far, with isozymes 1 to 6A, the production ratio of two oxidative hydrolysates produced in the NADPH+GSH-added group was 20 : 15 in either case, thus showing no change as compared to the production ratio in the NADPH-added group; however, with GST6B, Takatsuki and Yachiyo strain isozymes were 17 : 15 and 12 : 15, respectively, and the amount of despropylthio prothiofos oxon relatively declined in comparison to 2,4-dichlorophenol. As shown above, the isozyme implicated in the reaction of oxidative conjugation in the previous report was only GST6B from both strains. This change in the production ratio was considered to indicate an additional novel conjugation under oxidation besides the desethylation of prothiofos oxon by GST6B. As shown in Table 6, 2,4-dichlorophenyl phosphate was detected only in the GST6B reaction and the amount produced by Yachiyo strain 6B was about 2 times the amount produced by Takatsuki strain 6B, clarifying that the *in vitro* production of desethyl *S*-oxide as predicted by computational chemistry in our previous report was by GST6B.

5. Characteristics and role of housefly GST6A and 6B

One of the mechanisms of insect resistance to chemicals is the detoxification of the chemicals by GST overexpression. Previous studies have singled out particular GST isozymes for discussion. Recently, it was reported that MdGST6A from the Cornell-HR strain housefly was the most probable candidate for the detoxification of methyl parathion and lindane. In this study, desethyl products of diazinon oxon and prothiofos oxon were produced in large amounts by 6A as by 6B in this order. This indicated that GST6A and 6B detoxified many *O*-alkyl phosphate-type OP insecticides by desalkylation. In this case, 6A seems to play a distinct role. The amount of desethyl product from both oxons was more in Yachiyo strain 6A than in Takatsuki strain 6A, while the expression of Yachiyo strain 6A was exceedingly more than that of Takatsuki strain 6A, so it is well observed that isozyme 6A is a leading factor in the development of resistance; however, in the case of prothiofos oxon, GST6B rather than 6A seems to exert action while playing a different role from that of 6A. In other words, Takatsuki strain 6B is involved in detoxification by desalkylation, whereas Yachiyo strain 6B has hardly any effect, thus acting toward the suppression of detoxification. Its expression level was less than that of 6A, although in the ratio between the strains, like 6A, Yachiyo strain isozyme was higher than Takatsuki strain isozyme. Importantly, in both strains, 6B caused the GSH conjugation of oxon under oxidation *i.e.*, by *S*-oxide desethylation, GST6B was a factor in activation. The reaction proceeded to a greater extent for Yachiyo strain isozyme than for Takatsuki strain isozyme, which was in proportion to the expression level in 6B for both strains.

When prothiofos is administered to resistant insects possessing significant isozyme corresponding to Yachiyo strain 6B, detoxification through the desethylation of prothiofos oxon becomes ineffective, and activation by the desethylation of *S*-oxide proceeds. *S*-Propyl phosphorothiolate-type OP insecticide can kill insects resistant to *O*-alkyl phosphate-type OP insecticides at an inherent dose as a result, thus causing the death of resistant organisms and a decrease in its population ratio, enhancing the retention and prolongation of insecticidal activity, whereby prothiofos has yet to show insecticidal activity toward highly resistant insects.

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